MARJO SEPPÄNEN

Cytokines and Gynecological Cancer

In Vitro Effects of Cytokines on Ovarian, Endometrial and Vulvar Carcinoma Cells

University of Tampere
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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of
Building K, Medical School of the University of Tampere,
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on August 11th, 2000, at 12 o'clock.

University of Tampere
Tampere 2000

to Petri

CONTENTS

ABBREVIATIONS

ADU	arbitrary densitometric units
AP-1	activating protein 1
ATF	activating transcription factor
cAMP	cyclic adenosine monophosphate
CREBs	cAMP response element binding proteins
CTRL	control
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ER	estrogen receptor
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-etanesulphonic acid])
HPV	human papillomavirus
HRT	hormone replacement therapy
IFN-α	interferon alpha
IFN-β	interferon beta
IFN-γ	interferon gamma
IFN-δ	interferon delta
IFN-φ	interferon omega
IFN-τ	interferon tau
IL	interleukin
IκB	inhibitor of NF-κB
KOH	potassium hydroxide
mRNA	messenger ribonucleid acid
NF-κB	nuclear factor kappaB
NK-cells	natural killer-cells
PMSF	phenylmethylsulphonyl fluoride

PR	progesterone receptor
RH	Rel Homology
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
TNF-R	tumor necrosis factor-receptor
TRAIL	TNF-related-apoptosis-inducing-ligand

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original communications, referred to in text by their Roman numerals (I-V)

I Vihko KK, Seppänen M, Henttinen T, Punnonen J, Grenman S, Punnonen R (1997): Regulation of proliferation of UM-SCV-1A and UM-SCV-6 vulvar carcinoma cell by cytokines. Cancer Immunology Immunotherapy 43: 368-374.

II Seppänen M, Henttinen T, Lin L, Punnonen J, Grenman S, Punnonen R, Vihko KK (1998): Inhibitory effects of cytokines on ovarian and endometrial carcinoma cells in vitro with special reference to induction of specific transcriptional regulators. Oncology Research 10: 575-589.

III Seppänen M, Lin L, Punnonen J, Grenman S, Punnonen R and Vihko KK (2000): Regulation of UT-OC-3 ovarian carcinoma cells by cytokines: Inhibitory effects on cell proliferation and activation of transcription factors AP-1 and NF-κB. European Journal of Endocrinology 142:393-401

IV Seppänen M & Vihko KK: Activation of transcription factor NF-κB by growth-inhibitory cytokines in vulvar carcinoma cells. Immunology Letters (in press)

V Saarinen R, Lin L, Seppänen M, Grénman S, Punnonen R and Vihko KK (2000): Regulation of ovarian carcinoma cells by cytokines: effects on cell proliferation, activation of transcription factor AP-1 and programmed cell death. European Journal of Endocrinology (in press)

INTRODUCTON

Cytokines are multifunctional signaling peptide molecules which regulate numerous activities in the cells of the immune system, the haemopoietic system and of non-immune cells. An individual cytokine is able to mediate multiple biological activities within a given cell type. It has become apparent that cytokines, in addition to functioning as immunomodulatory proteins, also serve as paracrine, autocrine, and endocrine factors and modulate a number of cell functions ranging from proliferation to differentiation (Wadler and Schwatz 1994; Peschel and Aulitzky 1994).

Numerous investigations have demonstrated an important role of cytokines in the regulation of cell proliferation in malignant cells. It has been found that cytokines are able to interact with malignant cell growth either by direct inhibition of tumor cells or by inducing defense mechanisms of the immune system (Itri 1992; Miller et al. 1994). Beneficial growth-inhibitory effects of cytokines have also been observed in malignant cells originating from the female genital tract (Powell et al. 1993). In the case of ovarian cancer especially it has been suggested that the cells of immune system are important for the defense mechanisms against malignant cells, and several cytokines and growth factors have been shown to inhibit the proliferation of ovarian cancer cell lines (Jozan et al. 1992; Kost et al. 1996; Powell et al. 1993). However, the involvement of cytokines as growth-regulators in endometrial and vulvar carcinoma cells is relatively little understood.

Although there are some studies of cytokine-mediated growth regulation in gynecological carcinoma cells, the mechanisms by which cytokines regulate cell growth are poorly understood. AP-1 and NF-κB are transcription factors which have been shown to be involved in the regulation of cell proliferation and differentiation of several cell types (Angel and Karin 1991; Dejardin et al. 1999). It has been observed *e.g*. that TNF-α is able to inhibit cell proliferation and induce NF-κB in human myelomonoblastic leukemia cells (Chaturvedi et al. 1994), and that AP-1 is required for mitogenic as well as antimitogenic responses to TNF-α (Brach et al. 1993; Dixit et al. 1989). However, the knowledge of a connection between growth-inhibitory cytokines and transcription factors AP-1 and NF-κB in gynecological carcinoma cells is still in an early stage.

Programmed cell death (apoptosis) is a widely studied phenomenon and many hormone- and growth-factor responsive tissues and organs are subject to apoptosis upon addition or removal of the appropriate regulatory factor. Apoptosis is known to occur in neoplastic cells either spontaneously or in response to extracellular stimulus *e.g.* in colorectal carcinoma (Koshiji et al. 1998), squamous cell carcinoma (van Hogerlinden et al. 1999) and breast cancer (Sovak et al. 1997). Cytokines, *e.g.* TGF-β, IFN-γ and members of TNF-family have been found to be important mediators of apoptosis (Burke et al. 1997, Krupitza et al. 1998, Mathieu et al. 1995, Walczak and Krammer 2000).

Especially in the case of ovarian cancer, there is a need to develop new treatment methods, since surgical and combination chemotherapy does not provide cures in about half of the cases. Increased understanding of the molecular and cellular mechanisms underlying the effect of cytokines is essential for new strategies that aid in the treatment of gynecological malignancies. In addition to *in vitro* studies, there are some reports of clinical trials showing that the administration of certain cytokines has resulted in objective clinical responses in patients with gynecological malignancy (Pujade-Lauraine et al. 1996, Vanderkwaak and Alvarez 1999). The present study was carried out to investigate the effects of several cytokines on DNA synthesis of ovarian, endometrial and vulvar carcinoma cells *in vitro.* In order to better understand the molecular mechanisms of the observed growth-regulatory effects, the activation of transcription AP-1 and NF-κB by cytokines was studied. In addition, cellular DNA was extracted to study if any of these growth-inhibitory factors is capable of inducing apoptosis.

REVIEW TO THE LITERATURE

1 GYNECOLOGICAL MALIGNANCIES

1.1 *Ovarian carcinoma*

The ovary has the widest spectrum of neoplasia compared with any organ in the body. The most commonly used classification of ovarian tumors was defined by WHO in 1973 (Serov et al. 1973). This classification divides the primary tumors into epithelial, sex cord-gonadal and germ cell type. The epithelial tumors have subsequently been renamed *surface epithelial-stromal tumors*. Epithelial ovarian carcinoma account for 85-90 percent of all ovarian cancers (Weiss et al. 1977, Finnish Cancer Registry 1997).

Ovarian cancer is the most lethal form of the gynecological tumors and can occur in women of all ages, although there are differences in the histologic types during decades of life (Merino and Jaffe 1993). In Finland, the cancer of the ovary is the fourth most common cancer in women and has an incidence of 10.2 per 100 000 persons per year (Finnish Cancer Registry 1997). Although ovarian neoplasms may occur at any age, including infants and children, in young women the predominant types of ovarian lesions are benign or borderline type. High-grade aggressive carcinomas, on the other hand, are more frequently seen in elderly postmenopausal women (Merino and Jaffe 1993). A major cause of the high mortality rate from ovarian cancer is the late presentation of the disease. These neoplasms remain asymptomatic until massive ovarian enlargement causes compression of pelvic structures, ascites, abdominal distention or distant metastasis. At the time of diagnosis, two-thirds of the patients have stage III and IV disease, 25% stage I and about 15% stage II disease (Annual Report 1998).

Several factors have been implicated in the etiology of ovarian cancer, including a genetic predisposition, reproductive history, smoking, infections with mumps virus, and dietary factors (Piver 1987). However, the main risk factors for ovarian cancer are considered to be reproductive, that is high number of ovulations during the lifespan and nulliparity. The repeated proliferation of the epithelial cells of the ovary may increase the likehood of a genetic alteration that could contribute to the oncogene overexpression or inactivation of a suppressor gene (Berek and

Martinez-Maza 1994). The use of oral contraceptives protects against the development of ovarian cancer (Fathalla 1971, Whittemore 1994).

Chemotherapy either alone or in combination with platinum analogues plays a major role in the treatment of epithelial ovarian carcinoma although surgery is the mainstay of both the diagnosis and the treatment. Surgery alone is appropriate only in stage I disease. Most other cases receive chemotherapy, whereas radiotherapy is only rarely used in the primary treatment (Andersen et al. 1992, Herrin and Thigpen 1999)

1.2 *Endometrial carcinoma*

Endometrial carcinoma is the most common invasive malignancy of the female genital tract. In Finland, 655 new cancer cases are diagnosed every year (Finnish Cancer Registry 1997). Endometrial carcinoma is a tumor of elderly women and therefore its impact will increase as the population grows older.

The role of unopposed estrogens in endometrial carcinogenesis is well established. Estrogens stimulate mitotic activity of the endometrial cells, while progesterone reduces this activity. Therefore any factor that increases the exposure of the endometrium to unopposed estrogens such as hormone replacement therapy (HRT), obesity and irregular menstrual cycles tends to raise the risk. On the other hand, factors that lower exposure to estrogens or increase progesterone levels, such as oral contraceptives and smoking, decrease the risk (Parazzini et al. 1991, Villard et al. 1990).

Surgical treatment with or without radiation therapy is the established treatment modality in early-stage disease. Radiation therapy alone or in combination with chemotherapy, or hormonal treatmment, is used in more advanced cases (Quinn et al. 1992).

1.3 *Vulvar carcinoma*

Carcinoma of the vulva is a relatively rare malignancy with an incidence of 1 to 2 cases per 100 000 women (Crum 1992). Vulvar cancer is generally regarded as a disease of older women although

there appears to be a trend in patient population toward younger women presenting with squamous carcinoma of the vulva (Messing et al. 1995).

The etiology of vulvar carcinoma is largely unknown. It has been suggested that smoking is a strong risk for vulvar carcinoma (Brinton et al. 1990). Human papillomavirus (HPV) infection has been implicated by some studies as one of the risk factors associated with this disease. There appear to be two different types of vulvar cancer: keratinizing squamous cell carcinoma, the classic morphological type, which is rarely associated with human papillomavirus infection and is found in older women. Another type is the warty and basaloid carcinomas which usually are HPV related, found in younger women and are often, associated with HPV and vulvar intraepithelial neoplasia (Crum 1992, Hietanen et al. 1995a, Kaufman 1995).

Recently the interest in etiogical factors has been focused on the association between mutations of cellular oncogenes and tumor suppressor genes and the pathogenesis of vulvar cancer. The role of p53 in the genesis of vulvar cancer is not clear. Wild-type p53 is known to negatively regulate cell growth and division and is classified as a tumor suppressor gene. Mutation of p53 is frequently found in vulvar carcinoma cells (Hietanen et al. 1995b).

The surgical treatment varies from local excision to radical vulvectomy and bilateral inguinal and iliacal lymphadenectomy. Radiation therapy alone or in combination with chemotherapy is used in more advanced cases (Soutter 1992).

2 CYTOKINES

Cytokines, in addition to functioning as immunoregulatory proteins, also serve as paracrine, autocrine and endocrine factors in a variety of cell types in a non-specific manner. Cytokines and their receptors have been found to be expressed in human reproductive organs. Human endometrium has been found to express cytokines and their receptors both in benign and malignant situations. Cytokines may be derived from stromal cells, leukocytic cells in the stroma or from the endometrial epithelial cells (Dumont et al. 1995, Garcia et al. 1994, Gold et al. 1994, Giudice 1994, Tabibzadeh 1991, Van Le et al. 1991). In addition, it has been observed that in the case of advanced endometrial cancer, serum levels of cytokines have been elevated (Chopra et al. 1997). The function of the ovary is also under the influence of cytokines. Proteins, mRNA and receptors of cytokines have been localized *e.g.* in theca and granulosa cells, follicular fluid and in the corpus luteum of the normal ovary (Calogero et al. 1998, Srivastava et al. 1996, Tabibzadeh 1994). In a case of ovarian cancer cytokines have been found within solid tumor implants and ascites fluid (Erroi 1989, Jozan et al. 1992, Moradi 1993).

There are only few studies on the existence and regulatory role of cytokines in the vulvar area. It has been found that in vulvar vestibulitis, the levels of inflammatory cytokines are elevated in certain regions of the vulva (Foster and Hasday 1997). In addition, EGF has been found to inhibit cell proliferation in squamous cell carcinoma of the vulva *in vitro* (Bollag et al. 1992). The antitumor effect of locally injected IL-2 in bovine vulvar carcinoma has been observed (Hill et al. 1994). Clinical observations suggest the existence of interaction between the immune system and vulvar carcinoma. For example, abnormalities in lymphocyte function in patients suffering from vulvar carcinoma have been observed (Seski et al. 1978).

2.1 *Interleukins 6, 10 and 13*

Interleukin 6 is produced by a variety of cells including monocytes, lymphocytes and epithelial cells. It is a co-stimulator of T-cell immunity and induces antibody secretion by B cells. In addition, by the inducing of acute phase proteins it is involved in the control of infectious diseases and inflammation (Aulitzky et al. 1994). IL-6 exerts its action via a cell surface receptor complex consisting of an 80 kDa IL-6 binding protein (gp80) and a 130 kDa glycoprotein (gp130) involved in signal transduction. The measurable levels of IL-6 and its receptor have been found to be

expressed in the ovary (Buscher et al. 1999, Calogero et al. 1998, Keck et al. 1998) and the endometrium (Nasu et al. 1999, Vandermolen and Gu 1996, Yoshioka et al. 1999). In addition, IL-6 levels in the peritoneal fluids of patients with ovarian cancer have been found to be significantly higher than those with cervical or endometrial carcinoma (Punnonen et al. 1998).

There are several observations of the antiproliferative effect of IL-6. The antitumor effects of IL-6 against sarcoma and adenocarcinoma of the colon in mice have been demonstrated *in vivo* although it is unclear to what extent these effects are due to immune or inflammatory reactions and how much is caused by direct effect on tumor cells (Mulé et al. 1990). *In vitro* growth-inhibitory effect of IL-6 has been observed in human breast carcinoma cells (Chen et al. 1991) and normal endometrial stromal cells in menstrual cycle-specific fashion (Yoshioka et al. 1999, Zarmakoupis et al. 1995). The effect of IL-6 on the cell proliferation of ovarian cancer cells is not consistent: Wu et al. (1992) found that IL-6 is capable of stimulating ovarian cancer cell growth while Chen et al. (1991) have observed growth-inhibition of ovarian cancer cells by IL-6. Watson et al. (1990) were not able to find any effect of IL-6 on cellular proliferation of ovarian cancer cell lines.

Interleukin-10 is produced by a variety of cells including polymorphonuclear cells, B and T lymphocytes and monocytes. IL-10 is able to inhibit various immune functions such as cytokine production in various cell types (Howard and O´Garra 1992). The biological effects of IL-10 are mediated by interaction with cell-surface receptor, a molecular size of 40-120 kDa (Ho and Moore 1994). Expression of IL-10 in endometrium (Deniz et al. 1996, Stewart-Akers et al. 1998) and ovary (Moncayo et al. 1998, Pisa et al. 1992) has been observed.

Expression of IL-10 together with IL-6 in human carcinoma cell lines has been demonstrated, suggesting a role of these cytokines in anti-tumor immunity, although the exact biological relevance of IL-10 secretion by carcinoma cells remains unknown (Gastl et al. 1993, Rabinowich et al. 1996). The anti-tumor effect of IL-10, probably mediated by T or NK-cells, has been demonstrated *in vivo* in murine tumor models (Berman et al. 1996). In human melanoma cells, IL-10 caused antitumor and antimetastatic activity by inhibiting angiogenesis *in vivo* (Huang et al. 1999). IL-10 has been found also to have *in vitro* growth-inhibitory effect on leukemia cells (Iversen et al. 1997).

Interleukin 13 is T-cell-derived cytokine that exhibits both immunomodulatory and anti-inflammatory properties (Minty et al. 1993). IL-13 receptor is thought to consist of four different forms composed of different subunits (Jensen 2000). Type I and II receptors have identified in ovarian carcinoma (Murata et al. 1997a, Murata et al. 1997b). In addition, the presence of mRNA for IL-13 has been found in ovarian endometrioma (Odukoya et al. 1997). There are only few studies of the anti-tumor effects of IL-13. *In vitro* antiproliferative effect of IL-13 has been observed in breast cancer cells (Serve et al. 1996) and colorectal carcinoma cells (Schnyder et al. 1996). Transfection of tumor cells by IL-13 has been shown to prevent tumor growth in mice by stimulating the sensitization of specific memory T lymphocytes and by improving antigenic presentation of tumor membranes (Lebel-Binay et al. 1995). In addition, IL-13 has the capacity to induce a cytolytic activity in spleen cells from tumor-bearing rats (Reisser et al. 1996).

2.2 *IFN-α and IFN-γ*

IFNs are a multigene family of proteins produced by almost every kind of cells in response to a variety of stimuli. IFNs have an important role in defense against viral diseases but they also have immunoregulatory and antiproliferative properties. IFN-α, IFN-β, IFN-δ, IFN-φ and IFN-τ are classified as Type I interferons (Robets et al. 1998). They share a high degree of amino acid similarity and many of their biological effects are similar. IFN-α is a multigene product made primarily by lymphocytes and macrophages, whereas IFN-β is a single gene product expressed by fibroblasts, macrophages and epithelial cells. IFN-γ (Type 2 interferon) is unrelated to type 1 interferons in terms of structure and function. Two types (type I and II) of interferon receptors have been identified (Petska 1997). The type I (IFN-alpha/beta) receptor appears to consist of two chains, IFN-alphaR1 and IFN-alphaR2, which may be present in different forms. The receptor for human IFN-γ consists of two transmembrane chains, IFN-gammaR1 and IFN-gammaR2, both of which are required for activity. It shows little homology with any other receptor and is believed to be expressed by most cell types. IFN-γ is a single gene product that is synthesized primarily by T lymphocytes and, in some conditions, by natural killer cells. (Clemens and McNurlan 1985). Interferons produced by leukocytes have been found in ovarian cells (Odukoya et al. 1997, Pisa et al. 1992) and endometrial cells (Deniz et al. 1996).

The antiproliferative mechanisms of interferons have been the focus of the extensive study. For example, proliferation of normal ovarian epithelial cells has been observed to remain unaffected by

IFN-α or IFN-γ, whereas proliferation of ovarian carcinoma cells was reduced by both types of IFN (Marth et al. 1996). One mechanism by which interferons are able to inhibit cellular growth is effecting the phases of the cell cycle. In the case of fibroblasts and melanoma cells, interferons inhibit transition from G_0 to S phase. In some cases, all phases of the cell cycle appear to be prolonged in response to interferons. Interferon treatment of certain cells has been found to result in impairment of DNA polymerase activity, DNA ligation and degradation of newly replicated DNA. Furthermore, interferons are suggested to have a role in the inhibition of protein synthesis. (Clemens and McNurlan 1985).

In addition to functioning as cytostatic factors, interferons are able to induce cell death. In the case of ovarian and cervical carcinoma, IFN-α and IFN-γ have been found to inhibit cell growth by lysing cells when protein synthesis is inhibited beforehand (Massad et al. 1990, Powell et al. 1993a). The cytolytic activity of IFN-α is mediated through a signaling mechanism involving protein kinase C (Powell et al. 1993b). In addition, IFN-γ has been observed to induce apoptosis *e.g.* in colorectal carcinoma (Koshiji et al. 1997) and ovarian carcinoma cells (Burke et al. 1997). Moreover, IFN-β and IFN-α are able to enhance the apoptotic effect of IFN-γ in gastric adenocarcinoma cells (Nagao et al. 1997).

Numerous studies have shown that ovarian carcinoma cells are sensitive to the antiproliferative effect of IFNs *in vitro*, while in clinical trials only modest response rates have been reported (Pujade-Lauraine et al. 1996, Welander 1987). However, IFN in combination with cytotoxic agents may have a potential efficacy against ovarian cancer (Mäenpää et al. 1994, Wadler 1992, Windbichler et al. 2000). In addition to cytotoxic agents, the combination therapy *e.g.* with 13-cis-retinoid acid plus interferon-α achieved a major response in metastatic endometrial adenocarcinoma (Kudelka et al. 1993). In the case of hairy cell leukemia and AIDS-related Kaposi sarcoma, IFN-α has been approved for the treatment. In addition, IFN-α has an effect against melanoma and renal cell carcinoma *in vivo* (Wadler 1992).

IFN-γ has been found to inhibit the growth of ovarian carcinoma cells (Burke et al. 1999) and endometrial epithelial cells (Tabibzadeh 1994). In addition, the growth-regulatory role of IFNs is connected to interactions with steroid hormones. Both IFN-α and IFN-γ are able to increase the level of progesterone receptors of endometrial cancer cells (Angioli et al. 1993, Scambia et al.

1991). IFNs may in future have a role in the treatment of endometrial cancer since with high progesterone receptor level, endometrial cancer cells respond better to progesterone therapy.

2.3 *GM-CSF*

The granulocyte-macrophage colony-stimulating factor belongs to a group of molecules that regulate hematopoietic cell proliferation, differentiation and function. GM-CSF can be produced by many cell types including T-lymphocytes, monocytes, endothelial cells and fibroblasts. Two proteins have been described that serve as the receptors for this cytokine: one an α chain of 84 kDa and one a β chain of 130 kDa (Chiba et al. 1990). The main effect of GM-CSF is to increase neutrophil and monocyte production, and to prolong neutrophil survival (Steward 1993, Quesenberry et al. 1991). The therapeutic use of GM-CSF in oncology is to reduce the duration of severe neutropenia following conventional chemotherapy or high-dose chemotherapy with bone marrow transplantation (Costello 1993).

In addition to cells of hematopoietic origin, GM-CSF receptors are also expressed by other types of cells and solid tumors (Chiba et al. 1990). Expression of GM-CSF has been found in the endometrium (Deniz et al. 1996) and the ovary (Calagero et al. 1998, Pisa et al. 1992). The ability of GM-CSF to regulate cell growth in nonhematopoietic cells has been under investigation. There are a few studies showing that GM-CSF might exert antitumor activity against solid tumor cells. According to Salmon and Liu (1989), GM-CSF has some anti-proliferative efficacy against ovarian and breast cancer cells, while no effect of GM-CSF on most nonhematopoietic neoplams was observed. In contrast to these observations, an increase in the growth of endometrial and cervical carcinoma cells by GM-CSF has been observed by Herzog *et al*. (1996).

4.4 *TGF-β*

The transforming growth factor β is actually a family of peptide growth factors: three isoforms (TGF-β_1, TGF-β_2 and TGF-β_3) have been detected in humans, each encoded by a separate gene. TGF-β_1 is the best characterized isoform and displays a wide range of biological activities. The biological activities of TGF-β are mediated through binding to the high affinity cell surface receptors. Most cells contain two types of receptors, designed type I (65-70 kDa) and type II (85-110 kDa). The type III receptor (200-300 kDa) is a transmembrane proteoglycan that has been

thought to enhance binding of TGF-β to the type II receptor. Type IV receptor is limited to pituitary cells. (Derynck 1994, Dumont et al. 1995, Tabibzadeh 1994). Types I and II have been located in human ovarian carcinoma cells while all three types of TGF-β-receptors have been detected in human endometrial cells (Dumont et al. 1995).

Each isoform of TGF-β inhibits growth of a wide range of normal and neoplastic cells, including epithelial, lymphoid, hematopoietic cells and fibroblasts (Piu et al. 1993, Sporn et al. 1986). However, TGF-β is not restricted to growth control. It also affects differentiation and development. TGF-β sometimes causes opposing effects depending upon the origin of the target cell, the hormonal environment, the cellular density, and other local factors. In some cells (fibroblasts, osteoclasts) TGF-β can act as a mitogen, probably by stimulating the release of autocrine factors. However, in general, TGF-β inhibits cell cycle progression by prolonging or arresting the G_1 phase (Derynck 1994, Jozan et al. 1992). In addition, an important mechanism of the antiproliferative action of TGF-β is its ability to antagonize the mitogenic effects of other peptide growth factors (Sporn et al. 1986). In various cell types it seems probable that transcriptional regulation of growth-related genes (*e.g. jun, fos* and *myc*) may be a crucial component of the growth-inhibiting effects of TGF-β (Koo et al. 1992, Pertovaara et al. 1989, Piu et al. 1993). In addition, the involvement of transcription factor NF-κB has been observed in TGF-β-mediated growth regulation. The inhibition of constitutive NF-κB activation by stabilization of the inhibitory protein IκB plays an important role in the arrest of the proliferation of breast cancer cells by TGF-β (Sovak et al. 1999)

TGF-β has an important growth-regulatory role in both normal and transformed endometrial and ovarian cells, where TGF-β shows a primarily inhibitory effect on cell proliferation (Boyd and Kaufman 1990, Havrilesky et al. 1995, Jozan et al. 1992). In addition to inhibiting progression through the cell cycle, it has been shown that TGF-β can elicit programmed cell death under certain circumstances. TGF-β-mediated apoptosis has been observed in numerous types of cells including cells of the endometrium (Moulton 1994), cervix (Kim et al. 1998) and ovary (Havrilesky et al. 1995, Mathieu et al. 1995).

2.5 *TNF-α*

The tumor necrosis factor-family is composed of several members *e.g.* TNF, FasL and TRAIL. TNF-α, the protein originally called as cachectin, was described as early as in the late 1800s for its ability to induce hemorrhagic necrosis in tumors (Old 1985). TNF-α is produced primarily by monocytes and macrophages. Two distinct receptors for TNF-α have been defined with approximate molecular masses of 55 (TNF-R1) and 75 kDa (TNF-R2).

TNF-α has a significant role in the human ovarian-endometrial axis. Expression of both types of receptors and release of TNF-α by endometrial cells have been demonstrated (Garcia et al. 1994, Tabibzadeh 1995). The human ovary contains TNF-α both in normal and pathological situations (Moradi et al. 1993, Roby et al. 1990). However, the growth-regulatory role of TNF-α is not consistent in cells originating in the female genital tract: In endometrial cells, TNF-α was able to induce apoptosis (Tabibzadeh et al. 1994) but cell proliferation was increased by TNF-α in combination with fibroblast growth factor (Hammond et al. 1993). In endometrial carcinoma cells, TNF-α had either no effect on cell proliferation (Zhang et al. 1991) or growth inhibition was seen with high doses of TNF-α and growth stimulation with low doses of TNF-α (Lewis et al. 1987). TNF-α has been found both to stimulate the cell proliferation of normal and malignant ovarian cells (Wang et al. 1992, Wu et al. 1993) and to have antiproliferative effects on ovarian cancer cells by inducing cytolysis (Kost et al. 1996), eliciting apoptosis (Krupitza et al. 1998) and enhancing the antitumor activity of macrophages against malignant ovarian cells (Richters et al. 1993). In addition, IFN-γ has been found to be capable of enhancing the antiproliferative effect of TNF-α in cervical carcinoma cells (Lewis et al. 1987). The combination therapy of TNF-α and IFN-γ, when used together, had therapeutic potential against human ovarian cancer xenografts in mice (Balkwill et al. 1987).

The physiological mechanisms by which TNF-α exerts its growth-regulatory effects are largely unknown. Totpal et al. (1992) suggest that phosphatase inhibitors may be needed both for the proliferative and the antiproliferative effects of this cytokine. Transcription factors NF-κB and AP-1 may have a potential role in the signal transduction of growth regulation mediated by TNF-α (Brach et al. 1993, Dixit et al. 1989, Kelliher et al. 1998, Jabbar et al. 1994).

Table 1. Cytokines at a glance

cytokine	No. of amino acids and form	receptors	source	function
IL-6	184, monomer	IL-6R-complex: 80 kDa binding molecule + 130 kDa signal transducer	T-cells, macrophages, monocytes, fibroblasts, hepatocytes, endothelial cells, nerve cells	Induces proliferation and differentiation of T-, B-cells, hepatocytes, keratinocytes, nerve cells, acute phase protein production. Inhibits proliferation of some tumor cells.
IL-10	160, homodimer	IL-10R: 40-120 kDa	T and B cells, macrophages, keratinocytes	Inhibits production of other cytokines, induces B-cell proliferation. Anti-tumoral effect mediated by T and NK cells.
IL-13	132, monomer	IL-13Rα1, IL-13Rα2, IL-4α or γ chains	T cells	Inhibits production of other cytokines, induces B-cell proliferation. Inhibits proliferation of some tumor cells.
IFN-α	166, monomer	IFNα,βR: ~100 kDa	Monocytes, macrophages, fibroblasts, T and B cells	Antiviral activity, activates NK cell cytotoxicity, inhibits cell proliferation.
IFN-γ	143, monomer	IFN-γR: 90-100 kDa	T cells, NK cells	Antiviral activity, activates killing by phagocytes and NK cell cytotoxicity, inhibits cell proliferation.
TNF-α	157, trimer	TNFR1: 55 kDa TNFR2: 75-85 kDa	Monocytes,macrophages,leucocytes some tumor cells, endothelial cells, astrocytes, smooth muscle cells	Inhibits cell proliferation, stimulates cytokine production and acute phase protein production.
TGF-β	112, homo- and heterotrimers	TGF-βR1: 65 kDa TGF-βR2: 85 kDa TGF-βR3:200-300 kDa	T cells,monocytes, chondrocytes,osteoblasts, osteoclasts, platelets, fibroblasts	Inhibits cell proliferation, stimulates osteoblasts and inhibits osteoclasts, inhibits cytotoxic activity of NK cells and inhibits lymphocyte proliferation.
GM-CSF	127, monomer	GM-CSFRα: 84kDa GM-CSFRβ: 130kDa	leukocytes, macrophages, mast cells, endothelial cells, fibroblasts	Promotes the proliferation, maturation and activation of hematopoietic cells. Inhibits proliferation of some tumor cells.

3 APOPTOSIS

A cell undergoing necrosis exhibits changes including cellular osmotic swelling of the cytoplasm and organelles and lysis. In contrast to the injury-induced death of necrosis, there exists a spontaneous form of cell death that occurs in many different cells in various conditions. Programmed cell death is a physiological mechanism for deleting cells without inducing inflammation and subsequent damage to other cells in neighborhood. The process of programmed cell death occurs in two discrete stages. First the cell undergoes nuclear and cytoplasmic condensation, eventually breaking up into a number of membrane-bound fragments containing structurally intact organelles. The process of cellular fragmentation occurs extremely rapidly, within several minutes, and this along with the lack of inflammation, makes it difficult to observe the death process. In the second stage these cell fragments, termed apoptotic bodies, are phagocytozed by neighboring cells and rapidly degraded. (Farber 1994, Schwartzman and Cidlowski 1993). Apoptosis has been observed in a number of cell types upon addition or removal of the appropriate regulatory factor. In addition, apoptosis occurs spontaneously in many cells(Wyllie 1993).

Examination of the varied examples of apoptotic cell death shows that the mediators of apoptosis can be divided into three general classes. The largest group includes mediators that most likely induce apoptosis by binding to their respective receptors and eliciting changes in the intracellular environment. This group includes hormones and cytokines that regulate apoptosis in particular cell types. The second category is cell-mediated apoptosis within the immune system. In this case, target cells undergo apoptosis in response to intracellular changes caused by interaction with cytotoxic cells. The third class includes apoptosis in response to mildly adverse conditions, anticancer drugs, toxins and injury (Schwartzman and Cidlowski 1993).

Cytokines have been found to be important mediators of apoptosis. The members of the TNF-superfamily, especially TNF-α, FasL and TRAIL, induce apoptosis in a wide variety of malignant cells. Death receptors for TNF (TNF-R1), FasL (Fas) and for TRAIL (DR4 and DR5) contain an intracellular death domain which is essential for the transduction of the apoptotic signal (Hu W-H et al. 1999; Walczak and Krammer 2000). TRAIL and FasL-induced apoptosis involves caspase-activation and is regulated by numerous agents including tumor supressor gene p53, AP-1 and NF-κB (Houghton 1999).

To my knowledge, there are no studies about the existence of TRAIL-receptors in gynecological carcinoma cells, but the expression of Fas have been demonstrated in ovarian, endometrial and vulvar carcinoma cells (Das et al. 2000, Moers et al. 1999). The significant increase in apoptosis during the late secretory and menstrual phases in human endometrium may be attributed to the elevation of significant amounts of TNF-α induced by estrogen withdrawal (Tabibzadeh et al. 1993). The conclusion that TNF-α is connected to apoptotic response is further supported by the fact that the administration of TNF-α induced apoptosis in endometrial epithelial cells *in vitro* (Tabibzadeh et al. 1994). TNF-α has been shown also to induce apoptosis in endothelial cells (Robaye et al. 1991) and ovarian carcinoma cells (Krupitza et al. 1998). TNF-α in combination with IFN-γ induced apoptosis in luteal cells of the ovary (Jo et al. 1995) and colorectal adenocarcinoma cells (Koshiji et al. 1998). Moreover, IFN-γ has been found to induce cell cycle arrest and apoptosis in ovarian carcinoma *in vivo* (Burke et al. 1997). It has been observed that interferons are able to stimulate functional expression of TRAIL, and therefore it is likely that the apoptotic effect of IFN may be at least partially mediated by TRAIL-induced direct killing of tumor cells (Walczak and Krammer 2000).

TNF-α and FasL are efficient in killing a variety of tumor cells. However, they cause significant damage to normal tissues resulting in life-threatening toxicities. TRAIL has been shown to be selectively cytotoxic in inducing apoptosis against tumor cells and has minimal or no toxicity against normal tissues. Antiapoptotic proteins Bcl-2 and Bcl-x_L are often overexpressed in malignant cells, being the main reason for chemotherapy resistance. However, TRAIL can bypass their antiapoptotic efficacy (Gazitt et al. 1999). Thus TRAIL has potential as a novel therapy for cancer especially when achieving synergistic anti-tumor effect in combination with classic cytotoxic chemotherapeutic drugs.

TGF-β is also a well known inducer of apoptosis. TGF-β-induced apoptosis has been observed in the cells of the immune system (Andjelic et al. 1997, Buske et al. 1997), fibroblasts (Zhang and Phan 1999) and in transformed cells of the ventricle (Yanagihara H and Tsumuraya M 1992) and liver (Lin and Chou 1992). TGF-β controls apoptosis in endometrial stromal cells *in vitro* by an autocrine/paracrine mechanism (Moulton 1994). In cervical carcinoma cells, TGF-β induced the apoptotic response through down-regulation of *c-myc* gene (Kim et al. 1998). Ovarian cancer cells

that are growth-inhibited by TGF-β have also been found to undergo apoptosis (Havrilesky et al. 1995b, Mathieu et al. 1995).

In contrast to inducing apoptosis, cytokines have been shown to prevent cells from apoptotic response. TNF-α can both induce and inhibit apoptosis. Apoptotic cell death induced by TNF-α or the combination of TNF-α and IFN-γ has been found to be inhibited by IL-13 in different cell types (Manna and Aggarwal 1998, Wright et al. 1999). In addition to IL-13, several other cytokines (IL-1β, granulocyte-monocyte-CSF, IFN-α and IFN-γ) have been observed to prevent apoptosis (Liu et al. 1999, Lotem and Sachs 1995, Mangan and Wahl 1991). For example, IFN-γ has been observed to inhibit proliferation and apoptosis at the same time by arresting the cell cycle at the G1/S boundary (Xaus et al. 1999), whereas the growth-inhibitory IFN-α has been found to protect cells from apoptosis and concurrently activate transcription factor AP-1 (Liu et al. 1999).

A number of genes have been identified whose expression is altered in cells undergoing apoptosis, including the tumor suppressor gene p53, c-*jun*, c-*fos*, c-*myc*, *Bcl-x*$_L$ and *bcl*-2 (Colotta et al. 1992, Eliopoulos et al. 1995, Grimm et al. 1996, Preston et al. 1996). C-*fos* and c-*jun* proto-oncogenes code nuclear proteins of the Fos and Jun family that form the transcription factor AP-1. The role of AP-1 in apoptosis has been studied extensively in a variety of cell types. C-fos and c-jun especially have been connected with apoptotic cell death (Bossy-Wetzel et al. 1997, Colotta et al. 1992, Preston et al. 1996). However, there is also evidence that AP-1 is not essential for apoptosis. For example, it has been observed that programmed cell death can take place in embryos lacking both Fos and Jun (Roffler-Tarlov et al. 1996).

Transcription factor NF-κB also plays a significant role in the regulation of apoptosis (de Martin et al. 1999, Sonensheim 1997). Expression of NF-κB has recently been found to promote cell survival, inhibiting the induction of apoptosis (Wu et al. 1996). According to Mayo et al. (1997) the activation of NF-κB is connected to the suppression of a p53-independent apoptotic response. Nuclear, constitutive NF-κB activity in human breast cancer cells (Sovak et al. 1997) and in Hodgkin's lymphoma cells (Bargou et al. 1997) has been found to prevent cells from undergoing apoptosis. In addition, TNF-α-induced activation of NF-κB has recently been linked to the protection of multiple types of cells from apoptosis probably through transcriptional induction of apoptosis inhibitory genes (Beg and Baltimore 1996, Sumimoto et al. 1999, Wang et al. 1996).

However, others have demonstrated a lack of a role for NF-κB in TNF-α induced apoptosis (Cai et al. 1997) and moreover, Grimm et al. (1996) have observed that NF-κB is even able to induce apoptosis. Another member of the TNF-family, TRAIL, interacts with several receptors: TRAIL-R1 (DR4) and TRAIL-R2 (DR5) can induce apoptosis of cancer cells and activate NF-κB. TRAIL-R4 can activate NF-κB and protect cells from apoptosis in some cases (Hu et al. 1999). However, the study with T cells showed that neither NF-κB nor AP-1 activation is required for IL-2-mediated inhibition of apoptosis or cell cycle progression in activated primary human T cells (Iacobelli et al. 1999).

Wild-type p53 negatively regulates cell growth and division and is classified as a tumor-suppressor gene. In some cell lines, the loss of p53 function has been associated with induction of resistance to TGF-β-induced programmed cell death (Zhu et al. 1994). Mutations of p53 have been described recently in human ovarian carcinoma (Provencher et al. 1997) and other gynecological cancers (Rantanen et al. 1998). However, apoptosis and p53 status have not been found to correlate with each other in ovarian cancer cells (Diebold et al. 1996, Havrilesky et al. 1995a,b).

4 TRANSCRIPTION FACTORS

Eukaryotic genes are regulated by a variety of proteins called transcription factors. These factors localize to the regulatory promoter or enhancer elements of their target genes. Promoters are sequences participating in the initiation of transcription situated upstream of the transcription start site. Enhancers are frequently found upstream from promoter elements but also in introns or downstream of genes. These sequences enhance the activity of promoters. Several transcription factors participating in cytokine signaling have been identified, *e.g.* ISGF3, GAF and STAT proteins in interferon signaling cascades (David 1995, Levy et al. 1995).

4.1 *NF-κB*

NF-κB proteins are eukaryotic transcription factors that bind to target DNA elements and activate transcription of genes encoding proteins involved with immune or inflammation responses and with cell growth control. These proteins were first identified in B cells binding to DNA in the intronic κ light chain enhancer. Soon it became evident that these binding sites were not B-cell restricted but they could be found in nearly all cell types. Rel/NF-κB family is composed of a number of structurally-related, interacting proteins that bind DNA as dimers and whose activity is regulated by subcellular location. The family includes p50, p52, RelA(p65), RelB and c-Rel. Most of these can form DNA-binding homodimers or heterodimers. The best characterized heterodimer, so-called ”classic-NF-κB” is composed of p50 and p65 subunits. All Rel proteins contain a highly conserved, approximately 300 amino acids long amino-terminal domain called the Rel Homology (RH) domain, which contains sequences necessary for the formation of dimers, nuclear localization DNA binding and IκB binding (Baldwin 1996, Gilmore et al. 1996, Grimm and Bauerle 1993).

The IκB proteins include a group of related proteins that interact with Rel proteins and regulate their activities in a cell type-specific manner. In the best characterized example, IκB interacts with NF-κB to retain the complex in cytoplasm and to inhibit its DNA-binding activity (Liou and Baltimore 1993).

Most cells contain NF-κB in a transcriptionally inactive form within the cytoplasm where NF-κB is bound to an inhibitory IκB. Exposure of cells to a plethora of stimuli, including cytokines, UV-light, mitogens and viruses results in the dissociation of IκB from the NF-κB-complex through a

phosphorylation and degradation event. Subsequently, NF-κB translocate to the nucleus and bind to specific DNA sequences and activation of transcription may occur. The activation of NF-κB is independent of protein synthesis (Baldwin 1996).

Although in most cell types NF-κB is inactive, sequestered in the cytoplasm, recent studies have revealed constitutive NF-κB activity in several cell types including squamous cell carcinoma (Dong et al. 1999), ovarian carcinoma (Bours et al. 1994), breast cancer (Sovak et al. 1997) and Hodgkin's lymphoma (Bargou et al. 1997). The most studied cytokine in inducing NF-κB activity is TNF-α. The increased cytokine expression has been observed to be associated with an increase in constitutive and TNF-α-inducible activation of NF-κB in squamous cell carcinoma (Dong et al. 1999). Similarly the basic IL-6 expression has been found to be associated with DNA binding of NF-κB that was induced in response to TNF-α in ovarian carcinoma cells (Asschert et al. 1999). TNF-α is able to inhibit cell proliferation and induce NF-κB in human myelomonoblastic leukemia (Chaturvedi et al. 1994b). IFN-γ, which typically does not activate NF-κB, synergistically enhanced TNF-α-induced NF-κB nuclear translocation via a mechanism that involves the induced degradation of IkappaB (Cheshire and Baldwin 1997). TGF-β is also capable of inducing NF-κB activation in T cells (Han SH et al. 1998). In breast cancer cells, the inhibitory effect of TGF-β has been found to be mediated through the regulation of constitutive NF-κB expression (Sovak et al. 1999).

There is also evidence that cytokines are able to inhibit NF-κB activity. IL-10 and IL-13 have been found to suppress nuclear localization of NF-κB by augmenting I-κBα mMRA expression (Lentsch et al. 1997). Moreover, IL-10 signaling has been observed to block NF-κB activity by inhibiting the IkappaB kinases and through the inhibition of NF-κB DNA binding activity (Schottelius et al. 1999). Growth-inhibitory TGF-β has been found to inhibit NF-κB activity through the induction of IkappaB expression in human salivary gland cells (Azuma et al. 1999).

Constitutive nuclear NF-κB activity especially (Bargou et al. 1997, van Hogerlinden et al. 1999, Sonensheim 1997, Sovak et al. 1997) but also TNF-α induced NF-κB activity (Beg and Baltimore 1996, Sumitomo et al. 1999) has been linked in the prevention of cells from undergoing apoptosis. In addition, the maintenance of NF-κB/Rel expression has been found to protect B cells from TGF-β1-mediated apoptosis (Arsura et al. 1996).

It has been speculated that some proteins from the NF-κB family are involved in the development of cancer. v-Rel is a truncated and mutated version of avian c-Rel, and v-Rel containing viruses are highly oncogenic and cause aggressive tumors in young birds (Moore and Bose1988). In addition, v-Rel homodimers are the crucial effector complexes for the transformation of mouse lymphoid cells (Carrasco et al. 1996). However, it has been observed that transgenic mice overexpressing c-Rel, RelA and Rel B do not develop tumors (Perez et al. 1995, Weih et al. 1995, Carrasco et al. 1996). In fact, other than v-Rel, there have been only sporadic reports of Rel or IκB proteins causing malignant transformation *in vitro*.

Several associations of Rel and IκB proteins with regulation of cell growth have also been found (Hinz et al 1999), although Chaturvedi et al. (1994a) suggest that the activation of NF-κB is not a generalized mediator of signal transduction of most cytokines and further that NF-κB activation is not sufficient for antiproliferative effects mediated through certain cytokines in human histiocytic cells. However, certain anti-cancer agents for example, such as taxol and curcumin, can affect the induction of NF-κB binding activity (Han et al. 1999, Hwang and Ding 1995, Singh and Aggarwal 1995). The inhibitory effects of TGF-β1 on breast cancer cell proliferation have been observed to be mediated through the inhibition of constitutive NF-κB expression (Sovak et al. 1999). Similarly, the inhibition of NF-κB activity in T cells leads to a decrease of cell proliferation (Ferreira et al. 1999). In contrast to these observations, TNF-α-induced growth inhibition of human myeloid cells (Chaturvedi et al. 1994b, Hu et al. 1999) and hepatic cells (Gallois et al. 1998) has been observed to be associated with increase in NF-κB activation. Furthermore, it has been found that NF-κB activation in stratified epithelium is important for growth arrest (Seitz et al. 1998).

Several studies have revealed a connection between NF-κB, cytokines and steroid hormones. Estrogens are able to repress *IL-6* gene expression by inhibiting the DNA-binding activity of NF-κB by estrogen receptor (ER; Ray et al. 1997). Estrogen causes translocation of c-RelER (chicken c-Rel fused to the hormone-binding domain of the human estrogen receptor) to the nucleus and stabilizes its binding to DNA. The growth of fibroblasts with moderate expression levels of cRelER was stimulated by estrogen whereas the addition of estrogen to cells with high cRelER expression resulted in the inhibition of cell growth (Zurovec et al. 1998). A direct negative interaction between NF-κB and progesterone receptor (PR) has been evaluated: the activation of NF-κB by TNF-α

resulted in repression of PR, while PR was able to repress tumor necrosis factor-alpha-induced NF-κB activity (Kalkhoven et al. 1996).

4.2 *AP-1*

Transcription factor activating protein 1 (AP-1) is a complex composed of homodimers or heterodimers of different members of the Jun (v-Jun, c-Jun, JunB, JunD) and Fos (v-fos, c-Fos, FosB, Fra1, Fra2) families. AP-1 dimers recognize a *cis*-activating element, the phorbol 12-myristate 13-acetate (TPA)-responsive element (TRE:TGACTCA) and also have weaker affinity for a cyclic AMP-responsive element (CRE:TGACGTCA). AP-1 binding to DNA is sufficient and necessary for the transcriptional activation of AP-1-dependent genes following protein kinase C (PKC) activation. Dimerization between Fos and Jun proteins occurs via an α helical structure referred to as a leucine zipper that has also been found to mediate interactions between many transcription factors. Interactions between leucine zipper-containing proteins are very specific, owing to the distribution of charged amino acid residues within the leucine zipper domain. Therefore, members of the Fos family cannot dimerize with other Fos proteins, whereas Jun proteins can form both homodimers and heterodimers with other Jun proteins. Furthermore, different Jun and Fos proteins bind with different affinities to AP-1 site of DNA and heterodimers between Fos and Jun family proteins form the most stable complexes. (Angel and Karin 1991, Ryseck and Bravo 1991, Vandel et al. 1995) Each of the AP-1 proteins have a different ability to transactivate or repress transcription (Foletta 1996, Vandel et al. 1995). In addition, each protein and different dimer has a distinct effect on cell growth (Rezzonico et al. 1995). It has been observed that there are for example major differences in the expression pattern of AP-1 family members present in breast tumors (Bamberger et al. 1999).

In addition to the leucine zipper dimerization domain, AP-1 and a number of other transcription factors have another α helical region that mediates the DNA binding and dimerization. These b-zip (basic region leucine zipper) proteins have recently been added to the AP-1 family. This family includes the cAMP response element binding proteins (CREBs; also called activating transcription factors, ATFs), the C/EBP family, TEF/DBP, the Maf/Nrl family and NF-E2. There are many instances of cross-talk between b-zip protein families, and delineation of the nature of the AP-1 protein complexes involved in the regulation of expression of certain genes often requires analysis

of other b-zip families as well. AP-1 family members can also interact with non-b-zip transcription factors, such as NF-κB. (Foletta et al. 1998, Karin et al. 1997)

Changes in AP-1 activity in response to extracellular signals *e.g.* cytokines, tumor promoters, carcinogens and increased expression of various oncogenes is regulated both on the level of transcription of the *jun* and *fos* genes and by postranslational modification of pre-existing AP-1 by phosphorylation. Transcription of *jun* and *fos* genes occurs rapidly and does not require new protein synthesis. Transcription of the different subunits of AP-1 is regulated differently in response to extracellular signal. For example, in renal cancer cells, the expression of *junB* was augmented by TGF-β, while expression of c-*jun* was not altered (Koo et al. 1992). The third mechanism to regulate AP-1 activity is physical interaction with other cellular proteins. For example, the activated retinoic acid receptors have been found to inhibit AP-1 activity in ovarian carcinoma cells (Soprano et al. 1996). A variety of cytokines has been found to be able to regulate the activation of AP-1, including TGF-β (Pertovaara et al. 1989), TNF-α (Brach et al. 1993), GM-CSF (Adunyah 1993), IL-10 (Hurme et al. 1994), IL-13 (Manna and Aggarwal 1998), IFN-γ (Conant et al. 1998) and IFN-α (Liu et al. 1999).

AP-1 is thought to play a central role in the control of the proliferation of many different kinds of cells although results from different studies are contradictory. It has been observed that AP-1 activity is essential for fibroblasts to proliferate in response to serum (Riabowol et al. 1992), while *c-jun* was not essential for the proliferation of embryonic stem cells (Hilberg and Wagner 1992). In fibroblasts, *c-jun* was required for progression through the G1 phase of the cell cycle (Wisdom et al. 1999). In transformed mesenchymal cells, *c-jun* and *junB* serve as positive regulators and *junD* as a negative regulator for cell proliferation (Wang et al. 1996). However, *c-jun* has been found to be involved in positive and *junB* in negative growth control in fibroblasts, mammary carcinoma cells and pheochromocytoma cells (Schlingensiepen et al. 1993). Treatment of melanoma cells with the synthetic retinoid caused growth inhibition and induced apoptosis via activation of both *c-jun* and *c-fos* (Schadendorf et al. 1996). In addition, cyclic AMP has been found to stimulate AP-1 complex and inhibit cell proliferation (Rezzonico et al. 1995). In ovarian adenocarcinoma cells, retinoids have been found to inhibit cell growth by repressing AP-1 activity (Soprano et al. 1996). Moreover, there is evidence of an association between the antimitogenic action (Dixit et al. 1989) and mitogenic action (Brach et al. 1993) of TNF-α and AP-1. The autoinduction of TGF-β, that is a growth-inhibitory factor in several cell types, is

mediated by AP-1 complex (Kim et al. 1990). In addition, the enhanced expression of *junB* and c-*jun* by growth-inhibitory TGF-β has been observed in human lung adenocarcinoma cells (Pertovaara et al. 1989). In renal cell cancer, the expression of *junB* was enhanced by growth-inhibitory TGF-β and the expression of both *junB* and c-*jun* was augmented by growth-inhibitory TNF-α (Koo et al. 1992).

The growth and differentiation of endometrial cells is regulated by steroid hormones, estrogens and progestins. It has been observed that progesterone receptors stimulate AP-1 activity in endometrial adenocarcinoma cells (Bamberger et al. 1996). Cross-talk between estrogen receptors and members of the Fos/Jun family via protein-protein interactions may explain how anti-estrogens inhibit the mitogenic effect of growth-factors in breast cancer cells (Rochefort 1995, Webb et al. 1995). Therefore AP-1 may play a significant role in the regulation of cell growth of steroid-responsive tumor cells.

The role of AP-1 in apoptosis is not consistent. There is evidence that c-fos and c-jun are mediators of apoptotic cell death both in antiproliferative conditions (Colotta et al. 1992, Satomi et al. 1999, Schadendorf et al. 1996) and proliferative conditions (Sikora et al. 1993), although it seems that the role of c-jun in apoptosis is distinct from its role in cell proliferation (Wisdom et al. 1999). EGF-induced apoptosis of mammary carcinoma cells is preceded by the activation of AP-1 (Schaerli and Jaggi 1998). In addition, AP-1 is suggested to be a possible mediator of tumor necrosis factor-alpha-induced apoptotic response in mouse tumor cells (Singh et al. 1995). It has been suggested that different AP-1 proteins may play distinct roles both in triggering apoptosis and protecting cells from apoptosis (Jacobs-Helber et al. 1998). In fibroblasts, c-jun has been found both to induce apoptosis (Bossy-Wetzel et al. 1997) and, in contrast, by cooperating with NF-κB, to protect cells from apoptosis (Wisdom et al. 1999). In addition, c-fos has been observed to play an essential role in protecting thymocytes from apoptosis (Ivanov and Nikolic-Zugic 1997), whereas in embryonic cells, c-fos protein was able to induce apoptotic cell death (Preston et al. 1996). However, some data shows that neither c-fos nor c-jun is essential for apoptosis *in vivo* during normal development (Roffler-Tarlov et al. 1996) and that cells from the spleen and the thymus lacking c-fos may undergo normal apoptosis (Gajate et al. 1996).

AIMS OF THE STUDY

The development of carcinoma is thought to be a multi-step process starting from damage in the genome of transformed cells leading to various responses. It has been suggested that the cells of the immune system are important for the defense mechanisms against malignant cells, and several cytokines have been shown to inhibit the proliferation of different types of cancer cells. The beneficial growth-inhibitory effects of cytokines have also been observed in malignant cells originating from the female genital tract. However, the involvement of cytokines as growth-regulators especially in endometrial and vulvar carcinoma cells is relatively poorly understood. In addition, the mechanisms by which cytokines regulate cell proliferation in gynecological carcinoma cells are largely unknown. Increased understanding of the molecular and cellular mechanisms underlying the effects of cytokines is essential for new strategies that aid in the treatment of gynecological malignancies.

The present work was undertaken to study the effects of cytokines on ovarian, endometrial and vulvar carcinoma cells *in vitro*.

The specific aims were:

1. To study the effects of several cytokines on DNA synthesis using ^{125}I-deoxyuridine incorporation assay.

2. To investigate whether cytokines are able to induce apoptotic cell death

3. To evaluate effects of cytokines on the binding activity of transcription factors AP-1 and NF-κB

1. *Cytokines*

Recombinant human IFN-α (0.3-30 ng/ml), IFN-γ (0.3-30 ng/ml), and GM-CSF (1-100 ng/ml; Leukomax®) were all from the Schering-Plough Research Institute (Kenilworth, NJ). TNF-α (1-100 U/ml) and TGF-β_1 (0.3-30 ng/ml) and IL-6 (3-300 U/ml) were purchased from R&D systems (Minneapolis, MN). Recombinant human IL-10 (100 U/ml) and IL-13 (50 ng/ ml) were generously donated by Dr. Jan E. de Vries (DNAX Research Institute, Palo Alto, CA).

2. *Cell lines*

2.1 *Vulvar carcinoma cell lines*

UM-SCV-1A cell line has been established from a primary vulvar squamous cell carcinoma (Grenman et al. 1990). The tumor consisted of moderately well to poorly differentiated cells and displays hypotetraploid karyotype. The cells are estrogen receptor negative but show tamoxifen-induced inhibition of growth. UM-SCV-6 cells containing HPV were established from the primary tumor of a 43-year-old patient with invasive squamous epithelial cancer of vulva (Worsham et al 1991).

2.2. *Endometrial carcinoma cell line*

UM-EC-3 cell line has been established in the Cancer Research Laboratory of the University of Michigan from a primary tumor of a 60-year-old patient with endometrial papillary adenocarcinoma with focal clear cell and papillary morphological features (Rantanen et al. 1994).

2.3. *Ovarian carcinoma cell lines*

The UT-OC-5 cell line has been established in 1993 at Turku University Central Hospital from a metastatic omental tumor from a patient suffering from stage IV ovarian cystadenocarcinoma (Grénman et al. 1997)

The UT-OC-3 cell line has been established at the University of Turku from a primary tumor of a 60-year-old patient with well differentiated stage III ovarian serous cystadenocarcinoma (Engblom et al. 1999, Grénman et al. 1997)

The UT-OC-2 cell line has been established from a primary tumor of a 43-year-old patient with ovarian endometrioid cystadenocarcinoma, gradus III, stage IV (Grénman et al. 1997).

3. *Cell culture*

Cells were cultured in DMEM/ Ham´s F12 medium (Life Technologies; Gaithersburg, CA) containing 10 % heat-inactivated Fetal Bovine Serum (FBS; Gibco; Paisley, Scotland), penicillin and streptomycin (2000 U/l and 2 mg/l, respectively; both from Life Technologies, Gaithersburg, CA), L-glutamine (2 mmol/l; Life Technologies) and 1 % non-essential amino acids (Life Technologies) in 250 ml tissue culture plates (Greiner, Frickenhausen, Germany).

4. ^{125}I-*deoxyuridine incorporation assay*

Before the experiments, the cells were trypsinized and divided onto the wells of microtiter plates (Nunc; Roskilde, Denmark; 20-50 000 cells/ well), and were allowed to adhere for 16 hours followed by a 24 or 48 hours preincubation in presence or absence of cytokine. Subsequently, the cells were labeled with 5-(^{125}I-iodo)-2'-deoxyuridine (^{125}IUdR; Amersham, Buckinghamshire, UK) for 24 hours as described earlier (Asantila and Toivanen 1974). Finally, the cells were trypsinized and harvested. Incorporation of radioactivity into cellular DNA was measured with a γ-spectrometer (1272 Clinigamma, LKB-Wallac, Turku, Finland). All experiments were carried out in quadruplicate.

5. *Data expression and statistical analyses*

The effects of different factors on DNA synthesis of the cells cultured in the presence of cytokine were compared to the control. The results are expressed as disintegrations per minute (dpm). Statistical analyses of the results were performed by paired Student's *t*- test. A p value less than 0.05 was considered statistically significant.

6. *DNA fragmentation analyses*

These studies were carried out essentially as described earlier (Smith and Williams 1989). In brief, carcinoma cells were cultured for 48 or 72 hours in presence or absence of growth-inhibitory cytokines. 1-3 x 10^6 cells were washed and pelleted at +4°C (5 min at 2600 x *g*). Pellets were resuspended into 20 µl of 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM EDTA, 0.5% (w/v) sodiumlaurylsarkosinate and 0.5 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), and incubated at 50°C for 1 hour. Thereafter, RNase A (5 µg/ml; Boehringer Mannheim) was added and the incubation at 50°C continued for an additional hour. Samples were heated to 70°C, and 10 µl 10 mM EDTA (pH 8.0) containing 1% (w/v) low-gelling-temperature agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose were mixed with each sample before loading onto the wells of agarose gel (1%) containing 0.1 µg/ml ethidium bromide. Electrophoresis was carried out in 800 mM Tris-phosphate buffer (pH 7.8) containing 2 mM EDTA at 80 V for 4 hours. All experiments were carried out in quadruplicate.

7. *Extraction of nuclear proteins*

Nuclear proteins were isolated from carcinoma cells essentially as described by Andrews and Faller. Briefly, 3 x 10^6 cells were cultured in the presence or absence of cytokine for 30, 60, 120 or 180 minutes, while control cells were cultured in basal conditions. After culture, the cells were washed, pelleted and resuspended in 400 µl cold buffer A [10 mM HEPES-KOH (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride and 10 mM aprotinin]. The cells were allowed to swell on ice for 10 min and vortexed for 10 seconds. Samples were centrifuged (30s at 15000 x *g*) and the supernatant was discarded. The pellet was resuspended in 30 µl of buffer C [20 mM HEPES-KOH (pH 7.9) containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM dithiotreitol, 10 mM PMSF and 10 mM aprotinin] and incubated on ice for 20 min. Cellular debris was removed by centrifugation (2 min at 15000 x *g*) at +4°C and the supernatant fraction (containing DNA binding proteins) was stored at -70°C.

8. *Electrophoretic mobility shift assay (EMSA)*

The AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') and NF-κB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were purchased from Promega (Madison, WI, USA). Nuclear protein-DNA binding reactions were carried out in a volume of 20 μl containing 5 or 6 μg nuclear extract protein, 10 mM Tris-HCl (pH 7.5), 40 mM KCl, 1 mM EDTA, 1 mM dithiotreitol, 10% glycerol, 2 μg poly (dI-dC) as a nonspecific competitor and 10 fmol of ^{32}P-labeled consensus oligonucleotide probe. Binding reactions were performed at room temperature for 30 minutes. The specificity of the AP-1-DNA or NF-κB-DNA complex was confirmed by competition analyses using a 50-fold molar excess of unlabeled AP-1 or NF-κB oligonucleotide. The anti-p50, anti-p65, anti-c-jun and anti-c-fos antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA) and used according to the manufacturer's instructions. Samples were finally analyzed by electrophoresis on 4% nondenaturing low-ionic strength polyacrylamide gels in 0.25 x 22 mM Tris-borate containing 22 mM boric acid and 0.5 mM EDTA. The gels were dried and visualized by autoradiography. The band density was quantified by a Personal Densitometer (Molecular Dynamics Inc., Sunnyvale, USA). In densitometric analyses the arbitrary value (ADU) chosen for basal activity = integral (surface area) of the specific band in control after background calibration. All experiments were carried out in quadruplicate.

RESULTS

1. *Effects of cytokines on DNA synthesis*

The ^{125}I-deoxyuridine (^{125}IUdR) incorporation assay was used to study the antiproliferative effect of cytokines on carcinoma cells. Cytokines showed primarily growth-inhibitory effect on cell proliferation in all cell lines studied. In UM-SCV-1A vulvar carcinoma cells, the growth inhibitory effect was observed by IL-10, IL-13, TNF-α, TGF-β and IFN-γ after 48 and 72h, respectively. GM-CSF showed no statistically significant effects on proliferation of UM-SCV-1A cells. The effect of IFN-α was dose-dependent and biphasic: the growth inhibition was seen after 48 hours, while a stimulatory effect on DNA synthesis was observed after 72 hours (I).

The antiproliferative effects of IL-10, IL-13, TNF-α, TGF-β, GM-CSF and IFN-γ after 48 and 72 hours were observed in UM-SCV-6 vulvar carcinoma cells. IFN-α inhibited DNA synthesis after 48 hours while growth-stimulation was observed after 72 hours (I).

In UM-EC-3 endometrial carcinoma cell line, DNA synthesis was inhibited by GM-CSF, IL-10, IL-13, TGF-β_1, IFN-α and IFN-γ after 48 and 72 hours in culture, while TNF-α had no significant effect on cell proliferation in any of the experiments (II).

The inhibition of DNA synthesis was similarly observed in UT-OC-5 ovarian carcinoma cells by IL-10, TNF-α and IFN-γ after 48 and 72 hours, while IFN-α had no statistically significant effect. An inhibitory effect of GM-CSF was observed only after 48h and TGF-β after 72 hours in culture, respectively (II).

In UT-OC-3 ovarian carcinoma cells, TGF-β, TNF-α, IFN-α, IFN-γ, GM-CSF and IL-6 showed a clear inhibitory effect on ^{125}IUdR incorporation after 48 hours. IFN-α and IFN-γ were both growth-inhibitory after 72 hours in culture. Similarly, GM-CSF induced a slight inhibition, while TGF-β and TNF-α almost blocked DNA-synthesis after 72 hours. IL-6 had no statistically significant effect on cell proliferation after 72 hours (III).

In UT-OC-2 ovarian carcinoma cells the growth inhibition was observed by TNF-α, IFN-γ and TGF-β after 48 and 72 hours. IFN-α showed an inhibitory effect after 48 hours while GM-CSF did not affect cell proliferation (V).

Table 2. Effects of cytokines on DNA synthesis in ovarian, vulvar and endometrial carcinoma cells

	IL-6		IL-10		IL-13		IFN-α		IFN-γ		TGF-β		TNF-α		GM-CSF	
	48h	72h	48h	72h	48h	72h	48h	72h	48h	72h	48h	72h	48h	72h	48h	72h
UM-SCV-1A	not studied		- ***	- ***	- ***	- ***	- **	+ *	- ***	- ***	- *	- ***	- *	- *	+/-	+/-
UM-SCV-6	not studied		- ***	- ***	- ***	- ***	- ***	+ **	- ***	- ***	- ***	- ***	- ***	- ***	- ***	- ***
UM-EC-3	not studied		- *	- **	- ***	- ***	- ***	- ***	- ***	- ***	- *	- **	+/-	+/-	- ***	- **
UT-OC-2	not studied		not studied		not studied		- ***	+/-	- ***	- ***	- ***	- **	- ***	- **	+/-	+/-
UT-OC-3	- **	+/-	not studied		not studied		- **	- **	- ***	- **	- **	- ***	- **	- ***	- ***	- *
UT-OC-5	not studied		- ***	- **	not studied		+/-	+/-	- ***	- ***	+/- ***	-	- ***	- ***	- ***	+/-

- = inhibition
+ = stimulation
+/- = no effect

* = $p < 0.05$
** = $p < 0.005$
*** = $p < 0.001$

vulvar carcinoma : UM-SCV-1A and UM-SCV-6
endometrial carcinoma : UM-EC-3
ovarian carcinoma: UT-OC-2, UT-OC-3 and UT-OC-5

2. *Induction of apoptosis by cytokines*

In order to detect apoptosis, cellular DNA was extracted after the carcinoma cells were cultured in the presence of growth-inhibitory cytokines for 48 and 72h. The control cells were cultured in basal medium. In all experiments, with the exception of UT-OC-2 cell line, the electrophoretic analyses showed intact DNA, and no signs of DNA fragmentation, a feature typical of programmed cell death, were observed in any of these analyses. DNA extracted from cultured mouse thymocytes served as a positive control for apoptosis throughout these studies (I-III).

In UT-OC-2 ovarian carcinoma cells, analyses of extracted DNA showed fragmentation as a sign of apoptosis when cells were cultured in basal culture conditions. IFN-γ inhibited this constitutive apoptotic response while TNF-α and TGF-β did not affect the magnitude of apoptotic cell death (V).

3. *Activation of NF-κB by cytokines*

In order to analyze whether cytokines could have an influence on the binding activity of NF-κB, the carcinoma cells were cultured in the presence of growth-inhibitory cytokines before EMSA. The constitutive nuclear DNA-binding activity of NF-κB was observed in all carcinoma cell lines in basal culture conditions (II-V).

TNF-α was the only cytokine that increased the activity of NF-κB in UT-OC-3, UT-OC-5, UM-SCV-1A and UM-SCV-6 cells (II-IV). The binding activity of NF-κB was not altered by any of the cytokines in UM-EC-3 endometrial carcinoma (II). In UT-OC-2 endometrioid ovarian carcinoma cells, the activity of NF-κB was stimulated by IFN-γ (V). IL-10 and IL-13 inhibited DNA binding activity of NF-κB, in contrast to the effect of TNF-α, in UM-SCV-1A cells (IV).

The composition of the NF-κB complex bound to DNA was analyzed by pretreating the nuclear extracts with antibodies directed against the two major components of NF-κB, the p50 and p65 proteins. Supershift analyses showed that NF-κB is at least partially composed of p50/p65 heterodimers in the carcinoma cell lines studied (II-V).

Table 3. Activation of NF-κB by cytokines in vulvar, endometrial and ovarian carcinoma cell lines.

Cell line	IL-10	IL-13	TGF-β	TNF-α	IFN-α	IFN-γ
UM-SCV-1A	inhibition	inhibition	no effect	stimulation	not studied	no effect
UM-SCV-6	no effect	no effect	no effect	stimulation	not studied	no effect
UM-EC-3	no effect	no effect	no effect	no effect	no effect	no effect
UT-OC-2	not studied	not studied	no effect	no effect	no effect	stimulation
UT-OC-3	not studied	not studied	no effect	stimulation	not studied	not studied
UT-OC-5	no effect	not studied	no effect	stimulation	not studied	no effect

4. *Activation of AP-1 by cytokines*

The constitutive nuclear DNA-binding activity of AP-1 was observed in all carcinoma cell lines in basal culture conditions (II-V).

The binding activity of AP-1 was found to be stimulated by all growth-inhibitory cytokines: IL-10, (IL-13 in UM-EC-3 cells), TGF-β_1, IFN-α and IFN-γ studied in UM-EC-3 and UT-OC-5 cell lines (II). In UT-OC-3 ovarian carcinoma cells, the binding activity of AP-1 was found to be stimulated by growth-inhibitory cytokines TGF-β_1 and TNF-α (III). No effect on AP-1 was found by any of the cytokines in UM-SCV-1A and UM-SCV-6 vulvar carcinoma cell (IV). IFN-γ, TGF-β and TNF-α were able to stimulate the binding activity of AP-1 in UT-OC-2 ovarian carcinoma cells (V).

In order to analyze the subunits of AP-1, the nuclear extracts were treated with anti-c-jun or anti-c-fos antibodies before EMSA. Superhift analyses showed the appearance of new bands associated with preincubation with AP-1-specific antibodies, but AP-1-DNA-complex could not be supershifted completely suggesting that AP-1 is composed only partially of c-jun/c-fos proteins and the nature of other AP-1 complexes remains to be determined in endometrial, ovarian and vulvar carcinoma cells (II-V).

Table 4. Activation of AP-1 by cytokines in vulvar, endometrial and ovarian carcinoma cell lines.

Cell line	IL-10	IL-13	TGF-β	TNF-α	IFN-α	IFN-γ
UM-SCV-1A	no effect	no effect	no effect	no effect	not studied	no effect
UM-SCV-6	no effect	no effect	no effect	no effect	not studied	no effect
UM-EC-3	stimulation	stimulation	stimulation	not studied	stimulation	stimulation
UT-OC-2	not studied	not studied	no effect	no effect	not studied	stimulation
UT-OC-3	not studied	not studied	stimulation	stimulation	not studied	not studied
UT-OC-5	stimulation	not studied	stimulation	stimulation	not studied	stimulation

DISCUSSION

1 *Cytokines as growth-inhibitory factors in carcinoma cells*

Numerous studies on cytokines published in the last few years have revealed that cytokines are growth-inhibitory factors in various cell types, including malignant cells. Cytokines have been found to interact with malignant cell growth either by direct inhibition of tumor cells or by inducing defense mechanisms of the immune system (Itri 1992, Miller et al. 1994).

The role of cytokines as growth-regulators in gynecological carcinoma cells has been most intensively studied in the case of ovarian carcinoma. By contrast, the etiology of vulvar carcinoma is largely unknown and there are only few studies concerning the existence and the regulatory role of cytokines in the vulvar area. In the present study, the effects of several cytokines on DNA synthesis in ovarian, endometrial and vulvar carcinoma cell were tested. Cytokines displayed primarily growth-inhibitory effect on cell proliferation. the present study, the effect of several sytokines on DNA synthesis in ovarian, endometrial and vulvar carcinoma cell was tested.

Based on several studies of TNF-α, it could be concluded that the growth-regulatory role of TNF-α is not consistent in the cells originating in the female genital tract: TNF-α can both stimulate and inhibit cell proliferation of normal and malignant ovarian cells (Kost et al. 1996, Richters et al. 1993, Wang et al. 1992, Wu et al. 1993). In endometrial carcinoma cells TNF-α had either no effect on cell proliferation (Zhang et al. 1991) or growth inhibition was seen with high doses of TNF-α and growth stimulation with low doses of TNF-α (Lewis et al. 1987). However, in this study we have shown that TNF-α has a consistent growth-inhibitory effect on ovarian and vulvar carcinoma cells, whereas no statistically significant effect was observed in endometrial carcinoma cells.

Antiproliferative effects of interferons are well established in a variety of cell types including cervical, ovarian and endometrial cancer (Kudelka et al. 1993, Massad et al. 1990, Powell et al. 1993 a). Similarly, in the present study, IFN-α and IFN-γ showed an inhibitory effect on DNA synthesis in ovarian, endometrial and vulvar carcinoma cells, although the effect of IFN-α on DNA synthesis was biphasic, showing inhibitory effect after 48h and stimulatory effect after 72h in vulvar carcinoma cells.

GM-CSF has been found to have anti-proliferative effects against ovarian and breast cancer cells (Salmon and Liu 1989), and irradiated tumor cells secreting GM-CSF have been found to show specific anti-tumor immunity in a number of tumor models (Dranoff et al. 1993). In the present study, GM-CSF has been found to have some anti-proliferative efficacy in vulvar, endometrial and ovarian carcinoma cells.

In the present study the effect of IL-6 on DNA synthesis was found to be highly time-dependent and observed only after 48 hours exposure in UT-OC-3 ovarian carcinoma cells. The antitumor effects of IL-6 against sarcoma and adenocarcinoma of the colon have been demonstrated *in vivo* (Mule et al. 1990). Chen et al. (1991) have observed that IL-6 inhibits growth of human breast and ovarian carcinoma cells *in vitro*, although opposite observations have also been made (Wu et al. 1992). It seems likely that IL-6 is able to either inhibit or stimulate cell growth depending on the cell type used and different culture conditions.

The relatively new interleukins, IL-10 and IL-13, seem to be very potent regulators of DNA synthesis in vulvar, endometrial and ovarian (IL-10) carcinoma cells. There are a few studies supporting our findings. IL-10 has been found to have a growth-inhibitory effect *in vitro* on leukemia cells (Iversen et al. 1997). In addition, IL-10-producing monocyte subset, in the malignant ascites from a patient with ovarian carcinoma, was able to inhibit proliferation of T cells (Loercher et al. 1999). The anti-tumor effect of IL-10 has been demonstrated *in vivo* in murine tumor models (Berman et al. 1996) and in human melanoma cells (Huang et al. 1999). An *in vitro* antiproliferative effect of IL-13 has been observed in breast cancer cells (Serve et al. 1996) and colorectal carcinoma cells (Schnyder et al. 1996). Transfection of tumor cells by IL-13 has been shown to prevent tumor growth in mice by stimulating the sensitization of specific memory T lymphocytes and by improving antigenic presentation of tumor membranes (Lebel-Binay et al. 1995). In addition, IL-13 has a capacity to induce a cytolytic activity in spleen cells from tumor-bearing rats (Reisser et al. 1996).

2 *Induction of apoptosis*

Cytokines have been found to be important mediators of programmed cell death. TGF-β and TNF-α are probably the most important inducers of apoptotic cell death in cells originating from the female genital tract. Both TNF-α and TGF-β have been observed to induce apoptosis in normal endometrial carcinoma cells (Moulton 1994, Tabibzadeh et al. 1994) and ovarian carcinoma cells (Havrilesky et al. 1995b, Krupitza et al. 1998, Mathieu et al. 1995) *in vitro*. Although TGF-β and TNF-α show antiproliferative effects in various carcinoma cells, they were unable to induce apoptosis in the cell lines studied. It has been shown that cancer cells have a tendency to lose their ability to undergo apoptosis with time and therefore low passage cells were used throughout the experiments of the present dissertation (Dive and Wyllie 1993). The possibility cannot be excluded that apoptosis could have been induced in a small fraction of cells, but that this was below the detection threshold of the DNA fragmentation assay. However, the IFN-γ-regulated apoptosis in UT-OC-2 ovarian carcinoma cells suggests that the method described in this study is sensitive enough to detect apoptotic cell death.

Although the morphological changes in apoptosis have been clarified, little is known about the importance of apoptosis and its regulation in gynecological malignancies. The best characterized genes associated with apoptosis are *c-myc*, *bcl-2*, *ras*, p53 and the genes encoding proteins of transcription factor NF-κB. Wild-type p53 negatively regulates cell growth and division and is classified as a tumor-suppressor gene. In some cell lines, the loss of p53 function has been associated with induction of resistance to TGF-β-induced programmed cell death (Eliopoulos et al. 1995, Zhu et al. 1994). It appears that loss of p53 function either by mutation or by HPV is associated with the ethiopathogenesis of squamous epithelial cancers of the entire lower female genital tract. In fact, mutations of p53 are frequently found in vulvar carcinoma cells (Hietanen et al. 1995b, Rantanen et al. 1998) and also in endometrial and ovarian carcinomas (Diebold et al. 1996, Rantanen et al. 1998). However, the endometrial carcinoma cell line UM-EC-3 and vulvar carcinoma cell lines UM-CSV-1A and UM-CSV-6 (containing human papillomavirus 16) have recently been found to express wild-type p53 (Rantanen et al. 1998). This suggests that the inability of carcinoma cells to undergo apoptosis is not related to the p53 status of our cell models.

The present data show that vulvar, endometrial and ovarian carcinoma cells, when cultured under basal conditions, exhibit constitutive nuclear NF-κB-DNA binding activity. Recently NF-κB has been implicated in the control of apoptosis. The inhibition of the constitutive expression of NF-κB has been found to induce apoptosis in breast cancer cells (Sovak et al. 1997) and B cell lymphomas (Wu et al. 1996). In addition, the inhibition of NF-κB signaling has been observed to increase apoptotic cell death in squamous cell carcinoma (van Hogerlinden et al. 1999). This hypothesis of antiapoptotic role of NF-κB is further supported by the observation that IFN-γ, which increased the activation of NF-κB, was able to inhibit apoptotic cell death in UT-OC-2 ovarian carcinoma cells in the present study. Furthermore, activation of NF-κB by TNF-α has recently been linked to protection of multiple types of cells from apoptosis (Beg and Baltimore 1996, Wang et al. 1996). In addition, the maintenance of NF-κB/Rel expression has been found to protect B cells from TGF-β1-mediated apoptosis (Arsura et al. 1996). The constitutive and TNF-α induced activation of NF-κB may explain the finding of TNF-α and TGF-β being unable to induce apoptosis in the gynecological carcinoma cells investigated in the present study.

3 *Activation of NF-κB in carcinoma cells*

According to several observations, it has been suggested that NF-κB palys a significant role in the regulation of cell growth. However, to date, little is known about the connection between cytokines and transcriptional regulators in gynecological carcinoma cells. TNF-α is a well known inducer of NF-κB activation and it has been previously found that TNF-α is able to inhibit cell proliferation and to induce NF-κB *e.g.* in hepatic cells (Gallois et al. 1998). In addition, TNF-α-induced growth inhibition of human myeloid cells (Chaturvedi et al. 1994, Hu et al. 1999) has been observed to be associated with increase in NF-κB activation. It has moreover been found that NF-κB activation in stratified epithelium is important for growth arrest (Seitz et al. 1998). In accordance with these findings, in the present study, growth-inhibitory TNF-α increased the activation of NF-κB in vulvar and ovarian carcinoma cells, suggesting a role for NF-κB in cytokine-mediated growth-regulation in our cell models.

In UT-OC-2 ovarian carcinoma cells, NF-κB activity was increased by IFN-γ. According to the literature, this is a rare phenomenon, but it has recently been observed that IFN-γ synergistically enhanced TNF-alpha-induced NF-kappaB nuclear translocation via a mechanism that involves the induced degradation of IkappaB (Cheshire and Baldwin 1997). In addition to increasing the activation of NF-κB, IFN-γ was able to exert a supressive effect on the magnitude of apoptosis in UT-OC-2 cells. This observation supports the hypothesis of the antiapoptotic role of NF-κB in various cell types.

In contrast to the effect of TNF-α, IL-10 and IL-13 decreased the binding activity of NF-κB in UM-SCV-1A cells. It has been proposed that the biological effects of IL-10 vary in different cell types. In CD8 T cells, IL-10 is known to accelerate IL-2-dependent proliferation and is, in addition, known to potentiate the IL-2 induced activation of NF-κB (Hurme et al. 1997), while, in human monocytes, the NF-κB activation was inhibited by IL-10 (Wang et al. 1995). In addition, it has been observed that IL-13 suppresses TNF-induced NF-κB activation and apoptosis in myeloid and epithelial cells (Manna and Aggarwal 1998). These observations support the view that interleukins and TNF-α use different signaling pathways for the regulation of cell growth and for the activation of NF-κB (Bauerle and Baichwal, 1997). The previous studies have revealed that NF-κB is involved in the regulation of cell growth although the activation of NF-κB could equelly well be increased as decreased by growth-inhibitory factors (Ferreira et al. 1999, Han et al. 1999, Hwang and Ding 1995, Seitz et al. 1998). The data of the present study support the hypothesis that there is an association between cytokine-mediated growth-inhibition and activation of NF-κB in malignancies originating in the female genital tract.

4 *Activation of AP-1 in carcinoma cells*

AP-1 is thought to play an important role in the control of cell growth. Among numerous agents, cytokines have been observed to regulate AP-1 activity. However, different studies have shown that AP-1 activity may as well be increased as decreased by growth-inhibitory agents (Brach et al. 1993, Dixit et al. 1989). Furthermore, there are observations that different subunits of AP-1 complex have different roles in the control of cell growth. *JunB* especially has been considered as a negative growth-regulator in different cell types (Schlingensiepen et al. 1993). *C-jun* and *c-fos* have been found to participate both in growth-inhibition and induction of apoptotic cell death (Schadendorf et

al. 1996). Although direct evidence is lacking, the significant activation of AP-1 by growth-inhibitory cytokines in the present study suggests that AP-1 may be involved in mediating the antiproliferative effect of cytokines in endometrial and ovarian carcinoma cells.

It is obvious that the function of AP-1 and NF-κB is connected to several different signal transduction pathways. Interactions between steroid hormone receptors and transcription factors, especially in cells originating in the female genital tract, have been studied extensively. It has become evident that AP-1 and NF-κB are components of estrogen- and progesterone-induced signal transduction cascades which regulate target cell proliferation (Hyder et al. 1995, Kalkhoven et al. 1996). The differences in the involvement of transcription factors AP-1 and NF-κB in cytokine-mediated regulation may be related to the fact that endometrial and ovarian cancer are probably hormone-dependent malignancies while the etiology of vulvar cancer is more evidently related to non-hormonal factors such as viral infections.

SUMMARY AND CONCLUSIONS

Numerous investigations show that cytokines have a significant role in the regulation of cell growth. In addition, programmed cell death has been found to be induced by cytokines in numerous cell types. There is also increasing evidence for the role of transcription factors in the cytokine-mediated growth-regulation of cancer cells. This dissertation was undertaken to evaluate the significance of several cytokines as growth-regulatory factors in ovarian, endometrial and vulvar carcinoma cells *in vitro*. The effect of cytokines on DNA synthesis was measured by ^{125}I-deoxyuridine (^{125}IUdR) incorporation assay. In order to better understand the molecular mechanisms of the observed effects, the activation of DNA-binding proteins AP-1 and NF-κB was studied by electrophoretic mobility shift assay (EMSA). In addition, cellular DNA was extracted to determine if the most growth-inhibitory cytokines were able to induce apoptosis. Our results showed that IFNs, IL-10, TGF-β, TNF-α and GM-CSF are primarily growth inhibitory factors in ovarian, endometrial and vulvar carcinoma cells. IL-13 was also able to inhibit cell proliferation in vulvar and endometrial carcinoma cells.

Transcription factors NF-κB and AP-1 were constitutively activated in all cell lines studied. The binding activity of NF-κB was found to be further stimulated by TNF-α in vulvar carcinoma cells whereas activity of NF-κB was decreased by IL-10 and IL-13 in UM-SCV-1A cells. The binding activity of NF-κB was found to be increased by TNF-α in UT-OC-3 and UT-OC-5 cell lines, while in UT-OC-2 endometrioid ovarian carcinoma cells the activity of NF-κB was found to be stimulated by IFN-γ.

The binding activity of AP-1 was found to be stimulated by all growth-inhibitory cytokines studied in UT-OC-5 ovarian and UM-EC-3 endometrial carcinoma cell-lines. In UT-OC-3 ovarian carcinoma cells, the binding activity of AP-1 was found to be stimulated by growth-inhibitory cytokines TGF-β and TNF-α. No effect on AP-1 was found by any of the cytokines in vulvar carcinoma cells. Growth-inhibitory TNF-α, TGF-β_1, IFN-α and IFN-γ were able to stimulate the activity of AP-1 in UT-OC-2 cells.

In all experiments, with the exception of the UT-OC-2 cell line, the electrophoretic analyses after cytokine challenge showed intact DNA, and no signs of DNA fragmentation, a feature typical of programmed cell death, were observed in any of these analyses. DNA extracted from the UT-OC-2

ovarian carcinoma cells showed fragmentation as a sign of apoptosis under basal culture conditions. IFN-γ was found to inhibit apoptotic response whereas TNF-α and TGF-β showed no effect.

The findings of the present study suggests that AP-1 and NF-κB may play a role in regulating of cell proliferation and apoptosis in gynecological carcinoma cells studied. However, it is possible that rather than being specialized mediators of proliferative, antiproliferative or apoptotic responses to exogenous stimuli, these transcription factors may play a general role in signal transduction from the membrane to the nucleus by interacting directly or indirectly with other factors.

The role of cytokines, especially in endometrial and vulvar carcinoma, is poorly understood and therefore the present study offers new knowledge of the action of immunomodulatory factors in gynecological carcinoma cells. Although the results of the present study cannot be compared directly to *in vivo* situations, this *in vitro* study has a significance since recently characterized and low passage cell lines have been used. The enhanced understanding of the function of cytokines and increasing availability of recombinant cytokines may have important implications for novel therapeutic approaches in the future, especially for malignancies thus far having limited therapeutic options, *e.g.* ovarian carcinoma.

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ORIGINAL PUBLICATIONS (I-V)