

PATHOGENESIS OF LIVER INJURY:  
VIRUS-INDUCED AUTOANTIBODY RESPONSE AGAINST  
ASIALOGLYCOPROTEIN RECEPTOR IN WOODCHUCK  
(*Marmota Monax*) VIRAL HEPATITIS

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JINGYU DIAO







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ASIALOGLYCOPROTEIN RECEPTOR IN WOODCHUCK  
(*Marmota Monax*) VIRAL HEPATITIS**

by

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A thesis submitted to the School of Graduate Studies  
in partial fulfillment of the requirements for  
the degree of Master of Science

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## ABSTRACT

The purpose of this research was to recognize the induction and pathogenic potential of autoantibodies against a liver-specific asialoglycoprotein receptor (ASGPR) in the course of hepatitis B infection. Three sections of research work by using a woodchuck model of hepatitis B as an experimental system are presented in this thesis.

First, woodchuck hepatic ASGPR (wASGPR) was purified and its polypeptide subunit composition, ligand binding properties and antigenicity were characterized. The isolated receptor is a hetero-oligomeric complex constituted of two subunits with molecular masses of 40- and 47-kDa. Antisera were generated in guinea pigs by challenge with the affinity-purified, bioactive wASGPR and rabbit ASGPR (rASGPR) preparations and in woodchucks by immunization with purified rASGPR. With the help of these antisera, a strong antigenic cross-reactivity of the wASGPR polypeptides with the ASGPRs from other species was demonstrated. Further, using antisera raised against isolated rASGPR polypeptides, the distinct antigenic specificity of each of the wASGPR subunits was revealed. Surface expression of wASGPR on the woodchuck hepatocytes was demonstrated by immunofluorescent staining of the isolated cells. Detailed analyses of the purified woodchuck hepatic plasma membranes (HPMs) showed that both subunits of wASGPR were present in the HPM preparations, although the membrane-bound 47-kDa subunit was identifiable only after exposure of HPMs to the ligand of the ASGPR.

Second, the induction, dynamic patterns and specificity of autoantibodies to hepatic wASGPR were studied in animals with experimental woodchuck hepatitis virus (WHV) infection. Results showed that WHV infection induced anti-ASGPR autoantibodies or led to the rise in their levels in almost all animals experimentally infected with WHV (12 of 13). This demonstrated that the induction of this hepatocyte-specific autoreactivity was a common

consequence of WHV infection. The relationship between anti-ASGPR reactivity existing prior to WHV inoculation and the outcome of experimental WHV hepatitis was also investigated. The results revealed that the occurrence of chronic hepatitis was significantly greater in the group of animals with pre-existing anti-ASGPR autoantibodies (54.6%) than that among woodchucks without autoantibodies in preinoculation sera (15.6%;  $P < 0.05$ ), suggesting that the outcome of WHV infection might be influenced by the status of anti-ASGPR autoreactivity. Western blot analyses showed that WHV-induced anti-ASGPR autoantibodies recognized both 40- and 47-kDa subunits of wASGPR and rASGPR, implying that both receptor polypeptides may serve as targets for the pathogenic immune reactions. Furthermore, no antigenic cross-reactivity between wASGPR and WHV antigens could be identified by using antibodies against ASGPR or WHV envelope, suggesting that mechanisms other than viral protein mimicry could be involved in the induction of anti-ASGPR autoantibody in the course of WHV infection.

Third, *in vitro* investigations of the pathogenic effects of the WHV-induced anti-ASGPR autoantibodies showed that these antibodies had the ability to inhibit specific ligand binding to the hepatocyte surface ASGPR. Also, Ig fractions from the same anti-ASGPR reactive sera were hepatocytotoxic in the presence of active complement. These findings indicate that virus-induced autoantibodies against hepatic ASGPR have the potential to directly contribute to liver injury and to the distortion of the hepatocyte clearance of asialoglycoproteins in viral hepatitis. Further careful studies are required to determine the significance of anti-ASGPR autoimmunity in the pathogenesis of liver injury in viral hepatitis. The woodchuck model of virus-induced ASGPR-specific autoimmunity could play an important role in these investigations.

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## LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AIH	autoimmune hepatitis
ALT	alanine transaminase
AMA	anti-mitochondrial antibody
ANA	anti-nuclear antibody
ASFN	asialofetuin
ASGP	asialoglycoprotein
ASGPR	asialoglycoprotein receptor
ASOR	asialoorosomucoid
BCA	bicinchoninic acid
$B_{max}$	maximum binding
BSA	bovine serum albumin
CD	cluster of differentiation antigen
CDC	complement-dependent-cytotoxicity
CFA	complete Freund's adjuvant
CH	chronic hepatitis
Chaps	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CTL	cytotoxic T lymphocyte
DHBV	duck hepatitis B virus
DMEM	Dulbeccos' modified Eagle's medium
DOC	Sodium deoxycholate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Gal	D-galactose

HAV	hepatitis A virus
HBV	hepatitis B virus
HBSS	Hanks' balanced salt solution
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HL	hepatic lectin
HPM	hepatic plasma membrane
HTC	hepatoma tissue culture
IFA	incomplete Freund's adjuvant
IODO-GEN	1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril
K <sub>d</sub>	equilibrium dissociation constant
KDa	kilodalton
KPM	kidney plasma membrane
LKM	liver-kidney-microsomal
LPM	liver plasma membrane
MHC	major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NeuAc	N-acetylneuraminic acid
ORF	open-reading frame
ORM	orosomucoid ( $\alpha_1$ -Acid glycoprotein)
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
rASGPR	rabbit asialoglycoprotein receptor
RIA	radioimmunoassay

<b>RPMI medium</b>	Roswell Park Memorial Institute medium
<b>rtASGPR</b>	rat asialoglycoprotein receptor
<b>SD</b>	standard deviation
<b>SDS-PAGE</b>	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<b>SLAH</b>	self-limited acute hepatitis
<b>SMA</b>	anti-smooth muscle antibody
<b>SPM</b>	splenocytes plasma membrane
<b>TSHV</b>	tree squirrel hepatitis virus
<b>wASGPR</b>	woodchuck asialoglycoprotein receptor
<b>WHV</b>	woodchuck hepatitis virus

## GENERAL BACKGROUND

Viruses are known as the smallest microorganisms of all life forms. These microbes have to enter into host cells to propagate. The life cycle of a virus can be simply divided into three steps: attachment and entry into host cell, primary replication, and spread within the host. Each step of the viral life cycle can be harmful, sometimes lethal to the host, since the normal biological activities of the host are interrupted by the invader. On the other hand, host cells and host defence systems have developed protective barriers against viruses, such as physical barriers, non-specific natural and specific responses (Abbas et al., 1991). Interactions between host and virus are not completely understood; however, it is widely accepted that both virus and host defense responses are frequently involved in the process of viral diseases (Fields et al., 1990). The studies on the pathogenesis of human viral infection are usually carried out using cell or tissue cultures, as well as appropriate animal models. Clinical observations and analysis of human specimens (e.g., blood samples and tissues obtained by autopsy or biopsy) are also commonly used (Fields et al., 1996).

The functions and morphology of liver can be affected directly or indirectly by infections with both hepatotropic and non-hepatotropic viruses (Robinson, 1990). In humans, viral hepatitis is a disease with diffuse intralobular and portal inflammation which can be induced by infections with primary hepatotropic viruses, such as hepatitis A virus (HAV), hepatitis B (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), and the most recently discovered hepatitis G virus.

DNA viruses that exhibit prominent liver tropism with a tendency towards chronic infection have been referred to as the hepadnaviruses (Fields et al., 1990). Human hepatitis B virus (HBV) is a noncytotoxic DNA virus which is a prototype of the hepadnavirus family (*Hepadnaviridae*) (Fields et al., 1990). HBV infection and HBV-induced diseases are one

of the leading public health problems. About 300 million people worldwide are developing chronic HBV infection. It is estimated that half of the chronic carriers will develop liver cirrhosis or hepatocellular carcinoma (HCC) (Robinson, 1994; Sherlock et al., 1970). The pathogenic mechanisms of HBV infection are not well recognized (Chisari and Ferrari, 1995a), although research in this area has been carried out for nearly 30 years. One of the major obstacles to the study of this subject is the lack of propagation systems, such as efficient cell lines or appropriate animals, for HBV infection *in vitro* or *in vivo* (Chisari and Ferrari, 1995a).

Woodchuck hepatitis virus (WHV) is another member of the family *Hepadnaviridae*. This virus shows strong molecular and biological analogies with HBV (Summers et al., 1978; Galibert et al., 1982; Feitelson et al., 1984). Because of these similarities, experimental infection of woodchuck with WHV represents an important natural model for the study of HBV-induced diseases (Snyder et al., 1982; Korba et al., 1989). In the following sections, an overview of our current understanding of the general biological features of hepadnaviruses (especially HBV) and the pathogenesis of hepadnaviral infection will be presented and a general research proposal for this thesis will be given.

#### **A. Hepadnavirus Family.**

There are seven well-documented hepatotropic DNA viruses which belong to the Family *Hepadnaviridae*. These viruses are: HBV, WHV, ground squirrel hepatitis virus (GSHV), tree squirrel hepatitis virus (TSHV), arctic squirrel hepatitis virus (ASHV), duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV). Other candidate members of this family may be kangaroo hepatitis virus (KHV) and stink snake hepatitis virus (SSHV). Generally, these viruses can be divided into two subgroups, mammalian and avian hepadnaviruses (Fields et al., 1990, 1996).

Scientists began to study the viruses in the hepadnavirus family in the 1960s when

Blumber et al. (1965) discovered HBV envelope particles in the serum of an aboriginal Australian. Today, HBV is known as a principal etiologic agent causing varied clinical forms of infectious hepatitis, such as acute hepatitis, fulminant hepatitis and chronic hepatitis. Infection with HBV in adults is usually asymptomatic and self-limited. However, about 10% of HBV-infected adults and up to 90% of children infected with HBV during the neonatal period develop persistent infection (Beasley et al., 1981; Stevens et al., 1975). These people will have an increased risk of developing liver cirrhosis and HCC (Robison, 1994; Sherlock et al., 1970). No effective treatment is available to terminate the established HBV infection, although vaccines have been produced to prevent HBV infection in virus-naïve individuals (Chisari and Ferrai, 1995a; Hollinger, 1987a). Other viruses in the hepadnavirus family were described more recently. WHV was first found in association with liver necroinflammation and HCC in a woodchuck (*Marmota monax*) colony in the Philadelphia Zoo (Summers et al., 1978). Later, DHBV was discovered in the serum of Peking ducks (Mason et al., 1980). Subsequently, GSHV was found in wild-caught ground squirrels and IHIV in herons (Feitelson et al., 1984; 1986).

Viruses in the hepadnavirus family have a very narrow host range (Feitelson et al., 1984; 1986). For example, HBV can infect humans and chimpanzees but not other species. WHV infects woodchucks and ground squirrels. IHIV has only been found in Peking ducks and a few other domestic ducks under natural conditions. Among the viruses in this family, WHV is the virus most closely related to HBV, with a 65% sequence homology at the nucleotide level and sharing many antigenic determinants (Galibert et al., 1982). Both viruses infect the liver and the lymphoid system (Korba et al., 1989) and usually induce similar patterns of specific immune responses to viral antigens as well as non-organ specific autoimmune antibody responses (Fields et al., 1990; Dzwonkowski and Michalak, 1990). In addition, there are many similarities in the disease development after IHIV and WHV infection. For example, the progress from acute hepatitis to chronic hepatitis and then to

HCC also occurs in woodchucks with WHV infection, although virus-induced liver cirrhosis develops only in humans (Summers et al., 1978). In both HBV and WHV infections, 10% of infected adults develop chronic hepatitis, while nearly 100% of infected neonates do so. Presently, WHV infection in woodchucks is considered as the most relevant experimental system for the study of the pathogenic mechanisms of human HBV-induced liver diseases. This system also represents a valuable model for testing novel preventive and therapeutic approaches against HBV infection (Feitelson et al., 1986; Hornbuckle et al., 1985; Korba et al 1986).

## **B. Human Hepatitis B Virus and Immune Responses to HBV.**

### **B.1. Human Hepatitis B Virus.**

#### **B.1.1. HBV Virion.**

At least three different forms of HBV particles have been observed in human blood under the electron microscope (Hollinger, 1987b). Most of the particles are incomplete subviral spheres formed by the virus envelope lipoproteins with a size between 15-20 nm in diameter. Filaments of 20 × 20-200 nm are another form of free circulating HBV particles. The mature virions are spherical particles (42-47 nm in diameter) which contain virus envelope lipoproteins and an inner-core (20 nm in diameter). The inner-core, which can be released from the virion by treatment with detergent, carries viral DNA and other viral non-structural proteins.

#### **B.1.2. Organization of the HBV Genome.**

The HBV genome, with a circular DNA molecule of 3200 nucleotides, is one of the smallest genomes of identified viruses (Ganem and Varnus, 1987). The circular viral DNA is partially double-stranded with a single-stranded gap up to 50% of the circle length. At the beginning of DNA synthesis, a fully double-stranded circular DNA forms after being patched

by a DNA polymerase in the virion. Four overlapping open-reading frames (ORFs) have been identified in HBV DNA, which encode at least 7 different viral proteins or peptides. These ORFs are termed: S-ORF for surface proteins (HBpre-S/S), C-ORF for nucleocapsid protein (core protein, HBc) and e protein (HBe, a cleavage product of the core protein), P-ORF for polymerase protein, and X-ORF for X protein.

### B. 1.3. HBV Proteins.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) of HBV envelope reveals proteins with six different molecular sizes (Herrmann et al., 1984; Neurath et al., 1985; Stibbe and Gerlich, 1983). These proteins are called small (24-kDa protein and 27-kDa glycoprotein, or P24 and GP27 respectively), middle (GP33 and GP36) and large (P39 and GP42) HBV surface proteins (HBs protein, or HBs antigen, HBsAg). All three pairs of proteins have the same C-termini but different N-termini. The genes in S-ORF encoding these proteins are divided into three segments, termed preS1, preS2 and S. Small HBs protein is encoded by the S segment, middle HBs protein is encoded by preS2 and S genomic regions, while the large HBs protein is encoded by the entire S-ORF.

Understanding of the biological function of HBs proteins in the process of viral infection remains incomplete. The small HBs proteins are important for the assembly and secretion of virus (Lamb et al, 1983). A binding site for polymerized human serum albumin (pHSA) was found in the preS2 region, suggesting pHSA may act as a bridge for the interaction between HBV and hepatocytes since an albumin receptor is exhibited on the surface of human hepatocytes (Neurath et al., 1985). The preS1 appears to be more important for direct binding between virus and hepatocytes and probably other cells (e.g., peripheral blood mononuclear cells, PBMC) (Neurath et al., 1985; 1986). In addition, overexpression and accumulation of HBsAg in transgenic mice demonstrated that HBV envelope proteins in hepatocytes could be hepatocytotoxic (Gilles et al., 1992).

The core (nucleocapsid, HBc) protein has a molecular mass of 22 kDa and contains a typical amino acid-repeat sequence of DNA binding proteins (Hruska and Robinson, 1977). No free core proteins are observed in the serum of HBV-infected patients, but HBc proteins occur in the cytoplasm of hepatocytes where they self-assemble to nucleocapsid particles with the association of viral polymerase and viral DNA. HBe is another protein of HBV with molecular mass of 15 kDa, which has been identified as a proteolytic cleavage product of the core protein. HBe is a soluble protein and can be detected in the serum of HBV-infected patients (Budkowska et al., 1977; Petit and Pilot, 1985). HBc and HBe differ both in their biophysical features and antigenicity.

The HBV genome also encodes a polymerase protein (P protein) with RNA reverse transcriptase and RNase H activities (Toh et al., 1983). Another viral protein, the X-protein contains about 154 amino acids (Moriarty et al., 1985). Studies of transcription *in vitro* by expressing X-gene in cultured cells (Spandau and Lee, 1988) and *in vivo* by using transgenic mice (Balsano et al., 1994) suggested that the X-protein may modify viral and cellular gene expression.

## B.2. Immune Responses to HBV Antigens.

### B.2.1. Humoral Immune Response to HBV Antigens.

Antibodies to HBV proteins are important serologic markers for the diagnosis of HBV infection (Fields et al., 1990). In the course of infection, appearance of anti-HBc is a sign of viral replication, which can usually be detected within 2 - 4 weeks after the emergence of HBsAg (HBV surface antigen) in the circulation. Following anti-HBc, anti-HBe and anti-HBc appear, indicating the decline of viral replication and the start of disease resolution. Anti-HBs contains neutralizing antibodies that are considered a marker of the termination of HBV acute infection (Hoofnagle, 1981).

There is no direct evidence showing that antibodies to HBV proteins mediate the liver

injury. Indirect evidence shows that viral proteins (core, surface and e antigens of HBV) expressed on the surface of hepatocytes are recognized by specific antibodies (Schlicht and Schaller, 1989), suggesting that anti-viral antibodies may contribute to the injury of hepatocytes through antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). *In vitro* studies using isolated human hepatocytes from patients with chronic hepatitis have shown that anti-HBc could be cytotoxic in the presence of active complement (Michalak et al., 1995). This issue still requires intensive investigation. It is clear, however, that anti-viral antibodies are responsible for some of the extrahepatic diseases associated with HBV infection (e.g. immunocomplex diseases). On the other hand, the success of HBV vaccines using HBsAg peptide or proteins as immunogen to stimulate host humoral immune response indicates that anti-HBs has great protective value in the early phase of viral infection (Fields et al., 1996). In fact, it is thought that anti-HBV-protein antibodies contribute to the clearance of extracellular viral particles during viral infection (Hoofnagle, 1981).

#### B.2.2. Cellular Immune Response to HBV Antigens.

Major histocompatibility complex (MHC)-restricted T cells responding to multiple epitopes in HBV proteins (envelope, nucleocapsid and polymerase) have been identified in the peripheral blood and liver-infiltrating lymphocytes of patients with acute hepatitis (Chisari and Ferrari, 1995a). These cells include MHC class I-restricted CD8<sup>+</sup> cytotoxic T lymphocytes (CTL), MHC class II-restricted CD4<sup>+</sup> helper T cells and MHC class II-restricted CD4<sup>+</sup> CTL. In the early studies, these T cells were detected by testing their proliferative responses to purified soluble viral proteins. The detected cells were mainly CD4<sup>+</sup> T cells specific for HBcAg, but not for HBsAg or preS2 antigens. In recent years, a method was developed to establish T cell clones specific for HBV proteins, including *in vitro* stimulation of PBMC from patients with acute and chronic hepatitis B with synthetic peptides derived

from different HBV genes (i.e., C, S and P). Results from these studies show that HBs, HBe and P proteins contain T cell epitopes. In addition, evidence from the studies of HBV transgenic mice shows that CD8<sup>+</sup> CTLs most likely play a dominant role in viral clearance and the pathogenesis of liver injury. These cells are able to secrete lymphokines (i.e., IFN- $\gamma$ ) to inhibit HBV gene expression and replication and to induce HBV-infected cells to undergo apoptosis. The CD8<sup>+</sup> CTL can also induce non-viral specific inflammatory reactions (Gilles et al., 1992b; Guidotti et al., 1994).

### **C. Pathogenesis of Liver Injury in the Course of HBV Infection.**

Hepatitis due to HBV infection can be acute, chronic or fulminant. Distinguishing between acute and chronic hepatitis is based on the histological picture and the duration of the disease (Seeff, 1990). One of the major liver morphological features of acute viral hepatitis is intralobular spotty necrosis. At the fully developed stage, panlobular disarray, increased cellularity and pleiomorphism of hepatocytes can be observed. This is caused by a combination of liver changes consisting of hepatocellular degeneration and necroses, hepatocellular regeneration, sinusoidal cell activation and inflammation. In chronic hepatitis, the disease persists for at least 6 months associated with syndromes of hepatocellular damage, inflammation and fibrosis.

The pathogenic mechanisms that engage in viral diseases are multiple and complex (Notkins and Oldstone, 1986). Two basic mechanisms are frequently involved, one is direct cytopathic effect due to the biological interactions of the virus with the cell, and the other is the response of multiple host defence mechanisms to the virus infected cell. There is no final conclusion about the pathogenesis of liver injury during HBV infection. Since HBV is noncytopathic, host antiviral immune responses are considered as the primary pathway to induce the damage to the hepatocytes (Chisari and Ferrari, 1995a). As mentioned above, the CTL is thought to be the most likely candidate. Because endogenously synthesized viral

antigens are likely presented by MHC class I molecules on the surface of HBV-infected hepatocytes, these viral antigens may be recognized by class I - restricted CD8<sup>+</sup> CTL. One of the mechanisms of hepatocyte damage by CTL is to induce programmed cell death or inflammatory cell injury. CD4<sup>+</sup> helper T cells may have indirect cytotoxic effects, as they produce cytokines, (e.g., tumour necrosis factor), which may be able to directly induce liver injury. Antibodies to HBV proteins expressed on the surface of hepatocytes may also produce hepatocyte damage through ADCC and CDC mechanisms.

In addition to virus specific immune responses, participation of autoimmune responses triggered by viral infection is also considered in the pathogenesis of liver injury, since autoreactivity is a common feature of viral hepatitis (McFarlane, 1991; Schattner and Ragerzisman, 1990). Autoantibodies to liver-specific autoantigens, including asialoglycoprotein receptors (ASGPR), have been identified as the targets of autoimmune reactions in patients with autoimmune liver diseases or with HBV infection. General information on this subject will be provided in Part II of this thesis. To date, there are few studies addressing the role of virus-induced autoimmune responses in the pathogenesis of liver damage.

#### **D. Animal Models for Hepatitis B.**

Studies on the pathogenetic mechanisms of virus induced diseases require *in vitro* (cultured cells) or *in vivo* (susceptible animals) systems for viral propagation. Due to the lack of such systems, initial studies on the pathogenesis of disease related to HBV-infection were mainly based on the clinical analysis of patients (Fields et al., 1990; 1996). The problems associated with this approach are the difficulties of obtaining multiple samples from the same patients throughout the course of disease and the difficulties of monitoring the complete clinical histories of the patients (Korba et al., 1986).

Appropriate animal models are helpful in solving these problems by providing experimental systems with a well-controlled laboratory environment. Experimental animals

can be infected with defined doses of well-characterized virus pools.

Since the 1970s, scientists have been attempting to develop animal models for hepatitis B. Chimpanzees have been identified as highly susceptible animals for HBV (Fields et al., 1990; Korba et al., 1986). Experimental transmission of HBV to these animals has demonstrated the development of hepatitis with patterns of humoral immune responses resembling those occurring in infected humans. However, HBV-induced hepatitis in chimpanzees usually has only mild histological severity of liver damage. In addition, there is no evidence showing HCC development in HBV-infected animals. Therefore, the HBV-infected chimpanzee is a valuable model for evaluation of preventive strategies against virus but not for determination of viral pathogenic mechanisms of HBV oncogenesis.

With the development of microinjection technology, transgenic mice expressing HBV viral products and even replicating HBV have been introduced for studies on pathogenicity of HBV-related diseases (Chisari, 1995b). Compared to other animal models used in this research area, one of the benefits is that the immune system in mice has been well characterized, thus simplifying the analysis of the cellular immune responses. Current studies using this animal model have revealed many new aspects of HBV pathogenesis. It has been demonstrated that MHC class I-restricted CTL specific for viral epitopes, and cytokines induced by these CTL (e.g., IFN- $\gamma$ ) undoubtedly contribute to the hepatocyte injury in HBV infection (Gilles et al., 1992b; Guidotti et al., 1994). However, results with transgenic animals, although significant, may not necessarily explain viral infections in their natural hosts. Conclusions from the studies of HBV-transgenic mice can not be assumed to fully apply to the pathogenesis of HBV infection in humans.

The discovery of other mammalian and avian hepadnaviruses makes it possible to investigate the hepadnaviral pathogenesis in natural hosts (Feitelson et al., 1986). For example, woodchucks infected by WHV or Peking ducks infected by DHBV are available for such investigations and for the evaluation of new drug- or gene-therapies against HBV-related

diseases (Cote and Gerin, 1995). Because of the strong similarity between HBV and WHV infections in their specific hosts, in terms of the viruses themselves, the natural history of viral infection, host immune responses, and disease developments and outcomes, infection of woodchucks with WHV has been demonstrated as an excellent experimental model for the pathogenesis of HBV-related diseases (Korba et al., 1986; Summer et al., 1978). With respect to the pathogenesis of liver injury during WHV-infection, studies have demonstrated that WHV viral antigens (WHs, WHc and WHe) expressed in the hepatocyte plasma membranes of infected animals can be the target of host immune response. Recognition of these antigens by specific antibodies may lead to the destruction of hepatocytes (Michalak et al., 1990). PBMC responses to WHcAg, and cytokine production in these responses, have been identified as being important for the resolution of WHV infection, and may also be responsible for liver injury in the process of WHV infection (Cote and Gerin, 1995).

#### **E. General Proposal.**

The purpose of this research is to study the pathogenic role of autoantibodies in the induction of liver injury during hepadnavirus infection. Studies focus on a hepatocyte surface target antigen, asialoglycoprotein receptor (ASGPR). ASGPR has been considered as an important autoantigen in autoimmune hepatitis (AIH). Autoantibodies to ASGPR could be of particular importance in the pathogenesis of liver diseases, because they may be able to either influence ASGPR's biological function or contribute to destruction of hepatocytes. In the majority of patients with HBV-induced hepatitis, autoantibodies to ASGPR have been detected. However, the correlation between HBV invasion and the appearance of these autoantibodies remains controversial, due to the inability to clearly identify the onset of viral invasion, to determine the status of anti-ASGPR autoimmune response prior to viral infection and to conduct a detailed longitudinal study of infected patients. In addition, the role of autoantibody to ASGPR in the pathogenesis of hepatocyte-damage in patients with hepatitis

B is not established.

In this study, a woodchuck model of hepatitis B was employed to investigate the induction and the possible pathogenic significance of anti-ASGPR autoantibodies. Since the woodchuck ASGPR (wASGPR) has not been studied, current work also includes the identification and biological characterization of wASGPR. The study consists of the following three parts.

**Part I. Isolation, characterization and hepatocyte-surface expression of woodchuck ASGPR (wASGPR).**

The following questions are posed:

- a. What are the molecular size and biochemical features of wASGPR?
- b. What are the immunologic and antigenic properties of wASGPR?
- c. Is wASGPR expressed on the surface of hepatocytes? What are the structural and immunologic properties of hepatic plasma membrane-bound wASGPR?

**Part II. Induction of autoantibodies against ASGPR in experimental woodchuck viral hepatitis.**

Questions asked are:

- a. What is the frequency of autoantibodies against ASGPR in a normal, WHV-free woodchuck population?
- b. Is WHV invasion able to induce anti-ASGPR autoantibodies?
- c. What are the dynamics of autoantibody production against ASGPR in the course of WHV infection? Do their patterns have a value in prediction of the outcome of hepatitis?
- d. Does the status of anti-ASGPR autoimmunity influence the outcome of WHV-induced hepatitis?
- e. What is the receptor subunit specificity of anti-ASGPR autoantibodies induced by

WHV infection?

f. Are WHV and wASGPR immunologically cross-reactive?

**Part III. Pathobiological effects of anti-ASGPR antibodies.**

Questions asked are:

a. Do anti-ASGPR antibodies inhibit ligand binding by ASGPR?

b. Are anti-ASGPR antibodies cytotoxic to cells expressing ASGPR?

c. Is there any difference in terms of the biological effects between anti-ASGPR antibodies induced due to WHV infection and those from animals immunized with ASGPR?

**PART I**  
**ISOLATION, CHARACTERIZATION AND HEPATOCYTE-**  
**SURFACE EXPRESSION OF WOODCHUCK**  
**ASIALOGLYCOPROTEIN RECEPTOR**

**1.1. INTRODUCTION**

**1.1.1. Glycoproteins and Asialoglycoproteins.**

Glycoproteins are proteins containing one or more carbohydrate chains. The most common monosaccharides on the oligosaccharide chains of glycoproteins are galactose (Gal), glucose, mannose, fucose, N-acetylneuraminic acid (NeuAc), N-acetylgalactosamine (GalNAc), and N-acetylglucosamine (Kornfield and Kornfield, 1985). The carbohydrate chains are bound covalently to asparagine, serine or threonine of the protein by O- or N-glycosidic linkage. The most typical monosaccharide at the termini of oligosaccharide chains is NeuAc which is usually followed by subterminal Gal or GalNAc residue. The sialoglycoprotein becomes asialoglycoprotein (ASGP) when the sialic acids (NeuAc) are split and the subterminal Gal or GalNAc residues are exposed at the end of oligosaccharide chains. Liberation of the sialic acid is termed desialylation (Ashwell and Harford, 1982; Kjellen and Lindahl, 1991).

Glycoproteins exist in cells of both prokaryotic and eukaryotic cells, as well as in viruses. In eukaryotic cells, biosynthesis of glycoproteins is a process of glycosylation which takes place in the endoplasmic reticulum and Golgi complex (Hischberg and Sinder, 1987). After synthesis, some glycoproteins are transported to the cell plasma membranes (cellular or membrane glycoproteins), while others are released to the extracellular environment

(secretory or extracellular glycoproteins). In mammals, most of the plasma proteins, except albumin and some lipoproteins, are sialoglycoproteins which are mainly synthesized in the parenchymal cells of the liver (Spiess, 1990). Other cells or tissues also produce extracellular sialoglycoproteins, e.g., plasma cells synthesize immunoglobulins and thyroid glands produce thyroglobulin. Although sialoglycoproteins are widely distributed, native ASGPs are rarely found under *in vivo* conditions (Stockert, 1995). Perhaps, this is because they have a much shorter half-life than sialoglycoproteins. The first described natural ASGP was the so-called "slow-moving thyroxine-binding globulin" which was found in the circulation of patients with liver cirrhosis (Mashall et al., 1972). To date, a growing number of proteins appear to be cleared from circulation through specific receptor systems. Experimental desialylation of these proteins usually increases the clearance, suggesting that comparable processes occurs *in vivo* (Ashwell and Harford, 1982). ASGPs can be prepared *in vitro* by treatment of isolated native sialoglycoproteins with acid or neuraminidase which cleaves the sialic acid residues. Also, synthetic glycoconjugates can be obtained by chemical methods, such as the imidate coupling method and the reductive alkylation method to achieve a preparation with less microheterogeneity and fewer structural complexities than the natural glycoproteins (Lee et al., 1987a and 1987b).

Glycoproteins have been extensively studied over the past two decades. Increasing evidence reveals the wide diversity of their biological functions. However, understanding of the glycosylation and desialylation process is still incomplete. Recent studies indicate that oligosaccharide recognition is an important step for specific protein-protein interactions between different cell types (e.g., leukocytes and endothelial cells) (Stoolman, 1989). As for the secretory glycoproteins, the possible biological significance of glycosylation may be in the modification of the protein conformation. It has been found that specific saccharide moieties could contribute to the protein's conformational structure and, in consequence, change its biological activity (Rose et al., 1984). In addition, it is also possible that glycosylation may

control the half-life of glycoproteins through a mechanism of protecting the peptide chains from proteolytic cleavage or recognition by receptors for clearance. Ashwell and his colleagues (Morell et al., 1968; 1971) found that injected  $^{64}\text{Cu}$ -labelled asialoceruloplasmin could be rapidly cleared from serum and recovered from rabbit livers within 24 min, while the half-life of native ceruloplasmin in the circulation was 56 hours. Similarly rapid clearance of desialylated avian glycoproteins injected into rabbits was observed by Regocezi et al. (1975). These results suggest an important role for oligosaccharides in determination of the turnover rate of serum glycoproteins. The terminal sialic acid residues seem to act as elements preventing removal of the proteins from the circulation and their cleavage allows rapid clearance of glycoprotein (Drickerman, 1991). Since the different Gal-terminated branches of oligosaccharides are selectively recognized by ASGPR, the composition of the carbohydrate chains, the site and the mechanism of the removal of terminal sugar(s) may significantly contribute to the persistence of glycoproteins in the circulation (Rice et al., 1990).

### **1.1.2. Endocytosis.**

To maintain vital processes, cells develop ways to transport substances across the membrane. Endocytosis is one of the basic mechanisms for the transfer of macromolecules into cells. This process is mediated by specific receptor systems and can be divided into the following steps: binding of macromolecules (ligands) to receptors, formation of coated pits, internalization of ligand-receptor complex by coated vesicles, delivery of ligand-receptor complexes to the endosomal sorting compartment, dissociation of ligand-receptor complexes by acidification, degradation of ligand and recycling of the receptor to the cell surface (Schwartz, 1990; Wu and Wu, 1991). There are several different classes of receptors which are involved in endocytosis. For example, transcytotic receptor is for the transportation of polymeric immunoglobulin, growth factor hormone receptors (such as epithelial growth

factor) and recycling transport receptors for the transfer of low-density lipoproteins, transferrin and ASGPs. The receptor for ASGP (ASGPR) has been well-characterized as a model of receptor-mediated endocytosis.

### **1.1.3. Asialoglycoprotein Receptor (ASGPR).**

ASGPR is a receptor responsible for the clearance of desialylated glycoproteins from the circulation. This receptor is also called Gal lectin (or hepatic lectin, HL). As noted previously, initial knowledge about the biological properties of ASGPRs comes from observations of the metabolism of ceruloplasmin. Morell et al. (1968) found that the terminal sialic acid residues of ceruloplasmin were a key factor in controlling the turnover rate of this protein in the circulation of the rabbit. This study also showed that the disappearance of <sup>14</sup>C-labelled asialoceruloplasmin from the serum is accompanied by the appearance of the radioactive protein in the liver. Autoradiography of liver sections revealed that the parenchymal cells, but not Kupffer cells, were the sites of deposition of asialoceruloplasmin. Subsequent experiments demonstrated that binding sites specifically recognizing ASGPs, but not their sialylated derivatives, are anchored in the purified liver plasma membranes (Morell et al., 1971). The interaction between ASGP and liver plasma membranes requires the presence of calcium ions and occurs in a narrow pH range of 6.0 to 7.8. Based upon these features of ASGPR, a method for the isolation of ASGPR by using affinity chromatography on ASGPs or Gal has been established. This method was first employed for the purification of ASGPR from rabbit liver (rASGPR) (Hudgin et al., 1974). Since then, extensive studies on ASGPR have been performed and they have provided important insight into the structure and function of this membrane protein. The interaction between ASGPR and its ligands has been used as a molecular model for the study of receptor-mediated endocytosis. In addition, the prospects for clinical applications of ASGPR look promising, such as targeted diagnosis and therapy of liver diseases (Wu and Wu, 1991). In the following sections, the current

knowledge on the biochemical properties of ASGPR will be reiterated from several reviews on this issue (Ashwell and Harford, 1982; Breitfeld et al., 1985; Drickamer, 1991; Lodish, 1991; Schwartz, 1990; Spiess, 1990; Wu and Wu, 1991).

#### **1.1.3.1. Biochemical Properties of ASGPR.**

As an integral membrane protein (Morell and Scheinberg, 1972; Chiacchia and Drickamer, 1984), ASGPR has to be extracted by nonionic detergent (e.g., Triton X-100) from membranes followed by purification by ligand affinity chromatography (e.g., ASGP or Gal). Purified ASGPR preparations contain water-soluble, lipid-free proteins in association with 8 to 10% carbohydrate, and retain high ASGP binding activity. Such preparations also exhibit a high degree of protein aggregation which makes it difficult to determine the molecular size of the purified receptor. Currently, there are three main approaches for determination of receptor molecular masses. The classical methods are gel filtration and sedimentation equilibrium to directly determine the molecular size of ASGPR in aqueous solution. Detergents can be employed to disassociate the receptor proteins, and  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$  plus ligand (such as Gal) is usually added to maintain the stability of receptor protein. Molecular mass of receptor species measured by these methods reflect the size of the natural receptor molecule exhibited within the native plasma membranes. The most advanced approach to determine the functional size of receptors in their natural environment (i.e., within biological membrane) is radiation inactivation analysis. The concept of this technique is based on exposing receptor-containing plasma membranes to increasing doses of high-energy ionizing radiation to induce a radiation-dependent loss of receptor biological activity which can be measured by binding of a specific ligand. Results of this analysis will give the minimal functional size of the receptor molecule which is independent of the molecules' shape or volume. Nevertheless, the most common method to determine the number and the size of polypeptide subunits in purified ASGPR proteins is sodium dodecyl sulfate - polyacrylamide

gel electrophoresis (SDS-PAGE). This analysis requires protein denaturation by treatment with SDS and  $\beta$ -mercaptoethanol, and the results will reflect only the polypeptide constitution of the receptor molecule, not the size of the receptor with biological function. When all these biochemical methods fail, immunological and molecular biology approaches can be utilized to characterize ASGPR proteins. The usefulness of these different analytical techniques will also be addressed in the following sections of this introduction.

To date, the molecular composition of hepatic ASGPR from rabbit, rat, mouse, human and chicken has been characterized (see Table 1.1) (Anderson et al., 1982; Baenziger and Maynard, 1980; Bischoff and Lodish, 1987; Drickamer et al., 1984; Harford et al., 1982; Hong et al., 1988; Kawasaki and Ashwell, 1976; 1977; McPhaul and Berg, 1986; Schwartz et al., 1983; 1984; Steer et al., 1981; 1990; Tanabe et al., 1979). Overall, the studies indicate that the mammalian ASGPRs are composed of two or three polypeptide chains (subunits) with molecular masses ranging between 40- and 60-kDa. Most likely, these subunits form hetero- or homo-oligomeric complexes in hepatocyte plasma membranes, since the receptor functional size is always larger than that of the constituent subunits (Schwartz, 1984). The number of polypeptide subunits varies in ASGPRs derived from different species. Three subunits with distinct molecular masses were found in the affinity purified rat (rtASGPR or rat hepatic lectin, RHL) and mouse ASGPRs (mouse hepatic lectin, MHL), while only two subunits are observed in rabbit (rASGPR) and human ASGPRs (Table 1.1). The receptor subunits are distinguished as major and minor forms, depending on their relative abundance. In rASGPR, the ratio of major (40 kDa) to minor (48 kDa) subunits is found to be 2:1 (Kawasaki and Ashwell, 1976) and in human ASGPR, 5:1 (46-kDa to 50-kDa subunit, respectively) (McPhaul and Berg, 1986). The relative abundance of the major (43 kDa) and minor (54 and 64 kDa) subunits in rtASGPR is 8:1:1 (Drickamer et al., 1984). These ratios are based on measured protein staining of the affinity purified receptor preparations separated on SDS-PAGE and they may not reflect the real proportions of receptor subunits in particular

**Table 1.1. Molecular masses of hepatic asialoglycoprotein receptors  
from different species**

Species	Molecular mass (kDa) determined by different methods <sup>a</sup>		
	SDS-PAGE <sup>b</sup>	Gel filtration or sedimentation equilibrium <sup>b</sup>	Radiation inactivation <sup>c</sup>
Rabbit	40 and 48	230 - 612	109 ± 5
Rat	43, 54 and 64	264	104 ± 17
Mouse	42, 45 and 51	n.a. <sup>d</sup>	n.a.
Human	46 and 50	n.a.	140 (HepG2 cells) 70 (liver)
Chicken	26	250	62-85

<sup>a</sup> - according to the data reported in the past two decades, see details in text.

<sup>b</sup> - size of ASGPR molecules in the preparations purified by ligand affinity chromatography.

<sup>c</sup> - functional size of ASGPR molecules in hepatocyte plasma membrane.

<sup>d</sup> - n.a.: data not available.

locations of the cells (e.g., in hepatocyte plasma membranes). In addition, it is difficult to detect the existence and determine the molecular size of less abundant polypeptide species of the receptor. In this respect, the best example might be the identification of the minor subunit of human ASGPR. Initial studies consistently showed that only one subunit (H1, 46-kDa) could be detected in the receptor preparations derived from human liver or from a human hepatoma cell line (HepG2). However, examination of a cDNA library of human ASGPR revealed the existence of a clone encoding the second receptor subunit (H2) with an amino acid sequence showing 58% homology to H1 (Spiess and Lodish, 1985b). Because of this observation, the H2 with molecular mass of about 49 - 50-kDa was finally identified in HepG2 cells by Bischoff and Lodish (1987), using antibodies directed against a synthetic peptide with predicted sequence based on the cDNA sequence.

#### **1.1.3.2. Interspecies Homology Among ASGPR Nucleotide and Amino Acid Sequences.**

Since the 1980s, cDNA libraries of ASGPR from rat, mouse, human and chicken have been established (Drickamer, 1981; Leung et al., 1985; Monroe and Huber, 1994a; Sanford and Doyle, 1990; Spiess et al., 1985a). The existence of two ASGPR genes in mammals has been detected and their organization have been found quite similar to each other. The genes include six to nine exons corresponding to the DNA sequence encoding four distinct structural and functional domains of the receptor; the cytoplasmic tail, transmembrane sequence, extracellular neck region and  $Ca^{2+}$ -dependent ligand recognition domain. All the known mammalian ASGPRs are highly homologous, as shown by computer-assisted multiple sequence comparisons of the receptor amino acid and cDNA sequences of ASGPRs (Drickamer et al., 1984; 1987; 1988). One of the interesting findings was that identical genes encode the two different minor species of rASGPR (54- and 64-kDa polypeptides, RHL-2 and RHL-3, respectively) or mouse ASGPR (45- and 51-kDa polypeptides, MIL2 and MHL3, respectively) receptor subunits. The difference between these two minor subunits in

both receptors is the structure of their carbohydrate chains (Halberg et al., 1987). Further analysis of the amino acid sequences of ASGPRs from rat and human livers revealed that the identity of the amino acid sequences among all the subunit polypeptides is about 39%. A greater homology was found between the two major species (H1 vs. RHL-1: 80%) than between the minor species (H2 vs. RHL-2/3: 62%) or between the major and minor species (H1 vs. H2: 58%, H1 vs. RHL-2/3: 56%, RHL-1 vs. RHL-2/3: 52%, RHL-1 vs. H2: 50%). These results suggest that the most conserved region falls in the sequence of major subunits. Relatively high homology of ASGPRs between chicken and mammals is also observed (Bezouska et al., 1991; Drickamer et al., 1984).

Homology in the primary structures of ASGPRs from different species raises the possibility that common interspecies antigenic determinants exist in these receptors. Schwartz et al. (1981a) prepared a rabbit antiserum and a monoclonal antibody against a rtASGPR preparation to identify and quantify hepatic rtASGPR. They found that the monoclonal antibody could completely inhibit the binding of <sup>125</sup>I-labelled ligand to the immobilized receptor. Also, both the monoclonal and polyclonal antibodies precipitated all constituent polypeptides of rtASGPR. This observation suggests that the receptor subunits contain a common immunogenic domain which is responsible for the ligand binding. Another research group also found that a ligand binding site was presented on both subunits of rtASGPR and recognized by a monoclonal antibody to rtASGPR (Harford et al., 1981; 1982). Harford et al. (1981) prepared a subunit monospecific antiserum by immunization of a rabbit with the major 43-kDa subunit of rtASGPR purified by preparative SDS-PAGE. Interestingly, this antiserum recognized an antigenic determinant presented on all constituent polypeptides of rtASGPR but exhibited no inhibition of ligand binding by the purified receptors. According to the authors, this result might be due to the denaturation of receptor subunits during purification before the subunits were used for immunization.

In the human ASGPR system, the antigenicity of ligand binding sites or the structures

related to ligand-binding has also been demonstrated by specific antibodies to ASGPR. Schwartz and Diane (1983) demonstrated that the binding of  $^{125}\text{I}$ -labelled ligand to the ASGPR on HepG2 cells was blocked by antibodies raised either against purified human ASGPR, the SDS-denatured 46-kDa polypeptide of human ASGPR or rtASGPR. These antibodies differed by their affinities of inhibition. The  $I_{50}$  (50% inhibition) of anti-human ASGPR was approximately  $5 \times 10^{-8}$  M, whereas that of the anti-46-kDa subunit was  $5 \times 10^{-7}$  M and that of the anti-rtASGPR was  $1.5 \times 10^{-8}$ .

Taken together, current data indicate that ASGPRs are interspecies conserved molecules. There is a significant degree of homology in their ligand binding sites as well as in non-function-related regions.

#### **1.1.3.3. Subunit Interactions and ASGPR Ligand Binding Activity.**

Despite the amount of knowledge accumulated on the ASGPR structure and its biological function, there are still a number of unsolved issues related to the nature of the interactions between the major and minor subunits and their functions in receptor-ligand binding and ligand endocytosis. To address these questions, studies have been carried out in rat and human systems. Data appear to demonstrate that both the major and minor subunits are required for assembly of high affinity ligand binding sites on ASGPR molecules and to mediate efficient endocytosis.

In 1987, Halberg et al. developed a method to separate the major and minor subunits of rtASGPR. The concept was based on the observation that the minor RHL-2 and RHL-3 were more sensitive than the major RHL-1 unit to various oxidizing and reducing agents. Treatment of rtASGPR with  $\beta$ -mercaptoethanol for 1 hour at room temperature resulted in the loss of ligand binding activity displayed by RHL-2 and RHL-3, while RHL-1 still recognized the ligand. These results confirmed the earlier observation (Hsueh et al., 1986) that RHL-1 alone can specifically bind Gal residues on ASGP. Meanwhile, expression of

RHL-2/3 in an *in vitro* transcription/translation system created by construction of RHL-2/3-insulin fusion proteins, demonstrated that RHL-2/3 also contained a Gal-recognition domain. Moreover, cross-linking of surface receptor by antibodies showed that the RHL-1 and RHL-2/3 subunits self-associated into homo-oligomer. The major and the minor subunits were independent molecules and had no structural relationship with each other. In contrast to this observation, using immunoprecipitation technique, it was shown that the major and minor subunits of rat ASGPR interact noncovalently within plasma membranes (Sawyer et al., 1988). Similar conclusions were also obtained by Braiterman et al. (1989). In this last study, the authors performed co-transfection of rat hepatoma tissue culture (HTC) cells with RHL-1 and RHL-2/3. It was found that cells expressing RHL-1 alone could only bind synthetic ligand, Gal-Lysine (Gal-Lys), while the cells expressing both subunits could bind, uptake and degrade ASGPs (e.g., ASOR). The authors concluded that RHL-1 was sufficient to recognize ASGP and mediate the endocytosis of ASGP, while RHL-2/3 might play a supporting role in the formation of the high affinity ASGPR macromolecule in the plasma membranes. Therefore, in contrast to Halberg et al. (1987), they assumed that the native ASGPR is a hetero-oligomer. In the most recent studies using synthetic glycopeptides and photochemical cross-linking techniques, Rice et al. (1990) clarified the role of both major and minor subunits in ligand binding. They demonstrated that the precise binding geometries between the ligand and RHL-1, and the ligand and RHL-2/3 differed. They suggested that the structure of ASGPR with high affinity for ligand was constructed by all the constituent subunits of rtASGPR.

The relationship between the subunits of human ASGPR have been investigated in the HepG2 cell line (Bischoff and Lodish, 1987; Schwartz et al., 1981b; 1982; 1983). Using chemical cross-linking and enzyme-induced degradation of receptor molecules in plasma membranes, it has been found that the two subunits of human ASGPR (H1 and H2) form a hetero-oligomer in HepG2 (Bischoff and Lodish, 1987). Better understanding of this subject

came from the studies performed by transfection of murine NIH 3T3 fibroblasts with genes for H1 and H2 (Shia and Lodish, 1989). It was demonstrated that H1 could be expressed in the transfected fibroblast and transported to the cell surface, while H2 alone appeared to have a short half-life and could not be expressed as a mature molecule on the cell surface because of rapid degradation. However, the H2 degradation was overcome after co-transfecting both H1 and H2 into the same cell, suggesting that the stability of the receptor benefitted from the presence of both subunits. Interestingly, the presence of both H1 and H2 was also required for ligand binding, internalization and degradation in the transfected cells, as <sup>125</sup>I-ASOR could not be recognized by cells expressing only one receptor subunit. In contrast, Geffen et al. (1989) demonstrated that transfected H1 subunit could be functional without cotransfection of subunit H2. Further experiments performed by Henis et al. (1990) demonstrated the formation of the H1-H2 complexes in both human ASGPR transfected murine NIH 3T3 and HepG2 cells. The H1 and H2 subunits on the surface of HepG2 cells were associated to form stable hetero-oligomers and all the H1 bound with H2 on the surface of transfected fibroblasts. Therefore, it is possible that H2, like RHL-2/3, plays a role in the re-organization of receptor subunits to form a high affinity Gal-binding site.

In the most recent studies, it was shown that a single tyrosine (Tyr) in the cytoplasmic domains of H1 and RHL-1 was a determinant for phosphorylation and served for the signal transduction of ligand-receptor internalization or intracellular trafficking (Haynes et al., 1994). H2 also has a serine (Ser) residue available for phosphorylation, which is not necessary for endocytosis but does contribute to targeting the receptor-ligand complex to the basolateral region of the cell plasma membranes (Geffen et al., 1991).

#### **1.1.3.4. Distribution of ASGPR.**

It has been well demonstrated that desialylation of serum glycoproteins results in the deposition of ASGP on the surface of liver parenchymal cells (Tolleshaug et al., 1977).

Identification of subcellular distribution of ASGPR was accomplished by both biochemical and immunologic methods. Results obtained by Pricer and Ashwell (1976) and Tanabe et al. (1979) indicated that both plasma membranes and intracellular organelles (e.g., smooth endoplasmic reticulum and Golgi) of rat liver had the capability to bind ASGP. Subsequently, Bridges et al. (1982) and Weigel and Oka (1983) identified a large amount of intracellular ASGPR in isolated rat hepatocytes. Immunocytochemistry and immunoelectron microscopy analysis performed by using antibodies directed either against ligand or ASGPR showed that about 65% of the receptor pool was located in the intracellular compartment of rat hepatocytes. Within the intracellular compartment, 20% of the receptors were associated with the rough endoplasmic reticulum, 30% with Golgi apparatus, and 50% with the smooth endoplasmic reticulum and present in the compartment of uncoupling of receptor and ligand vesicles (CURL).

Subcellular localization of human hepatic ASGPR has been studied in HepG2 cells (Schwartz et al., 1982). In contrast to the distribution of rtASGPR in rat hepatocytes, 87% of the ligand binding sites were plasma membrane-associated. Intracellular ASGPR accounted for only 13% of the total ASGPR. However, the results of immunohistochemical staining of sections from human liver (Sipos et al., 1989) was in good agreement with the findings in rat liver and showed that between 40 and 90% of the ASGPRs were located intracellularly.

It is worth noting that the ASGPRs on the hepatocyte surface are not evenly distributed but show polarity. This polarity has been visualized by using immunocytochemistry and electron microscopy techniques. The density of staining of ASGPR ligand binding sites was 70-fold greater on the sinusoidal (blood-facing) surface of the hepatocyte plasma membrane than on other membrane regions (i.e., lateral and bile canalicular) (Wall, et al., 1981). Geuze et al. (1983) have also found that ASGPRs on resin sections of parenchymal cells from rat liver are mainly located in the sinusoidal plasma

membrane (85%), the concentration being nearly 10 times that in the lateral and bile canalicular membranes. In the case of human ASGPR, the receptor was also found to be mainly distributed on the sinusoidal surface of hepatocytes from patients without liver diseases (Burgess et al., 1992). Significantly less ASGPR was detected on the lateral surface, and ASGPR was absent on the canalicular plasma membranes. A similar distribution was found by Mizuno et al. (1993). Interestingly, both research groups reported that such polarity of ASGPR exhibition changed in livers of patients with cirrhosis or poorly-differentiated hepatocellular carcinoma (HCC). In these cases, a decreased expression of ASGPR was found on the sinusoidal plasma membrane of hepatocytes. Since the presence of ASGPR on the sinusoidal surface could make ASGPR-mediated endocytosis more efficient, it is postulated that a change in the polarity of ASGPR distribution may influence liver function regarding the clearance of ASGP from the circulation.

The distribution of hepatic ASGPR differs between mammalian species. Chang and Chang (1988) measured ligand binding by hepatocytes, as well as liver homogenates originating from different species. The results showed that the amount of ASGPR on the hepatocytes or in the liver from different species has the following rank order: rabbit > rat > guinea pig, indicating a species-difference of ASGPR expression on hepatocytes.

During the past few years, the classical concept of "hepatic" ASGPR has been challenged by the observations that ASGPR or ASGPR-like substances also exist in other tissues. Studies in this area initiated in the late 1980s when investigators were trying to identify the receptors on the surface of spermatogenic cells which could bind to the zona pellucida of eggs (Abdullah and Kierszenbaum, 1989). Results of cDNA sequence analysis, mRNA detection, and analysis of the receptor antigenic and ligand specificities (Monroe and Huber, 1994b; Mu et al., 1993) showed that rat or mouse genes encode RHIL-related or MHL-related proteins in the spermatids and epididymal sperm of rat and mouse. These proteins are capable of binding ligand of ASGPR. In addition, rat peritoneal macrophages

also express Gal binding receptors with 77% amino acid sequence identity with RHL-1 (Masayuki et al., 1988; Ozaki et al., 1992). More recently, Pacifico et al. (1995) showed that both RHL-1 and RHL-2/3 mRNA were expressed in the liver, kidney, brain and thyroid, but not detectable in the rat spleen. It is not excluded that hepatic and extrahepatic ASGPRs may be different in their gene transcription, expression and receptor biological activity. However, current evidence does raise some doubts about the strict liver-specificity of the ASGPR-delivery system for gene and drug target therapy.

## **1.2. MATERIALS AND METHODS**

### **1.2.1. Animals.**

Woodchucks (*Marmota monax*, males and females), rabbits (New Zealand white, adult female), rats (Sprague-Dawley, adult females) and guinea pigs were used in this project. All the animals were maintained in the Animal Care Facility, Memorial University of Newfoundland. Experiments with animals were carried out according to the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

Woodchucks from a woodchuck colony maintained in the Liver Research Laboratory, Faculty of Medicine, Memorial University of Newfoundland were divided into two groups: healthy, WHV-nonexposed woodchucks, and woodchucks with experimentally induced WHV infection. Inoculation with the virus, and serological and histological evaluations of the status of WHV infection in animals were done by other investigators from this laboratory, according to the methods and diagnostic criteria previously described in detail (Michalak et al., 1989; 1990).

### **1.2.2. Cells.**

#### **1.2.2.1. Hepatocytes Isolated from Liver Tissue.**

Hepatocytes were isolated from woodchuck and rat livers using a collagenase perfusion technique (collagenase digestion) or a mechanical disruption method according to previous descriptions (Michalak et al., 1988; 1994) with some minor modifications.

#### **Materials:**

- (1) Phosphate-buffered saline (PBS): 150 mM sodium chloride (NaCl) in 0.02 M phosphate buffer, pH 7.4.
- (2) 1 × Hanks' balanced salt solution (HBSS; Gibco BRL, Life Technol., Inc., Grand Island, NY.).

- (3) Washing solution: 0.2% (w/v) ethylenediaminetetraacetic acid (EDTA) (Baker Chemical Co., NJ.) in  $1 \times$  RPMI 1640 medium (INC., Biomedicals Inc., Cost Mesa, CA.).
- (4) Perfusion solution: 150 mM NaCl, 5 mM potassium chloride (KCl) and 9 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (Hepes, sodium salt; Sigma Chemical Co. St.Louis, MO.) adjusted to pH 7.4 by 1 N HCl.
- (5) Collagenase solution: 0.05% (w/v) collagenase (type IV) (Sigma) in  $1 \times$  HBSS.
- (6) Culture medium: 0.1% (w/v) bovine albumin (Sigma), 10  $\mu$ g/ml insulin (Sigma), 60  $\mu$ g/ml penicillin and 100  $\mu$ g/ml streptomycin (ICN Biomedicals Inc., Horsham, PA.) in  $1 \times$  RPMI 1640 medium (INC., Biomedicals Inc.)
- (7) Trypan blue solution: 0.2% (w/v) trypan blue (Sigma) in 0.85% NaCl.
- (8) Fresh liver tissues obtained from woodchuck by liver biopsy or at autopsy, and rat autopsy were stored in sterile PBS or HBSS on ice for transfer.

Methods:

- (1) For mechanical isolation of hepatocytes, liver tissue was cut into small pieces of 0.25 to 0.5  $\text{cm}^3$  and washed 3 times using about 10 volumes of washing solution, 5 min for each wash. Subsequently, the liver tissue was minced in cold HBSS and then pushed through a cell dissociation sieve with 50 mesh screens using the plunger of a 5-ml syringe. All these procedures were done on ice. Hepatocytes were collected from the resulting cell suspension and purified 2 to 3 times by centrifugation at  $200 \times g$  for 5 min in cold HBSS. Microscopy revealed that the purity of hepatocytes was above 95% and the cells had typical hepatocyte morphology. Trypan blue staining revealed that most of the cells were nonviable.
- (2) For collagenase perfusion method, the liver tissue was also cut into small pieces of 1 to 1.5  $\text{cm}^3$  and washed once with washing solution for about 5 min. First perfusion was performed for 15 min using 10 volumes of circulating oxygenated perfusion solution preheated to 37°C. The perfusion was carried out on a cell dissociation sieve by using a 25G 5/8" needle to puncture the liver tissue at different sites and at a speed of 20 ml perfusion

solution per min. Similar perfusion was performed twice using circulating collagenase solution, 10 volumes of fresh solution for 10 min per each perfusion. Subsequently, the liver tissue was minced and gently pushed through the sieve into HBSS. The cells were washed and purified by centrifugation as above. The purity of hepatocytes from this preparation was above 95% according to microscopy examination and the viability was 95 to 99% according to trypan blue exclusion.

#### 1.2.2.2. Hepatocyte Cell line.

The human hepatocellular carcinoma cell line HepG2 (ATCC HB 8065), kindly provided by Dr. C.H. Ford (Faculty of Medicine, Memorial University of Newfoundland), was maintained according to the instructions of the American Type Culture Collection (ATCC). Culture medium contained Dulbecos' modified Eagle's medium (D-MEM)(Gibco BRL, Life Techol., Inc., Grand Island, NY.) with fetal bovine serum (10%), sodium pyruvate (66 mg/L), 100 × nonessential amino acids (6 ml/L) penicillin (60 IU/L), and streptomycin (60 µg/L) (all these materials were purchased from ICN Biomedical Inc., Horsham, PA.). Cells were cultured in plastic tissue culture dishes at 37°C, in a controlled environment of 5% CO<sub>2</sub> (v/v, in air) and 100% humidity, till full monolayer growth. The cells were harvested by using trypsin-EDTA (10 ×, Bibco BRL) digestion and washed with culture medium. The separated cells were used for another seeding or for fixation (see section 3.2.).

HepG2 cells cultured on tissue culture slides (Miles Scientific, Inc., Naperville, IL.) under the same conditions for 48 hours were used for immunofluorescent staining (see section 1.2.11).

#### 1.2.3. Hepatic Plasma Membranes.

Liver and hepatocyte plasma membranes (hepatic plasma membranes, HPMs) were isolated from liver homogenate or purified hepatocytes using a method described by Aronson

and Touster (1974) and Michalak et al. (1988; 1989). Splenocyte and kidney plasma membranes (SPMs and KPMs, respectively) used in control experiments were prepared using the similar method and kindly provided by other investigators from this laboratory.

**Materials:**

- (1) Protease inhibitor solution: 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma), 50,000 units/ml aprotinin (Calbiochem-Novabiochem Co., La Jolla, CA.) and 2 mM  $\alpha$ -amino-n-caproic-acid (Sigma) in PBS.
- (2) Sucrose solution A: 250 mM sucrose in 5 mM Tris-HCl buffer, pH 8.0.
- (3) Sucrose solution B: 37% (w/w) sucrose in 5 mM Tris-HCl buffer, pH 8.0.
- (4) Sucrose solution C: 57% (w/w) sucrose in 5 mM Tris-HCl buffer, pH 8.0.
- (5) Liver tissues obtained from autopsies of woodchucks, rabbits and rats.
- (6) Woodchuck hepatocytes isolated from woodchuck livers using the collagenase perfusion technique (see section 1.2.2.1.).

**Method:**

Animal liver tissues were extensively washed with cold protease inhibitor solution, placed into cold sucrose solution A on ice, and then minced into small pieces. For isolated woodchuck hepatocytes, the cells were washed twice with cold solution A. Subsequently, tissue fragments or hepatocytes suspended in 5 volumes of solution A were homogenized 6 times, 15 seconds each time using a knife homogenizer (Brinkmann Instruments, Rexdale, Ont., Canada). The suspension was filtered through three layers of prewetted surgical gauze (16 threads/cm<sup>2</sup>). The solution was again homogenized with 1 to 2 up-and-down strokes of a pestle homogenizer (Con-torque, Eberbach Corporation, MI.). This homogenate was centrifuged at 1000  $\times$  g for 10 min to remove nuclei and cellular debris and the supernatant was recentrifuged at 33,000  $\times$  g for another 10 min to pellet mitochondria and lysosomes. The resulting supernatant was spun at 78,000  $\times$  g for 100 min and the microsomal pellet was then suspended in 2.5 volumes of solution C and homogenized with three up-and-down

strokes of the pestle homogenizer. Ten ml of the suspension was placed on the bottom of ultra-clear centrifuge tubes (Beckman). About 20 ml of solution B was carefully layered above the suspension and then about 10 ml of sucrose solution A was used to fill the tube. After centrifugation at  $75,500 \times g$  for 16 hours, the plasma membrane fraction was collected from the interface between solution B and solution A overlay. The collected plasma membranes were washed once in solution A by centrifugation at  $75,500 \times g$  for 1 hour.

#### **1.2.4. Liver Acetone Powder.**

Materials:

- (1) Acetone precooled to  $-10^{\circ}\text{C}$  before using.
- (2) Liver tissue: fresh or frozen liver tissue fragments kept at  $-70^{\circ}\text{C}$ .
- (3) Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England).
- (4) Solid sodium hydroxide (NaOH).

Method:

Liver tissue fragments were minced with scissors and then homogenized three times, 30 seconds each time, in 10 volumes of cold acetone using a kitchen knife blender. The suspension was filtered through Whatman paper under reduced pressure and washed with another 10 volumes of cold acetone. A dark brown liver mass formed on the filter paper was blended again in acetone, filtered and transferred to a glass plate. After evaporating acetone at  $4^{\circ}\text{C}$  for 1 hour, liver powder was prepared by pushing the liver mass through a sieve with 60 mesh screens. The powder was allowed to dry in a desiccator over solid NaOH for 4 days at  $4^{\circ}\text{C}$ .

#### **1.2.5. Preparation of Affinity Chromatography Matrices.**

##### **1.2.5.1. Preparation of Asialofetuin-Sepharose 4B.**

Asialofetuin (ASFN) was coupled to Sepharose beads using CNBr-activated

Sephacryl S4B, according to the manufacturer's instructions.

**Materials:**

- (1) CNBr-activated Sepharose 4B supplied as freeze-dried powder by Pharmacia LKB (Biotechnology AB, Uppsala, Sweden).
- (2) Asialofetuin (ASFN; Sigma).
- (3) Hydrochloric acid (HCl) solution: 1 mM HCl.
- (4) Coupling buffer: 500 mM NaCl in 100 mM sodium carbonate buffer, pH 8.3.
- (5) Tris-HCl buffer: 100 mM Tris-HCl, pH 8.0.
- (6) Alternating pH buffer A: 500 mM NaCl in 100 mM sodium acetate buffer, pH 4.0.
- (7) Alternating pH buffer B: 500 mM NaCl in 100 mM Tris-HCl buffer, pH 8.0.
- (8) Column storage buffer: protease inhibitor solution as described in section 1.2.3 plus 0.02% NaN<sub>3</sub> in 100 mM Tris-HCl buffer, pH 7.6.
- (9) 0.02% sodium azide (NaN<sub>3</sub>) in water.

**Method:**

15 g of CNBr-Sepharose 4B was suspended in 100 ml of HCl solution and washed with 3 L of the same HCl solution on a sintered glass filter for 15 min. Then the matrix was resuspended in 80 ml of coupling buffer. 250 mg of ASFN was dissolved in 20 ml of coupling buffer and added to the matrix. The mixture was rotated at 4°C overnight. Excess ASFN was washed away with 400 ml of coupling buffer. The remaining active groups on the matrix were blocked with Tris-HCl buffer for 2 hours at room temperature. The conjugate was washed with 3 cycles of alternating pH buffers, each cycle consisted of a wash with 300 ml of alternating pH buffer A followed by another wash with 300 ml of alternating pH buffer B. The prepared matrix was packed into a column in storage buffer at 4°C. After being used, the ASFN-Sepharose can be regenerated by washing with 3 changes of alternating pH buffers.

### 1.2.5.2. Preparation of D-Gal-Sepharose 4B.

Preparation of D-Gal-Sepharose 4B was carried out according to Fornsted and Porath (1975) with some modifications.

#### Materials:

- (1) Sepharose 4B (Pharmacia).
- (2) Carbonate buffer A: 500 mM sodium carbonate buffer, pH 11.0.
- (3) Carbonate buffer B: 500 mM sodium carbonate buffer, pH 10.0.
- (4) Bicarbonate solution: 500 mM bicarbonate in distilled water adjusted to pH 8.5 by 1 N NaOH.
- (5) D-Gal solution: 20% (w/v) D-galactose (D-Gal; Sigma) in carbonate buffer B.
- (6) Divinyl sulphone (DVS) (Sigma).
- (7) 2-mercaptoethanol (Sigma).
- (8) 0.02% (w/v)  $\text{NaN}_3$  in distilled water.

#### Method:

100 ml of Sepharose 4B was washed with 500 ml of distilled water on a glass filter and then with 200 ml of carbonate buffer A, and immediately suspended in carbonate buffer A to a final volume of 100 ml. The Sepharose was then incubated with 10 ml DVS under stirring at room temperature for 70 min. The DVS-activated gel was carefully washed with distilled water and resuspended in 100 ml of D-Gal solution and incubated overnight at room temperature. Subsequently, the Gal-coupled Sepharose 4B was extensively washed with water and suspended in 100 ml of bicarbonate solution. The remaining DVS groups were blocked by adding 2 ml of 2-mercaptoethanol and stirring for 2 hours at room temperature. The resulting product was extensively washed with water and stored in water with 0.02%  $\text{NaN}_3$ .

### **1.2.6. Isolation of ASGPR from Liver Acetone Powder.**

ASGPR isolation was carried out according to Hudgin et al. (1974).

#### **1.2.6.1. Solubilization of Liver Acetone Powder.**

**Materials:**

- (1) Liver acetone powder: see section 1.2.4.
- (2) Solubilization buffer: 200 mM NaCl, 10 mM EDTA and protease inhibitors (see section 1.1.3.) in 100 mM sodium acetate-acetic acid buffer, pH 6.0.
- (3) Distilled water.
- (4) Extraction buffer: 400 mM KCl, 1% (v/v) Triton X-100 and 0.02% (w/v)  $\text{NaN}_3$  in 10 mM Tris-HCl buffer, pH 7.8.
- (5) Solid calcium chloride ( $\text{CaCl}_2$ , Sigma).
- (6) Solid Tris base (Sigma).

Note: all the solutions were cooled at 4°C before using.

**Method:**

15 g of liver acetone powder was suspended in 15 to 20 volumes of solubilization buffer and homogenized with five up-and-down strokes of a pestle homogenizer. After incubation on ice for 30 min, the suspension was centrifuged for 10 min at  $20,000 \times g$ . The pellet was washed once with the solubilization buffer and then with distilled water by re-homogenization of the pellet and re-centrifugation. The final pellet was suspended and solubilized in 300 ml of extraction buffer at 4°C for 1 hour. Unsolubilized fragments were removed by centrifugation at  $100,000 \times g$  for 2 hours. Finally, solid  $\text{CaCl}_2$  was added into the crude receptor extract to a concentration of 50 mM and the pH was adjusted to 7.8 by adding small amounts of solid Tris base.

#### **1.2.6.2. Isolation of ASGPR by Ligand Affinity Chromatography.**

**Materials:**

- (1) ASFN- or D-Gal-Sepharose 4B matrix (see sections 1.2.5.1 and 1.2.5.2)
- (2) Loading buffer A: 50 mM  $\text{CaCl}_2$ , 1.25 M NaCl, 0.5% (v/v) Triton X-100 and protease inhibitors (section 1.2.3) in 10 mM Tris-HCl buffer, pH 7.8.
- (3) Loading buffer B: loading buffer A without Triton X-100.
- (4) Elution buffer A: 1.25 M NaCl and 0.05% Triton X-100 in 20 mM ammonium acetate - acetate acid ( $\text{NH}_4\text{Ac-HAc}$ ) buffer, pH 5.0-5.2.
- (5) Elution buffer B: 500 mM NaCl in 20 mM  $\text{NH}_4\text{Ac-HAc}$  buffer, pH 5.2.
- (6) NaOH solution: 10 N NaOH in distilled water.
- (7) Solid Tris base.
- (8) Saturated ammonium sulfate: 90 g of ammonium sulfate,  $(\text{NH}_4)_2\text{SO}_4$  was added to 100 ml of distilled water, heat-solubilized, cooled to room temperature and adjusted to pH 6.5 with solid Tris base.
- (9) PBS: section 1.2.2.1.
- (10) Tris-HCl buffer: 25 mM Tris-HCl buffer, pH 7.8.

**Method:**

The procedure for ASGPR isolation and purification includes following three major steps:

- (1) Isolation of ASGPR using a large ligand affinity column. About 50 ml of ASFN-Sepharose 4B or D-Gal-Sepharose 4B gel was washed several times with loading buffer A on a glass filter. The gel was immediately transferred to the prepared crude receptor extract (200-300 ml) and gently stirred on a shaker at 4°C overnight. After careful washing with loading buffer on a glass filter, the receptor bound gel in 100 ml of the same buffer was carefully packed into a chromatography column (50 cm  $\times$  5 cm  $\phi$ ) and further washed with 3 bed volumes of loading buffer till there was no protein washed out. Bound receptor was eluted with elution buffer A and fractions of 5-ml were collected. After protein determination by using Bicinchoninic Acid Assay (BCA, see section 1.2.16), the protein-containing fractions

were pooled and the  $\text{Ca}^{2+}$  concentration was re-adjusted to 50 mM and pH to 7.8.

(2) Purification of ASGPR using small affinity chromatography column. Receptor-containing solutions obtained from the large column were applied to a small ligand affinity column (10 cm  $\times$  1.5 cm  $\phi$ ) with about 5 ml of gel which was pre-equilibrated with loading buffer B. Upon adsorption at 4°C for 3 hours, the Triton X-100 was removed by washing the column with 50 ml of loading buffer B and the bound receptor was eluted with elution buffer B. Fractions of 1- or 0.85-ml were collected for protein determination by absorbency at 280 nm or, in some instances, by BCA (section 1.2.16). Fractions containing proteins were pooled and pH was adjusted to 6.5 by NaOH solution.

(3) Concentration of purified ASGPR. Purified ASGPR obtained from the small ligand affinity column was precipitated by adding an equal volume of cold saturated  $(\text{NH}_4)_2\text{SO}_4$  followed by incubation on ice for 2 hours. Receptor protein was recovered by centrifugation at  $50,000 \times g$  for 30 min, dissolved in 1 ml of PBS or 25 mM Tris-HCl buffer, dialyzed against PBS or Tris-HCl buffer, and lyophilized using a Freeze Dry-5 apparatus (Labconco, K.C., MO.).

In some instances, purified receptor protein was dialyzed against 10-fold diluted PBS or Tris-HCl buffer and directly concentrated by lyophilization without prior ammonium sulfate precipitation.

### **1.2.7. Isolation of ASGPR Subunit Polypeptides by Electro-Elution.**

#### **Materials:**

- (1) Materials for SDS-PAGE are described in section 1.2.16.
- (2) Electro-elution buffer: 15 mM glycine and 20 mM Tris base, pH 8.3.
- (3) Dialyser tubing for protein dialysis (Fisher Scientific Co., Pittsburgh, PA.).
- (4) PBS: section 1.2.2.1.

#### **Method:**

Affinity purified rASGPR proteins (section 1.2.6) were subjected to SDS-PAGE (section 1.2.17) in parallel with prestained molecular mass markers. The gel slices containing bands of receptor subunits with appropriate molecular sizes were cut out from the gel and placed into prewetted dialyzer tubing with 2 ml of electro-elution buffer. Then, the membrane tubing was closed tightly and placed into the upper reservoir of a Mini-PROTEAN II apparatus (Bio-Rad). After adding electro-elution buffer to the upper and lower reservoirs, electro-elution was carried out at 180 mA for about 45 min. Eluted subunit polypeptides were collected from the elution buffer in the dialyzer tubing, dialyzed against PBS and concentrated by lyophilization.

#### **1.2.8. Immunization of Animals with Purified Receptors and Receptor Subunit Polypeptides.**

Materials:

- (1) Animals: healthy guinea pigs and woodchucks.
- (2) PBS: section 1.2.2.1; sterilized.
- (3) Adjuvant: complete and incomplete Freund's adjuvant (CFA and IFA, respectively) (Gibco).
- (4) Antigens: affinity-purified rASGPR, wASGPR, and electro-eluted 40-kDa and 47-kDa polypeptides of rASGPR (see sections 1.2.6 and 1.2.7). Antigens were dialyzed against PBS prior to immunization.

Method:

Before immunization, antigen in PBS in a glass syringe was mixed with an equal volume of adjuvant (CFA or IFA) in another syringe. An emulsion was produced by pushing the plungers of the two glass syringes which were connected through a three-way valve.

For the immunization of guinea pigs with the affinity-purified wASGPR, rASGPR, 40-kDa or 47-kDa polypeptides of rASGPR, three doses of antigen were administered

intramuscularly or subcutaneously at multiple sites for the first injection, and intramuscularly for booster injections over a 6- to 8-week period. Each dose contained 50 µg of wASGPR, rASGPR or 40-kDa subunit protein, or 25 µg of 47-kDa subunit protein in 300 µl volume. Antigen emulsified in CFA was used for the first injection and in IFA for the second and third injections. Animals were bled out two weeks after the final injection.

For the immunization of woodchucks with affinity-purified rASGPR, 50 µg of antigen per dose was used. Antigen was administered in two doses within a 2-week period. For the first injection, rASGPR was emulsified in CFA and for the second in IFA. Serial serum samples were collected before, during and after completion of the injections at weekly or biweekly intervals. This immunization was handled by other researchers in this laboratory and the resulting antisera were kindly provided and contributed to part of the current work for this thesis.

Sera from healthy woodchucks and guinea pigs were used as controls. All antisera were separated and stored in small volumes at -70°C.

### **1.2.9. Solid Phase Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-ASGPR Detection.**

ELISA was carried out according to McFarlane et al. (1983) and Treichel et al. (1990) with some modifications.

#### **Materials:**

- (1) Affinity-purified rASGPR and wASGPR (see section 1.2.6.).
- (2) PBS: section 1.2.2.1.
- (3) Blocking buffer: 3% (w/v) bovine serum albumin (BSA) (Sigma) and 5 mM EDTA in PBS.
- (4) Loading buffer: 25 mM NaCl, 5 mM EDTA and 0.05% (v/v) Tween-20 in 10 mM Tris-HCl buffer, pH 7.6.

- (5) Reaction buffer: 250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.0.
- (6) Rabbit anti-woodchuck IgG and IgM: prepared by other researchers in this laboratory (Michalak et al., 1990).
- (7) Enzyme-labelled antibody A: alkaline phosphatase-conjugated goat anti-guinea pig IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA.).
- (8) Enzyme-labelled antibody B: horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad laboratories, Hercules, CA.).
- (9) Alkaline phosphatase substrate: p-Nitrophenyl phosphate (pNPP tablets, Sigma FAST, Sigma).
- (10) Horseradish peroxidase substrate: TMB peroxidase EIA substrate kit (Bio-Rad).
- (11) Washing buffer (PBST): 0.2% (v/v) Tween-20 in PBS.
- (12) Woodchuck sera and guinea pig sera for testing: see section 1.2.8.
- (13) 1 M H<sub>2</sub>SO<sub>4</sub>.
- (14) 1 N NaOH.

**Method:**

In the preliminary experiments, purified rASGPR proteins were diluted in PBS, 50 ng, 100 ng, 200 ng and 1  $\mu$ g/50  $\mu$ l, and coated on the microplates (96 flat-bottom wells plates, ICN Biomedical Inc., Horsham, PA) at 50  $\mu$ l per well. After incubation at 4°C overnight, the plates were washed with PBST and then blocking buffer was added, 300  $\mu$ l/well, for overnight incubation at 4°C. The plates were washed again. For the final experiments, plates coated with 100 ng receptor protein/well were chosen.

Subsequently, woodchuck sera or guinea pig sera collected from animals immunized with ASGPR and the receptor subunit polypeptide preparations (see section 1.2.8) were diluted to 1:50 or serially 2-fold diluted from 1:50 to 1:1,638,400 in loading buffer. Diluted serum (50  $\mu$ l/well) was added to the ASGPR coated plates and the reaction was carried out at 4°C overnight. The plates were carefully washed with PBST three times, 5 min per wash.

For the detection of woodchuck antibodies, the plates were further incubated with 1:50 rabbit anti-woodchuck IgG and IgM in loading buffer (50  $\mu$ l/well) at room temperature for 2 hours. After washing, 50  $\mu$ l of enzyme-labelled antibody B at 1:250 in loading buffer was added to the plates for 1 hour reaction at room temperature. The plates were extensively washed in PBST and the bound enzyme activity was determined by adding horseradish peroxidase substrate according to the manufacturer's instructions. After stopping the reaction by adding 1 M H<sub>2</sub>SO<sub>4</sub>, the product of the reaction was determined by reading the plates at 450 nm, using a microplate reader (model 3553, BIO-RAD).

The guinea pig antibodies were detected by enzyme-labelled antibody A at a dilution of 1:10,000 in reaction buffer following the manufacturer's specifications. After 2 hours incubation at room temperature, the plates were washed extensively in reaction buffer and then developed by alkaline phosphatase substrate, according to the manufacturer's instructions. The reaction was ended by adding 1 N NaOH and the results were read from the plates at 405 nm.

Each serum sample was tested in duplicate. Mixtures of two or three sera from nonimmunized woodchucks or guinea pigs were used as negative controls.

#### **1.2.10. Absorption of Anti-rASGPR Antiserum Using Purified rASGPR.**

Materials:

- (1) Blocking buffer: 3% (w/v) BSA in loading buffer (see section 1.2.9.).
- (2) Nitrocellulose membrane: 0.45  $\mu$ m trans-blot transfer medium (Bio-Rad).
- (3) Guinea pig anti-rASGPR antiserum: section 1.2.8. The serum was diluted to 1:100 in loading buffer (see section 1.2.9) before absorption.
- (4) PBS: section 1.2.2.1.
- (5) BSA solution: 1 mg/ml BSA in PBS.

Method:

About 45 µg of affinity-purified rASGPR protein was spotted onto 1 cm<sup>2</sup> of nitrocellulose membrane. The spots were air-dried and then incubated in blocking buffer at room temperature for 1 hour. One ml of diluted guinea pig anti-rASGPR antiserum was incubated with the nitrocellulose blot at 4°C overnight and then at ambient temperature for 1 hour. The aqueous solution containing ASGPR-absorbed serum was collected for further testing. Antiserum which was incubated with nitrocellulose membrane spotted with BSA instead of ASGPR was used as a control.

### **1.2.11. Immunofluorescent Staining.**

#### **Materials:**

- (1) Cells: isolated woodchuck hepatocytes and cultured HepG2 cells (section 1.2.2).
- (2) PBS: section 1.2.2.1.
- (3) HBSS (ICN Biomedical Inc.).
- (4) Antisera: guinea pig anti-ASGPR and anti-subunits of ASGPR antisera (section 1.2.8).
- (5) Fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG (H+L) (Jackson ImmunoResearch Labs, Inc., West Grove, PA.).

#### **Method:**

To assess the expression of ASGPR on the surface of isolated or cultured cells, approximately  $1 \times 10^6$  of mechanically isolated woodchuck or rat hepatocytes as controls in 5-ml glass tubes, and HepG2 cells growing on tissue culture slides were washed with HBSS and then incubated with anti-ASGPR antisera at 1:10 or 1:20 dilution in cold PBS for 30 min on ice. After washing with three changes of cold PBS, the bound guinea pig antibodies were identified by incubation with FITC-conjugated antibodies diluted to 1:25 in PBS for 30 min. The cells were washed again in cold PBS and examined for immunofluorescence using a Leitz Diaplan microscope (Leitz Co., Germany). Sera from non-immunized guinea pigs were used as first layer antibody control. Cells incubated with PBS and then with FITC-conjugated

antibodies were also used as additional controls.

### 1.2.12. Radioiodination of Proteins.

Iodination of purified ASGPR, ASFN, HPM and hepatocytes was carried out according to the IODO-GEN method described by Markwell and Fox (1978).

Materials:

- (1) IODO-GEN solution: 0.1% (w/v) of 1,3,4,6 tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril (Pierce Inc., Rockford, IL.) in chloroform (Fisher Scientific Co., Fair Lawn, NJ).
- (2) PBS: section 1.2.2.1.
- (3) Phosphate buffer (PB): 20 mM PB, pH 8.0.
- (4) Tris-HCl buffer: 25 mM Tris-HCl buffer, pH 7.6.
- (5) Nitrogen gas.
- (6) Carrier-free Na<sup>125</sup>I: activity 100  $\mu$ Ci/ $\mu$ l (Amersham Canada Ltd, Lachine, Que., Canada)
- (7) BSA solution: 5% BSA in PB or Tris-HCl buffer.
- (8) Sephadex G-25 (Pharmacia).
- (9) Materials for radiolabelling: affinity-purified ASGPRs, asialofetuin and asialoorsumuroid\*, and purified woodchuck HPMS as described in sections 1.2.2 and 1.2.6.
- (10) 2% SDS in PB.
- (11) Cell culture medium: section 1.2.2.
- (12) TCA solution: 25% (w/v) trichloroacetic acid in PB or Tris-HCl buffer.

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\* Asialoorsumuroid (ASOR) was prepared by digestion of 7 mg of orsumuroid (ORM, Sigma) with 0.6 g insoluble neuraminidase (neuraminidase from *Clostridium perfringens* type VI attached to agarose beads at 40 units/g agarose, Sigma) in 10 ml of 0.15 M NaAc-HAc buffer (pH 5.0) at 37°C for overnight and then by removal of the beads by centrifugation at 500  $\times$  g for 10 min. Prepared ASOR was dialyzed against PBS and concentrated before use.

**Method:**

(1) Preparation of samples for radiolabelling. Purified ASGPRs, HPMS, ASFN and ASOR were diluted in PBS at a concentration of 1 mg/ml. In some instances, ASGPRs and HPMS were solubilized by boiling in 2% SDS for 3 min prior to labelling.

(2) Preparation of IODO-GEN coated tubes. 10 or 100  $\mu$ l of freshly prepared IODO-GEN - chloroform solution was used to coat the inner-surface of a 5-ml glass tube. Coating was carried out at room temperature under a stream of nitrogen gas for evaporating the chloroform. The coated tubes were stored in a desiccator at room temperature and used within 1 year.

(3) Preparation of Sephadex G-25 columns. Sephadex G-25 beads were swollen in PB and transferred to a 10 ml disposable syringe with a glass fibre cushion. The prepared column was equilibrated with 1 ml of BSA solution and then washed with 10 ml of PB or Tris-HCl buffer.

(4) Iodination using IODO-GEN method. 10 to 150  $\mu$ g proteins were reacted with 200  $\mu$ Ci or 500  $\mu$ Ci of  $\text{Na}^{125}\text{I}$  at ambient temperature for 10 to 15 min, in IODO-GEN coated tubes. The reaction was stopped by adding 1 ml of PB or Tris-HCl buffer. Labelled proteins were separated from free iodine by gel filtration on Sephadex G-25 columns, eluting with PB or Tris-HCl buffer. Fractions of 1 ml were collected and their radioactivities were measured by a gamma counter (1277 Gammamaster, Pharmacia). High radioactivity-containing fractions were pooled together, divided into small samples and stored at 4°C.

Specific radioactivity for preparations ranged from  $2.5 \times 10^3$  to  $2.5 \times 10^4$  cpm/ng protein. The concentrations of labelled proteins ranged from 2.5 ng/ $\mu$ l to 44 ng/ $\mu$ l.

(5) Determination of radioactivity incorporated into proteins. The method used was described by McFarlane (1984). Briefly, 5  $\mu$ l of radiolabelled proteins, 495  $\mu$ l of BSA solution and 500  $\mu$ l of TCA solution were mixed in a tube. After incubation on ice for 1 hour, the mixture was centrifuged at  $1,000 \times g$  for 5 min. Then, 500  $\mu$ l of the upper supernatant was transferred into another tube and both tubes, containing the upper and lower parts of the

solution respectively, were counted in a gamma counter. The percentage, calculated according to the following equation, represented the radioactivity incorporated into labelled proteins:  $\text{Radioactivity bound to protein} = \frac{(\text{lower} - \text{upper})}{(\text{lower} + \text{upper})} \times 100\%$

Radioabelled proteins were used for other experiments only when the percentages (as calculated above) were above 90%.

### 1.2.13. Solid Phase Receptor-Ligand Binding Assay.

The ligand binding activity of purified wASGPR and rASGPR preparations was determined according to Quesenberry and Drickamer (1992) and Schwartz et al. (1981) with some modifications.

#### Materials:

- (1)  $^{125}\text{I}$ -labelled ASFN or ASOR (section 1.2.12): approximately 20 to 44 ng/ $\mu\text{l}$  in Tris-HCl buffer with a specific radioactivity of  $1.36\text{-}1.42 \times 10^3$  cpm/ng.
- (2) Receptor proteins: affinity-purified wASGPR and rASGPR (section 1.2.6).
- (3) ASFN, ASOR or ORM solution: 10-20 mg/ml asialofetuin (ASFN, Sigma), asialoorosumucoid (ASOR) (section 1.2.12) or orosumucoid (ORM, Sigma) in dilution buffer (see below).
- (4) PBS: section 1.2.2.1.
- (5) Dilution buffer: 25 mM Tris-HCl buffer, pH 7.6.
- (6) Blocking buffer: 0.2% (v/v) Tween-20 and 5 mM EDTA in dilution buffer.
- (7) Washing buffer: 1.25 M NaCl and 25 mM  $\text{CaCl}_2$  in dilution buffer.
- (8) Reaction buffer ( $10 \times$ ): 1.5 M NaCl, 250 mM  $\text{CaCl}_2$  and 0.2% (v/v) Triton X-100 in dilution buffer.
- (9) Falcon flexible plates: 96-flat-bottom-well micro-test assay plates (Becton Dickinson Co., Oxnard, CA).

#### Method:

(1) In the preliminary experiments, to establish assay conditions, falcon flexible plates were coated overnight at 4°C with affinity-purified ASGPR at concentrations ranging from 25 to 125 ng per 50 µl of PBS per well. After rinsing wells with washing buffer, the plates were incubated with blocking buffer at 4°C overnight and washed again. Then, increasing amounts of <sup>125</sup>I-ASFN ranging from 40 to 100 ng was pipetted into the wells. The reaction volume was adjusted to 50 µl per well by adding 1/10 volume of reaction buffer (10<sup>-6</sup>) and dilution buffer to reach a final concentration of 150 mM NaCl, 25 mM CaCl<sub>2</sub> and 0.02% Triton X-100 in 25 mM Tris-HCl buffer. Following incubation at 4°C overnight, the unbound <sup>125</sup>I-ASFN was removed in several changes of cold washing buffer. Subsequently, each well was cut out and the bound radioactivity counted using a gamma counter.

(2) To determine the specificity of ASGPR-ligand reaction, a binding assay was carried out in the presence of unlabelled ASGPs or glycoproteins. Briefly, affinity-purified wASGPR in PBS was used for coating the plates (100 ng/50 µl/well) as described above. <sup>125</sup>I-ASOR ranging from 17 to 102 ng per 50 µl was applied for the binding under similar conditions as above (1). Parallel experiments were carried out with unlabelled ASOR or ORM at 850 ng or 8500 ng/well; this was 5- to 450-fold excess to labelled ASOR.

(3) To measure the binding kinetics between ligand and purified ASGPR, plates coated with 100 ng/well of ASGPR protein were used. Increasing amounts of <sup>125</sup>I-ASFN from 25 to 450 ng/well were added. Non-specific binding was determined by parallel incubations in the presence of 100-fold excess of unlabelled ASFN. Specific binding of <sup>125</sup>I-ASFN to ASGPR was calculated by subtraction of non-specific binding from the corresponding total binding.

(4) All the above determinations were performed at least in triplicates. A nonlinear regression program (GraphPad InPlot, GraphPad Software, Inc., CA.) was applied to fit the data obtained from the kinetic study. The data for saturation determination were fitted to an equation:  $\text{Ligand bound} = B_{\text{max}} \times [\text{ligand}] / (K_d + [\text{ligand}])$ , where  $K_d$  was the concentration of ligand at which half-maximal binding was obtained and  $B_{\text{max}}$  was the maximal binding under

the conditions described in this assay.

#### **1.2.14. Radioimmunoprecipitation assay.**

**Materials:**

- (1) <sup>125</sup>I-labelled HPM: section 1.2.12.
- (2) Loading buffer: 50 mM Tris-HCl buffer, pH 7.6, containing 0.3 M NaCl, 0.4 M KCl, 5 mM EDTA, 1 mM PMSF and 1% (v/v) Triton X-100.
- (3) Sera: guinea pig antisera and normal guinea pig serum as described in section 1.2.8.
- (4) Protein A beads: 10% (w/v) staphylococcal protein A immobilized on Sepharose CL-4B (Sigma) swollen in loading buffer.
- (5) Washing buffer: 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.25% (w/v) gelatin, 0.1% (v/v) Triton X-100 and 0.025% (w/v) NaN<sub>3</sub>.
- (6) Sample buffer: 3% SDS, 10% glycerol and 5% 2-mercaptoethanol in 62.5 mM Tris-HCl buffer, pH 6.8.

**Method:**

10<sup>5</sup> cpm of radiolabelled HPMs in 1 ml of loading buffer was precleared by incubation with 10 µl of normal guinea pig serum and 50 µl of protein A beads for 3 hours at ambient temperature. Then, the samples were incubated with 10 µl of anti-ASGPR antiserum per sample at 4°C overnight, followed by precipitation with 50 µl of protein A beads at room temperature for 1 hour. The resulting beads were washed in several changes of loading buffer and treated with sample buffer to release the bound proteins which were further analyzed by SDS-PAGE and autoradiography (see sections 1.2.17 and 1.2.19).

#### **1.2.15. Ligand Precipitation Assay.**

To examine the ligand binding activity of ASGPR in purified HPM, an affinity precipitation assay was carried out according to a method described by Mu et al. (1993) with

some modifications.

**Materials:**

- (1) Dilution buffer: section 1.2.13.
- (2) Reaction buffer (10 ×): section 1.2.13. Reaction buffer (1 ×) was diluted from concentrated buffer with dilution buffer.
- (3) Plasma membrane preparations: woodchuck, rabbit and rat HIPMs, and woodchuck KPMs and SPMs as controls. See section 1.2.3 for details.
- (4) D-Gal beads: 10% (w/v) D-Gal-Sepharose 4B (see section 1.2.5.2) in reaction buffer.
- (5) Ligand-free beads: 10% (w/v) Sepharose 4B in reaction buffer.
- (6) Chaps solution (5 ×): 50 mM 3-[(3-cholanidopropyl) dimethylammonio]-1-propanesulfonate (Chaps, Sigma) in dilution buffer.
- (7) DOC solution (5 ×): 0.5% (w/v) sodium deoxycholate (Sigma) and 150 mM NaCl in 50 mM Tris-HCl buffer, pH 8.4.
- (8) Sample buffer: section 1.2.17.

**Method:**

Plasma membrane preparations (340 µg protein per sample) were treated with 1/10 volume of reaction buffer (10 ×), 1/5 volume of Chaps solution (5 ×) or 1/5 volume of DOC solution (5 ×) in dilution buffer. After incubation at room temperature for 30 min, 100 µl of D-Gal beads were added to the membrane solution and the total volume was adjusted to 1 ml with reaction buffer. Reaction was carried out at 4°C overnight with gentle mixing on a rotating tray, followed by centrifugation at 320 × g for 10 sec and 5 washes with reaction buffer (1 ×, in dilution buffer). Bound proteins were released by boiling for 3 min in 50 µl of sample buffer and used for SDS-PAGE separation and Western blot analysis.

For control experiments, solubilized woodchuck HIPMs were preincubated with an excess of free D-Gal prior to the incubation with D-Gal beads, as well as membrane preparations were incubated with ligand free beads instead of D-Gal beads.

### **1.2.16. Bicinchoninic Acid Assay for Protein Content Determination.**

#### **Materials:**

- (1) Bicinchoninic acid assay (BCA) kit (Sigma).
- (2) Protein standard (BSA, 1 mg/ml) (Sigma).

#### **Method:**

The procedure recommended by the manufacturer was used. Fresh protein determination reagent was prepared for each assay. Protein standards (BSA) ranging from 10 µg to 100 µg were made in PBS or in the solution used for sample dilution (e.g., Tris-HCl buffer) and tested to establish a standard curve. Tested samples were diluted in PBS, or in original solution in which sample was kept, to 100 µl and incubated with 2 ml of protein determination reagent at 37°C for 30 min. After cooling to room temperature, the absorbency was detected at 562 nm. Protein content was determined according to the standard curve established from the protein standards.

### **1.2.17. SDS-PAGE.**

The method used was according to Harlow and Lane (1988).

#### **Materials:**

- (1) Sample buffer: 3% SDS, 10% glycerol and 5% β-mercaptoethanol in 62.5 mM Tris-HCl buffer, pH 6.8.
- (2) Samples for testing: all the preparations for the determination of subunit composition and polypeptide molecular sizes or for autoradiography and Western blotting analyses.
- (3) Protein molecular mass standards: low range standards containing rabbit muscle phosphorylase B (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.5 kDa) (Bio-Rad); low range prestained standards containing phosphorylase B (106.0 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean

trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa) (Bio-Rad); high range prestained standards containing myosin (205.0 kDa),  $\beta$ -galactosidase (116.0 kDa), bovine serum albumin (80.0 kDa) and ovalbumin (49.5 kDa) (Bio-Rad);  $^{14}\text{C}$ -methylated protein standards containing myosin (200 kDa), phosphorylase-b (97 kDa), BSA (69.0 kDa), ovalbumin (46.0 kDa), carbonic anhydrase (30.0 kDa) and lysozyme (14.0 kDa) (Amersham).

(4) 30% acrylamide mix: 29.2% (w/v) acrylamide (Bio-Rad) and 0.8% (w/v) N,N'-methylene-bis-acrylamide in distilled water.

(5) Lower Tris-HCl buffer: 0.4% (w/v) SDS and 1.5 M Tris-HCl buffer, pH 8.8.

(6) Upper Tris-HCl buffer: 0.4% (w/v) SDS and 0.5 M Tris-HCl buffer, pH 6.8.

(7) Ammonium persulfate (AP) solution: 10% ammonium persulfate (Bio-Rad) in distilled water. This solution was freshly prepared before use.

(8) TEMED: N,N,N',N'-tetramethylethylenediamine (Bio-Rad).

(9) Running buffer: 25 mM Tris-base and 200 mM glycine with 2% SDS in distilled water, pH 8.7.

#### Methods:

(1) 12.5% separating gel solution was prepared by combining 4 ml of 30% acrylamide mix, 3.35 ml of water, 2.5 ml of lower Tris-HCl buffer, 150  $\mu\text{l}$  of AP solution and 2.5  $\mu\text{l}$  of TEMED. Upper stacking gel solution contained 500  $\mu\text{l}$  of acrylamide mix, 1.25 ml of upper Tris-HCl buffer, 3.17 ml of water, 75  $\mu\text{l}$  of AP solution and 5  $\mu\text{l}$  of TEMED. The gel was polymerized in 0.75 or 1 mm  $\times$  7 cm  $\times$  10 cm glass plates (mini-gel) and assembled for a Mini-Protean II apparatus (Bio-Rad) or in 1.5 mm  $\times$  15 cm  $\times$  20 cm (large gel) for a Protean II apparatus (Bio-Rad) following the manufacturer's instructions.

(2) All samples for SDS-PAGE analysis were treated with sample buffer by boiling for 3-5 min and loaded into the gel wells using approximately 0.25 to 10  $\mu\text{g}$  proteins per lane for mini-gels and 100  $\mu\text{g}$  proteins per lane for large gels. Electrophoresis was carried out at 200 V for about 45 min or 4 hours in running buffer. Protein standards were applied in parallel

for each run.

#### **1.2.18. Coomassie Blue Staining.**

**Materials:**

- (1) Staining solution: 0.05% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid in distilled water.
- (2) Destaining solution: 5% methanol and 7.5% acetic acid in distilled water.
- (3) Proteins separated on SDS-PAGE gels: section 1.2.17.
- (4) Filter paper (Whatman International Ltd.).

**Method:**

Proteins separated on SDS-PAGE slab gels were placed in staining solution for 30 min at room temperature with gentle shaking and then destained in destaining solution at room temperature till the dye color of the background was removed. Stained gels were stored in destaining solution for photography or dried on filter paper using a slab-gel dryer (Hoefer Scientific Instruments, San Francisco, CA.) for long-term storage.

#### **1.2.19. Autoradiography.**

**Materials:**

- (1) X-ray film: Kodak scientific imaging film (XAR-5) or Kodak diagnostic film (XRP-1) (Eastman Kodak Company, Rochester, NY.).
- (2) <sup>125</sup>I-labelled proteins separated on SDS-PAGE gels: section 1.2.17.

**Method:**

SDS-PAGE slab gels were dried and exposed to X-ray film for the appropriate length of time (from 16 hours to several weeks, depending on the sample radioactivity) at 4°C or at -70°C in a light-proof cassette. The film was developed in a Kodak automatic developing machine.

#### **1.2.20. Densitometer Quantitation for the Gels and Autoradiograms.**

Wet SDS-PAGE slab-gels and autoradiograms were analyzed by densitometric quantitation using an Ultrascan XL laser densitometer with a LKB 2220 recording integrator (Pharmacia-LKB Biotechnology) according to the manufacturer's instruction.

#### **1.2.21. Western Blotting.**

##### **Materials:**

- (1) Transfer buffer: 39 mM glycine, 48 mM Tris-base, 0.037% (w/v) SDS and 20% (v/v) methanol in distilled water.
- (2) Nitrocellulose membrane: 0.45  $\mu$ m trans-blot transfer medium (Bio-Rad).
- (3) Other buffers and reagents included loading buffer, reaction buffer, blocking buffer, PBST, rabbit anti-woodchuck IgG and IgM, enzyme-labelled antibodies. For detail see section 1.2.9.
- (4) Alkaline phosphatase substrate: bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP/NBT) tablets (25 mg substrate/tablet) (Sigma). According to the manufacturer's instructions, 1 tablet of BCIP was dissolved in 0.5 ml of dimethylformamide (Sigma) and 1 tablet of NBT was dissolved in 1 ml of distilled water. BCIP/NBT solution was freshly prepared by adding 330  $\mu$ l of NBT solution and 33  $\mu$ l of BCIP solution into 10 ml of 100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl and 5 mM magnesium chloride ( $MgCl_2$ ).
- (5) Horseradish peroxidase substrate: 3% (w/v) of 4-chloro-1-naphthol (Sigma) in absolute ethanol. Before developing the reaction, 100  $\mu$ l of chloronaphthol and 10  $\mu$ l of 30% hydrogen peroxide ( $H_2O_2$ ) were mixed with 10 ml of 50 mM Tris-HCl buffer, pH 7.6
- (6) Samples for SDS-PAGE: section 1.2.17.

##### **Method:**

Proteins separated on SDS-PAGE slab-gels were transferred onto nitrocellulose membrane as described by Michalak and Lin (1994), by using a semi-dry transfer unit

BioTransMaxi (Gelman Sciences, Ann Arbor, MI).

(1) After SDS-PAGE, the slab gel was soaked in distilled water for 5 min and then assembled in a sandwich in the following order: 3 layers of filter paper presoaked in transfer buffer, 1 nitrocellulose membrane presoaked with distilled water, the slab gel and another 3 layers of transfer buffer-wetted filter paper. The sandwich was placed in the transfer unit, with the anode on the membrane side and the cathode on the gel side. Electrotransfer was carried out at  $0.8 \text{ mA/cm}^2$  of gel for 30 to 45 min. The efficiency of transfer was verified according to the prestained protein standards or by the protein trace on the post-transferred gel visualized with Coomassie blue.

(2) After transfer, the membrane blots were disassembled from the apparatus, air-dried for about 10 min at room temperature, and then incubated with blocking buffer for 1 hour at room temperature. Being rinsed in PBST twice for 5 min each, the blots were incubated with primary antibodies (for guinea pig anti-ASGPR antisera, dilution was 1:400 or greater; for woodchuck antisera, dilution was 1:10 or greater) in loading buffer at  $4^\circ\text{C}$  overnight and followed by incubation at room temperature for 1 hour. After washing the blots in PBST, goat anti-guinea pig IgG (H+L) conjugated with alkaline phosphatase was used to detect the bound guinea pig antibodies, whereas a mixture of rabbit antisera recognizing woodchuck IgG and IgM and horseradish peroxidase-conjugated goat anti-rabbit IgG were employed to identify binding of woodchuck antibodies, using the same conditions as described in section 1.2.9. After extensively washing the blots with PBST, the enzyme activity was developed by adding the corresponding substrate solution to obtain an insoluble product. Reaction with alkaline phosphatase substrate identified the activity of guinea pig antibodies, whereas the reaction with horseradish peroxidase substrate identified the activity of woodchuck antibodies. Molecular masses of visualized proteins were determined using curves established from the relative mobilities of prestained molecular standards run on each gel.

### 1.3. RESULTS

#### 1.3.1. Isolation and Characterization of Woodchuck Hepatic ASGPR (wASGPR).

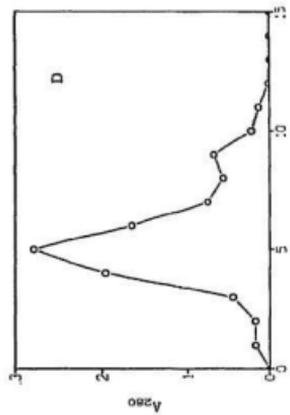
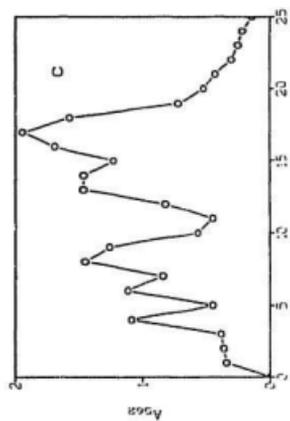
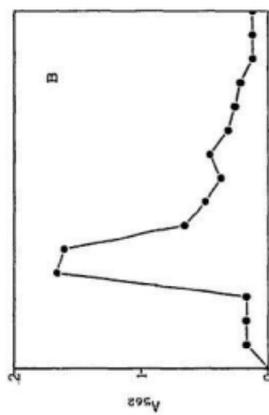
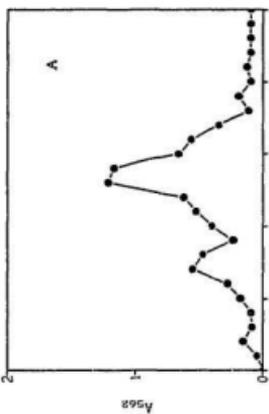
##### 1.3.1.1. Purification of wASGPR.

According to an original procedure established by Hudgin et al. (1974), ASGPR was extracted from acetone liver powder with 1% Triton X-100. The crude receptor preparation was further purified by ligand-affinity chromatography, by using two successive (one large and one small) ASFN affinity columns or, in some instances, D-Gal columns. Typical profiles of the elution of binding receptor proteins from the ASFN columns are detailed in Fig. 1.1. As shown (Fig. 1.1 A and B), purified woodchuck receptor proteins were recovered in the secondary bed volume of elution buffer after the chromatography in the small column. These results are in good agreement with data reported by Hudgin et al. (1974). Fig. 1.1 (C and D) also shows corresponding chromatography profiles of rabbit ASGPR proteins, revealing patterns similar to those of wASGPR.

Five preparations of wASGPR, four of rASGPR and one of rtASGPR were purified in the course of the present work. The average yields of these receptor preparations are summarized in Table 1.2, which show that the yield of wASGPR is lower than that of rASGPR, but similar to that of rtASGPR. Considering the possible difference in the interactions between ligand and ASGPRs derived from different species, a few modifications to the receptor purification procedure were made in an attempt to improve the yield of wASGPR. Grant and Kaderbhai (1986) reported that factors, such as the concentration of Triton X-100 and NaCl, markedly influence the ligand binding to the purified rtASGPR or the plasma membrane-associated ASGPR. Based on this report, the original procedure was modified by adjusting the pH of the elution buffer from 6.0 to 5.0 and the concentrations of NaCl and Triton X-100 from 1.25 M to 150 mM and from 1% to 0.1%, respectively. However, despite these modifications, there was no improvement in the yield of wASGPR.

**Figure 1.1. Purification of ASGPR by ligand-affinity chromatography.** Chromatograms of wASGPR (A and B) and rASGPR (C and D) were obtained by using two successive ASFN-Sepharose 4B columns (A and C: large columns, approximately 50 ml beads per column; B and D: small columns, approximately 10 ml beads per column) as described in the text (section 1.2.6.). The initial chromatographies were performed on the large columns by subjecting 200-300 ml of Triton X-100 extracts of liver acetone powders from normal woodchuck (A) and rabbit (C). The columns were eluted with elution buffer A (see section 1.2.6.2.) at a flow rate of 25 ml/h. Fractions (5 ml/each) were collected and monitored for protein content using the BCA method (see section 1.2.16.). The protein-containing fractions (in A, fractions 5 to 20 and in C, fractions 4 to 20) were pooled and subsequently applied to the small columns (B and D, respectively). For the small columns, the elution was performed with elution buffer B (see section 1.2.6.2.) at a flow rate of 15 ml/h. The collected fractions (3 ml/each) were assayed for protein content by BCA for wASGPR (B) and by absorbance at 280 nm for rASGPR (D). Fractions zero are the points where elution buffers were applied on the columns.

This figure reveals that the affinity chromatographic profiles of wASGPR are similar to those of rASGPR.



Fractions (5 ml/each)

Fractions (3 ml/each)

**Table 1.2. Yields of acetone liver powder and affinity-purified hepatic ASGPRs from woodchucks, rabbits and a rat**

Species	Number of preparations	Yield (per 100 g liver)	
		Acetone liver powder (g) <sup>a</sup>	ASGPR protein (μg) <sup>a</sup>
Woodchuck	4	24 ± 4	150 ± 103
Rabbit	4	17 ± 2	784 ± 181
Rat	1	19	216

<sup>a</sup> - mean ± standard deviation (SD).

This confirmed our initial observation that the overall amount of ASGPR in woodchuck liver was lower than that in rabbit liver.

### 1.3.1.2. Biochemical properties of wASGPR.

#### 1.3.1.2.1. Receptor polypeptide composition.

Examination of the affinity-purified wASGPR by SDS-PAGE and Coomassie blue staining (Fig. 1.2) demonstrated that the receptor was composed of two major polypeptides with apparent molecular masses of 40 and 47 kDa. According to the densitometric scanning of the gels, the approximate ratio of 2-3:1 was found between the 40-kDa and the 47-kDa polypeptides in the purified receptor. Rabbit hepatic ASGPR also yielded the same pattern of protein bands on SDS-PAGE gel with a subunit ratio of 40 kDa to 47 kDa at 2:1. In contrast, as described by other researchers (Table 1.1), purified rat ASGPR showed one prominent polypeptide with molecular mass of about 44 kDa and minor bands with approximate molecular masses of 52 kDa and 65 kDa. In addition, several bands with molecular masses of about 80-105 kDa and above 200 kDa were consistently seen on the Coomassie blue-stained gels of both wASGPR and rASGPR. Long term storage of the purified receptor protein resulted in additional bands with approximate molecular masses of 25 to 35 kDa which are considered to be degraded receptor proteins (e.g., in Fig. 1.3 and Fig. 1.4). Overall, the wASGPR polypeptide profile closely resembles that of the rASGPR, but is distinct from the molecular pattern of the rASGPR subunits.

The high molecular weight species (higher than 100 kDa) seen in the purified ASGPR preparations are most likely the result of receptor subunit self-aggregation, as described by Tanabe et al. (1979) and shown in Table 1.1. In the course of this study, the presence of protein bands with high molecular weight was also consistently observed in the isolated ASGPR subunits. As shown in Fig. 1.3, 40-kDa subunits of rASGPR isolated by electroelution displayed the formation of homo-oligomer polypeptides with approximate molecular

**Figure 1.2. Comparison of molecular polypeptide profiles of the affinity-purified wASGPR, rASGPR and rtASGPR proteins by SDS-PAGE.** wASGPR (lane 2), rASGPR (lane 3) and rtASGPR (lane 4) were separated at 10  $\mu$ g protein per lane on SDS-12.5% polyacrylamide gel and the polypeptide bands were visualized by Coomassie blue staining. See sections 1.2.17 and 1.2.18 for details of the methods. Non-prestained protein molecular mass markers (lane 1) in kilodaltons are indicated on the left side of the panel.

This figure shows that the molecular composition of wASGPR is similar to that of rASGPR and different from that of rtASGPR.

m.wt.  
kDa

1 2 3 4

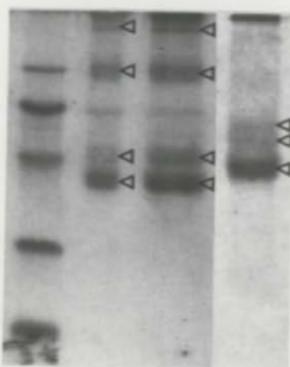
97.4 -

66.2 -

45.0 -

31.0 -

21.5 -



**Fig. 1.3. The relation between monomeric and oligomeric species of ASGPR subunits analyzed by Western blotting.** Receptor subunits were isolated from affinity-purified rASGPR by SDS-PAGE and gel electro-elution (see section 1.2.7 for details). The purified 40-kDa subunit polypeptides (lane 1, 0.2  $\mu$ g protein per lane), 47-kDa subunit polypeptides (lane 2, 0.2  $\mu$ g protein per lane) and the affinity-purified rASGPR whole proteins (lane 3, 0.5  $\mu$ g protein per lane) were subjected to Western blot analysis using guinea pig antiserum against rASGPR. See section 1.2.21 for details of the method. The molecular masses of prestained protein standards (lane 4) are shown on the right of the panel.

This figure shows that purified 40-kDa subunit polypeptides form homo-oligomers with the molecular mass of 80-kDa, whereas the 47-kDa subunit polypeptides self-aggregate into 90-kDa homo-oligomers. Degraded species with approximate molecular mass of 30 kDa are also shown in the preparation of 40-kDa subunit.

m.wt  
kDa

1

2

3

4

m.wt.  
kDa

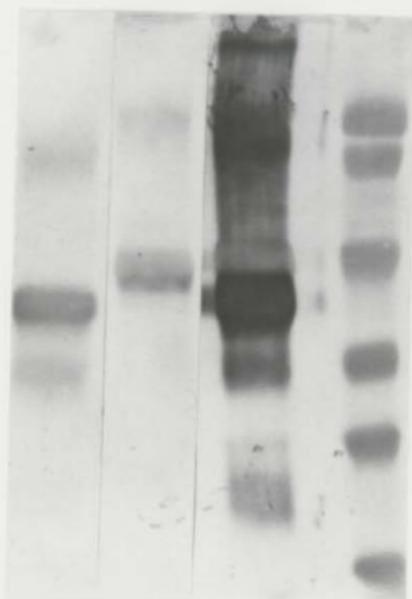
90 -

80 -

47 -

40 -

30 -



- 106.0

- 80.0

- 49.5

- 32.5

- 27.5

- 18.5

**Fig. 1.4. The interaction between constituent subunits in affinity purified ASGPR preparations analyzed by SDS-PAGE and autoradiography.** Surface <sup>125</sup>I-labelled affinity-purified native rASGPR (lane 1, 15 ng protein/5 × 10<sup>5</sup> cpm per lane; lane 2, 6 ng protein/2 × 10<sup>5</sup> cpm per lane) and 2% SDS-solubilized rASGPR (lane 3, 6 ng protein/5 × 10<sup>5</sup> cpm per lane) were separated by SDS-PAGE and visualized by autoradiography exposing overnight at -70 °C. See sections 1.2.17 and 1.2.19 for details of the methods. The molecular masses of <sup>11</sup>C-labelled molecular standards (lane 4) are indicated on the right side of this panel.

This figure shows that the 47-kDa subunit of the purified native rASGPR was poorly labelled by surface iodination and became well labelled after treatment of the receptor with detergent prior to radiolabelling.

m.wt.  
kDa

1

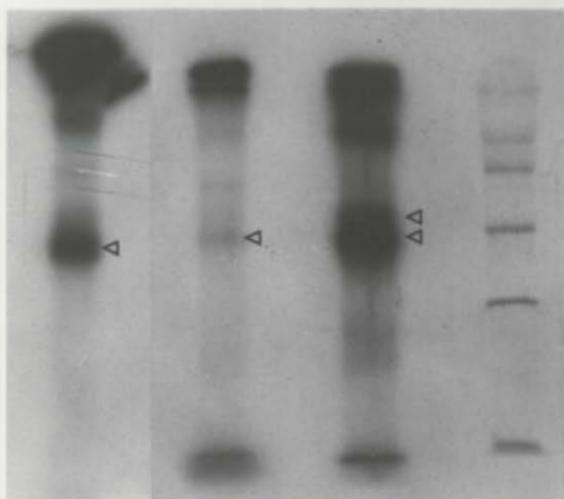
2

3

4

m.wt.  
kDa

47 -  
40 -



- 200

- 97

- 69

- 46

- 30

- 14

mass of 80 kDa, whereas the 47-kDa subunits self-aggregated into 90-kDa polypeptides.

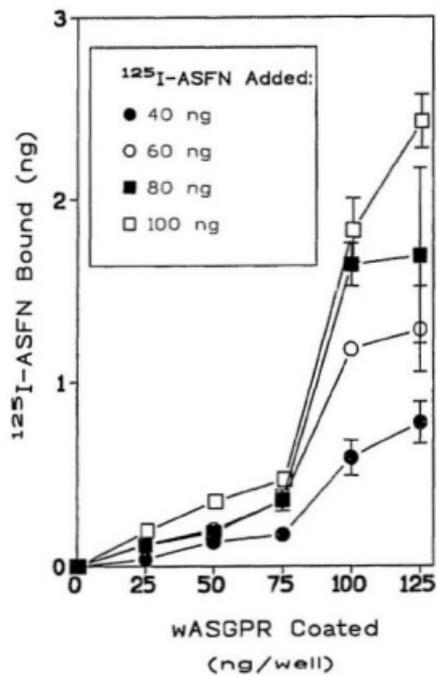
The presence of self-aggregates in the purified receptor preparations raised some concern about the immunological properties of the native (or detergent untreated) ASGPR macromolecules, as these preparations were used as antigen for immunization of animals and for antibody detection. During the investigation of the physical relationship between the subunits, it was noted that the 40-kDa polypeptides of rASGPR were preferentially labelled after surface-specific iodination of the purified, native rASGPR (Fig. 1.4). Densitometric tracing of the autoradiograms of these receptor preparations showed that the 40-kDa and 47-kDa subunit had a ratio of 7 : 1. To disrupt the intact receptor macromolecules, receptor was solubilized in detergent (2% SDS) prior to the radiolabelling. Results showed that under such conditions, 40-kDa and 47-kDa subunits of rASGPR could be labelled at an approximate ratio of 2 : 1. This ratio closely resembled that found for the subunits from the native receptor separated by SDS-PAGE and stained with Coomassie blue. These results suggest that the 47-kDa polypeptides are partially hidden in the purified intact rASGPR macromolecules. Since there is a strong similarity between the woodchuck and rabbit receptors, we proposed that the same structure relationship may exist between the polypeptides of isolated native wASGPR.

#### 1.3.1.2.2. Ligand binding activity and kinetic studies of the purified wASGPR.

The ASGPR purified by affinity chromatography from woodchuck liver or, as a control, from rabbit liver was examined for the ligand binding activity using solid-phase binding assays. In the initial set of experiments, optimal conditions for ligand-ASGPR binding were established by using microtiter plates coated with increasing amounts of purified wASGPR and subsequently exposed to increasing amounts of <sup>125</sup>I-ASFN. Fig. 1.5 shows the amounts of <sup>125</sup>I-ASFN bound to the immobilized wASGPR. At a coating concentration of 100 ng of wASGPR protein per well, the binding values fall into a region in which the binding

**Figure 1.5. Determination of ASGPR coating concentration for solid phase-ligand binding assay.** Flexible plates coated with affinity-purified wASGPR in PBS at different concentrations ranging from 25 ng to 125 ng/50  $\mu$ l per well were incubated with increasing amount of  $^{125}$ I-ASFN ( $1.36 \times 10^3$  cpm/ng) ranging from 40 ng to 100 ng. After washing out excess  $^{125}$ I-ASFN, the bound radioactivity was counted and presented as amount of  $^{125}$ I-ASFN bound in ng. See section 1.2.13 for details of the method. Each point represents the mean of triplicate determinations (mean  $\pm$  SD).

This figure shows a dose-dependent ligand binding by wASGPR coated at 100 ng protein per well.



kinetic profile showed a linear relation between the concentrations of coated wASGPR and bound  $^{125}\text{I}$ -ASFN. Therefore, this coating concentration (100 ng per well) was chosen as a standard for the studies of ligand and purified ASGPR binding.

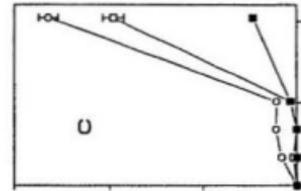
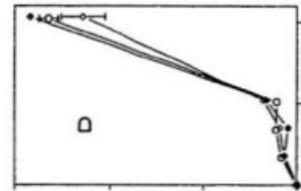
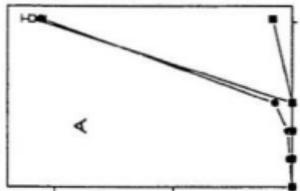
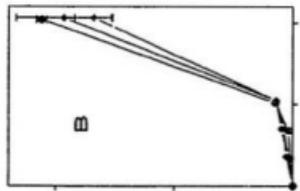
The specificity of the ligand binding to the purified wASGPR or rASGPR was determined by adding 5- to 425-fold excess of an unlabelled ASOR (asialylated glycoprotein) or ORM (sialylated derivative of ASOR, control), as competitors, to the reaction of ASGPR and  $^{125}\text{I}$ -ASOR. As shown in Fig. 1.6, results revealed that ASGP (ASOR), but not sialoglycoprotein (ORM) inhibited (>80%) the interaction between  $^{125}\text{I}$ -ASOR and ASGPR. This result demonstrates that purified wASGPR recognizes an asialylated glycoprotein ligand but not a sialoglycoprotein, indicating a strict ligand specificity of the purified wASGPR.

To access the ligand binding capability of the affinity-purified wASGPR, the kinetics of  $^{125}\text{I}$ -ASFN binding were examined. Incubation of increasing amounts of  $^{125}\text{I}$ -ASFN with a constant amount of immobilized wASGPR gave a monophasic, concentration-dependent curvilinear pattern of the binding kinetics. This pattern suggests that the interaction is mediated by a single class of saturable receptor. Fitting the data to a one-site binding model by a computerized nonlinear regression program produced the ligand-binding kinetic curve (Fig. 1.7) and Scatchard plot (Fig. 1.7, insert), which shows the dissociation constant ( $K_d$ ) for the binding was  $7.4 \times 10^{-14}$  M and the maximum binding ( $B_{\max}$ ) was  $1.2 \times 10^{-11}$  M under the assay conditions used. These data support the conclusion that a single class of receptor is involved in the interaction between wASGPR and its ligand, ASFN.

Under the same assay conditions, binding between purified rASGPR and ASFN gave an estimated  $K_d$  and  $B_{\max}$  value of  $5 \times 10^{-12}$  M and  $2.3 \times 10^{-11}$  M, respectively. Comparison of these data with those for wASGPR suggests that although the wASGPR displays a 100-fold lower number of ligand binding sites than rASGPR per amount of receptor protein, its ligand binding affinity is approximately 100-fold higher than that of the rabbit receptor.

**Figure 1.6. Specificity of ligand binding to the affinity-purified wASGPR and rASGPR determined through competition of  $^{125}\text{I}$ -ASOR attachment by asialylated and sialylated glycoproteins.** Immobilized wASGPR (A and B) or rASGPR (C and D) was incubated with  $^{125}\text{I}$ -ASOR ( $1.42 \times 10^3$  cpm/ng) ranging from 17 ng to 102 ng per well (controls). Parallel experiments were carried out under the same assay conditions with the additional unlabelled ASOR (A and C) or ORM (B and D) present. The bound radioactivity on the plates was counted and presented as the amount of  $^{125}\text{I}$ -ASOR bound (ng). See section 1.2.13 for details of the method. Each point represents the mean of triplicate determinations.

This figure shows that binding between the affinity-purified ASGPR and  $^{125}\text{I}$ -ASOR is specifically inhibited by an asialylated glycoprotein, ASOR, but not by a sialylated glycoprotein, ORM.



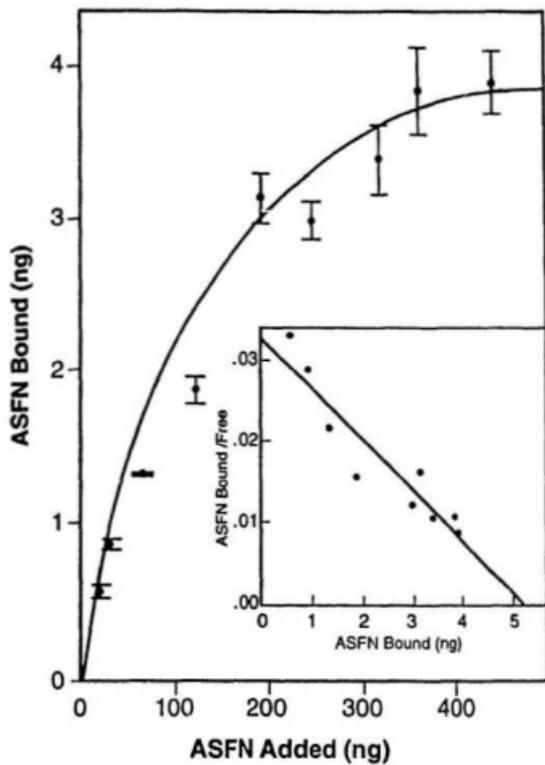
■ Controls (binding by wASGPR)  
 □ - ASOR (0.85 ug/well)  
 ● - ASOR (8.5 ug/well)  
 ◆ - ORH (0.85 ug/well)  
 • - ORH (8.5 ug/well)  
 ○ Controls (binding by rASGPR)

125I-ASOR Bound (ng)

125I-ASOR Added (ng/well)

**Figure 1.7. Binding of  $^{125}\text{I}$ -ASFN to the affinity-purified wASGPR as a function of ligand concentration.** Increased amounts of radiolabelled ASFN ( $1.36 \times 10^3$  cpm/ng) were incubated with constant amounts of immobilized wASGPR (coated at 100 ng protein per well) with or without a 100-fold excess of unlabelled ASFN. Each point represents the mean of triplicate determinations of specific binding. *Insert.* Scatchard plot of binding data indicating graphically the presence of a single saturable receptor site. The lines of best fit to the data points was determined by a computerized program. See section 1.2.13 for details of the method.

This figure shows a concentration-dependent pattern of reaction between purified wASGPR and its ligand, suggesting that the binding is mediated by a single class of saturable receptor.



### 1.3.1.3. Immunological properties of ASGPRs.

In the course of current studies, guinea pigs were immunized with purified wASGPR, rASGPR and its subunits (40-kDa and 47-kDa polypeptides) in order to prepare specific antibodies for the detection of receptor subunits and the analysis of receptor antigenic properties. In addition, two woodchucks were challenged with purified rASGPR, and their sera were tested to determine whether rASGPR was immunogenic in woodchucks despite its apparent structural and antigenic similarities to wASGPR. A reference guinea pig antiserum to rASGPR was kindly provided by Dr. B.M. McFarlane from the Institute of Liver Studies, King's College School of Medicine and Dentistry, London, U.K. This serum was used as a positive control.

#### 1.3.1.3.1. Immunogenic and antigenic properties of wASGPR analyzed by using guinea pig anti-ASGPR antisera.

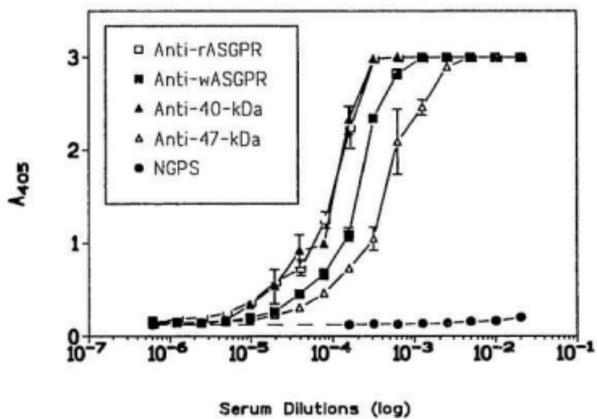
Titration of the antisera from immunized guinea pigs using microplates coated with rASGPR (Fig. 1.8) showed that challenge with both the entire ASGPR preparations and isolated receptor subunits induced high levels of specific antibodies, demonstrating a high immunogenicity of the receptor preparations derived from either woodchuck or rabbit liver. The titers of these antisera were as high as 1:819,200, while the non-immunized guinea pig sera (NGPS) used as controls were negative. Titration of the antisera against rASGPR was also performed by radioimmunoassay (RIA) (this method is described and discussed in Part II of this thesis). Both assays, ELISA and RIA, gave similar results.

Using these guinea pig antisera (i.e., anti-wASGPR, anti-rASGPR, anti-40-kDa and anti-47-kDa), the antigenic properties of the affinity-purified ASGPRs and their subunits were analyzed by Western blotting. The following results were obtained:

(1) All the constituent subunits of ASGPR preparations derived from different species were recognized by both anti-rASGPR and anti-wASGPR antisera.

**Figure 1.8. Titration of guinea pig anti-ASGPR antisera by ELISA.** Sera from guinea pigs immunized with the affinity-purified rASGPR, wASGPR, or isolated 40-kDa- and 47-kDa-subunits of rASGPR were serially diluted from 1:50 to 1:1,638,400. The sera were tested for the reactivity with rASGPR by ELISA, as described in section 1.2.9. Results are presented as the absorbance at 405 nm. A pool of sera from non-immunized guinea pigs (NGPS) were used as a control. Each point represents the mean of duplicate determinations (mean  $\pm$  SD).

This figure shows that all the anti-ASGPR antisera raised in guinea pigs against the entire ASGPR molecules (anti-wASGPR and anti-rASGPR) or the isolated subunit polypeptides (anti-40-kDa and anti-47-kDa) react with purified ASGPR with similar titers.



All the polypeptides of wASGPR, rASGPR and rtASGPR were recognized by anti-rASGPR antisera, indicating that a strong antigenic cross-reactivity exists among the affinity-purified ASGPRs derived from these three different species (Fig. 1.9A). The ASGPR-specificity of anti-rASGPR was confirmed (Fig. 1.10, lane 10) by complete elimination of the antibody activities after absorption with purified rASGPR performed as outlined in section 1.2.10.

Essentially identical results to those found with guinea pig anti-rASGPR were obtained when guinea pig anti-wASGPR antiserum was used for immunoblot detection (Fig. 1.9B). Both subunit polypeptide bands of either wASGPR or rASGPR were recognized with a comparable density by this antiserum, further demonstrating the strong antigenic cross-reactivity between these two receptors.

(2) Distinct antigenic specificity of ASGPR subunits were recognized by anti-40-kDa and anti-47-kDa antisera.

The antigenic specificity of ASGPR subunits was also analyzed by Western blotting using guinea pig antisera raised against 40- or 47-kDa polypeptides of rASGPR. Results revealed that immunization with the 40-kDa polypeptide led to appearance of antibodies with exclusive reactivity towards the 40-kDa polypeptides of either wASGPR or rASGPR (e.g., Fig. 1.10, lane 5). This antiserum also recognized the 44-kDa protein band of the rtASGPR preparation (not shown). Also, antiserum raised against the 47-kDa polypeptide displayed a specific reactivity towards the 47-kDa of both wASGPR and rASGPR at a dilution higher than 1:400 (e.g., Fig. 1.10, lane 8) and recognized 52-kDa and 65-kDa polypeptides of the affinity-purified rtASGPR (data not shown). Thus, these results clearly demonstrated that the 40- and 47-kDa subunits of wASGPR or rASGPR are antigenically distinct by using the subunit mono-specific antisera. On the other hand, the corresponding subunits of wASGPR and rASGPR with the comparable molecular masses appear to have very similar antigenic properties. This conclusion is in good accord with the reports on the protein sequence

**Figure 1.9. The specificity of guinea pig antisera against affinity-purified ASGPR preparations analyzed by Western blotting.** Affinity purified wASGPR (lane 2), rASGPR (lane 3) and rtASGPR (lane 4) were separated at 0.2  $\mu$ g protein per lane on SDS-PAGE, electrotransferred onto nitrocellulose and identified by immunoblotting with guinea pig antisera against affinity-purified hepatic ASGPR from rabbit (**A**) and woodchuck (**B**). See section 1.2.21 for details of the method. The molecular masses of prestained protein standards (lane 1) in kilodaltons (kDa) are indicated on the left side of the panels.

This figure shows that guinea pig antisera against either rASGPR or wASGPR recognize all the constituent subunits of the affinity-purified wASGPR, rASGPR and rtASGPR.

**A**m.wt.  
kDa

1 2 3 4

106.0 -

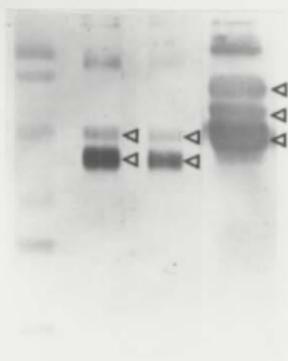
80.0 -

49.5 -

32.5 -

27.5 -

18.5 -

**B**m.wt.  
kDa

1 2 3

106.0 -

80.0 -

49.5 -

32.5 -

27.5 -

18.5 -



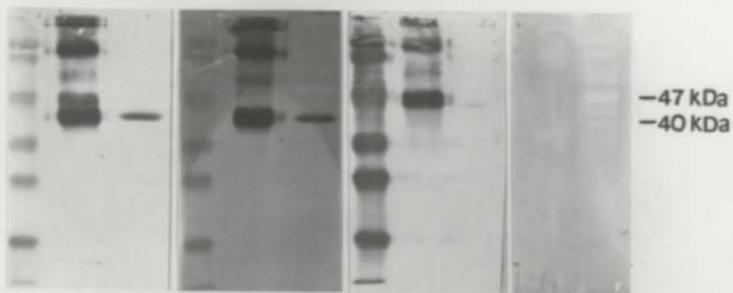
**Figure 1.10. The subunit-specificity of guinea pig antisera raised against affinity-purified ASGPR and isolated ASGPR polypeptides analyzed by Western blotting.** Affinity-purified rASGPR (0.3  $\mu$ g protein per lane; lanes 2, 5, 8 and 10) and woodchuck hepatic plasma membranes (HPMs) (10  $\mu$ g protein per lane; lanes 3, 6, 9 and 11) were separated by SDS-PAGE and analyzed by immunoblotting using guinea pig antisera against wASGPR (lanes 2 and 3), the 40-kDa subunit (lanes 5 and 6) or the 47-kDa subunit (lanes 8 and 9) or with antiserum against rASGPR preabsorbed with purified rASGPR (lane 10 and 11). See section 1.2.21 for details of the method. Prestained protein standards are shown in lanes 1, 4 and 7 and their molecular masses indicated on the left of the panel.

This figure shows that guinea pig anti-ASGPR subunit monospecific antisera recognize relevant receptor subunits in the purified receptor and woodchuck HPM preparations, and that antisera against the entire ASGPRs react with both 40- and 47-kDa subunit polypeptides of affinity-purified ASGPR. It is also shown that neither anti-wASGPR nor anti-47-kDa antibodies recognize the 47-kDa polypeptides in woodchuck HPM.

m.wt.  
kDa

1 2 3 4 5 6 7 8 9 10 11

106.0 —  
80.0 —  
49.5 —  
32.5 —  
27.5 —  
18.5 —



homology between ASGPR subunits from different species (Drickmamer et al., 1984; Bezouska et al., 1991).

#### 1.3.1.3.2. Immunogenic properties of ASGPR analyzed using antisera from woodchucks immunized with rASGPR.

The above immunoblot analyses using guinea pig antisera have shown the strong antigenic cross-reactivity between affinity-purified wASGPR and rASGPR. It is of interest to know whether rASGPR is immunogenic to woodchucks. Sera from two woodchucks injected with two doses of rASGPR were collected at different times before, during and after immunization. Titration of these woodchuck sera by RIA (for method, see section 2.2.3) revealed that the titers of anti-ASGPR in these sera ranged between 1:800 and 1:1600. As the immunizations of woodchucks were carried out at a lower antigen dose per body-weight than those of guinea pigs, it is difficult to precisely evaluate the strength of the immune response to ASGPR by comparing the titer of woodchuck anti-ASGPR antisera with that of guinea pig anti-ASGPR antisera. However, these two woodchucks did show that rASGPR was immunogenic to woodchucks, despite the antigenic similarity between wASGPR and rASGPR. This result indicates that rASGPR can be used as an antigen to stimulate anti-liver-specific antibody responses in woodchucks. Therefore, immunization of woodchucks with purified ASGPR may provide a convenient experimental system to study the autoimmune response to hepatic ASGPR and viral hepatitis in the same animals. This system may be of significant value in the study of the pathogenic role of anti-ASGPR autoimmune responses in the woodchuck model of hepatitis B.

Receptor subunit-specificity of the woodchuck antisera was also analyzed by Western blotting. Results are summarized in Table 1.3 and illustrated in Fig. 1.11. These results revealed that challenge with rASGPR led to the appearance of antibodies specific for both 40- and 47-kDa subunits of rASGPR, indicating that both subunits of rASGPR are immunogenic

**Table 1.3. Western blotting analysis of the antisera from woodchucks collected at various times after immunization with rASGPR\***

Antigen (ASGPR subunit)	Animal	Time of serum collection (d.p.i.) <sup>b</sup>				
		0	10	30	42	71
40-kDa	A	-	+	+	+	+
47-kDa		-	-	+	+	+
40-kDa	B	-	+	+	n.t. <sup>c</sup>	+
47-kDa		-	-	-	n.t.	+

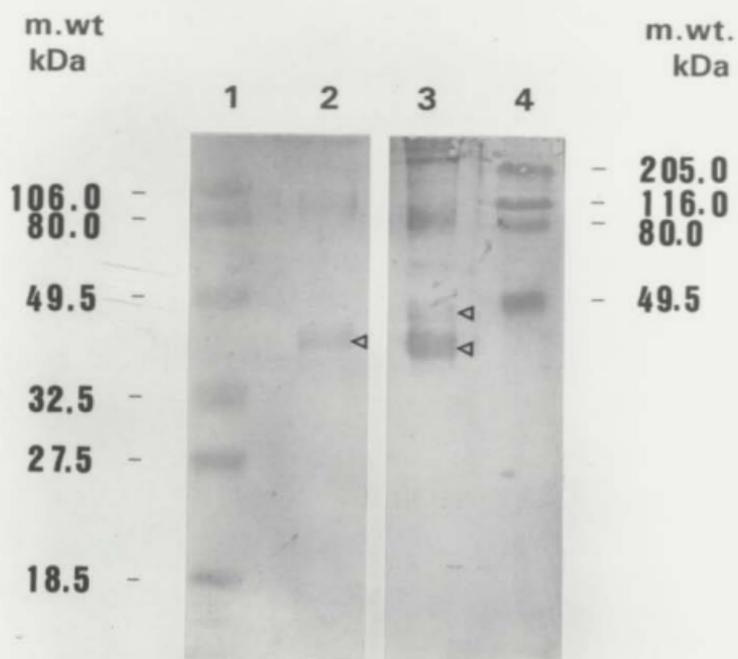
\* - The animals were injected with rASGPR on days 0 and 16. See section 1.2.8 for details of the method.

<sup>b</sup> - d.p.i.: days post first immunization with rASGPR.

<sup>c</sup> - n.t.: not tested.

**Figure 1.11. The ASGPR subunit specificity of antisera raised in woodchucks by immunization with rASGPR.** Affinity-purified rASGPR (0.3 µg protein per lane; lanes 2 and 3) were separated by SDS-PAGE, transferred onto nitrocellulose and separately analyzed by Western blotting using sera (1:10) from woodchuck A (see Table 1.3) obtained at 10 days (lane 2) and 42 days (lane 3) after administration of the first dose of rASGPR. See section 1.2.21 for details of the method. The molecular masses of prestained protein standards (lanes 1, low range; and lane 4, high range) are indicated on the left and right sides of the panels.

This figure shows that immunization of a woodchuck with purified rASGPR produced a specific antibody response which initially was directed against the receptor 40-kDa subunit.



to woodchucks. Interestingly, antibodies with reactivity against the 40-kDa polypeptide could be detected within 10 days after immunization with the first dose, while antibodies to the 47-kDa polypeptide emerged approximately 2 weeks after the booster immunization. This result may be interpreted as showing that the major 40-kDa subunit of rASGPR is more immunogenic than the minor 47-kDa subunit when the affinity-purified, whole receptor macromolecules are used for immunization.

### **1.3.2. Hepatocyte Surface Expression of wASGPR.**

#### **1.3.2.1. Immunocytochemical localization of the wASGPR on hepatocytes.**

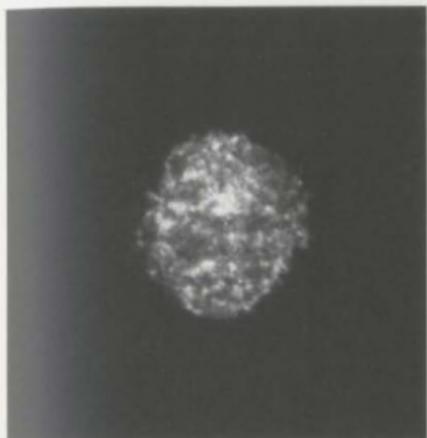
Expression of wASGPR on isolated woodchuck hepatocytes was examined by indirect immunofluorescent staining with guinea pig antisera directed to the entire wASGPR or rASGPR molecules, or to the subunit polypeptides of rASGPR. Fig. 1.12A shows that the guinea pig antibodies to ASGPR are granularly deposited on hepatocytes, indicating the reaction between anti-ASGPR antibodies and wASGPR on hepatocytes. No difference in the pattern of the ASGPR localization was observed by staining woodchuck hepatocytes with either anti-ASGPR antisera or anti-subunit specific antisera. Incubation of the hepatocytes with a pool of sera from non-immunized guinea pigs resulted in no immunofluorescent staining (Fig. 1.12B), confirming the specificity of this reaction.

ASGPR expression was also determined on the surface of cultured HepG2 cells, a cell line derived from human HCC. The results showed that the ASGPR displayed on HepG2 cells was very well recognized by our guinea pig anti-ASGPR antisera (e.g., Fig. 1.12C). The specificity of this reaction was confirmed by incubation of the HepG2 cells with a pool of sera from non-immunized guinea pigs (data not shown). These results imply that an antigenic cross-reactivity not only exists between wASGPR and rASGPR but also exists between wASGPR and human ASGPR.

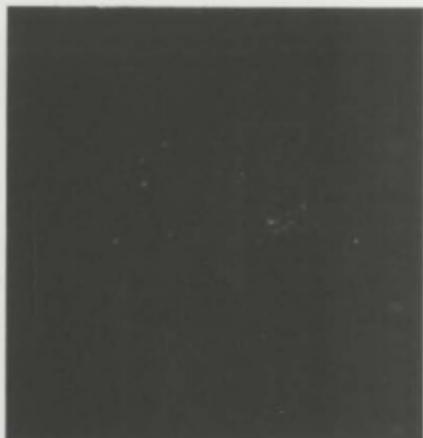
**Figure 1.12. Expression of ASGPR on hepatocytes determined by indirect immunofluorescent staining.** Isolated woodchuck hepatocytes (A) and cultured HepG2 cells (C) were incubated with 1:20 guinea pig antiserum against rASGPR. After washing, the cells were stained with FITC-conjugated goat anti-guinea pig IgG (1:25). As a control, woodchuck hepatocytes (B) were incubated with a pool of normal guinea pig sera (1:20) instead of anti-rASGPR antiserum. See section 1.2.11 for details of the method. Original magnifications, A and B,  $\times 400$  and C,  $\times 250$ .

This figure shows that both woodchuck and human hepatocytes express ASGPR on the cell surface which is readily recognized by anti-rASGPR antiserum.

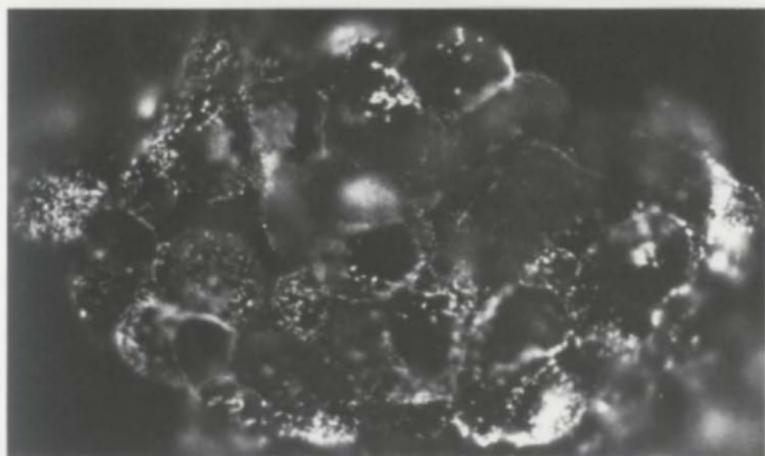
A



B



C



### 1.3.2.2. Hepatic plasma membrane expression of wASGPR subunits.

#### 1.3.2.2.1. Immunologic properties of hepatic plasma membrane-associated wASGPR.

Although results from immunofluorescent staining have shown the presence of wASGPR molecules at the surface of isolated woodchuck hepatocytes, it was necessary to determine the receptor subunit distribution in the purified hepatic plasma membranes (HPM), since the immunocytochemical staining does not provide the opportunity for such detailed analysis. For this purpose, plasma membranes were purified from whole liver homogenate or from isolated hepatocytes of healthy woodchucks, as well as WHV-infected animals which developed CH or HCC (see section 1.2.3). Subsequently, the purified membranes were analyzed by Western blotting for the expression of the membrane-associated wASGPR subunits.

Results from Western blot analysis showed that only one band with approximate molecular mass of 40 kDa was consistently detectable in the purified HPMs from healthy woodchucks, using guinea pig antisera against wASGPR, rASGPR or against the 40-kDa subunit (Fig. 1.10, lanes 3 and 6; Fig. 1.13, lane 5). Identical results were also obtained when HPMs from woodchucks with WHV-induced CH or HCC were examined by using the same antisera (Fig. 1.14). As controls, plasma membranes purified from woodchuck splenocyte (SPM) and kidney homogenate (KPM) were probed. Results revealed no recognition of SPM (Fig. 1.13, lane 2) or KPM (not shown) by these antisera, suggesting that the protein bands identified in woodchuck HPM were in fact liver specific. In addition, the specificity of this immunoreaction was confirmed by the elimination of recognition of the HPM-associated 40-kDa-band following the absorption of the anti-ASGPR antiserum with the affinity-purified ASGPR (Fig. 1.10 lane 11). This indicates that the 40-kDa polypeptide band in woodchuck HPM is antigenically closely related, if not identical, to the 40-kDa subunit of the affinity-purified wASGPR.

It was surprising that a protein band corresponding to the 47-kDa-subunit of

**Figure 1.13. Comparison of molecular polypeptide profiles of ASGPR in hepatic plasma membranes isolated from woodchuck, rabbit and rat.** Isolated HPMs (10 µg protein per lane) from a healthy woodchuck (lane 5), rabbit (lane 3) and rat (lane 4), and control plasma membranes from woodchuck splenocyte (lane 2) were separated by SDS-PAGE, electrotransferred onto nitrocellulose and analyzed by immunoblotting using guinea pig antiserum against rASGPR. See section 1.2.21 for details of the method. The molecular masses of prestained protein standards (lanes 1 and 6) are indicated on the left side of the panel.

This figure shows that in woodchuck HPM only the 40-kDa polypeptide, which is liver-specific and corresponds to the major subunit of the affinity-purified wASGPR (see Fig. 1.2, lane 2) was recognized by antiserum against rASGPR. Nevertheless, the same antiserum identified the polypeptides corresponding to all the constituent subunits of rASGPR and rtASGPR in HPMs from the respective hosts. This result indicates an apparent lack of the 47-kDa subunit reactivity in the purified woodchuck HPMs when analyzed by a conventional Western blotting.

m.wt.  
kDa

1 2 3 4 5 6

106.0 —

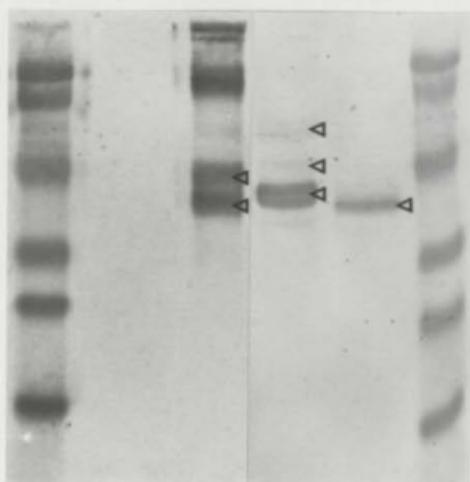
80.0 —

49.5 —

32.5 —

27.5 —

18.5 —



**Figure 1.14. Identification of wASGPR subunits in hepatic plasma membranes from healthy woodchucks and woodchucks with chronic hepatitis and WHV-induced hepatocellular carcinoma by Western blotting.** Isolated HPMs from healthy animals (lanes 6 and 7) and animals with experimentally induced CH (lanes 1, 2, 3 and 4) or with HCC (lane 5) were separated by SDS-PAGE at 10  $\mu$ g protein per lane, transferred onto nitrocellulose and analyzed by immunoblotting using guinea pig anti-rASGPR antiserum. See section 1.2.21 for details of the method. The molecular masses of prestained protein standards (lane 8) are indicated on the right side of the panel.

This figure reveals that only the 40-kDa subunit polypeptide of wASGPR was detectable by Western blot using anti-ASGPR antibodies in the HPMS from woodchucks with or without WHV-induced liver pathology.

m.wt.  
kDa

1

2

3

4

5

6

7

8

m.wt.  
kDa

- 106.0

- 80.0

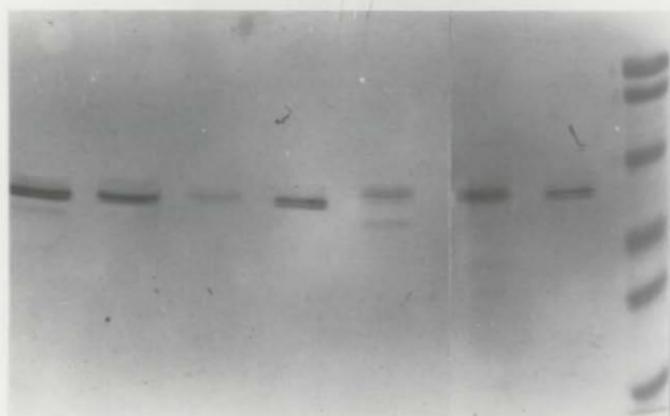
- 49.5

- 32.5

- 27.5

- 18.5

40 -



wASGPR could not be detected in any of the HPM preparations (Fig. 1.10, Fig. 1.13 and Fig. 1.14). Increasing the amount of the membranes tested by 10-fold (from 10  $\mu\text{g}$  to 100  $\mu\text{g}$  membrane proteins per lane) for Western blot analysis did not lead to the detection of the 47-kDa-band (Fig. 1.15, lanes 1, 2 and 3). In contrast, the same panel of antibodies clearly demonstrated expression of all the constituent ASGPR polypeptides in either rabbit or rat hepatic plasma membranes when 10  $\mu\text{g}$  of membrane protein per lane was used for the immunoblotting test (Fig. 1.13, lanes 3 and 4 and Fig. 1.15, lane 4).

In an attempt to detect the 47-kDa subunit of wASGPR in woodchuck HPM, a radioimmunoprecipitation assay was employed to analyze  $^{125}\text{I}$ -labelled native (detergent untreated) HPMs or SDS-solubilized woodchuck LPMs. After precipitation by antisera against wASGPR, 40-kDa or 47-kDa polypeptide, the precipitates of both native or solubilized HPMs displayed three bands with approximate molecular masses of 30, 40 and 47 kDa, as it is shown for native HPM in Fig. 1.16. Polypeptides of similar molecular sizes to these bands had been detected in purified ASGPR preparations by protein staining of SDS-PAGE separated gels and by anti-ASGPR antisera in Western blotting, as shown in Fig. 1.2 and Fig. 1.9. Therefore, these plasma membrane bands most likely represented the wASGPR subunits in the membrane preparations. In addition, controls prepared by precipitation of radiolabelled-HPMs with protein A-Sepharose beads alone or with non-immunized guinea pig sera did not reveal such polypeptide patterns. Fig 1.16 also shows that identical patterns were obtained with all the antisera tested, irrespective of whether the antiserum was raised against the whole receptor or against the isolated subunits. It is possible that there are cross-linkages among the subunits of wASGPR in the native plasma membranes and therefore, these subunits could be co-precipitated by antibodies specific for any one of the subunits. Such cross-linkage of ASGPR subunits has been observed in the receptors from other species (Harford et al., 1982). Taken together, these observations suggest that both 40- and 47-kDa subunit polypeptides of wASGPR are expressed in the woodchuck hepatic plasma membranes.

**Figure 1.15. Western blot analysis of wASGPR subunit expression in woodchuck HPMS using guinea pig anti-ASGPR antiserum.** HPMS isolated from healthy woodchucks (lanes 1, 2 and 3) and rabbit (lane 4, control) were separated by SDS-PAGE at 100 µg protein per lane, transferred onto nitrocellulose and analyzed by immunoblotting using anti-rASGPR antiserum. See section 1.2.2.1 for details for the method. The molecular masses of the detected polypeptide species are indicated on the right side of the panel.

This figure shows that only the 40-kDa subunit polypeptide was detected by antibodies against rASGPR even when relatively large amounts of woodchuck HPMS (100 µg membrane proteins per lane) were employed for determination.

1

2

3

4

kDa

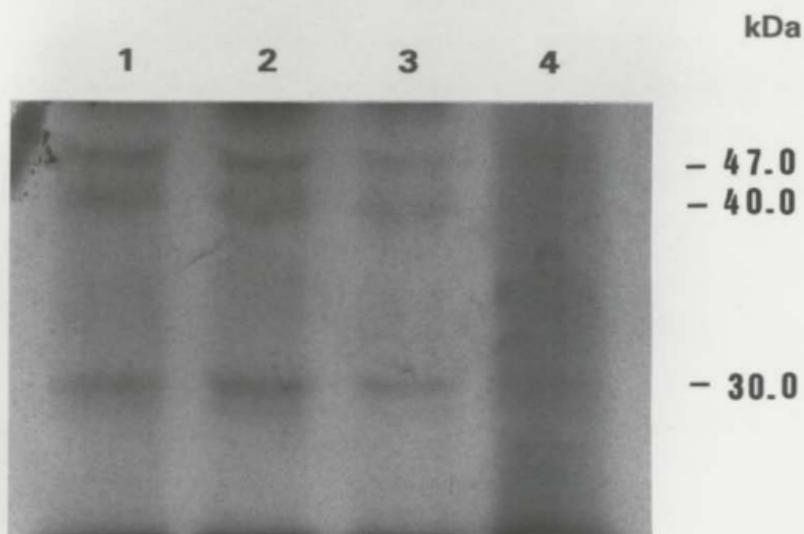
- 47.0

- 40.0



**Figure 1.16. Radioimmunoprecipitation analysis of the expression of wASGPR subunits in woodchuck HPMs using anti-wASGPR and monospecific anti-rASGPR-subunit antisera.**  $10^5$  cpm of  $^{125}\text{I}$ -labelled native (non-detergent treated) woodchuck HPMs (specific activity,  $2.5 \times 10^3$  cpm/ng) were precleaned by incubation with normal guinea pig serum and then incubated with guinea pig antisera against wASGPR (lane 3), 40-kDa (lane 2) and 47-kDa (lane 1) subunits of rASGPR. Subsequently, the immunocomplexes were precipitated by protein A-Sepharose 4B. Immunoprecipitates were separated by SDS-PAGE and the radioactivity on the gel was detected by autoradiography. Precleaned HPMs precipitated with protein A beads alone was used as control (lane 4). See section 1.2.14 for details of the method. The molecular masses of the precipitated polypeptides are indicated on the right side of the panel.

This figure shows that both 40- and 47-kDa subunit polypeptides of wASGPR were detected by guinea pig antisera against wASGPR or against subunits of rASGPR



#### 1.3.2.2.2. Biological properties of hepatic plasma membrane-bound wASGPR.

To determine whether the ASGPR bound to woodchuck HPMs is biologically active, purified membranes in the presence of mild ionic and zwitterionic detergents were examined by using a ligand affinity precipitation method. After incubation of woodchuck HPMs with Sephrose-4B beads coated with D-Gal followed by extensive washing, proteins bound to the beads were released by SDS-PAGE sample buffer and analyzed by Western blotting. Results presented in Fig. 1.17A show that the 40-kDa subunit polypeptide of wASGPR in the purified woodchuck HPMs were precipitated by D-Gal-Sepharose and then recognized by antisera against ASGPR. This result indicates that ASGPR existed in the purified woodchuck HPMs has biological activity. Results also show that there were no differences between the HPMs treated with various detergents, suggesting that the ligand binding sites on ASGPR molecules within the purified native plasma membranes are readily accessible for binding of ligand. The specificity of this reaction was confirmed in the control experiment performed in the presence of free D-Gal which acted as a competitor to the D-Gal immobilized on Sepharose. This control experiment revealed that the precipitation was extensively inhibited by free D-Gal (data not shown). In additional control experiments, incubation of native and solubilized woodchuck HPMs with Sepharose-4B beads, or incubation of woodchuck KPMs with D-Gal-Sepharose beads did not result in the recovery of any protein species with ASGPR activity (data not shown).

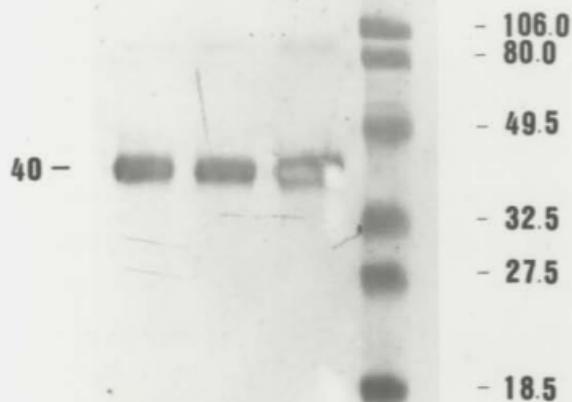
Interestingly, the membrane-bound 47-kDa subunit became detectable when the HPMs were precipitated with D-Gal beads and the blots carrying released wASGPR proteins were probed with monospecific antisera against receptor subunits. As illustrated in Fig. 1.17B, the woodchuck HPM-bound 47-kDa polypeptide band of wASGPR was unveiled by antiserum against the 47-kDa subunit (lanes 5 and 6), while the 40-kDa polypeptide band was recognized by anti-40-kDa-subunit antiserum (lanes 1 and 2). In a control experiment, incubation of woodchuck KPMs with D-Gal beads did not show any detectable precipitates

**Figure 1.17. Identification of ASGPR subunits in woodchuck hepatic plasma membranes by ligand-affinity precipitation.** HPM preparations (340  $\mu$ g per assay) in 0.02% Triton X-100 (lanes A1, B1, B2, B5 and B6), 10 mM Chaps (lane A2) or 0.1% DOC (lane A3) were precipitated with D-Gal-Sepharose beads. Plasma membranes purified from woodchuck kidney (KPMs) were also precipitated by D-Gal beads, as controls (lanes B3 and B7). Bead-bound proteins were released by sample buffer, separated by SDS-PAGE and analyzed by immunoblotting using guinea pig antisera against rASGPR (lanes A1, A2 and A3), the 40-kDa subunit (lanes B1, B2 and B3) and the 47-kDa subunit of rASGPR (lanes B5, B6 and B7). See section 1.2.15 for details of the method. The molecular masses of prestained protein standards (lanes A4, B4 and B8) are indicated on the right side of the panels.

These figures show that both 40- and 47-kDa subunits of wASGPR are detectable in woodchuck HPM by antibodies after precipitation of the membranes with D-Gal conjugated to Sepharose beads.

**A**m.wt.  
kDa

1 2 3 4

m.wt.  
kDa**B**

1 2 3 4 5 6 7 8

m.wt.  
kDa47 -  
40 -

- 106.0  
- 80.0  
- 49.5  
- 32.5  
- 27.5  
- 18.5

with ASGPR specific activity (Fig. 1.17B, lanes 3 and 7). This result indicates that the functional active 47-kDa subunit of wASGPR is expressed in woodchuck HPMs and that, in fact, it appears to present in numbers comparable to the 40 kDa subunit species.

Taken together, the above findings indicate that biologically active 40- and 47-kDa subunits of wASGPR are expressed in woodchuck hepatic plasma membranes. Additional evidence to support this conclusion will be provided in Part III of this thesis, which will show that ligand binding activity of wASGPR on the surface of isolated woodchuck hepatocytes can be inhibited by monospecific antibodies to both 40 and 47-kDa receptor subunits. The evidence will confirm that both subunits of wASGPR are expressed on the surface of woodchuck hepatocytes and are accessible to both natural ligand as well as specific antibodies.

#### 1.4. DISCUSSION

Numerous studies on the characterization of mammalian ASGPRs have revealed that ASGPRs from rabbit, rat and human livers contain Gal-binding sites which can interact with the terminal Gal residues of ASGP. Amino acid sequence comparisons and immunological analysis identified the evolutionary homology between these receptors. Taking these common biochemical and immunologic properties of mammalian ASGPRs into account, we employed ligand affinity chromatography to isolate and purify ASGPR from woodchuck livers. This method was first established for the isolation of ASGPR from rabbit liver and later widely applied for ASGPR isolation from rat and human livers. Using wASGPRs affinity-purified by chromatography on an ASGP (i.e., ASFN) or D-Gal column, we were able to analyze the biochemical and immunologic properties of woodchuck hepatic ASGPRs. The receptors were analyzed both in the purified water-soluble form and in their natural environment as plasma membrane-associated proteins. The data presented here demonstrate that wASGPR consists of two subunits with apparent molecular masses of 40- and 47-kDa, respectively. Both polypeptides are immunogenic to guinea pigs and are immunologically closely related to the rASGPR and rtASGPR. The wASGPR is specifically expressed on the surface of woodchuck hepatocytes and is detectable in purified woodchuck hepatocyte plasma membranes. Although both 40- and 47-kDa subunits of wASGPR exist in the HPM, mainly the 40-kDa subunit polypeptides were detected directly by Western blotting.

A major finding in the present studies is that wASGPR is closely related to rASGPR in terms of receptor subunit constitution, immunological properties and ligand binding specificity. Very similar, if not identical, molecular masses of receptor subunit polypeptides (i.e., 40- and 47-kDa) are consistently seen in all the preparations purified from woodchuck and rabbit livers; purified rtASGPR displayed three subunits with molecular masses of 44, 52 and 66 kDa after separating the receptor proteins by SDS-PAGE (Fig. 1.2).

However, wASGPR differs from rASGPR by characteristics of ligand binding kinetics. The  $K_d$  and  $B_{max}$  of wASGPR is lower than that of rASGPR under the conditions tested. The classical method for the kinetic analysis of receptor-ligand interaction is usually carried out in liquid-phase. The critical step in this method is to separate receptor bound ligand from free ligand (B and F, respectively). For ASGPR analysis, the receptor-ligand complex can be precipitated by  $(NH_4)_2SO_4$  and harvested on glass-fibre discs (Hudgin et al., 1974). The disadvantage of this method is that the non-specific background can be too high (Grant, 1986). Solid-phase assay for receptor and ligand reaction is also used for the binding kinetic studies of membrane receptors (Quesenberry and Drickamer, 1992). The principle for the separation of B and F is simply by pre-immobilization of receptor on plastic plates. Obviously, if the conformation of the receptor is changed when coating the receptor onto the solid phase, the binding between receptor and ligand will be influenced. Due to our inability to decrease the background in the liquid-phase assay, we used a solid-phase assay for the kinetic analysis of wASGPR and  $^{125}I$ -ASFN interaction. Therefore, it is possible that our values could be somehow lower than the true kinetic values for wASGPR. This point will be further addressed in Part III of this thesis (sections 3.3.2 and 3.3.4). That wASGPR had lower  $K_d$  and  $B_{max}$  than rASGPR can be a result due to the species specificity. In this regard, it is clear that hepatic ASGPR distribution is different among mammals (Chang and Chang, 1988). We also found that the recovery of wASGPR from woodchuck livers was much lower (approximately 6-10 fold) than that from rabbit livers. According to Chang and Chang, the total ASGPR concentration in the liver of different mammals varies with a rank order of rabbit ( $423.4 \pm 24.8$  pmol/g tissue) > rat ( $300.2 \pm 26.6$  pmol/g tissue) > guinea pig ( $159.8 \pm 7.1$  pmol/g tissue). From our experience, wASGPR is more likely close to ASGPR from rat or guinea pig rather than from rabbit.

With respect to the antigenic cross-reactivity between wASGPR and rASGPR, it has been shown, as summarized in Table 1.4, that not only can anti-rASGPR or anti-subunits of

**Table 1.4. Summary of the results of Western blotting analysis of purified ASGPR and membrane-bound ASGPR using guinea pig antisera**

Source of ASGPR*	ASGPR subunit polypeptides identified on blots using antiserum against:			
	wASGPR	rASGPR	40-kDa subunit of rASGPR	47-kDa subunit of rASGPR
<b>Woodchuck</b>				
purified ASGPR 40-/47-kDa	+/+	+/+	+/-	-/+
IIPM-bound ASGPR 40-/47-kDa	+/-	+/-	+/-	-/- (-/+) <sup>b</sup>
<b>Rabbit</b>				
purified ASGPR 40-/47-kDa	+/+	+/+	+/-	-/+
IIPM-bound ASGPR 40-/47-kDa	+/+	+/+	+/-	-/+
<b>Rat</b>				
purified ASGPR 44-/52-/66-kDa	+/+/+	+/+/+	+/-	-+/+
IIPM-bound ASGPR 44-/52-/66-kDa	n.l. <sup>c</sup>	+/+/+	n.l.	-+/+

\* - purified ASGPRs or IIPMs are separated on SDS-PAGE gel and transferred onto blot membranes.

<sup>b</sup> - 47-kDa subunit polypeptide of wASGPR in IIPM ASGPR can be recognized by anti-47-kDa antiserum after exposure of IIPM to D-Gal-bands.

<sup>c</sup> - n.l.: not tested.

rASGPR recognize wASGPR, but also that anti-wASGPR reacts with rASGPR. These results indicate that wASGPR and rASGPR share antigenic epitopes. Such cross-reactivity exists between the corresponding subunits from different species, but not between the subunits from the same species; anti-40-kDa-subunit specific antibody recognizes only 40-kDa polypeptide while the antibody to the 47 kDa polypeptide only reacts with the 47-kDa polypeptides of ASGPR. These observations are in good agreement with previous findings that ASGPRs are very conserved molecules with high homology among receptors from different species. According to the amino acid sequence analysis (Drickamer et al., 1984; Spiess and Lodish, 1985b), there is 70-80% homology between the corresponding subunits of rat and human (RHL1 versus HHL1 and RHL2/3 versus HHL2) while only approximately 50 % homology between different subunits from the same species. Thus, it is not surprising that when the composition of rASGPR and wASGPR are similar, they will also have similar antigenic epitopes and, likely, have homologous primary structure. In addition, as identical ligand binding specificity exists in all ASGPRs, the structure of the ligand binding site itself or structures related to the binding site may be similar. This point will be addressed further in Part III of this thesis.

Surface expression of wASGPR on hepatocytes was demonstrated by immunofluorescent staining as well as by ligand binding assay (see Part III for more details). To understand in detail the subunit distribution and receptor function in woodchuck HPMS, purified woodchuck HPMS were analyzed by Western blotting using guinea pig anti-ASGPR or anti-subunit antisera. Results showed that only the polypeptide bands with molecular masses corresponding to those of the affinity purified wASGPR were recognized by these antisera. The specificity of the immunoblot reactions were confirmed by several controls. It was found that recognition of the purified ASGPR and the polypeptides in woodchuck HPM was abolished when the antiserum had been pre-absorbed with affinity-purified ASGPR proteins, indicating that this antiserum was specifically directed against ASGPR and that the

recognized polypeptide band in woodchuck HPM was antigenical cross-reactive with the purified ASGPR (Fig. 1.10, lanes 10 and 11). It was also found that the ASGPR-related polypeptide band recognized by anti-ASGPR antisera was present only in HPMs, but not in the plasma membranes from other organs. Woodchuck KPM and SPM were not reactive with antisera against ASGPR or ASGPR subunits (e.g., Fig. 1.13 and Fig. 1.17).

The biological activity of the HPM-associated wASGPR proteins was examined by D-Gal bead precipitation. The ligand-bound proteins were analyzed by Western blotting using anti-rASGPR and anti-subunit antisera (Fig. 1.17). Controls for the D-Gal precipitation assay were set up by using ligand-free beads or addition of free D-Gal as a competitor. Based on the results of these experiments, we conclude that functional wASGPR molecules are present in the purified woodchuck HPMs. Since both subunits were detectable in woodchuck HPM by this method, both 40- and 47-kDa subunits are most likely involved in the ASGPR function for ligand binding. Alternatively, it is possible that at least one of the subunits is able to bind ligand, but because of the cross-linkage between the two subunits, both of them can be precipitated by the D-Gal beads.

Interestingly, analysis of the woodchuck HPM directly by Western blotting showed only the expression of 40-kDa subunit. The 47-kDa polypeptide could not be directly detected (Fig. 1.10, Fig.13, Fig.14, and Fig.15), unless the membrane-bound receptor had been precipitated from HPMs by D-Gal beads (Fig. 1.17B). At this point, this result may suggest that ASGPR subunit-distribution in woodchuck HPMs is different from that in HPMs of other species tested, since all the subunits of ASGPR in rabbit and rat HPM preparations were directly detectable by Western blotting.

It is worthy of mention that the antigenicity of the 47-kDa subunit in wASGPR prepared from liver homogenate appears to be different from that in membrane-bound wASGPR. The 47-kDa subunit in affinity-purified wASGPR preparations could react with anti-rASGPR antibodies (Fig. 1.9), while the 47-kDa subunit in woodchuck HPMs could not

(Fig. 1.17A) although the HPM had also been exposed to ligand on beads. This difference may suggest that the change in the expression of the antigenic epitopes on the 47-kDa subunits (e.g., due to a conformational modification in the course of the receptor transportation from the cytoplasm to the cell surface).

Ligand-coated beads appeared to have the ability to enrich the 47-kDa polypeptide from woodchuck HPM (Fig. 1.17B). Thus, we can speculate that the higher ratio of 47-kDa:40-kDa subunits than their initial ratio in ligand-free membrane preparations is due to receptor subunit re-organization when receptor binds to the ligand on beads. The ligand may function as a bridge to link several 47-kDa subunits together. Probably, the 47-kDa subunits of wASGPR have high affinity for the ligand although they are present in low amounts in the membranes. If this speculation holds true *in vivo*, the 47-kDa subunit may function as a key regulator, at the level of the plasma membrane, to control the rate of ASGP binding as well as the speed of endocytosis. Of note is that pre-fixed hepatocytes are also able to bind ligand (for details, see Part III). This may suggest that some of the specific ligand binding does not require such re-organization. According to the literature, subunit re-organization has been observed in both human and rat ASGPR gene transfected cells (Braiterman et al., 1989; Henis et al., 1990). It is postulated that re-organization is responsible for the formation of a high affinity ligand binding site. Our observation in wASGPR may provide new evidence to support this notion.

On the other hand, according to the results from the radioimmunoprecipitation assays (Fig. 1.16), both 40- and 47-kDa polypeptides were detectable in woodchuck HPMs and had comparable densities in the autoradiograms, after being precipitated or co-precipitated by antisera against wASGPR, 40-kDa or 47-kDa polypeptides of rASGPR. These results indicate comparable iodinations on both subunits of the membrane-bound wASGPR and suggest that equal amounts of 40- and 47-kDa subunits may exist in the woodchuck HPMs.

Therefore, alternatively, the observation that the 47-kDa polypeptide in woodchuck

HPM became detectable by anti-47-kDa antibodies after precipitation by ligand (Fig. I.17B) could suggest that the 47-kDa subunit undergoes a ligand-dependent conformation change during this precipitation. Such a conformational change could expose the antigenic epitopes which were recognized by our antibodies. In other words, our antisera to the entire ASGPR molecules or ASGPR 47-kDa subunit might not recognize the 47-kDa polypeptide of membrane-bound ASGPR, unless the receptor's structure has been modified by ligand. Such modification could be achieved by using ligand (ASFN or D-Gal) conjugated on beads, but not by free D-Gal since after treatment of woodchuck plasma membrane with free D-Gal the 47-kDa subunit of wASGPR in HPM was still not recognized by any of our antisera (data not shown). Just as configuration is important to the activity of an enzyme, so a change in configuration or conformation of a receptor could be critical for the activation of the receptor. It has been found that receptor-ligand binding involves ligand-dependent conformational changes of the receptor (Leng et al., 1993; Ryu et al., 1993; Zanolari et al., 1992), which may lead to the conversion of the receptor from an inactive (or less active) to an active form and induce some other functions of the receptor, such as signal transduction. In the studies of ASGPR, it has been found that  $Ca^{2+}$  concentration and pH influence the ligand binding activity of chicken ASGPR by modulating receptor conformation (Loeb and Drickamer, 1988). This conformation change is found to correlate with the rate of protease resistance of ASGPR. It is, therefore, likely that such a change may also introduce new antigenic epitopes which can be distinguished by antibodies. Our observations suggest such a ligand-dependent change(s) in the antigenicity of the HPM-bound wASGPR.

## 1.5. CONCLUSIONS

The work on the molecular composition and immunologic properties of the affinity-purified wASGPR reveals that wASGPR is composed of two subunits, with approximate molecular masses of 40-kDa and 47-kDa. Both subunits are immunogenic and share antigenic cross-reactivities with the corresponding subunits of ASGPR from other mammalian species. It is also demonstrated that both subunits of wASGPR expressed on the surface of woodchuck hepatocytes or purified HPMs are accessible to ASGPR-specific antibodies. Further, despite close structural and antigenic similarities between woodchuck and rabbit ASGPRs, woodchucks are able to produce an antibody response against rASGPR. This observation can be utilized in further studies on the determination of the contribution of anti-ASGPR specific immune responses to the pathogenicity of hepadnavirus-induced liver injury in a woodchuck model of hepatitis B.

These studies also suggest that the structure of the 47-kDa-subunit in woodchuck HPM may be important for the antigenic properties and function of wASGPR. The antigenic properties of the hepatocyte membrane-associated ASGPR could be modified due to ligand binding. This could be the first demonstration of a ligand-dependent change in the antigenicity of the hepatocyte surface receptor. Further work remains to be done to clearly understand the biological nature of the 47-kDa-subunit in its natural environment and to reveal the differences of the mechanisms for the induction of antibody responses to the 40-kDa- and the 47-kDa subunits.

**PART II**

**INDUCTION OF AUTOANTIBODIES AGAINST ASGPR  
IN EXPERIMENTAL WOODCHUCK VIRAL HEPATITIS**

**2.1. INTRODUCTION**

**2.1.1. General Mechanisms of Autoimmune Response Induced by Viral Infection.**

Many factors, such as environmental, genetic and hormonal factors, contribute to the induction of autoimmune responses and the development of autoimmune diseases. Viruses are usually considered important environmental agents related to the pathogenesis of autoimmune diseases in their host. Evidence to support this concept has been collected from several experimental animal models of virus-induced autoimmunity, as well as from various clinical investigations. Based on these findings, mechanisms for virus-induced autoimmunity have been suggested (for reviews, see McFarlane, 1991; Schattner and Rager-Zisman, 1990).

The initial studies on this subject began in the 1970s. Clyde and Thomas (1973) found that turkeys infected with mycoplasma developed disease which might be generated by an autoimmune reaction between host antigen and antibody against mycoplasmal antigen. Kuzumaki et al (1974) also found that rats infected with leukemiaigenic viruses would develop autoimmune hemolytic anemia. Following these observations, several experimental models of virus-induced autoimmunity have been established to study the pathogenesis of autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM), myocarditis, polymyositis, demyelinating disease and chronic active hepatitis etc. One example is that a high titer of retrovirus was found in the New Zealand black (NZB) mouse model of systemic lupus erythematosus. The involvement of this virus in the pathogenesis of this autoimmune disease has been demonstrated (Theofilopoulos and Dixon, 1985). Another example is that

experimental inoculation of young mice with coxsackie B3 virus induced persistent myocarditis (Rose et al., 1988). In these mice, heart-reactive autoantibodies directed against cardiac myosin heavy chain, but not against the virus, were detected in the circulation and heart, suggesting an autoimmune mechanism for the induction of myocarditis. Interestingly, the cardiac myosin did not cross react with coxsackie virus. However, immunization of the susceptible animals with cardiac myosin antigen could induce syndromes similar to that of virus-induced myocarditis, suggesting that exposure of this autoantigen after viral infection might be responsible for the induction of autoantibodies in myocarditis.

In addition to studies using animal models, clinical investigations have indicated that viral infections or even virus vaccinations in individuals are associated with the development of autoimmunity. Autoantibodies (e.g., antibodies against nuclear antigens, lymphocytes, and smooth muscle) are commonly detectable in the serum of patients with some viral infections (e.g., Epstein-Barr virus, cytomegalovirus, HBV, HAV, HCV, influenza virus, measles virus, coxsackie virus, human immunodeficiency virus, etc.) (Schattner and Rager-Zisman, 1990). Some autoimmune diseases have been found to be associated with the administration of virus vaccines. For example, the Guillain-Barre' syndrome developed in some individuals after vaccination with influenza or measles vaccines (Grose and Spigland, 1976). Uveitis and myasthenia gravis were also observed in individuals after hepatitis B vaccinations (Fried et al., 1987). On the other hand, a past or ongoing viral infection in patients with autoimmune diseases is commonly observed, such as persistent measles virus genome and high level of antibody to measles in patients with HBV-negative autoimmune chronic hepatitis (Robertson et al., 1987). It was proposed that rheumatoid arthritis is associated with infection by Epstein-Barr virus, parvovirus B19, or retroviruses (Schattner and Rager-Zisman, 1990).

However, a definite demonstration that viruses have a primary role in the pathogenesis of autoimmune diseases is still lacking. In addition, it is still a matter of debate as to how viruses are involved in the process of autoimmunity in their hosts. One possibility is that host

autoimmune responses are triggered by virus through polyclonal B cell activation, effects on immuno regulatory cells (e.g., T suppressor) or generation of cytokines (IFN, TNF, etc.). For example, it has been demonstrated that Epstein-Barr virus infected B cells are functionally activated and can produce autoantibodies recognizing autoantigens in multiple organs (Garzelli et al., 1984). Another important mechanism of viral-induced autoimmunity is molecular mimicry. Antibodies against viral antigens recognize self-antigens due to the antigenic cross-reactivity between virus and host components. The appearance of cross-reactive antibody could lead to tissue injury, formation of immune complexes with self and viral antigens, and generation of anti-idiotypic antibodies. For example, a sequence of 8 to 10 amino acids in the encephalitogenic site of myelin basic protein is found to be identical with a segment of measles virus P3 and HBV polymerase. This myelin basic protein determinant has been identified capable of eliciting autoreactivity and autoimmune disease (i.e., experimental allergic encephalomyelitis) (Fujinami and Oldstone, 1985). Finally, infection by virus can also modify the expression of autoantigens by exposure of sequestered autoantigens, modification of antigen on the cell surface and induction of novel antigens. Such alteration may induce the induction of autoimmune responses and lead to the damage of infected cells. For example, a cell surface protein was found to be modified by influenza virus and then recognized by influenza virus-specific cytotoxic T cells (Bennink et al., 1982). Changes in the plasma membrane of skin fibroblasts were also observed after coxsackie virus B3 infection (Lutton and Gauntt, 1986).

### **2.1.2. Autoantibody Response in Liver Disorders.**

In 1957, Gajdusek found that antibodies in the sera of patients with liver disorders could react with the extract of normal human liver (Gajdusek, 1957). Since then, many research groups have studied the occurrence and pathogenic role of autoantibodies in hepatic and other disorders. Autoimmune hepatitis (AIH) (Johnson and McFarlane, 1993), which

was also called autoimmune chronic active hepatitis (ai-CAH), and primary biliary cirrhosis (PBC) are the typical autoimmune diseases in which the liver becomes the primary target organ for the autoimmune responses induced by unknown etiologic factors. Liver-specific or non-organ-specific autoantibodies are associated with these diseases and some of these antibodies have been used as diagnostic markers, although their pathogenic role and their relation to the severity of liver injury are not clear (Meyer zum Buschenfeld et al., 1990). Autoantibodies are also frequently detected in patients with viral hepatitis, in particular, induced by HBV infection (McFarlane, 1991). These autoantibodies, generally, can be divided into two categories according to their antigenic specificity: non-organ and liver-specific.

#### 2.1.2.1. Non-organ-specific autoantibodies.

Non-organ-specific autoantibodies identified in the sera of patients with liver disorders mainly include antibodies to nuclear antigens (anti-nuclear antibodies, ANA), smooth muscle (anti-smooth muscle antibodies, SMA), liver-kidney microsomal antigens (anti-liver-kidney microsomal antibodies, LKM antibodies) and mitochondrial antigens (anti-mitochondrial antibodies, AMA). Most of these antibodies react with intracellular antigens which are not accessible to the antibodies, unless they are occasionally exhibited on the cell surface or released into the circulation. In the following paragraphs, a general description of these non-organ specific autoantibodies observed in patients and, relevant to the present study, in woodchucks with WHV infection will be given according to several reviews (Meyer zum Buschenfelde et al., 1990; McFarlane, 1991; Peters et al., 1990) and report (Dzwonkowski and Michalak, 1990).

ANA is one of the autoantibodies most frequently detected in sera or  $\gamma$ -globulin fractions from patients with different liver disorders, such as AIH, PBC, viral hepatitis and some drug-associated hepatic disorders. A high frequency of ANA is also observed in

systemic lupus erythematosus (SLE). Double-stranded DNA, RNA and histone have been identified as the autoantigen for ANA. So far, there is no direct evidence that ANA can induce autoimmune liver diseases.

SMA were first detected in the sera of patients with chronic active hepatitis. Since then, they have been found in patients with different hepatic or non-hepatic diseases. Up to 80% of patients with acute and 50% of patients with chronic HBV infection were found to be SMA-positive, while 50% of patients with AIH developed SMA. It has been demonstrated that the major autoantigen recognized by sera of patients with AIH type 1 is F-actin (actin filaments), a microfilament protein. Because these antibodies are common in the sera of patients with a variety of autoimmune diseases, it is unlikely that there can be a liver-specific etiologic factor involved in the pathogenesis of liver injury.

LKM antibodies have been found in patients with different liver disorders. Microsomal antigens, cytochrome P-450 2D6 or cytochrome P-450 2C9, were identified as the autoantigens for LKM-1 and LKM-2 autoantibodies. Another microsomal antigen in family I uridine diphosphate-glucuronosyl transferases (UGT) is recognized by LKM-3 autoantibodies. Appearance of LKM antibodies is considered a marker for a particular type of autoimmune hepatitis. However, the role of LKM antibodies in the pathogenesis of the disease remains controversial. The presence of cytochrome P-450 on isolated rat HPMs was detected by Loeper et al. (1990), suggesting that LKM antibody-mediated hepatocyte lysis may contribute to the liver injury. In contrast, Yamamoto et al. (1993) found that the cytochrome P-450 2D6 reacting with LKM-1 autoantibodies could not be expressed on the surface of the hepatocytes. Therefore, the mechanisms of LKM antibody-mediated liver damage remains to be clarified.

A high frequency (80-95%) of AMA was detected in PBC and connective tissue disorders, while a low frequency (10-25%) was observed in chronic active hepatitis, and occasionally in patients with acute hepatitis. A 70-kDa protein identified as the E2

component of a pyruvate dehydrogenase multienzyme complex of mitochondria and a 48-kDa protein on the inner mitochondrial membrane were found to be the autoantigens for AMA. These autoantigens are highly conserved during evolution and cross-react with enterobacteria. Therefore, it is suggested that bacterial, parasitic or viral infection may induce AMA due to antigenic mimicry.

Although all the above mentioned autoantibodies have been encountered in liver diseases induced by HBV infection, the spectrum, prevalence, and dynamics of related autoimmune response are difficult to assess in patients. This is mainly because of inability to determine precisely the moment of virus invasion and the status of autoimmunity prior to infection, and to conduct longitudinal studies in a clinical situation. In 1990, Dzwonkowski and Michalak evaluated the significance of the non-organ specific autoantibodies (e.g., SMA, AMA, ANA and antibodies against the brush border of proximal kidney tubules) in predicting the occurrence and outcome of viral infection in a woodchuck model of hepatitis B. In this investigation, the authors analyzed 646 sequential serum samples from 18 animals with experimental WHV infection and 8 with natural WHV infection. Results demonstrated that WHV invasion always induced non-organ specific autoimmune response, especially SMA. The appearance of SMA preceded the occurrence of serological markers of viral infection, suggesting that the induction of the autoantibodies is unlikely a consequence of WHV-induced liver injury, but is mediated by other mechanisms (e.g., virus-induced polyclonal B cell activation). In addition, it was found that the SMA dynamics did not correlate with viral serological markers and disease development, indicating that the dynamics of SMA, as well as other non-organ-specific autoantibodies are not good predictors of the outcome of acute infection or the progression to chronic hepatitis.

#### 2.1.2.2. Liver-specific autoantibodies.

As mentioned in the above section, antibodies against non-organ-specific autoantigens

are detectable in a variety of disorders. It is therefore unlikely that these antibodies may be responsible for liver-specific injury. When the autoantigens are liver-specific, the consequence of having autoantibodies may be different. Appearance of liver-specific autoantibodies could be a marker for the damage of liver cells or for the disorder of immunoregulation. On the other hand, liver-specific autoantibodies may contribute to the pathogenesis of hepatocyte injury, especially when they are directed to an autoantigen expressed on the surface of liver cells. In an attempt to identify liver-specific autoantigens, isolated hepatocytes, purified hepatocyte plasma membranes and purified hepatocyte cytoplasmic components have been used as substrates to test the autoimmune reactivities of sera from patients with various liver disorders (e.g., Hopf et al., 1974, 1975). So far, only a few liver-specific components have been described as important potentially pathogenic autoantigens.

One of the liver-specific components is "liver specific proteins" (LSP), which has been purified from human liver homogenate (Meyer et al., 1972). Antibodies specific for such a preparation were correlated with the clinical severity and histological changes of liver tissue in patients with acute and chronic liver diseases (Manns et al., 1980). High frequency of anti-LSP autoantibodies has been detected in patients with autoimmune hepatitis, viral hepatitis and other liver disorders. The autoantigens in LSP preparations were identified as a mixture of components, so called liver-specific membrane lipoproteins. In this mixture, the macromolecular antigens could be species-specific, species cross-reactive, liver-specific and non-organ-specific (Lebwohl and Gerber, 1981; Manns et al., 1980; McFarlane and Williams, 1980). It has also been shown that anti-LSP can serve as a serologic marker to predict the ongoing relapse of AIH after cancellation of immunosuppressive treatment (McFarlane et al., 1984b). So far, ASGPR is the only component in the macromolecule mixture of LSP that has been identified. Anti-ASGPR autoantibodies will be discussed later in detail.

In addition, a protein in purified hepatocyte plasma membranes was proposed as a

candidate target autoantigen for antibodies in the sera of patients with AIH (Swanson et al., 1990). This protein has an apparent molecular mass of 60 kDa and seems to be liver-specific, but not species-specific. Another protein in purified liver plasma membranes has a molecular mass of 26-kDa, and it is also recognized by antibodies in sera from patients with AIH (Hopf et al., 1990). This protein was identified as a species cross-reactive antigen without any relation to other known autoantigens in liver. The role of these proteins in the immunopathogenesis of liver disorders is unknown and the characterization of these proteins remains to be done.

### **2.1.3. Autoimmune Responses Against ASGPR.**

#### **2.1.3.1. Autoantibodies against ASGPR.**

The relationship between autoimmune responses against cellular receptors and autoimmune diseases has been a very interesting subject, because reactions between antibodies and receptors may inhibit receptor biological functions (e.g., ligand binding, signal transmission), induce destruction of cells (e.g., antibody-mediated cytotoxicities can be induced when antibodies react with receptors expressed on the surface of cells) or induce stimulation. Studies in many autoimmune diseases suggest that anti-receptor autoantibodies do play important pathogenic roles. For example, it is postulated that antibodies to thyroid-stimulating hormone receptor in thyroid diseases (Graves' hyperthyroidism), antibodies to acetylcholine receptor in myasthenia gravis, and antibodies to insulin receptor in insulin-resistant diabetes mellitus play the primary roles in the pathogenesis of these autoimmune diseases (Abbas, et al., 1991). It is well known that hepatocytes are not only the producer of a variety of physiologically important compounds, but also the target and the metabolic center of many substances. Thus, a large number of receptors are presented on the surface of hepatocytes, such as receptors for vitamins, hormones and many metabolites. Since LSP was identified as the plasma membrane protein complex (Hopf et al., 1974), it would contain

some of the hepatocyte-specific receptors. ASGPR became the first candidate in the search for these liver-specific and autoantigenic receptors, because it was a receptor clearly identified as being present on the surface of hepatocytes and appeared to be expressed mainly in the liver. McFarlane et al. (1984a) addressed this subject and identified ASGPR as a component of LSP. They purified ASGPR from rabbit and human livers and tested their antigenic properties using antibodies from guinea pigs immunized with human or rabbit LSP. Results from both RIA and ELISA indicated that anti-LSP antibodies reacted strongly with purified ASGPR. This reactivity could be abolished after absorption of the antibodies with either LSP or ASGPR proteins, but not with kidney homogenate, indicating that ASGPR was a liver-specific, species cross-reactive antigen and a constitutional component of LSP. Subsequently, ASGPR was shown to be an autoantigen which could be recognized by autoantibodies with LPS reactivity from patients with liver disorders (McFarlane et al., 1985; Treichel et al., 1990). Of note is that *in situ* perfusion of isolated rat livers with polyclonal antibodies against purified rASGPR showed that ASGPR on the hepatocyte surface was in fact accessible for recognition by specific antibodies in *in vivo* conditions (McFarlane et al., 1990). Furthermore, the deposition of antibodies showed a localization pattern which was similar to that of piecemeal and intralobular hepatocellular necrosis observed in AIH and chronic active hepatitis B, suggesting the possibility that anti-ASGPR autoantibodies may contribute to these hepatocellular injuries.

In the past few years, the occurrence of anti-ASGPR antibodies in patients has been extensively investigated. Using purified rabbit ASGPR, McFarlane et al. (1986) detected anti-ASGPR activity by RIA in sera from 129 European patients with acute or chronic liver diseases. 83% of patients with AIH had anti-ASGPR in their sera. The titer of these antibodies appeared to be related to the inflammatory activity of the disease, based on the histological examination of liver biopsies. A similar frequency (73%) of anti-ASGPR was detected in patients with chronic active hepatitis B. The titers of anti-ASGPR in patients with

chronic active hepatitis B were found to be lower than those in patients with AIH. Also, anti-ASGPR antibodies were detected in the sera of patients with acute HBV hepatitis (35%) and other liver disorders (22%) (e.g., PBC). Another group of patients (421 individuals with different forms of inflammatory liver diseases and 288 individuals with other extrahepatic disorders) were investigated by Treichel et al. (1990) using an ELISA with purified human, rat and rabbit ASGPRs as target antigens. They found a high occurrence of autoantibodies against human ASGPR in patients with AIH (50%), especially in those with high inflammatory activity (88%). Only 4 to 15% of patients with other liver disorders (e.g., PBC, viral hepatitis and alcoholic liver disease) had antibodies to human ASGPR detectable by ELISA. On the other hand, antibodies to rat or rabbit ASGPR were detected more frequently in patients with non-AIH liver disorders. Their occurrence appeared to be correlated with inflammatory activity and was not related to the disease etiology. In respect to HBV infection, the occurrence of autoantibodies to human, rabbit and rat ASGPRs was 4%, 21% and 26% in patients who were HBsAg-positive. In addition, up to 10% of patients with non-hepatic autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus and systemic vasculitis) were found to be anti-ASGPR-positive. The authors suggested that different epitopes on ASGPRs from different species were recognized by the anti-ASGPR autoantibodies from patients with different liver disorders. Thus, it is speculated that the mechanisms for the induction of anti-ASGPR antibodies in AIH must differ from that in other liver diseases (Poralla et al., 1991). Since antibodies to non-species-specific epitopes on ASGPR were detected in patients with liver disorders without etiologic-specificity, it is more likely that these autoantibodies could be induced in a non-specific manner, such as by polyclonal B-cell stimulation. In contrast, anti-ASGPR, specific for human epitopes, appears to be more restricted to a specific disease (i.e., AIH) and thus, more likely induced by immune responses to specific stimulation, such as hepatocyte damage. This speculation may be indirectly supported by a recent observation that the immunoglobulin subclass of anti-human

ASGPR antibodies are IgG2 in patients with AIH and IgG4 in patients with HBV or hepatitis C virus-related chronic hepatitis (Treichel et al., 1993).

The specificity of anti-ASGPR autoantibodies from patients has also been studied by Western blotting (Treichel et al., 1990). Results show that these autoantibodies recognized the 46-kDa subunit of human ASGPR. One interesting observation (Treichel et al, 1992) is that anti-ASGPR antibodies in patients with different hepatic diseases are heterogeneous. Thus, the autoantibodies from patients with AIH and viral hepatitis appear to recognize different antigenic epitopes on the human ASGPR. This finding may further suggest that the mechanisms of the processing of ASGPR antigens and the generation of anti-ASGPR autoantibodies could be different in different liver diseases.

#### 2.1.3.2. Cellular immune responses to ASGPR.

In contrast to the studies on humoral immune response to ASGPR, there has been much less progress in the investigation of the cellular immune responses to this receptor. O'Brien et al. (1986) first detected circulating T lymphocytes specific for ASGPR in patients with AIH by using a lymphocyte migration inhibition assay. ASGPR-specific T cell clones were later established from the lymphocytes of patients with AIH (Li et al., 1990).

ASGPR-specific T cells were also detected in intrahepatic lymphocyte infiltrations (Lohr et al., 1991; Treichel et al, 1992a), suggesting that local T cells might play a role in the induction of specific immune responses to ASGPR. In this respect, it is worth noting that most of the established T cell clones were CD4-positive cells and some of them were able to induce spontaneous anti-ASGPR antibody production. Some of the T-cell autoactivities were HLA class II-restricted, since recognition of autologous antigen-presenting cells by these T cells could be blocked by monoclonal antibody to HLA class II molecules (Lohr, et al., 1991). The role of ASGPR-specific T cells in the pathogenesis of hepatocellular injury requires extensive study both in *in vitro* and *in vivo* experimental systems.

## **2.2. MATERIALS AND METHODS**

### **2.2.1. Woodchucks and Serum Samples.**

Woodchucks (n=55, 20 male and 35 female) were maintained as described in section 1.2.1. Woodchucks (n=43) were experimentally infected with WHV by intravenous inoculation with a WHV infectious pool (Michalak and Churchill, 1988). According to the criteria described previously, the diagnosis of the status of viral infection and disease development were based on the serological investigations of the WHV DNA, WHV surface antigens (WHsAg), antibodies to WHsAg and WHV core antigen (WHcAg) (anti-WHs and anti-WHc, respectively), as well as the histological examination of liver biopsies (Michalak et al., 1988; 1990; Pardoe and Michalak, 1995). Acute WHV infection was diagnosed when WHsAg and anti-WHc were detected in the circulation of animals after inoculation with WHV. Resolution of acute WHV infection upon clearance of WHsAg from the circulation within six months after viral inoculation was classified as self-limited acute hepatitis (SLAH) (n=32). Chronic viral hepatitis (CH) (n=11) was diagnosed when WHsAg remained in the circulation for more than six months after the first appearance of the antigen. CH was also confirmed by histopathological examination of liver biopsies obtained at 6- to 12-month intervals or at autopsy.

Serum samples (n=55) collected from the above group of 55 animals before inoculation of WHV were designated as zero samples. All these sera were WHV DNA negative by a nested polymerase chain reaction with WHV core gene specific primers (Pardoe and Michalak, 1995) and nonreactive for anti-WHc by specific ELISA (Churchill and Michalak, unpublished). Serum samples (n = 218) of 13 animals from the above group, were collected sequentially at bi-weekly or monthly intervals for a period of up to 22 months (62 ± 20 weeks) after experimental inoculation with WHV. Among these 13 animals, 7 (3 male and 4 female) displayed SLAH and 6 (3 male and 3 female) developed CH. All sera were

stored at -20°C until testing.

### **2.2.2. Protein A Double-Diffusion Assay.**

According to Jensen et al. (1978) and McFarlane et al. (1985), insoluble protein A could be used to precipitate the immunocomplexes of ASGPR and anti-ASGPR antibody in a radioimmunoassay. This method was originally developed to determine anti-ASGPR activity in human sera. To identify whether protein A can be used to precipitate immunoglobulins (Ig) in woodchuck sera, the binding between protein A and sera from different species were tested by a modified Ouchterlony double-diffusion assay (Harlow and Lane, 1988).

#### **Materials:**

- (1) Sera from woodchucks, humans, guinea pigs and rats were centrifuged at  $3000 \times g$  for 10 min to remove possible aggregated debris. Protein concentration was determined (see section 1.2.16) and adjusted to 1.0 mg/ml using PBS. In some instances, sera were two-fold diluted in PBS from 1 mg protein/ml.
- (2) PBS (section 1.2.2)
- (3) BSA solution: 1.0 mg/ml BSA in PBS.
- (4) Protein A solution: 0.2% (w/v) soluble protein A (Sigma) in PBS.
- (5) Agarose solution: 1.4% (w/v) agarose (Sigma) in PBS.
- (6) Staining solution: Coomassie brilliant blue solution (section 1.2.18)
- (7) Destaining solution: section 1.2.18.

#### **Method:**

An Ouchterlony slide was prepared by pipetting 3 ml of melted agarose gel onto a clean glass slide (76 × 26 mm) to form a layer of about 2 mm thick. After the agarose gelled at room temperature, small wells were carefully cored in the gel in a pattern of equal distance between the center well and the surrounding wells.

Serum samples (5  $\mu$ l each) were added in each of the surrounding wells, while 5  $\mu$ l of protein A solution was applied in the center. PBS and BSA solution were used as controls and added in the surrounding wells. After incubation at 37°C in a humid atmosphere overnight till the appearance of precipitin lines, proteins on the gel were washed and stained by Coomassie brilliant blue (see section 1.2.18). The gel was stained for 15 min, then gently rinsed using three changes of destaining solution for about 30 min, and air-dried at room temperature.

### 2.2.3. Radioimmunoassay (RIA) for Detection of Woodchuck Anti-ASGPR Antibodies.

This assay was employed to detect anti-ASGPR antibodies in woodchuck sera or in the antisera from guinea pigs immunized with wASGPR or rASGPR, following the method described by Jensen et al. (1978) and McFarlane et al. (1985).

Materials:

- (1) Reaction buffer: 1 M NaCl, 5 mM EDTA, 0.1% (w/v) BSA, 0.2% (v/v) Triton X-100 and 0.02% (w/v)  $\text{NaN}_3$  in 50 mM Tris-HCl buffer, pH 7.8.
- (2) Sucrose solution: 250 mM sucrose in reaction buffer.
- (3) Insoluble protein A suspension: 1% (w/v) staphylococcal dried cells (*S. aureus* Newman D<sub>1</sub>C, Sigma) in reaction buffer. The cells were suspended in 100 volumes of reaction buffer, washed twice and adjusted to 1% (w/v) with the same buffer. Suspensions were prepared immediately before use.
- (4) Serum samples: 10  $\mu$ l of woodchuck serum was diluted to 1:50 in reaction buffer. Samples were prepared on the day of the assay and were heat-inactivated at 56°C for 30 min before the assay. In some instances, serum was serially diluted in reaction buffer from 1:25 to 1:800.
- (5) Positive controls: guinea pig anti-rASGPR antiserum (section 1.1.8), guinea pig anti-rASGPR antiserum kindly provided by Dr. B.M. McFarlane (Institute of Liver Studies, King's

College School of Medicine and Dentistry, London, UK) and woodchuck sera found anti-ASGPR reactive when pre-tested by Dr. B.M. McFarlane using the same RIA protocol.

(6) **Negative controls:** a pool of sera from two healthy guinea pigs and woodchuck sera without anti-ASGPR antibody reactivity which had been pre-tested by Dr. B.M. McFarlane.

(7) **<sup>125</sup>I-rASGPR solutions:** 1 ng <sup>125</sup>I-rASGPR with specific radioactivity of  $1 \times 10^5$  cpm/ng protein (section 1.2.12) was diluted in 25  $\mu$ l of reaction buffer. In some instances, the concentration of <sup>125</sup>I-rASGPR ranged from 0.5 ng to 2 ng/25  $\mu$ l.

**Method:**

25  $\mu$ l of tested serum samples, control serum samples or reaction buffer (as blank) were incubated with 25  $\mu$ l of <sup>125</sup>I-rASGPR in a 5-ml plastic tube at 4°C overnight. Then, after adding 100  $\mu$ l of insoluble protein A suspension, the reaction continued 1 hour at room temperature. Finally, the reaction volume was adjusted to 1 ml with addition of 850  $\mu$ l of cold sucrose solution. After centrifuging at  $1,130 \times g$  for 5 min, 500  $\mu$ l of the upper supernatant (U) of the reaction was transferred to another tube and both the upper and remaining solution with pellet (R) were counted in a gamma counter (LKB).

Total antibody activity in each sample was expressed as percent of <sup>125</sup>I-rASGPR binding according to the following calculation: percent binding (%) =  $(R - U)/(R + U) \times 100$ .

All serum samples and control samples were tested in triplicate. For each determination, the background radioactivity detected in the blanks containing only reaction buffer was subtracted. The intra- and inter-assay variation of the mean values obtained from positive and negative controls were 10% or less in the experiments performed at each time.

#### **2.2.4. Western and Dot Blot Analyses.**

To determine specificity of woodchuck anti-ASGPR autoantibodies against ASGPR subunits, a Western blotting technique was carried out as described in section 1.2.21.

A similar Western blot procedure was employed for the analysis of the cross-reactivity

between ASGPR and WHV proteins. For this purpose, 0.25  $\mu\text{g}$  rASGPR protein/lane, 0.5  $\mu\text{g}$ /lane of WHsAg or WHV virion proteins were applied for analysis. The affinity-purified wASGPR and rASGPR were prepared as described in section 1.2.6.2. The WHsAg and WHV virions were isolated from the sera of chronic WHV carriers by other researchers in this laboratory (Michalak et al., 1988, 1989). A dot-blot assay was also performed to examine the possible cross-reactivity between ASGPR and WHV antigens. The assay was carried out in the same way as described for Western blotting, with the exception that the samples were immobilized onto nitrocellulose membranes by microfiltration using a Bio-Rad apparatus. Briefly, 0.36 ~ 0.8  $\mu\text{g}$  proteins of wASGPR, rASGPR, WHsAg or WHV virions in 100  $\mu\text{l}$  of PBS were spotted per dot. In these experiments, the nitrocellulose membranes were probed with guinea pig anti-rASGPR antiserum (1:400) or rabbit anti-WHs antiserum (1:400). The reaction conditions and reagents used in this experiment were described in section 1.2.21.

#### **2.2.5. Statistical Analysis.**

Student's nonpaired *t* test was used to compare the mean values of anti-ASGPR antibody activity for tested woodchuck groups. Wilcoxon signed rank test was used to compare the data of anti-ASGPR antibody activity in the samples at each time point during the course of follow-up of animals with WHV infection. Fisher's exact test was used to compare the ratio of disease development in different groups. In all tests, two-sided *P* values less than 0.05 were considered significant. Analyses were performed using the GraphPad InStat software package (Graph Software, San Diego, CA).

## 2.3. RESULTS

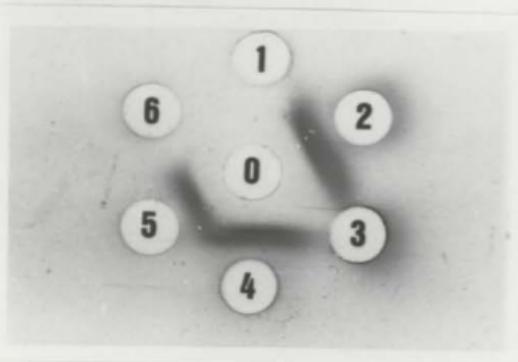
### 2.3.1. Detection of Anti-ASGPR Autoantibodies in Woodchuck Sera by RIA.

#### 2.3.1.1. Establishment of the assay conditions.

RIA was originally developed for the detection of human anti-ASGPR (McFarlane, *et al.* 1985) and was adopted in the course of the present study for the identification of anti-ASGPR activity in woodchuck sera. In one of the preliminary experiments, the reactivity between woodchuck serum and protein A was determined by using a double-diffusion assay in which sera from woodchuck and other species were tested. The results, for example in Fig. 2.1, showed that protein A recognizes woodchuck, guinea pig and human serum (positive controls) with similar efficiency, while, as expected, it did not recognize the rat sera (the negative control). The results from protein A affinity chromatography experiments (see section 3.3.1) further confirmed the highly efficient binding between protein A and woodchuck Ig fractions. To determine the appropriate dilution of woodchuck serum and the optimal concentration of radiolabelled ASGPR for the RIA, another two preliminary experiments were performed. First, the anti-ASGPR-positive and negative woodchuck sera at dilutions ranging from 1:25 to 1:800 and the  $^{125}\text{I}$ -ASGPR at concentrations ranging from 0.5 to 2 ng per assay were tested. Some of the sera used in these experiments had been pretested by Dr. Barbara M. McFarlane (Institute of Liver Studies, King's College School of Medicine and Dentistry, London, U.K.) using a RIA for human anti-ASGPR detection (McFarlane *et al.*, 1985) and served as positive or negative controls. The affinity-purified rASGPR radiolabelled with  $\text{Na}^{125}\text{I}$  ( $2.5 \times 10^4$  cpm/ng, see section 1.2) was used as the target antigen. The reasons for choosing rASGPR instead of wASGPR were that rASGPR is more readily available than wASGPR and that both receptors are highly antigenically cross-reactive (see results in section 1.3.1, Table 1.2 and Fig. 1.2). Results of these preliminary experiments revealed that RIA was a reliable method to identify anti-ASGPR activity in woodchuck sera,

**Figure 2.1. The reaction between woodchuck serum and protein A detected by double-diffusion assay in agarose.** Serum samples (1 mg serum protein/ml) and protein A (0.2%, w/v) were added to the wells (5  $\mu$ l/each) on the Ouchterlony slide. 0, soluble protein A; 1, PBS (blank); 2, human serum (positive control); 3, rat serum (negative control); 4, woodchuck serum; 5, guinea pig serum (positive control); 6, BSA (negative control, 1 mg/ml). After incubation at 37°C overnight, the gel was stained with Coomassie blue, destained and dried, as described in section 2.2.2.

This figure shows that protein A recognizes a component in woodchuck serum with an efficiency similar to that of the reaction between protein A and IgG fractions in human and guinea pig sera.

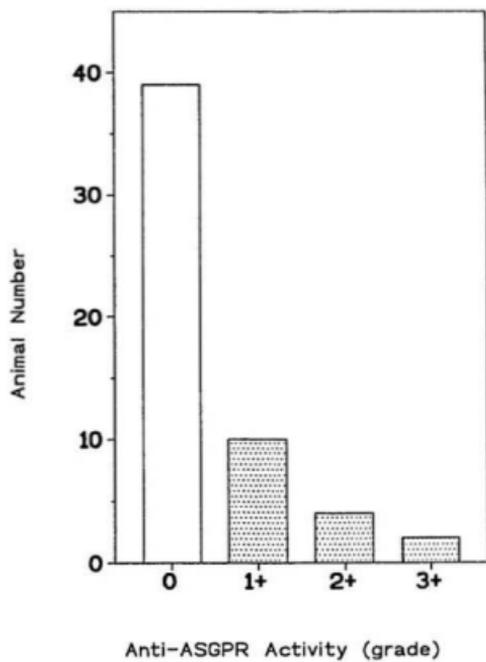


since the positive and negative control serum samples had been well distinguished and the obtained values were highly reproducible. On the basis of these evaluations, standard assay conditions for the testing of anti-ASGPR in woodchuck sera were used (see section 2.2.3 for details). In general, these assay conditions were similar to those determined for the detection of anti-ASGPR in human sera (McFarlane et al., 1985), thus 1 ng of  $^{125}$ I-rASGPR and woodchuck serum at a dilution of 1:50 were selected.

#### 2.3.1.2. Determination of the prevalence of anti-ASGPR antibodies in a healthy, WHV-noninfected woodchuck population.

To evaluate the prevalence of anti-ASGPR autoantibodies in woodchucks with experimental WHV infection, we measured the anti-ASGPR activity in healthy WHV-free animals to establish a normal value as a cut off line. Fig. 2.2 gives the distribution of anti-ASGPR autoantibody activity in the sera (zero samples) of 55 woodchucks at different ages without previous exposure to WHV. The mean of anti-ASGPR activity in this group of animals, as detected by RIA and expressed as percentage of  $^{125}$ I-rASGPR bound, was 3.3%  $\pm$  1.2 (mean  $\pm$  2  $\times$  standard error of mean, SEM). On this basis, we defined values above 4.5% (mean + 2 SEM) as anti-ASGPR autoantibody positive. As shown in Fig. 2.2, the majority of healthy, WHV-noninfected animals (39 of 55 animals; 70.9%) were anti-ASGPR autoantibody negative. Sera from these woodchucks gave values below 4.5%, which corresponding to an antibody titer below 1:50 (or grade 0). Some animals (10 of 55; 18.2%) were moderately positive for anti-ASGPR ranging between 4.5 and 8.0%, corresponding to titers below 1:200 (or grade 1+). Four woodchucks (7.6%) exhibited strong ASGPR-binding activity ranging between 8.1 and 15%, corresponding to titers below 1:400 (or grade 2+). Two others (3.6%) showed very high anti-ASGPR activity with values above 15% which corresponded to titers above 1:400 (or grade 3+). Overall, the results revealed that the prevalence of anti-ASGPR autoantibodies in WHV-noninfected animals was 29.1%.

**Figure 2.2. Distribution of anti-ASGPR autoantibody activity in the sera of 55 healthy woodchucks without previous exposure to WHV.** Anti-ASGPR antibody activity in woodchuck sera was determined by RIA using <sup>125</sup>I-rASGPR as a target antigen, as described in section 2.2.3. The blank bar represents the number of animals whose sera were anti-ASGPR negative (grade 0) and the speckled bars represents the number of animals whose sera were anti-ASGPR positive (grades 1+ to 3+), as assessed according to the cut off value of anti-ASGPR activity (4.5% of ASGPR binding) as defined in section 2.3.1.2.



### 2.3.1.3. Determination of the induction and dynamics of anti-ASGPR autoantibodies after WHV inoculation and in the course of WHV infection.

To study the relation between WHV invasion and the appearance of anti-ASGPR autoantibodies, serial serum samples from 13 animals collected before and after inoculation with WHV were analyzed by RIA for the activity of anti-ASGPR autoantibody. These animals were at different ages. Based on disease development, the animals were divided into two groups. One group included 7 woodchucks that displayed a serologic pattern indicating recovery from acute WHV infection (SLAH) and the other included 6 animals that developed CH (see section 2.2.1 for details of the animals).

Table 2.1 summarizes the characteristics and occurrence of autoantibodies against ASGPR in relation to the appearance of WHsAg in these animals. Examples of the changes over time in the activity pattern of autoantibodies against ASGPR in individual animals are illustrated in Fig. 2.3. The comparison of anti-ASGPR activity before and 6 to 8 weeks after WHV inoculation in the sera of all the animals is shown in Fig. 2.4. The overall patterns of serum anti-ASGPR activity in animals with SLAH and those with CH during the follow-up period are shown in Fig. 2.5 which are the computer generated graphs of the mean values of anti-ASGPR autoantibody activity. The following issues were revealed.

(1) As shown in Table 2.1 and Fig. 2.4, among the 13 tested animals, 9 (i.e., WM.1 to WF.6, and WF.8 to WM.10) were anti-ASGPR nonreactive before WHV injection. After WHV inoculation, anti-ASGPR became detectable in 8 of these animals, the other one (WF.6) which developed SLAH tested negative throughout the entire follow-up. In general, in these woodchucks, anti-ASGPR response was induced after WHV infection at a ratio of 88.9% (8 of 9). In addition, the overall difference between the values of anti-ASGPR activity before inoculation and that from postinoculation was statistical significant (*t* test,  $P < 0.05$ ).

**Table 2.1. Occurrence of WIsAg and autoantibodies against ASGPR in the sera of woodchucks with experimental WHIV infection**

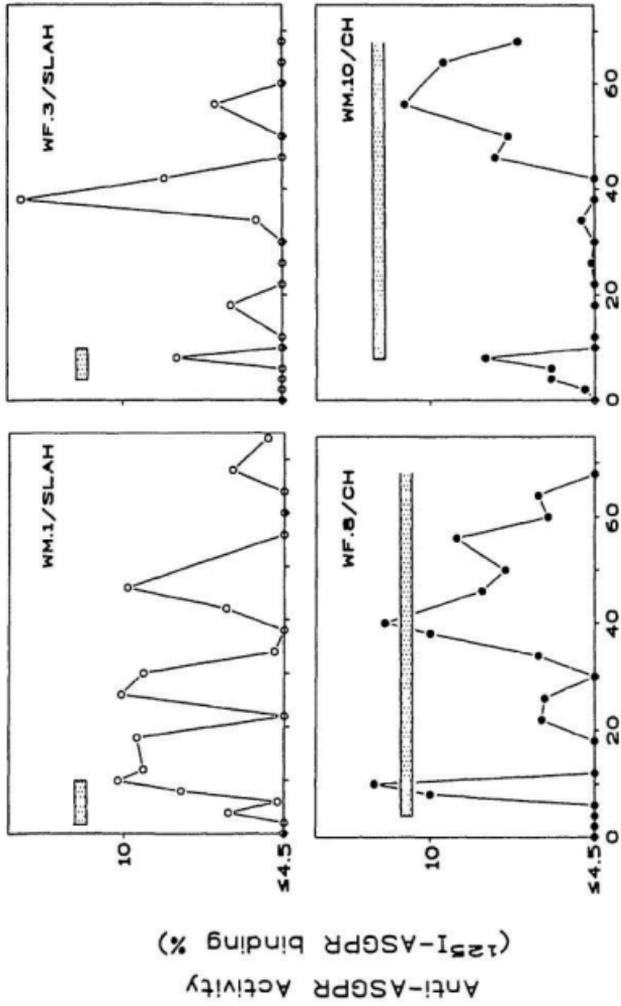
Category of disease and animal No.*	Observation period (weeks)	Appearance of WIsAg (w.p.i.) <sup>b</sup>	Anti-ASGPR antibodies	
			Appearance (w.p.i.)	Number of sera positive / total tested
<b><u>SLAH</u></b>				
WM.1	74	2	4	13/19
WF.2	74	4	4	9/20
WF.3	68	4	8	6/19
WF.4	74	4	22	5/20
WM.5	42	4	2	13/13
WF.6	74	8	n.d. <sup>c</sup>	0/20
WM.7	42	4	0	11/12
<b><u>CH</u></b>				
WF.8	68	4	8	13/19
WF.9	40	6	4	9/12
WM.10	68	8	2	11/18
WF.11	96	2	0	8/13
WF.12	72	6	0	7/19
WF.13	18	2	0	3/14
mean ± SD	62 ± 20	4 ± 2	4 ± 6	

\* - Diagnosis of WHIV infection was based on the criteria described in the text (section 2.2.1.). SLAH, Self-limited acute hepatitis; CH, Chronic hepatitis; WM, male woodchuck; WF, female woodchuck.

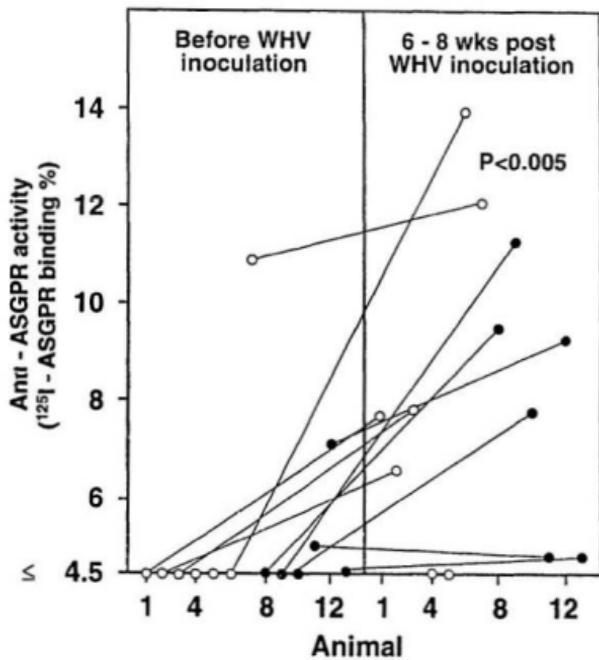
<sup>b</sup> - w.p.i., weeks post WHIV inoculation.

<sup>c</sup> - n.d., not detected.

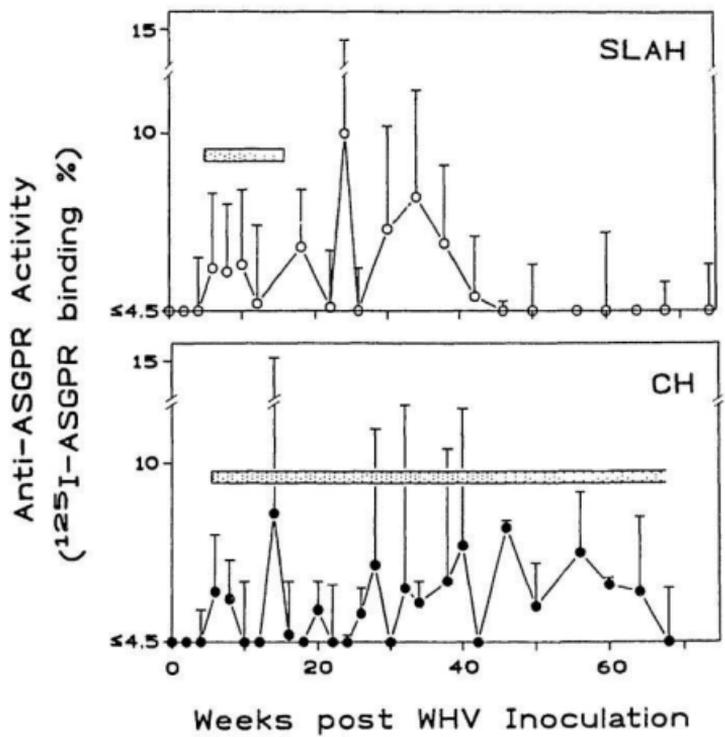
**Figure 2.3. Dynamic patterns of anti-ASGPR autoantibody during follow-up of four woodchucks with experimentally induced WHV infection.** WM.1 and WF.3, animals with a self-limited episode of acute infection (SLAH). WF.8 and WM.10, animals with acute WHV infection which progressed to chronic hepatitis (CH). The values of anti-ASGPR antibody reactivity were determined by RIA, as described in section 2.2.3. Values at point zero reflect the anti-ASGPR antibody reactivity in the serum samples collected before inoculation with WHV. Horizontal bars show the time of the appearance and duration of WHs-antigenemia.



**Figure 2.4. Comparison of anti-ASGPR activity before WHV inoculation and 6 to 8 weeks after in the sera of 13 woodchucks with experimental acute hepatitis. Open symbols show the mean values detected in each of 7 animals which finally recovered from acute hepatitis, whereas shaded symbols represent the mean values found in each of 6 animals in which acute infection progressed to chronic hepatitis.**



**Figure 2.5. Computer generated graphs of the means of anti-ASGPR autoantibody reactivity in woodchucks with experimental WHV infection.** Anti-ASGPR autoantibodies in woodchuck sera were determined by RIA, as described in section 2.2.3. Graphs present the mean of anti-ASGPR autoantibody reactivity in the sera obtained from woodchucks with SLAH (n = 7) and animals with CH (n = 6) after experimental WHV inoculation. Values shown at zero represent the means of anti-ASGPR autoantibody reactivity in sera collected before inoculation with WHV. Horizontal bars show the mean time of the appearance and duration of WHs-antigenemia. Comparison of the data at each time point of the follow-up between these two groups by Wilcoxon signed rank test did not show significant difference ( $P > 0.05$ ).



(2) Table 2.1 also showed that in 5 (WM.1 to WF.4, and WF.8) of the 8 animals (62.5%) with anti-ASGPR response induced after viral inoculation, the anti-ASGPR activity emerged concomitantly with (WF.2) or 2 to 18 weeks later (WM.1, WF.3, WF.4 and WF.8) than the appearance of WHsAg. Since WHsAg appearance in the circulation is an indicator of well-established viral replication and/or viral hepatitis and is accompanied by active liver necroinflammation in the majority of animals, the present finding may suggest that the anti-ASGPR autoantibody response depends on the damage of hepatocytes due to WHV infection. In three other animals (WM.5, WF.9 and WM.10) (37.5%), anti-ASGPR autoantibodies appeared between 2 to 6 weeks prior to that of WHsAg. As WHV DNA had been detected in these animals 10 to 20 days after WHV inoculation by PCR (data are not shown), the emergence of anti-ASGPR in these groups still followed the establishment of viral replication. Therefore, WHV infection was necessary for all these 8 woodchucks to produce ASGPR-specific autoantibody response.

(3) Further, Table 2.1 and Fig. 2.4 revealed that in 4 (WM.7, WF.11, WM.12 and WM.13) of these 13 animals, the zero samples (preinoculation sera) were positive for anti-ASGPR activity. However, WHV infection in 2 (WM.7 and WM.12) of the 4 animals was associated with a moderate rise in the autoantibody activity during the first 8 weeks post WHV inoculation (Fig. 2.4 and Fig. 2.5). The remaining two woodchucks displayed no change in the autoantibody levels until in the late phase of follow-up when increases in the level of anti-ASGPR were finally observed. Therefore, it appeared that WHV infection induced a rise of anti-ASGPR antibody levels in the majority of animals, even in those that had preexisting anti-ASGPR antibodies. Most interestingly, 3 (WF.11, WM.12 and WM.13) of these 4 animals developed chronic viral hepatitis, while only one (WM.7) recovered from acute infection. This observation raises the possibility that animals with preexisting autoimmune response to liver-specific ASGPR may be at higher risk to progress to chronic WHV infection. Further analysis of this issue will be presented in section 2.3.2.

(4) As shown in Figs. 2.3 and 2.5, anti-ASGPR autoantibodies remained detectable through the entire follow-up period in 12 of the 13 animals (WF.6 did not generate anti-ASGPR), even after the disappearance of serological markers of active WHV infection (e.g., WHsAg). The patterns of anti-ASGPR occurrence showed alternate periods of increases and falls which did not parallel the fluctuation of WHsAg and progression or recovery from acute hepatitis (e.g., in Fig 2.3, WM.1 and WF.3). In the animals that recovered from viral infection (SLAH), the clearance of WHsAg from the circulation and the appearance of antibodies directed to WHsAg (anti-WHs) were typically detectable, as determined by other investigators in this laboratory. This indicates a complete serological resolution of WHV infection. Statistical analysis, by comparisons of the anti-ASGPR activity data at each time point during the whole follow-up period, showed that the dynamic difference was not significant (Wilcoxon signed rank test,  $P = 0.47$ ) between SLAH and CH animals (Fig. 2.5). It appeared that the activities of anti-ASGPR autoantibody after 50 w.p.i tended to decline, even to undetectable levels, more frequently in animals with SLAH than in those with CH (Fig. 2.5). However, the difference in the autoantibody dynamics between these two groups in the late observation period was still not quite significant (Wilcoxon signed rank test,  $P = 0.063$ ). These observations suggest that evaluation of anti-ASGPR autoantibody patterns in the course of hepadnaviral hepatitis does not have a prognostic value in the prediction of the outcome of acute hepatitis, i.e. progression to chronic disease or recovery from acute infection.

### **2.3.2. Investigation of the Relationship Between Anti-ASGPR Response and Disease Patterns in WHV-Infected Woodchucks.**

As mentioned in the previous section (2.3.1.3), the occurrence of anti-ASGPR autoantibodies prior to WHV inoculation appeared to predispose to the development of chronic liver disease in 3 of 4 woodchucks. This observation led us to explore in a larger

woodchuck population whether there is any relationship between pre-existing anti-ASGPR autoantibody activity and the disease pattern of WHV infection. Among the 55 healthy woodchucks whose serum samples were used to establish the normal anti-ASGPR autoantibody reactivity value (section 2.3.1.2.), 43 animals, including 13 woodchucks described in detail in section 2.3.1.3, had been experimentally infected with WHV. The serum and liver biopsy samples had been evaluated for WHV infection markers and morphological status of liver injury by other investigators in this laboratory. Therefore, the available data were retrospectively reviewed for the purpose of the present studies.

Table 2.2 summarizes the outcome of WHV infection in these 43 animals, in which 11 (25.6%) were found anti-ASGPR autoantibody positive and the remaining 32 (74.4%) were anti-ASGPR autoantibody negative prior to WHV inoculation. It was found that 32 of the 43 woodchucks (74.4%) developed AH and finally recovered from hepatitis (SLAH), while 11 animals (25.6%) developed CH. Interestingly again, among these 11 animals that developed CH, 6 (54.6%) were anti-ASGPR positive prior to WHV inoculation. In contrast, the rate of the CH development in 32 animals that were anti-ASGPR negative before viral inoculation was only 15.6% (5 of 32). Statistical analysis revealed a significant difference in the CH development rate between these two groups of animals (Fisher's exact test,  $P < 0.05$ ). This result supports our preliminary observation described in section 2.3.1.3 and suggests a possibility that anti-ASGPR autoimmunity existing prior to hepadnaviral infection predisposes to the development of chronic hepatitis in woodchucks.

### **2.3.3. Receptor Subunit Specificity of WHV-induced Autoantibodies to ASGPR.**

To determine the ASGPR subunit specificity of anti-ASGPR autoantibodies induced by WHV infection and detected by RIA, Western blot analysis was performed by using purified wASGPR and rASGPR. Fig. 2.6 shows that both the 40- and 47-kDa subunits of ASGPR from woodchuck and rabbit livers were specifically recognized by the anti-ASGPR

**Table 2.2. Outcome of experimentally induced WHIV infection in animals with or without anti-ASGPR autoantibodies in sera prior to viral inoculation**

Status of anti-ASGPR prior to WHIV inoculation <sup>a</sup>	Number of animals	Number of animals with disease <sup>b</sup>	
		(percent)	
		SLA11	C11
positive	11	5 (45.5%)	6 (54.5%)
negative	32	27 (84.4%)	5 (15.6%)

<sup>a</sup> - Anti-ASGPR activity was determined using RIA as described in section 2.2.3

<sup>b</sup> - Diagnosis based on criteria described in section 2.2.1.

**Figure 2.6. The ASGPR subunit specificity of anti-ASGPR autoantibodies induced by experimental WHV infection analyzed by Western blotting.** Affinity-purified hepatic ASGPRs (0.3  $\mu$ g protein per lane) from woodchuck (lane 2) and rabbit (lane 3) were separated by SDS-PAGE and analyzed by immunoblotting using a pool of sera with anti-ASGPR activity determined by RIA collected from WF.7 and WF.9 (see Table 2.1) after experimental WHV infections. See section 2.2.4 for details of the method. The molecular masses of prestained protein standards (lane 1) are indicated on the left side of the panel.

This figure shows that anti-ASGPR autoantibodies induced due to WHV infection react with the 40- and 47-kDa subunits of hepatic ASGPR from both woodchuck and rabbit.

m.wt.  
kDa

1

2

3

m.wt.  
kDa

106.0 -

80.0 -

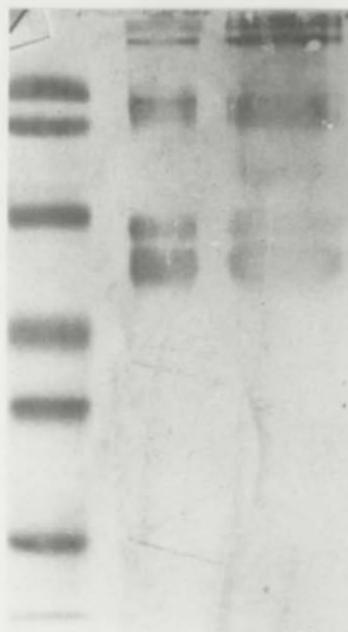
49.5 -

32.5 -

27.5 -

18.5 -

- 47  
- 40



positive sera from WHV-infected animals. This result indicated that both subunits of wASGPR and at least the cross-species-reactive determinants in wASGPR and rASGPR were autoimmunogenic in the animals with WHV infection. Interestingly, identical results were obtained by using anti-ASGPR positive sera from different periods of WHV infection, even using the sera in which anti-ASGPR became positive for the first time by RIA after WHV inoculation (data not shown). This result indicated that the anti-40-kDa and anti-47-kDa activity appeared at the same time following WHV invasion. This finding varied from our previous observation that the anti-40-kD appeared prior to anti-47-kDa in woodchucks immunized with affinity-purified rASGPR (Table 1.3 and Fig. 1.11). This finding may suggest that induction of autoantibodies to ASGPR by WHV infection could involve a mechanism different from that through active immunization.

#### **2.3.4. Assessment of WHV-ASGPR Immunological Cross-Reactivity.**

To test if there is any antigenic cross-reactivity between w-ASGPR and WHV proteins, immunodot blot and Western blot analyses were performed by using affinity-purified wASGPR and rASGPR preparations, and isolated WHV envelope (WHsAg) proteins and complete virion particles as target antigens. In these experiments, guinea pig anti-ASGPR and rabbit anti-WHs antibodies were used. The obtained results revealed the lack of antigenic cross-reactivity between ASGPR and WHV proteins, as summarized in Table. 2.3. In addition, there was no correlation between the patterns of anti-ASGPR and anti-viral antibody (i.e., anti-WHs) responses in the course of WHV infection (see section 2.3.1.3.). This further suggests that it is unlikely that there are antigenic determinants shared by wASGPR and WHV structural proteins.

**Table 2.3. Analysis of the antigenic cross-reactivity  
between WHV and ASGPR proteins by dot and Western blotting**

Antigen	Antisera		
	Rabbit anti-WHVs (dot blot) <sup>a</sup>	Guinea pig anti-rASGPR <sup>b</sup>	
		dot blot	Western blot
WHV virions	+	-	-
WHVsAg	+	-	-
w-h-ASGPR	-	+	+

<sup>a</sup> - Rabbit anti-WHVs antibodies were visualized by HRP<sup>o</sup>-labelled goat antibodies to rabbit IgG(1H4).

<sup>b</sup> - Guinea pig anti-rASGPR antibodies was visualized by AP<sup>o</sup>-labelled goat antibodies to guinea pig IgG(1H4).

## 2.4. DISCUSSION

Two immunodetection methods, RIA and ELISA, have been established for the detection of human anti-ASGPR autoantibodies (McFarlane et al., 1985; 1986; Treichel et al., 1990; 1994a). There are several disadvantages of the anti-ASGPR ELISA method, compared with the RIA method. Firstly, a relatively high amount of purified receptor is required as an antigen for antibody detection. Considering the time-consuming procedure of ASGPR isolation and the relatively limited yields of purified ASGPR, it is more convenient to choose a method in which less ASGPR protein is consumed. Secondly, ASGPR must be immobilized on the plastic plates in anti-ASGPR ELISA. However, isolated native ASGPR occurs in highly aggregated forms in aqueous solutions. Detergents can not be applied to dissociate these aggregates during the coating of the ASGPR onto the ELISA plates, since they interfere in the efficiency of protein attachment to plastics. In addition, conformation changes of the ASGPR molecules may take place due to the interaction between ASGPR protein and plastic. Thus, it is possible that some important domains (e.g., antigenic epitopes or ligand binding sites) may not be accessible to antibodies. As an example, our finding (section 3.3.2) showed that subunit-specific antibodies against the 47-kDa subunit did not inhibit ligand binding to ASGPR immobilized on plates, but did inhibit ligand binding to ASGPR on hepatocytes. This suggests that the binding site on the 47-kDa polypeptide of ASGPR may not be accessible to specific antibody when purified native ASGPR is coated on plastic plates. Therefore, considering these factors, a liquid-phase RIA was mainly used in the course of the present studies. In RIA, purified ASGPR reacts with antibodies in the presence of a low concentration of Triton X-100. Therefore, it is likely that ASGPR aggregates can be dissociated and the majority of antigenic epitopes on ASGPR are available for antibody recognition. The possible drawback of RIA is that protein A has to be used to precipitate ASGPR-anti-ASGPR immunocomplexes, although the affinity of different Ig

classes and subclasses to protein A varies. With regards to the present study, the affinity of different woodchuck Ig classes or subclasses to protein A remain unidentified. Therefore, it is possible that some anti-ASGPR autoantibodies may not be detected in this assay system. However, according to the results from our double-diffusion assay, the reactivity of woodchuck Ig with protein A appears similar to that of human or guinea pig Igs. In addition, our results have shown that woodchuck Ig can be isolated by using a protein A-affinity column and that the purified woodchuck Ig behaves as IgG (see section 3.3.1; Michalak et al., 1990). So, it is likely that anti-ASGPR RIA used for the study of woodchuck sera may be as sensitive as that used for the study of human sera.

Comparison of the rates of anti-ASGPR detection in the same group of patients by the liquid-phase RIA using protein A and by the solid-phase ELISA has been done in a recent study (Treichel et al., 1994). The results of this study show a high degree of agreement between these two assays. The final conclusion of these authors is that both RIA and ELISA are highly reliable for the detection of anti-ASGPR autoantibody.

In the course of this study, we found that anti-ASGPR autoantibodies were detectable in the sera of some healthy, WHV-noninfected animals. Occurrence of autoantibodies to ASGPR in these animals may suggest some physiologic function of these autoantibodies. Natural autoantibodies in the circulation are thought to be involved in the clearance of free autoantigens (e.g., following cell death) (Cohen and Cooke, 1986). With regard to this scenario, it has been found that a soluble ASGPR exists in human serum (Yago et al., 1995). This human serum ASGPR was identified, after being absorbed on an anti-ASGPR monoclonal antibody affinity column, as proteins with molecular masses of 35 to 40 kDa. The ASGPR levels in the serum appear to be related to the status of liver degeneration or regeneration. Thus, it is quite possible that anti-ASGPR may be induced by these soluble ASGPR proteins under both natural and pathogenic conditions.

The normal level ranges of human autoantibodies against ASGPR in sera has been

investigated in small groups of patients by using both RIA and ELISA. McFarlane et al. (1985) determined the anti-ASGPR autoantibody activity by RIA in 12 patients without liver disorder or organ-specific autoimmune disorders. Treichel et al. (1990) analyzed serum samples from 31 healthy controls. In both of these reports, the mean of the normal values plus two standard deviation (SD) was used as a cut off value to determine the activity of anti-ASGPR in the tested samples. Unlike our result obtained in healthy woodchucks, the normal values of autoantibody against ASGPR in human control sera were within a narrow range; all the values were below the cut off value. In the present study, the prevalence of autoantibodies against ASGPR was found to be 29.1% in healthy woodchucks. This high incidence of anti-ASGPR antibody in apparently healthy woodchucks may be a species specific event. A previous study in this laboratory has shown that the prevalence of non-organ specific SMA in healthy woodchucks is as high as 55%, while the prevalence of SMA among normal human subjects is only 12% (Dzwonkowski and Michalak, 1990). Nevertheless, since all the woodchucks were wild-caught, it is also possible that some of the woodchucks with high anti-ASGPR activity may have had a previous exposure to some pathogenic factors other than WHV (e.g., bacterial or parasitic infections, or chemical toxins).

The important finding in this study is that autoantibody response to ASGPR was induced (in 8 of 9 animals, 88.9%) or boosted (in 4 of 4 animals) due to WHV infection (Table 2.1). This finding provides, for the first time, a direct evidence that hepadnavirus invasion triggers liver-specific autoimmune response. In addition, observations in the present study give some new insights on the relationship between hepadnavirus infection and anti-ASGPR autoimmune response. Firstly, the induction of anti-ASGPR antibodies in WHV-infected animals appeared to be a consequence of well-established WHV infection. The appearance of anti-ASGPR autoantibodies usually followed the emergence of serum WHsAg which is an indicator of progressing WHV infection. In contrast to this observation, WHV-infection induced non-organ specific autoantibodies, in particular SMA, usually appear prior

to the serum WHsAg (Dzwonkowski and Michalak, 1990). Therefore, it is likely that anti-ASGPR response is a consequence of virus-induced liver damage rather than a direct effect of hepadnavirus on the immune system, a mechanism postulated in the case of WHV-induced SMA response. Second, the levels of autoantibodies to ASGPR showed rapid changes during the course of follow-up. The dynamic patterns of anti-ASGPR autoantibodies in animals with SLAH could not be distinguished from that in animals with CII, which also showed a strong resemblance to the dynamics of non-organ specific autoantibody responses in WHV-infected woodchucks (Dzwonkowski and Michalak, 1990). Furthermore, like the non-organ-specific autoantibody response, WHV-induced anti-ASGPR autoantibodies persisted in SLAH animals. This type of viral-related autoimmune responses could be the result of a "hit-and-run" event (Oldstone, 1987), in which autoantibodies remain when viruses had been long removed. This type of autoimmune response could also be the outcome of continuous stimulation of the immune system due to life-long persistence of WHV replication and residual liver necroinflammation in some SLAH woodchucks (Michalak, et al., 1994; Pardoe, et al., 1996).

The occurrence rate (88.9%) of anti-ASGPR in experimental WHV infected animals was only slightly higher than that in patients with HBV infection (73%) (McFarlane et al., 1986). This finding may suggest similar pathways leading to the anti-ASGPR autoimmune responses in WHV and HBV infections. On the other hand, there is a possibility that a higher rate of response in woodchucks may be due to infection under experimental conditions. Inoculation of animals with WHV was carried out by intravenous administration of a relatively high dose of virulent virus. Thereby, infection could be more severe than under natural conditions and induce more efficiently autoimmune response to ASGPR.

One of the most interesting and unexpected observations in this study was that the presence of anti-ASGPR in preinoculation sera was associated with the higher rate of development of chronic WHV hepatitis. It was found that 6 of 11 woodchucks (54.5%) with

anti-ASGPR autoantibody before inoculation of WHV developed CH, while the rate of CH development in anti-ASGPR non-reactive animals was only 15.6% (Table 2.2).

Viral persistence is one of the major subject in the study of viral pathogenesis. The balance between viral invasion and host defense controls the outcome of viral infection. Several mechanisms have been suggested based on HBV research (for review see Chisari and Ferrari, 1995a). With regards to virus, production of defective viral particles and spontaneous mutations are commonly observed in patients with HBV chronic infection. It is thought that this alteration of viral replication may help HBV escape from immune surveillance. Also, as for the host immune defense mechanisms, immune surveillance can be influenced by viral infection since HBV not only attacks hepatocytes but also cells of the immune system (e.g., lymphocytes and macrophages). Therefore, the functions of immune cells may also be destroyed. Meanwhile, high concentration of HBsAg in serum is thought to be able to induce a state of immune tolerance in the host. Moreover, cytokine production (e.g.,  $\gamma$ -interferon) can be suppressed in patients with chronic hepatitis. One of the interesting studies on anti-viral CTL response have shown that patients with chronic HBV infection usually have weaker (even undetectable) virus-specific CTL responses than those patients who successfully recover from acute HBV infection (Ferrari et al., 1990). Taken together, the current studies indicate that the host immune system may significantly influence the development of chronic viral infection. Relevant to our observation, anti-ASGPR activity occurring prior to WHV-inoculation could be one of the signs of an abnormal responses induced by the host immune system. Under such abnormal conditions, it would not be surprising if a higher rate of viral persistence occurs in these animals (Table 2.2). Further work should continue to investigate the course and the outcome of WHV infection by using anti-ASGPR positive woodchucks inoculated with WHV. The anti-ASGPR antibody reactivity in the experimental animals could be induced by immunization with purified ASGPR or possibly by some "natural agents" (e.g., bacteria or parasite infection). Such experiments

are in progress in this laboratory and the results may provide some clarification on this subject in the future.

According to Poralla et al. (1991), in patients with AIH, species-specific epitopes in human ASGPR are thought to be the major epitopes recognized by anti-ASGPR autoantibodies. In contrast, non-species-specific epitopes of ASGPR are mainly recognized by anti-ASGPR autoantibodies from patients with HBV infection. Our results indicated that at least non-species-specific epitopes expressed on both wASGPR and rASGPR could be autoimmunogenic in the animals with WHV infection (Fig. 2.6). This observation appears to be comparable to the results from clinical studies. Whether there is any strictly wASGPR specific autoantibody response in woodchucks with WHV-infection still needs further investigation.

In addition, sera from animals in different stages of WHV infection (i.e., AIH and CH1) showed identical patterns of ASGPR polypeptide reactivity (Fig. 2.6), including the sera in which anti-ASGPR was detected by RIA for the first time after inoculation with WHV. This result is different from our observation in woodchucks challenged with affinity-purified rASGPR (Table 1.3 and Fig. 1.11), where an anti-40-kD response occurred prior to anti-47-kDa response. This difference suggests that the pathway of anti-ASGPR antibody induction in WHV infection could be different from that through the immunization routes.

Molecular mimicry between viral peptide and self peptide is one of the important mechanisms for the induction of autoimmune responses (Oldstone, 1987). With respect to the autoimmune phenomena occurring during HBV infection, there has been no reports regarding the cross-reactivity between HBV and ASGPR proteins. According to computer-assisted multiple sequence comparisons using the PC GENE program (IntelliGenetics, Mountain View, CA), we found that the identity between the DNA or amino acid sequences of HBV proteins and human ASGPR protein is very low. At the nucleotide sequence level, the overall identity between HBV genes and the gene of the human ASGPR H1 subunit is

36% and that between the HBV gene and the H2 gene of human ASGPR is 37%. Although, at the amino acid sequence level, the identity between HBV proteins and H1 or H2 is 3% or 4%, respectively. Our results showed a lack of cross-reactivity between antigenic domains of ASGPR and WHsAg (Table 2.3). However, it should be noted that induction of antibody production requires antigen-processing by antigen-presenting cells. This process involves proteolysis of antigens, and thus, some epitopes of cross-reactivity and hidden inside the antigen molecules could be exposed. To verify this, further study of cross-reactivity between WHV and ASGPR should include limited digestion of ASGPR and WHV with proteolytic enzymes, treatment with detergents or denaturation of the ASGPR or WHV proteins. Whether WHV and wASGPR share common T cell epitopes also remains to be elucidated.

## 2.5. CONCLUSION

The major observation from this part of the work is that experimental hepadnavirus infection evidently triggers an autoantibody response against host hepatic ASGPR. These WHV-induced autoantibodies recognized both constituent subunits of wASGPR, suggesting that both receptor subunits expressed on the hepatocyte surface may serve as targets for liver-specific autoimmune reaction. WHV and ASGPR seemingly do not contain cross-reactive epitopes, suggesting that induction of the anti-ASGPR autoantibody response is unlikely to be a consequence of viral antigenic mimicry. Since autoantibodies against ASGPR were usually detected after the establishment of WHV infection, it is more likely that generation of these antibodies is related to hepatocyte damage. Thus, the induction of anti-ASGPR autoantibodies may be caused by exposure of autoantigenic ASGPR epitopes to the host immune system after WHV infection, rather than through a virus-induced polyclonal stimulation which may be responsible for the induction of non-organ specific autoantibodies.

The existence of anti-ASGPR autoantibodies in preinoculation sera of woodchucks appeared to be associated with the development of chronic WHV infection. The apparent relationship between the pre-existing anti-ASGPR autoantibodies and the development of chronic hepatitis in woodchucks needs further study. In addition, judging from current data, it is possible that a similar situation may occur in patients with chronic HBV infection since the disease progresses similarly after WHV and HBV invasion (Summers et al., 1978). Thus, clinical studies of the incidence of chronic hepatitis B in patients with pre-infection anti-ASGPR positive autoimmune responses may be of considerable interest. If this hypothesis is correct, strategies designed to control or terminate ASGPR-specific autoimmune responses in patients with non-viral-related liver disorders (e.g., AIH) may prevent or reduce the development of chronic HBV infection.

## PART III

### PATHOBIOLOGICAL EFFECTS OF ANTI-ASGPR ANTIBODIES

#### 3.1. INTRODUCTION

In comparison to the considerable progress which has been made in the recognition of biochemical properties of ASGPR, current understanding of the physiological and pathological roles of ASGPR is very limited. Because of this limitation, the pathogenic significance of autoimmune responses specific for ASGPR is also limited. In the following sections, a summary of our knowledge on the physiological function of ASGPR and the ASGPR behavior in pathological conditions are presented. Finally the postulated mechanisms of the pathogenic action of antibodies to ASGPR are discussed.

##### 3.1.1. The Physiological Functions of ASGPR.

The original thinking about the physiological function of ASGPR is that this receptor system mediates the homeostasis of biological macromolecules. It is accepted that glycoproteins after desialylation need to return from the circulation to the liver for catabolism. These glycoprotein molecules include protein complexes (e.g., heptoglobin), carrier proteins for smaller molecules (e.g., ceruloplasmin, thyroglobulin and transferrin), modulators of cellular responses (e.g., interferon, follicle stimulation hormone), and immunoglobulin or immunocomplexes (see review by Aswell and Harford, 1982). It is thought that the major function of the ASGPR system is to perform specific receptor-mediated endocytosis and, in turn, to regulate the lifetime, metabolism and biological activity of these molecules (Drickamer, 1991).

Besides the desialylated proteins, some cells can also be cleared by ASGPR-involved phagocytosis. Studies on programmed cell death show that immature glycan structures without terminal sialic acid moieties are exposed on the surface of apoptotic cells (Savill et al., 1990). More recent studies employing both *in vitro* and *in vivo* systems, indicate that ASGPRs are involved in the removal of apoptotic cells in the liver (Dini et al., 1992; 1993). By using primary cultures of neonatal rat liver cells (which mainly contain hepatocytes and stromal cells), it was found that apoptotic liver cells which expressed desialylated glycans were recognized by ASGPR. These apoptotic cells could be cleared by bystander cells through ASGPR-dependent phagocytosis, since anti-ASGPR antibody and the ASGPR-specific ligands (e.g., Gal, GalNAc and ASFN) inhibited the cell clearance. Further, in an experimental liver hyperplasia induced by lead nitrate injections in rats, it was also found that apoptotic hepatocytes were internalized by hepatocytes as well as sinusoidal cells, including Kupffer and endothelial cells. In addition, ASGPR expression at both mRNA and protein levels were greatly increased during the apoptosis, suggesting that exhibition of ASGPR can be modulated to respond to the cell death or damage. It is well-known that apoptosis is one of the features of programmed cell death under physiological conditions. Thus, receptor-mediated clearance of apoptotic cells should be considered one of the physiological functions of ASGPR.

The function of ASGPR in some pathological conditions has been examined in a few laboratories. Sawamura et al. (1981) found that accumulation of ASGP in serum could be experimentally produced after the induction of acute hepatocellular damage in rats treated with galactosamine. Later, Sawamura et al. (1984) observed that patients with cirrhosis and liver cell carcinoma have high levels of plasma asialoglycoproteins (hyperasialoglycoproteinemia). In both human and rat systems, the authors found a significant correlation between the accumulation of ASGP in the circulation and the decrease of ASGPR activity in the liver (about 28% of the normal activity). These findings indicate

that ASGPR plays an important physiological role and suggest that in acute or chronic liver diseases, the receptor function can be disrupted.

However, one of the most recent reports reveals some contradictory evidence on the physiological relevance of ASGPR (Ishibashiet al., 1994). Using ASGPR-deficient mice which were established by targeted disruption of the MHL-2 gene, it was found that the expression of MHL-1 in the liver was extensively decreased and the injected asialoglycoprotein (<sup>125</sup>I-ASOR) could not be removed from the circulation. Nevertheless, the accumulation of plasma ASGP or other proteins was not detected in the circulation of these knockout mice. Although it is not excluded that the expression of MHL-1 may still be sufficient to remove some of the glycoproteins, this result still appears to argue against the possible function of ASGPR in the homeostasis of glycosylated plasma components.

### **3.1.2. ASGPR as a Potential Cell Receptor for Viruses.**

Based on the evidence that has emerged during the past two years, it has become apparent that ASGPR may function as a potential liver-specific receptor for viruses. It is well known that the envelope of many viruses contains sialoglycoproteins which originate from host cells or are the products of viral gene transcription. These glycoproteins play an important role in recognition of host cell receptors. As the sialoglycoproteins can be easily desialylated by acid or enzymes under normal biological conditions, the Gal or GalNAc residues on viral surface proteins can become the potential ligands recognized by ASGPR. To examine this possibility, Treichel et al. (1994) tested whether HBV particles can bind to purified human ASGPR or cultured hepatocyte cell lines. They found that one domain on the preS1 region of the virus envelope could be recognized by ASGPR. This binding site appeared to be sensitive to a detergent (Lubrol), and was only expressed by the native viral particles which were isolated from the sera of chronic HBV carriers. In addition, two human hepatocyte cell lines, HepG2 and HuH7, expressing ASGPR were able to internalize HBV

particles, suggesting that ASGPR could be a hepatocyte-specific receptor for HHBV. Another hepatotropic virus, Marburg (MBG) virus isolated from patients with haemorrhagic fever, was investigated by Becker et al. (1995). The envelope of this virus contains highly glycosylated proteins. Unlike other glycoproteins, the terminal sugar residues of native MBG virus glycoprotein lack sialic acid (Will et al., 1993). Becker et al. (1995) demonstrated that such an unusual glycosylation pattern could lead to the specific binding of MBG virus to ASGPR on cells and subsequently resulted in the internalization of virus by HepG2, as well as by ASGPR-transfected fibroblasts. Although these experiments were carried out *in vitro*, it is possible that ASGPR may also have the ability to bind some viruses and facilitate viral invasion of the liver *in vivo*.

### **3.1.3. The Biological Effects of Antibodies Against ASGPR.**

The common occurrence of anti-ASGPR autoantibodies in patients with liver disorders, particularly in AIH and viral hepatitis, raises an assumption about their pathogenic relevance. Since the antibodies are directed to a receptor naturally occurring on the hepatocyte surface, the binding of antibody to ASGPR may lead to several pathological outcomes. In the following paragraphs, a few potential mechanisms by which these antibodies could be involved in the hepatocyte dysfunction or damage will be addressed

#### **3.1.3.1. Induction of ASGPR loss from the cell surface by anti-ASGPR antibodies.**

Schwartz et al. (1986) revealed that ASGPR expression on the surface of HepG2 cells would be rapidly decreased after exposure of the cells to anti-ASGPR antibodies at 18°C. This investigation showed that the loss of ASGPR was not caused by protein degradation in lysosome, but by an unknown non-lysosomal mechanism. Whole antibodies to ASGPR, but not their Fab fragments, could induce cross-linking of ASGPR and clustering of ASGPR molecules on the plasma membranes. Such linkage might lead to a reduction in the recycle

rate of ASGPR and the surface expression of ASGPR. Although the mechanisms of these processes remain unknown, these observations suggest that anti-ASGPR antibodies may play a role in regulation of ASGPR expression on the surface of hepatocytes.

#### 3.1.3.2. Anti-ASGPR antibody-mediated inhibition of receptor-ligand binding.

It is well recognized that monoclonal antibodies against ASGPR and antisera produced by immunization with purified rat or human ASGPR proteins or subunit polypeptides contain antibodies recognizing the ligand binding site on ASGPR molecules. These antibodies can inhibit ligand binding to both purified ASGPR and ASGPR associated with hepatocyte plasma membranes *in vitro*. The studies on these antibodies have been summarized in the introduction of Part I (see section 1.1.3.2) of this thesis.

Recently, few studies have reported on the biological or pathogenic effects of autoantibody against ASGPR. Treichel et al. (1992b) published an abstract reporting that autoantibodies to ASGPR from patients with AIH could inhibit ligand binding to ASGPR and the ligand uptake through ASGPR into HepG2 cells. This study suggests that the ligand binding site on human ASGPR is autoimmunogenic. Therefore, it is possible that anti-ASGPR autoantibody binding to ASGPR on the hepatocyte may block the endocytosis of ASGPR *in vivo*.

#### 3.1.3.3. Anti-ASGPR-mediated complement-dependent cytotoxicity.

Early in the 1950s, Roitt and Doniach (1958) described the involvement of the complement system in the pathogenesis of autoimmune thyroid diseases. Complement components represent a biological system which greatly contributes to the effectiveness of humoral immune responses and inflammation. After activation, the proteolytic fragments of complement components mediate many biologic events, such as complement-dependent cytotoxicity (CDC), opsonization and phagocytosis, immuno-complex clearance,

immunoregulation and inflammation. Complement activation can be induced by many factors. Activation by antibody - antigen complexes is one of the well-documented pathways.

Theoretically, anti-ASGPR binding to ASGPR on hepatocytes may activate complement and subsequently lead to the lysis of target hepatocytes. The ability of antibody to fix complement is one of the key factors controlling complement activation. At the beginning of complement activation, one of the complement components C1q will bind to the Cγ2 domain on the Fc region of human IgG or Cμ3 domain on the Fc region of human IgM. The affinity of C1q binding depends on the IgG subclass, with an affinity order of IgG3>IgG1>IgG2>IgG4 in human Ig (Dean et al., 1983). In this context, Treichel et al. (1993) found that the Ig class of anti-ASGPR autoantibodies varies in patients with different liver disorders. IgM class was found in patients with AIH with severe inflammation and in patients with chronic viral hepatitis. The major IgG subclass of anti-ASGPR antibodies is IgG2 in AIH and IgG4 in viral-related liver disorders. Thus, these data suggest that anti-ASGPR autoantibodies from the patients in many cases belong to the Igs which are able to fix complement.

Under normal circumstances, complement activation is also highly controlled by regulatory factors to avoid damage of autologous cells and tissues, unless these factors are deficient. In respect to CDC in the liver, the microenvironment around the target hepatocytes needs to be considered. It is known that hepatocytes are the major type of cells which produce complement components including their regulators (Perlmutter and Colten, 1986; Ramadori et al., 1985). Thus, liver could be a more susceptible organ for complement activation, because of the *in situ* supplement of complement; or it could be better protected from complement pathological effects, since regulators are synthesized locally. Ikeda and Kurebayashi (1991) demonstrated that deposition of C3 on the surface of hepatocytes could be induced *in vivo* due to the reaction between hepatocytes and a monoclonal antibody against liver-specific antigen. Deposition of homologous complement accompanied by

HBsAg and Igs in liver tissue has also been described in patients with hepatitis B (Nowoslawski, et al., 1972). All these results suggest that antibody-antigen complex-induced complement activation does occur in liver.

It is not known whether anti-ASGPR antibody-mediated hepatocyte lysis may occur in liver. However, cytotoxic effect of anti-ASGPR under *in vitro* conditions has been detected by Michalak et al. (1995). Using purified hepatocytes from patients with chronic hepatitis B, it was shown that guinea pig antibodies to ASGPR were highly cytotoxic (with cytotoxicity of  $90.2 \pm 8.9\%$ ) to isolated hepatocytes in the presence of active heterologous complement. This suggests that isolated hepatocytes are not well protected from complement-mediated injury. Therefore, it is likely that anti-ASGPR-mediated CDC may occur in liver *in vivo*.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Chromatography of Immunoglobulin (Ig) on Protein A Column.

A method adapted from Ey et al.(1978) was used for purification of Ig fractions from woodchuck and guinea pig sera.

#### Materials:

- (1) Sera: guinea pig antisera raised against wASGPR, rASGPR, 40-kDa- or 47-kDa-subunit of rASGPR; sera from healthy, non-immunized guinea pigs (see section 1.2.8) and sera from WHV-infected animals (see Table 2.1) with and without anti-ASGPR activities were used.
- (2) Protein A-Sepharose CL-4B: protein A immobilized on Sepharose CL-4B (Sigma).
- (3) 1 M Tris-HCl buffer, pH 8.0.
- (4) 100 mM Tris-HCl buffer, pH 8.0.
- (5) 10 mM Tris-HCl buffer, pH 8.0.
- (6) 25 mM Tris-HCl buffer, pH 7.6.
- (7) 100 mM glycine, adjusted pH to 3.0 with HCl.
- (8) Storage buffer: 0.02% (w/v)  $\text{NaN}_3$  and protease inhibitors (see section 1.2.3) in 100 mM Tris-HCl buffer, pH 7.6.
- (9) Washing buffer: 2 M urea and 1 M LiCl in 100 mM glycine-HCl buffer, pH 2.5.

#### Method:

- (1) Protein A-Sepharose CL-4B was suspended and swollen in 1 M Tris-HCl buffer, pH 8.0. About 1 ml of the beads were packed into a 3 ml disposable syringe stopped with glass fibers. Before using, the matrix was equilibrated with 1 M Tris-HCl buffer, pH 8.0. The column could be re-used after washing with 5 ml of washing buffer and followed by equilibration with 1 M Tris-HCl buffer, pH 8.0.
- (2) To isolate Ig fractions, 1 to 2 ml of serum was adjusted to pH 8.0 using 1 M Tris-HCl buffer and applied to a protein A column. The column was washed with 10-bed-volumes of

1 M Tris-HCl buffer and then with the same volume of 100 mM, and 10 mM Tris-HCl buffer. Subsequently, bound proteins were eluted with 100 mM glycine, pH 3.0. 500- $\mu$ l or 1-ml fractions were collected into tubes containing 50  $\mu$ l or 100  $\mu$ l of 1 M Tris-HCl buffer, pH 8.0. Finally, the protein content in each fraction was determined by spectrophotometry at 280 nm. The purity of the isolated Ig fractions was assessed by SDS-PAGE (see section 1.2.17). Purified Ig fractions were stored at 4°C in the presence of 0.02% NaN<sub>3</sub> or concentrated by lyophilization.

### **3.2.2. Ammonium Sulfate Precipitation of Ig Fractions.**

Materials:

- (1) Sera: see section 3.2.1.
- (2) Saturated ammonium sulfate: 761 g ammonium sulfate in 1 L of distilled water. The pH of this solution was adjusted to 7.8 using solid Tris base.
- (3) Loading buffer: 10 mM Tris-HCl buffer, pH 8.5.

Methods:

Serum for precipitation was centrifuged at 3,000  $\times$  g for 30 min to remove large protein aggregates. While the serum was gently stirred on a magnetic stirrer, an equal volume of saturated ammonium sulfate solution was added slowly. After incubation on ice for 1 hour or overnight at 4°C, this serum solution was centrifuged at 3,000  $\times$  g for 30 min. The pellet was resuspended in 0.5 ml of loading buffer and then extensively dialyzed against the same buffer.

### **3.2.3. Chromatography of Ammonium Sulfate Precipitated Ig Fraction on DEAE-Matrix.**

Materials:

- (1) Ig fractions prepared by ammonium sulfate precipitation of guinea pig or woodchuck sera

(see section 3.2.2).

(2) DEAE-matrix: DEAE-Sepharcl beads, preswollen cellulose ion exchanger (Pharmacia Fine Chemicals).

(3) 0.5 N HCl.

(4) 0.5 N NaOH.

(5) Loading buffer: 10 mM Tris-HCl buffer, pH 8.5.

(6) 50 mM NaCl in loading buffer.

(7) 100 mM NaCl in loading buffer.

(8) 200 mM NaCl in loading buffer.

(9) 500 mM NaCl in loading buffer.

(10) 25 mM Tris-HCl buffer, pH 7.6

(11) 10% (w/v)  $\text{NaN}_3$  in distilled water.

Method:

(1) 10 ml of DEAE-matrix was washed with 0.5 N HCl and then 0.5 N NaOH on a glass filter. The beads were equilibrated with loading buffer till the pH reached 8.5. A small column (10 cm  $\times$  1 cm  $\phi$ ) was packed with the beads and washed with 3 bed volumes of loading buffer.

(2) Ig solution was applied to the column and then the column was washed with 10-bed-volumes of loading buffer. Igs were eluted with increasing concentrations of NaCl in loading buffer, beginning with 50 mM, then 100 mM, 200 mM and finally 500 mM (using approximately 25 to 30 ml of each of the eluting solutions). Protein content was determined in fractions (5.5 ml each) at  $A_{280}$  and the Ig purity was identified by SDS-PAGE (see section 1.2.17 for method). The protein peak fractions were combined and dialyzed against 25 mM Tris-HCl buffer, pH 7.6. Purified Ig fractions were stored at 4°C with addition of  $\text{NaN}_3$  or concentrated by freeze-drying.

### **3.2.4. Fixation of Isolated Hepatocytes and Cultured Cells.**

Cell fixation was performed according to Harford et al. (1982).

#### **Materials:**

- (1) Cells: woodchuck hepatocytes mechanically isolated from liver tissue by a method described in section 1.2.2.1 and cultured HepG2 (see section 1.2.2.2).
- (2) PBS (see section 1.2.2.1)
- (3) Paraformaldehyde solution: 5% (w/v) paraformaldehyde (Sigma) in PBS. The paraformaldehyde was solubilized in PBS by heating the solution at 70°C and adding a few drops of 1N NaOH. The pH was adjusted to 7.6-7.8 by adding 1 N HCl and the solution was cooled on ice before using.
- (4) Glycine solution: 1 M glycine (Sigma) in water.
- (5) Washing buffer: 5 mM glycine and 0.02% (w/v)  $\text{NaN}_3$  in PBS.

#### **Method:**

Isolated hepatocytes and harvested HepG2 cells were washed with cold PBS and counted. The cell number was adjusted to  $10^5$  to  $10^7$ /ml in PBS. Paraformaldehyde solution was added slowly into the cell suspension until a concentration of 0.5% was reached. Then the suspension was gently stirred on ice for 10 min. Subsequently, glycine solution was added to a final concentration of 170 mM, the cells were kept on ice for another 10 min and then washed three times with washing buffer. The fixed cells were stored in washing buffer at 4°C and used for ASGPR-ligand binding studies within one week.

### **3.2.5. Antibody-Mediated ASGPR-Ligand Binding Inhibition Assays.**

Inhibition of ligand binding to ASGPR by anti-ASGPR antibodies was investigated using paraformaldehyde-fixed woodchuck hepatocytes and HepG2 cells, or affinity-purified wASGPR and rASGPR preparations. The assays were performed according to the method of Harford et al.(1982), with some modifications.

#### Materials:

- (1) Paraformaldehyde-fixed cells: isolated woodchuck hepatocytes and cultured HepG2 cells were fixed as described in section 3.2.4.
- (2) ASGPR-coated flexible microtiter plates: plates were coated with 100 ng of affinity-purified wASGPR or rASGPR in 50  $\mu$ l of PBS per well (see section 1.2.13).
- (3) Antibodies: Ig fractions purified by DEAE- or protein A-chromatography from guinea pig antisera raised against wASGPR, rASGPR, 40-kDa- and 47-kDa-subunits of rASGPR, and Ig fractions purified by protein A-chromatography from the anti-ASGPR-positive sera of woodchucks with WHV infection. Ig fractions purified from non-immunized guinea pig sera and from the anti-ASGPR-negative sera of woodchuck with WHV infection were used as controls (see sections 3.2.1 and 3.2.3 for details of the purification of Ig).
- (4) Dilution buffer: 25 mM Tris-HCl buffer, pH 7.6.
- (5)  $^{125}$ I-ASFN: radiolabelled ASFN in dilution buffer (section 1.2.12).
- (6) ASFN solution: 10 mg/ml asialofetuin (Sigma) in dilution buffer.
- (7) Reaction buffer ( $10\times$ ): 1.5 M NaCl, 250 mM  $\text{CaCl}_2$  and 0.2% (v/v) Triton X-100 in dilution buffer.
- (8) Hanks' reaction solution: 1.7 mM  $\text{CaCl}_2$  in  $1\times$  HBSS.

#### Methods:

- (1) Antibody inhibition assay using fixed cells. Paraformaldehyde-treated cells were washed and adjusted to approximately  $5\times 10^5$  cells/ml in Hanks' reaction solution. Then, 100  $\mu$ l of cells were incubated with 20  $\mu$ g of guinea pig Ig or 60  $\mu$ g of woodchuck Ig on ice for 90 min. In some instances, increasing amounts of woodchuck Ig ranging from 2  $\mu$ g to 500  $\mu$ g of Ig were added to determine a dose-dependent inhibitory effect of the Ig from the anti-ASGPR-positive sera derived from WHV-infected woodchucks. For the detection of a direct  $^{125}$ I-ASFN binding to hepatocytes, cells without added Ig were used. For the detection of nonspecific binding, cells were incubated with 2.2  $\mu$ g unlabelled ASFN instead of Ig.

Subsequently, 220 ng of  $^{125}\text{I}$ -ASFN was added to the mixtures and the final volume was adjusted to 300  $\mu\text{l}$  with Hanks' reaction solution (0.73  $\mu\text{g}$   $^{125}\text{I}$ -ASFN/ml). The mixture was kept for 90 min on ice and the volume of the reaction was adjusted to 1 ml with Hanks' reaction solution. The cells were centrifuged at  $3,000 \times g$  for 5 min and 500  $\mu\text{l}$  of the upper supernatant was transferred to another tube. The radioactivity presented in the tubes containing upper supernatant (U) and the remaining solution plus the cell pellet (R) was counted using a gamma counter (LKB). The percent of binding was calculated according to the following calculation: binding percent (%) =  $(\text{R}-\text{U})/(\text{R}+\text{U}) \times 100\%$ .

Each reaction was tested in triplicate and the mean percent binding was calculated. Only the mean value with a standard deviation less than 10% would be used. The  $^{125}\text{I}$ -ASFN binding to hepatocytes (woodchuck hepatocytes or HepG2 cells) in the presence of Ig was considered as the binding after adding Ig for inhibition (I). The binding present in the absence of Ig (T) was assessed by incubation of hepatocytes with  $^{125}\text{I}$ -ASFN in Hanks' reaction solution. Nonspecific binding (N) was defined as the binding occurring in the presence of 100-fold excess of unlabelled ASFN instead of Ig. The percent of inhibition by antibodies was calculated by the following formula: percent inhibition (%) =  $[(\text{T}-\text{N})-(\text{I}-\text{N})]/(\text{T}-\text{N}) \times 100\%$ .

(2) Antibody inhibition assay using purified ASGPR. Plates coated with 100 ng affinity-purified wASGPR or rASGPR were preincubated with DEAE-purified guinea pig Ig (10  $\mu\text{g}$  protein/well; 0.2 mg protein/ml) or serial two-fold dilution of the Ig (from 1.5625 to 200  $\mu\text{g}$  protein/well or 31.25  $\mu\text{g}$  protein/ml to 4 mg protein/ml). Then,  $^{125}\text{I}$ -ASFN (220 ng protein/well or 4.4  $\mu\text{g}$  protein/ml) was added to each well and the reaction was carried out in a total volume of 50  $\mu\text{l}$ /well in  $1 \times$  reaction buffer at  $4^\circ\text{C}$  overnight. The binding in the absence of Ig and the non-specific binding were performed in the same way as described above for fixed cells. Subsequently, the plates were washed and the wells were cut off. The bound radioactivity was counted and the percent of antibody inhibition was calculated as described above.

### 3.2.6. Antibody-Mediated Complement-Dependent Cytotoxicity (CDC) Assay.

As there is no woodchuck hepatocyte cell line currently available, human HepG2 cells were employed as target cells to study the complement-dependent cytotoxic effects of antibodies directed against ASGPR. A Microculture Tetrazolium (MTT) assay was employed to measure the cytotoxic effects, according to the method of Alley et al. (1981).

#### Materials:

(1) HepG2 cells: HepG2 cells were seeded at  $1 \times 10^3$ ,  $1.5 \times 10^3$  or  $3.0 \times 10^3$  in 100  $\mu$ l medium/well in tissue microculture plates. After 48 hours of culture (see section 1.2.2.2 for details), the medium was removed from the cell monolayer and 50  $\mu$ l of fresh medium was added. The cells were then immediately used for the cytotoxic assay.

(2) Antibodies: Ig fractions purified by DEAE or protein A-chromatography from guinea pig antisera raised against wASGPR, rASGPR, 40-kDa- and 47-kDa-subunit of rASGPR and from anti-ASGPR-positive sera of animals with WHV-infection were used. Ig fractions purified from the sera of non-immunized guinea pigs and from the anti-ASGPR-negative sera of woodchucks with WHV-infection were used as controls (see section 3.2.1 and 3.2.3).

(3) Active complement (AC): low-toxic rabbit complement (Cedar Line Laboratories Limited, Hornby, Ont., Canada). Each lot was pre-tested for toxicity against HepG2 cells before using. As a control, complement was also heat-inactivated at 56°C for 30 min (inactive complement, IC).

(4) Cell culture medium: D-MEM (see section 1.2.2.2 for details).

(5) MTT solution: 2% (w/v) 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma) in PBS.

(6) DMSO: dimethylsulfoxide (Sigma).

#### Method:

The following standard CDC assay conditions were established after preliminary experiments performed for testing the number of cells, amounts of complement and incubation

time used in each step of the assay.

HepG2 cells were seeded in 96-well plates ranging from  $10^3$ ,  $1.5 \times 10^3$  and  $3 \times 10^3$  per well. Then, the medium was changed (50  $\mu$ l of fresh medium/well) and the cells in the fresh medium were incubated at 37°C with 60  $\mu$ g of purified Ig and 10  $\mu$ l of AC or IC. Total reaction volume was adjusted to 100  $\mu$ l/well with fresh medium. After 2 hour incubation, MTT solution was added (100  $\mu$ l/well) and incubation was continued at 37°C for another 3 hours. Then the medium was carefully removed and DMSO was added (100  $\mu$ l/well) to solubilize the MTT metabolites. Results were read using a microplate reader (model 3553, Bio-RAD) at  $A_{595}$ . The final reading was taken from the mean of 4 to 5 wells tested, with a standard deviation lower than 10% of the mean. The absorbance values (A) obtained from the wells in the presence of AC and in the presence of IC were used for the determination of percent cytotoxicity (%):

$$\text{percent cytotoxicity (\%)} = (A_{IC} - A_{AC})/A_{IC} \times 100\%.$$

### 3.2.7. Statistical Analysis.

$\chi^2$  test was used to determine significance in the data for inhibition and cytotoxicity mediated by the woodchuck or guinea pig Ig fractions with or without anti-ASGPR antibody activity. *P* values less than 0.05 were considered significant.

### 3.3. RESULTS

#### 3.3.1. Purification of Ig Fractions from Woodchuck or Guinea Pig Sera.

Before examining the biological effects of anti-ASGPR antibodies induced in woodchucks by WHV infection or raised in guinea pigs by immunizations, two methods, DEAE- and protein A-chromatographies, were employed for the purification of the antibody-enriched Ig fractions. The purpose of the purification was to eliminate the influence of other serum components. In our preliminary experiments, it was found that serum elements present in both anti-ASGPR reactive and non-reactive sera interfere with the analysis of the effects of anti-ASGPR antibody in assays (i.e., antibody-mediated ASGPR-ligand binding inhibition assay and anti-ASGPR antibody-mediated CDC assay). This interference was eliminated when the Ig fractions of the antisera were used.

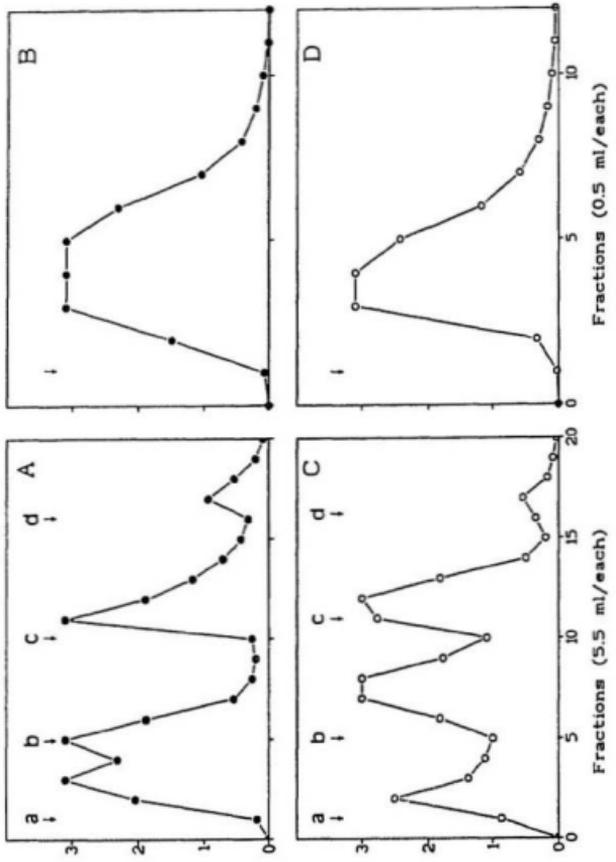
Ig fractions purified from woodchuck sera by either DEAE or protein A chromatography gave protein elution profiles which were very similar to those from guinea pig sera (Fig. 3.1). The sera for Ig isolation were selected from three woodchucks (No. 2, 3 and 4 which are WF4, WF8 and WM12 in Table 2.1, respectively). From each animal, three serum samples obtained at different stages of WHV infection (i.e., AH and CH) were used. The anti-ASGPR activity in these sera ranged from 4.9% to 7.9% as determined by <sup>125</sup>I-rASGPR binding in anti-ASGPR RIA (section 2.2.3), corresponding to approximate titers of 1:50 to 1:200. Ig fractions were also isolated from the mixture of two serum samples of a WHV-infected animal without anti-ASGPR response and used as an anti-ASGPR-negative control sample. In addition, Ig fractions were isolated from the sera of guinea pigs immunized with affinity-purified wASGPR (anti-wASGPR), rASGPR (anti-rASGPR), 40-kDa (anti-40-kDa) or 47-kDa (anti-47-kDa) polypeptides of rASGPR (see section 1.3.1.3 for details). Ig fractions were also obtained from a pool of non-immunized, normal guinea pig sera (NGPS). SDS-PAGE analysis demonstrated that all the purified Ig fractions contained

**Figure 3.1. Isolation of immunoglobulin (Ig) fractions from woodchuck and guinea pig sera by DEAE- and protein A-chromatography.** Elution patterns obtained by chromatography of crude Ig preparations on DEAE-Sepharcel columns (**A** and **C**). The crude Ig was prepared by precipitation of the woodchuck or guinea pig sera with 50% ammonium sulfate (see section 3.2.2 for details). These preparations of woodchuck (**A**) or guinea pig (**C**) Ig were applied separately on columns. After washing out unbound proteins, the bound Ig was eluted successively with 2.5 to 3-bed volumes of 50, 100, 200 and 500 mM NaCl in 10 mM Tris-HCl buffer, pH 8.5, as indicated by arrows a, b, c and d, respectively. The fractions were collected and the protein content was monitored by absorbance at 280 nm. See section 3.2.3 for details of the method.

Elution profiles obtained by chromatography of sera from woodchucks and guinea pigs on protein A columns (**B** and **D**). Sera from woodchuck (**B**) and guinea pig (**D**) (2 ml/each) was adjusted to pH 8.0 and applied to the column. After washing to remove excess proteins, elution was performed with 100 mM glycine-HCl buffer, pH 3.0. The collected fractions were immediately neutralized by 1/10 volume of 1 M Tris-HCl buffer, pH 8.0. Protein content in each fraction was determined by the absorbance at 280 nm. The arrows indicate the point where elution buffer was applied on the columns. See section 3.2.1. for details of the method.

These figures show that the chromatography patterns of woodchuck Ig and guinea pig Ig were similar.

A<sub>280</sub>



polypeptides with approximately the same molecular masses of 25 kDa and 55 kDa which reflected the existence of the Ig light and heavy chains (data not shown). After dialysis, the Ig fractions were used in the ASGPR-ligand binding inhibition assay and CDC experiments.

### 3.3.2. Inhibition of Receptor-Ligand Binding by Anti-ASGPR Antibodies.

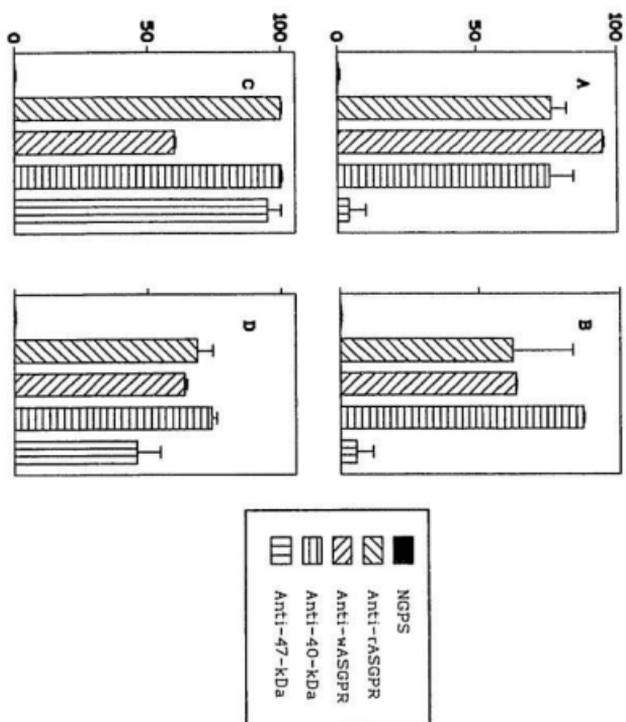
#### 3.3.2.1. Guinea pig anti-ASGPR antibody-mediated inhibition of ASFN binding to purified ASGPR

Guinea pig Ig fractions purified by DEAE-chromatography were tested for their ability to inhibit the binding of  $^{125}\text{I}$ -ASFN to affinity-purified ASGPR. Results in Figs. 3.2 A and B show that all the Ig fractions from guinea pig sera with anti-ASGPR reactivity exhibited a high inhibitory effect on the  $^{125}\text{I}$ -ASFN binding to wASGPR or rASGPR, except the Ig from anti-47-kDa antiserum. At a concentration of 10  $\mu\text{g}$  per assay (0.2 mg/ml), when the inhibition by Ig fractions from anti-wASGPR, anti-rASGPR and anti-40-kDa antisera reached 57.3% to 90%, the inhibition by anti-47-kDa was only 12%. In a control experiment, the inhibition of  $^{125}\text{I}$ -ASFN binding to either wASGPR or rASGPR by the Ig fractions from NGPS was less than 1%. Statistical analysis showed a significant difference ( $\chi^2$  test,  $P < 0.05$ ) between inhibitory effects of the Ig fractions from anti-wASGPR, anti-rASGPR or anti-40-kDa and that of the Ig fractions from NGPS, as well as between the inhibitory effects of Ig fractions from anti-47-kDa and that of the Ig fractions from other anti-ASGPR antisera. Fig. 3.2 also shows that there is no difference in the inhibition of ligand binding to wASGPR and to rASGPR by Igs (Fig. 3.2A vs Fig. 3.2B), suggesting that both receptors behave in a similar way in terms of the ligand and antibody bindings.

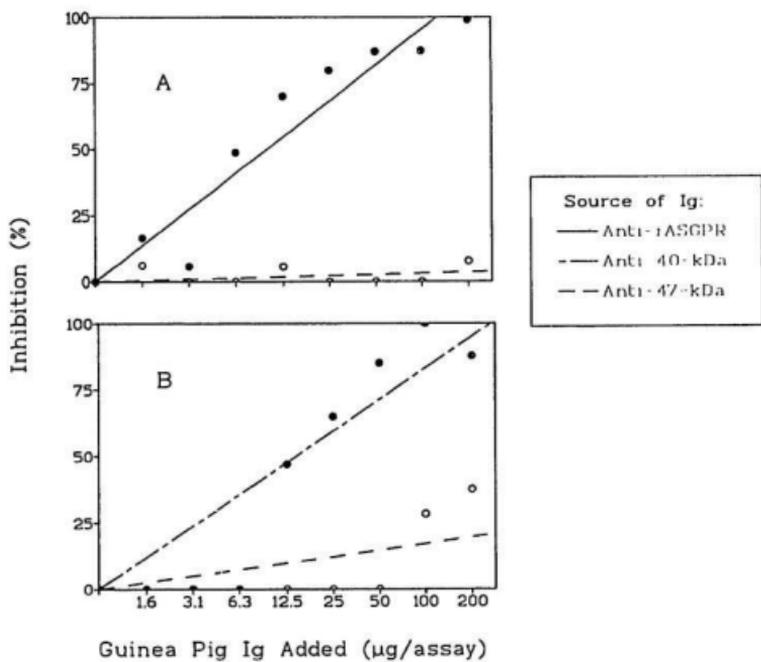
To confirm the specificity of the above observation, an inhibition assay was performed by serial double dilution of Ig fractions from the same antisera. Fig. 3.3 demonstrated the dose-dependent inhibition of the binding of  $^{125}\text{I}$ -ASFN to wASGPR (Fig. 3.3A) or to rASGPR (Fig. 3.3B) by Ig fractions from antisera raised against rASGPR (Fig. 3.3A), 40-kDa (Fig.

**Figure 3.2. Inhibition of ligand binding to purified ASGPR or hepatocyte surface-bound ASGPR by guinea pig antibodies raised against ASGPR and ASGPR subunits.** Affinity-purified wASGPR (A) and rASGPR (B) immobilized on microtitre plates (100 ng protein in 50  $\mu$ l/well) were incubated with immunoglobulin (Ig) fractions purified from guinea pig antisera against rASGPR, wASGPR, or 40-kDa and 47-kDa subunits of rASGPR or from a pool of normal guinea pig sera (NGPS) by DEAE-chromatography. Woodchuck hepatocytes (C) and cultured HepG2 cells (D) prefixed in paraformaldehyde and washed were also incubated with these Ig fractions (approximately  $2.4 \times 10^5$  or  $5 \times 10^4$  cells/assay). The concentration of Ig was 200  $\mu$ g protein/ml. After 90 min on ice,  $^{125}$ I-ASFN (220 ng/well; specific activity  $1.42 \times 10^3$  cpm/ng) was added to each assay and the incubation continued at 4°C. See section 3.2.5 for details of the method. Percent inhibition was calculated with specific binding of  $^{125}$ I-ASFN in the absence of Ig taken as 100%. Bars represent the mean values  $\pm$  SD of triplicate determinations.

# Inhibition (%)



**Figure 3.3. Dose-dependent inhibition of ligand binding to purified ASGPR by guinea pig antibodies against rASGPR and subunit polypeptides of rASGPR.** Affinity-purified wASGPR (**A**) and rASGPR (**B**) immobilized on microtitre plates (100 ng protein in 50  $\mu$ l/well) were incubated with increasing amounts of guinea pig immunoglobulin (Ig) ranging from 1.56 to 200  $\mu$ g in 50  $\mu$ l/assay. These Ig fractions were purified from guinea pig antisera directed against rASGPR (closed symbols in **A**), 40-kDa (closed symbols in **B**) and 47-kDa (open symbols in **A** and **B**) subunits of rASGPR by DEAE-chromatography. The procedure was followed as described in the legend to Figure 3.2. The results are expressed as the percent inhibition of ligand binding as described in Figure 3.2.



3.3B) and 47-kDa (Figs. 3.3A and B) polypeptides of rASGPR. Ig fractions from both anti-rASGPR and anti-40-kDa antisera inhibited the binding by approximately 80-100% at a concentration of 100-200  $\mu$ g Ig protein per assay (2-4 mg Ig protein/ml). The inhibition by Ig from anti-47-kDa at the same concentration was less than 10% when wASGPR was used for ligand binding or about 35% using rASGPR. These results suggest that ligand binding to purified ASGPR under this solid-phase assay conditions appears to be mainly mediated by the 40-kDa but not the 47-kDa subunit of ASGPR.

#### 3.3.2.2. Guinea pig anti-ASGPR antibody-mediated inhibition of ASFN binding to ASGPR on hepatocytes.

It is common knowledge that conformation of the purified soluble proteins can be different from that of the proteins distributed on the cell surface. Because ASGPR is a transmembrane protein, some of the receptor antigenic epitopes may be inaccessible to the specific antibodies. Therefore, it is necessary to test whether ligand binding to ASGPR on hepatocytes can be inhibited by antibodies against ASGPR. For this purpose, paraformaldehyde-fixed woodchuck hepatocytes and HepG2 cells were used as targets for the inhibition assays, as described by Harford et al. (1982). The results indicated that the Ig fractions from guinea pig antisera against the whole ASGPR molecule or the receptor subunits displayed 60.9% to 100% inhibition of specific ligand ( $^{125}$ I-ASFN) binding to woodchuck hepatocytes (Fig. 3.2 C). The same Ig fractions exhibited 54.4% to 76.3% inhibition of ligand binding to human HepG2 cells (Fig. 3.2 D). The results also revealed that  $^{125}$ I-ASFN binding to human ASGPR on hepatocytes could be inhibited by Ig fractions from anti-wASGPR antiserum, suggesting that the ligand-binding sites on wASGPR and human ASGPR are antigenically cross-reactive. This suggestion is supported by our previous observation (see section 1.3.1.3) that hepatic wASGPR is antigenically closely related to hepatic ASGPR from other species.

The interesting finding was that the Ig fraction from anti-47-kDa antiserum gave 54.4% (Fig. 3.2 D) to 100% (Fig. 3.2 C) inhibition of ligand binding to hepatocytes, suggesting that, in contrast to purified ASGPR, the 47-kDa-subunit of the membrane-bound ASGPR also contributes to <sup>125</sup>I-ASFN binding to the hepatocyte surface. Moreover, this result implies that both subunits of wASGPR may contribute equally to ligand binding to the plasma membrane-associated wASGPR, because Ig fractions from either anti-40-kDa or anti-47-kDa antisera alone could completely inhibit <sup>125</sup>I-ASFN binding to woodchuck hepatocytes.

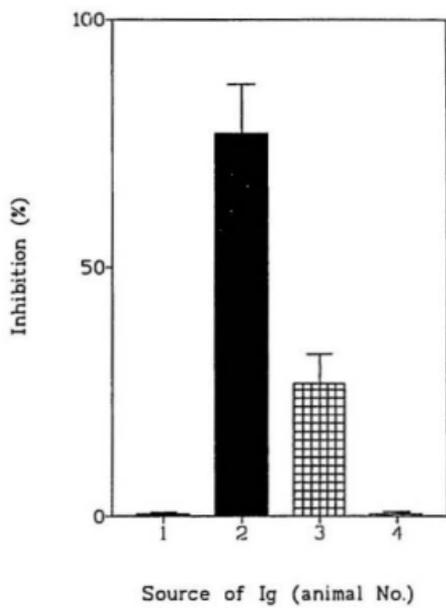
Results also revealed that control Ig fractions from non-immunized guinea pig sera did not have any inhibitory effect on <sup>125</sup>I-ASFN binding to hepatocytes (Figs.3.2 C and D). This strengthens the conclusion that the inhibitory effects of anti-ASGPR antibodies from anti-ASGPR and anti-subunit antisera were ASGPR specific.

### 3.3.2.3. Inhibition of ASFN-ASGPR binding by Ig fractions from woodchuck sera with anti-ASGPR activity induced by WHIV infection.

To test whether autoantibodies to ASGPR induced in the course of WHIV infection have an inhibitory effect on ligand-ASGPR binding, Ig fractions purified by protein A-chromatography from the anti-ASGPR positive sera of WHIV-infected woodchucks were examined in an inhibition assay using pre-fixed HepG2 cells. Fig. 3.4 shows that Ig fractions with anti-ASGPR activity, derived from two of three WHIV-infected animals (animals No.2 and No.3), displayed the ability to inhibit by 86.6% and 32.4%, respectively, the ASFN binding to HepG2 cells at an Ig concentration of 60 µg per assay (0.6 mg/ml). Under the same conditions, the Ig fraction from one of the woodchucks with WHIV-induced anti-ASGPR autoantibodies (animal No.4) did not display any inhibitory effect. A control Ig fraction from anti-ASGPR nonactive serum of a WHIV-infected woodchuck (animal No.1) also gave no inhibition of <sup>125</sup>I-ASFN binding to HepG2 cells.

The specificity of the anti-ASGPR antibody-mediated inhibition was confirmed using

**Figure 3.4. Inhibition of  $^{125}\text{I}$ -ASFN binding to HepG2 cells by Ig fractions from woodchucks with WHV-induced anti-ASGPR autoantibodies.** Paraformaldehyde-fixed HepG2 cells ( $2.4 \times 10^5$  cell/assay) were incubated with Ig fractions (60  $\mu\text{g}$ /assay, 600  $\mu\text{g}/\text{ml}$ ) purified from woodchuck sera by chromatography on a protein A column. The sera were obtained from woodchucks experimentally infected with WHV which subsequently developed anti-ASGPR autoantibodies (animals No.2, No.3 and No.4) or remained anti-ASGPR nonreactive (animal No.1) as determined by anti-ASGPR RIA (see section 2.2.3 for details). After 90 min on ice, 220 ng of  $^{125}\text{I}$ -ASFN was added to the cells and the reaction was continued for an additional 90 min. See section 3.2.5 for details of the method. Results are expressed as percent inhibition calculated by taking specific binding of  $^{125}\text{I}$ -ASFN to HepG2 cells in the absence of Ig as 100%. Bars represent the mean values  $\pm$  SD of triplicate determinations.

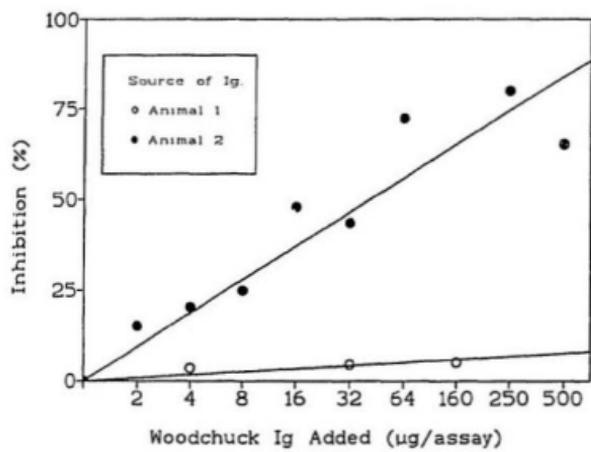


two-fold serial dilutions of the Ig fractions. Fig. 3.5 demonstrates that the inhibitory effect of woodchuck Ig from animal No.2, with anti-ASGPR activity induced due to WHV infection, was dose-dependent. The highest degree of inhibition reaching up to 80% was observed at the concentration of 256  $\mu\text{g}$  Ig protein per assay (2.56 mg Ig protein/ml). At the same Ig protein concentration, the inhibition of  $^{125}\text{I}$ -ASFN binding to HepG2 cells by Ig fractions from control animal No.1, with active WHV infection but without anti-ASGPR antibodies, was lower than 10%.

### **3.3.3. Complement-Dependent-Hepatocytotoxicity Induced by Anti-ASGPR Antibodies.**

To determine whether the antibodies to ASGPR may have the potential to induce liver tissue damage, *in vitro* experiments were performed to measure the complement-dependent cytotoxicity (CDC) caused by anti-ASGPR antibodies. The antibodies were produced in guinea pigs by immunization with affinity-purified ASGPR or induced in woodchucks by WHV infection. The guinea pig and woodchuck Ig fractions with known inhibitory activity on ligand-ASGPR binding, which had been tested as presented in section 3.3.2, were used in these studies. As there is no woodchuck hepatocyte cell line available, freshly isolated woodchuck hepatocytes were initially employed in this experiment. However, due to overall low viability of woodchuck hepatocytes isolated by the mechanical method and the low expression of ASGPR on the surface of hepatocytes isolated by the collagenase digestion method (even after 24 hours' incubation), a human hepatocyte cell line (HepG2) was finally used. These cells were also selected for this experiment based on the fact that both the expression and biological properties of ASGPR on HepG2 cells had been well established by other investigators. In addition, in the course of the present studies, the reactivity of HepG2 cells with antibodies raised against whole ASGPRs or ASGPR subunits had been documented (i.e., Fig. 1.12, Fig. 3.2, Fig. 3.3 and Fig. 3.4).

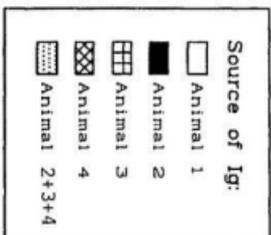
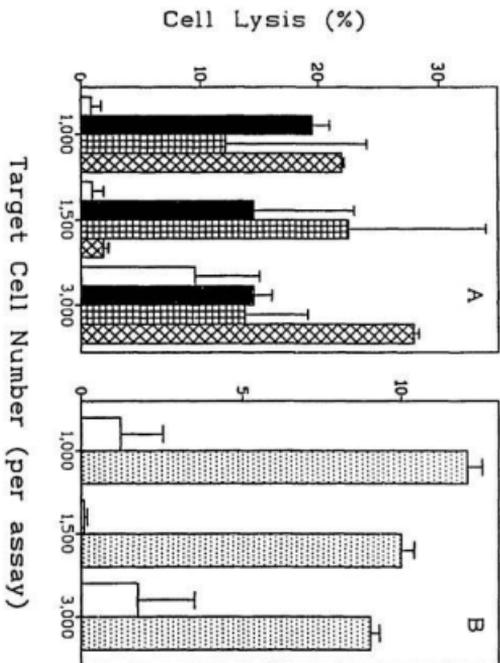
**Figure 3.5. Dose-dependent inhibition of  $^{125}$ I-ASFN binding to HepG2 cells by woodchuck Ig fractions with anti-ASGPR autoantibodies induced by WHV infection.** Paraformaldehyde-treated HepG2 cells ( $2.4 \times 10^5$  cells/assay) were incubated with increasing amounts of woodchuck Ig ranging from 2 to 500  $\mu$ g protein/assay (20  $\mu$ g/ml to 5 mg/ml). The Ig fractions were purified from the sera of WHV-infected woodchucks with (animal No.2) or without (animal No.1) anti-ASGPR autoantibody activity. Experimental procedure as described in the legend to Figure 3.4. Results are expressed as percent of inhibition.



Cultured HepG2 cells were seeded at three different concentrations (1,000, 1,500, and 3,000 cells/well) and allowed to attach to the wells for 48 hr before the experiments. These cell concentrations were selected based on a preliminary experiment performed in order to determine the cell concentration at which there was a linear relationship between cell number and the formation of MTT metabolites. As the amounts of available woodchuck anti-ASGPR-positive sera were limited, a single concentration of anti-ASGPR reactive Ig fractions at 60 µg Ig protein per assay (1.2 mg/ml) was selected for investigation. Selection of this Ig concentration was based on a previous report evaluating the CDC effect of human anti-thyroglobulin autoantibodies (Chiovato et al., 1993). In another control experiment, HepG2 cells were incubated with AC or IC by using different concentrations of rabbit complement at 1:5, 1:10 and 1:20 to determine the possible cytotoxic effect of complement alone. It was found that at concentrations of 1:5 and 1:10, cell metabolism of MTT was not influenced in the presence of either AC or IC alone. Therefore, the complement concentration of 1:10 was chosen for our standard CDC assay. Results from CDC assays are presented as percent cell lysis.

Results shown in Fig. 3.6 A indicate that all tested Ig fractions purified by protein A-chromatography from the sera of woodchucks with WIV-induced anti-ASGPR activity (animal No.2, No.3 and No.4) were cytotoxic for HepG2 cells in the presence of AC. The induced effective cell killing ranged from 25% to 12.5%. Results also show that the Ig fractions from different animals produced different degrees of cytolytic effect at the same number of target cells. The mean percent cell lysis when using these Ig fractions ranged from  $13.1\% \pm 10.3$  (mean  $\pm$  SD) to  $18.8\% \pm 8.1$ . In addition, Ig fractions from the same animal also produced different degrees of cytolytic effect when different number of target cells were used. The mean percent cell lysis induced by each Ig fraction at different concentrations of target cells ranged from  $16.2\% \pm 5.5$  to  $17.9\% \pm 11.2$ . There was no linear relation between the cytotoxic effects of the tested Ig fractions and the number of target cells used in the assay.

**Figure 3.6. Complement-dependent cytotoxicity mediated by woodchuck Ig fractions with anti-ASGPR activity induced by WHV infection.** Woodchuck Ig fractions (60  $\mu\text{g}$  protein in 100  $\mu\text{l}$ /assay) purified by chromatography on protein A column (A) or DEAE column (B) were added to HepG2 cells seeded at 1,000, 1,500 or 3,000 cells/well. The Ig fractions were derived from sera of WHV-infected woodchucks with anti-ASGPR activity (animals No.2, No.3 and No.4) or no anti-ASGPR activity (animal No.1), as described in the legend of Fig. 3.4. Subsequently, 10  $\mu\text{l}$  of active (AC) or heat-inactivated (IC) rabbit complement was also added. After incubation for 2 h at 37°C, 100  $\mu\text{l}$  of 2% MTT in PBS was added and the cells incubated for an additional 3 h at 37°C (see section 3.2.6 for details). Results are presented as the percent cytotoxicity of woodchuck Ig fractions in the presence of AC with cell survival in the presence of IC taken as 100%. The bars represent the mean values  $\pm$  SD from two experiments each done with 4 to 5 samples.



Overall, these results suggest that there is a high individual variability in the CDC assay of the Ig fractions from the woodchucks with WHV-induced anti-ASGPR activity.

Fig. 3.6A also shows that the control woodchuck Ig fraction (derived from anti-ASGPR-nonactive sera of WHV-infected animal No. 1) did not produce cytotoxic effect in the presence of AC (less than 3%), except when 3,000 target cells were used for the test (9.4%). Statistical analysis of the values of percent cell lysis induced by Ig fractions of anti-ASGPR positive sera and those from control Ig fractions revealed that the difference between them was significant ( $\chi^2$  test,  $P < 0.05$ ).

Since the Ig fractions of woodchuck sera purified by protein A-chromatography may mainly contain IgG-like Ig, it was of interest to examine whether other Ig classes or subclasses may contribute to the hepatocytolytic effect. For these purpose, woodchuck Ig fractions purified by DEAE-chromatography, which should contain all the classes of woodchuck Igs, were used for testing. Fig. 3.6B shows that the percent cell lysis produced by these Ig fractions from the pool of anti-ASGPR-reactive sera ranged from  $8.9\% \pm 0.3$  to  $12.1\% \pm 0.6$  (mean  $\pm$  SD). In contrast, the control Ig fractions purified by DEAE-chromatography from anti-ASGPR-nonreactive sera produced a percent lysis of  $0.1\% \pm 0.1$  to  $1.7\% \pm 1.5$ . Statistical analysis showed a significant difference in percent cytolysis between cells tested with anti-ASGPR positive versus negative Ig fractions ( $\chi^2$  test,  $P < 0.05$ ). Comparison of the cytolysis percentages obtained using Ig fractions isolated by different methods (3.6A and Fig.3.6B) indicated that the overall cytotoxic effects of Ig fractions purified by protein A chromatography (the mean of cytolysis ranged from 13.1 to 18.8%.) were higher than those of fractions purified by DEAE-chromatography (the mean of cytolysis ranged from 8.9 to 12.1%). This suggest that the cytotoxic antibodies capable of complement activation are enriched in the IgG-like class in the sera with anti-ASGPR-reactivity induced by WHV infection.

It was very surprising that guinea pig Igs isolated from the antisera raised against

wASGPR, rASGPR and rASGPR subunits did not reveal any complement-mediated cytotoxicity to HepG2 cells under the assay conditions described above. However, it is worth mentioning that a CDC effect of Ig fractions from anti-wASGPR antiserum has been observed by other investigators in this laboratory when 8,000 target HepG2 cells were used. Experience gained in the course of the present work suggests that CDC induced by Ig fractions from anti-ASGPR positive woodchuck sera is highly dependent on the assay conditions (e.g., number of target cells). It is possible that a similar situation may exist in the case of Ig fractions from guinea pig antisera.

### 3.4. DISCUSSIONS

#### 3.4.1. Anti-ASGPR Antibody-Mediated Inhibition of Ligand Binding to Purified and Hepatocyte Surface-Bound ASGPR.

Ligand binding sites on ASGPR molecules in human and rat origin are immunogenic (Harford et al., 1982, Schwartz et al., 1981a, 1983). Some of the antibodies directed to the whole receptor or receptor subunits can inhibit ligand binding to both purified and hepatocyte plasma membrane-associated ASGPR. Our findings revealed that antibodies raised against purified wASGPR, rASGPR and the 47-kDa subunit of rASGPR had the ability to inhibit ASGP binding to affinity purified receptors, as well as to ASGPR on woodchuck hepatocytes and human HepG2 cells. These results suggest that the dominant immunogenic epitopes of the ligand binding sites or the epitopes related to the ligand binding on hepatic ASGPR from woodchuck, rabbit and human are similar to each other. This notion is consistent with the observation that human, rat, mouse, as well as chicken ASGPRs have homologous carbohydrate binding domains which are accessible on the extracellular side of hepatocytes (Spies, 1990).

In addition, two other interesting results were obtained in the course of this study. First, the 40- and 47-kDa subunits of wASGPR and rASGPR are immunogenically distinct. Anti-40-kDa antiserum only recognized the 40-kDa subunit and the anti-47-kDa antiserum reacted only with the 47-kDa polypeptides of the ASGPR, either purified soluble or membrane-bound receptors, as shown by Western blotting (see summary in Table 1.4). However, the Ig fractions from both anti-subunit (anti-40-kDa or anti-47-kDa) antisera could completely (100%) inhibit ASGP binding to ASGPR on hepatocytes (Fig. 3.5.). This indicates that blocking one of the subunits by antibody is sufficient to abolish ligand binding to membrane-associated ASGPR. On this basis, we conclude that both subunits of wASGPR on woodchuck hepatocytes form the ligand binding site of the receptor. Alternatively, it is

also possible that there is a distinct binding site on each of the subunits of wASGPR, but the special configuration for ligand binding is such that binding of antibody to one of the subunits blocks or modulates the ligand binding activity of the second subunit. These possibilities are supported by the findings that the expression of both the major and minor subunits of human ASGPR on the surface of transfected murine fibroblast cells is required for the binding of  $^{125}$ I-ASOR (Shia and Lodish, 1989).

Second, it appears that the ligand binding site or a structure related to the ligand binding site on the 47-kDa-subunit of the purified wASGPR is not accessible to antibodies. It was demonstrated in Fig. 3.2 and Fig. 3.3 that Ig fractions from anti-47-kDa antiserum could not block ligand binding to the purified wASGPR or rASGPR, although the Ig fractions from anti-40-kDa antiserum did so. This inaccessibility might be caused by a conformation change due to receptor purification or when receptor was immobilized on the microtiter plates for ligand binding study. It has been reported that the sensitivity to reducing agents is different among the subunits of rat ASGPR (Halberg et al., 1987). The minor subunits can be denatured more easily. So, it is possible that the binding site on the 47-kDa subunit of wASGPR or rASGPR might have been denatured in the course of receptor purification or due to coating the receptor onto the plates. However, it is even more likely that the ligand binding site on the 47-kDa subunit of wASGPR or rASGPR was hidden due to the fact that purified ASGPR molecules are highly aggregated. This possibility is supported by our previous observation that there is a low expression of 47-kDa polypeptides on the surface of purified rASGPR macromolecules (Fig. 1.4).

In the context of the above interpretation and in view of the results showing that Ig fractions from anti-40-kDa antiserum could completely inhibit ligand binding to the affinity-purified ASGPR, it is reasonable to consider that the observed ligand-receptor binding kinetics (Fig. 1.7) may reflect the activity of the 40-kDa subunit only. In other words, the ligand binding values (i.e.,  $K_d$  and  $B_{max}$ , see section 1.3.1.2.2) determined using a solid-phase

assay may only characterize the binding between ligand and the 40-kDa subunit of purified wASGPR or rASGPR, but not the whole natural receptor molecule. It could be expected that binding mediated by the hepatocyte plasma membrane-bound ASGPR would be more efficient than that expressed by purified receptors. In the former case, both subunits of wASGPR or rASGPR would contribute to ligand binding.

Our results revealed that the Ig fractions from woodchuck sera with anti-ASGPR autoantibody induced by WHV infection also inhibited ligand binding to membrane-bound ASGPR. This suggests that the ligand binding sites of wASGPR are autoimmunogenic. Therefore, it is possible that the autoantibodies to ASGPR can interfere with wASGPR recognition of specific ligands *in vivo* and, thereby, may contribute to inhibition of ASGPR endocytosis and accumulation of serum ASGPR. Hyperasialoglycoproteinemia has been observed in patients with cirrhosis and liver cell carcinoma (Sawamura et al., 1984). Administering a single dose of galactosamine could also experimentally induce hyperasialoglycoproteinemia in the rat (Sawamura et al., 1981). The mechanism responsible for this phenomenon is not clear at present. However, the authors found that the levels of ASGPR expressed on the hepatocytes of either patients or experimental rats were decreased compared with the control values. In addition, the distribution of hepatic ASGPR was also found abnormal in the patients with cirrhosis and HCC by other researchers (Burgess et al., 1992; Hyodo et al., 1993). Moreover, anti-ASGPR autoantibody could be detected in patients with chronic liver disorders (McFarlane et al., 1986). Therefore, any factors leading to the reduction of ASGPR expression on hepatocytes, dysfunction of ASGPR and induction of ASGPR loss may contribute to the development of hyperasialoglycoproteinemia. According to our observations and the report of Schwartz et al. (1986), antibody binding to ASGPR on HepG2 cells *in vitro* could result in the blocking of ligand-receptor binding or antibody-induced ASGPR loss. However, in the course of the present study it was also observed that the ability for ligand binding still remained in the wASGPR associated with

purified hepatic plasma membranes from WHV-infected animals (data not shown in this thesis). Analysis of one of these animals showed a high level of anti-ASGPR antibodies in the serum. Moreover, that the anti-ASGPR antibody could bind to the membrane-associated wASGPR *in vivo* was demonstrated in this woodchuck (data not shown). These observations suggest that WHV-induced anti-ASGPR autoantibodies may block the ligand binding sites of ASGPR on some of the hepatocytes, but probably not on all of the hepatocytes. Therefore, probably the majority of the ligand binding sites on ASGPR molecules in the whole liver need to be blocked in order to produce any serious effect. The *in vivo* pathogenic effect induced by autoantibody to ASGPR in the course of hepadnaviral infection requires further studies, particularly in relation to ligand-ASGPR binding.

In addition, we also found that there appeared to be some diversity among the animals in the autoantibody-response to the ligand binding sites of wASGPR. Fig. 3.4 shows that Ig fractions from animal No.4 did not display any inhibitory effect on ligand binding to HepG2 cells. Since the anti-ASGPR-reactivity in the sera of animal No.4 ( $5.6\% \pm 0.9\%$ , mean  $\pm$  SD, ASGPR binding activity determined by RIA) was similar to that of animal No.2 ( $6.1\% \pm 0.9\%$ ) and No.3 ( $7.6\% \pm 1.3\%$ ), one explanation for this result may be that a very low level of or even no anti-ASGPR autoantibody specifically directed against the ligand binding sites was triggered in this animal. Mechanisms of induction of ligand-binding-site-specific autoantibodies in the course of hepadnaviral infection require further detailed analysis by using the woodchuck experimental system.

### **3.4.2. Hepatocytotoxicity of Anti-ASGPR Autoantibodies Induced in the Course of WHV Infection.**

One of the mechanisms by which autoantibody may induce cell killing is by complement-dependent-cytotoxicity (CDC). It has been found that organ-specific autoantibodies (e.g., directed to pancreatic islets, thyroid epithelial, gastric parietal and

adrenal fasciculata) of IgG class can fix complement (Dean et al., 1983), suggesting that these autoantibodies may induce target cell killing through CDC. The CDC effect of autoantibodies is usually measured as cell lysis by using labelled target cells (e.g.,  $^{51}\text{Cr}$  or europium labelling) (Cui and Bystry, 1992; Cui et al., 1993). Interestingly, the value of cell lysis determined by using these methods is usually lower than 50%. For example, Chiovato et al (1993) used  $^{51}\text{Cr}$ -labelling of primary cultures of human thyroid cells as the targets to analyze the specific CDC effect of autoantibodies (IgG fraction) from sera of patients with atrophic thyroiditis and goitrous Hashimoto's thyroiditis. The authors found that specific lysis of the target cells ranged from 5 to 15% when concentrations of 1 or 2 mg Ig protein/ml were used. The interassay coefficient of variation was very high (40%) in their experiments.

In our studies, Ig fractions with anti-ASGPR-reactivity from sera of woodchucks with WHV infection displayed cytotoxicity to HepG2 cells ranging between 12 and 25% cell lysis at an Ig concentration of 1.2 mg/ml. This result was comparable or even exceeded that given by other organ-specific autoantibodies analyzed by Parkes et al. (1994). These low values of cytotoxicity may suggest that the degree of CDC mediated by naturally occurring autoantibody tested under *in vitro* conditions are unlikely to reach very high values. One of the possible reasons can be that antibody binding to ASGPR on HepG2 cells resulted in the receptor loss from the surface, as described by Schwartz (1986), or induced receptor-mediated antibody endocytosis. Another reason can be that some of the target cells are expressing lower amounts of antigen on the surface than others. For cultured cells employed in the studies, it was also possible that not all the cells were at the same stage of the cell cycle. Therefore, target antigen expression might vary among these cells. In the case of ASGPR expression on HepG2 cells, we have analyzed the cells by immunofluorescent staining using anti-ASGPR antibodies (see section 1.3.2) and frequently observed that some of the cells had much lower staining intensity than others, suggesting a heterogeneity in ASGPR expression among these cells. To confirm this, further work should be done by comparing the numbers

of ASGPR molecules expressed on the surface of hepatocytes before and after antibody treatments. To distinguish the possibility of antibody-induced receptor loss or receptor-mediated antibody endocytosis, the incubations should be done at a lower temperature (e.g., 10°C).

Like the ligand binding inhibition effects, the CDC effects of Ig fractions from different woodchucks varied. Such variability could be caused by differences in affinity of the autoantibodies produced by different animals. In addition, results from CDC experiments using the guinea pig Ig fractions enriched with antibodies against wASGPR, rASGPR and subunits of rASGPR revealed that antibodies derived from immunized animals did not show evident cytotoxic effects on HepG2 cells. One probable explanation for such results may be the differences in Ig class composition or epitope-specificity of the anti-ASGPR antibodies produced by guinea pigs immunized with ASGPR compared with woodchuck autoantibodies induced by WHV infection. It is also possible that our assay conditions were not suitable for the detection of CDC effects induced by guinea pig antibodies. In fact, in a most recent series of experiments, it was found that some guinea pig antibodies (e.g., anti-wASGPR) displayed CDC effects under modified assay conditions (i.e., using  $8 \times 10^3$  cultured woodchuck hepatocytes/assay). It has also been demonstrated that guinea pig antiserum to rASGPR were cytotoxic to hepatocytes isolated from the liver biopsies of patients with chronic hepatitis B in the presence of active complement using a microcytotoxicity assay (Michalak et al., 1995).

### 3.5. CONCLUSION

These *in vitro* studies attempted to determine the possible pathobiological effects of antibodies to the ASGPR, either induced after hepadnavirus invasion or generated due to immunization with the affinity-purified receptor. The investigations revealed two mechanisms by which the antibodies may contribute to liver dysfunction.

Firstly, both the hepadnavirus- and ASGPR-induced antibodies were able to recognize the receptor ligand binding sites or structures located in the proximity of the binding sites and specifically inhibit asialoglycoprotein binding to the purified ASGPR and, more importantly, to the receptor associated with the hepatocyte surface. This finding suggests that the naturally occurring anti-ASGPR autoantibodies may interfere with recognition and endocytosis of the circulating ASGP by hepatocytes. According to this scenario, it is reasonable to expect that under some extreme conditions, the autoantibody may lead to such disorder of the physiological removal of desialylated proteins that hyperasialoglycoproteinemia may result.

Secondly, anti-ASGPR autoantibodies induced in the course of WHV infection have the ability to activate complement and mediate complement-dependent killing of hepatocytes *in vitro*. This mechanism may directly contribute to liver injury in viral hepatitis. The true pathogenic significance of this finding requires further evaluation in an *in vivo* system. In addition, other cytotoxic mechanisms of virus-induced ASGPR specific autoimmunity (i.e., T cell mediated responses) need to be elucidated in further investigations. The woodchuck model of WHV-triggered ASGPR-specific autoimmunity should contribute to our better understanding of the complexity of the mechanisms leading to hepatocyte damage and the development of chronic liver disease in viral hepatitis.

## PART IV

### FUTURE WORK

Although it is generally accepted that viral infections can initiate or enhance autoimmune phenomena or autoimmune diseases, the pathogenic mechanisms of this process and the relationship between viral invasion and autoimmunity remains under investigation. Studies involved in this thesis have touched on a few of the questions about the biological and pathological consequences of hepadnaviral infection and the virus-induced liver-specific autoantibody response. Based on the results of the present work and current knowledge on this issue, further *in vivo* studies on two of the above topics which should be addressed are proposed .

#### **4.1. *In Vivo* Experiments to Study Anti-ASGPR-Mediated Liver Injury Through Enlargement of Autoantibody Response Against ASGPR.**

One of the major purposes of the present work was to evaluate the appearance, specificity and biological or pathological effects of autoantibodies against hepatic ASGPR in woodchucks with WHV infection. Results presented in this thesis show that the experimentally induced WHV infection triggers the appearance of anti-ASGPR autoantibodies. Results also revealed that wASGPR was expressed on the surface of hepatocytes and is accessible to both ligand (e.g., D-Gal and ASGPs) and specific antibodies, suggesting that hepatocyte injury or dysfunction might be induced by anti-ASGPR antibodies when plasma membrane associated hepatic wASGPR becomes the target of autoantibody response. Of considerable interest in this regard was our finding that the *in vitro* reactions between ASGPR on HepG2 cells and specific antibodies resulted in the lysis of hepatocytes

in the presence of complement and in the inhibition of ligand binding. However, whether similar CDC effects toward woodchuck hepatocytes *in vitro* could be produced and whether pathobiological effects of anti-ASGPR autoantibodies could occur *in vivo* remains to be determined.

Animal models of liver injury are usually established by treatment with chemicals (e.g., CCl<sub>4</sub>, endotoxin, galactosamine and acetaminophen) (Arthur, et al., 1985, 1988; Laskin, 1990; Winwood and Arthur, 1993). Attempts to establish animal models of liver lesions mediated by liver-specific autoimmune reactions, also called experimental autoimmune hepatitis, have been made by injection of animals with liver antigens in CFA (for review see Lohse, 1991). A model of chronic autoimmune hepatitis was established by repeated injection of rabbits with human liver antigens in CFA. Antibodies against hepatic tissue antigens were detected both in the circulation and on the isolated hepatocytes. Lymphocyte proliferation in response to liver antigen preparations was also observed in these animals. The target antigens were identified as LSP or its subfraction LP2. Experimental autoimmune hepatitis has also been established in mice by immunization with allogenic or syngenic liver homogenate as immunogen. Results of passive transfer experiments suggest the importance of T cells in the pathogenesis of autoimmune hepatitis.

*In vivo* studies on the liver damage caused by antibodies are usually carried out by intravenous injection of antibodies into animals. Poralla *et al* (1987) prepared a monoclonal antibody against rabbit liver-specific protein with a molecular mass of 43 kDa. After intravenous injection of this antibody into rabbits, an increase of liver enzyme (LDH, an indicator of liver damage) levels in the sera and hepatocyte necrosis were found, suggesting hepatic damage induced by this antibody. The hepatotoxicity of another monoclonal antibody raised against rat liver-specific membrane component with a molecular mass of 105 kDa was demonstrated *in vivo* by Ikeda and Kurebayashi (1990). They injected intravenously anti-105-kDa antibody to rats and detected the appearance of acute massive hepatocellular necrosis

which was associated with an increase in liver enzyme (ALT and AST) activities and a decrease in complement concentration in the sera of these rats. C3 deposition in the hepatocyte cytoplasm or at the surface of degenerated and necrotic liver cells was also identified. These results suggested that hepatocyte injury caused by this monoclonal antibody was due to complement-mediated immune attack.

The *in vivo* studies of antibodies against ASGPR through *in situ* perfusion of rat livers with polyclonal guinea pig anti-ASGPR antibodies or monoclonal anti-human-ASGPR antibody were performed by McFarlane *et al* (1990). They showed the deposition of anti-ASGPR antibodies on liver cells in the periportal areas. The authors postulated that the periportal liver cell necrosis in patients with AIH might be caused by an equivalent deposition of natural anti-ASGPR autoantibodies. However, they did not provide histological or immunohistochemical evidence to demonstrate hepatocyte injury after *in situ* perfusion of the anti-ASGPR antibodies. In addition, they reported that no hepatocyte damage was observed in livers of guinea pigs immunized with rASGPR, although these animals produced high levels of anti-ASGPR antibodies. In our present studies on the woodchucks with experimental WHV infection, it was found that the serum autoantibodies against ASGPR were detectable after recovery from acute viral hepatitis. Also, no biochemical evidence of liver injury could be detected in these SLAH animals, although minimal inflammatory changes were observed. Therefore, if there was a pathogenic effect of virus-induced anti-ASGPR autoantibodies, it is likely that such effect could be transient and not detectable using the current monitoring technique.

To verify the pathological significance of anti-ASGPR autoantibodies induced by WHV infection, valuable information may be obtained by injection of WHV-infected animals with affinity-purified ASGPR. This may enhance the autoimmune responses to ASGPR in these animals. For this purpose, two sets of *in vivo* experiments could be proposed. First, to enhance the degree of anti-ASGPR autoantibody response originally induced by WHV

infection, animals with chronic viral hepatitis will be injected with affinity-purified ASGPR in adjuvant (CFA for the first injection and IFA for the remaining injections). Serial sera will be collected weekly or biweekly and tested for anti-ASGPR activity, marker enzymes for liver injury (e.g., ALT and LDH) and serum complement levels. A liver biopsy will be performed at the time when biochemical indicators of liver injury appear. Autopsy will be considered in some of the animals to examine histological changes and to identify the specific deposition and biological effects of anti-ASGPR antibodies on the hepatocytes. A control group will be set up by injection of chronically infected animals with adjuvants alone. Second, to examine the specificity of hepatocyte damage in woodchucks, a passive transfer experiment will be performed by using anti-ASGPR antibodies triggered by WHV infection. These antibodies will be purified by affinity chromatography on ASGPR and injected into healthy woodchucks or animals with WHV infection. Following injection, serum samples will be collected for the detection of ALT, complement and anti-ASGPR antibody levels. Histological examination of the liver tissue will be done to confirm the liver damage. Results from these *in vivo* experiments may provide some evidence to reveal the pathogenic effects of anti-ASGPR autoantibodies in the course of viral hepatitis.

#### **4.2. *In Vivo* Experiments and Clinical Investigation to Study the Relationship Between Anti-ASGPR Autoantibody Response and Viral Persistence.**

The evidence to date is very strong that a causative relationship exists between host immune response and chronic hepatitis B (for a review, see Chisari and Ferrari, 1995a). It has been shown that the strength and specificity of the T cell response to HBV could be important for viral clearance or viral persistence, although the mechanisms controlling the strength and specificity in different individuals are unknown. In view of the association between the presence of autoantibody reactivity to ASGPR in preinoculation sera and the development of chronic WHV infection, it would be intriguing to study viral persistence in

animals with liver-specific autoimmune responses. Due to the preexistence of liver-specific autoimmune responses, the host immune system may not respond to viral antigens in a manner that would limit the spread of infection. Experiments testing the WHV infection rate in animals with pre-existing anti-ASGPR activity should be performed as discussed in part II. In these *in vivo* experiments, perhaps, the most difficult part is to produce experimental autoimmune response to ASGPR in healthy woodchucks. According to our current experience, anti-ASGPR antibodies can be induced by immunization of woodchucks with purified rASGPR. The produced antibodies recognize epitopes on both wASGPR and rASGPR. It is expected that induction of an autoimmune response to ASGPR may also be achieved by introducing bacteria, parasites or chemical agents into woodchucks. However, whether the same ASGPR epitopes will be recognized by the autoantibodies produced under these artificial conditions and by WHV-infection still needs to be determined. The results from these experiments will help us to ask the right questions and to understand the underlying basic mechanisms regarding the pathogenic role of ASGPR-specific autoimmune responses.

Last, but not least, the conclusions from all these *in vivo* experiments should eventually lead us to perform clinical studies, such as investigating the incidence of chronic hepatitis B in individuals with the preexisting ASGPR-specific autoimmune responses, and hopefully allow the development of new preventive and therapeutic strategies for chronic hepatitis B.

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