5-FLUOROURACIL-INDUCED APOPTOSIS AND ALTERED EXPRESSION OF APOPTOSIS-REGULATING PROTEINS IN A MODEL SYSTEM FOR MULTISTAGE CERVICAL CARCINOGENESIS

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5-FLUOROURACIL-INDUCED APOPTOSIS AND ALTERED EXPRESSION OF APOPTOSIS-REGULATING PROTEINS IN A MODEL SYSTEM FOR MULTISTAGE CERVICAL CARCINOGENESIS

By

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ABSTRACT

Cervical cancer is the second most frequent cancer among women in the world. It develops by a multistage pathogenesis. However, the molecular mechanism of cervical carcinogenesis remains unclear. Apoptosis, or programmed cell death, has received widespread attention during the past decade due to its essential role as an effective defense against the emergence of cancer. Anticancer agents are effective through the induction of apoptosis specifically in cancer cells. 5-Fluorouracil (5-FU) is a commonly used, effective anticancer drug. In addition, our laboratory has developed a model system for multistage cervical carcinogenesis. To obtain a better understanding of how the progression of cervical cancer may occur in women, we studied whether and how 5-FU may differentially induce apoptosis in the multiple cellular stages.

To determine whether 5-FU induces apoptosis in our multistage model system composed of primary human ectocervical cells (HEC), HPV 18-immortalized HEC (HEC-18), and CSC-transformed HEC-18 cells (HEC-18T), we treated these cells with 5-FU. 5-FU induced apoptosis in HEC, HEC-18, and HEC-18T, as evidenced by morphologic changes, DNA ladder formation, and flow cytometry analysis. Growth inhibition assay results further indicated that 5-FU-induced apoptosis occurred in a dose- and time-dependent manner. In addition, comparison of HEC with HEC-

18 and HEC-18T revealed that both of the latter cell lines were less sensitive to 5-FU-induced apoptosis than HEC when cultured in serum-free medium KGM. However, a higher level apoptosis induced by 5-FU was observed when HEC-18 and HEC-18T were grown in serum-containing medium DMEM compared with KGM.

The expression level of apoptosis-regulating proteins without treatment with apoptotic stimuli is known to correlate with the sensitivity of cells to apoptosis. To examine whether such a correlation exists in our model system for multistage cervical carcinogenesis, a panel of apoptosis-regulating proteins was analyzed by Western blot analysis without 5-FU treatment. The results revealed that the levels of pro-apoptotic p53, Bak, and Bax proteins were lower in HEC-18 and HEC-18T compared to those in HEC, whereas anti-apoptotic Bcl-2 and BAG-1 p33 isoform, but not Bcl-x_L and BAG-1 p50, p46 and p29, were found in enhanced levels. These findings suggest that immortalization by HPV 18 results in decreased sensitivity to 5-FU-induced apoptosis, possibly due to underexpression of pro-apoptotic proteins and overexpression of specific anti-apoptotic proteins. On the other hand, pro-apoptotic p53 and Bax, but not Bak, showed higher expression levels in HEC-18 and HEC-18T in DMEM than KGM, whereas anti-apoptotic Bcl-2, but not Bcl-x_L and BAG-1 isoforms, was lower in expression. These findings suggest that the two media differentially affect the sensitivity of cells to 5-FU-induced apoptosis,

possibly involving the overexpression of p53 and Bax pro-apoptotic proteins and underexpression of anti-apoptotic Bcl-2.

Apoptosis is of special interest for chemotherapy for human cancers. Extensive studies have been performed towards the understanding of the mechanism for the initiation of apoptosis. Various apoptosis-regulating proteins have been identified and studied. To investigate whether the expression of apoptosis-regulating proteins is involved in the initiation of apoptosis induced by 5-FU treatment, a panel of such proteins was assayed by Western blot analysis in 5-FU-treated HEC-18 and HEC-18T grown in DMEM. Of the apoptosis-regulating and related proteins, p53, p53 target proteins p21 and PCNA but not MDM-2, and Bak but not Bax displayed enhanced expression, whereas anti-apoptotic Bcl-2 and BAG-1, but not Bcl-x_L, exhibited decreased expression. These results suggest that the dysregulated expression of certain apoptosis-regulating proteins is involved in the initiation of 5-FU-induced apoptosis.

Resistance to anticancer agents is the major limitation to chemotherapy for cancers. It has become clear that the sensitivity of cells to apoptosis reflects the sensitivity to being killed by anticancer drugs. Therefore, any method that can increase the sensitivity of cells to apoptosis may be effective in chemotherapeutic strategies. As described above, higher susceptibility of HEC-18 and HEC-18T to 5-FU-induced

apoptosis was observed when the medium used was DMEM instead of KGM. To study in more detail methods to increase susceptibility to apoptosis, low-density lipoproteins (LDL) or elevated Ca^{2+} concentration was employed to study the factors involved in the difference between susceptibility to apoptosis in KGM versus DMEM. Both HEC-18 and HEC-18T exhibited more apoptosis induction by 5-FU treatment when they were cultured in KGM plus LDL than in only KGM or KGM plus Ca^{2+}. This suggests that elevated delivery of 5-FU may play an important role in the differential sensitivity to apoptosis in the two media. Furthermore, both Western blot analysis and flow cytometry results showed that differential expression of LDL receptor on cell surfaces was not involved in the differential apoptotic response.

In summary, this study showed that 5-FU induces apoptosis in our model system for multistage cervical carcinogenesis, discovered that expression level changes of apoptosis-regulating proteins correlate with sensitivity changes of cells to 5-FU-induced apoptosis, implied how 5-FU-induced apoptosis might be initiated and regulated in human ectocervical cells, and also provided information suggesting a feasible approach for clinical application of 5-FU in cervical cancer chemotherapy.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AIDS	acquired immunodeficiency syndrome
ALL	acute lymphocytic leukemia
AP1	activator protein 1
Bcl	B-cell lymphoma
BH	Bcl-2 homology
bp	base pair
C	cytosine
Ca^{2+}	calcium
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
CIS	carcinoma *in situ*
CKII	casein kinase II
CSC	cigarette smoke condensate
DM	double-minute
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FUdR	5'-deoxy-5-fluorouridine
G	guanosine
HEC	human ectocervical cells
HEN	human endocervical cells
HPV	human papillomavirus
Hsp	heat shock protein
HSR	homogeneous-staining region
HSV	herpes simplex virus
HTLV	human T-cell leukemia virus
IC_{50}	50% cell viability inhibiting concentration
ICE	interleukin-1β-converting enzyme
IL	interleukin
IRF-1	interferon regulatory factor 1
Kb	kilobase
kDa	kilodalton
KGM	keratinocyte growth medium
KRF1	keratinocytic-specific transcription factor 1
LCR	long control region
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor

LTR	long terminal repeat
mAb	monoclonal antibody
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCR	non-coding region
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP) ribose polymerase
PBS	phosphate-buffered saline
PCD	programmed cell death
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	propidium iodide
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
Rb	retinoblastoma
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
snrp	small nuclear ribonucleo protein
SV40	simian virus 40

TEMED	N,N,N',N'-tetra-methylethylenediamine
TF	transactivation factor
TGFβ1	transforming growth factor β1
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
URR	upstream regulatory region
UV	ultraviolet

CHAPTER 1 INTRODUCTION

1.1 Carcinogenesis

1.1.1. Introduction

Tumor cells show a number of features differentiating them from normal cells. They have lost the finely controlled balance, which is found in normal cells, between growth promoting and growth restraining so that proliferation normally occurs only when required. Consequently, in tumor cells, continued cell proliferation occurs, loss of differentiation may be found, and the normal process of programmed cell death (apoptosis) may not operate. Specifically: (1) Tumor cells are no longer as dependent on growth factors as normal cells and therefore their proliferation is continuously stimulated. (2) Normal cells require extracellular contact for growth, whereas tumor cells do not. (3) Normal cells respond to the presence of other cells and form a monolayer in culture due to contact inhibition, whereas tumor cells often grow over or under each other. (4) Tumor cells are less adhesive than normal cells. (5) Normal cells stop proliferating once they reach a certain density, but tumor cells continue to proliferate (MacDonald *et al.*, 1997).

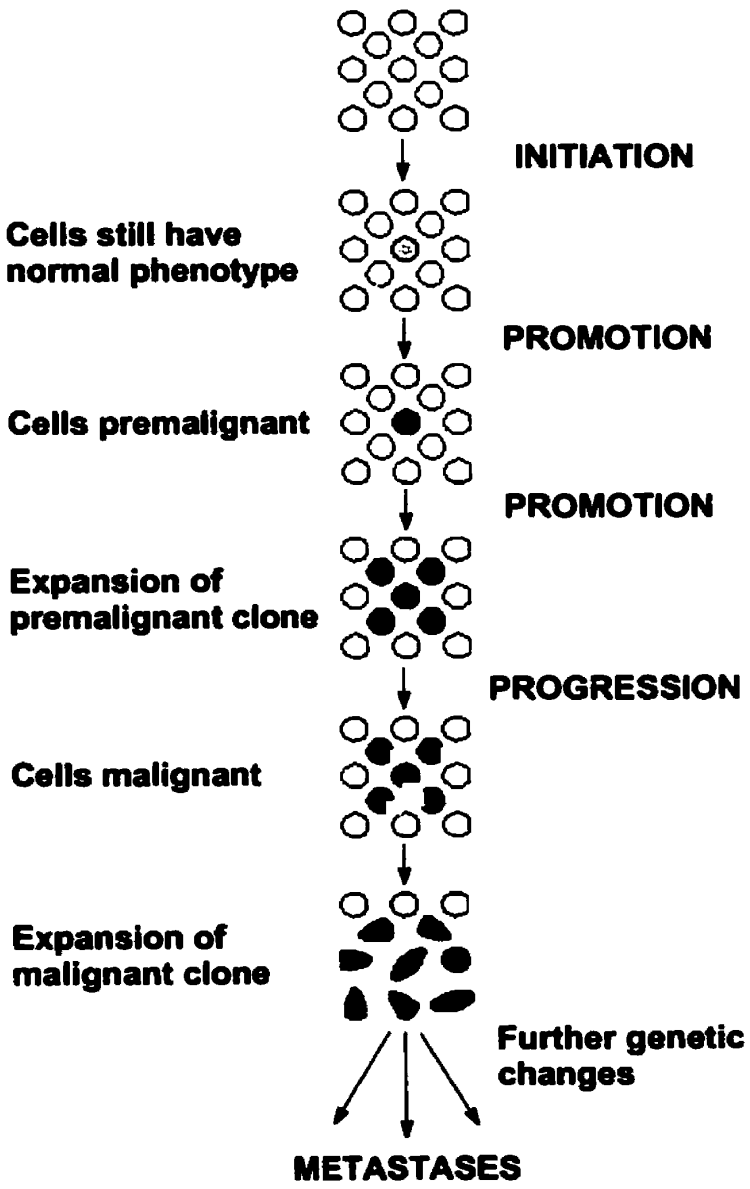
It has been recognized for many years that cancer is a genetic disease. Cancers arise from a single cell as a result of genetic mutation.

The initial mutation will cause cells to produce a genetically homogeneous population of cells. Additional mutations will in turn occur, giving rise to subclones with different properties within the tumor. Therefore, most tumors are heterogeneous.

Cancer development exhibits a multistage nature. Berenblum and Shubik (1949) concluded that carcinogenesis was at least a two-stage process. Armitage and Doll (1954) took this observation a step further and suggested that carcinogenesis is a six or seven stage process. Additionally, Foulds (1957) showed that tumor progression occurred in a stepwise fashion. Although each of these steps is not always found in specific tumors, it is clear that malignancy occurs via multistage progression (Figure 1.1). The initial step is believed to be caused by some form of genotoxic agents such as radiation, chemical carcinogens or viruses. The cells at this stage are phenotypically normal although they have altered DNA levels. Further mutation events lead to the emergence of clones with additional properties associated with tumor cell progression. Finally, additional changes allow the outgrowth of clones with metastatic potential. Animal model studies enabled these steps to be classified as initiation, promotion, malignant progression and metastasis (Nowell, 1976).

Various factors are associated with the development of human cancers. They fall into two types: biophysical and biochemical factors.

Figure 1.1 Multistage progression to malignancy (adapted from Nowell, 1976).


INITIATION
Cells still have normal phenotype
PROMOTION
Cells premalignant
PROMOTION
Expansion of premalignant clone
PROGRESSION
Cells malignant
Expansion of malignant clone
Further genetic changes
METASTASES

Biophysical factors consist of physical factors such as ultraviolet (UV) light and radiation and chemical factors such as chemical carcinogens. Biochemical factors include oncogenes, tumor suppressor genes and cell cycle control genes. The biochemical factors are of special interest for oncologists, since they provide much valuable information about the pathogenesis of human cancers.

1.1.2. Oncogenes

The origin for what we know today about oncogenes comes from studies with viruses. Many DNA viruses, such as SV40, and RNA viruses, such as the retrovirus, Rous sarcoma virus are associated with animal cancers, and some are implicated in human diseases (Table 1.1). These viruses have also been used to reveal the molecular basis for human carcinogenesis (Cheng, 1992). Another useful method, DNA transfection assay, was used to identify oncogenes in the human genome that are specific to tumor cells and responsible for uncontrolled cell proliferation (Shih *et al.*, 1981). However, only about 20% of tumors contained oncogenes that could be identified this way. Alternative strategies were required for identifying additional oncogenes. It has long been clear that some tumors carry a consistent chromosome translocation. In some examples the chromosome breakpoints were shown to coincide with the location of oncogenes already identified by retroviral studies, making chromosomal translocation analysis another useful

Table 1.1 Viruses associated with human cancers and their oncogenes (adapted from MacDonald and Ford, 1997)

Virus	Associated tumors
DNA viruses	
Epstein-Barr	Burkitt's lymphoma
	Nasopharyngeal cancer
Hepatitis B	Liver cancer
Papilloma virus	Benign warts
	Cervical cancer
RNA viruses	
Human immunodeficiency virus(HIV-1)	Kaposi's sarcoma
Human T-cell leukemia virus Type 1(HTLV-1)	Adult T-cell leukemia
HTLV-2	Hairy cell leukemia
HTLV-5	Cutaneous T-cell leukemia

method for identifying oncogenes (Heim *et al.*, 1987). Two other chromosome abnormalities observed in tumors, homogeneous-staining regions (HSRs) and double-minute chromosomes (DMs), have been used to locate cellular oncogenes (Schwab *et al.*, 1990). Currently, 60-100 different cellular proto-oncogenes have been identified, and they exhibit their oncogenic activities when activated. In normal cells, the expression of these proto-oncogenes is tightly controlled and they are transcribed at the appropriate stages of growth and development of cells. However, alterations in these genes or their control sequences lead to inappropriate expression, contributing to malignant progression.

Proto-oncogenes have been predicted to be involved in the basic essential functions of the cell related to control proliferation and differentiation. Cells are stimulated by external signals, such as growth factors and hormones, which bind to cell surface receptors, thereby activating their function as signal transducers. In turn, this stimulates intracellular signaling pathways, eventually leading to alternations in gene expression. The proto-oncogenes function at each of these steps; therefore, mutations in any of the genes can result in their abnormal activation, cell growth promotion in the absence of external stimuli and malignant transformation. Growth factors act at the first step of the pathway and exist as polypeptides, oligopeptides or steroid hormones (Aaronson, 1991). They bind to specific receptors to stimulate or inhibit

cell growth acting via alterations in gene expression. Two models, autocrine model and paracrine model, have been established to suggest auto-stimulation and reciprocal stimulation of growth factor production, respectively (Sporn *et al.*, 1980). A second group of proto-oncogenes encode either growth factor receptors themselves or their functional homologs. The growth factor receptors link the information from the extracellular signals to intracellular signaling pathways. With respect to cancer development, the transmembrane receptor tyrosine kinases play the most important role (Aaronson, 1991). The third group of proto-oncogenes produce proteins which are capable of interacting with activated tyrosine kinases and are involved in cell signaling (Schlessinger, 1994). The final group of proto-oncogenes are those involved in the control of gene expression by their function as transcription factors. This is the last step of signal transduction and is the level at which control of growth and proliferation ultimately operates. Several proto-oncogene proteins, such as MYC (Amati *et al.*, 1994; Harrington *et al.*, 1994), JUN and FOS (Angel *et al.*, 1991), have been shown to bind to DNA and presumably control the transcription of genes. Since the various groups of proto-oncogenes perform major functions in the cell, any disruption in their expression would therefore be expected to result in disruption in the control of that cell, leading to its death or transformation.

Certain mechanisms have been reported by which proto-oncogenes are activated. The first mechanism is the production of an abnormal product which can occur by point mutations (McCormick, 1995; Rodrigues *et al.*, 1994), chromosomal translocation (Rabbitts, 1994), or deletion of part of the protein (Seidel-Dugan *et al.*, 1992). The second mechanism is the over-production of the normal protein by amplification of the proto-oncogene (Schwab *et al.*, 1990). The third mechanism of activation is described as loss of control mechanisms (Spencer *et al.*, 1991). There is no single consistent mechanism of activation of these genes, but the final result is to give a protein that can cause abnormal growth.

Oncogenes have been found in most groups of cancers so far examined. A few of the most common cancers and associated oncogenes are listed in Table 1.2 (Cooper, 1990; MacDonald *et al.*, 1997). Mutations in proto-oncogenes are very rarely the cause of familial cancers, since they are likely to be lethal. In many tumors such as Burkitt's lymphoma, the presence of the oncogene is likely to have an important, but not necessarily primary, role in the cancer development. In the majority of other cancers it is not clear exactly what role the oncogene has to play in the carcinogenesis. It was also reported that one gene might be able to destablize the cell sufficiently to provide an environment in which further changes in other oncogenes are likely (Wyllie *et al.*, 1987). A single mutational event usually is not enough for a tumor to develop, and much

evidence indicates that there is collaboration between different oncogenes necessary to produce the fully transformed phenotype. In model systems it was initially demonstrated that whereas a single oncogene was insufficient for transformation, collaboration between genes could result in full transformation. More direct observations have been made in transgenic mice (Sinn *et al.*, 1987). Since carcinogenesis is a multistage process, model systems with components representing different stages involved in malignant developments are usually crucial for more detailed investigation on mechanistic studies.

1.2. Human papillomavirus (HPV)

Cervical cancer is a major health issue for women, because it is the third most common and fourth most deadly malignancy among women in the world (Parkin *et al.*, 1999; Pisani *et al.*, 1999). The global estimates are for 452,000 new cases and more than 234,000 deaths from cervical cancer each year around 2000(Miller *et al.*, 2000). Screening for cervical cancer has undoubtedly led to an overall decline in incidence and mortality in many countries. However, mortality rates are leveling off or increasing in women under 40 years of age in most countries (Beral *et al.*, 1994). Consequently, developing therapeutic strategies for treating cervical cancer continues to be an important objective.

Table 1.2 Oncogenes in human tumors (adapted from MacDonald and Ford, 1997)

Tumor	Associated oncogenes
Bladder	*HRAS, KRAS, INT-2*
Brain	*ERBB1, SIS*
Breast	*ERBB2, HRAS, MYC, INT-2*
Cervical	*MYC, HRAS, ERBB2*
Colorectal	*HRAS, KRAS, MYB, MYC*
Gastric	*ERBB1, HST, MYB, MYC, NRAS, YES*
Lung	*ERBB1, HRAS, KRAS, MYC, LMYC, NMYC*
Melanoma	*HRAS*
Neuroblastoma	*NMYC*
Ovarian	*ERBB2, KRAS*
Pancreas	*KRAS, MYC*
Prostate	*MYC*
Testicular	*MYC*
Leukemia	*ABL, MYC, BCR, BCL1, BCL2*

1.2.1. Introduction

Sexual activity has been recognized as one of the most important risk factors involved in the development of cervical carcinoma, and numerous infectious agents have been suggested to play a causative role (Davies *et al.*, 1993; Vousden, 1989). Currently, the most likely candidates among those are human papillomaviruses (HPVs). The hypothetical link between HPVs and cervical carcinoma was first suggested in the 1970s (zur Hausen, 1975; zur Hausen, 1976). Less than 10 years after the hypothesis, the DNAs of HPV types were cloned and characterized (zur Hausen, 1989). Moreover, these DNAs are regularly and frequently found in biopsies obtained from cervical cancer patients throughout the world (Dürst *et al.*, 1983; Boshart *et al.*, 1984).

There is a large virus family known as papovaviradae consisting of polymaviruses, simian virus 40 (SV 40), and human papillomaviruses (HPVs) (Salsman, 1986). HPVs are small DNA viruses known to be capable of inducing papillomas or warts in a number of higher vertebrates including human beings (Table 1.3). Infection by HPVs is restricted to epithelial cells of the epidermis or squamous epithelial cells of the oral or genital mucosa. Infection is believed to occur by the entry of the virus into basal epithelial cells exposed by microlesions or local abrasions of the skin, or into proliferating cells that are exposed at the squamocolumnar border of the cervix uteri (Subramanian, 1993).

Table 1.3 Naturally occurring cancers associated with papillomaviruses (adapted from Shah *et al.*, 1990)

Species	Cancer	Predominant viral types	Cofactors
Humans	Skin carcinomas in EV patients	HPV-5, -8	Sunlight, genetic defect
	Lower-genital-tract cancers	HPV-16, -18, -31, -33	Not known
	Malignant progression of respiratory papillomas	HPV-6, -11	X-irradiation
Cattle	Alimentary-tract carcinoma	BPV-4	Bracken fern
	Eye and skin carcinoma	Not characterized	Sunlight
Sheep	Skin carcinoma	Not characterized	Sunlight
Cottontail rabbit	Skin carcinoma	Cottontail rabbit papillomavirus(CRPV)	Not known

1.2.2. Classification

More than 70 HPV types have been isolated and characterized so far, among which 30 types are associated with anogenital tract lesions including cervical carcinomas (DeVilliers, 1989). According to infection region, HPVs can be divided into two groups: those that cause cutaneous lesions and those that cause lesions of the oral or genital mucosa (Orth, 1986). According to malignant potential of lesions they induce, HPVs are classified into two other groups. The low-risk group is associated with benign lesions, whereas the high-risk group is associated with malignant lesions (zur Hausen *et al.*, 1994). A majority of HPVs causing mucosal lesions have been found to be associated with lesions of the genital tract. Following the initial evidence implicating HPVs related to cervical carcinogenesis, further investigations identified the first high-risk type HPV16 DNA and the second high-risk type HPV18 DNA in 70-80% of cervical tumors and tumor cell lines (Pater *et al.*, 1985). Approximately 85% of all cervical carcinomas are conservatively estimated to be high-risk HPV positive. Epidemiological studies have shown that infection with a high-risk HPV is a significant risk factor for developing cervical cancer (Munger, 1995).

1.2.3. Genome organization

Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. The papillomavirus particles are 52-55 nm in diameter and consist of an icosahedral outer shell or capsid made up of viral structural proteins enclosing a core that contains the viral DNA complexed with host histones forming a nucleosome structure similar to that of the host cellular DNA. Two viral proteins constitute the capsid. The major protein has a molecular weight of approximately 55 kDa and represents approximately 80% of the total structural protein. The minor protein has a molecular weight of approximately 70 kDa (Howley, 1991).

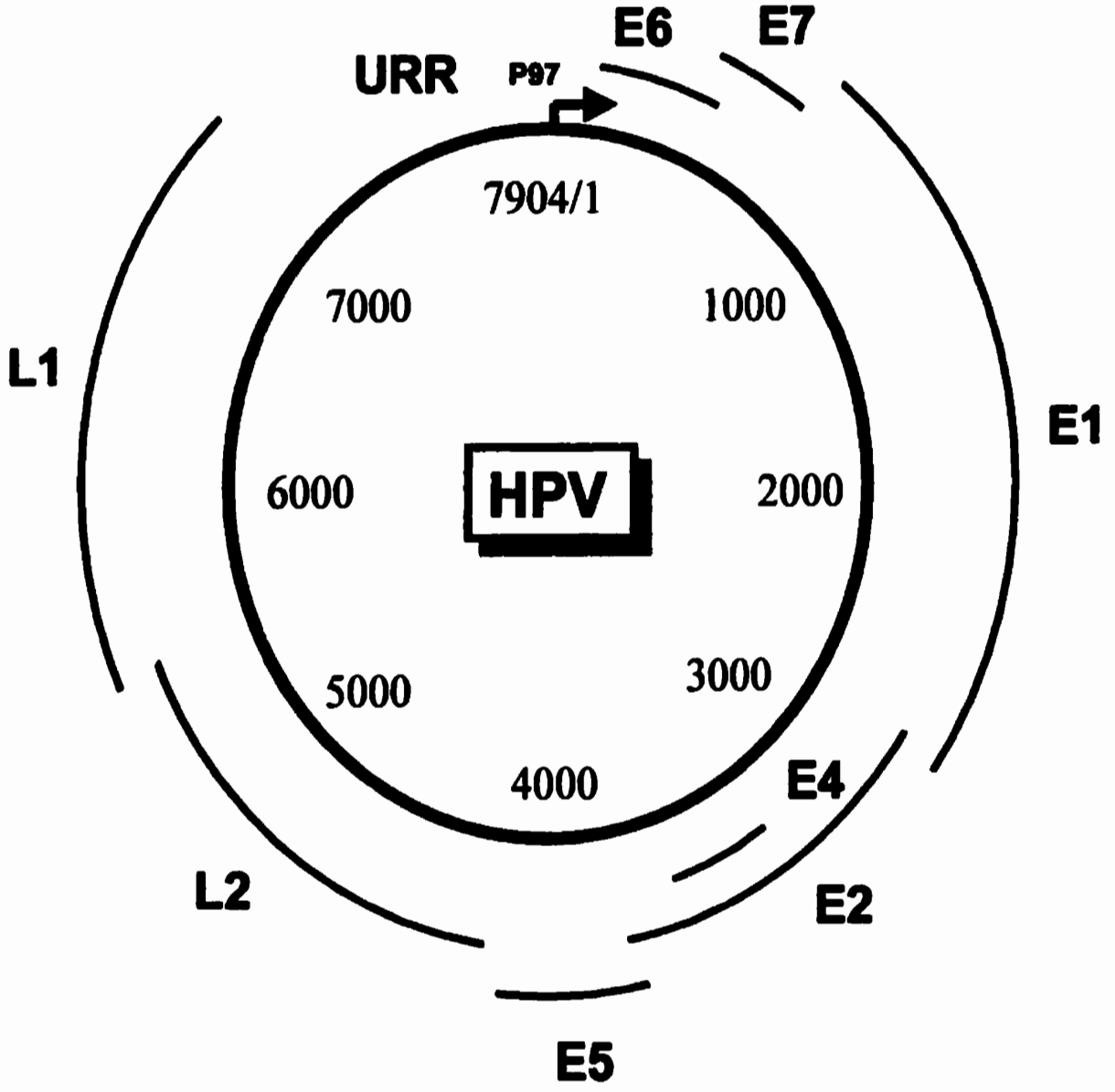
Full HPV particles contain the viral genome in a double-stranded circular DNA form. All HPVs have a genome with a size of approximately 8,000 base pairs (bp) and a molecular weight of 5.2×10^6 Daltons. The guanosine (G) : cytosine (C) content is approximately 42%. The DNA constitutes approximately 12% of the virion by weight, accounting for the density in cesium chloride of approximately 1.34 g/ml (Cranford *et al.*, 1963).

Despite the significant differences in target cell specificity of HPVs, the structural genome organization (Figure 1.2) and functions of each genomic region (Table 1.4) of all of the known HPVs are similar. The viral

Table 1.4 Functions of HPV encoded proteins (adapted from Davies *et al.*, 1993)

Early proteins	
E1	Control of replication
E2	Control of transcription
E5	Transformation (not expressed in cancers)
E6	Immortalisation/transcriptional control
E7	Immortalisation/transcriptional control
Late proteins	
L1	Structural protein
L2	Structural protein
E4	Disruption of cell keratin cytoskeleton
Non-coding region	
URR	Cis acting transcription and replication control

Figure 1.2 Genomic organization of HPVs (adapted from Park *et al.*, 1995).


E6
E7
URR
P97
7904/1
7000
1000
L1
E1
6000
HPV
2000
5000
3000
4000
E4
L2
E2
E5

genome is generally organized into three major regions: early region (E), late region (L), and upstream regulatory region (URR).

1.2.3.1. Upstream regulatory region

Upstream regulatory region (URR), also called long control region (LCR) or non-coding region (NCR), is a 400 bp DNA segment adjacent to the origin of viral replication. URR does not encode protein, but instead, contains a complex array of overlapping binding sites for many different transcriptional repressors and transcriptional activators (Turek, 1994). These include activator protein 1(AP1), keratinocytic-specific transcription factor 1 (KRF1), and nuclear factor (NF-I/CTF) as well as virally derived transcriptional factors encoded by the early region. Functioning as enhancer elements, URR regulates transcription from the early and late regions and controls the production of viral proteins and infectious particles. It is generally believed that URR plays a critical role in determining the host range of specific types of HPV because of its potential for binding a wide array of specific transcriptional factors (Hoppe-Seyler *et al.*, 1994).

1.2.3.2. Early region

The early region is downstream of the URR and consists of six open reading frames (ORFs), designated E1, E2, E4, E5, E6, and E7. Open reading frames are DNA segments that are transcriptional units and are capable of encoding proteins. Two of the early region ORFs, E6 and E7, encode oncoproteins critical for viral replication as well as host cell immortalization and transformation. In several malignant lesions and in cell lines derived from cervical cancer (SiHa, CaSki and HeLa), HPV DNA is integrated in the cellular genome in single or multiple copies. This viral DNA integration results in the disruption of several viral genes with preservation of only E6 and E7 genes (Baker *et al.*, 1987a; Matsukura *et al.*, 1986; Munger *et al.*, 1992; Schwarz *et al.*, 1985). Both E6 and E7 proteins are reported to have an immortalizing and transforming activity *in vitro* (Tommasino *et al.*, 1995). The E1-encoded protein of HPV 11 has ATPase activity (Bream *et al.*, 1993). The full-length E2-encoded protein acts as a transcriptional activator that binds to specific DNA sequences in URR to increase transcription of the early region, whereas a smaller E2-encoded protein inhibits transcription of the early region (Ward *et al.*, 1989). On the other hand, the E4 protein appears to be important for the maturation and replication of the virus and, like the L1 and L2 capsid proteins, is expressed in later stages of infection when complete virions are being assembled (Brown *et al.*, 1994). However, less is known about

the function of E5 in the viral life cycle. The E5 protein interacts with cell membrane receptors, and this may stimulate cell proliferation in HPV-infected cells (Conrad *et al.*, 1994).

1.2.3.3. Late region

The late gene region contains two separate ORFs, termed L1 and L2, which encode the viral capsid proteins. The L1 encodes the major viral capsid protein, which is highly conserved among different HPVs. Antibodies made against bovine papillomavirus L1 capsid proteins cross-react with HPVs and have been used to identify HPV capsid proteins in human tissues. *In vitro* vaccinia virus and baculovirus expression systems have been used to produce HPV L1 capsid protein that self-assemble to form empty viral-like particles and results showed that HPV type-specific antibodies occur in HPV-infected women (Galloway, 1994; Kinnbauer *et al.*, 1994). The L2 encodes the minor capsid protein, which has considerably more sequence variation between HPV types than does the L1 protein and has been used as a source of antigen for type-specific HPV antibodies. Transcription from the L1 and L2 ORFs occurs when complete virions are being assembled. Transcription appears to be regulated by cell-derived transcriptional regulators that are only produced by the more differentiated epithelial cells in the upper layers of infected epithelium (Baker *et al.*, 1987b).

1.3. Apoptosis

Apoptosis or programmed cell death (PCD) is a genetically controlled response for cells to commit suicide (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; White, 1996). It is recognized to play critical roles in a wide variety of normal physiologic processes, including fetal development, immune cell education, and tissue homeostasis (Reed, 1994; 1998). Moreover, dysregulation of apoptosis contributes significantly to the pathogenesis of many human diseases, including those such as cancer, autoimmunity, and restenosis characterized by insufficient cell death, as well as those involving excessive cell death, such as acquired immunodeficiency syndrome (AIDS), stroke, myocardial infarction, and some neurodegenerative diseases (Thompso, 1995).

Apoptosis differs from necrosis or accidental cell death in a number of important ways. Firstly, it is an active process as opposed to an unplanned process induced by cell injury. Secondly, apoptotic cells are recognized by phagocytes and removed before they disintegrate. As a consequence, there is no surrounding tissue damage or induction of inflammatory responses. In contrast, in necrosis, cells become leaky, release macromolecules and rapidly disintegrate, thereby inducing inflammation (Darzynkiewicz *et al.*, 1998).

Table 1.5 Changes in cell morphology and gene expression during apoptosis (adapted from Darzynkiewicz *et al.*, 1998)

Morphological Changes
• Cell shrinkage
• Cell shape change
– Nuclear chromatin condensation
– Loss of visually recognizable nuclear structure (framework)
• DNA hyperchromicity
• Dissolution of nuclear envelope
• Nuclear fragmentation
• Condensation of cytoplasm
• Loss of cell surface structures (pseudopodia, microvilli)
• Formation of apoptotic bodies ("budding", "blebbing")
• Detachment of cells in cultures
• Phagocytosis of the apoptotic cell remains

Biochemical and Molecular Events of Apoptosis

- Increased ratio of apoptosis promoters vs inhibitors of Bcl-2 family
- Mitochondrial changes
  - Decrease in the transmembrane potential
  - Leakage of cytochrome C
  - Oxidative stress (formation of ROI)
- Intracellular Ca^{2+} rise
- Cell dehydration
- Loss of asymmetry in plasma membrane phospholipids
- Activation of serine protease(s)
- Cascade-activation of caspases
  - Proteolysis of the "death substrates"
- Degradation of F actin and proteins other than "death substrate"
- Loss of DNA stability to denaturation
- Presence of ss DNA sections
- Endonucleolytic DNA degradation
  - 50-300 Kb fragments
  - Cleavage in internucleosomal DNA
- Activation of transglutaminase

1.3.1. Characteristics

Table 1.5 lists the morphological and molecular characteristics that appear during apoptosis. Many of these features serve as markers to identify and quantify apoptotic cells.

1.3.1.1. Morphological

One of the early events of apoptosis is cell dehydration. Loss of intracellular water leads to condensation of the cytoplasm followed by cell shape and size changes. As a consequence, the cells become elongated or acquire irregular shape and become smaller. Another change is condensation of nuclear chromatin, which is one of the most characteristic features of apoptosis. The condensation starts at the periphery of the nucleus with the areas of condensed chromatin often acquiring a concave shape resembling a half-moon or horseshoe. The condensed chromatin has a very uniform, smooth appearance, with no evidence of any texture normally seen in the interphase nucleus (Darzynkiewicz *et al.*, 1997; Szabo *et al.*, 1987).

During further progression of apoptosis the nuclear envelope disintegrates, lamin is proteolytically degraded, and the nucleus undergoes fragmentation. Nuclear fragments are then scattered throughout the cytoplasm. Together with constituents of the cytoplasm, the nuclear fragments are packaged and enveloped by fragments of the

plasma membrane. These structures, called apoptotic bodies subsequently detach from the surface of the cell by the process often defined as "blebbing", which rather resembles the mechanism of "budding" in yeast (Majno *et al.*, 1995). Loss of cell surface structures is also a characteristic feature of apoptosis (Endersen *et al.*, 1995). Cells detach during apoptosis and float in the medium when they grow in tissue culture. When apoptosis occurs, *in vivo* apoptotic bodies are phagocytized by neighboring cells, including those of epithelial or fibroblast origin, without triggering an inflammatory reaction or scar formation in the tissue (Arends *et al.*, 1995; Wyllie, 1992).

1.3.1.2. Molecular

Changes in the expression level ratio of apoptosis promoters of the Bcl-2 protein family, such as Bax, Bik, and Bad, over apoptosis inhibitors such as Bcl-2, Bcl-x_L, and Bcl-w, appear to be among the earliest molecular events of apoptosis (Golstein, 1997; Kumar, 1997; Nagata, 1997). These proteins dimerize with one another, which is modulated by their phosphorylation (Gajewski *et al.*, 1996). However, little is known about the mechanism by which the Bcl-2 proteins execute their function in promoting or inhibiting apoptosis. The structure of Bcl-x_L suggests that it may form pores in biological membranes and interactions between the antagonistic members of the Bcl-2 family may regulate the pore's

permeability (Muchmore *et al.*, 1996). The loss of mitochondrial trans-membrane potential (Petit *et al.*, 1995), the appearance of reactive oxidative intermediates (ROIs) within the cell (Hedley *et al.*, 1996), and the release of cytochrome C (Kluck *et al.*, 1997) and Ca^{2+} from mitochondria (McConkey *et al.*, 1989) are all presumed as consequences of the opening of pores in the mitochondrial membrane. These events can activate downstream caspases, and appear to be the earliest events, triggering the cascade of the apoptotic process (Krippner *et al.*, 1996; Wu *et al.*, 1997).

The most characteristic change in the plasma membrane is the loss of asymmetry of the phospholipids on the plasma membrane leading to exposure of phosphatidylserine on the surface (Fadok *et al.*, 1992). Exposure of phosphatidylserine on the out leaflet of the plasma membrane preconditions remnants of the apoptotic cells to become a target for phagocytizing cells. Loss of pseudopodia or microvilli is paralleled by degradation of F actin (Endersen *et al.*, 1995). In addition, activation of transglutaminase results in a change in the physical properties of the cell, making them stiffer and resistant to deformation under pressure (Piacentini *et al.*, 1995).

Emerging evidence demonstrates the important role of a family of aspartate specific cysteine proteases recently termed caspases in apoptosis (Alnemri *et al.*, 1996). The cascade-like activation leads to

proteolytic degradation of selected proteins. These substrate proteins include poly(ADP)ribose polymerase(RARP), lamin, actin, U1 small nuclear ribonucleo protein (snrp), rho-DGI, and DNA-dependent protein kinase (Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995). Some specificity in degradation of the proliferation-associated nuclear proteins Ki-67, p120, and proliferating cell nuclear antigen (PCNA) has also been observed (Gorczyca *et al.*, 1993).

The activation of an endonuclease(s) is another very characteristic event of apoptosis (Compton, 1992). Initially, DNA is cleaved at the sites of attachment of chromatin loops to the nuclear matrix (Oberhammer *et al.*, 1993). Subsequently, DNA is preferentially cleaved between nucleosomes. The products are discontinuous DNA fragments representing nucleosomal- and oligonucleosomal- sized DNA sections. They form a characteristic "ladder" pattern during agarose gel electrophoresis (Gong *et al.*, 1994). However, in many cell types, DNA cleavage during apoptosis does not proceed to inter nucleosomal-sized sections but rather proceeds only to 300-350 Kb size DNA fragments (Ormerod *et al.*, 1994; Zakeri *et al.*, 1993; Zamai *et al.*, 1996).

1.3.2. Inducers

Apoptosis is imperative for host survival since it discards unwanted, damaged, and atypical cells. This process is implicated in the continuous

regulation of development, differentiation, and homeostasis. Furthermore, apoptosis is a response to physiological and pathological factors that disrupt the balance rates of cell proliferation and death (Dixon *et al.*, 1997). Both the activation and inhibition of apoptosis are genetically controlled. Additionally, they are influenced by physiological and nonphysiological stimuli (Fesus *et al.*, 1991).

Loss of protective signals, such as hormones and cytokines, is an important physiological trigger of apoptosis. Several growth factors and hormones have been shown to promote cell survival. Apoptosis occurs in normal tissues when there is a loss of these hormonal signals. Loss of androgen stimulated by castration of male nude mice or rats leads to prostate atrophy (Kyprianou *et al.*, 1988; 1991). Glucocorticoids are steroid hormones that are involved in the induction of apoptosis in immature T-cells in the thymus. The cell death induced in this case requires protein synthesis (Cohen *et al.*, 1984). Adhesion molecules (intergins) have also been shown to promote cell viability upon binding to specific cell surface receptors (Ruoslahti *et al.*, 1994).

Cytokines are low molecular weight signaling molecules that bind to high affinity receptors on target cells. They may provide signals for cell survival, proliferation, differentiation or death. Death inducing cytokines, such as FasL and tumor necrosis factors (TNF), can act as membrane bound ligands or soluble secreted factors. Fas and TNF receptor ligation

induces apoptosis in the absence of RNA or protein synthesis (Itoh *et al.*, 1991; Yonehara *et al.*, 1989). TNF mediates a subset of mature T cells to undergo apoptosis through the p75 TNF receptor (Zheng *et al.*, 1995). Interleukin (IL)-4 was shown to trigger apoptosis in immature B cells (Manabe *et al.*, 1994). On the other hand, IL-6 inhibited the apoptotic response of myeloid cells induced by transfected wild type p53 and in malignant plasma cells (Lichtenstein *et al.*, 1995; Yonish-Rouach *et al.*, 1991).

One of the most important initial observations with respect to drug-induced apoptosis was that etoposide caused rapid internucleosomal fragmentation (Kaufmann, 1989). This chemotherapeutic agent was thus recognized as a potent inducer of apoptosis. The spectrum of diverse acting cytotoxic agents known to trigger an apoptotic response includes inhibitors of protein and RNA synthesis, dihydrofolate reductase inhibitors, topoisomerase I and II targeting drugs, nucleoside analogues, microtubule poisons, alkylating agents, cisplatin, ionizing radiation, and hydrogen peroxide (Fisher, 1994; Hannun, 1997). Many of these agents have been shown to provoke consistently an apoptotic response in a variety of cell types. Another nonphysiological factor is Ca^{2+} concentration elevation (McConkey *et al.*, 1990). Ca^{2+} concentration control is critical to cell viability and function. However, uncontrolled Ca^{2+} increase can mediate cell killing (Orrenius *et al.*, 1989). Current studies demonstrate that it is

possible that both physiological and nonphysiological inducers utilize common molecular mediators in the induction of apoptotic cell death.

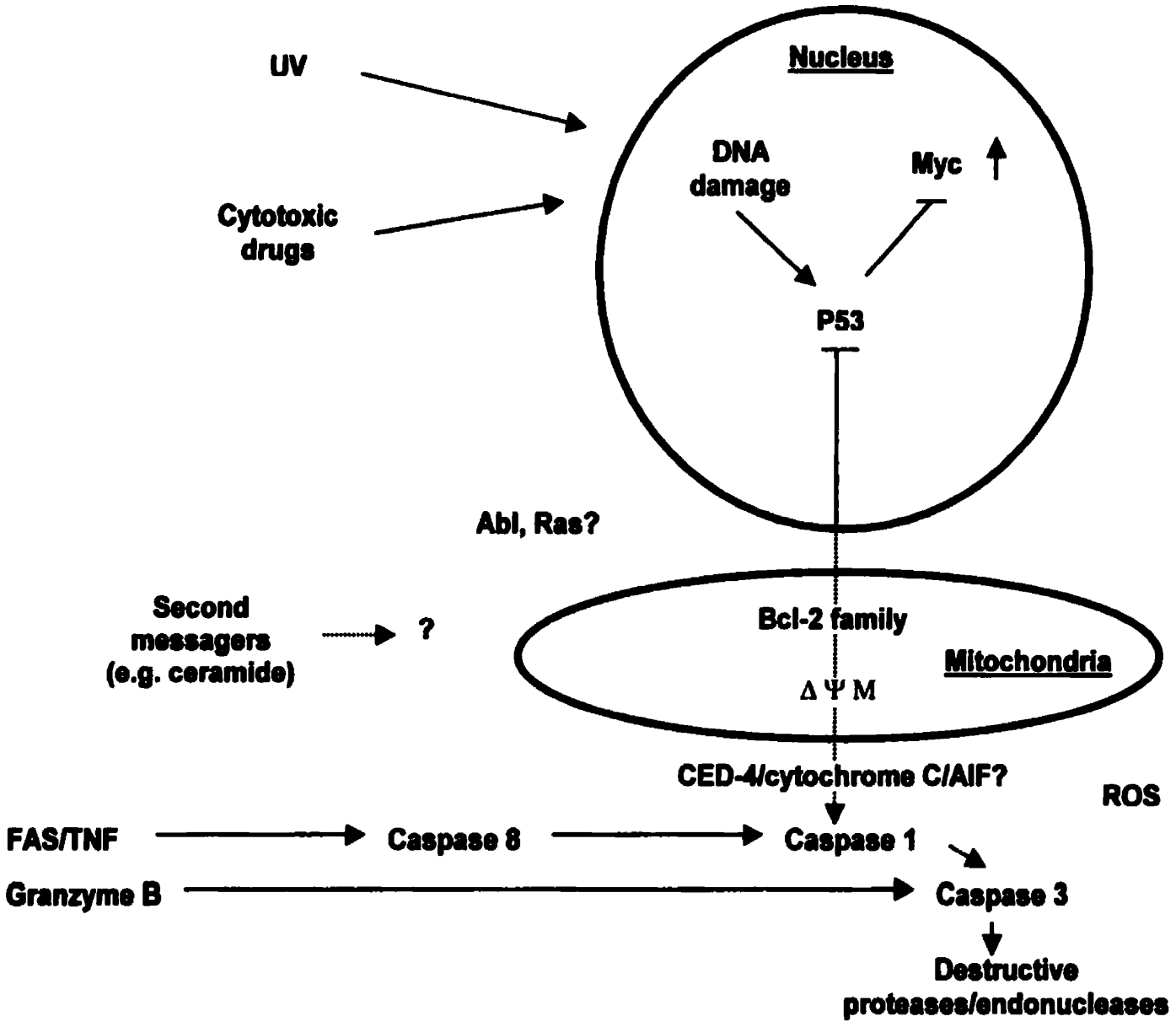
1.3.3. Regulation

A large number of apoptosis regulators have been identified, and several gene families largely comprised of cell death mediators have emerged. In addition, a variety of previously identified oncogenes or tumor suppressor genes have been shown to modulate pathways for apoptosis. Functional and comparative studies with some cell death regulators have enabled a limited amount of molecular ordering in the apoptotic pathway as shown in Figure 1.3 (McKenna *et al.*, 1998).

1.3.3.1. Bcl-2 protein family

Bcl-2 protein family includes both pro-apoptotic proteins, such as Bax, Bak, Bad, and Bik, and anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L. Family members interact to form homodimers and heterodimers with other related or non-related proteins. Bcl-2/Bcl-2 homodimerization involves a head-to-tail association in which sequences located in the first approximately 80 amino acids of the protein where the BH4 domain resides form an interaction with sequences located in the more remote

Figure 1.3 Gene regulation of apoptosis (adapted from McKenna *et al.*, 1998)


UV
Cytotoxic drugs
Nucleus
DNA damage
Myc
P53
Abl, Ras?
Second messagers (e.g. ceramide)
?
Bcl-2 family
Mitochondria
ΔΨM
CED-4/cytochrome C/AIF?
ROS
FAS/TNF
Caspase 8
Caspase 1
Granzyme B
Caspase 3
Destructive proteases/endonucleases

portions of the protein where BH1, BH2, and BH3 domain are located (Sato *et al.*, 1994). Therefore, it is not a surprise that Bcl-2 mutants with the deletion of BH4, BH1, or BH2 do not form homodimers with themselves but still bind the wild-type Bcl-2 protein affording mutant/wild-type heterodimers (Hanada *et al.*, 1995). These mutants are deficient in anti-apoptotic function in mammalian cells, suggesting that Bcl-2/Bcl-2 homodimerization is important for function (Borner *et al.*, 1994). Recent data suggest that deletion of BH4 domain of Bcl-2 converts the protein to a dominant inhibitor of the wild-type Bcl-2 protein, suggesting that mutant/wild-type heterodimers may be dysfunctional dimmers.

In contrast to Bcl-2/Bcl-2 homodimerization, the BH4 domain of Bcl-2 is completely expendable for dimerization with Bax. Actually, the first approximately 80 amino acids of Bcl-2 can be removed without impairing heterodimerization with Bax. Although BH4 is not required for heterodimerization with Bax, the BH1 and BH2 domains are necessary (Hanada *et al.*, 1995). BH4 domain of Bcl-2 is postulated to sterically interfere with the binding of Bax to some other death effector protein or to modulate somehow post-translational modifications of the Bax protein. However, it is also possible that this domain is required for Bcl-2/Bcl-2 homodimerization or for interactions of Bcl-2 with other proteins that require the BH4 domain for their association with Bcl-2/Bax complexes (Wang *et al.*, 1994).

Further studies show that the BH1 and BH2 domains of Bax are expendable for both homodimerization with Bax and heterodimerization with Bcl-2. In contrast, the BH3 domain of Bax is absolutely required for binding to both wild-type Bax and Bcl-2 (Zha *et al.*, 1996). Bax/Bcl-2 heterodimerization appears to occur through a tail-to-tail interaction. N-terminal truncation mutants of Bax can also homodimerize with themselves, indicating that Bax/Bax homodimerization also occurs via tail-to-tail interaction (Zha *et al.*, 1996).

Data show that many other Bcl-2 family members can form homodimers with themselves and heterodimers with other proteins. Cell susceptibility to apoptosis is generally thought to be largely influenced by the relative ratios of pro-apoptotic and anti-apoptotic Bcl-2 family proteins.

1.3.3.2. Caspase family

Subsequent to the description of the pro-apoptotic function of CED-3, interleukin-1β-converting enzyme (ICE), and murine Nedd2, a family of homologues have been identified, all of which are cysteine proteases that share the requirement for cleavage at aspartate residues. These proteases have been termed caspases (Yuan *et al.*, 1993). There are 11 caspases being identified and described so far.

Caspases are produced as inactive pro-enzymes, and they are activated by proteolytic cleavage, which generates two subunits that form

a tetramer. Emerging evidence suggests the caspases containing a long pro-domain are the first to be activated in response to apoptotic stimuli, while those with short or absent pro-domains are activated subsequently and are the effector proteases responsible for cleaving the cellular substrates that mediate apoptotic cell death (Harvey *et al.*, 1998).

The genetic evidence from *C. elegans* implying a central effector role for proteases was supported by the fact that over-expression of caspases induces apoptosis in a variety of cell types (Ellis *et al.*, 1986). Moreover, specific inhibitors of caspases block apoptosis induced by a wide range of stimuli. Caspase inhibitors include viral gene products and short synthetic polypeptides. Viral gene products, crmA and p35, can inhibit apoptosis induced by both Fas and TNF, suggesting that caspases are downstream mediators of their cytotoxicity (Tewari *et al.*, 1995). It is also shown that inhibition of apoptosis by over-expression of Bcl-2 or Bcl-x_L also inhibits activation of caspases. Bcl-2 is unable to prevent substrate cleavage by active caspases (Kumar, 1997).

1.3.3.3. Tumor suppressor genes

The p53 tumor suppressor gene encodes a protein that is critical for maintaining the integrity of the genome. It is the most frequently mutated gene in human tumors (Vogelstein, 1990; Hollstein, 1991). Reintroduction of p53 into transformed cells can induce apoptosis (Yonish-Rouach *et al.*,

1991). p53 accumulates and directs apoptosis in response to DNA damage in skin, thymocytes, and intestinal epithelium (Clarke *et al.*, 1993; 1994; Merritt *et al.*, 1994; Ziegler *et al.*, 1994). Endogenous p53 expression was shown to significantly accelerate apoptosis in myeloid 32D cells upon withdrawal of IL-3 (Blandino *et al.*, 1995). Additionally, anti-sense p53 oligonucleotides reduced the level of apoptosis in growth factor-dependent leukaemia cells after withdrawal of growth factor (Zhu *et al.*, 1994). The mechanism whereby p53 leads to G1 arrest following DNA damage can be attributed to its role as a transcription factor. It is reasonable to suspect that a similar transcription-dependent mechanism might exist for apoptosis. In that case, p53 might activate the transcription of death genes (Bax) or repress the transcription of survival genes (Bcl-2) (Miyashita *et al.*, 1994). Furthermore, p53 has been reported to stimulate Bax expression in promoter-reporter assays in cultured cells (Miyashita *et al.*, 1995). These results indicate that up-regulation of Bax transcription is a means by which p53 induces apoptosis.

Rb is another tumor suppressor gene, which is inactivated by deletion or mutation in many different cell types (Lee, 1991). The connection between Rb and apoptosis came from several directions. Induction of apoptosis by E1A was genetically inseparable from the ability of E1A to stimulate DNA synthesis and bind p300 and Rb, suggesting that

some aspect of deregulation of cell growth control by E1A is responsible for apoptosis (White *et al.*, 1987; 1991). A loss of Rb function during development apparently produces inappropriate DNA synthesis and apoptosis, suggesting that Rb function is required in development to inhibit cell cycle progression, thereby avoiding the apoptotic response (Morgenbesser *et al.*, 1994; Pan *et al.*, 1994). Overriding Rb by enforced E2F expression induces both DNA synthesis and apoptosis (Qin *et al.*, 1994; Kowalik *et al.*, 1995).

1.3.3.4. Fas and TNF receptor family

Many cellular responses such as proliferation, differentiation and survival are initiated by extra-cellular signals such as cytokines acting through specific cell surface receptors. It is not surprising that specific receptor-ligand binding can also mediate apoptosis. Since these external stimuli are only part of the information influencing the decision between self-destruction and survival, intracellular signaling pathways, such as the Fas-mediated pathway, are of particular importance in controlling apoptosis. Cell-surface receptor-mediated mechanisms involve the stimulation of the receptor, the activation of protein kinase/phosphatase cascades, and the release of second messengers to upregulate or suppress the transcription of specific genes.

Fas and TNF receptors belong to the expanding tumor necrosis factor receptor (TNFR) family of cell-surface receptors (Nagata *et al.*, 1995). Induction of apoptosis through aggregation of cell surface Fas, eg. by binding to Fas-ligand, is particularly important in regulation of the immune system. It appears that apoptosis induced by engaging the T-cell receptor on T-lymphocyte cell lines is mediated through interaction between Fas-ligand and Fas. Both Fas and TNF receptors are functionally dependent on a specific sequence of 60-70 amino acids in their carboxyl terminus termed the "death domain" (Golstein *et al.*, 1995). It is noted that this region is homologous to the protein reaper (White *et al.*, 1994), which is essential for most programmed cell death in *Drosophila*. This suggests that Fas and TNFR were produced in evolution after gene fusion of exons encoding an inducer of apoptosis related to reaper with those encoding a surface receptor.

Several proteins that may form part of the intracellular pathway induced by Fas have been identified recently, including MORT1/FADD (Chinnaiyan *et al.*, 1995), TRADD (Hsu *et al.*, 1995), and RIP (Stanger *et al.*, 1995). Over-expression of any of these three genes in transient expression systems induces apoptosis.

1.3.3.5. Oncogenes

The Myc family of proto-oncogenes encodes DNA binding proteins that can heterodimerize with other factors to form transcriptional activators or suppressors. They have been implicated in the regulation of genes involved in proliferation, differentiation, and apoptosis (Amati *et al.*, 1994). Normal proliferating fibroblasts maintain c-Myc expression through the cell cycle. Constitutive high-level expression of c-Myc abolishes cell cycle arrest in fibroblasts following serum deprivation, and results in concomitant proliferation and apoptosis (Evan *et al.*, 1992). It is shown that the insulin-like growth factors and platelet-drived growth factor (PDGF) could inhibit Myc induced apoptosis in low serum (Harrington *et al.*, 1994). Moreover, anti-apoptotic proteins such as Bcl-2 and mutant p53 can inhibit Myc induced cell death, enabling proliferation (Lotem *et al.*, 1995). However, mice with Myc alone did not show hyperplasia, whereas Myc/Bcl-2 double transgenic mice rapidly developed malignant lymphomas (Cory *et al.*, 1994).

Ras genes encode a family of small GTP-binding signal transducing proteins associated with apoptosis (Izquierdo Pastor *et al.*, 1995). Ras genes become oncogenic when activated by mutation, and can cooperate with other genes such as p53, Myc, EIA, and SV40 in cellular transformation (Barbacid, 1987). In the presence of interferon regulatory factor 1 (IRF-1) cDNA, cells with activated ras undergo apoptosis,

suggesting that IRF-1 may act like a tumor suppressor gene as it can mediate the death of an oncogenic activated cell (Tanaka *et al.*, 1994). Other studies suggested that the consequence of ras activation is dependent on the activity of protein kinase C (PKC). Activated ras induced apoptosis in a T-lymphoblastoid cell line, when PKC activity was suppressed. Apoptosis induced by ras in this system could be blocked by Bxl-2 (Chen *et al.*, 1995). In contrast, other groups have reported that activation or over-expression of H-ras could have a protective effect on apoptosis (Lin *et al.*, 1995).

1.4. Multistage cervical carcinogenesis

Like all the other human cancers, the development of cervical cancer is a multistage process. The current concept of squamous carcinoma of the cervix is that it is a multistage disease developing over a period of 10 to 15 years and arising as a result of mutational event in the metaplastic epithelium of the transformation zone in early adult life. Three stages are recognized as preinvasive, microinvasive and invasive stage, respectively. The characteristic histological feature of the preinvasive lesions is that the malignant cells are confined to the epithelium. The terminology "cervical intraepithelial neoplasia" (CIN) is used to describe this initial stage. The second stage, microinvasive cancer, is believed to represent the earliest stage of invasion. Invasive cancer, which is the

final stage, occurs once vascular and lymphatic channels have been breached by tumor cells (Coleman *et al.*, 1986).

Evidence to support this sequence of events has been obtained from studies of the natural history of CIN and invasive cancer. Epidemiological studies have shown that the risk factors for both preinvasive and invasive lesions are identical. Multiplicity of sexual partners, early age at first coitus, venereal disease and smoking are important determinants of risk for squamous cervical cancer (Greenberg *et al.*, 1985; Vessey *et al.*, 1983).

The more important evidence linking preinvasive and invasive cancer comes from retrospective studies of untreated patients with precancerous lesions of the cervix. It was found that 57% of the women with untreated precancerous lesions of the cervix developed invasive cancer within 10 years. Furthermore, 19% of women with preinvasive lesions may develop invasive cancer if they remain untreated for two years or more (Kinlen *et al.*, 1978; Peterson, 1956).

However, the evidence linking these two forms of disease is largely circumstantial, and objective evidence of a biological connection between the different stages of the disease is obviously needed. For this sake, studies of the role of HPVs in cervical carcinogenesis may reveal this link efficiently.

1.4.1. HPV

Normal cells *in vitro* replicate only a limited number of times before they stop dividing and undergo senescence. Immortalized cells have overcome this limitation of life span and can be propagated indefinitely in tissue culture. Normal cells suspended in fluid or in a semisolid agar gel stop proliferating and eventually undergo senescence. In contrast, tumor-derived cells demonstrate anchorage independency of cell growth and continue to grow and form colonies when suspended. The ability to grow in the absence of attachment is a characteristic feature of fully transformed cells that is clearly different from immortalization (Park *et al.*, 1995).

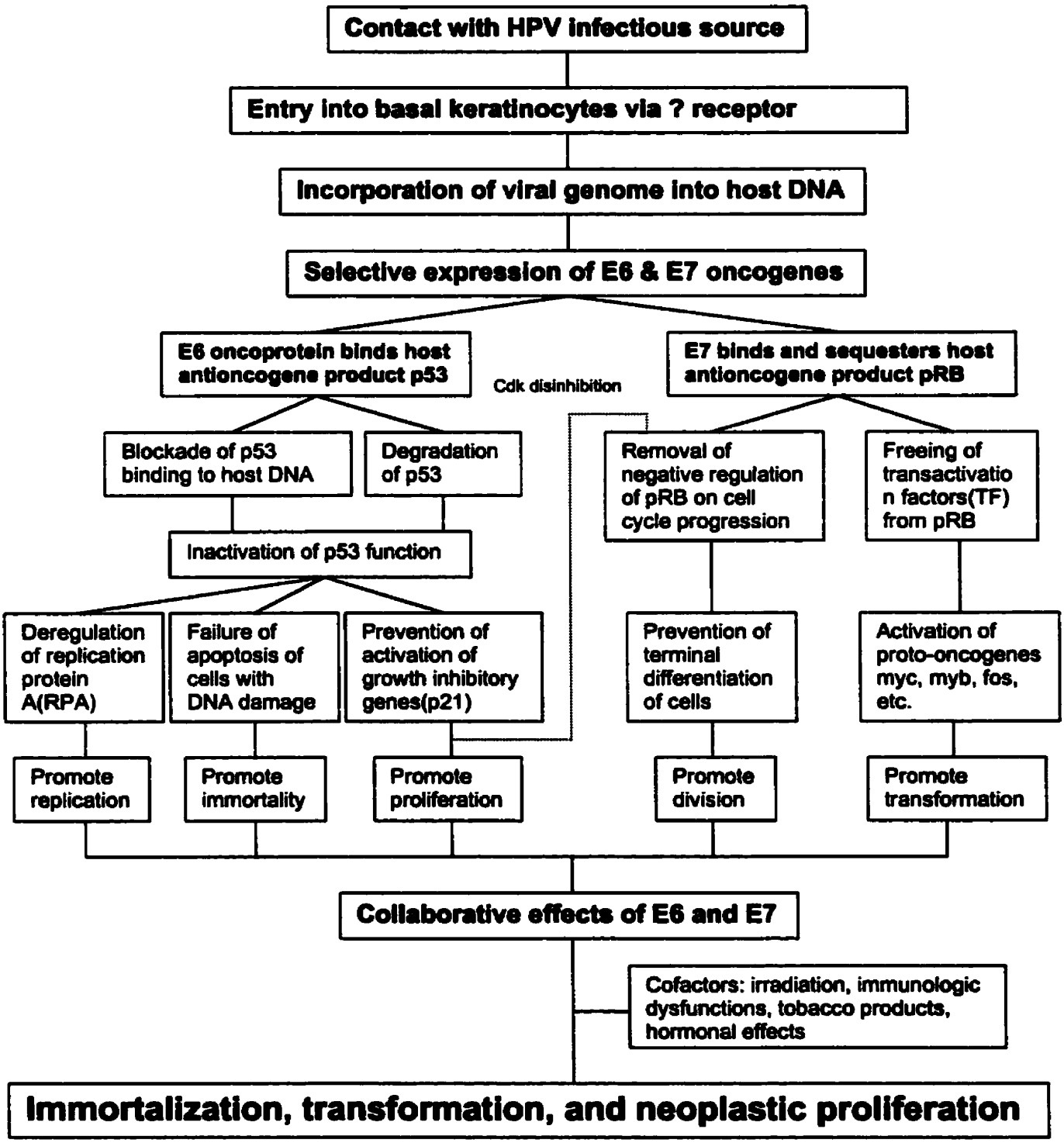
Introduction of HPV 16 or 18 DNA into rodent cell lines that are already immortalized causes the recipient cells to develop the capacity for anchorage independency of growth and the capacity to form tumors when injected into mice (Bedell *et al.*, 1987; Crook *et al.*, 1988). However, when HPV 16 or 18 DNA is introduced into early passage, nonimmortalized cells, a different result is observed. The recipient cells become immortalized but are not transformed and cannot form tumors in experimental animal systems (McCance *et al.*, 1988; Woodworth *et al.*, 1988).

HPV positive cervical cancers and cervical cancer-derived cell lines contain mRNA transcripts of the E6 and E7 ORFs, suggesting that the

viral genes are required for maintenance of the transformed phenotype. The hypothesis that E6 and E7 are the major transforming proteins gained further evidence with the demonstration that both proteins have transforming and immortalizing activities *in vitro* (Vousden, 1990). The amino terminal of E7 protein has been shown to play a role in the ability to form a complex with the cell encoded retinoblastoma (Rb) gene product and also provide the recognition signals for phosphorylation by casein kinase II (CK II). In turn Rb is associated with transcription factors, such as E2F, transactivation factors (TF) and c-Myc during certain stages of the cell cycle, thereby disrupting cell cycle control (Chellappan *et al.*, 1992). Unlike E7, E6 exerts its oncogenic activity through interaction with the cellular protein, p53, which appears to possess both positive and negative functions in regulating cell growth (Levine *et al.*, 1990). The mechanism by which HPV E6 proteins contribute to abnormal cell growth appears to be by binding and rapidly targeting the p53 protein for degradation via the ubiquitin pathway (Scheffner *et al.*, 1990).

In summary, HPVs possess immortalizing and transforming functions by defeating intracellular defense mechanisms against neoplastic proliferation (Mansur *et al.*, 1993). As shown in Figure 1.4, the most critical is the double inactivation of the two anti-oncogene products pRb and p53, thus initiating cascades of events along two oncogenic pathways.

Figure 1.4 Summary of the cascades of oncogenic pathways of high-risk HPVs (adapted from Kao *et al.*, 1994).


Contact with HPV infectious source
Entry into basal keratinocytes via ? receptor
Incorporation of viral genome into host DNA
Selective expression of E6 & E7 oncogenes
E6 oncoprotein binds host antioncogene product p53
Cdk disinhibition
E7 binds and sequesters host antioncogene product pRB
Blockade of p53 binding to host DNA
Degradation of p53
Removal of negative regulation of pRB on cell cycle progression
Freeing of transactivatio n factors(TF) from pRB
Inactivation of p53 function
Deregulation of replication protein A(RPA)
Failure of apoptosis of cells with DNA damage
Prevention of activation of growth inhibitory genes(p21)
Prevention of terminal differentiation of cells
Activation of proto-oncogenes myc, myb, fos, etc.
Promote replication
Promote immortality
Promote proliferation
Promote division
Promote transformation
Collaborative effects of E6 and E7
Cofactors: irradiation, immunologic dysfunctions, tobacco products, hormonal effects
Immortalization, transformation, and neoplastic proliferation

1.4.2. HPV cofactors

Association of HPVs with cervical infections and malignancies has been indicated by epidemiological, pathological clinical and *in vitro* data. However, the long latency period between the initial HPV infection and cancer development indicates that HPV infection alone is insufficient for cervical cacinogenesis. In addition, HPV-associated carcinogenesis is an inefficient process, because only a small fraction of women who are infected with high-risk HPVs will ever develop invasive cervical cancer. This suggests that additional factors might also be required for cervical carcinogenesis. Indeed, a number of endogenous and exogenous factors were reported to contribute directly or indirectly to the development of CIN and malignancy in HPV-infected individuals (Herrington, 1995). Exogenous cofactors with HPVs are more applicable for therapy than the endogenous factors and are proposed to play important roles in the process of initiation and progression, including cell DNA mutations, transregulation of HPV and cell transcription, regulation of cell growth, differentiation and death, virus neutralization and cell killing. Exogenous agents can be classified into physical-chemical cofactors, hormones, microbiological cofactors, and immune response cofactors (Khare *et al.*, 1995).

As one of physical-chemical cofactors, cigarette smoke and tobacco have been evidenced to be associated with cervical cancer (Brisson *et al.*, 1994; Daling *et al.*, 1992). Also, clinical studies of the cervix found tobacco components in the endocervical mucus and DNA adducts from cigarette smoke constituents in the cells, implicating the chemical carcinogens in smoke as a cofactor in cervical cancer development (Sasson *et al.*, 1985; Simons *et al.*, 1995).

Many exogenous hormones bind to a growing family of nuclear protein receptors and regulate viral and cellular transcription in epithelial cells. Progesterone and glucocorticoid endocrine steroid hormones have a profound influence on the differentiation of the cervical epithelium (Mittal *et al.*, 1993; Reagon *et al.*, 1983).

Microbiological cofactors can be grouped as viruses and bacteria. The transforming region of herpes simplex virus-2 was associated epidemiologically with high grade CIN and cervical cancer and all the lesions containing this region were also positive for HPV (Yamakawa *et al.*, 1994). Although the association and mechanism are uncertain, the bacteria may promote progression to cancer by lowering the pH of the cervix or by inducing an inflammatory immune response (Guijon *et al.*, 1992).

The relatively immune system-inaccessible epithelial target of HPVs and the external release of virions reduce the effectiveness of the immune

response. The cell-mediated response is apparently compromised for CINs containing oncogenic HPVs, as evidenced by the decrease in the number of Langerhans' cells (Hawthorne *et al.*, 1988). Additionally, natural killer cell activity was increased significantly in CINs during regression of the lesions and loss of the HPV infection (Garzetti *et al.*, 1995).

1.4.3. Model systems

As described previously, cancer development is a multistage process, and cervical carcinogenesis, with no exception, also exhibits this multistage feature. It is generally postulated that the infection with high-risk HPVs, such as HPV 16 and HPV 18, can initiate CIN lesions and, in the presence of some cofactors, then result in the progression of CIN to invasive cervical cancer. Therefore, hypothetical model systems are mandatory components for *in vitro* studies of multistage cervical carcinogenesis.

A number of groups have reported the immortalization of human cervical epithelial cells by transfection with HPV 16 and HPV 18 DNA (Pecoraro *et al.*, 1989; Woodworth *et al.*, 1988). Our laboratory discovered that both endocervical and ectocervical cells are targets for immortalization by either HPV 16 or HPV 18 (Sun *et al.*, 1992; Yokoyama *et al.*, 1994). Moreover, the metaplastic cells were immortalized by HPV

16 and featured CIN III in nude mice xenotransplants (Tsutsumi *et al.*, 1993). The cell lines were named as HEC-18, HEC-16, and HEN-16, after immortalization by HPV 18 or HPV 16 (Pater *et al.*, 1994; Sun *et al.*, 1993; Tsutsumi *et al.*, 1992; 1994; Yokoyama *et al.*, 1995). They were subsequently transformed by cigarette smoke condensate (CSC) treatment to afford cell lines HEN-16T, HEN-16-2T, and HEC-18T (Nakao *et al.*, 1996; Yang *et al.*, 1996; Yoshifumi *et al.*, 1996). All of the cell lines provide effective model systems representing the final 2 stages of malignant progression of cervical cells for investigations on multistage carcinogenesis (Yang *et al.*, 1997).

1.5. Cancer chemotherapy

1.5.1. Introduction

Regulation of apoptosis is essential for normal development and is an important defense against the emergence of cancer. Too much cell death can lead to impaired development and degenerative diseases, whereas too little cell death can result in cancer (White, 1996). In human cancers, there is disequilibrium between the rates of cell division and cell death, which is reflected by genetic alterations in apoptosis regulators stated previously.

Bcl-2 is the acronym for the B-cell lymphoma/leukemia-2 gene. As implied by its name, Bcl-2 was first discovered because of its involvement in chromosomal translocations in B-cell lymphomas (Tsujimoto *et al.*, 1985). Bcl-2 expression at abnormally high levels or in aberrant patterns is found in approximately 50% of human cancers, suggesting that deregulation of this proto-oncogene represents one of the most common events associated with human malignancy (Reed, 1995a). Bcl-2 is reported to be capable of blocking apoptosis induced by all types of anti-cancer drugs by preventing drug-induced damage from being efficiently translated into cell death. Thus, tumor cells that contain high levels of Bcl-2 still experience the cell cycle inhibitory effects of drugs, but they remain viable for protracted periods of time, resulting in enhanced clonigenic survival in many cases (Delia *et al.*, 1995; Miyashita *et al.*, 1994b). Furthermore, overexpression of Bcl-2 has been associated with poor responses to chemotherapy and shorter survival for patients with some types of lymphomas, acute myelogenous leukemias, and prostate cancer (Reed, 1995b).

As stated before, both oncogene Myc and tumor suppressor gene p53 are involved in the control of apoptosis. Overexpression or deregulation of Myc in tumors has important implications for the control of apoptosis. The importance of p53 has been demonstrated in "knockout" mice in which both copies of p53 are absent. A high rate of tumors is

observed in these animals and in addition they are highly resistant to the induction of apoptosis (Angel *et al.*, 1991).

1.5.2. Apoptosis

Inhibition of apoptosis, either by suppression of genes that induce cell death or by activation of genes that cause cell survival, contributes to the development of cancer. There is considerable interest in this area not only to better understand the mechanism of carcinogenesis but also to reveal novel targets for the therapy of cancers. Chemotherapy significantly improves survival rates in certain tumors, such as testicular cancer and acute lymphocytic leukemia (ALL). Cancer cells are relatively resistant to apoptosis (Carson *et al.*, 1993; Stellar, 1995). The key to effective therapy strategies using programmed cell death is that there is a highly regulated pathway with a myriad of points where intervention could occur. Tumor cells can undergo apoptosis under nonphysiological conditions, such as chemotherapy, leading to the suppression of tumor growth and micrometastases (Thompson, 1995). Pharmacologic agents capable of inducing apoptosis, therefore, may be worth investigating.

1.5.3. Fluoropyrimidines

Fluoropyrimidines are a rationally developed group of anticancer drugs, and are of special interest since they are transformed into oral formulations, which improve their anticancer activity and minimizes their toxicity (Lamont *et al.*, 1999). Fluoropyrimidines exert their antitumor effects through several mechanisms including inhibition of RNA synthesis and function, inhibition of thymidylate synthase activity, and incorporation into RNA, leading to DNA strand breaks (Grem, 1996). The pattern and extent of cell damage induced by fluoropyrimidines in human cancer cells have been suggested to depend on pathways downstream from drug-target interactions that, once triggered, will initiate apoptosis (Canman *et al.*, 1992; Lowe *et al.*, 1993). Indeed, fluoropyrimidines have been demonstrated to be capable of inducing programmed cell death in human cancer cell lines, such as human colon cancer cell lines, human colorectal tumor cell lines and HeLa cells (Dusenbury *et al.*, 1990; Elliott *et al.*, 1998; Nita *et al.*, 1998).

1.6. Purposes of this study

Cervical cancer is the second most common cancer among women worldwide. Cervical cancer, like all the other human cancers, possesses

a multistage nature during its development. HPV and many cofactors are closely associated with the multistage carcinogenesis of cervical cells. However, the mechanism of this multistage, multifactor carcinogenesis towards full malignant conversion of human cervical cells still remains unclear. To better understand this process, several model systems for multistage cervical carcinogenesis have been established in our laboratory. One of them, which consists of primary human ectocervical cells (HEC), HPV-18-immortalized HEC (HEC-18), and a cell line subsequently transformed with CSC from HEC-18 (HEC-18T), is chosen for my study.

Apoptosis is genetically controlled events to remove unwanted cells without disrupting normal cells, and therefore, usually serves as a potent defense against the emergence of human cancers. As a consequence, apoptosis has been widely investigated to gain better understanding of the mechanisms involved in multistage development of human cancers. Additionally, apoptosis has also been used as an ideal target for chemotherapy of cancer. Among a variety of anticancer drugs, fluoropyrimidines appear to be very promising. The most common fluoropyrimidine, 5-fluorouracil (5-FU), was the first synthesized in an attempt to exploit the increased avidity of tumor cells for uracil (Heidelberger *et al.*, 1957). Although 5-FU has been reported to induce apoptosis in many tumor cell types, to the best of our knowledge, it was

only once investigated for apoptosis in cervical carcinoma cells (Ueda *et al.*, 1997). Even less is known about its molecular basis, such as its effects on expressions of apoptosis-related proteins. Experimental data suggested the involvement of p53, Bcl-2 family, and BAG-1 in chemotherapy-induced apoptosis (Harris, 1996; Jaattela, 1999). The first purpose of my study is to investigate the apoptosis induced by 5-FU in our model system for multistage cervical carcinogenesis. In addition to the morphological changes, molecular changes including modulations of p53, p53-related proteins, some Bcl-2 family members, and BAG-1 have been examined to afford implications for involved mechanisms in apoptosis and multistage cervical carcinogenesis.

During the past 10 years, interest of basic scientists and clinicians in the influence of apoptosis on the sensitivity of tumors to anticancer treatment has risen and continues to rise dramatically (Brown *et al.*, 1999). The present view is that the level of apoptosis and/or genes controlling apoptosis affects the sensitivity of cancer cells to being killed by genotoxic agents (McGill *et al.*, 1997; Weinberg, 1996). Tumor cells are relatively resistant to apoptosis. Therefore, the best expectation of chemotherapies based on apoptosis is increasing the sensitivity of tumor cells to anticancer drug-induced apoptosis because this means that cells are sensitive to anticancer agents as well. As expected, we have primarily found that immortalized cell line HEC-18 and transformed cell

line HEC-18T were less sensitive to 5-FU-induced apoptosis than primary HEC cell line. We have also found that the cell lines in our model system for multistage cervical carcinogenesis, which were insensitive to 5-FU-induced apoptosis, became sensitive upon the medium change from KGM to DMEM. As implied by these findings, another purpose of my study is to investigate the role of HPV-18 immortalization and medium change in the sensitivity change of cell lines in our model system to 5-FU-induced apoptosis. This would be expected to reveal more details in mechanisms of sensitivity change, and, if possible, to provide some predictive value for clinical application of 5-FU.

CHAPTER 2 MATERIALS AND METHODS

2.1. Materials

Keratinocyte growth medium (KGM) and Dulbecco's modified Eagle medium (DMEM) were purchased from GIBCO-BRL and ICN, respectively. GIBCO-BRL was the supplier for the fetal calf serum (FCS) and trypsin-ethylenediamine tetraacetic acid (EDTA). Penicillin-streptomycin was obtained from ICN. HEC-18 and HEC-18T cells were established as described previously (Yokoyama *et al.*, 1994; Nakao *et al.*, 1996, respectively). HeLa cell was purchased from ATCC.

Taq DNA polymerase, 0.1 M DTT, restriction endonucleases and and superscript reverse transcriptase with their respective 10× reaction buffers were obtained from GIBCO-BRL.

GIBCO-BRL supplied the 1 Kb and 100 bp DNA ladder markers. Low melting point agarose, agarose, acrylamide, N,N'-methylenebisacrylamide, urea and 10 mM dNTPs were purchased from GIBCO-BRL. Baker Inc., Bio-Rad, and Carnation were the suppliers of 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED) and skim milk powder, respectively.

5-Fluorouracil (5-FU), and 5'-deoxy-5-fluorouridine (5-FUdR) were purchased from Sigma Chemical Co.. RNase cocktail and proteinase K were obtained from Ambion Co..

Mouse anti-BAG-1 monoclonal antibody (mAb) was generated and prepared in our laboratory (Yang *et al.*, 1998). Rabbit polyclonal antibodies for Bak and mouse mAbs for Bcl-x_L and p21 were obtained from Santa Cruz Biotechnology. Mouse mAbs for Bax, PCNA, MDM-2, and LDLR were purchased from Oncogene Co.. DAKO Co. supplied p53 and Bcl-2 mouse mAbs. Other mouse mAbs used were: anti-β-actin (Sigma Chemical Co.) and secondary antibodies (Amersham Co.). Rainbow protein markers and biotinylated protein marker were purchased from Amersham Co. and New England Biolabs Inc., respectively.

The MTT assay kit was purchased from Promega. Annexin V-FITC apoptosis assay kit was obtained from PharMingen Co.. The DC protein assay kit was purchased from Bio-Rad Co.. ECL system and Hybond nitrocellulose membrane was obtained from Amersham Co.. Kodak was the supplier of X-ray film.

Four-well and eight-well tissue chamber slides; 35-mm, 60-mm and 100-mm tissue culture plates; 6-well plates; 96-well microplates and culture tubes were obtained from NUNC. Eppendorf microcentrifuge test tubes for PCR were obtained from Fisher Scientific Co..

2.2. Cell culture

Primary human ectocervical cells (HEC) were obtained after dissection of pathologically normal cervical tissues derived from

hysterectomies performed for benign conditions and were maintained in serum-free medium for keratinocytes, as described previously (Boyce *et al.*, 1985). HEC-18 and HEC-18T were cultured in serum-free KGM containing 1% penicillin/streptomycin. HeLa was cultured in DMEM containing 10% FCS and 1% penicillin/streptomycin.

All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. After three days or when the cells had reached approximately 80% confluence, the medium was aspirated from the plates and the cells were washed with phosphate-buffered saline (PBS). Then, 2 ml of trypsin-EDTA was added into each plate, which was placed in the incubator for 15 minutes. For cells cultured in KGM, 8 ml of PBS containing 10% FCS was added to the trypsinized cells to quench the activity of trypsin. The cells were then suspended and centrifuged at 1,000 rpm for 10 minutes. The cell pellets were resuspended with KGM and passaged at a dilution of 1:10, 1:4, or 1:2 into plates. For cells cultured in DMEM containing 10% FCS, 8 ml of this medium was added directly to the trypsinized cells, which were further passaged at 1:10, 1:4, or 1:3 into fresh plates. The cell numbers were counted with a hemocytometer.

2.3. Growth rate and saturation density assay

Exponentially growing primary HEC, HEC-18 and HEC-18T cells were trypsinized, centrifuged, and resuspended in 2 ml medium. Approximately 2 × 10^4 cells were dispensed into 60-mm plates for each cell line. The cell numbers were counted from each of three plates every two days for 8 days.

The saturation density of each cell line was measured by the same procedure 5 days after the cells reached confluence.

2.4. Soft agar or anchorage-independent growth assays

The 0.7% agarose underlying gel was prepared by mixing equal volumes of 2× DMEM containing 20% FCS and low melting point agarose melted in sterilized water, dispensing 2.5 ml into 60-mm plates, and leaving the plates at room temperature until the gel solidified. Subsequently, the cells were trypsinized, resuspended in 2× DMEM, and counted using a hemocytometer. The 0.35% agarose overlaying gel was prepared by mixing approximately 10^5 cells in 2.5 ml of 2× DMEM with 2.5 ml of 0.7% low melting point agarose in sterilized water, pouring the mixture into the 60-mm plates containing the 0.7% underlying gel. The plates were placed at 4 °C for 5 minutes, and then incubated at 37 °C. One week later, 1 ml DMEM was carefully added onto the surface of the soft agar to replenish nutrients. The colony formation was monitored

every two days for 2-4 weeks. Generally, triplicate assays were performed for each cell line. Representative photographs were taken for documentation.

2.5. Morphology analysis

For light microscopic analysis of morphology, cells in either DMEM or KGM were cultured in 4-well tissue culture chamber slides. Medium was aspirated from approximately 80% confluent cells and the cells were washed with PBS. The chamber frame was released from the slides and the cells were examined under light microscopy. Cell morphology was documented with Kodak TMX 100 film.

2.6. Drug treatment

5-FU and 5-FUdR were prepared as 100 mM stocks in DMSO and stored at -20°C. Immediately prior to cell treatment, the drug was diluted in medium to the concentration as indicated. Log phase cells were supplemented with the drug and they continued to be incubated for indicated periods. Control cells received equivalent solvent treatment.

2.7. Cytotoxicity assay

Cell drug sensitivity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay kit (Promega Co.) after 72 hr continuous drug incubation. Briefly, 10^4 cell aliquots in 100 µl of growth medium/well were uniformly seeded into 96-well microplates for 24 hr before exposure to drug. Each concentration was performed in quadruplicate. The absorbance of each of four wells was measured by a microplate reader at 590 nm. Control cells received equivalent solvent treatment. The average growth inhibition rates compared with the control were calculated from the results of three or more independent experiments. The 5-FU or 5-FUdR concentrations causing a 50% growth inhibition compared with the controls (IC_{50}) were calculated from a semilogarithmic dose-response curve by linear extrapolation.

2.8. Cell viability assay

Cell viability was evaluated using trypan blue dye exclusion assay. In brief, 10^5 cells/well were seeded in 6-well plates, incubated for 24 hr, and then treated with drugs. Untreated and treated cells were trypsinized, and equal volumes of cell suspensions and 4% trypan blue were mixed gently. Cell viability was examined immediately by microscopy. Viable cells were unstained whereas dead cells stained blue. Cell viability was

calculated as the percentage of treated cells compared with untreated cells that excluded trypan blue dye.

2.9. Apoptosis assays

2.9.1. Morphological analysis

Cells were cultured in triplicate on 4-well tissue culture chamber slides for 24 hr, and then incubated with drug for 6 hr. Then, the chamber frame was removed from slides, and cells were examined using phase-contrast microscopy. Apoptotic cells were documented with Kodak TMX 100 film.

2.9.2. Flow cytometry apoptosis analysis by annexin V-fluorescein isothiocyanate (FITC) assays

Apoptosis was analyzed using an annexin V-FITC assay kit (PharMingen Co.), according to the manufacturer's instructions. Briefly, approximately 10^5 cells/plate in 6-well plates were incubated for 24 hr. Cells were treated with drug, and both floating dead cells and attached viable cells were harvested. Approximately 10^5 cells were incubated with Annexin V-FITC and 5 µg/ml propidium iodide (PI) in binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at room temperature in the dark for 15 min. Apoptotic cells were quantified using

a FACStar Plus flow cytometer (Becton Dickinson). Data analysis was performed with the standard Cell Quest software (Becton Dickinson). Combination Annexin V-FITC/PI staining was used for simultaneous detection of viable (-/-), apoptotic (+/-) and necrotic cells (+/+).

2.9.3. DNA fragmentation assays

Cells were seeded into 6-well plates and incubated with drug for 24 hr. Aliquots of cells were washed in PBS and lysed in DNA lysis buffer (10 mM Tris/HCl, pH 7.4, 400 mM NaCl, 1% sodium dodecyl sulfate (SDS), and 0.2 mg/ml proteinase K) at 37 °C for 4 hr. Samples were treated further with DNase-free RNase A/T_1 (0.2 mg/ml) at 37 °C for 1 hr. The resulting extracts were loaded onto 1.5% agarose gels containing ethidium bromide (0.5 µg/ml). Electrophoresis was performed at 60 V at room temperature for 3 hr. The gel was viewed and photographed on an ultraviolet (UV) transilluminator.

2.10. Western blot

For protein extraction, cells were washed twice with ice-cold PBS, and proteins were extracted from approximately 10^7 cells by lysis in 1 ml ice-cold extraction buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5%

sodium deoxycholate, 200 g/ml phenylmethylsulfonyl fluoride (PMSF) and 20 g/ml aprotinin) for 30 min and centrifuged at 4 °C for 10 min, after which the supernatant was transferred into another fresh microcentrifuge tube and stored at -70 °C. Protein concentration was quantified using a DC Lowry protein assay kit (Bio-Rad), as instructed by the manufacturer.

Protein extracts, usually 10 µg, were boiled in 2× SDS-polyacrylamide gel electrophoresis (PAGE) gel loading buffer (200 mM Tris/HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol) for 3 min and resolved in an SDS-PAGE gel. The running gel was prepared with 8-12% acrylamide, 375 mM Tris/HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate, and 6 µl of TEMED. The stacking gel was composed of 5% acrylamide, 125 mM Tris/HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 5 µl of TEMED. Electrophoresis was at 200V in SDS-PAGE running buffer (25 mM Tris, 250 mM glycine) using a Protein II minigel apparatus (Bio-Rad).

After electrophoresis, the gel was incubated at room temperature in Towbin transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 10 min. Subsequently, the proteins were transferred to Hybond enhanced-chemiluminescence nitrocellulose membranes (Amersham Co.) under semidry conditions at 20 V for 50 min using a Trans-Blot SD transfer apparatus (Bio-Rad). After transfer, the membranes were air-dried and probed immediately or wrapped in Saran Wrap and stored at 4

°C prior to probing. To eliminate nonspecific binding of antibodies, membranes were blocked by gentle shaking in 5% skim milk powder in TBST (20 mM Tris/HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20) for 1 hr at room temperature. Subsequently, membranes were incubated with the primary antibody diluted in 5% skim milk powder in TBST overnight at 4 °C with shaking. After washing thrice with TBST for 10 min, membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted in 5% skim milk powder in TBST for 1 hr at room temperature, and then washed in TBST for three times. Signals were detected using the enhanced chemilumiscence (ECL) system (Amersham Co.) and subsequently exposing the membranes to ECL film, as instructed by the manufacturer.

After primary signals were detected with the ECL system, they were removed from the membranes by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7) at 50 °C for 30 min with occasional agitation. Then, membranes were rinsed with TBST and reprobed with anti-β-actin mAb and the signals were detected as for the primary signal.

Signal intensities were quantified by densitometry of bands with an Eagle Eye II Still Video system (Stratagene).

2.11. Flow cytometry

The expression of LDLR on 10^6 cells was quantified by flow cytometry. Cells were washed in washing buffer (PBS, 0.2% bovine serum albumin and 0.02% sodium azide) and incubated at room temperature in 50 µl of washing buffer containing 2.5 µg/ml normal mouse immunoglobulin for 15 min. Next, anti-LDLR antibody (IgG_{2a}) was added to the final concentration of 2.5 µg/ml. After being incubated for 30 min, cells were washed, resuspended in 50 µl of washing buffer containing 2.5 µg/ml of FITC-conjugated goat-anti mouse IgG_{2a}, and incubated for 15 min at 4 °C. Negative controls, cells that were LDLR-negative cells, were treated only with FITC-conjugated goat-anti mouse IgG_{2a}, treated with nonspecific normal mouse IgG antibodies. After staining, cells were washed once and resuspended in PBS containing 2% formaldehyde. Stained cells were analyzed in a FACStar Plus flow cytometer (Becton Dickinson).

2.12. Statistical analysis

Statistical analysis was conducted using the Student's t-test. Differences with a value of $p < 0.05$ were considered to be significant.

CHAPTER 3 RESULTS

3.1. Morphology of HEC, HEC-18, and HEC-18T

It has been reported that the morphology of cultured cervical epithelial cells indicated the differentiation potential and oncogenicity of the normal or abnormal tissue from which the cells are derived (Turyk *et al.*, 1989; Vooijs, 1991). Compared with normal cells, premalignant and tumor cells usually have obvious, progressive morphological abnormalities *in vitro*. In addition, it has been observed that cellular morphology depended on the culture medium (Nakao *et al.*, 1996; Yang *et al.*, 1996). Therefore, the morphology of HEC, HEC-18, and HEC-18T grown in KGM or DMEM was compared.

In monolayer cultures, cells were grown either in KGM, a serum-free medium containing 0.15 mM calcium, or in DMEM, a medium containing 10% fetal calf serum (FCS) and a physiological level of calcium (1.5 mM). In KGM, HEC (Figure 3.1 A), HEC-18 (Figure 3.2 A), and HEC-18T (Figure 3.3 A) formed monolayers consisting of polygonal, keratinocyte-like cells. HEC grew faster than HEC-18 and HEC-18T, as suggested by cell densities after the same period of culture time.

Figure 3.1 Morphology of primary human ectocervical cells (HEC) grown in KGM and DMEM. Panel A represents HEC grown in KGM. Panel B represents HEC grown in DMEM. (Magnifications: ×100)

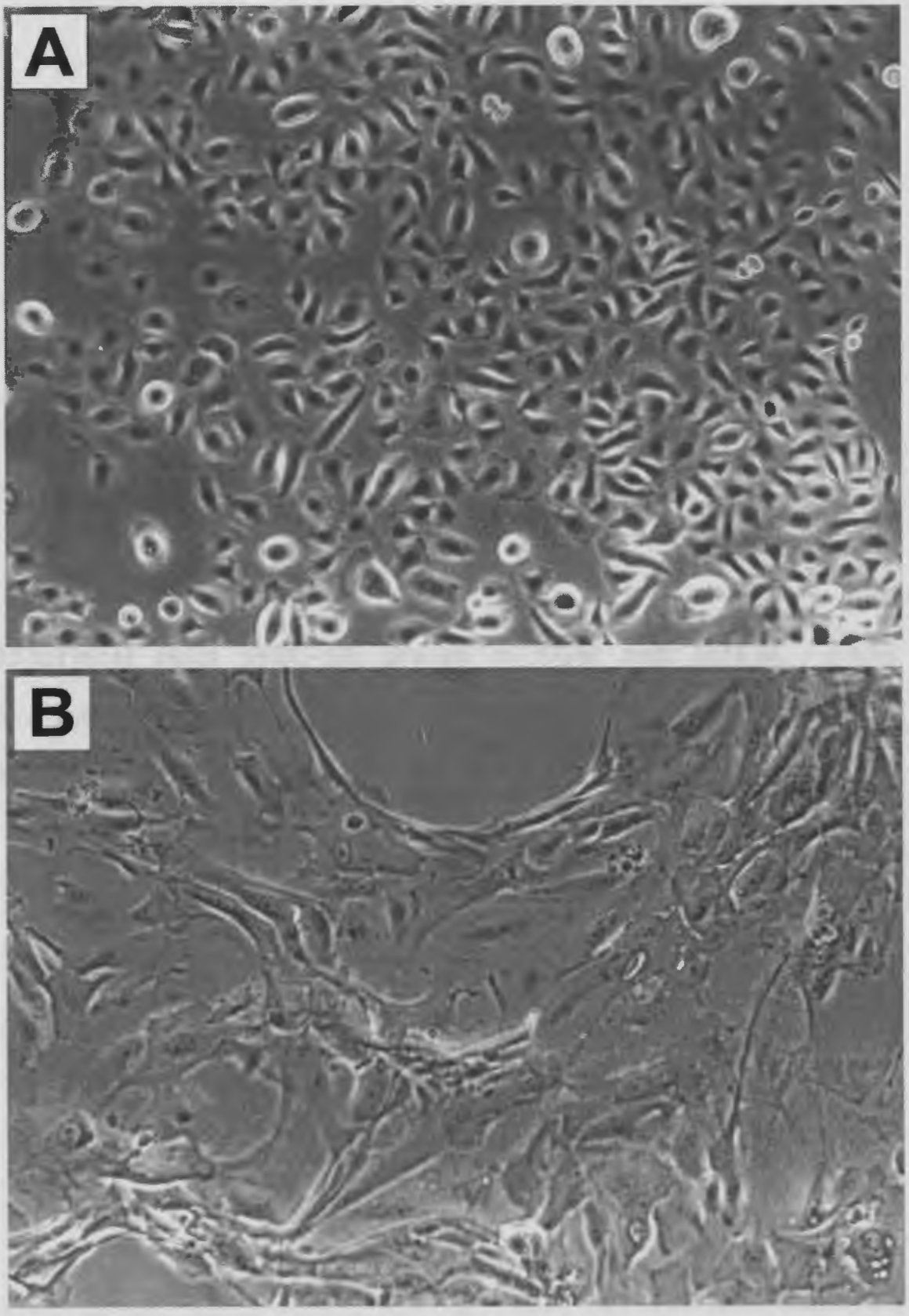

A
B

Figure 3.2 Morphology of HPV 18-immortalized human ectocervical cells (HEC-18) grown in KGM and DMEM. Panel A represents HEC-18 grown in KGM. Panel B represents HEC-18 grown in DMEM. (Magnifications: ×100)

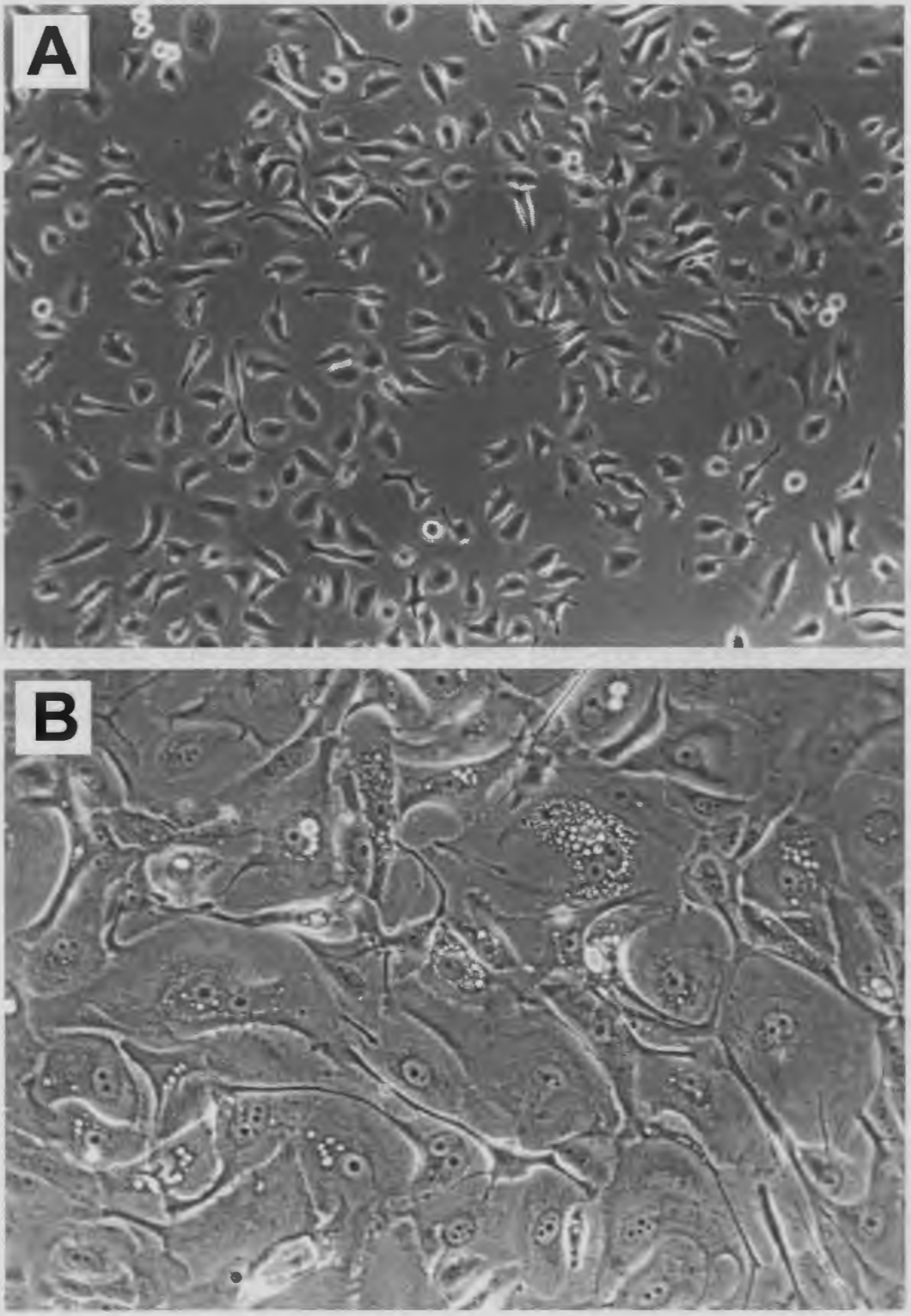
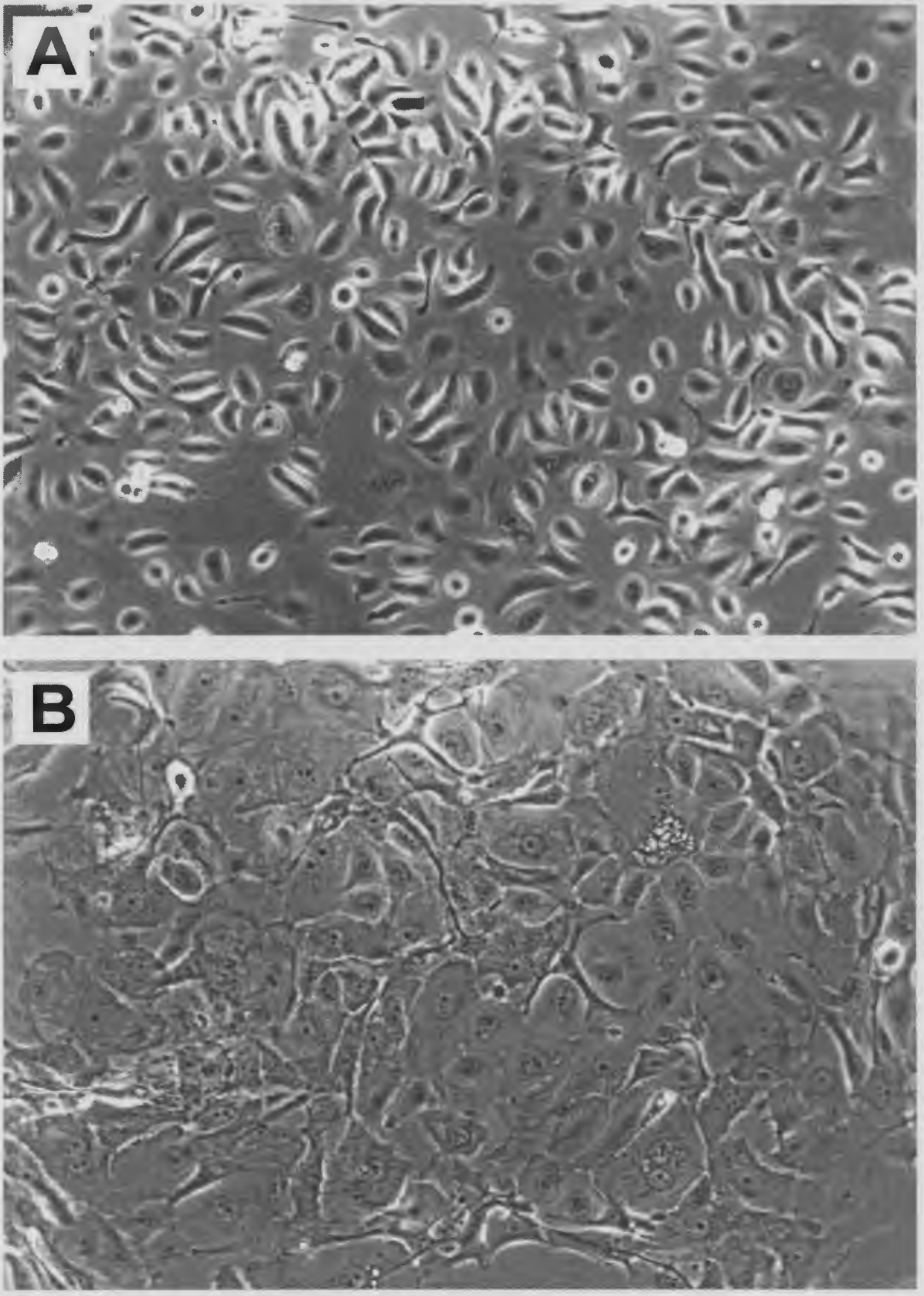

A
B

Figure 3.3 Morphology of CSC-transformed HPV 18-immortalized human ectocervical cells (HEC-18T) grown in KGM and DMEM. Panel A represents HEC-18T grown in KGM. Panel B represents HEC-18T grown in DMEM. (Magnifications: ×100)


A
B

However, when cells were adapted to and grown in DMEM, HEC (Figure 3.1 B) could do neither, stopped growing and died after 2 weeks. The other two cell lines, HEC-18 (Figure 3.2 B) and HEC-18T (Figure 3.3B), were slowly growing. Initially, all the three cell types became flattened, dendritic (having branched cytoplasm), uneven-sized cells and distributed heterogeneously in the culture plates. Moreover, the cells displayed a smaller size, and an increasingly higher nucleus/cytoplasm ratio for HEC, HEC-18, and HEC-18T. The morphology of the three cell lines is obviously different in DMEM from that in KGM in each case.

3.2. Growth characteristics of HEC, HEC-18, and HEC-18T

The growth rate, the saturation density and the potential of anchorage-independent growth in soft agar are three important *in vitro* indicators of the stages of multistep carcinogenesis (Li *et al.*, 1992). To characterize the growth potential of HEC, HEC-18, and HEC-18T, their proliferation either in both KGM and DMEM was examined.

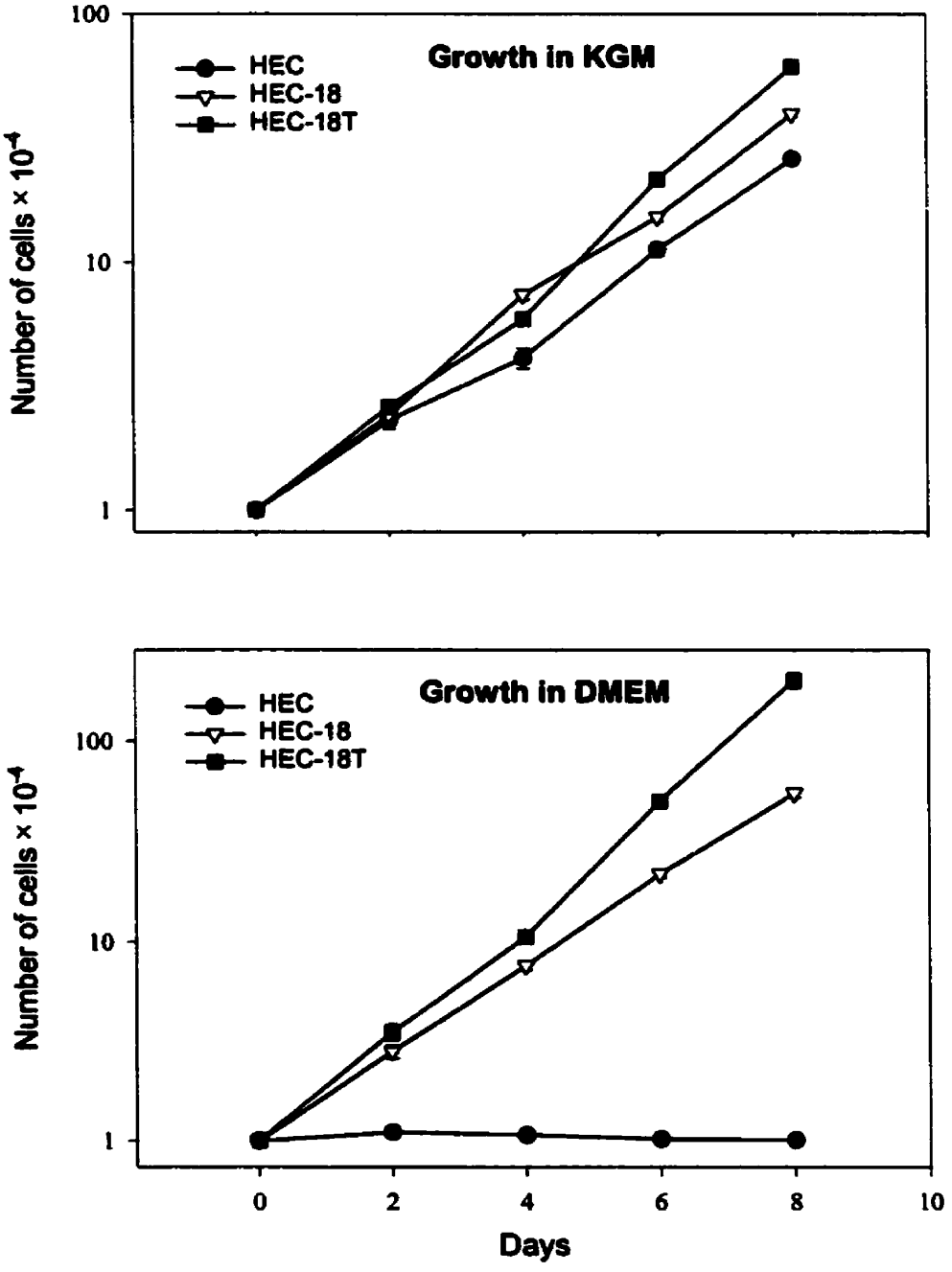
In KGM, transformed cell line HEC-18T proliferated slightly faster than its parental immortalized counterpart HEC-18 and HEC-18, in turn, proliferated faster than HEC (Figure 3.4; Table 3.1). The doubling times of HEC, HEC-18, and HEC-18T in KGM were 39, 36, and 32 hr, respectively. However, HEC did not proliferate in DMEM. The other two

Table 3.1 Growth characteristics of ectocervical cells

Growth characteristics	Ectocervical cells					
	HEC		HEC-18		HEC-18T	
	KGM	DMEM	KGM	DMEM	KGM	DMEM
Doubling time [1]	39±6	NG	36±2	32±2	32±2	25±3
Saturation density [2]	0	0	157±26	92±9	287±35	174±21
Soft agar growth [3]	—	—	—	—	+	+

[1] The doubling time in hours ± standard error is presented. NG, no growth. [2] The number of cells ± standard error × 10^{-5} is presented as the average number of duplicate counts of triplicate 100 mm dishes. [3] -, 5 negative dishes; +, 5 positive dishes.

Figure 3.4 Proliferation of multistage cervical carcinogenesis model cells in serum-free medium and high calcium, serum containing medium. Cells were cultured in 100 mm plates in serum-free medium (KGM) and high calcium, serum-containing medium (DMEM). Values represent the mean +/- the standard deviation from three independent experiments. See text for details.


Growth in KGM
HEC
HEC-18
HEC-18T
Number of cells x 10⁻⁴
100
10
1
Growth in DMEM
HEC
HEC-18
HEC-18T
Number of cells x 10⁻⁴
100
10
1
0
2
4
6
8
10
Days

cell lines, HEC-18 and HEC-18T proliferated much slower in DMEM than in KGM. Predictably, HEC-18T proliferated faster than HEC-18. The doubling times of HEC, HEC-18 and HEC-18T in DMEM were no growth, 32 hr and 25 hr, respectively. Additionally, transformed cell line HEC-18T obtained much higher saturation density than its immortalized counterpart HEC-18 in both media. The saturation densities in 100 mm dishes of HEC-18 and HEC-18T were 1.57×10^7 and 2.87×10^7 cells in KGM, and 9.2×10^6 and 1.74×10^7 cells in DMEM, respectively.

Oncogenicity is correlated with anchorage-independent growth of tumor cells. To characterize the oncogenic phenotype, anchorage-independent growth in soft agar was assayed, in which HEC-18 and HEC-18T were growth adapted to either KGM or DMEM before soft agar assays, and HeLa were first growth adapted to DMEM. Generally, the formation of colonies was observable after one week of incubation, and the colonies were unequivocally identified after 2-4 weeks. HEC-18 (Figure 3.5 A and B) remained as single cells after adaptation to both media, whereas HEC-18T (Figure 3.6 A and B) formed colonies that were smaller than those formed by HeLa (Figure 3.5 C and Figure 3.6 C).

Figure 3.5 Anchorage-independent growth assays of HPV 18-immortalized human ectocervical cells (HEC-18). A, HEC-18 initially grown in KGM; B, HEC-18 initially grown in DMEM; C, HeLa cells used as the control. See text for details.

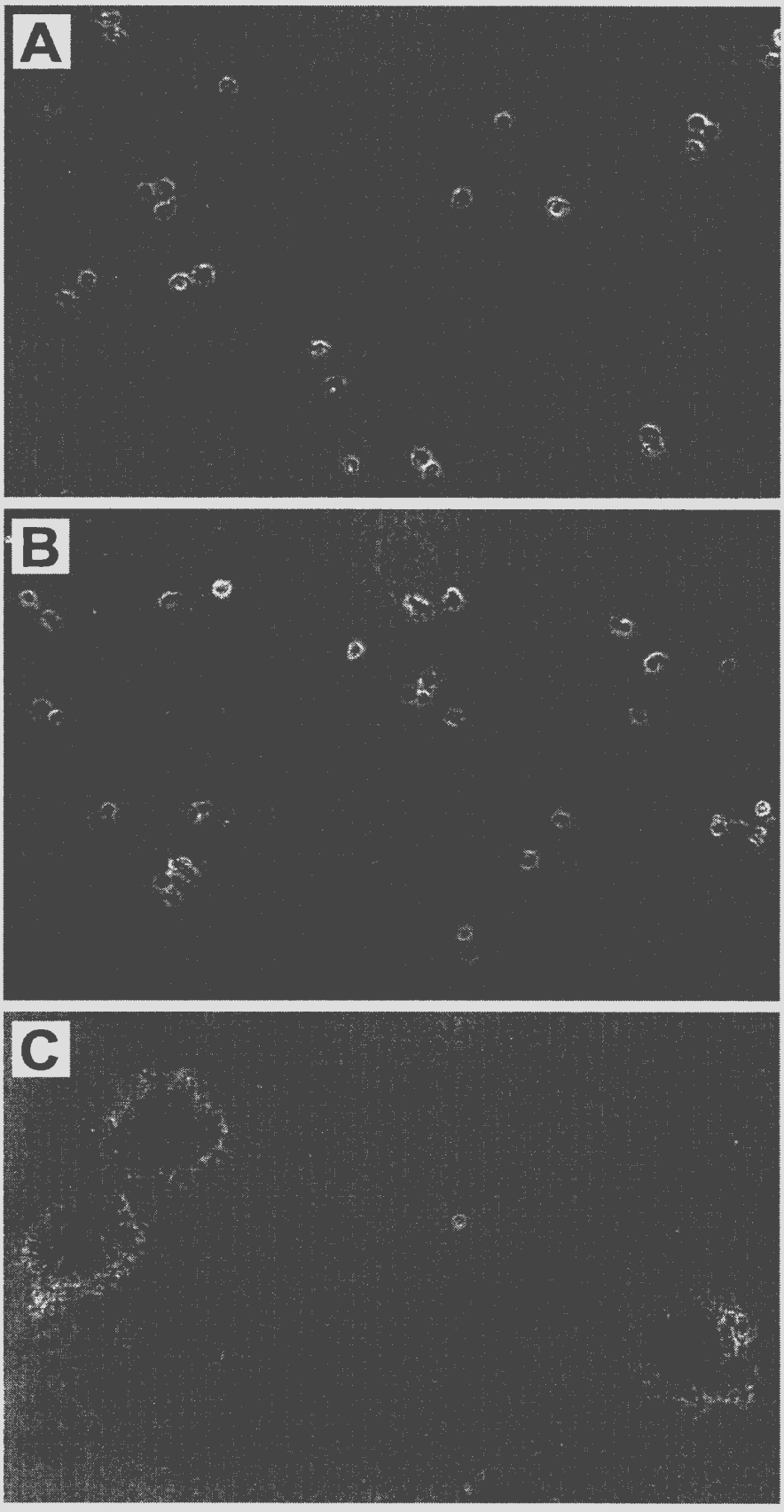
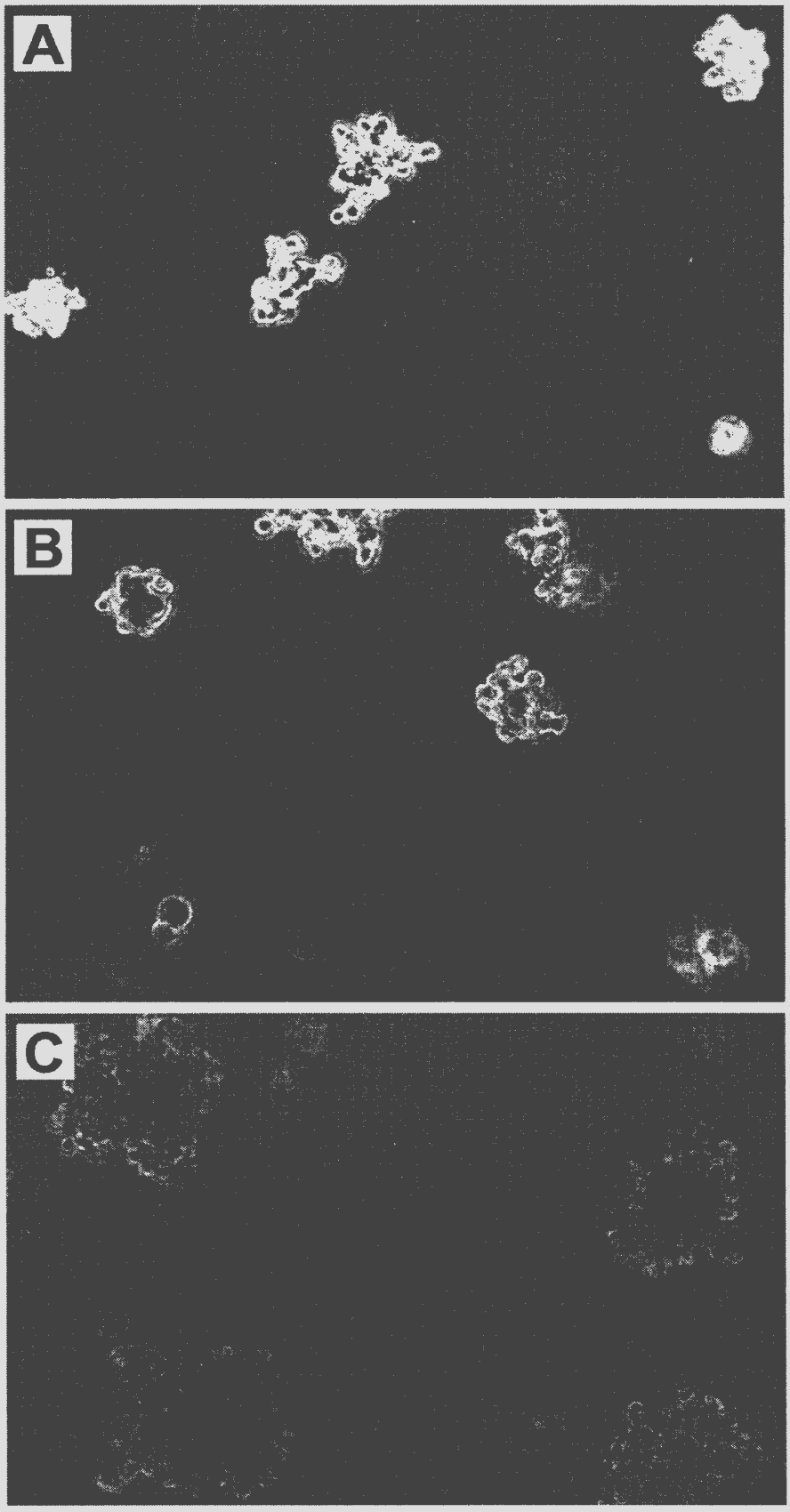

A
B
C

Figure 3.6 Anchorage-independent growth assays of CSC-transformed HPV 18-immortalized human ectocervical cells (HEC-18T). A, HEC-18T initially grown in KGM; B, HEC-18T initially grown in DMEM; C, HeLa cells used as the control. See text for details.


A
B
C

3.3. Morphological modifications of HEC, HEC-18, and HEC-18T treated with 5-FU

Morphological changes often serve as markers of apoptotic cell death. To evaluate whether 5-FU induces apoptosis in HEC, HEC-18, and HEC-18T, cells were examined using phase contrast microscopy after incubation with 5-FU in the concentration of IC_{50} for 6 hr. Initially, HEC, HEC-18, and HEC-18T cultured in KGM were analyzed after 5-FU treatment (Figure 3.7). Apoptotic characteristics, including cell shrinkage due to cell dehydration, irregular shape, smaller size, and apoptotic bodies (blebbing of cytoplasmic and nuclear membranes), were observed in all cases.

HEC-18 and HEC-18T were then adapted to DMEM, and subsequently treated with 5-FU for 6 hr. Apoptotic characteristics were again observed in both (Figure 3.8).

3.4. Flow cytometry analysis of apoptosis induced by 5-FU

By staining cells with a combination of Annexin V-FITC and PI, it is possible to detect apoptotic cells by flow cytometry (Koopman *et al.*, 1994). To confirm the apoptosis induced by 5-FU in HEC-18 and HEC-

Figure 3.7 Morphological changes of cells grown in KGM undergoing apoptosis induced by 5-FU. A-B, HEC; C-D, HEC-18; E-F, HEC-18T. Left panel represents controls. Right panel represents cells treated with 5-FU for 6 hr. Morphology was examined by phase contrast microscopy.

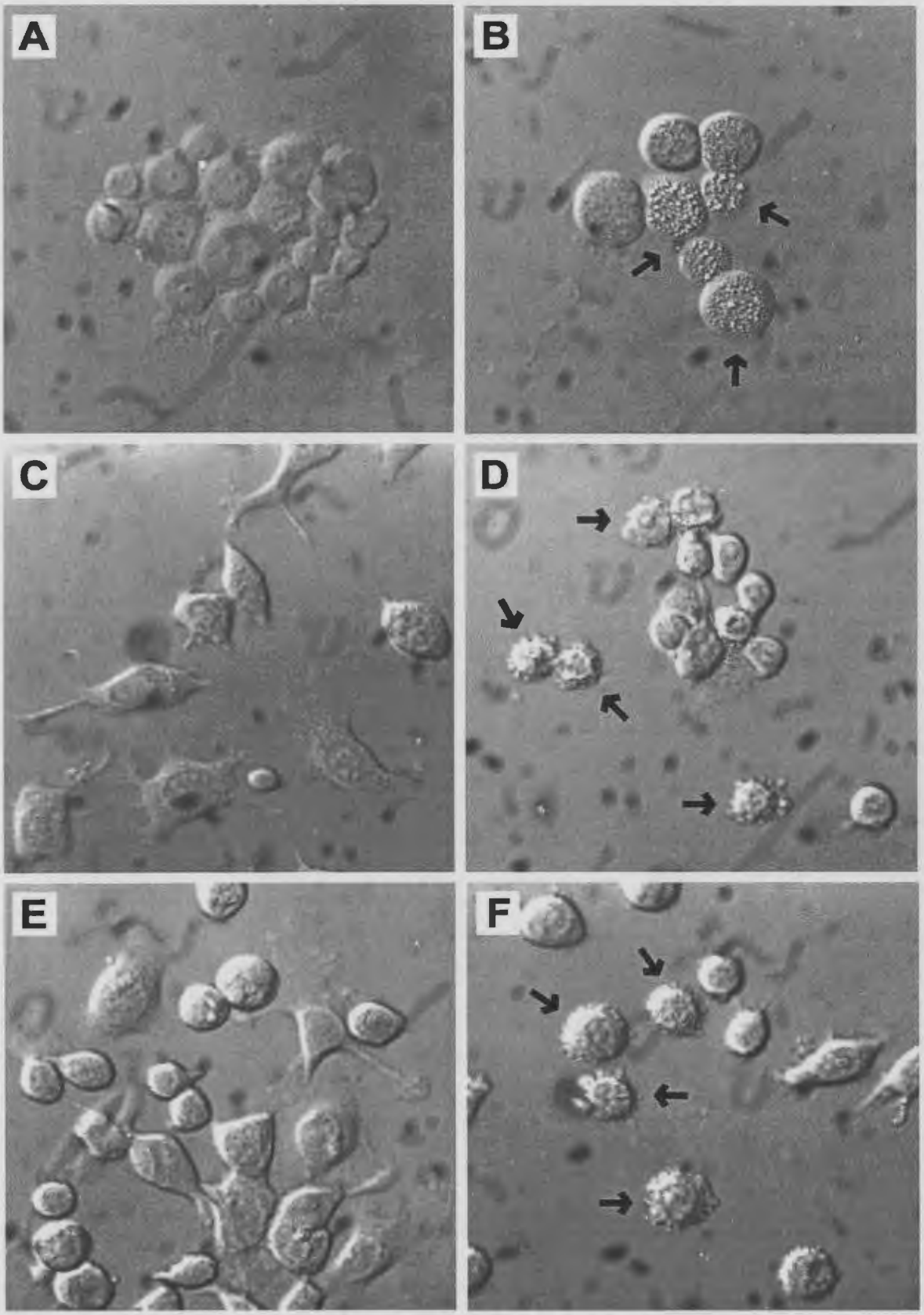

A
B
C
D
E
F

Figure 3.8 Morphological changes of cells grown in DMEM undergoing apoptosis induced by 5-FU. A-B, HEC; C-D, HEC-18; E-F, HEC-18T. Left panel represents controls. Right panel represents cells treated with 5-FU for 6 hr. Morphology was examined by phase contrast microscopy.

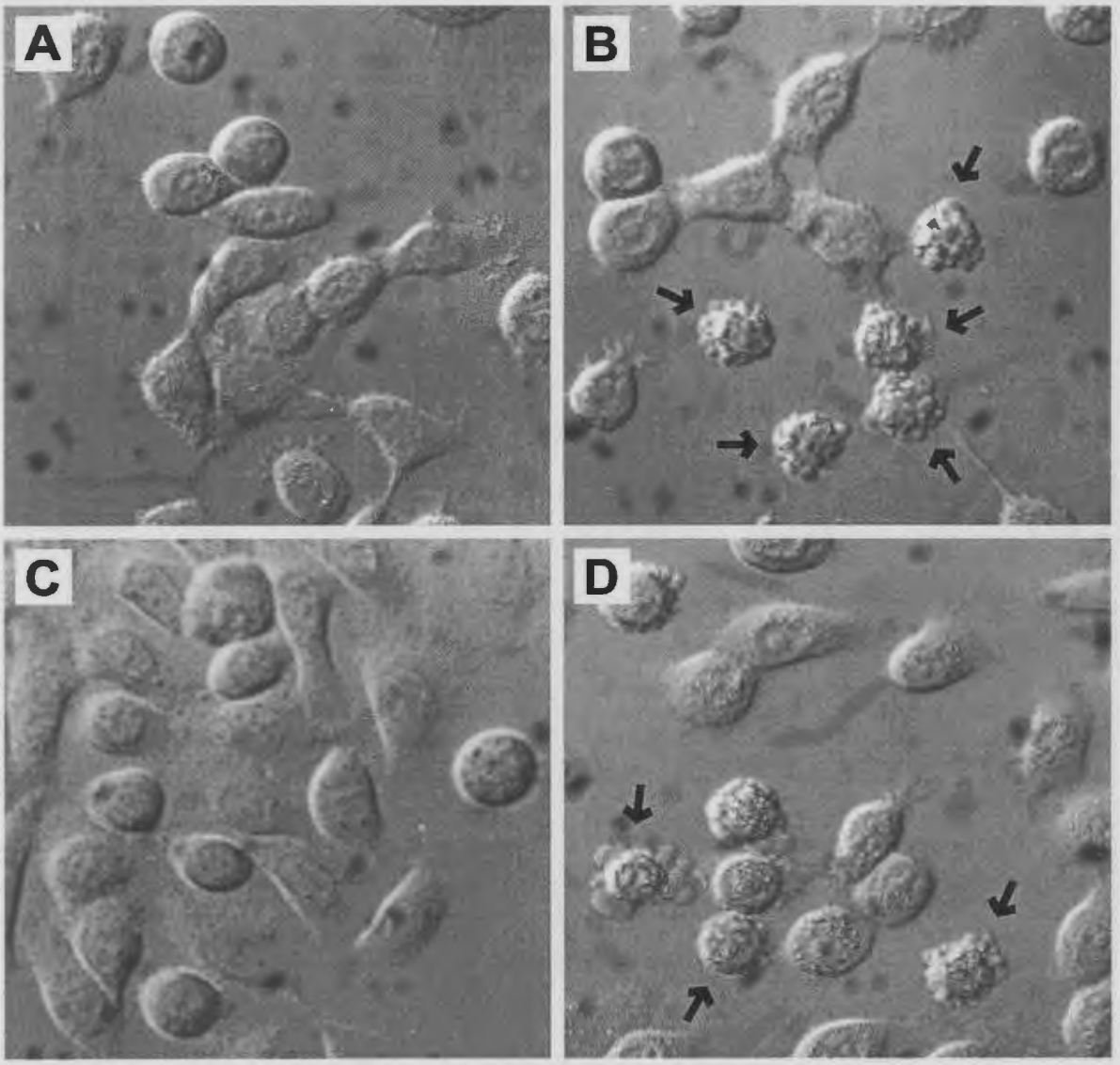
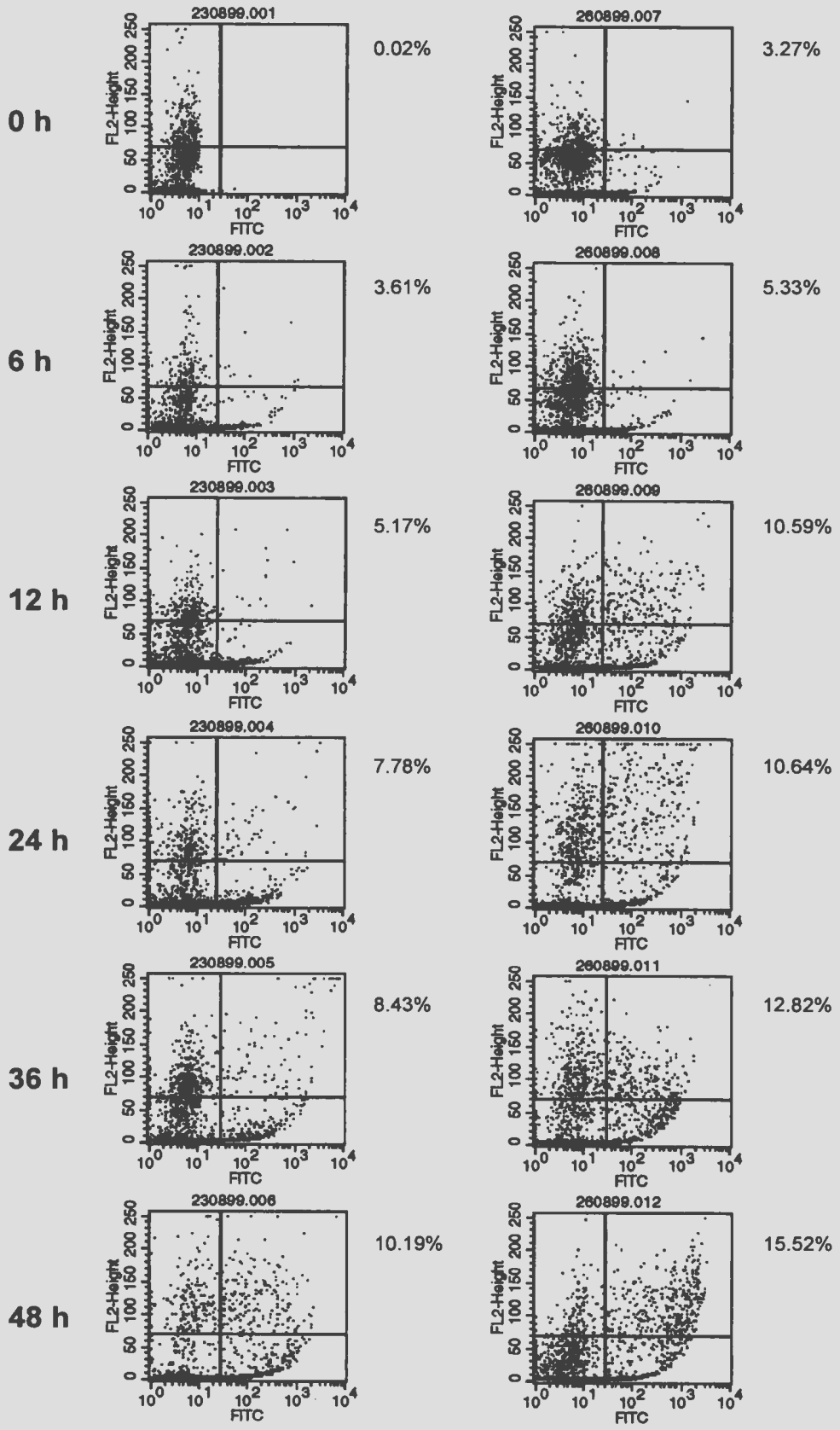

A
B
C
D

Figure 3.9 Analysis of apoptosis in HEC-18 and HEC-18T treated with 5-FU. Cells were treated with 5-FU for 0, 6, 12, 24, 36, and 48 hr, respectively, stained with Annexin V-FITC and PI, and analyzed by flow cytometry as described in Materials and Methods. Apoptotic cells are represented by the numbers indicating Annexin V-FITC positivity (right panels). The upper right panel indicates late-apoptotic or necrotic cells that have become permeable to PI. The data are representative of three separate experiments.


HEC-18
HEC-18T
0 h
6 h
12 h
24 h
36 h
48 h
230899.001
230899.002
230899.003
230899.004
230899.005
230899.006
260899.007
260899.008
260899.009
260899.010
260899.011
260899.012
0.02%
3.61%
5.17%
7.78%
8.43%
10.19%
3.27%
5.33%
10.59%
10.64%
12.82%
15.52%
FL2-Height
FITC

18T, flow cytometric analysis was performed using Annexin V-FITC assay. As shown in Figure 3.9, the majority of HEC-18 remained viable. The proportion of apoptotic cells was increased along with the increase of treatment time with 5-FU. Similarly, Figure 3.9 also shows that the apoptotic effect of 5-FU on HEC-18T also exhibited time dependence.

3.5. DNA fragmentation analysis of apoptosis induced by 5-FU

Activation of endonuclease cleavage of DNA into discrete fragments is considered to be a hallmark of apoptosis (Oberhammer *et al.*, 1993). Therefore, this phenomenon provides a strong indication of apoptotic cell death. To further confirm the apoptosis induced by 5-FU, the cellular DNA of 5-FU-treated and untreated HEC, HEC-18, and HEC-18T was extracted and assayed on 1.5% agarose gels. At first, experiments were performed on cells incubated in KGM for 72 hr (Figure 3.10). Obvious ladder-pattern DNA fragmentation, a characteristic feature of apoptosis, was observed in all 5-FU-treated cells. No ladder formation was detected in cells not exposed to 5-FU. Consistent with results from flow cytometry analysis, 5-FU-induced apoptosis occurred in a time-dependent fashion. More importantly, the induction of apoptosis by 5-FU was more pronounced in HEC than in HEC-18 and HEC-18T, implying that immortalization may decrease the sensitivity of cells to apoptosis, as reported (Yang *et al.*, 1998b).

Subsequently, HEC-18 and HEC-18T cultured in DMEM were also analyzed. As shown in Figure 3.11, discrete DNA fragments were apparent for both 5-FU-treated cell lines. Again, the extent of apoptosis increased with treatment time. It was observed that apoptosis induced for cells adapted to and grown in different culture media appeared to affect the extent of cleavage, as shown in Figure 3.11 compared with Figure 3.10.

These results encouraged us to directly compare the differences in the level of apoptosis induced by 5-FU in HEC and HEC-18 and HEC-18T cultured in different media for the optimal time. Cells, initially cultured in different media, were incubated with 5-FU for 72 hr, and analyzed. Indeed, as shown in Figure 3.12, apoptosis was more significant in DMEM than in KGM for both HEC-18 and HEC-18T, and almost equivalent to that of HEC. This suggested that growth medium affected the sensitivity of immortalized cells to 5-FU-induced apoptosis.

Figure 3.10 Effects of 5-FU-treatment on DNA fragmentation in human ectocervical cells. Cells (10^6) cultured for 24 hr in KGM were treated with 5-FU. Cells without 5-FU treatment were used as controls. DNA was extracted and analyzed by 1.5 % agarose gel electrophoresis and ethidium bromide staining, as described in Materials and Methods.

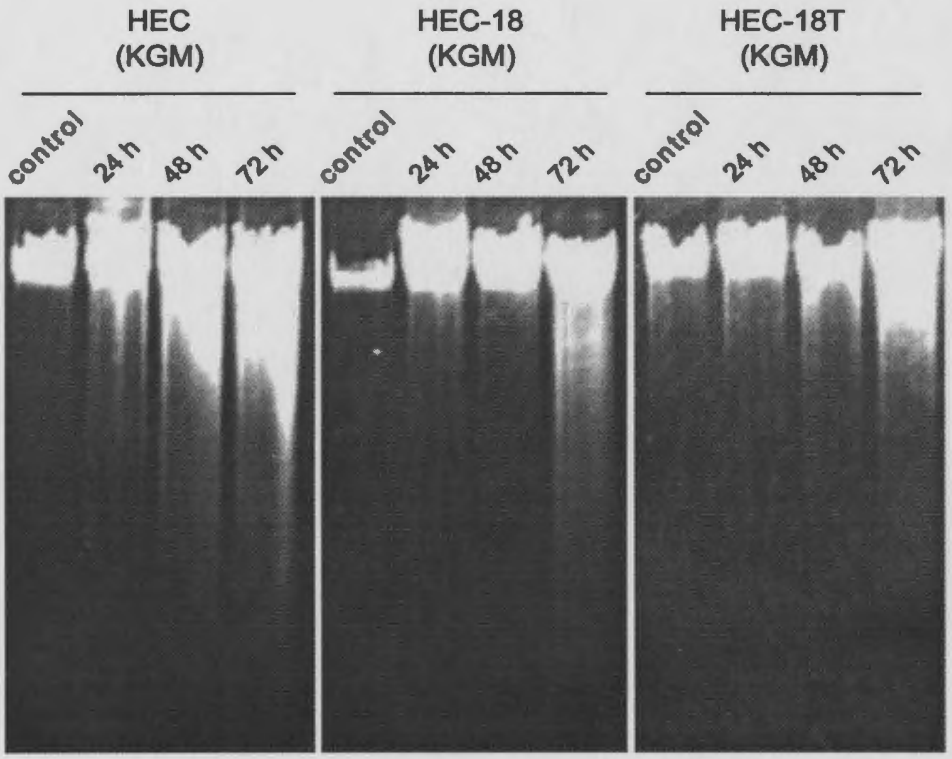

HEC
(KGM)
HEC-18
(KGM)
HEC-18T
(KGM)
control
24 h
48 h
72 h
control
24 h
48 h
72 h
control
24 h
48 h
72 h

Figure 3.11 DNA fragmentation assays of human ectocervical cells treated with 5-FU. Each cell line is indicated on the top of the figure. Cells (10^6) were cultured for 24 hr in DMEM, then 5-FU was added and the incubation period continued for another 24, 48, and 72 hr, respectively. Cells without 5-FU treatment are used as controls. DNA was extracted and analyzed by agarose gel electrophoresis in the presence of ethidium bromide, as described in Materials and Methods.

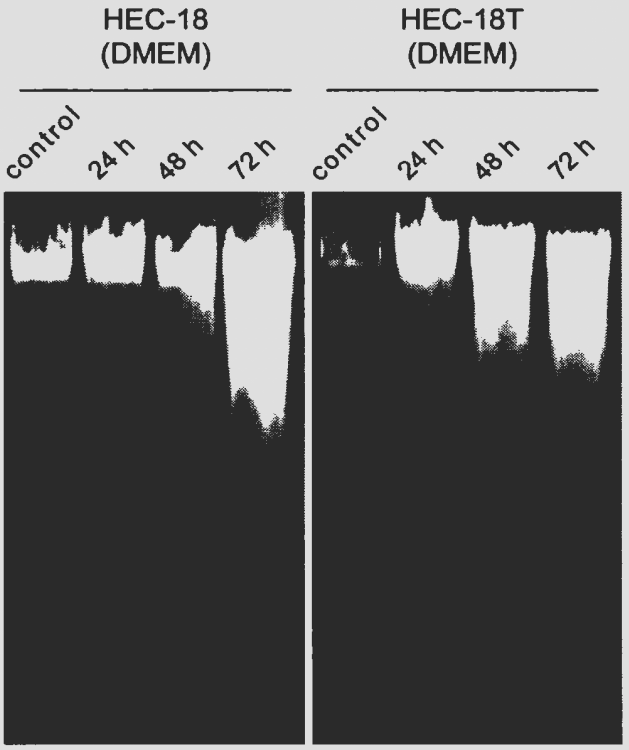
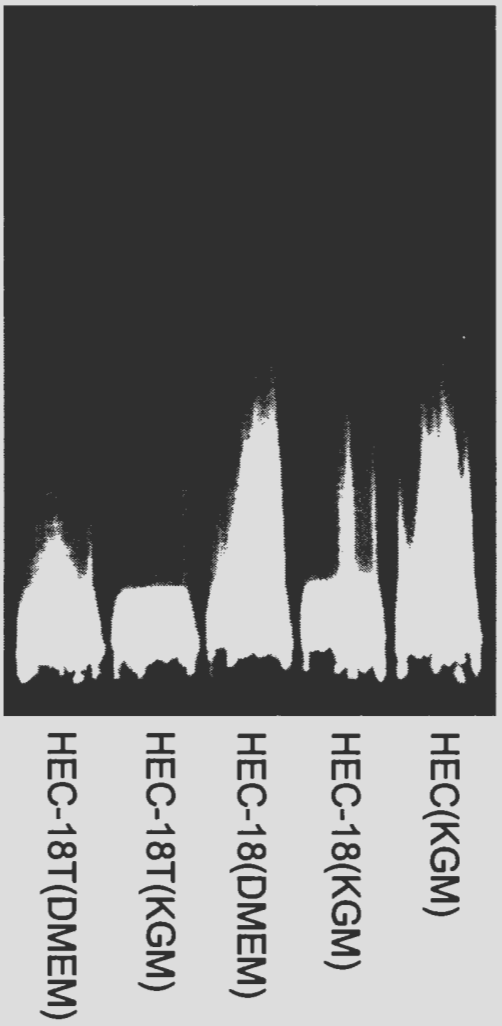

HEC-18
(DMEM)
HEC-18T
(DMEM)
control
24 h
48 h
72 h
control
24 h
48 h
72 h

Figure 3.12 DNA fragmentation assays of human ectocervical cells treated with 5-FU. Each cell line is indicated on the top of the figure. Cells (10^6) were cultured for 24 hr in either KGM or DMEM, then 5-FU was added and the incubation period continued for another 72 hr. DNA was extracted and analyzed by agarose gel electrophoresis in the presence of ethidium bromide, as described in Materials and Methods.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)

3.6. Effects of 5-FU treatment on apoptosis in HEC, HEC-18, and HEC-18T

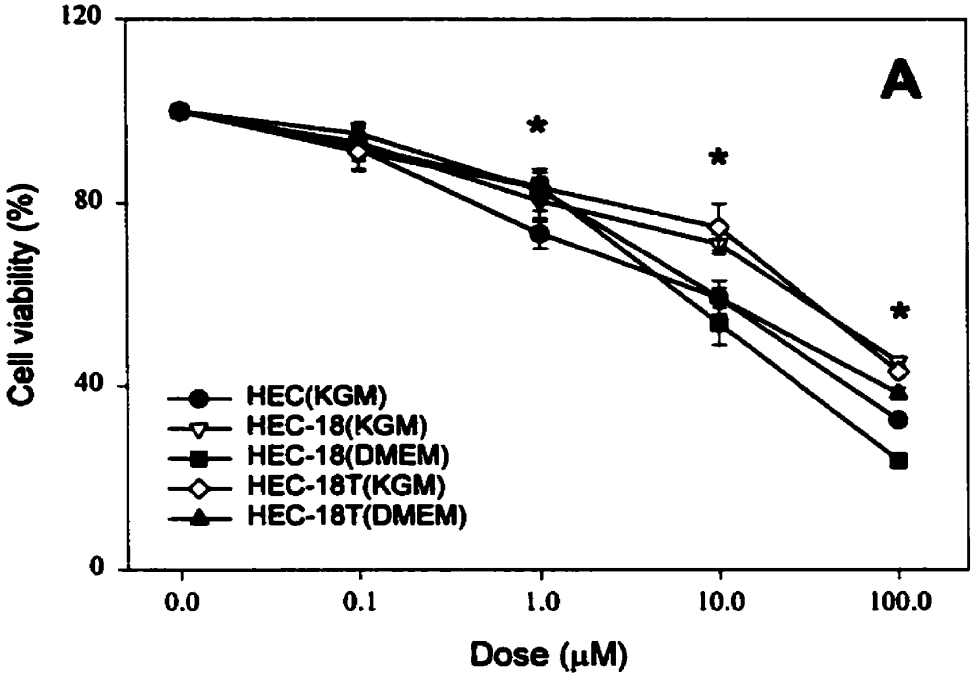
As described above, immortalization decreased the sensitivity of the ectocervical cells to apoptosis, and growth medium affected apoptosis determined by DNA fragmentation assay. To examine a different measure of apoptosis for confirmation, a marker of membrane integrity, apoptosis was assessed with the trypan blue dye exclusion assay (Figure 3.13). Ectocervical cells of the model system cultured in different media were treated with 5-FU at different concentrations for constant times (Figure 3.13 A), or treated with 5-FU at the same concentrations for different periods of time (Figure 3.13 B). In both cases, the same trend was observed: HEC-18 and HEC-18T culture in KGM were less sensitive than HEC to 5-FU-induced apoptosis, and HEC-18 and HEC-18T were more sensitive in DMEM than in KGM to 5-FU-induced apoptosis. Moreover, apoptosis induced by 5-FU in all cultures was time-dependent and dose-dependent.

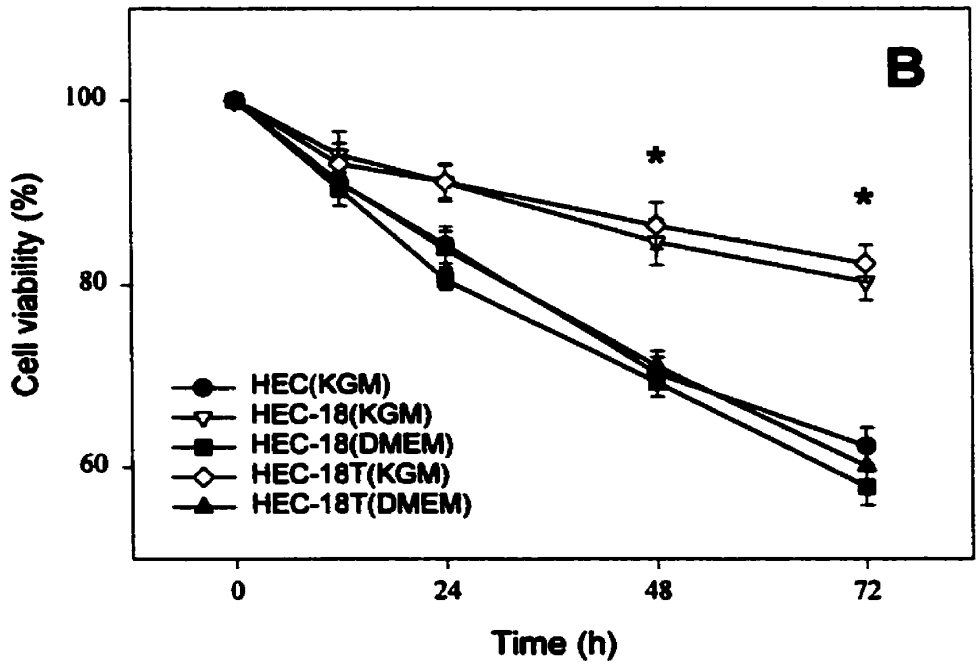
Furthermore, drug sensitivity was also determined by MTT cytotoxicity assay. The 5-FU concentrations causing a 50% growth inhibition (IC_{50}) compared with untreated cells were calculated from a dose-response curve by linear interpolation, and summarized in Table 3.2. A fluoropyrimidine prodrug, 5'-deoxy-5-fluorouridine (FUdR), was tested

Table 3.2 Concentrations of 5-FU and FUdR inhibiting proliferation by 50% (IC_{50}) of human ectocervical cells cultured in KGM or DMEM

Cells	IC_{50} (μM)	
	5-FU	FUdR
HEC (KGM)	23.1 ± 1.5	17.7 ± 1.2
HEC-18 (KGM)	48.7 ± 3.7	30.9 ± 2.1
HEC-18T (KGM)	72.3 ± 4.3	48.3 ± 2.5
HEC-18 (DMEM)	19.9 ± 1.8	10.2 ± 0.9
HEC-18T (DMEM)	20.3 ± 1.1	15.2 ± 1.3

Figure 3.13 Dose- and time-dependent induction of apoptosis in human ectocervical cells induced by 5-FU. The percentage of viable cells was determined by trypan blue dye exclusion assays. Results represent the mean from three independent experiments. The bars indicate standard deviation (SD). Panel A represents cells treated with 5-FU for 72 hr. Panel B represents cells treated with IC_{50} concentrations of 5-FU.


A
120
80
40
0
Cell viability (%)
HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
*
*
*
0.0
0.1
1.0
10.0
100.0
Dose (μM)


B
100
80
60
Cell viability (%)
HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
*
*
0
24
48
72
Time (h)

for comparison. In KGM, IC_{50} values for HEC, HEC-18 and HEC-18T were 23.1 μM, 48.7 μM and 72.3 μM, respectively. This is consistent with the finding that immortalization decreased the sensitivity of cells to 5-FU-induced apoptosis. For HEC-18, and HEC-18T, IC_{50} values were 19.9 μM and 20.3 μM, respectively in DMEM. These overall results showed that the sensitivity to 5-FU-induced ectocervical cell death was greater in immortalized than transformed cells, greater in immortalized cells in DMEM than KGM and equal in transformed cells. For FUdR-induced cytotoxicity, the pattern was the same except that it was less in transformed cells and it was greater for all values.

3.7. Expressions of apoptosis-regulating proteins in HEC, HEC-18, and HEC-18T

It has been hypothesized that defects in apoptosis-regulating genes may cause human cancer (Williams, 1991). This hypothesis has been supported by several reports of inactivation of genes inducing apoptosis, such as p53, and activation of genes inhibiting apoptosis, such as Bcl-2, in various human cancers (Haldar *et al.*, 1994; Hirose *et al.*, 1997; Rampino *et al.*, 1997). To investigate whether there is a correlation of expression of apoptosis–regulating proteins with sensitivity changes of cells to 5-FU-induced apoptosis, which may in turn closely relate to multistage cervical carcinogenesis, the expression of p53 in KGM, some

Bcl-2 family members, and BAG-1 proteins were examined by Western blot analysis.

3.7.1. Apoptosis-promoting proteins

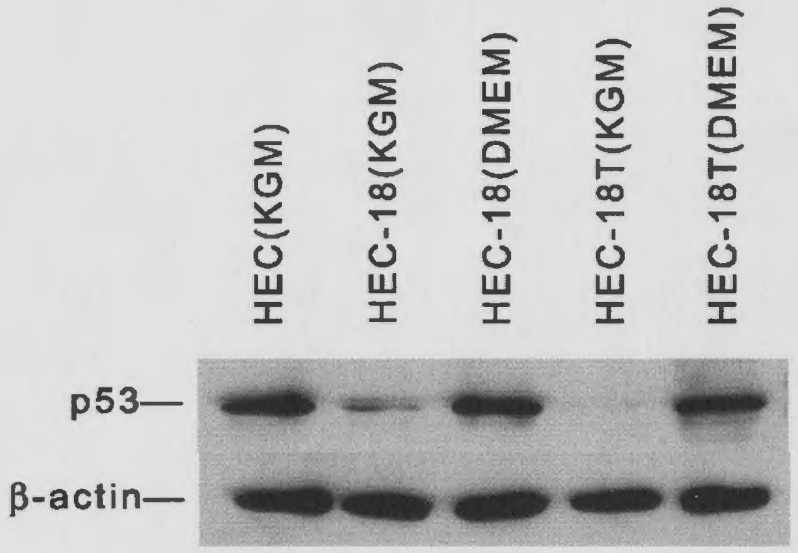
The apoptosis-promoting protein, p53, is known to suppress tumor growth and progression by induction of apoptosis. The expression of p53 protein was 6-fold lower in HEC-18 and 33-fold lower in HEC-18T compared with that in HEC (Figure 3.14, and Table 3.3). However, when cells were in DMEM compared with KGM, the levels of p53 were dramatically enhanced in both HEC-18 and HEC-18T.

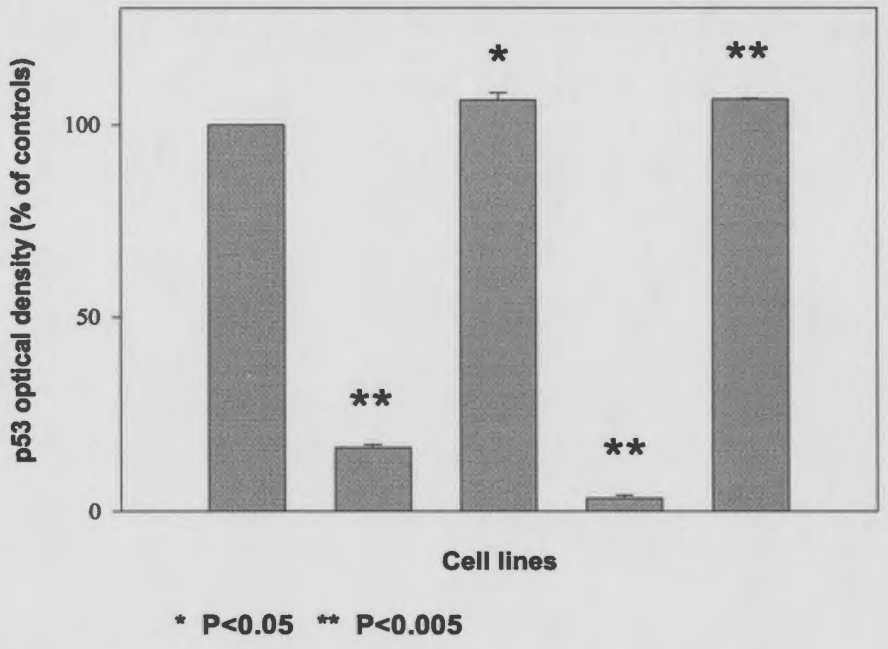
In KGM, the expression levels of Bak were lower in HEC-18 than in HEC (Figure 3.15). Nevertheless, Bak levels did not show significant difference when the immortalized and transformed cells were compared and culture medium was KGM or DMEM in both HEC-18 and HEC-18T.

Similar to p53, Bax was expressed 2-fold lower in HEC-18 and 5-fold lower in HEC-18T than in HEC when cells were cultured in KGM (Figure 3.16, and Table 3.3). The Bax levels increased 2-fold in HEC-18 and 7-fold in HEC-18T when cultured in DMEM.

These results suggest that there is a correlation of expression of apoptosis-promoting proteins with sensitivity changes of cells to 5-FU-induced apoptosis and cytotoxicity. The reduced levels of p53, Bak, and Bax are correlated with decreased sensitivity of

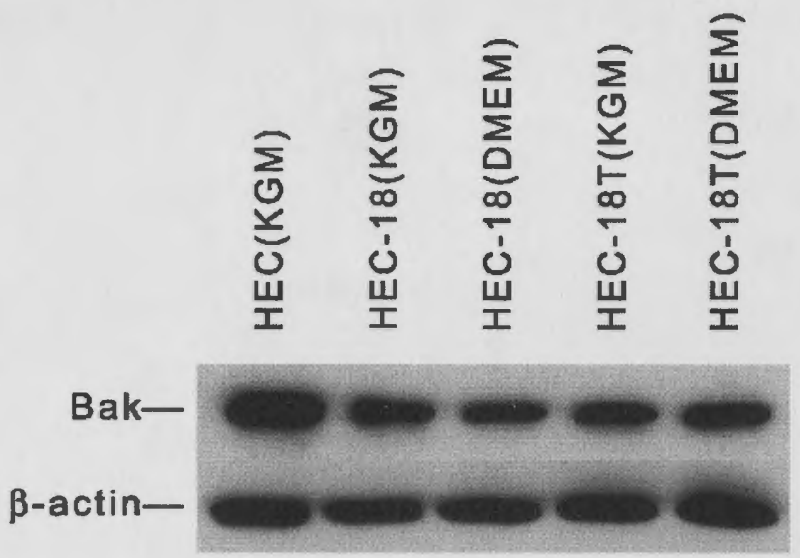
Figure 3.14 Expressions of p53 in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in p53 expression between untreated and treated cells.

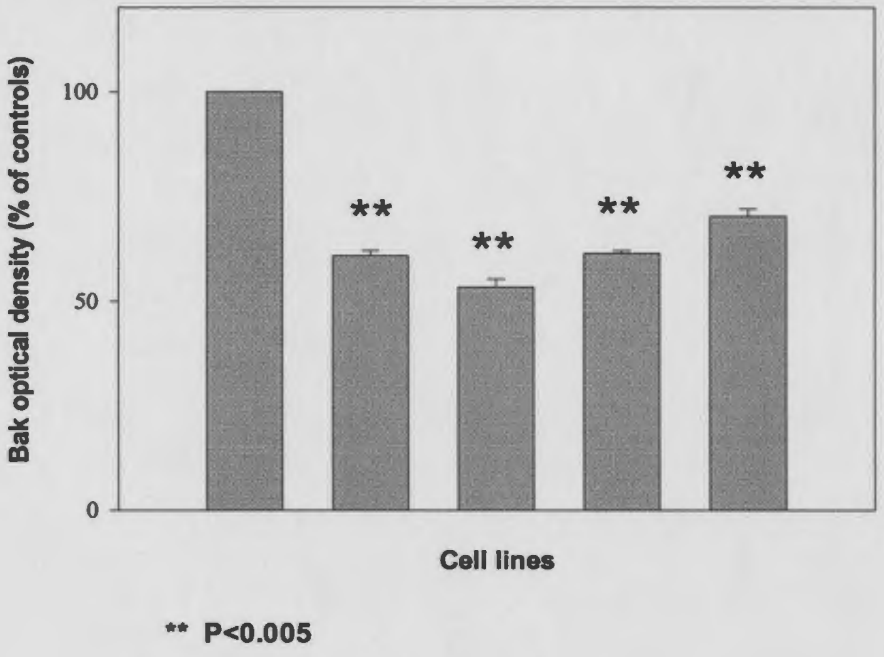

HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
p53—
β-actin—


p53 optical density (% of controls)
100
50
0
*
**
**
**
Cell lines

* P<0.05 ** P<0.005

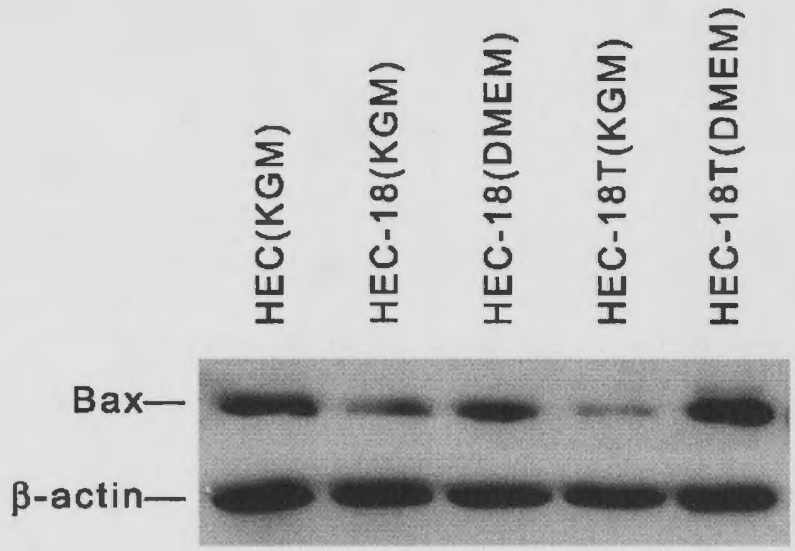
Figure 3.15 Expressions of Bak in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in Bak expression between untreated and treated cells.

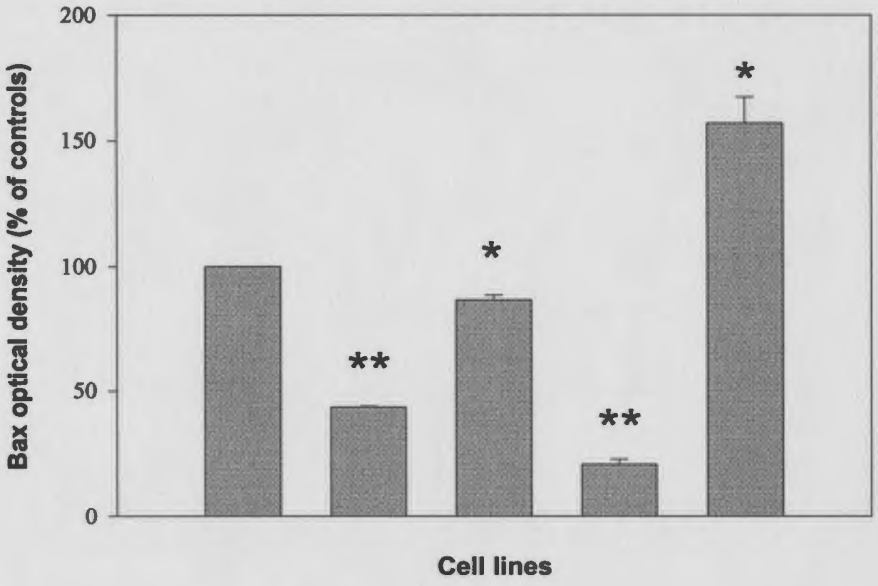

HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
Bak—
β-actin—


Bak optical density (% of controls)
100
50
0
**
**
**
**
Cell lines

** P<0.005

Figure 3.16 Expressions of Bax in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in Bax expression between untreated and treated cells.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
Bax—
β-actin—


Bax optical density (% of controls)
200
150
100
50
0
*
**
*
**
*
Cell lines

* P<0.05 ** P<0.005

HEC-18 and HEC-18T compared with HEC to 5-FU-induced apoptosis and cytotoxicity. The enhanced levels of p53 and Bax, but not changed level of Bak, are correlated with increased sensitivity of HEC-18 and HEC-18T in DMEM compared with in KGM to 5-FU-induced apoptosis.

3.7.2. Apoptosis-inhibiting proteins

In KGM, the expression of Bcl-2 increased more than 7-fold and 14-fold in HEC-18 and HEC-18T compared with the cells of the previous stage of carcinogenesis (Figure 3.17, and Table 3.3). However, the Bcl-2 levels decreased progressively in both HEC-18 and HEC-18T when the culture medium was changed from KGM to DMEM.

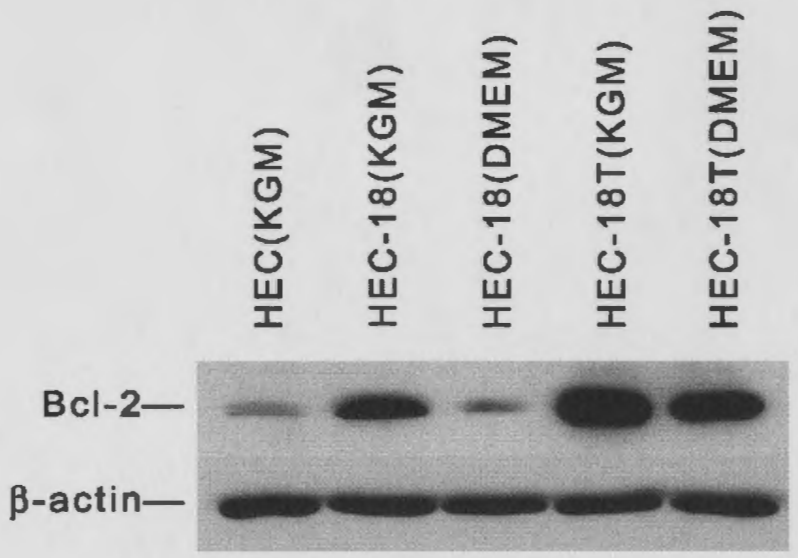
Since it was increased after immortalization in both cell lines and again after transformation in the HEN multistage carcinogenesis model (Yang *et al.*, 1998), it was surprising that there were no significant differences for the Bcl-x_L expression levels in all three cell lines and both media (Figure 3.18).

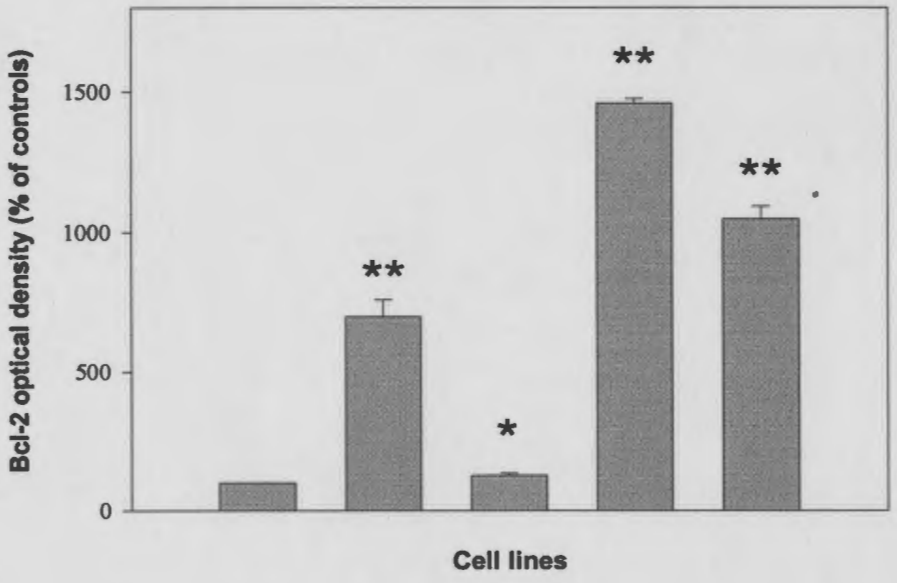
In the case of BAG-1, previous results have shown that p50, p46 and p33 of the four isoforms of BAG-1 were overexpressed in all the cervical carcinoma cell lines. They were variably detected in cervical carcinomas and p29 was not detected in any samples (Yang *et al.*, 1999). In contrast, BAG-1 p46 and p50 in the current model system cells were not detectable in any sample in three experiments. In KGM, BAG-1 p29

isoform was clearly expressed and showed reduced expression in HEC-18 and HEC-18T compared with HEC (Figure 3.19). Its expression was higher in HEC-18 and HEC-18T cultured in DMEM compared with in KGM. Although not apparent in the autoradiograph for HEC-18, densitometric comparison with actin control showed the difference to be significant ($P < 0.05$). However, another BAG-1 isoform p33 increased remarkably in HEC-18 and HEC-18T compared with HEC (Figure 3.20). In addition, p33 increased even further in HEC-18 and HEC-18T when the medium was DMEM. The overall level of BAG-1, which can be obtained by combining the results shown in Figure 3.19 and Figure 3.20, increased in HEC-18 and HEC-18T compared with HEC, and further increased when HEC-18 and HEC-18T were adapted to DMEM.

Therefore, for apoptosis inhibiting proteins, the enhanced levels of Bcl-2, total BAG-1 and p33 BAG-1 isoform, as summarized in Table 3.3, are correlated with decreased sensitivity of HEC-18 and HEC-18T compared with HEC to apoptosis. The reduced level of Bcl-2 and BAG-1 p29 and increased level of total BAG-1 and BAG-1 p33 are correlated with increased sensitivity of HEC-18 and HEC-18T in DMEM compared with in KGM, respectively, to apoptosis.

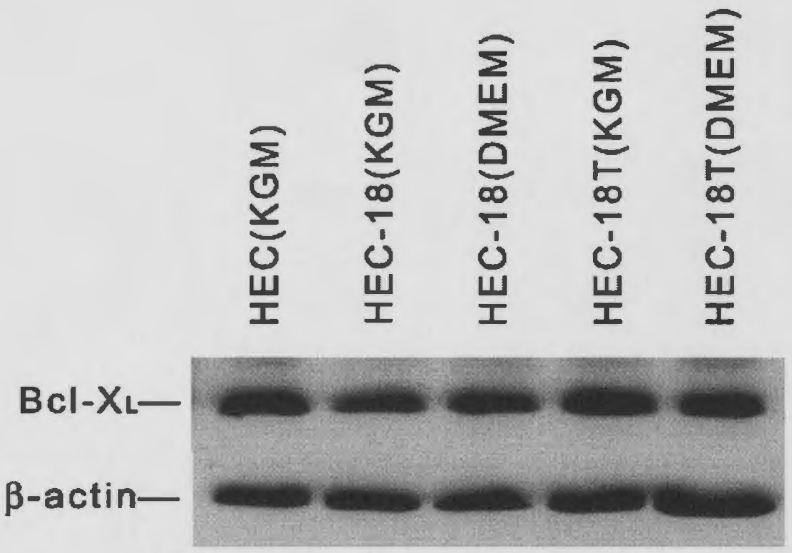
Figure 3.17 Expressions of Bcl-2 in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in Bcl-2 expression between untreated and treated cells.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
Bcl-2—
β-actin—


Bcl-2 optical density (% of controls)
1500
1000
500
0
**
*
**
**
Cell lines

* P<0.05 ** P<0.005

Figure 3.18 Expressions of Bcl-x_L in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in Bcl-x_L expression between untreated and treated cells.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
Bcl-XL
β-actin

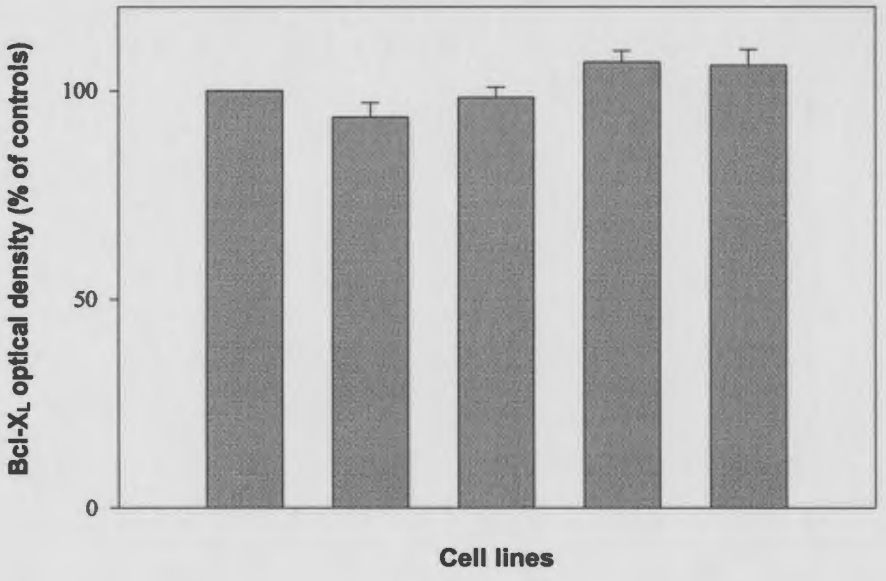
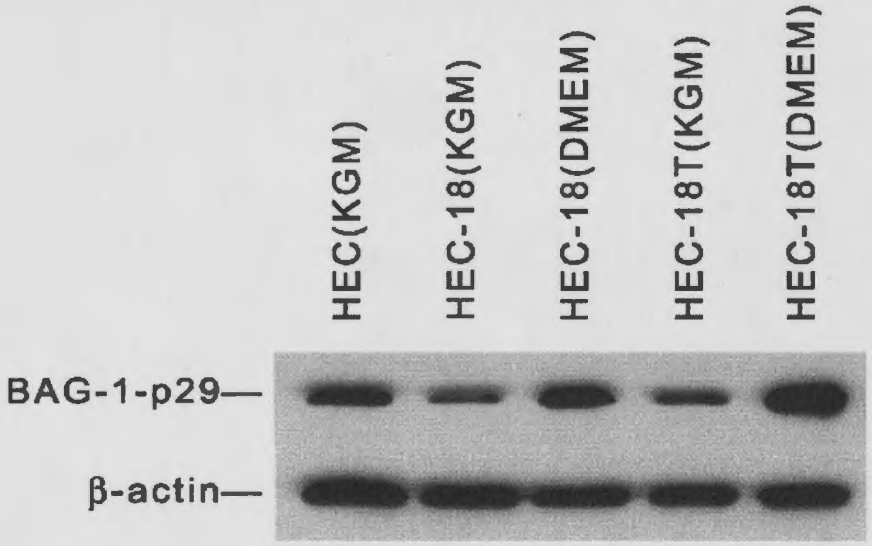
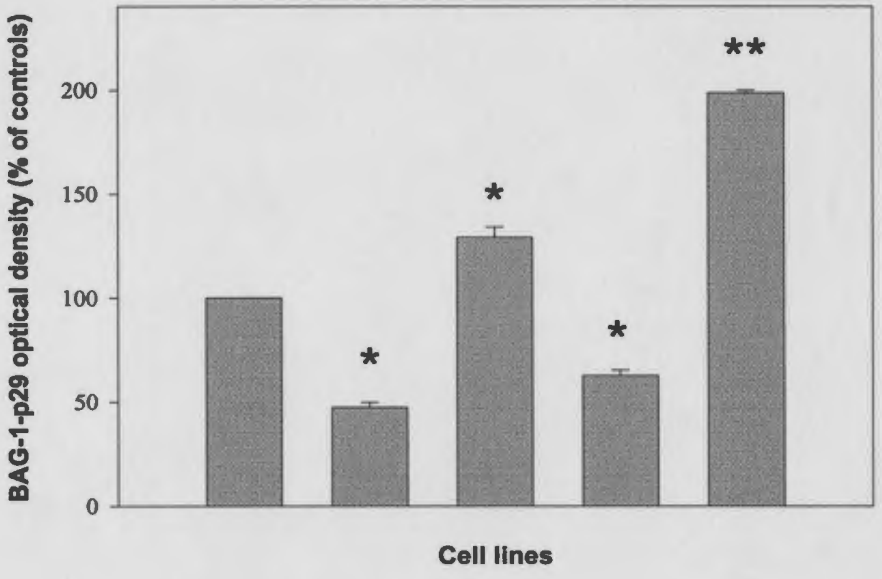

Bcl-XL optical density (% of controls)
100
50
0
Cell lines

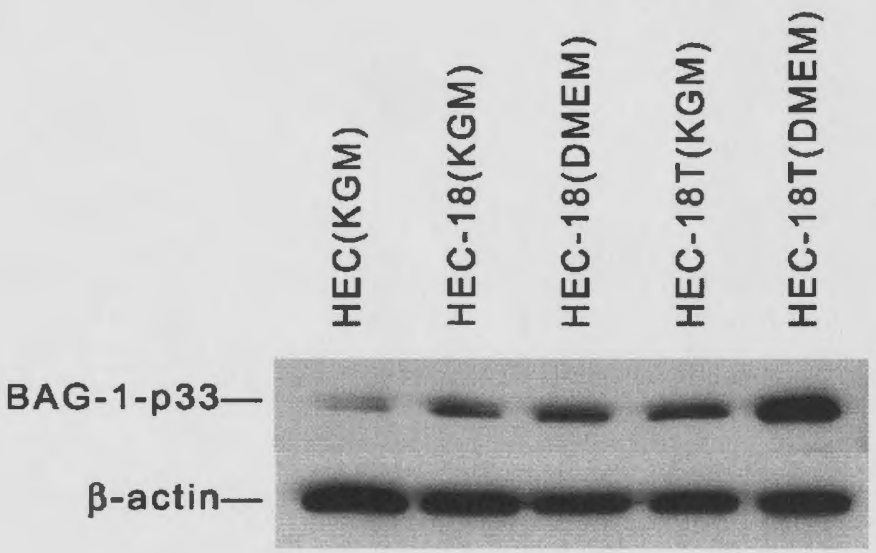
Figure 3.19 Expressions of BAG-1-p29 in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in BAG-1-p29 expression between untreated and treated cells.

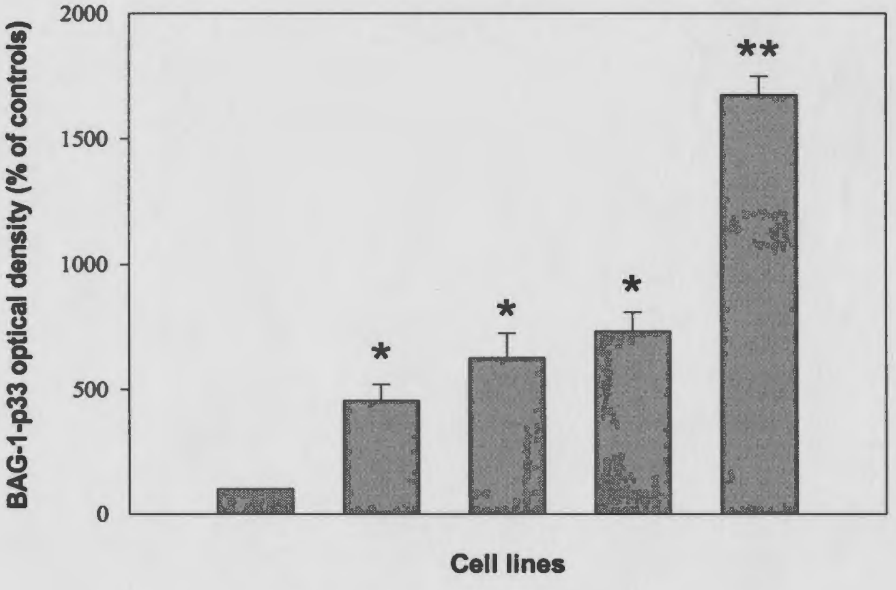

HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
BAG-1-p29—
β-actin—


BAG-1-p29 optical density (% of controls)
0
50
100
150
200
*
*
*
**
Cell lines

* P<0.05 ** P<0.005

Figure 3.20 Expressions of BAG-1-p33 in human ectocervical cells. The top panel shows Western blot analysis of cells grown in KGM. The bottom panel presents the corresponding densitometry results. Values represent the mean ± the standard deviation of three independent experiments. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in BAG-1-p33 expression between untreated and treated cells.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
BAG-1-p33—
β-actin—


BAG-1-p33 optical density (% of controls)
2000
1500
1000
500
0
*
*
*
**
Cell lines

* P<0.05 ** P<0.005

Table 3.3 Relative expressions of apoptosis-regulating proteins in HEC, HEC-18, and HEC-18T[1]

Proteins	HEC	HEC-18		HEC-18T	
		KGM	DMEM	KGM	DMEM
p53	100.0±0.0	16.3±1.2	106.4±2.7	3.3±1.2	106.7±0.2
Bak	100.0±0.0	61.0±1.7	53.5±2.7	61.6±0.9	70.4±2.5
Bax	100.0±0.0	43.7±0.3	86.4±2.8	20.7±2.8	156.9±14.7
Bcl-2	100.0±0.0	697.0±86.0	129.8±10.6	1459±21.0	1047±63.3
Bcl-x_L	100.0±0.0	93.7±4.8	98.4±3.4	106.8±3.8	106.0±5.4
BAG-1 p29	100.0±0.0	47.6±3.3	129.0±7.3	62.8±3.5	198.9±1.7
BAG-1 p33	100.0±0.0	454.6±80.9	624.6±142.3	731.9±107.8	1677±104.4

[1] The levels of cellular proteins were quantified relative to those of HEC after normalization to the actin control. Protein expression was quantified by measuring the optical density of bands at medium exposure on X-ray film. The data are shown as the mean ± the standard deviation of three experiments.

3.8. Effects of 5-FU treatment on expressions of apoptosis-regulating proteins in HEC, HEC-18, and HEC-18T

The molecular mechanism implicated in apoptosis has been partially elucidated and the induction of apoptosis is partly regulated intracellularly by several genes, such as p53 and Bcl-2 (Chiarugi *et al.*, 1994). Results of DNA fragmentation analysis (section 3.5.) showed that HEC-18 and HEC-18T were somewhat resistant to 5-FU-induced apoptosis compared with HEC when cultured in KGM. More notably, HEC-18 and HEC-18T were highly sensitive to 5-FU-induced apoptosis when they were cultured in DMEM. The mechanism of this enhanced susceptibility of cells to anticancer drug-induced apoptosis might offer informative clues for apoptosis-based chemotherapy of human cancers. To investigate whether 5-FU regulates apoptosis-associated genes, thus inducing apoptosis, a panel of protein products of apoptosis-associated genes, including p53, Bcl-2, and their related genes, were examined after 5-FU treatment, and the results were summarized in Table 3.4.

3.8.1. Apoptosis-promoting proteins

As shown in Figure 3.21, 5-FU treatment upregulated p53 in both cell lines cultured in DMEM. Levels of a p53 target protein, p21, were also examined. As shown in Figure 3.22, the expression of p21 tumor

promoter protein was upregulated more than 2-fold in HEC-18. The same trend was observed in HEC-18T, but the result was not as pronounced as that in HEC-18.

MDM-2 proto-oncoprotein is also a transcriptional target of p53 (Barak *et al.*, 1993). p53 increases MDM-2 expression whereas MDM-2 suppresses p53 activity, creating an autoregulatory feedback loop. Figure 3.23 shows our studies on MDM-2 protein with 5-FU treatment. It remained unchanged in HEC-18, but was remarkably increased in HEC-18T by 5-FU treatment.

PCNA transcription is suppressed by p53 (Shivakumar *et al.*, 1995). However, PCNA displayed a pattern (Figure 3.24) that was the same as that of p53 (Figure 3.21) in HEC-18 and HEC-18T after 5-FU treatment.

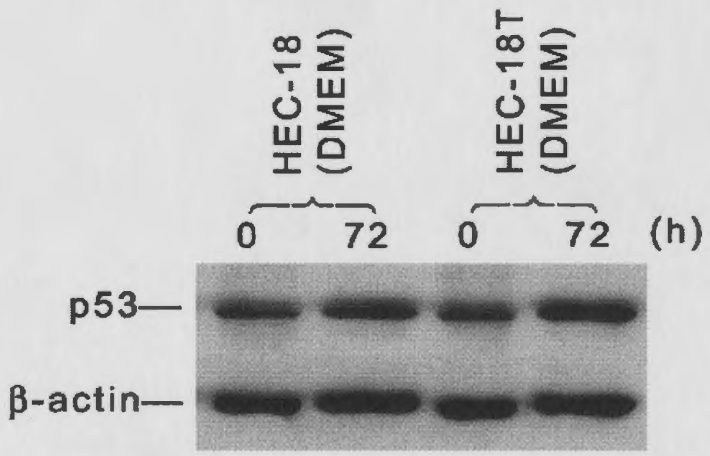
Bax is also the target of p53 and the protein upregulates apoptosis by binding with Bcl-2 (Oltvai *et al.*, 1993). Our experiments did not show any significant changes either in HEC-18 or in HEC-18T after 5-FU treatment (Figure 3.25).

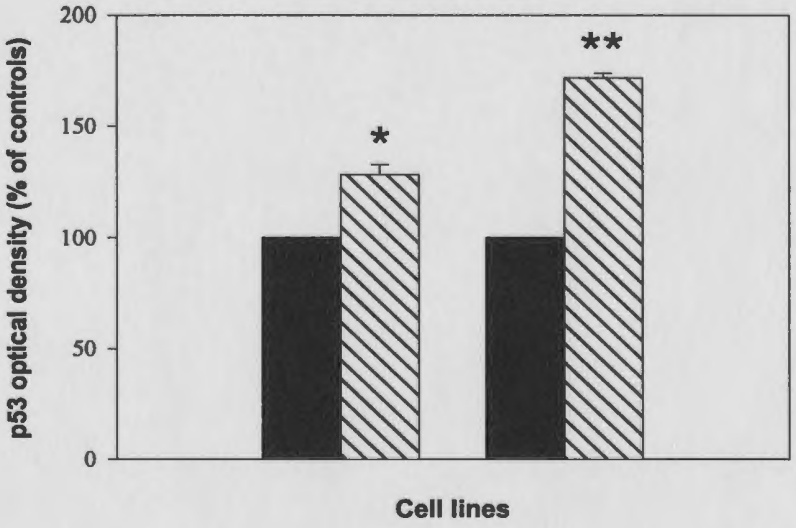
Bak, an apoptosis-promoting protein, was upregulated by 5-FU in HEC-18 and more significant in HEC-18T (Figure 3.26).

3.8.2. Apoptosis-inhibiting proteins

Among apoptosis inhibiting proteins, are members of the Bcl-2 family. The prototype, Bcl-2, is transcriptionally downregulated by p53 (Miyashita *et al.*, 1994b). Figure 3.27 shows that Bcl-2 was

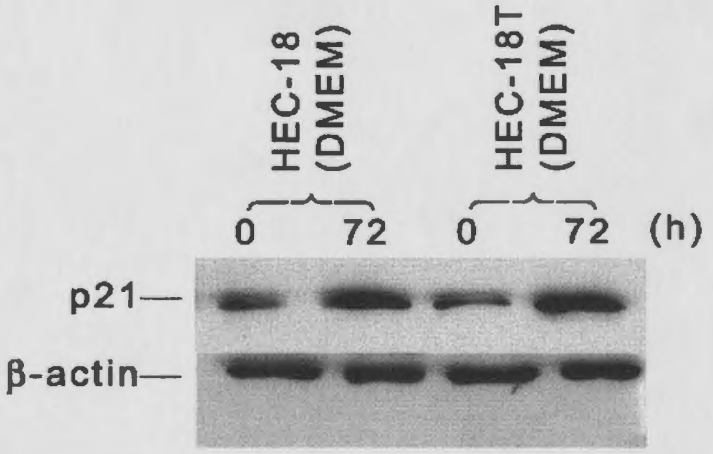
Figure 3.21 Expressions of p53 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-p53 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. *P < 0.05 and **P < 0.005 are the statistical significance of the difference in expression of p53 between each 5-FU-treated cell line and control untreated cells.

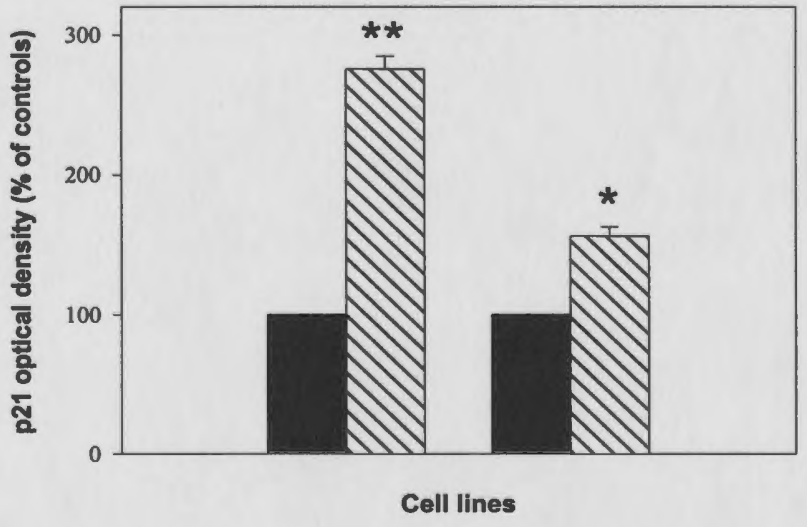

HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
p53—
β-actin—


200
150
100
50
0
p53 optical density (% of controls)
*
**
Cell lines

* P<0.05 ** P<0.005

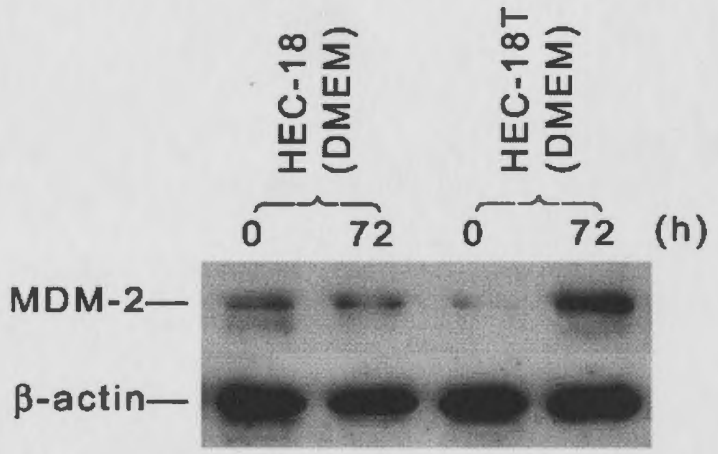
Figure 3.22 Expressions of p21 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-p21 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. *$P < 0.05$ and **$P < 0.005$ are the statistical significance of the difference in expression of p21 between each 5-FU-treated cell line and control untreated cells.

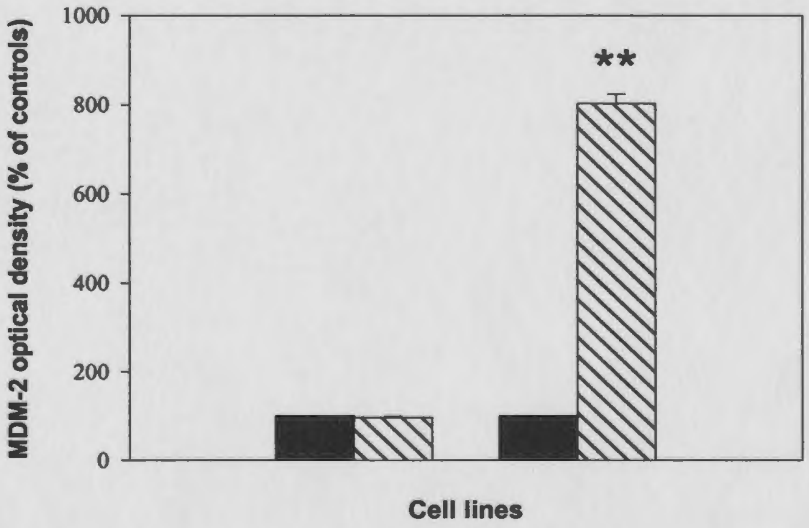

HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
p21—
β-actin—


p21 optical density (% of controls)
300
200
100
0
**
*
Cell lines

* P<0.05 ** P<0.005

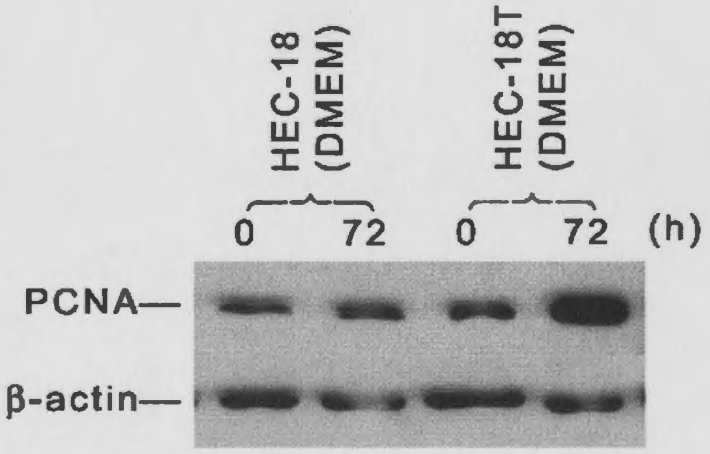
Figure 3.23 Expressions of MDM-2 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-MDM-2 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. $^{**}P < 0.005$ is the statistical significance of the difference in expression of MDM-2 between each 5-FU-treated cell line and control untreated cells.

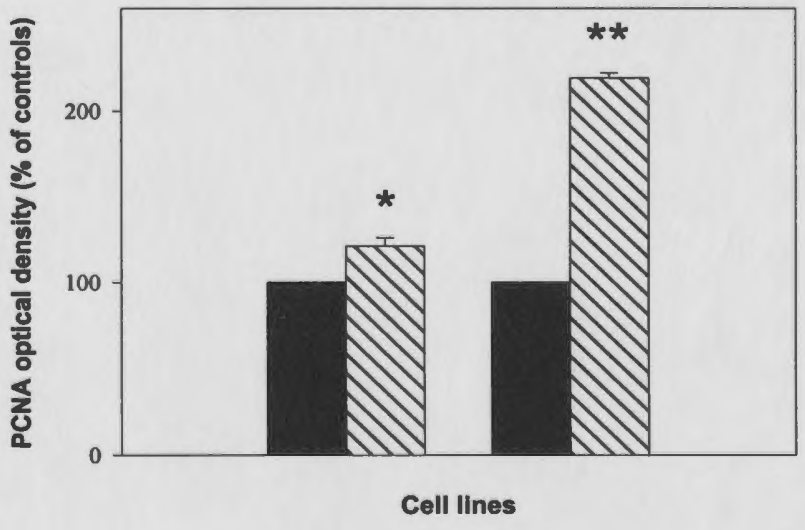

HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
MDM-2—
β-actin—


MDM-2 optical density (% of controls)
1000
800
600
400
200
0
**
Cell lines

** P<0.005

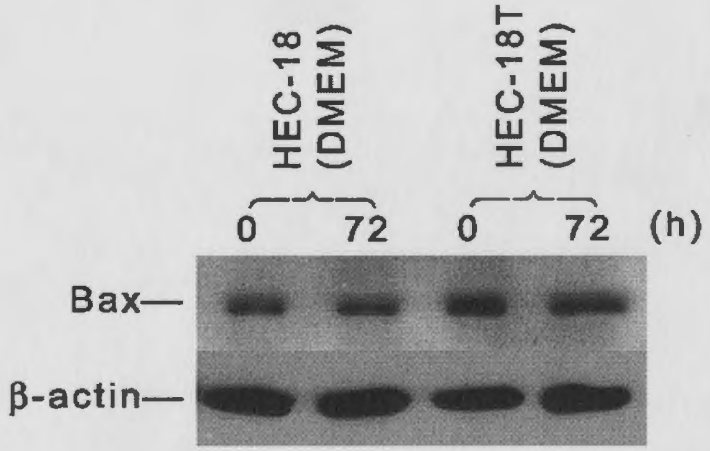
Figure 3.24 Expressions of PCNA in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 µg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-PCNA antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. *$P < 0.05$ and **$P < 0.005$ are the statistical significance of the difference in expression of PCNA between each 5-FU-treated cell line and control untreated cells.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
PCNA—
β-actin—


PCNA optical density (% of controls)
200
100
0
*
**
Cell lines

* P<0.05 ** P<0.005

Figure 3.25 Expressions of Bax in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-Bax antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
Bax—
β-actin—

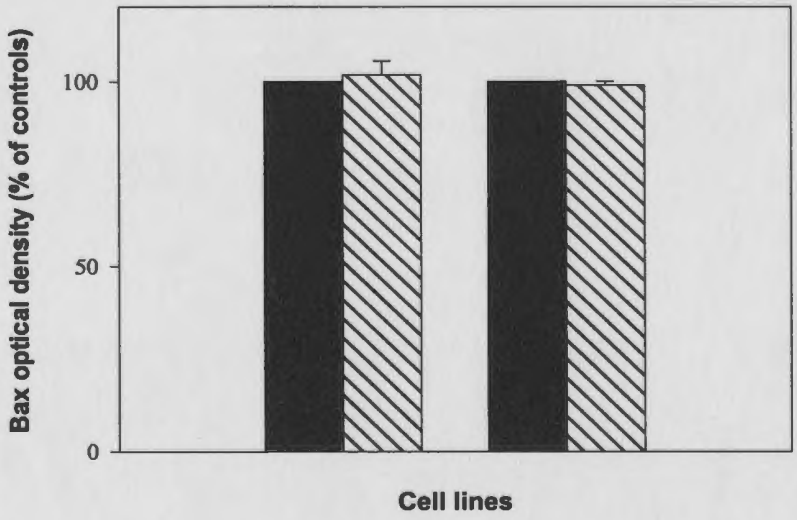
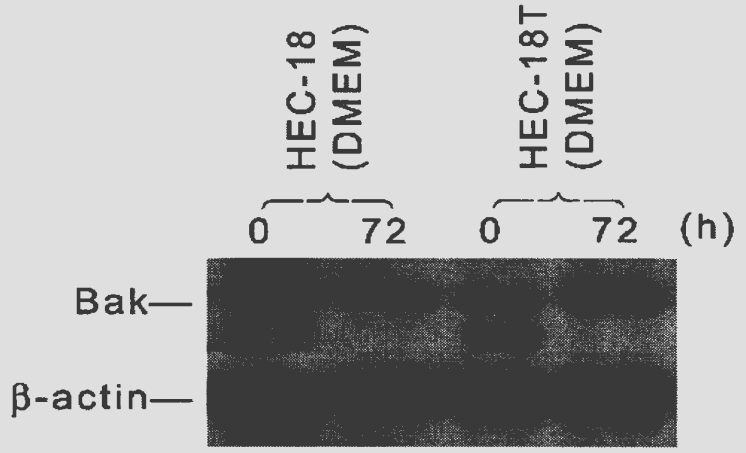
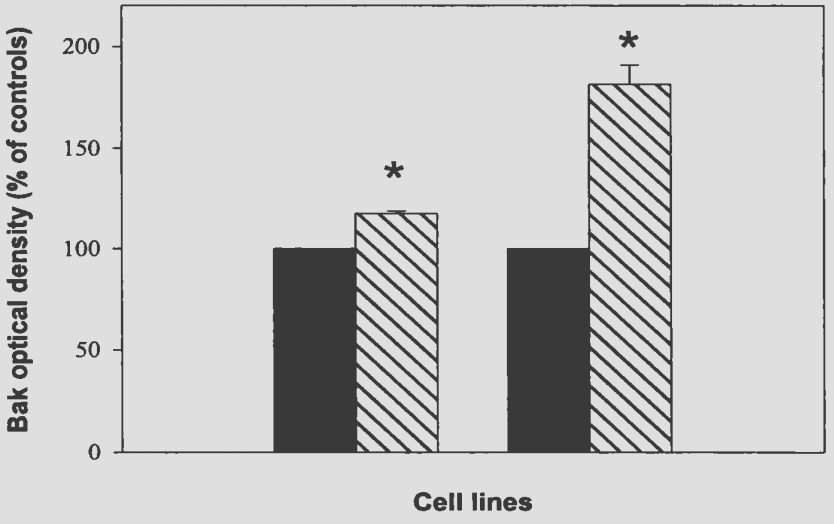

Bax optical density (% of controls)
100
50
0
Cell lines

Figure 3.26 Expressions of Bak in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the rabbit polyclonal IgG anti-Bak antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. *$P < 0.05$ is the statistical significance of the difference in expression of Bak between each 5-FU-treated cell line and control untreated cells.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
Bak—
β-actin—


Bak optical density (% of controls)
200
150
100
50
0
*
*
Cell lines

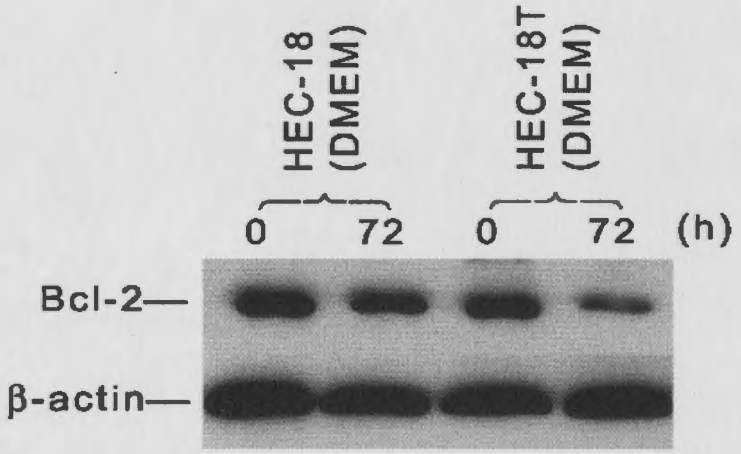
* P<0.05

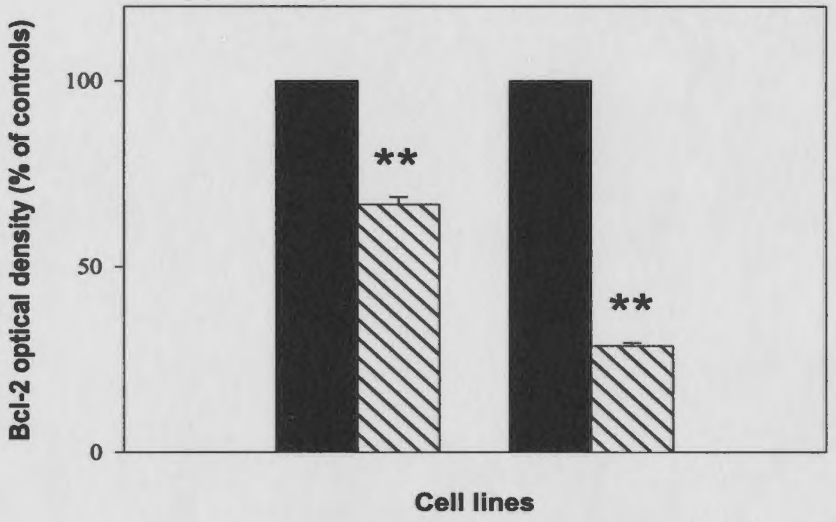
downregulated by 5-FU in both HEC-18 and HEC-18T. Therefore, Bcl-2 exhibited an opposite trend to that of p53 (Figure 3.21).

Upon treatment of 5-FU, Bcl-x_L appeared to be not significantly regulated in both cell lines (Figure 3.28).

BAG-1, known as an apoptosis-inhibiting gene, was isolated by the interaction of its protein with Bcl-2 (Takayama *et al.*, 1995). As shown in Figure 3.29, BAG-1 isoform p29 was downregulated by 5-FU treatment in both HEC-18 and HEC-18T. Another of the four BAG-1 isoforms, p33, was also downregulated in HEC-18T (Figure 3.30). However, p33 remained the same in HEC-18 after 5-FU treatment. Although not expressed in KGM, the use of DMEM as a medium led to induction of weak expression of BAG-1 p46 and p50 isoforms in HEC-18 and moderate expression in HEC-18T (Figure 3.31). They did not change in HEC-18 with treatment of 5-FU. On the other hand, they were both downregulated in HEC-18T by 5-FU. In total, BAG-1 proteins showed a trend of downregulation by the treatment with 5-FU.

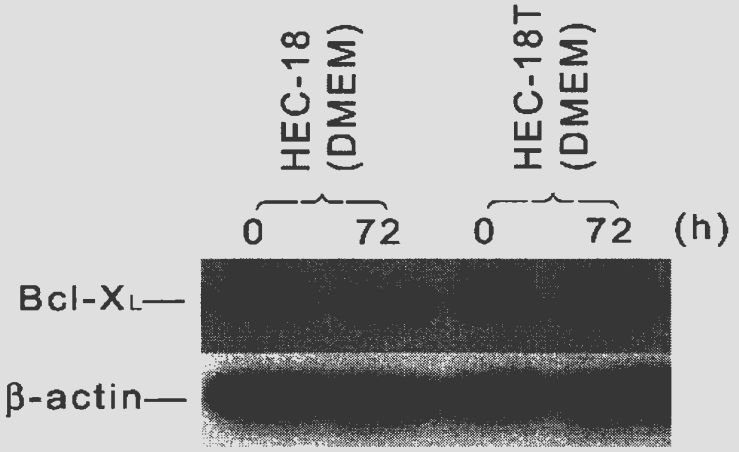
Figure 3.27 Expressions of Bcl-2 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 µg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-Bcl-2 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. $^{**}P < 0.005$ is the statistical significance of the difference in expression of Bcl-2 between each 5-FU-treated cell line and control untreated cells.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
Bcl-2—
β-actin—


Bcl-2 optical density (% of controls)
100
50
0
**
**
Cell lines

** P<0.005

Figure 3.28 Expressions of Bcl-x_L in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-Bcl-x_L antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
Bcl-XL—
β-actin—

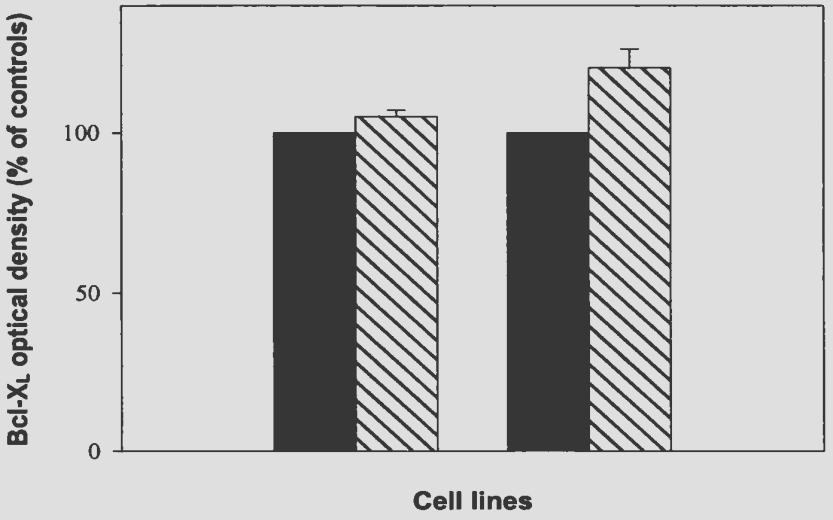
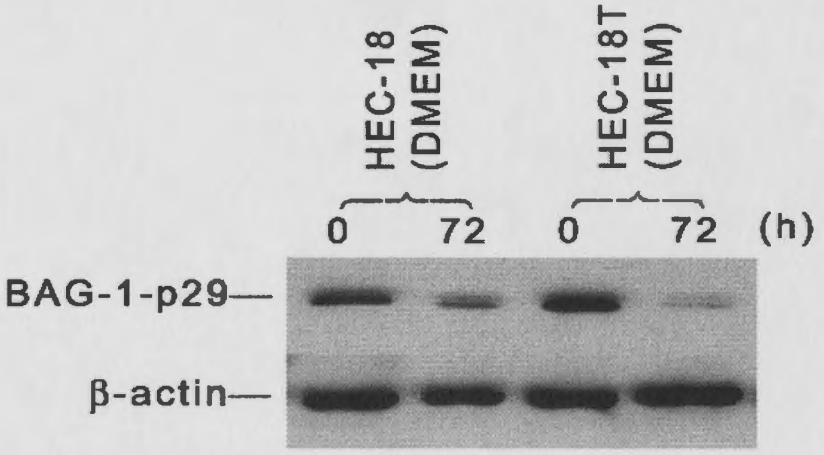
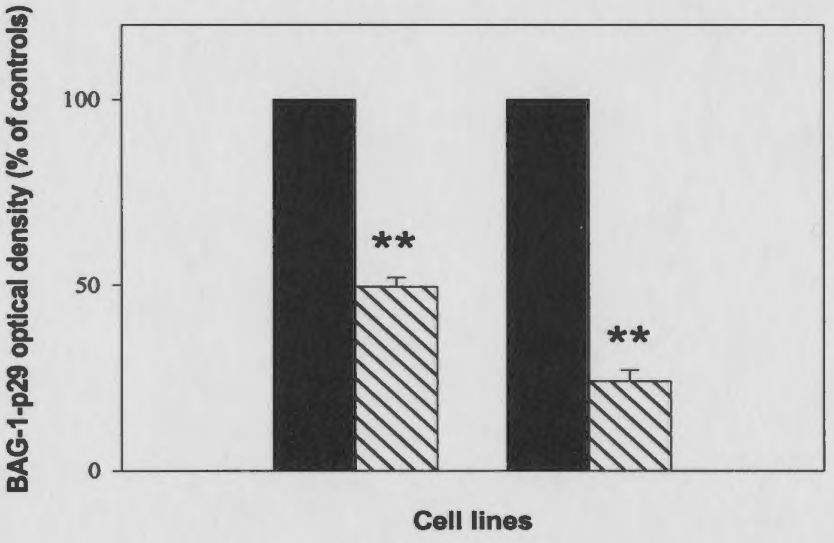

Bcl-XL optical density (% of controls)
100
50
0
Cell lines

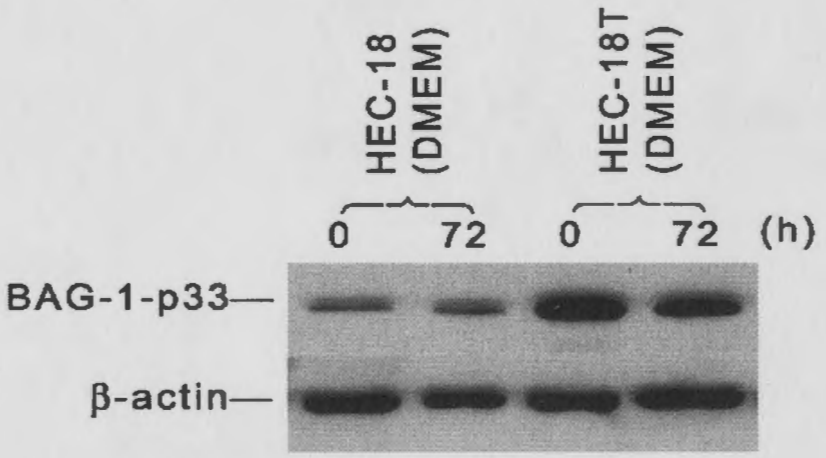
Figure 3.29 Expressions of BAG-1-p29 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-BAG-1 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. $^{**}P < 0.005$ is the statistical significance of the difference in expression of BAG-1-p29 between each 5-FU-treated cell line and control untreated cells.

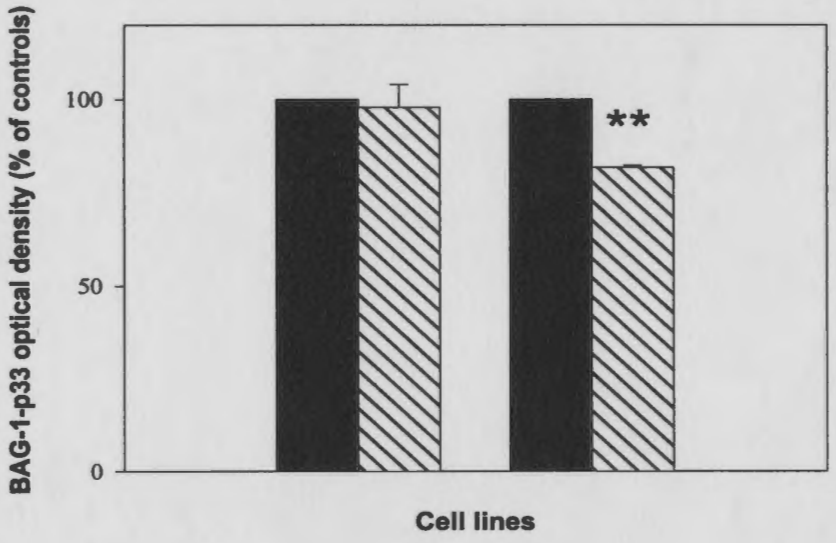

HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
BAG-1-p29
β-actin


BAG-1-p29 optical density (% of controls)
100
50
0
**
**
Cell lines

** P<0.005

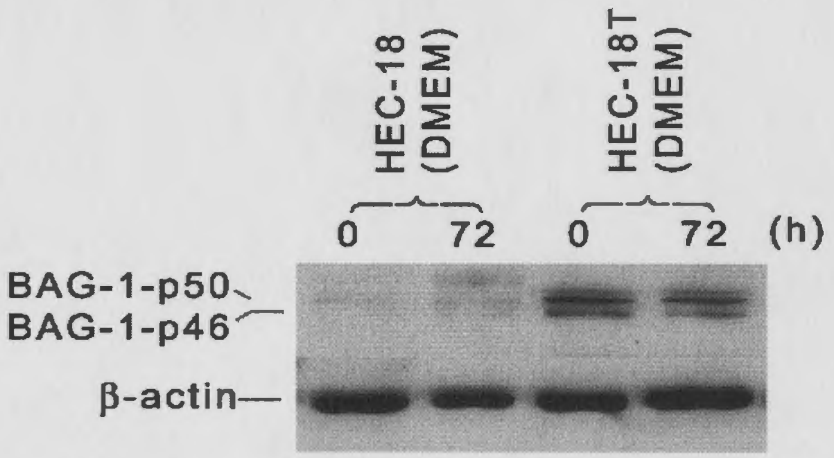
Figure 3.30 Expressions of BAG-1-p33 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-BAG-1 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. $^{**}P < 0.005$ is the statistical significance of the difference in expression of BAG-1-p33 between each 5-FU-treated cell line and control untreated cells.

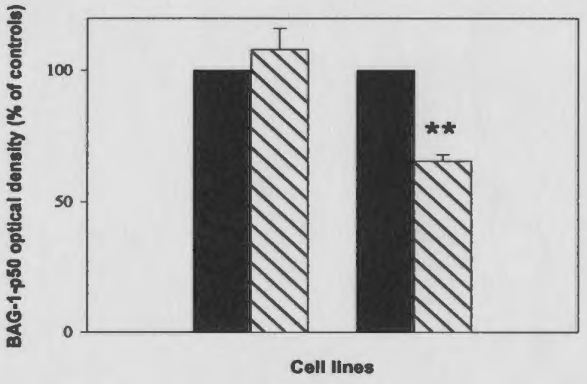

HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
BAG-1-p33—
β-actin—

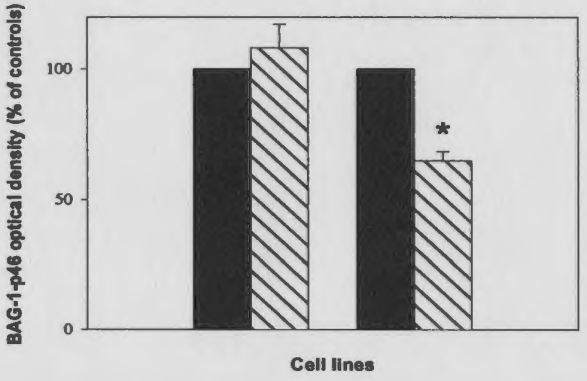

BAG-1-p33 optical density (% of controls)
100
50
0
**
Cell lines

** P<0.005

Figure 3.31 Expressions of BAG-1-p46 and p50 in human ectocervical cell lines detected by Western blot analysis after treatment with 5-FU. Each cell line is indicated on the top of the figure. 10 μg of protein from each cell-free extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane as described in Materials and Methods. Western-blots were probed with the mouse monoclonal IgG anti-BAG-1 antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Columns and bars represent mean values ± SD, respectively. *$P < 0.05$ and **$P < 0.005$ are the statistical significance of the difference in expression of BAG-1-p46 and p50 between each 5-FU-treated cell line and control untreated cells.


HEC-18 (DMEM)
HEC-18T (DMEM)
0
72
0
72
(h)
BAG-1-p50
BAG-1-p46
β-actin


BAG-1-p50 optical density (% of controls)
100
50
0
**
Cell lines


BAG-1-p46 optical density (% of controls)
100
50
0
*
Cell lines

* P<0.05 ** P<0.005

Table 3.4 Expressions of apoptosis-regulating proteins in HEC-18 and HEC-18T after treatment of 5-FU[1]

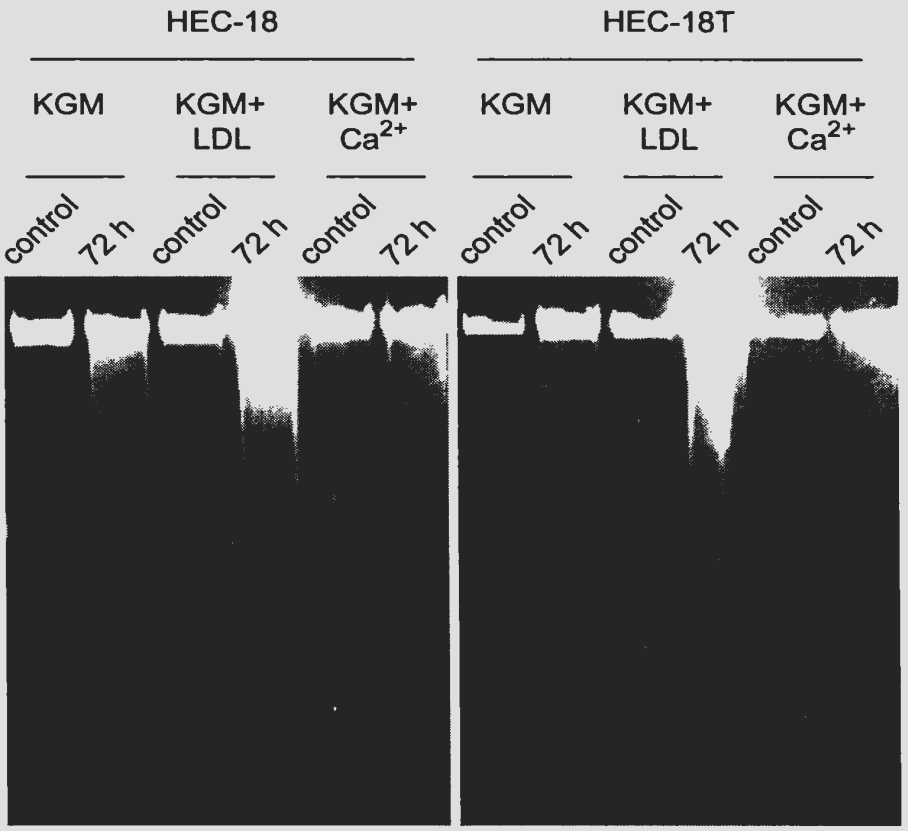
Proteins	HEC-18		HEC-18T	
	0 hr	72 hr	0 hr	72 hr
p53	100.0±0.0	128.2±4.5	100.0±0.0	171.7±2.1
p21	100.0±0.0	275.8±9.1	100.0±0.0	156.2±6.8
MDM-2	100.0±0.0	96.5±3.0	100.0±0.0	802.9±21.0
PCNA	100.0±0.0	121.2±4.8	100.0±0.0	219.4±2.8
Bax	100.0±0.0	101.8±3.8	100.0±0.0	99.0±1.0
Bak	100.0±0.0	117.5±1.3	100.0±0.0	181.5±9.4
Bcl-2	100.0±0.0	66.9±2.0	100.0±0.0	28.5±0.9
Bcl-x_L	100.0±0.0	105.1±2.0	100.0±0.0	120.3±6.0
BAG-1 p29	100.0±0.0	49.5±2.5	100.0±0.0	24.0±3.0
BAG-1 p33	100.0±0.0	97.8±6.1	100.0±0.0	81.8±0.5
BAG-1 p46	100.0±0.0	108.2±9.0	100.0±0.0	64.9±3.4
BAG-1 p50	100.0±0.0	108.0±8.0	100.0±0.0	65.5±2.5

[1] The levels of cellular proteins were quantified relative to those of the corresponding cell lines without 5-FU treatment after normalization to the actin control. Protein expression was quantified by measuring the optical density of bands at medium exposure on X-ray film. The data are shown as the mean ± the standard deviation of three experiments.

3.9. Effects of low-density lipoprotein (LDL) on 5-FU-induced apoptosis

It is well known that both high calcium concentration (McConkey *et al.*, 1990) and serum deprivation (Bryckaert *et al.*, 1999; Trimble *et al.*, 1999) are two nonphysiological conditions that induce apoptosis. Serum contains LDL, which delivers cholesterol to cells, especially to rapidly growing cancer cells. In studies reported in this thesis, the experimental data show that ectocervical cells were more sensitive to 5-FU-induced apoptosis in DMEM than in KGM. The major differences between these two media are calcium concentration and serum. DMEM contains a 10-fold higher calcium concentration than that in KGM. DMEM contains 10% fetal calf serum, and KGM is a serum-free medium. LDLs are a serum component and are of interest for carcinogenesis, because they provide the cancer cells with cholesterol, which is required for cell membranes during their faster growth compared with normal cells. For example, cervical cancer cells metabolize 50-fold more LDL than normal gynecological tissue (Gal *et al.*, 1981). The same requirement applies to faster proliferating cells in multistage carcinogenesis.

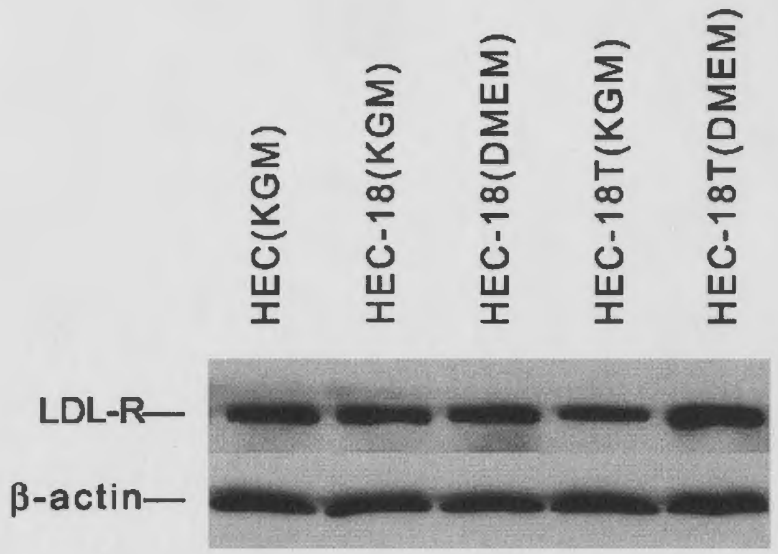
Figure 3.32 Agarose gel electrophoresis of DNA extracted from HEC-18 and HEC-18T treated with 5-FU for 72 hr. Each culture medium is indicated underneath the cell line. Cells (10^6) were cultured for 24 hr in indicated medium, then 5-FU was added and the incubation period continued for another 72 hr. Cells without 5-FU treatment are used as controls. DNA was extracted and analyzed by agarose gel electrophoresis in the presence of ethidium bromide, as described in Materials and Methods.


HEC-18
HEC-18T
KGM
KGM+ LDL
KGM+ Ca2+
KGM
KGM+ LDL
KGM+ Ca2+
control
72 h
control
72 h
control
72 h
control
72 h
control
72 h
control
72 h

To investigate whether calcium or the LDL pathway are involved in the differential susceptibility of ectocervical cells to 5-FU-induced apoptosis in the two media, apoptosis was examined in HEC-18 and HEC-18T cultured for 72 hr in KGM, KGM with added LDL, and KGM with the physiological level of calcium. Then, DNA fragmentation was analyzed (Figure 3.32). There was little difference in apoptotic DNA fragmentation in either HEC-18 or HEC-18T in KGM with or without added calcium, implying that calcium concentration does not play an important role in ectocervical cellular sensitivity to 5-FU-induced apoptosis. On the other hand, when cells were cultured in KGM containing LDL, the apoptotic effect of 5-FU was dramatically higher than those in KGM not containing LDL.

To examine whether the LDLR plays a role in multistage cervical carcinogenesis or the differential sensitivity in KGM and DMEM of the cells to apoptosis induced by 5-FU, the expression of LDLR on HEC, HEC-18, and HEC-18T was analyzed by Western blot (Figure 3.33). No differences were observed between either different cells or same cell line cultured in different media. Analysis by flow cytometry showed a same trend (Figure 3.34). These results suggest that the presence of LDL in serum is an important factor involved in the differential susceptibility in the two media of HEC-18 and HEC-18T to 5-FU-induced apoptosis.

Figure 3.33 Expressions of LDLR in human ectocervical cells. Western-blots were probed with the mouse monoclonal IgG_{2a} anti-LDLR antibody. The blots were also analyzed for expression of β-actin protein as controls for protein loading. The bottom graph represents the corresponding densitometry for results from Western blot analysis. Values represent the mean ± the standard deviation from three independent experiments.


HEC(KGM)
HEC-18(KGM)
HEC-18(DMEM)
HEC-18T(KGM)
HEC-18T(DMEM)
LDL-R
β-actin

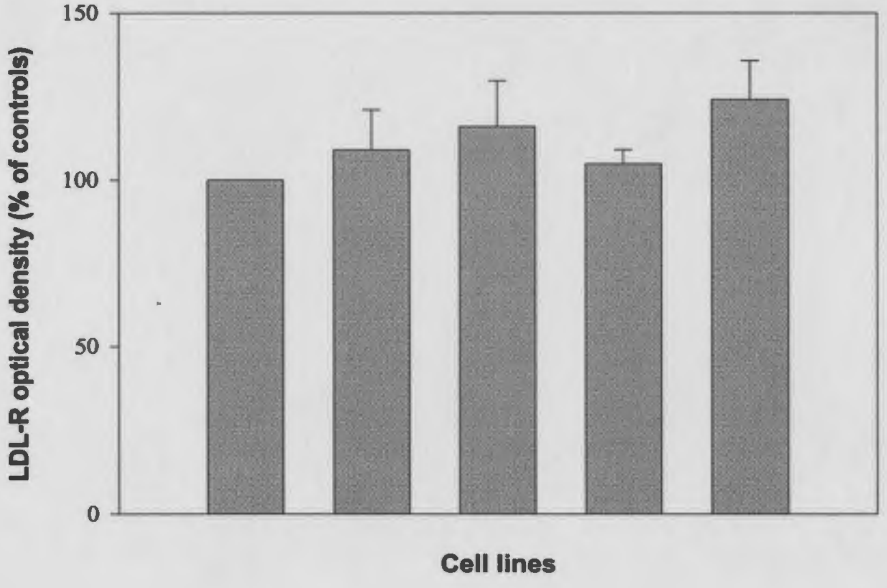
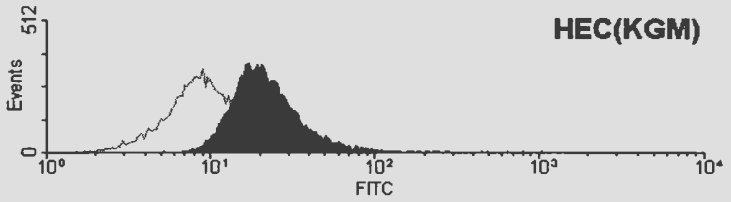
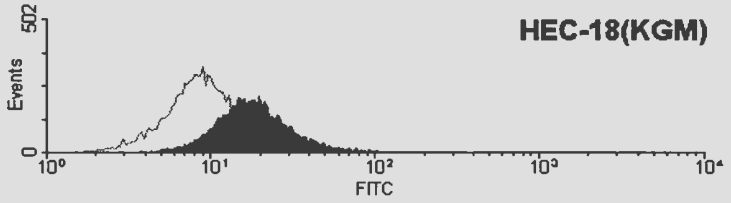
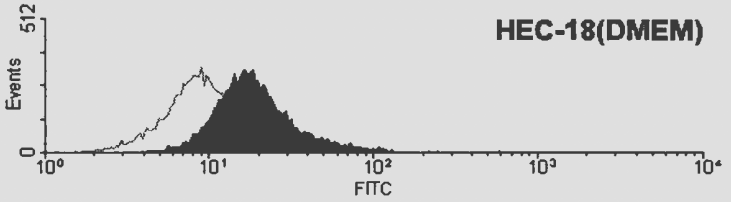
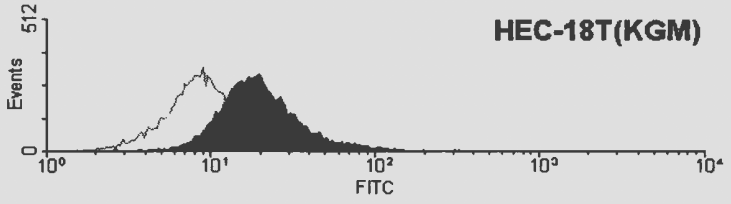

150
100
50
0
LDL-R optical density (% of controls)
Cell lines

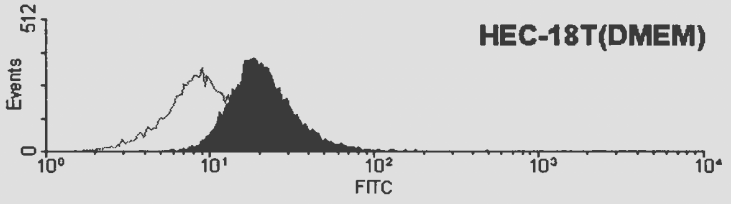
Figure 3.34 LDLR expressions in human ectocervical cells. Each cell line is indicated to the right of the figure. Flow cytometry analysis of cells stained with anti-LDLR antibody (black peaks) or negative control anti- IgG_{2a} (open peaks) followed by staining with a secondary FITC-conjugated antibody.


HEC(KGM)
512
Events
0
10⁰
10¹
10²
10³
10⁴
FITC


HEC-18(KGM)
502
Events
0
10⁰
10¹
10²
10³
10⁴
FITC


HEC-18(DMEM)
512
Events
0
10⁰
10¹
10²
10³
10⁴
FITC


HEC-18T(KGM)
512
Events
0
10⁰
10¹
10²
10³
10⁴
FITC


HEC-18T(DMEM)
512
Events
0
10⁰
10¹
10²
10³
10⁴
FITC

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS

4.1. Multistage cervical carcinogenesis and effects on growth *in vitro* and *in vivo*

Cervical carcinogenesis is considered to be a multistage process. To perform *in vitro* studies, a model system that mimics the *in vivo* system was required. In this study, a model system was chosen for the malignant progression of cervical cells consisting of HEC, HEC-18, and HEC-18T. HEC-18 was derived from HEC by transfection and immortalization by HPV 18 DNA (Yokoyama *et al.*, 1994). Northern blot analysis using HEC-18 DNA showed the expression of E6-E7 mRNA, which is known to be a template for E6 and E7 oncoprotein synthesis. HEC-18 exhibited a squamous phenotype. They formed monolayers of typical keratinocyte-like polygonal cells, which were similar to HEC in KGM (Figure 3.2 A). Some growth characteristics of HEC-18 were also tested, and the results indicated that there were no significant differences from those of HEC (Figure 3.4 and Table 3.1). HEC-18 can be readily subcultured, indicating that they are immortalized. However, HEC-18 did not display anchorage-independent growth assayed by soft agar, indicating an absence of full transformation (Table 3.1 and Figure 3.5). Furthermore, tumorigenicity of HEC-18 had been tested on nude mice. After 7-14 days, small nodules appeared at the site of injection, but they subsequently regressed

(Yokoyama *et al.*, 1994). Therefore, HEC-18 was nontumorigenic in nude mice. Because cervical carcinogenesis is a multistep process, HPV 18 may not be sufficient for the full neoplastic transformation of primary human ectocervical cells, although the immortalization by HPV 18 DNA remains a prerequisite to the development of tumorigenicity. The enhanced life span of HEC-18 would be expected to lead to a greater accumulation of genetic changes, and HPV E6 and E7 proteins are known to induce chromosomal instability. Consequently, HEC-18 is useful for the investigation of factors involved in early multistep progression of cervical cancer and of oncogenic events that follow HPV 18-immortalization.

Since HEC-18 was not tumorigenic in nude mice (Yokoyama *et al.*, 1994), to employ a full model system mimicking every major stage in multistep progression of cervical cancer, experiments herein used HEC-18T. It was established by our laboratory using CSC treatment on HEC-18 (Nakao *et al.*, 1996). The CSC treatment, representing cigarette smoking as a late-acting cofactor for cervical cancer, has been frequently employed in our laboratory group to develop malignantly transformed human cervical cell lines from cells immortalized by HPV 16 or HPV 18 (Yang *et al.*, 1996; Nakao *et al.*, 1996). Morphological studies of HEC-18T did not evidence significant differences from those for HEC and HEC-18 (Figure 3.1, Figure 3.2, and Figure3.3); neither did growth potential analysis (Figure 3.4). Northern blot analysis, Southern blot analysis and RT-PCR all indicated that malignant transformation in HEC-18T had

no effect on the physical state of HPV 18 and little effect on the gene expression of HEC-18T compared to HEC-18. However, HEC-18T reconstructed severe dysplasia / carcinoma *in situ* (CIS) in the organotypic (raft) culture system and produced invasive squamous cell carcinomas on nude mice (Nakao *et al.*, 1996). Furthermore, anchorage independence was observed in soft agar growth of HEC-18T (Table 3.1 and Figure 3.6). Therefore, an *in vitro* model system of HPV 18-related squamous cell carcinomas derived directly from the uterine ectocervix, consisting of normal (HEC), immortalized (HEC-18) and transformed (HEC-18T) cell lines, has been established and chosen to mimic multistep cervical carcinogenesis for further elaboration in this study.

4.2. Apoptosis in the cervical oncogenesis model system

Apoptosis became a central focal point of my experiments. It is a highly organized physiologic mechanism that kills abnormal cells, such as cancerous cells. Regulation of apoptosis is, therefore, an important defense against the emergence of cancer. In early carcinogenesis, target cell populations become predisposed to genetic lesions leading toward malignancy. Apoptotic destruction of predisposed cells may limit the number of cells available for malignant progression. This hypothesis may be applied to neoplasms known to be indolent, such as follicular lymphoma. The cancer typically acquires genetic defects that allow it to become more aggressive in later stage (Wyllie, 1992). Many changes

associated with malignant transformation, such as mutations, occur at important checkpoint controls or activate cell survival genes, such as Bcl-2. This allows cells to proliferate and survive when normal mechanisms would trigger the initiation of apoptosis (Dixon *et al.*, 1997).

An important part of these studies of cervical oncogenesis was to further examine the specific roles in apoptosis of cellular genes. Despite the myriad of apoptosis-inducing agents, the apoptotic pattern is conserved in diverse organisms. This conservation suggests that there may be a limited number of shared genes regulating apoptosis (Dixon *et al.*, 1997). Since the known genes all participate in regulating apoptosis, their expression and interactions have far reaching implications in the control and possible treatment of human cancers. However they are influenced by a multitude of cellular and environmental factors.

4.3. Effects of 5-FU on growth in the multistage cervical oncogenesis model

5-FU is an effective anticancer drug that is also used for cervical oncogenesis. To better understand the mechanism of 5-FU induced apoptosis and its role in multistage cervical carcinogenesis, in this study, the effects of 5-FU on growth have been investigated in our model system, HEC, HEC-18, and HEC-18T, for multistage cervical carcinogenesis.

In initial studies, HPV 18 DNA in both immortalized cell line HEC-18 and transformed cell line HEC-18T had dramatically decreased the response to apoptotic stimuli 5-FU (Figure 3.10 and Figure 3.12). Similarly, HPV 16 DNA in both immortalized cell line HEN-16 and transformed cell line HEN-16T has also been reported to reduce the response to various apoptotic stimuli (Yang *et al.*, 1998b).

Cell morphology can change by growing in a different medium (Nakao *et al.*, 1996; Yang *et al.*, 1996). This is also true in our cases when HEC, HEC-18, and HEC-18T (Figure 3.1, Figure 3.2, and Figure 3.3, respectively) were cultured in KGM or in DMEM. Additionally, transformed oral keratinocytes demonstrated enhanced growth potential when the medium was shifted from KGM to DMEM (Li *et al.*, 1992). Growth potential (Figure 3.4) and growth characteristics (Table 3.1) of HEC, HEC-18, and HEC-18T were different when the cells were grown in different media, either KGM or DMEM. These results encouraged experiments investigating the apoptotic effects of 5-FU on cell lines cultured in KGM, as well as in DMEM.

Cell morphology is one of the most common and reliable criteria for characterizing apoptotic cell death (Huschtscha *et al.*, 1996). Initially, cell morphological changes were compared before and after treatment with 5-FU. In KGM, apoptotic characteristics, such as cell shrinkage and surface blebbing, were observed after 5-FU treatment in all of three cell types (Figure 3.7). In DMEM, similar phenomena were observed in cell lines HEC-18 and HEC-18T

(Figure 3.8). Subsequently, flow cytometry analysis (Figure 3.9) confirmed the apoptotic effects of 5-FU. HEC was not tested when grown in DMEM, since they did not proliferate in this medium.

The strongest evidence, indicating the cell death caused by 5-FU treatment was apoptotic came from ladder-pattern DNA fragmentation. Since DNA fragmentation analysis can function as qualitative evaluation for apoptosis levels, cell lines cultured either in KGM (Figure 3.10) or in DMEM (Figure 3.11) were investigated after treatment with 5-FU for different times. In KGM, HEC showed significant apoptosis in a time-dependent manner, whereas HEC-18 and HEC-18T did not exhibit apparent DNA fragmentation until the longest 5-FU treatment period. This indicated that immortalized HEC-18 and transformed HEC-18T were at least resistant to 5-FU-induced apoptosis to some extent (Figure 3.10). However, surprisingly, the two cell lines were contrarily susceptible to 5-FU-induced apoptosis when they were cultured in DMEM (Figure 3.11). After comparing apoptosis levels in different cells grown in different media, susceptibility of HEC-18 and HEC-18T to 5-FU-induced apoptosis was caused by medium change from KGM to DMEM (Figure 3.12). Furthermore, the results from trypan blue exclusion (Figure 3.13) and MTT cytotoxicity assays (Table 3.2) were consistent with those from DNA fragmentation analysis regarding the sensitivity evaluation of cell lines to 5-FU-induced apoptosis.

Taken together, the data led to: (1) 5-FU induced apoptotic cell death in HEC, HEC-18, and HEC-18T. (2) HPV 18 immortalization and cigarette smoke

condensate transformation decreased the sensitivity of cell lines to 5-FU-induced apoptosis. (3) Medium change from KGM to DMEM increased the sensitivity of HEC-18 and HEC-18T to 5-FU-induced apoptosis.

4.4. Effects on apoptosis-regulating proteins by 5-FU in cervical oncogenesis model

Cellular events can be often rationally explained by molecular events. 5-FU is an anticancer drug that functions effectively in cancer prevention and treatment. 5-FU usually specifically affects the genetic and biochemical signals of apoptosis. However, the molecular basis involved in 5-FU-induced apoptosis on human cervical cancer cell lines had not been investigated. Therefore, examining the induction of apoptosis in multistage carcinogenesis was considered to be important for studying the mechanism in the current investigations and gene modulations were consequently examined before and after treatment with 5-FU.

To measure the correlation between the apoptosis sensitivity and medium change, a panel of apoptosis-regulating proteins was tested in HEC-18 and HEC-18T, first, without treatment with 5-FU. The relationship between 5-FU treatment and apoptosis-regulating proteins in ectocervical cells was examined and compared by Western blot analysis in HEC, HEC-18, and HEC-18T without treatment with 5-FU.

p53 is a phosphoprotein composed of 393 amino acids, and it resides in the nucleus of the cell. p53 is one of the tumor suppressors and it appears to occupy a pivotal role in deciding the fate of cells that have been stressed. Activation of p53 drives a series of events that culminate in either cell cycle arrest or apoptosis (Steele *et al.*, 1998). p53 protein level decreased in HPV 16-immortalized HEN and CSC-transformed HEN, which correlated with resistance of cell lines to apoptosis induced by DNA-damaging agents, staurosporine and cisplatin (Yang *et al.*, 1998b). It was reported that transfection of wild-type p53 gene enhanced the cytotoxicity of cisplatin and cisplatin-induced apoptosis in HeLa cells (Miragawa *et al.*, 1999). In this study, p53 level decreased remarkably in HEC-18 and HEC-18T, resulting in decreased sensitivity to 5-FU-induced apoptosis. It also increased remarkably in HEC-18 and HEC-18T when the medium was switched to DMEM, accounting for increased sensitivity to 5-FU-induced apoptosis (Figure 3.14).

Bcl-2 family consists of pro-apoptotic and anti-apoptotic genes and it is the balance in expression between these gene lineages that may determine the death or survival of a cell. Bak was isolated based on its sequence homology to Bcl-2 within the BH1 and BH2 regions and its ability to interact with the E1B 19K protein (Kiefer *et al.*, 1995). Bak is expressed widely, and in most cases, accelerates cell death. Therefore, it

is one of the pro-apoptotic genes in Bcl-2 family. A downregulation of Bak was reported to facilitate the accumulation of neoplastic cells in the early stages of colorectal tumorigenesis (Krajewski *et al.*, 1996). Our data showed that Bak level decreased greatly after immortalization and transformation by HPV 18, which correlated with the reduced sensitivity of HEC-18 and HEC-18T to 5-FU-induced apoptosis (Figure 3.15). However, Bak level did not change significantly when HEC-18 and HEC-18T were adapted to DMEM, implying that a Bak-independent mechanism might be involved in sensitivity to 5-FU-induced apoptosis change upon medium switch. The role of Bak in this process remains elusive.

Bax was the first Bcl-2-associated protein to be identified. It is homologous to Bcl-2 in sequence and coimmunoprecipitates with Bcl-2 in cell extracts and *in vitro* (Oltvai *et al.*, 1993). In functional assays, Bax suppresses the ability of Bcl-2 to block apoptosis. Bax knockout mice develop normally but display lymphoid hyperplasia consistent with a role for Bax in the promotion of apoptosis (Knudson *et al.*, 1995). It was shown that overexpression of Bax gene by transfection of Bax vector sensitized erythroleukemia cells to chemotherapeutic agent-induced apoptosis (Kobayashi *et al.*, 1998). Another study reported that Bax enhanced apoptosis in ovarian cancer cell lines that were transfected with Bax (Strobel *et al.*, 1996). Enhanced Bax expression was demonstrated to sensitize human breast cancer cells to radiation-induced apoptosis

(Wagener *et al.*, 1996). Consistently, our studies also showed the correlation between sensitivity to apoptosis and expression level of Bax. As shown in Figure 3.16, the down-regulation of Bax is associated with decreased sensitivity to 5-FU-induced apoptosis in HPV 18-immortalized and -transformed cell lines, while up-regulation is associated with increased sensitivity to apoptosis due to medium change.

The Bcl-2 gene was first discovered as part of the most common translocation in human B cell follicular lymphoma (Bakhshi *et al.*, 1985). Bcl-2 has been shown to enhance cell survival by inhibiting apoptosis induced under a wide variety of circumstances, suggesting that it is a ubiquitous inhibitor of cell death triggered by multiple routes (White, 1996). It was reported that dephosphorylation played an important role in anti-apoptosis potential of Bcl-2 (Haldar *et al.*, 1994). Overexpression of Bcl-2 delayed apoptosis induced by various treatments in a HeLa cell line in which the expression of Bcl-2 was controlled by the concentration of tetracycline (Yin *et al.*, 1995; 1996). In addition, transfection-enforced overexpression of Bcl-2 was demonstrated to inhibit chemotherapeutic agent-induced apoptosis and enhance clonogenic survival (Elliott *et al.*, 1998; Kobayashi *et al.*, 1998). More recently, another study showed that renal epithelial cells with overexpression of Bcl-2 were significantly less sensitive to cisplatin and S-(1,2-dichlorovinyl)-L-cysteine-induced apoptosis (Zhan *et al.*, 1999). Similarly, greatly enhanced expression of

Bcl-2 in HEC-18 and HEC-18T was associated with their resistance to 5-FU-induced apoptosis. On the other hand, reduced expression of Bcl-2 in HEC-18 and HEC-18T when grown in DMEM resulted in increased sensitivity to 5-FU-induced apoptosis (Figure 3.17).

Bcl-x gene has high homology to Bcl-2 and also functions to regulate apoptosis (Boise *et al.*, 1993). Bcl-x transcripts are alternatively spliced into long (L) and short (S) forms. The protein product of the long form, Bcl-x_L, resembles Bcl-2 and is also a potent inhibitor of apoptosis. The protein product of the short form, Bcl-x_S, antagonizes apoptosis inhibition by the Bcl-x_L and Bcl-2 (Boise *et al.*, 1993). Bcl-x_L was reported to block apoptosis induced by immunosuppressants in a B lymphoid cell line (Gottschalk *et al.*, 1994). Furthermore, it was suggested that IL-6 can upregulate expression of Bcl-x_L, and this may correlate with protection against apoptosis in murine myeloma cells (Schwarze *et al.*, 1995). Bcl-x_L expression in malignant plasma cells strongly correlated with decreased response rates in patient groups treated with melphalan and prednisone or vincristine, adriamycin, and dexamethasone. In addition, myeloma cells with overexpression of Bcl-x_L demonstrated a generalized resistance to apoptosis that was induced by several different agents (Tu *et al.*, 1998). However, the present study did not show any significant changes in the model system (Figure 3.18). Bcl-x_L does not seem to be involved in mechanisms regarding sensitivity changes to 5-FU-induced apoptosis

caused by HPV 18 immortalization, transformation or medium change. Nevertheless, it is generally accepted that the relative ratios of anti-apoptotic and pro-apoptotic members of the Bcl-2 protein family determine the ultimate sensitivity or resistance of cells to various stimuli or circumstances that trigger apoptosis. It was reported that Bcl-x_L to Bax ratio significantly correlated with chemosensitivity to 5-FU (Nita *et al.*, 1998). Combined with results shown in Figure 3.16, Bcl-x_L to Bax ratio displayed an increasing trend in HEC-18 and HEC-18T, correlating with decreased sensitivity to 5-FU-induced apoptosis, and a decreasing trend upon medium change, correlating with increased sensitivity to 5-FU-induced apoptosis.

BAG-1 is a novel anti-apoptotic protein identified as a Bcl-2-binding protein (Takayama *et al.*, 1995). It is a multi-isoform protein with four isoforms, which have molecular masses of 29, 33, 46, and 50 kDa, respectively. Although most Bcl-2-binding proteins contain BH domains, BAG-1 has no homology with Bcl-2 in its structure. Analysis of transfectants overexpressing BAG-1 has revealed that BAG-1 cooperates with Bcl-2 to prolong cellular survival against various apoptotic stimulations (Takayama *et al.*, 1995). In contrast to Bcl-2, only limited data are available so far as to the function of BAG-1. BAG-1 has been found to bind the receptors for steroid hormones and prevent their apoptosis-inducing function. Therefore, one mechanism through which

BAG-1 inhibits apoptosis might be regulation of hormone pathways (Kullmann *et al.*, 1998). As expected, the overall level of BAG-1 exhibited an increasing tendency in HEC-18 and HEC-18T compared to that in HEC, possibly accounting for their decreased sensitivity to 5-FU-induced apoptosis (Figure 3.19 and Figure 3.20). However, to our surprise, BAG-1 expression once again increased in HEC-18 and HEC-18T when cultured in DMEM. These results suggest that something more complicated occurred and may be due to BAG-1 functions other than anti-apoptosis.

4.5. Modulation of apoptosis-regulating proteins by 5-FU treatment

An important focus of this study on the effect of 5-FU was programmed cell death, commonly termed apoptosis. Apoptosis is regulated by a finely controlled equilibrium between anti-apoptotic genes (involved in inhibiting apoptosis) and pro-apoptotic genes (involved in triggering apoptosis). A wide variety of studies demonstrate that the sensitivity of cells to apoptosis usually can reflect by expression levels of anti-apoptotic genes, pro-apoptotic genes or ratio of anti- to pro-apoptotic genes. Our studies, as discussed above, also provide supporting evidence for these correlations. This is of special interest, since it is generally accepted that the sensitivity of cancer cells to apoptosis affects the sensitivity to killing by anticancer agents, i.e., the more sensitive the cancer cells are to apoptosis, the more sensitive they are likely to be to

anticancer agents. Because resistance to apoptosis is now thought to be a major problem limiting the utility of anticancer therapy, factors that can sensitize cancer cells to apoptosis would be expected to facilitate the development of effective anticancer therapy (St. Croix *et al.*, 1997). However, high level endogenous expression of pro-apoptotic genes and/or low level endogenous expression of anti-apoptotic genes are by themselves insufficient to initiate apoptosis, even though they exhibit correlations with cell sensitivity to apoptosis. Additional physiological or nonphysiological triggers are required to initiate programmed cell death. Anticancer drugs are common exogenous stimuli to induce apoptosis. It was, therefore, very important to understand whether anticancer drugs modulate apoptosis-associated genes and how these genetic modulations finally lead to apoptosis.

Induction of tumor suppressor gene p53 and inhibitor of cyclin-dependent kinases p21 played an important role. p21 is associated with phosphorylation of Bcl-2. Phosphorylation of Bcl-2 and the elevations of p21 lead to apoptosis (Wang *et al.*, 1999). It has become evident that apoptosis can be regulated through either p53-dependent or p53-independent pathways (Liebermann *et al.*, 1995). The tumor suppressor gene p53 has become a major player in the context of studying the molecular mechanisms of apoptosis was observed to suppress cell growth and induce apoptosis in several cell types (Shaw *et al.*, 1992).

Experimental evidence showed that MDM-2 and p21 were direct target genes of p53.

p21 was found in a quaternary complex that included not only cyclin and CDK, but also PCNA, to inhibit the kinase activity and result in apoptosis (Henderson *et al.*, 1994). The spectrum of known p53 target genes has then broadened to include Bcl-2 and Bax (Miyashita *et al.*, 1994). It was shown that Bcl-2 protein family interacts to form various homo- and hetero-dimers to either accelerate or inhibit apoptosis (Boise *et al.*, 1993). On the other hand, treatment of M1 cells with transforming growth factor β1 (TGFβ1) induced rapid growth arrest and apoptosis through a p53-independent pathway (Selvakumaran *et al.*, 1994). The comparison of the kinetics indicated that apoptosis through the p53-dependent pathway was more rapid than through p53-independent pathways. Ectopic expression of Bcl-2 at levels that block TGFβ1-induced apoptosis of M1 cells delayed, but did not block, p53-induced apoptosis. Moreover, both p53 and TGFβ1 down-regulated endogenous Bcl-2 expression, but only p53 up-regulated Bax expression. Thus, the p53-mediated up-regulation of Bax provides at least a partial explanation for the more rapid apoptosis induced by p53 compared to TGFβ1, as well as for the ineffectiveness of ectopic Bcl-2 to abrogate p53-mediated apoptosis (Selvakumaran *et al.*, 1994). Apoptosis through the p53-

dependent pathway has been receiving extensively increased interest (Meikrantz *et al.*, 1995).

As discussed before, our studies on expression of apoptosis-regulating proteins without 5-FU treatment suggested that the sensitivity of cells to apoptosis correlated with expression level of p53 (Figure 3.14), giving a strong implication that 5-FU-induced apoptosis in HEC, HEC-18, and HEC-18T is p53-dependent. To test this hypothesis, a range of apoptosis-regulating proteins, including p53 and some p53 target gene products, were examined after 5-FU treatment. Since immortalization by HPV 18 and transformation decreased the sensitivity of cells to 5-FU-induced apoptosis while medium change to DMEM increased the sensitivity, HEC-18 and HEC-18T grown in DMEM were a better system for studies on genetic modulation by 5-FU treatment.

Anticancer agents, such as 5-FU, etoposide, and adriamycin, have been reported to be capable of inducing p53-dependent apoptosis (Lowe *et al.*, 1993). Radiation was also demonstrated to induce p53 upregulation and result in apoptosis in testicular germ cancer cells (Burger *et al.*, 1998). Additionally, 5-FU increased p53 protein levels and cause programmed cell death in colonic adenoma cell lines (Piazza *et al.*, 1997). It was reported that 5-FU triggered p53-dependent apoptosis in oncogenically transformed mouse embryonic fibroblasts (Lowe *et al.*, 1993). Similarly, in the present study, p53 was upregulated significantly

by 5-FU treatment in HEC-18 and HEC-18T that were cultured in DMEM (Figure 3.21), supporting the idea that 5-FU induced apoptosis in cervical cells through a p53-dependent pathway.

The activated p53 protein shows sequence-specific DNA-binding activity. By binding to its target DNA sequence in upstream promoter regions, p53 transcriptionally activates a range of genes, including the p21 gene (Cox, 1997). p21 is thought to exert its effect by inhibiting CDKs that are required to drive the cell division cycle (Henderson *et al.*, 1994). Overexpression of p21 leads to apoptosis and has been shown to effectively suppress tumor growth *in vitro* and *in vivo* (Gartel *et al.*, 1996). Radiation has been reported to cause a p53-dependent upregulation of the p21 gene in testicular germ cell tumor lines with wild type p53, but not in those with mutant p53, and result in apoptosis (Burger *et al.*, 1998). In breast cancer cells, apoptosis was found induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Also, upregulation of p21 was observed in TPA-treated cells, implying the involvement of p21 in TPA-induced apoptosis (Li *et al.*, 1998). A prodrug of 5-FU, FUdR, was demonstrated to inhibit cell growth, which was associated with increased expression of p21 protein in human colon carcinoma cells (Huang *et al.*, 1998). Moreover, 5-FU treatment induced apoptosis in both intestinal and midcolonic crypts, and this has been shown to correlate with a prolonged, p53-dependent expression of p21 (Pritchard *et al.*, 1998). More recently,

a strict p53-dependence of mRNA up-regulation for p21 was reported following ionizing irradiation in mice (Bouvard *et al.*, 2000). Similarly, p21 up-regulation was observed in both HEC-18 and HEC-18T following 5-FU treatment (Figure 3.22). Therefore, our study showed that 5-FU treatment had a similar effect on p21 to that on p53 (Figure 3.21), implying an association of p53-dependent up-regulation of p21 with 5-FU-induced apoptosis.

p53 could function as a transcription factor, and MDM-2 gene is one of its targets (Barak *et al.*, 1993). On the other hand, the MDM-2 gene encodes a protein whose binding to p53 inhibits its transactivation function and negatively regulates its stability in cells (Kubbutat *et al.*, 1999). As a result, p53 increases MDM-2 expression whereas MDM-2 suppresses p53 activity, creating an autoregulatory feedback loop. Interestingly, MDM-2 has also been found to bind to Rb (Xiao *et al.*, 1995). It was reported that MDM-2-Rb interaction played an important role in regulating the stability and apoptotic function of p53, which could be achieved by Rb-MDM-2-p53 trimeric complex (Yap *et al.*, 1999). Upregulation of MDM-2 was found to be p53-dependent in irradiated mice (Bouvard *et al.*, 2000). The loss of MDM-2 allowed the unregulated p53 protein to continuously exert its growth-suppressing activity, which either led to a complete G1 arrest or induced p53-dependent apoptosis (de Rozieres *et al.*, 2000). In the present study, the expression level of MDM-

2 remained the same in HEC-18, and increased remarkably in the case of HEC-18T after 5-FU-treatment (Figure 3.23). This could be explained by the special relationship between MDM-2 and p53 based on an autoregulatory feedback loop. A distinct difference of MDM-2 level between HEC-18 and HEC-18T was observed by comparing them by Western blot analysis before 5-FU treatment (Figure 3.23). The MDM-2 level in HEC-18 was much higher than that in HEC-18T before 5-FU treatment. The level of MDM-2 did not change at all in HEC-18 after 5-FU treatment, probably because the endogenous MDM-2 in HEC-18 was sufficient to inhibit upregulated p53 by 5-FU treatment. On the other hand, MDM-2 was upregulated along with the regulation of p53 by 5-FU until its level was high enough to keep the equilibrium in the autoregulatory feedback loop.

Another p53 target gene, PCNA, plays an essential role in nucleic acid metabolism as a component of the replication and repair machinery (Kelman, 1997). PCNA is believed to interact with p21 in a quaternary complex with CDKs and cyclins, suppressing CDK functions (Henderson *et al.*, 1994). An elevation in PCNA expression was mediated by p53 protein, which was shown to activate transcription from the promoter of the PCNA gene (Morris *et al.*, 1996). UV irradiation was able to up-regulate PCNA expression (Zeng *et al.*, 1994). As expected, an up-regulation of PCNA expression was observed along with the up-regulation

of p53 by 5-FU treatment in both HEC-18 and HEC-18T, suggesting the possibility of PCNA's involvement in the mechanism of 5-FU-induced apoptosis in cervical cells (Figure 3.24).

Bax, another key gene transcriptionally activated by p53, functions as an apoptosis promoter (Steele *et al.*, 1998). Bax expression is induced in a variety of biologically and clinically relevant situations associated with apoptosis, including treatment with chemotherapeutic drugs or radiation. The induction of Bax expression following genotoxic stress has been attributed to p53, which binds to typical recognition elements located in the Bax gene promoter and directly transactivates this pro-apoptotic gene (Reed, 1998). The ability of radiation and chemotherapeutic drugs to increase Bax expression may explain why some types of tumors with high levels of Bax protein, such as low-grade non-Hodgkin's lymphomas and small-cell lung cancers, initially respond well to therapy (Kitada *et al.*, 1996). 5-FU and interferon-γ were reported to independently or additively upregulate Bax expression level and induce apoptosis in colorectal carcinoma cells (Koshiji *et al.*, 1997). The up-regulation of Bax was also proposed to be involved in the molecular mechanisms of TPA-induced apoptosis in breast cancer cells (Li *et al.*, 1998). Furthermore, a p53-dependent mRNA upregulation of Bax was detected in ionizing irradiated mice, and apoptosis was induced (Bouvard *et al.*, 2000). In the present study, there was no significant difference for Bax expression levels before

and after treatment with 5-FU in either HEC-18 or HEC-18T (Figure 3.25). It is generally accepted that ratios of anti-apoptotic to pro-apoptotic Bcl-2 family members determine ultimate sensitivity to cell death stimuli. Changes in expression of both Bcl-2 and Bax in breast cancer may provide an explanation for the seemingly paradoxical observation that lower levels of Bcl-2 have been associated with better prognostic features in some types of malignancies (Reed, 1998). A lower Bcl-2 to Bax ratio can be actually obtained by combining the results for Bcl-2 (Figure 3.27), indicating an expected effect from 5-FU-induced apoptosis.

Similar to Bax, Bak appears to antagonize Bcl-2 function, thus abrogating the ability of Bcl-2 to promote cell survival. A high level of Bcl-2 relative to Bak promotes survival; however, an excess of Bak relative to Bcl-2 results in apoptosis (Chittenden *et al.*, 1995). 5-FU was reported to remarkably increase Bak expression level in human colon cancer cells and induce apoptosis (Nita *et al.*, 1998). According to our data, 5-FU significantly upregulated the Bak expression level in HEC-18 and HEC-18T (Figure 3.26), suggesting that Bak was involved in the signaling transduction pathway for 5-FU-induced apoptosis.

As one of the most important apoptosis regulating genes, Bcl-2 has the ability to inhibit apoptosis. Antimicrotubule drugs were reported to induce apoptosis, which resulted from the inactivation of Bcl-2 function through phosphorylation (Wang *et al.*, 1999a). The induction of Bcl-2

phosphorylation is known followed by loss of its ability to form heterodimers with Bax. It was reported that there is a significant decrease in the formation of Bcl-2 / Bax heterodimers after induction of Bcl-2 phosphorylation, even though the level of Bax protein is increased in MCF-7 cells exposed to vinorelbine (Wang *et al.*, 1999b). It was shown that 5-FU induced apoptosis in colorectal carcinoma cells in a dose- and time-dependent manner, which was correlated with the down-regulation of Bcl-2 (Koshiji *et al.*, 1997). The tumor suppressor p53 is a negative regulator of Bcl-2 expression. The Bcl-2 promoter alone coupled to a reporter gene does not show regulation by p53, but a 195 bp segment of the 5' untranslated region of the Bcl-2 gene confers negative regulation by p53 (Miyashita *et al.*, 1994). We also found an inverse correlation between the expression of Bcl-2 (Figure 3.27) and p53 (Figure 3.21) upon 5-FU treatment. This result suggested that p53 down-regulates Bcl-2 expression. Both effects would be expected consequences from the treatment with anticancer agent 5-FU.

Similar to Bcl-2, Bcl-x_L was shown to confer resistance to apoptosis induction following exposure to apoptotic stimuli. Bcl-x_L and Bcl-2 are complementary in function as apoptosis inhibitors in mouse development (White, 1996). Recent results indicated that ischaemia-induced acute renal failure is associated with upregulation of anti-apoptotic Bcl-2 and Bcl-x_L in the damaged distal tubule (Gobé *et al.*, 1999). Ionizing radiation

has been demonstrated to induce apoptosis, which resulted from the downregulation of Bcl-2 and Bcl-x_L, in acute lymphoblastic leukemia cells (Findley *et al.*, 1997). Insight into the role of Bcl-x_L in regulating apoptosis was also obtained from Bcl-x deficient mice (Motoyama *et al.*, 1995). The Bcl-x knockout embryos displayed extensive apoptosis involving post-mitotic neurons of the developing brain, spinal cord, dorsal root ganglia, and hematopoietic cells in the liver. My results showed that 5-FU did not modulate Bcl-x_L expression level in either HEC-18 or HEC-18T (Figure 3.28). However, the involvement of Bcl-x_L in 5-FU-induced apoptosis cannot be excluded, since it could be explained by the decreasing trend of expression ratio of Bcl-x_L to pro-apoptotic Bak (McPake *et al.*, 1998; Figure 3.26). Further investigation is needed.

As an anti-apoptotic gene, BAG-1 was found overexpressed in tumor cells, such as breast cancer cells (Takayama *et al.*, 1998). Its anti-apoptotic function has been extensively studied (Adachi *et al.*, 1998). BAG-1 has been reported to have a general regulatory function in signal transduction pathways involved in cell survival (Kullmann *et al.*, 1998). The BAG-1 protein was recently identified as a chaperone cofactor of the constitutively expressed Hsc70 and the heat-inducible Hsp70 in mammalian cytosol and nucleus (Höhfeld *et al.*, 1997). Furthermore, gene transfer-mediated elevations of the level of BAG-1 isoforms cause a variety of cellular phenotypes, possibly through a modulation of

Hsc70/Hsp70 activity, including increased resistance to apoptosis, enhanced cell proliferation, and altered transcriptional activity of steroid hormone receptors (Lüders *et al.*, 2000). However, less is known about the status of BAG-1 during apoptosis and the effects of exogenous apoptosis inducers on BAG-1 expression. In the present study, it was first presented how an anticancer agent, 5-FU, would modulate BAG-1 expression during apoptosis in cervical cancer cell lines. In agreement with the fact that BAG-1 is an apoptosis-inhibiting gene, 5-FU down-regulated the expression level of BAG-1 in both HEC-18 and HEC-18T, favoring 5-FU-induced apoptosis (Figure 3.29, Figure 3.30, and Figure 3.31). However, the p33 isoform, but not other isoforms, was enhanced. Various levels of changes were observed in each BAG-1 isoform, indicating that they may have different functions in regulating apoptosis.

4.6. Effects of LDL on 5-FU-induced apoptosis

The observation reported herein that the response to 5-FU of cells in the model was medium-dependent may provide informative clues for chemotherapies with 5-FU. Two key differences in the media were Ca^{2+} level (Kasturi *et al.*, 1993) and presence of serum. Regarding the LDL in the serum, a number of cervical tumor cell lines were found to have higher LDL receptor activity than normal cells (Gal *et al.*, 1991). The reason for these observed differences described herein in different media thus

became the next focus. It was shown that reversal of 5-FU susceptibility is correlated with the expression level of apoptosis-regulating proteins upon medium change. However, the question about more detailed factors causing the reversal susceptibility still was required to be addressed by continuing study concerning the differences between KGM and DMEM.

DMEM contains Ca^{2+} with a concentration 10 times higher than that in KGM. Being a ubiquitous factor in cell biology, Ca^{2+} has been reported to have effects on the complex process of apoptosis (McConkey *et al.*, 1997). In fact, both increases and decreases in cellular Ca^{2+} levels have been shown to promote apoptotic cell death. In general, the prevailing view is that elevations in intracellular Ca^{2+} may be one of the key signals leading to the promotion of apoptosis (Mason, 1999). To examine whether Ca^{2+} plays a role in the reversal susceptibility to 5-FU-induced apoptosis, experiments were performed using KGM plus Ca^{2+} with the same concentration as in DMEM. When HEC-18 and HEC-18T were cultured in above-mentioned medium, there was no significant difference in apoptotic response observed after exposure to 5-FU for 72 hr (Figure 3.32). These results suggested that Ca^{2+} level difference in these two media was not involved in the differential susceptibility of cervical cells to 5-FU-induced apoptosis.

Another major difference between KGM and DMEM is serum. DMEM contains fetal calf serum, while KGM does not. Apparently, serum

deprivation is not the reason to change the sensitivity since DMEM contains serum and sensitizes cells to 5-FU-induced apoptosis. However, one of the principle components in serum, LDL (about 130 mg/dl), may have had some effect on 5-FU-induced apoptosis, considering its wide-accepted role as an effective vehicle for chemotherapeutic drugs (Gal *et al.*, 1981). LDLs are particles that enable intracellular uptake of drug, and are known to affect chemotherapeutic drug uptake (Wasan *et al.*, 1996).

To investigate the role of LDLs in the reversal susceptibility to 5-FU-induced apoptosis, KGM plus an appropriate amount of LDLs was employed as the culture medium to imitate this difference between KGM and DMEM. The apoptotic levels of HEC-18 and HEC-18T greatly increased when cells were subject to the KGM plus LDL medium after 5-FU treatment (Figure 3.32). Therefore, LDLs may play an important role in the sensitivity change of HEC-18 and HEC-18T to 5-FU-induced apoptosis. Since LDLs usually accelerate the delivery of chemotherapeutic drugs through LDLR pathway, the expression level of LDLR on the cell surfaces of each cell line was detected by Western blot analysis (Figure 3.33) and flow cytometric examination (Figure 3.34). Both results showed that LDLRs are comparably expressed on cell surfaces of all types of cells, suggesting that LDLR levels are not differential as part of the mechanism involved in 5-FU-induced apoptosis.

4.7. Future directions

This study involves investigations on apoptosis, and effects of anticancer agent 5-FU, in our model system for multistage cervical carcinogenesis and on correlations with molecular basis from mechanistic point of view. The results provide valuable information for the mechanisms involved in multistage cervical carcinogenesis, chemosensitivity of tumor cells, and anticancer agent-induced apoptosis. On the other hand, continuing study is always necessary for extensive understanding of a molecular biological process.

The following are additional experiments that could be done in the future:

(1) To further understand the role of apoptosis in cervical carcinogenesis, the genetic modulations of E6 and E7 oncogenes and their targets in HEC-18 and HEC-18T could be analyzed before and after 5-FU treatment.

(2) To confirm the correlations of apoptosis-regulating protein expression with cellular sensitivity to 5-FU-induced apoptosis, overexpressions of concluded proteins could be achieved by transfecting expression vectors for their genes and examining the result and sensitivity to 5-FU-induced apoptosis.

(3) To supply more useful clues for chemotherapy of cervical cancer, synergistic effects of 5-FU with cytokines, such as TNF-α, IL-1α, and INF-γ, and growth factors, such as EGF and TGF-α, could be measured.

(4) To investigate the resistance mechanism of cervical cancer cells to anticancer drug-induced apoptosis, 5-FU resistant cell lines could be established and the effects could be examined on apoptosis-regulating genes in experiments parallel to those of this thesis.

(5) To obtain further insight into the role of the LDLR pathway in 5-FU-induced apoptosis, LDL-5-FU complexes could be prepared and employed on our model system for apoptotic studies.

(6) To link theoretic studies with clinic applications, 5-FU-induced apoptosis and expression of apoptosis-regulating proteins could be examined in *in vivo* systems, such as samples from normal and cervical cancer patients or mice.

(7) To study why BAG-1 expression was increased in HEC-18 and HEC-18T when cultured in DMEM, other BAG-1 functions that irrelated to apoptosis could be examined.

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