OPIOID INHIBITION OF THE GROWTH OF THE TRANSPLANTABLE ANDROGEN-RESPONSIVE (AR) SHIONOGI MOUSE MAMMARY CARCINOMA (SC115)

by

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ABSTRACT

We investigated the effects of steroid hormones, growth factors, and anti-growth factor antibodies on the growth of cells in primary culture from the transplantable androgen-responsive (AR) Shionogi mouse mammary carcinoma (SC115). In addition, the effects of opioid agonists and antagonists on the growth of SC115 tumor cells in primary culture were examined. The possible role of opioids in mediating the differential tumor growth rates of mice exposed to experimental housing groups was also examined. SC115 tumor cells were cultured at 5 x 10^4 cells/cm^2 onto collagen-coated 96-well microtiter tissue culture plates. The cells were incubated in medium containing 5% fetal bovine serum (FBS), 2% dextran charcoal-treated FBS (DCTFBS), or seurm-free medium, with or without different concentrations of dihydrotestosterone (DHT), hydrocortisone (HC), 17ß-estradiol (E_2), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), anti-bFGF antibody with either DHT or bFGF, or anti-EGF antibody with either E_2 or EGF. After 48 h, some of the cultures were exposed to 10^{-14}-10^{-7} M of either ß-endorphin (ß-EP), which binds to the δ opioid receptor, cyclazocine (CZ), which binds to the *k* receptor, or morphine sulfate (MS), which binds to the μ receptor. These opioids were added daily for 3 d. In some experiments, 10^{-8} and 10^{-6} M of naloxone, an opioid receptor antagonist, were added with the opioids. On day 5, the cultures were terminated and cell numbers determined by the tetrazolium dye reduction assay.

The results show that AR SC115 tumor cells in serum-containing medium were significantly stimulated to grow in a dose-dependent manner with DHT ranging from 3.5×10^{-10} - 3.5×10^{-6} M and bFGF ranging from 1-100 ng/ml and in serum-free medium were significantly stimulated to grow by bFGF at all concentrations examined (1-500 ng/ml). HC ranging from 10^{-9}-10^{-5} M also significantly stimulated SC115 tumor cell growth. In contrast, E_2 (10^{-9}-10^{-6} M) and EGF (1-100 ng/ml) at all concentrations examined significantly inhibited SC115 tumor cell growth in serum-containing medium, whereas EGF had no effect on SC115 tumor cell growth in serum-free medium at any of these concentrations. Anti-bFGF antibody had a significant inhibitory effect on the DHT- or bFGF-stimulated SC115 tumor cell growth in serum-free medium. Anti-EGF antibody also significantly inhibited SC115 tumor cell growth with or without E_2 or EGF in serum-free medium. All 3 opioids at concentrations higher than 10^{-12} M significantly inhibited SC115 tumor cell growth (up to 40%) in medium that maximally stimulated these cells (DHT at 3.5×10^{-8} M, HC at 10^{-6} M, or 5% FBS), although inhibition also occured, albeit to a lesser degree, in steroid hormone-free medium. ß-EP at concentrations of 10^{-10}-10^{-7} M also inhibited SC115 tumor cell growth when added to medium containing DCTFBS and 10 ng/ml bFGF. When naloxone was added with ß-EP or MS, the inhibitory effects of the opioids were partially or totally blocked. Both ß-EP and CZ also significantly inhibited growth of SC115 tumor cells from mice exposed to different social housing conditions. Overall, ß-EP inhibited growth of SC115 tumor cells from IG mice (smaller

tumors) to a greater degree than SC115 tumor cells from GI mice (larger tumors) in 2% DCTFBS-containing and HC-containing media, whereas the result was reversed in DHT-containing medium. CZ inhibited growth of SC115 tumor cells from GI mice to a greater degree than cells from IG mice in 2% DCTFBS-containing and DHT-containing media, whereas the result was the same in HC-containing medium.

The results suggest that the growth of AR SC115 tumor cells is sensitive to different steroid hormones and growth factors in primary culture. Opioid peptides may be involved in regulating endocrine control of growth of the AR SC115 carcinoma and the inhibitory effects of opioids may be mediated by multiple opioid receptors. In our animal-tumor model, opioids may also play a role in the differential tumor growth rates of mice exposed to the experimental housing groups.

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LIST OF ABBREVIATIONS

α-,ß-,γ-MSH	alpha, beta, gamma-melanocyte-stimulating hormone
ACTH	adrenocorticotropic hormone
AI	androgen-independent
ANOVA	analysis of variance
AR	androgen-responsive
ß-EP	beta-endorphin
bFGF	basic fibroblast growth factor
ß-LPH	beta-lipotropin
BSA	bovine serum albumin
CA	cyproterone acetate
CM	conditioned medium
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
CZ	cyclazocine
DCT	dextran charcoal-treated
DCTFBS	dextran charcoal-treated fetal bovine serum
Dex	dexamethasome
DHT	dihydrotestosterone
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
E_2	17ß-estradiol
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EOP	endogenous opioid peptide(s)
ER	estrogen receptor

ER+	estrogen receptor positive
ER-	estrogen receptor negative
ETOH	ethyl alcohol
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
HC	hydrocortisone
HCl	hydrochloric acid
IFN	interferon
IGF	insulin-like growth factor
IGF-I	insulin-like growth factor-I
IL	interleukin
iv	intravenous
kDa	kilodalton
LH	luteinizing hormone
LPS	bacterial lipopolysaccharide
MS	morphine sulfate
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide
NK cell	Natural Killer cell
PDGF	platelet-derived growth factor
PgR+	progesterone receptor positive
PHA	phytohemagglutinin
POMC	proopiomelanocortin
PWM	pokeweed mitogen
sc	subcutaneous
SC115	Shionogi mouse mammary carcinoma
SC-3	AR cell line derived from the SC115 tumor

SRBC	sheep red blood cell
STV	saline-trypsin-versene
T	testosterone
TGF	transforming growth factor
TP	testosterone propionate

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I. Introduction

1.1 General Introduction

Since the endorphin-like drugs have become increasingly useful for managing pain in patients with advanced cancer, it is important to determine if these same drugs affect tumor growth, thereby ultimately affecting patient survival. This research project was designed to investigate the effects of opioids on the growth of cells from the transplantable androgen-responsive (AR) Shionogi mouse mammary carcinoma (SC115) in primary culture.

The AR SC115 tumor, established by Minesita and Yamaguchi in 1964, has been considered an excellent model to study the proliferative effects of steroid hormones as it contains several steroid hormone receptors, including androgen receptors (Bruchovsky & Rennie 1978; Matsumoto *et al.* 1982), estrogen receptors (Noguchi *et al.* 1984; 1985b), and glucocorticoid receptors (Watanabe *et al.* 1982) and has been shown to be sensitive to androgens (Darbre & King 1987; Jung-Testae *et al.* 1988; Matsumoto *et al.* 1982; Yates & King 1978), estrogens (Noguchi *et al.* 1984; 1985a; 1985b; 1987), and glucocorticoids (Omukai *et al.* 1987; Watanabe *et al.* 1982). This tumor originated spontaneously in a female mouse of the DD/S strain. After 19 passages in male mice, an AR variant arose and grew more rapidly in males than in females. This AR SC115 tumor has maintained its responsiveness to physiological concentrations of androgens *in vivo* (Matsumoto *et al.* 1982) and *in vitro* (Desmond *et al.* 1976; Yates *et al.* 1980; Yates & King 1978) for many years. Furthermore, the growth of the SC115 tumor is also stimulated by

pharmacological doses of glucocorticoids both *in vivo* (Omukai *et al.* 1987; Watanabe *et al.* 1982) and *in vitro* (Omukai *et al.* 1987) and by pharmacological doses of estrogens *in vivo* (Noguchi *et al.* 1987). The growth-stimulatory effects of androgens, estrogens, and glucocorticoids are mediated through their receptors. Using a competitive [^{3}H]-binding assay, androgen receptor complexes can be isolated from both nuclear and cytosol fractions of SC115 tumor cells, which are taken from tumors or culture (Matsumoto *et al.* 1982). These androgen receptor complexes of SC115 tumor cells have similar binding properties to receptors isolated from normal androgen target tissues. The antiandrogen, cyproterone acetate (CA), which by itself is inactive, completely suppresses the androgen-induced effects on growth *in vitro* (Jung-Testae *et al.* 1985; 1988). Recent evidence suggests that the stimulatory effects of androgens and glucocorticoids on SC-3 cell proliferation (an AR cell line derived from the SC115 tumor) may be mediated by the synthesis of a fibroblast growth factor-like polypeptide that acts on the cells in an autocrine or paracrine manner (Lu *et al.* 1989; Nakamura *et al.* 1989; Nonomura *et al.* 1988; 1989). This mouse mammary tumor was selected for studing as it is similar to some human breast cancers in its sensitivity to steroid hormones.

1.2 Human Breast Cancer

It is well known that 1 in 9 women in America will develop breast cancer sometime during their lifetime. This fact has stimulated a major research effort to develop curative procedures

for this disease. Historically, treatments applied specifically to breast cancer have been based on altering the endocrine environment of the tumors (Wakeling 1990). Estrogen receptors (ER) can be detected in 60-80% of human breast cancers. The presence of ER in breast cancer is used as a marker for predicting likely responses to endocrine (*i.e.*, antiestrogen) therapy. Of patients with ER positive (ER+) tumors, 50% will show a beneficial response; of those with ER+ and progesterone receptor positive (PgR+) tumors, 75% will respond to therapy (Horwitz 1987). The presence of ER has also been known to reflect the grade of this tumor; less differentiated, more aggressive tumors tend to be ER negative (ER-; Horwitz 1987). Endocrine treatment of breast cancer classically involves both removal of the ovaries (*i.e.*, reducing estrogens) in premenopausal women and administration of pharmacological doses of estrogens in postmenopausal women (Sutherland 1987). More recently, the antiestrogen tamoxifen has become the initial hormone treatment of choice for both premenopausal and postmenopausal women (Patterson *et al.* 1981). The major antiestrogen effects of tamoxifen are due to competition with estrogen for the high-affinity ER. Binding of tamoxifen to this receptor leaves the cells refractory to further estrogen stimulaton (Dickson & Lippman 1989). Hormonal treatment on relapse involves either the administration of progestins, androgens, or glucocorticoids, or the surgical ablation of the adrenal or pituitary glands. These methods act to decrease estrogen stimulation either directly (oophorectomy), through removal of the precursor necessary for

estrogen formation (adrenalectomy), or through removal of the trophic hormones, adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), or luteinizing hormone (LH), necessary for estrogen formation (hypophysectomy).

Estrogens are mitogens for both normal and malignant breast epithelium (Lippman & Dickson 1989). *In vitro*, it has been observed that many ER+ human breast cancer cell lines, including MCF-7, ZR-75-1, and T-47-D, are growth stimulated by physiological concentrations of 17ß-estradiol (E_2) and inhibited by pharmacological concentrations of E_2, whereas ER- lines do not show these effects (Darbre *et al.* 1983). In the well-characterized MCF-7 human breast cancer cell line, physiological concentrations of E_2 induce cell growth, mitogenic enzyme activity, and metabolic events (Dickson & Lippman 1987). Pharmacological concentrations of E_2 inhibit these events to a level similar to one that is seen with the antiestrogen tamoxifen (Lippman *et al.* 1976). However, tamoxifen can block cell growth in the absence of E_2, suggesting either that tamoxifen might have antimitogenic effects independent of ER (Wakeling 1988), or that the phenol-red containing medium used to culture these cells might have a weak estrogenic effect (Bindal *et al.* 1988). In another ER+ and PgR+ line, T-47-D, E_2 has a biphasic effect on cell growth (Chalbos *et al.* 1982). E_2 at concentrations of less than 10^{-8} M stimulates cell proliferation and at higher concentrations progressively inhibits cell proliferation. The antiestrogen tamoxifen inhibits the E_2-induced stimulation and decreases the growth of control cells. E_2 increases ZR-75-1 cell

growth with half maximal stimulation of cell proliferation at 5 x 10^{-12} M (Labrie *et al.* 1990).

In addition to ER, glucocorticoid receptors have also been found in human mammary epithelial cells and in some human breast cancer cell lines, such as MCF-7 and ZR-75-1 (Horwitz *et al.* 1975). Glucocorticoids have stimulatory effects on normal human mammary epithelial cell growth in primary culture. Yang *et al.* (1980, 1982) demonstrate that hydrocortisone (HC) stimulates mammary epithelial cell growth in a dose-responsive manner. Glucocorticoids have both stimulatory and inhibitory effects on breast cancer cell line growth. A synthetic glucocorticoid, dexamethasome (Dex), inhibits the E_2-stimulated cell proliferation of the MCF-7 cell line in a dose-dependent fashion, and this is antagonized by an antiglucocorticoid (Zhou *et al.* 1989). Although glucocorticoids have inhibitory effects on the proliferation of MCF-7 cells in the presence of E_2 (Lippman *et al.* 1976), it has been reported that HC augments basal and insulin-enhanced DNA synthesis (Linebaugh & Rallerma 1977). Darbre and Daly (1989) report that in the absence of phenol-red, glucocorticoids (*eg.* Dex) have stimulatory effects on the growth of several cell lines, including MCF-7, ZR-75-1, and T-47-D, but this effect is less than the effect of E_2. Glucocorticoids have inhibitory effects on the growth of these cell lines in the presence of phenol-red (Darbre & King 1987). Glucocorticoids and androgens have cumulative inhibitory effect on ZR-75-1 cell proliferation (Labrie *et al.* 1990).

Androgen receptors can be detected in 30-40% of human breast

tumors (Poortman *et al.* 1975). The specific effects of androgens on breast cancer cell proliferation have also been investigated (Labrie *et al.* 1990; Poulin *et al.* 1988). In the ZR-75-1 cell line, physiological concentrations of androgens markedly decrease basal and E_2-induced cell proliferation as well as cell saturation density through their androgen receptors (Poulin *et al.* 1988). Furthermore, the potent antiproliferative effect of androgens is additive to that achieved by antiestrogens. Dihydrotestosterone (DHT) has a biphasic effect on the proliferation of these cells incubated in the absence of E_2. Concentrations of DHT ranging from 10^{-11}-10^{-8} M inhibit basal cell growth to a maximam of 50% in a dose-dependent manner. When concentrations of androgens are increased to 10^{-8}-2 x 10^{-6} M, the inhibitory effects gradually decrease, although cell numbers remain below control levels. The inhibitory effect of DHT is neutralized by coincubating ZR-75-1 cells with an antiandrogen. Testosterone (T) has the same inhibitory effect on the growth of these cells. The mechanism of androgen's inhibitory effect may be due to the fact that androgens exert a potent negative effect on ER expression in this breast cancer cell line. The direct action of androgens on breast cancer cells is also illustrated by the demonstration of an inhibitory effect of androgens on E_2-induced PgR levels in MCF-7 cells (MacIndoe & Etre 1981). It is also illustrated by their stimulation of the secretion of $Zn^{\alpha 2}$-glycoprotein (Chalbos *et al.* 1982) and their stimulation of the expression of the 15 kilodalton (kDa) gross cystic disease fluid protein (GCDFP-15) in T-47-D cells (Chalbos *et al.* 1982). In the

absence of phenol-red, androgens (*eg.* T) have stimulatory effects on growth of several cell lines, including MCF-7, ZR-75-1, and T-47-D, but the effects are less than that of E_2 (Darbre & Daly 1989).

The effects of E_2 on the growth of normal and malignant human breast epithelial cells in primary culture in the presence or absence of serum have also been studied. E_2 at physiological concentrations can stimulate the growth and at pharmacological concentrations inhibit the growth of human breast cells in the presence of serum (Emerman *et al.* 1987). In contrast, E_2 has no growth-stimulatory effects in serum-free medium (Gabelman & Emerman, 1992). This may be due to the absence of serum-born factor(s) required for estrogenic stimulation of proliferation. There is considerable evidence that E_2-stimulated growth is mediated by the production of growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF), and/or their receptors (Barker & Vinson 1990; Dickson *et al.* 1986; Huff *et al.* 1988). An increase in EGF and/or IGF receptor levels by E_2 could enhance the effect on growth of EGF and/or IGF present in serum.

1.3 Effects of Androgens on the Growth of the SC115 Tumor

Androgen is the major steroid hormone that affects SC115 tumor growth. Originally, the AR subline of the transplantable SC115 tumor grew only in intact male mice. It failed to grow in either female or castrated male mice unless these mice were given exogenous androgens (Bruchovsky & Rennie 1978; Emerman & Worth

1985). However, due to the process of progression, the SC115 tumor now also consists of a heterogeneous population of AR and androgen-independent (AI) cells (Buchovsky & Rennie 1978; Emerman & Seimiatkowski 1984; Emerman & Worth 1985). The predominant subpopulation of the SC115 tumor in intact male mice is AR; whereas the predominant subpopulation of the SC115 tumor grown in female or castrated male mice is AI. These two subpopulations of cells have different growth rates and morphologies (Emerman & Siemiatkowski 1984; Emerman & Worth 1985). The AR tumors are generally palpable in intact males 1 wk after tumor-cell injection and grow to a weight of approximately 3 gm by 3 wk. In female or castrated males, the AI tumors are palpable after about 1 mo and grow to almost 1 gm by 2 mo following tumor-cell injection. The AR SC115 tumor grown in intact males is a soft, undifferentiated medullary carcinoma showing a compact polyhedral cell pattern characteristics of epithelial cells. In contrast, the AI tumors lose their cohesive growth pattern, and cells are dispersed into loose sheets and irregular strands which grow in loose stroma. In addition, the AI tumors are relatively hard. Rowse *et al.* (1990b) show that tumors maintained in female mice contain osteoid-like regions which stain positive for sialic acid and sulphate moieties. It has also been shown by others that 83% of the tumors grown in females are slow growing and designated as "hard" tumor due to the remarkable bone formation among these spindle-shaped cells (Kitamura *et al.* 1979). The hard tumors grow equally well in both male and female mice. The cells contain cytosolic androgen receptors but show a postreceptor defect for

androgen actions (Bruchovsky & Rennie 1978).

There are two possibilities that may explain the development of the AI spindle-shaped tumors (Emerman & Worth 1985): (a) The SC115 tumor is composed of two different types of cells, AR and AI cells. The latter becomes predominant with androgen depletion. (b) AR cells change into AI cells in the absence of androgens. A small population of the latter derived from the AR cells can survive and proliferate with androgen depletion. In addition, a large tumor mass leads to accelerated proliferation and autonomy from androgens, perhaps by leading to constitutive production of tumor-specific growth factors, the production of which is initially triggered by androgens (Kitamura *et al.* 1979; Luthy *et al.* 1988).

AR growth of SC115 tumor cells is also maintained in cell culture. Since serum is an undefined mixture of hormones and growth factors, Stanley *et al.* (1977) developed a dextran-charcoal method to remove all the endogenous steroid hormones, including androgens, estrogens, and glucocorticoids, from the serum. A cell line derived from the SC115 tumor cells cultured in medium containing dextran charcoal-treated (DCT) serum show a dose-dependent proliferative response to androgens (Yates & King 1978; 1981). However, after 1 wk of androgen deprivation, the response of SC115 cells to androgen is greatly reduced, and after 2 wk, there is no growth stimulation of androgen-deprived cells by T. Restoration of T to the medium of short-term androgen-deprived cells results in an increase in the growth response to T, eventually reaching the level observed for cells maintained in

androgen. Long-term androgen deprivation causes an irreversible loss of the androgen response.

Androgen withrawal leading to a loss of androgen responsiveness in these cells is accompanied by morphological changes, increased sensitivity to serum, increased density dependency (lower saturation density), and increased anchorage dependency (Yates *et al.* 1980). AR SC115 tumor cells are elongated and rounded, fibroblastic in appearance, grow in an irregular manner and overlap to form multilayered foci (Yates & King 1981). In the absence of androgen, however, the cells are flatter and more epithelial-like with little overlapping. Although the cells are stimulated to grow by androgens, this obviously does not reflect the SC115 tumor grown *in vivo*. Emerman and Worth (1985) demonstrate that the growth rates, morphologies, and tumorigenic potentials characterizing the AR SC115 tumor and its AI variant *in vivo* persist when cells are cultured on collagen gels rather than on plastic. It has been shown that normal and malignant mammary epithelial cells from several species retain their morphological and functional characteristics when grown on a collagen substrate (Emerman *et al.* 1990; Emerman & Pitelka 1977; Emerman & Worth 1985). Cells from the AR SC115 tumor cultured in DHT-containing medium grow faster than cells in DHT-deprived medium and cells from AI tumors. AR tumor cells maintained in medium containing DHT form a confluent pavement, similar to the tumors *in vivo*. The culture morphologies of AR tumor cells grown in the absence of DHT and of AI tumor cells cultured in the presence or absence of DHT resemble the

histologic pattern of AI tumors *in vivo*. Cells form small clumps or cords of cells or remain isolated. Tumors arising from injection into male mice of cells from freshly dissociated AR tumors or cells of AR tumors cultured in the presence of DHT appear more rapidly and grow faster in intact males than in females or castrated males. Tumors arising from cells cultured in the absence of DHT or from freshly dissociated or cultured cells of AI tumors have identical rates of appearance and growth in all hosts.

Cells of the AR and AI SC115 tumors have been characterized by flow cytometric analysis of their DNA content and by karyotypic analysis of metaphase spreads (Emerman & Kalousek 1987). Both tumors have diploid and near tetraploid populations of cells. However, data suggest that AR and AI malignant cells both appear to be polyploid. A decrease in the polyploid population of the AR tumor accompanies tumor regression following castration, but this population is restored when tumor growth resumes. Karyotypic analysis of metaphase spreads of cells from both AR and AI tumors shows a range of 55-88 chromosomes and they contain the same chromosome anomalies. Differencies between the cell populations of the AR and AI tumors remain to be elucidated.

Cells from the AR tumor cultured in androgen have the same proportion of diploid and polyploid cells as the AR tumor *in vivo* (Emerman *et al.* unpublished results). If androgen is removed from the culture, growth slows and the percentage of polyploid cells decreases. When growth resumes following replacement of androgen, the polyploid population increases comparable to that *in vivo*.

The ratio of diploid to polyploid cells in cultures of cells from the AI tumor is similar to that of the AI tumor *in vivo* in medium with or without androgen. The karyotype is also maintained in the polyploid populations in culture.

1.4 Effects of Glucocorticoids on the Growth of the SC115 Tumor

Watanabe *et al.* (1982) studied the effect of glucocorticoids on the growth of the SC115 tumor *in vivo*. When castrated male mice are treated with Dex for 2 wk at daily doses of 1-5 mg/kg starting 3 d before tumor inoculation, the tumor weight as well as transplantability are significantly increased. Daily injections of high doses of Dex (4 mg/kg), but not physiological doses (160 μg/kg) markedly stimulate tumor growth, approaching the growth rate found in a normal male mouse (Omukai *et al.* 1987). The SC115 tumor grown in Dex-treated castrated male mice has morphplogical, biochemical, and biological characteristics similar to those of the SC115 tumor grown in intact male mice. Dex does not interact with androgen receptors. It may act directly through glucocorticoid receptors or its action may be mediated in part by the suppression of the immune system of the host. Glucocorticoids have been shown to have effects on virtually every component of the immune system in both animals and humans (Munck *et al.* 1984). Generally, they have immunosuppressive and anti-inflammatory effects at pharmacological doses. There are two indirect pieces of evidence that the immunological status of the host may affect the growth of the SC115 tumor. Kitamura *et al.* (1979; 1980) demonstrate that

the SC115 tumor grows in both castrated male and female nude athymic mice. In addition, Nohno *et al.* (1986) reports that a single administration of S. aureus increases host immunity and decreases the transplantability of the SC115 tumor. It has recently been shown that the presence of the SC115 tumor has an effect on the immune system (Rowse *et al.* 1990a). A study was undertaken to determine if natural killer (NK) cell activity is involved in mediating the effects of differential housing conditions on SC115 tumor growth rate (see below). Splenic NK cell activity was assayed at 24 h, 3 d, and 1 wk post-injection in both tumor- and vehicle-injected animals. Significant stimulation of splenic NK cell activity occurs 3 d post-injection of SC115 cells. This study demonstrates that the SC115 tumor is capable of stimulating NK cell activity. However, it is not yet clear if modulation of the NK cell activity has an effect on mediating the growth rate of this tumor from mice in different housing conditions.

In addition to androgen receptors, glucocorticoid receptors have also been found in the cytosol of SC115 cells (Waranabe *et al.* 1982). In serum-free cell culture, the proliferation of SC-3 cells is markedly (up to 25 fold) stimulated by 10^{-11}-10^{-8} M T, whereas proliferation is only slightly (up to 3.3 fold), but significantly stimulated by 10^{-8}-10^{-5} M Dex (Omukai *et al.* 1987). Both androgens and glucocorticoids bind to their own receptors, but do not bind with each other's receptors. Dex shows both inhibitory and stimulatory effects on androgen-induced proliferation of SC115 cells in culture (Hiraoka *et al.* 1987).

Stimulation of SC-3 cells induced by greater than 10^{-8} M T is significantly inhibited by the addition of 10^{-8}-10^{-5} M Dex in a dose-dependent manner, whereas a lesser degree of stimulation induced by less than 10^{-10} M T is significantly enhanced by the addition of Dex. The mechanism of the biphasic effect of Dex is not clear at this point, but it might occur at the post-receptor level. It has been hypothesized that Dex-glucocorticoid receptor complexes inhibit the binding of the more active T-androgen receptor complexes to the acceptor sites on the chromatin. This hypothesis is further supported by the finding that 10^{-8} M Dex stimulates the synthesis of five secretory proteins in SC-3 cells that are identical to those induced by 10^{-8} M T (Nakamura *et al.* 1987a).

The interaction between physiological doses of androgens and pharmacological doses of glucocorticoids in stimulating the proliferation of the SC115 tumor is very interesting. AR cells are sensitive to both androgens and glucocorticoids, whereas AI cells are unresponsive to both steroids. Darbre and King (1987) find that the two steroids can interact to prevent the progression of steroid insensitivity that occurs when one hormone is removed. SC115 cells can be protected against a loss of response to either androgens or glucocorticoids with either steroid alone. That is, androgen protects against loss of glucocorticoid sensitivity and vice versa. Yates and King (1978) demonstrated that Dex has a biphasic effect on a cell line from the AR SC115 cell growth in the absence of T *in vitro;* at 10^{-8} M,

it inhibits proliferation by 30% but at 10^{-6} M, it stimulates growth by 235%.

1.5 Effects of Estrogens on the Growth of the SC115 Tumor

Noguchi *et al.* (1984) and Nohno *et al.* (1982) found ER in the cytosol of subcutaneous (sc)-grafted SC115 tumors. When E_2 at a daily dose of 2 mg/kg is given to intact male mice bearing the SC115 tumor, tumor growth as well as the weight gains of seminal vesicles and prostate glands are inhibited. Estrogen binds to ER but also binds to androgen receptors which is likely responsible for its inhibitory effects on growth. In contrast, Noguchi *et al.* (1984) found a stimulatory effect of E_2 on SC115 tumor growth. Although a physiological concentration of E_2 (40 μg/kg) does not significantly enhance tumor growth, daily injections of 160-400 μg/kg of E_2 significantly stimulate growth in a concentration-dependent manner (Noguchi *et al.* 1984). The growth rate stimulated by 4 mg/kg of E_2 approaches the level induced by 4 mg/kg testosterone propionate (TP) (physiological dose). Low doses of androgens alone (400 μg/kg of TP) fail to maintain the SC115 tumor (Noguchi *et al.* 1985a). Tumor growth in castrated male mice treated with a low dose of TP (400 μg/kg) is similar to that of the AI tumor developed from the original SC115 tumor after androgen removal. A dose of 160 μg/kg E_2 can act synergistically with low doses of TP (400 μg/kg) to stimulate the growth of the SC115 tumor. Addition of 400 μg/kg E_2 to 400-1000 μg/kg TP significantly augments growth.

The morphology of tumors was also examined (Noguchi *et al.* 1985b). Tumors grown in castrated males are composed of medullary cells if stimulated by daily injections of 4 mg/kg of TP whereas tumors consist of spindle-shaped cells if the host is injected with vehicle only. If the SC115 tumors are grown in mice injected with 400 or 1000 μg/kg of TP or 400 μg/kg of E_2, about one half of the tumors are of the spindle-cell type and the other half, of mixed type. In contrast, most tumors are composed of the medullary type when E_2 is added to 400 or 1000 μg/kg of TP.

The stimulatory effect of estrogen-induced proliferation of SC115 cells is shown to be mediated by the ER but not the androgen receptor (Noguchi *et al.* 1985b). Daily injection of high doses of diethylstilbestrol, which does not bind to the androgen receptor even at high concentrations, significantly stimulates tumor growth in a dose-dependent manner. Moreover, the anti-androgen, CA, has no inhibitory effect on E_2-induced growth of the SC115 tumor but significantly inhibits in a dose-dependent manner the TP-induced growth of the SC115 tumor and of seminal vesicles.

In 1976, Jung-Testae *et al.* and King *et al.* found ER in SC115 cells in culture, but did not correlate E_2 with a proliferative response of the cells. In cell culture (Nakamura *et al.* 1987b; Noguchi *et al.* 1987), E_2 (10^{-12}-10^{-6} M) has no effect on SC-3 cells in serum- supplemented medium, whereas E_2 (10^{-9} - 10^{-6} M) as well as CA (10^{-8}-10^{-6} M) inhibit the growth-stimulatory effect of T (10^{-10} M) on SC-3 cells in a dose-dependent manner in medium with DCT serum. This is probably due

to the competitive inhibition of T binding to its receptor by E_2 and CA.

1.6 The Proliferation of Androgen-induced SC115 Tumor Cells may be Mediated by a FGF-like Polypeptide through an Autocrine Mechanism

It is suggested that androgen-induced growth of SC115 cells may be mediated by the production of a growth factor(s). Using [^{35}S]-methionine labeling of SC115 cells in primary culture, Jung-Testae *et al.* (1988) have shown several intracellular and secreted proteins with molecular weights of 55, 40, and 15 kDa specifically induced by T. Furthermore, the culture medium conditioned by T-treated SC115 cells has significant mitogenic activity on L929 mouse fibroblasts. However, the growth factor(s) is not characterized (Jung-Testae & Baulieu 1985). Using serum-free (Lu *et al.* 1989; Nakamura *et al.* 1987b; 1989; Nonomura *et al.* 1988) and protein-free (Tanaka *et al.* 1990) media, investigators have been able to demonstrate that androgen- and glucocorticoid-induced proliferation of SC115 tumor cells is caused by a fibroblast growth factor (FGF)-like polypeptide in an autocrine fashion. Nakamura *et al.* (1987b) found that both cell numbers and DNA synthesis of SC-3 cells are significantly stimulated by conditioned medium (CM) from SC-3 cells exposed to T, whereas the CM from cells not exposed to T has no growth stimulatory effects. The CM was fractionated using gel filtration. Specific fractions markedly stimulate the proliferation of SC-3 cells without T and the morphology changes

from epithelial to spindle-shaped (Sato *et al.* 1988). The activity present in CM is not antagonized with the anti-androgen CA at 10^{-6} M, eliminating the possibility that a residual amount of T remaining in the CM induces these events (Nakamura *et al.* 1987b; Sato *et al.* 1988). The purified androgen-induced growth factor from the CM can be associated with FGF receptor on SC-3 cells, strongly suggesting that this growth factor is an FGF-like polypeptide (Nonomura *et al.* 1990). The androgen-induced growth factor eluted from the heparin-Sepharose column reacts with anti-FGF antibody when examined by radioimmunoassay and specifically inhibited the binding of [^{125}I] bFGF to FGF receptor and these activities are exactly in parallel to the growth-stimulatory activity (Nonomura *et al.* 1989).

Nakamura *et al.* (1989) further examined the effects of various growth factors, including EGF, transforming growth factor-alpha (TGF-α), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), insulin, TGF-ß, bFGF, and aFGF on the growth of the SC-3 cell line in serum-free medium. The proliferation of SC-3 cells reaches a plateau with 10^{-8} M T (up to 200-fold), 10^{-7} M Dex (up to 30-fold), and 1 ng/ml of FGF (up to 50-fold). In contrast, all concentrations tested of progesterone, E_2, EGF, PDGF, IGF-I, insulin, and TGF-α have no effects. However, the addition of EGF (1 ng/ml) significantly enhances the T-induced growth of SC-3 cells (Nakamura *et al.* 1987a; Noguchi *et al.* 1987). TGF-ß (1 ng/ml) slightly stimulates the growth (up to 5-fold) of these cells but markedly inhibits the growth stimulation induced by T (Yamanishi *et al.* 1990).

Although the T-induced growth is almost completely inhibited by TGF-ß, b- or a-FGF-like peptide-induced growth is only partially inhibited (45%) by TGF-ß. This difference can be explained by the fact that TGF-ß decreases the amount of T-induced FGF-like peptide secreted from SC-3 cells to 18% of control. This inhibitory effect is found to be reversible.

Androgen-induced proliferation of SC-3 cells (either directly or by CM) is neutralized by anti-bFGF antibody (Luthy *et al.* 1988; Nonomura *et al.* 1989; Tanaka *et al.* 1990). Anti-bFGF antibody can significantly inhibit 10^{-8} M T-, 10^{-7} M Dex-, and 0.1 ng/ml bFGF-induced proliferation of SC-3 cells by 70%. This is dose-responsive within the concentrations of 100-600 ng/ml of anti-bFGF antibody. These results provide strong evidence that the physiological doses of androgens and pharmacological doses of glucocorticoids produce a FGF-like polypeptide that stimulates SC-3 cell growth through an autocrine mechanism.

1.7 The Endogenous Opioid Peptide (EOP) System

Since 1975, when EOP were first isolated from the brain (Hughes *et al.* 1975), their anatomical distribution and physiological functions have been extensively studied. In mammals, these peptides can be derived from at least 3 distinct genes: the ACTH/ß-endorphin (ß-EP) precursor (proopiomelanocortin or POMC), the enkephalin precursor (proenkephalin A), and the dynorphin/neo-EP precursor (proenkephalin B) (Akil *et al.* 1984; 1988). Using molecular biology techniques, the POMC gene, first discribed by Nakanishi *et al.* (1979), has been found to contain

the 31 amino acid peptide ß-EP at its carboxy terminus and the 91 amino acid precursor ß-lipotropin (ß-LPH, which contains ß-melanocyte stimulating hormone (MSH) in some species); the midregion of this precursor contains $ACTH_{1-39}$ (which can be cleaved into α-MSH and CLIP or $ACTH_{18-39}$). At the amino terminus of this precursor is the active ACTH/MSH core, known as γ-MSH. In the proenkephalin A precursor, all of the known active peptides are opioid in nature. It contains 7 peptides with the Met- or Leu-enkephalin active core. Four of the 7 peptides produced are simple Met-enkephalin, two are carboxyl extended Met-enkephalin-Arg-Phe-Arg-Ghy-Leu, and the final one is Leu-enkephalin (Comb *et al.* 1982; Noda *et al.* 1982). The proenkephalin B precursor produces 3 main Leu-enkephalin-containing peptides: α/ß-neo-EP, dynorphin A, and dynorphin B (Kakidani *et al.* 1982). All of these peptides have one structural feature in common, namely, a N-terminal sequence beginning with the same four amino acids (Tyr-Gly-Gly-Phe).

The EOP are widely distributed in both central and peripheral nervous systems. The major location of POMC is the pituitary (Bloom *et al.* 1977). In man, it is found mainly in the anterior lobe, but in most other species, it is found primarily in the intermediate lobe. There are two cell groups in the brain that produce ß-EP/ACTH peptides: one is in the region of the arcuate nucleus of the medial basal hypothalamus (Bloch *et al.* 1978) and the other is in the nucleus of the solitary tract and the nucleus commissuralis (Schwartzberg & Nakane 1981). Proenkephalin A is also widespread in the hypothalamus,

pituitary, and central nervous system. In the brain, it is found at every level from the cortex to the spinal cord (Elde *et al.* 1976). Unlike POMC peptides, proenkephalin-like peptides are also found in the adrenal medulla, the gastrointestinal tract, the autonomic nervous system (with the catecholamines), and several other structures. Recently, the pro-neo-EP/dynorphin precursor has been found in the gut, posterior pituitary, and brain. It is found in scattered cell groups throughout the brain stem and in several hypothalamic nuclei including vasopressin-producing cells of the magnocellular portion of the paraventricular nuclei (Khachaturian *et al.* 1986; Watson *et al.* 1982).

The final products produced by and stored within a given neuron depend not only on the genetic code for the precursor, but also on the program that directs enzymes to process the precursor in certain ways---the post-translational processing events. The precursor is translated from its mRNA using ribosomal machinery and emerges in the form of a protein. The opioid precursors have a molecular weight of about 25-30 kDa. The precursor is then processed by proteolytic enzymes, which produce individual peptide domains. POMC cells of the anterior pituitary and those of the intermediate lobe of the pituitary process the precursor quite differently (Akil *et al.* 1981; Zakarian & Smyth 1982). In the anterior lobe, $ACTH_{1-39}$ is the major product; whereas in the intermediate lobe, this peptide is further processed to produce the highly modified α-MSH [N-acetyl $ACTH_{1-13}$-NH_2] and CLIP or $ACTH_{18-39}$. All of the ß-LPH in the anterior lobe is converted to ß-EP in the intermediate lobe. The potent opioid peptide ß-EP_{1-31}

undergoes α-N-acetylation at the tyrosine residue, or it is cleaved at the carboxyl-terminus to remove the last four to five residues, or both. Thus the main product in the intermediate lobe is not ß-EP_{1-31}, but rather N-acetyl-ß-EP_{1-27}, a peptide with greatly decreased receptor affinity, which has been proposed as a possible endogenous antagonist.

The processing of POMC in the brain is less understood and controversial. The ß-EP_{1-27} form exists in substantial quantities. While N-acetylation occurs in some brain regions, it is less common than in the intermediate lobe of the pituitary (Evans *et al.* 1982; Zakarian & Smith 1982).

The post-translational processing of proenkephalin in the brain often yields the enkephalin pentapeptides. In the adrenal gland, post-translational processing may yield large peptides, such as peptide E and peptide F which contain the enkephalin sequences at each terminus (Kimura *et al.* 1980).

Processing of prodynorphin yields two possible neo-EPs, α and ß, which differ by a lysine residue (Kanagawa *et al.* 1981), as well as dynorphin A_{1-17}, which further cleaves to dynorphin A_{1-8}. The latter is the major product in the brain, but not in the pituitary (Weber *et al.* 1982). Prodynorphin processing in the pituitary is less well known.

The multiple opioid receptor system was first postulated by Martin *et al.* (1976). The basic opioid receptors including mu (morphine), kappa (ketocyclazocine), delta (enkephalin), and a specific ß-EP receptor (epsilon), show different degrees of selectivity for the different peptides (Akil *et al.* 1988).

Pharmacological studies show that multiple opioid receptors interact with the multiple opioid peptides. ß-EP, except for its unique epsilon receptor, recognizies both mu and delta sites, with a slight preference for delta. All of the proenkephalin-related peptides show delta receptor activity ranging from Leu-enkephalin, which is primarily delta, to the enkephalin octapeptides, which appear to be equally mu and delta. All dynorphins and neo-EPs show a preference for the kappa receptor. Dynorphin$_{1-8}$, however, retains delta capability, whereas dynorphin$_{1-23}$ acts potently at both mu and kappa receptors. Most of the opioid peptides and drugs perform their functions by interacting with at least one of the four receptor types.

1.8 EOP Interaction with Endocrine Functions

EOP are important in the control of many body functions, including analgesia (Jaffe & Martin 1980)), behavioral (Jaffe & Martin 1980), endocrine (Van Augt & Meites 1980), and immune functions (Fischer 1988; Fischer & Falke 1984; Weber & Pert 1984), as well as in tumor biology (Lewis *et al.* 1983; Zogan & McLaughlin 1981a). The effect of morphine, an opioid agonist, in analgesia was found a century ago. The dense concentrations of opioid receptors in the hypothalamus and pituitary suggest that they have endocrine functions, that is, they may play a physiological role in regulating pituitary hormone release (Van Augt & Meites 1980).

In general, the EOP, morphine, and related drugs exert similar effects on acute release of pituitary hormones (Van Augt

& Meites 1980). A high dose of opioids produces a rapid increase in the release of prolactin, growth hormone, ACTH, antidiuretic hormone, and possibly MSH, and a decrease in gonadotropins, thyrotropin, and oxytocin. Continous administration of opioids may lead to attenuation or even loss of their influence on secretion of some pituitary hormones (Van Augt & Meites 1980). Rivier *et al.* (1977) confirm that ß-EP (120 µg/kg) and morphine (4 mg/kg) injected intravenously (iv) into male rats stimulate prolactin and growth hormone release; this effect can be reversed by the opioid antagonist, naloxone (1 mg/kg). Naloxone (250 mg/h, iv, for 8 h) alone has been shown to reduce basal serum levels of prolactin and growth hormone and to elevate serum levels of LH and FSH, as well as to decrease a stress-induced rise of prolactin in rats (Armstrong *et al.* 1988; Rivier *et al.* 1977). Further, Seigel *et al.* (1982) demonstrate that acute naloxone (5 mg/kg) administration produces a basal and stress-induced hypersecretion of ACTH and corticosterone in adult male rats. It is generally believed that opioid peptide regulation of pituitary hormone release occurs at the level of the hypothalamus, mainly via hypothalamic neurotrasmitters (dopamine, norepinepherine, serotonin, acetylcholine, and others) which regulate release of the hypothalamic-releasing hormones into the pituitary portal vessels (Van Augt & Meites 1980).

1.9 EOP Interaction with Immune Functions

Evidence accumulated in the past few years suggests that opioids may influence the immune system. The EOP have been found

to influence the functions of most of the major cell types in the immune system. The different EOP have different immunomodulatory functions even though they have identical NH_2-terminals. Opioid peptides affect various immunocyte-mediated events such as NK cell activity (Faith *et al.* 1984; Mattews *et al.* 1983), antibody production (Johnson *et al.* 1982), mononuclear cell chemotaxis (Van Epps *et al.* 1983), and the generation of cytotoxic T cells (Carr & Klimpel 1986). Some effects can be blocked by an opioid receptor antagonist, and some cannot, suggesting that both opioid specific and non-specific ligands exist on the immune cells.

Data indicate that lymphocytes, phagocytic leukocytes, and terminal comlexes of complement possess opioid receptors (Carr *et al.* 1988; Hazum *et al.* 1979; Schweigerer *et al.* 1983). Early work demonstrates the presence of high affinity opioid receptors on murine splenocytes and human leukocytes (Johnson *et al.* 1982; Mehrishi & Mills 1983)). Using SUPERFIT, Carr *et al.* (1988) demonstrate that both human and murine splenic and peripheral leukocytes possess specific binding sites for the σ-class ligand, a specific receptor ligand for σ-receptor.

(1) Antibody Production

Johnson *et al.* (1982) report that α-endorphin (>=0.05 μM) as well as Met- and Leu-enkephalin (>=0.02 nM) are potent suppressors of antibody production. In contrast, ß- and γ-EPs have no effect on antibody production. Suppressive effects of α-EP, Met-, and Leu-enkephalins can be partially inhibited by naloxone (3 μM) or ß-EP (12 μM). Others (Heijnen & Ballieux 1986)

have found that if added to the medium at the appropriate time, ß-EP enhances the number of antibody-forming cells and that this effect is initiated by the COOH-terminal end of the peptide.

(2) SRBC-Rosetting

Met- and Leu-enkephalins increase the percentage of T cells that form active rosettes with sheep red blood cells (SRBC), but depress total T cell rosette formation (Miller *et al.* 1983; Wybran *et al.* 1979). Morphine (0.1-100 nM) reduces the frequency of active and total SRBC rosette formation. This effect of morphine is reversed by naloxone (Bayer *et al.* 1990).

(3) Lymphocyte Proliferation

Gilman *et al.* (1982) investigated the effects of α- and ß-EP and [D-Ala-Met]-enkephalin in modulating the proliferative response of splenic T lymphocytes to mitogenic stimulation. ß-EP at doses of 1-100 ng/ml (rat physiological concentrations) enhances the proliferative response of rat splenic cells to the T cell mitogens Concanavalin A (ConA) and phytohemagglutinin (PHA) but not to the B cell mitogens bacterial lipopolysaccharide (LPS) and dextran sulfate. This is not reversed by 10 μM naloxone. Neither α-EP nor [D-Ala-Met]-enkephalin has this effect. These data suggest that the enhanced T cell proliferative response to mitogens may be mediated by a non-opioid but ß-endorphin-specific ligand. However, others have reported (Kusnecov *et al.* 1987) that ß-EP (10^{-12}-10^{-9} M) induces a dose-responsive enhancement of the proliferative response of rat splenic T cells to ConA, which is

inhibited by naloxone (10^{-6} M). The question of whether or not the effects of opioid peptides on the immune system are mediated by opioid or non-opioid receptors needs further investigation. In contrast, in humans it has been shown that ß-EP has a suppressive effect on PHA-induced lymphocyte proliferation by a stimulating suppressor T cells and that this effect is not naloxone-reversible (McCain *et al.* 1982). Conflicting results may be due to the differences in species, lymphocyte source, and concentrations used by particular researchers.

Generally, *in vitro*, data suggest that high pharmacological concentrations of morphine and methadone (0.1 mM or more) depress the PHA-, ConA-, or pokeweed mitogen (PWM)-induced blastogenesis of both T and B lymphocytes (Bocchini *et al.* 1983), whereas lower concentrations of morphine (0.1 µM) appear to have inconsistent effects (Maravelias & Contselinis 1984).

(4) Cytotoxic Activity

Opioid peptides enhance the generation of cytotoxic T lymphocytes (CTL, Carr & Klimpel 1986) as well as the expression of NK cell-mediated cytotoxicity (Faith *et al.* 1984; Mandler *et al.* 1986). ß-EP as well as Met- and Leu-enkephalins have been demonstrated to enhance NK cell activity (Faith *et al.* 1984; Mattews *et al.* 1983). These effects can be inhibited by the opioid antagonist, naloxone, suggesting that enhanced activity is mediated via an opioid receptor. In normal volunteers and cancer patients, Plotnikoff *et al.* (1986) found that Met-enkephalin (1-250 µg/kg) significantly increases total lymphocytes, T cell

subset, and NK cell activity and increases mitogen-stimulated lymphocyte blastogenesis with PHA, ConA, and PWM. Interestingly, Williamson *et al.* (1987) found that ß-EP_{1-31} has a biphasic effect on human NK cells. Preincubation of effector human lymphocytes with 10^{-11}-10^{-7} M ß-EP containing an unmodified N-terminal sequence increases NK cell activity. In contrast, preincubation with lower concentrations (10^{-18}-10^{-13} M) of ß-EP reduces NK cell activity, which bind only to non-opioid receptors. Shavit *et al.* (1987) report that a single exposure to the opioid form of footshock stress or a single high dose (35-50 µg/kg) of morphine induces suppression of rat splenic NK cell cytotoxicity. This effect appears 3 h after treatment, returning to normal by 24 h. The effect of morphine is blocked by naltrexone, another opioid antagonist. Naltrexone-reversible suppression of NK cell activity has been seen in cells derived from the spleen, bone marrow, and peripheral blood, suggesting that this suppression does not result from a selective egress of NK cells from the spleen. In contrast, Mandler *et al.* (1986) demonstrated that ß-EP (10^{-10}-10^{-7} M), but not α-EP or γ-EP, significantly augments human NK cytolytic activity by 63% in a naloxone-reversible manner.

Overall, the opioid peptides can both increase and reduce lymphocyte cytotoxicity depending on the opioids, the concentrations used, and whether binding occurs at the N- or C-terminal sequence.

(5) Chemotactic Response of Monocytes and Neutrophils

Opioid peptides are able to stimulate chemotaxis of monocytes and neutraphils both *in vivo* and *in vitro*. Van Epps *et al* (1983) show that ß-EP and Met-enkephalin (10^{-8} M) can interact with and stimulate leukocyte migration, particularly monocytes, lymphocytes, and possibly neutrophils. Furthermore, in the case of monocytes, this stimulation of migration is directional, indicating that ß-EP has chemotactic activity. Migration can be blocked by naloxone, suggesting opioid receptor involvement.

(6) Factors Released by Lymphocytes

Another effect of opioid peptides on the immune system is that they can modulate production of lymphokines such as interferon-γ (IFN), interleukin-2 (IL-2), and lymphocyte chemotactic factors. Brown and Van Epps (1986) demonstrate that ß-EP and Met-enkephalin (10^{-14}-10^{-10} M) significantly enhance IFN production by human peripheral lymphocytes incubated with ConA. This effect is not inhibited by naloxone (10^{-8} M) and varies from donor to donor. There is also no absolute correlation between an enhanced response to ß-EP and an enhanced response to Met-enkephalin. Gilmore and Weiner (1988) investigated the effect of ß-EP on IL-2 production. ß-EP (10^{-6} M) enhances the production of IL-2 from ConA-stimulated, unfractionated murine splenocytes as well as from a mouse T cell lymphoma. Met- and Leu-enkephalins (10^{-12}-10^{-6} M) do not have this effect. ß-EP-stimulated IL-2 production is not reversed by naloxone and dependent on the integrity of the C-terminal amino acids. The *in vitro*

proliferative expression of both CTL and NK cells is regulated by IL-2. IL-2 production may be a mechanism by which the cytotoxic activity of CTL and NK cells is increased by ß-EP.

1.10 Opioids and Tumors

Evidence suggests that opioid peptides are also involved in tumor biology (Lewis *et al.* 1983; Zagon & McLaughlin 1981a). Opioid peptides have the ability to retard normal cell growth (Slotkin *et al.* 1980; Willson *et al.* 1976), particularly in developing neural tissues. Mechanisms involved include antimitotic and cytotoxic activity, opioid-induced damage to chromosomes or suppression of cell division, and alterations in polyamine, nucleic acid or protein systhesis. Several tumor cell lines, including neuroblastomas (Kazmi & Mishra 1986), MCF-7, a breast cancer cell line, (Maneckjee *et al.* 1990), and small cell carcinomas (Roth & Barchas 1986) have been found to contain multiple opioid receptor subtypes.

Opioid agonist-modulation of the growth of neuroblastoma has been well studied. Chronic administration of heroin (3-15 mg/kg daily), a narcotic opioid compound, effectively retards tumor growth and prolongs survival time in mice with transplanted neuroblastoma (Zagon & McLaughlin 1981a). The antitumor effect is blocked by simultaneous administration of the opioid antagonist, naloxone. Heroin's antitumor effect has been confirmed by *in vitro* investigations (Zagon & McLaughlin 1981c). The addition of heroin in concentrations ranging from 10^{-8}-10^{-2} M to S20Y (a neuroblastoma cell line) 24 h after cell seeding, produces a

dose-dependent inhibition of growth. This growth-retarding effect is reversible, since removal of heroin allows cells to resume normal growth. Analysis of mitotic figures reveals that heroin affects cell division. The action of heroin in perturbing cell growth is also blocked by concomitant administration of naloxone (10^{-4} M). Moreover, opioid antagonists alone may also have stimulatory or inhibitory effects on neuroblastoma growth depending on the dosage used (Zagon & McLaughlin 1981a, 1981c, 1985). For example, naloxone (5-20 mg/kg daily), a non-addictive opioid antagonist, exerts a similar effect on neuroblastoma growth in mice as heroin itself (Zagon & McLaughlin 1981b). Naltrexone, another opioid antagonist that is eight times as active and three times as long-acting as naloxone (Blumberg & Dayton 1973), administered once daily at 10 mg/kg (a dose which blockes the opioid receptor for 24 h), stimulates tumor growth and shortens survival time in mice innoculated with neuroblastoma cells. In contrast, a dosage of 0.1 mg/kg (which blocks the opioid receptor for 4-6 h) has remarkable antitumor effects (Zagon & McLaughlin 1983).

Besides neuroblastoma, opioid agonists and antagonists are also involved in other carcinogenic processes, including mammary carcinoma, lymphoma, melanoma, and tumor matastasis (Aylsworth *et al.* 1979; Murgo 1985). Pretreatment with naloxone and naltrexone significantly inhibits growth of carcinogen-induced mammary cancer in rats (Aylsworth *et al.* 1979) and causes a complete regression in mice with spontaneous and transplanted mammary tumors (Tsunshima *et al.* 1982). Similarly, a well characterized

human breast cancer cell line, MCF-7, has been found to possess multiple opioid receptor subtypes (Maneckjee *et al.* 1990). Opioid ligands specific for the different receptor subtypes significantly inhibit the growth of MCF-7 cells in a dose-related manner. This inhibition is reversed by concomitant addition of naloxone to cell culture. Met- and Leu-enkephalins significantly inhibit the growth rate of the B16-BL6 tumor in mice and decrease the numbers of metastases in lung (Murgo 1985; Scholar *et al.* 1987). Furthermore, clinical studies show that a high percentage of primary ductal carcinoma of the breast contains scattered tumor cells with opioid peptide immunoreactivity (Scopsi *et al.* 1989) and two small cell carcinoma cell lines contain opioid peptide receptors (Roth & Barchas 1986).

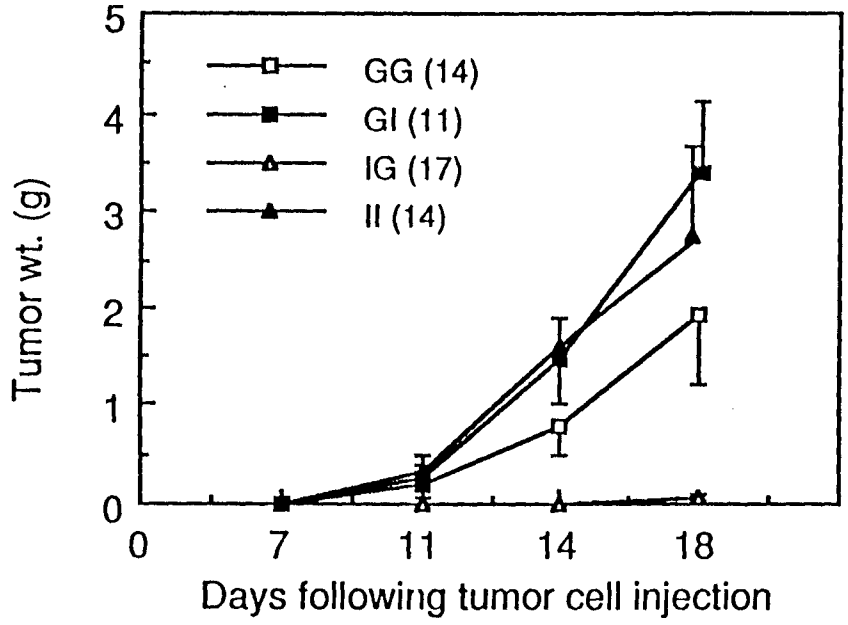
1.11 Animal-tumor Model for Studing Effects of Stressors on Mammary Tumor Growth

An animal-tumor model has been developed in our laboratory to examine the effects of social housing condition on growth of the SC115 tumor (Weinberg & Emerman 1989). Following weaning at 3 wk of age, male mice of the DD/S strain are housed individually (I) or in groups of 3 (G). At 2-4 mo of age, animals are injected with tumor cells or vehicle and experimental groups formed as follows. Mice reared individually remain individually housed (II) or are rehoused in groups of 5 (IG). Those reared in groups remain in their groups (GG) or are rehoused individually (GI). Interactive effects of changes in housing conditions and an acute psychological stressor (daily exposure to a novel environment)

are also examined. Thus half of the animals in each housing condition are exposed for 15 min/day to 1 of 5 different novel environment (see 2.1.4 below). Data demonstrate that animals reared individually and remaining individually housed (II) or reared in a social group and then singly housed (GI) following tumor cell injection show markedly increased tumor growth rate compared to that in mice remaining in the rearing group (GG), if animals are also exposed to acute novelty stress. In contrast, mice reared individually and then moved to a large social group (IG) following tumor cell injection show markedly reduced tumor growth, both in the presence or absence of acute daily novelty stress (Fig.1, Weinberg & Emerman 1989).

As noted above, androgens and glucocorticoids, both stress-related hormones, have significant effects on the growth rate of SC115 tumor cells. Therefore, it is hypothesized that an alteration in the endocrine function of the mice may be involved in the differential tumor growth rates observed in this model. A study was undertaken (Rowse *et al.* 1992) to investigate the possibility that an effect of housing condition on plasma levels of androgens and glucocorticoids may, in part, mediate the differential tumor growth rates observed in this model. Plasma T and corticosterone levels were assayed 24 h, 3 d, and 1 wk post tumor cell/vehicle injection and group formation. Basal levels of plasma T are elevated in mice of the GG, GI, and II groups, but not in mice of the IG group, at 1 d post-injection and group formation. At 3 d and 7 d, T levels of mice in the GG and II condition decline, whereas basal T levels of mice in the GI

Fig.1 Tumor growth in male mice in the four housing groups: GG, raised and maintained in silbing groups of two to three; GI, raised in sibling groups of two to three, then seperated and housed singly; IG, raised singly housed, then rehoused in nonsibling groups of four to five; II, raised and maintained singly housed. () = *n* per group. On Day 18 following tumor cell injection, GI = II > GG > IG, $p < .05$ (Weinberg & Emerman 1989).


GG (14)
GI (11)
IG (17)
II (14)
Tumor wt. (g)
0
1
2
3
4
5
0
7
11
14
18
Days following tumor cell injection

condition remain elevated. In contrast, basal levels of plasma corticosterone are significantly elevated in IG animals compared with animals in all other groups on all 3 test days. Overall, plasma corticosterone levels show significantly greater elevations at 1 d post injection than at 1 wk post injection although levels are within the physiological basal range at all times. This experiment demonstrates that changes in plasma hormone levels may be important mediators of the differential tumor growth rates observed in mice housed under the different housing conditions in our model.

Another possible mediator of the differential tumor growth rates observed in this model is a shift in the responsiveness of the tumor cells to hormones. As noted previously, the SC115 tumor is heterogeneous, containing AR and AI cells (Emerman 1988; Emerman & Worth 1985). It has been demonstrated that growing SC115 tumor cells in an androgen deprived environment (in a female mouse or *in vitro*) results in the selection of AI cells (Darbre & King 1987; Emerman 1988; Emerman & Worth 1985). Thus it is possible that selection for cells with greater (AR) or lesser (AI) hormone sensitivity may occur in animals in the 4 housing conditions, resulting in the differential tumor growth.

In a recent study, this possibility was investigated. *In vitro* proliferation of tumor cells from IG and GI animals in response to DHT and HC was examined (Rowse *et al.* 1992). Data show that cells from both groups are significantly stimulated by both DHT and HC. In fact, tumor cells from IG animals have a significantly greater response to DHT and HC stimulation than do

tumor cells from GI animals. This result suggests that the slower growth rates of tumors in mice of the IG conditions result from alterations in the internal environment of the mice rather than from a decrease in the tumor cells' ability to respond to hormones.

1.12 The Objectives of the Thesis

There are two main objectives of this thesis:

(1) The first objective was to extend the study of hormone modulation of SC115 tumor growth. Most of the previous *in vitro* work characterizing the SC115 tumor has been done using an AR cell line derived from the SC115 tumor. The present study is unique in that SC115 primary cultures were examined. The effects of steroid hormones, growth factors, and anti-growth factor antibodies on the growth of SC115 tumor cells in serum-containing and serum-free medium in primary culture were investigated. In addition, this is the first study to examine the effects of opioid agonists and antagonists on the regulation of AR SC115 tumor growth.

(2) The second objective of this thesis was to examine the possible role of opioid peptides in modulating the differential tumor growth rates of mice from our experimental housing groups. For this study, tumors were taken from mice in GI (largest tumors) and IG (smallest tumors) conditions, dissociated to single cells and cultured in serum-containing medium. *In vitro* response to steroid hormones and opioids of AR SC115 tumor cells from mice in GI and IG conditions was studied.

Two major sets of experiments have been completed.

I. Preliminary experiments to develop precedures

(1) to choose the better procedure for measuring cell growth: the MTT assay versus hemacytometer counts.

(2) to choose the optimal seeding density for the cell culture studies.

(3) to find the appropriate serum-free medium for growing SC115 tumor cells.

II. Primary Experiments

(1) to study the effects of steroid hormones and growth factors on the growth of SC115 tumor cells in serum-containing and serum-free medium.

(2) to study the effects of anti-bFGF and anti-EGF antibodies on the growth of SC115 tumor cells in serum-free medium.

(3) to study the effects of opioid agonists and antagonists on the growth of SC115 tumor cells.

(4) to study the effects of opioids on the growth of SC115 tumor cells from mice in GI (largest tumors) and IG (smallest tumors) conditions.

II Materials and Methods

2.1 General Methods

2.1.1 Materials

(1) Steroid Hormones and Growth Factors

Dihydrotestosterone (DHT), hydrocortisone (HC), 17ß-estradiol (E_2), epidermal growth factor (EGF), mouse anti-EGF antibody, and insulin were purchased from Sigma Chemical Co. (St. Louis, MO). Basic fibroblast growth factor (bFGF) (human recombinant) was purchased from Upstate Biotechnology, Inc. (UBI, Lake Placid, NY). IgG fraction of bFGF antibody was purchased from R & D Systems, Inc. (Minneapolis, MN). The anti-bFGF antibody was prepared in rabbits by injection of highly-purified native bovine brain bFGF. Rabbit IgG used as the control was a kind gift from Dr. J. H. Ledsome, Dept. of Physiology, University of British Columbia (Vancouver, B. C. Canada).

DHT, HC, and E_2 were prepared by dissolving in 95% ethyl alcohol (ETOH, Sigma) in 10, 5, and 2 mg/ml stock solution, respectively, diluted to working solutions of 100, 500, and 20 µg/ml, aliquoted at 1 ml/tube into polypropylene tubes (1.8 ml size, Falcon, Lincoln Park, NJ), and then stored at -20°C. The final concentration of ETOH in the culture medium was less than 0.05%. Insulin dissolved in 0.005 M HCl was stored in a working solution of 0.5 mg/ml at 4°C for 2 wk. Basic FGF was diluted to 125 ng/µl in serum-free medium containing 0.5% bovine serum albumin (BSA, pH 7.0-7.5, Sigma), aliquoted to 10-20 µl/tube (Falcon, 1 ml polypropylene tubes), and stored at 4°C for 1 mo. EGF at a concentration of 10 ng/µl in distilled H_2O was aliquoted

to 100 µl/tube and stored at -20°C. Lyophilized bFGF antibody (1 mg/vial) was reconstituted in 48.5 µl dH_2O and used immediately. Mouse anti-EGF antibody (0.1 ml/vial) was diluted to a working solution of 1:10 in serum-free medium, aliquoted to 0.5 ml/tube (1.8 ml size polypropylene, Falcon), and stored at 20°C for 1 wk.

(2) Opioids

ß-Endorphin (ß-EP), cyclazocine (CZ), and morphine sulfate (MS) were supplied by Peninsula laboratories (San Carlos, CA), Sterling Drug Co., and BDH Inc. (Toronto, Ontario), respectively. ß-EP was dissolved in 0.005 M acetic acid, aliquoted at 2-4 µg/glass tube (12 x 17 cm), lyophilized, and stored at 4°C. ß-EP solution was made fresh with each medium change. CZ was dissolved in 0.005 M HCl at a concentration of 1 mg/ml. MS was dissolved in dH_2O at a concentration of 10 mg/ml. Both CZ and MS stock solutions were aliquoted at 1 ml/tube (1.8 ml) and kept at 4°C. Naloxone (Sigma) was dissolved in 0.005 M HCl at a concentration of 4 mg/ml, aliquoted at 1 ml/tube (1.8 ml), and stored at 4°C.

2.1.2 Maintaining the SC115 Tumor

The androgen-responsive (AR) Shionogi mouse mammary carcinoma (SC115) was maintained in our laboratory by serial transplantation in intact male mice (mice that are not castrated) of the DD/S strain. The dissociation of tumors was as described in our standard laboratory protocol (Emerman 1988; Emerman & Worth 1985). Tumors weighing approximately 2 g were dissected free of subcutaneous (sc) tissue and finely minced. The pieces

were transferred to a flask containing 0.05% trypsin (1:250; Grand Island Biological Co. [GIBCO], Burlinton, Ontario) and 0.25% ethylenediamine tetraacetic acid (EDTA) (Sigma) in Ca^{2+}- and Mg^{2+}-free saline A, pH 7.3. The flask was shaken at 37°C for two 7 min periods. At the end of each peroid, the supernatant was decanted and cells in suspension were collected by centrifugation at 80 x g for 4 min. The pellets were resuspended in Dulbeco's modified Eagle's medium (DMEM, Terry Fox Laboratory, Vancouver, B.C.), combined, and then passed through a 150 μm Nitex (Tetko Inc., Elmsford, N.Y.) to collect single cells or small cell aggragates. Viable cells, determined by trypan blue exclusion, were counted on a hemacytometer (described below). Suspensions of 3 x 10^6 cells in 0.1 ml DMEM were injected sc into the interscapular region of intact male mice 2-4 mo old. The remaining cells were resuspended in freezing medium (Appendix 1), frozen in 1.8 ml tubes at concentrations of 1-1.5 x 10^7 cells/ml/tube, and stored in liquid nitrogen for use in cell culture studies.

Cell counting was conducted using a hemacytometer. One drop of trypan blue was added to a cell suspension (10 ml) and mixed. A drop of cell suspension was placed onto both side wells of the hemacytometer. Viable cells were counted from the 4 corner squares of both wells.

2.1.3 Harvesting Cells

In the initial experiments, cell viability was determined by trypan blue exclusion and viable cells were counted using a

hemacytometer. In order to do this, cells had to be cultured onto 35 mm or 16 mm tissue culture dishes (Falcon). Not only did this procedure require a large number of cells, which was sometimes difficult to obtain from dissociations of fresh tumors, but it was also time consuming.

The tetrazolium dye reduction (MTT) assay (Carmichael *et al.* 1987) modified for our laboratory procedures (described below; Stingl *et al.* 1992) was then used to measure viable cell number. This dye is a soluble yellow compound that is cleaved by the dehydrogenase enzymes of mitochondria in living cells to form an insoluble purple formazan product. Therefore, the amount of formazan product is proportional to the number of viable cells in the population. The cells were cultured in 96-well microtiter plates and the plates were counted using a 96-well microtiter reader (model EL 311, Biotek Instruments Inc., Winooski, Vermont). This assay was used in order to process large numbers of samples rapidly. There was a linear relationship between cell counts and the MTT assay, so that the MTT assay was used routingly in subsequent experiments. Results were expressed as a percentage of the controls (control conditions specified in each experiment).

(1) Hemacytometer Counts

SC115 tumor cells were cultured onto collagen-coated 35 mm dishes or 16 mm wells. At the end of the experiments, media were removed and 1-2 ml of collagenase (Appendix 2) was added to each dish for 5 h in order to detach the cells from the plates. This

was followed by adding 1 ml of saline-trypsin versene (STV, Appendix 3) for another 30 min. Cell viability was determined by trypan blue exclusion and viable cells were counted on a hemacytometer.

(2) The MTT Assay

[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT, Sigma) was dissolved in phenol red-free F12/DMEM/H (Sigma) and filter sterilized using a 0.22 μm filter (Falcon). Media were removed and 1 mg/ml of MTT solution was added to each well at 100 μl/well for 5 h at 37°C. At the end of this incubation, 100 μl of 20% formal in saline was gently added to each well for 30 min at room temperature to fix the cells to the plates. After removing the MTT-formal-saline solution, 100 μl of isopropanol (Sigma) was added to each well for 1 h to dissolve the formazan crystals. The plates were read under a 96-well microplate reader. The amount of formazan crystals formed was determined by absorbance at 540 nm. Results were expressed as a percentage of the controls.

2.1.4 Animal-tumor Model for Studying Effects of Stressors on Mammary Tumor Growth

The colony of DD/S mice was maintained in our laboratory according to our standard protocol (Weinberg & Emerman 1989). Mice were reared in sibling groups of 3 from weaning (3 wk of age) until adulthood. Animals were housed under conditions of controlled temperature (22°C) and lighting (12 h light; 12 h

dark) in a colony room that was protected from extraneous laboratory noise.

Experimental conditions designed to study the effects of stressors on SC115 tumor growth were described as in Weinberg & Emerman (1989). Mice were reared either individually housed or in sibling groups of 3 from weaning until adulthood (2-4 mo of age). At the start of the experiment, animals were injected sc in the interscapular region with a single cell suspension of 3×10^6 SC115 cells in 0.1 ml of DMEM. Two groups, balanced for age, were formed immediately following tumor-cell injection:

(1) IG---male mice raised singly housed were rehoused into groups of 5 and injected with tumor cells.

(2) GI---male mice raised in sibling groups of 3 were separated, singly housed, and injected with tumor cells.

These different housing conditions reliably result in differential tumor growth rates (Weinberg & Emerman, 1989). In addition, animals in all groups were subjected to acute daily novelty stress (being placed into 1 of the 5 novel environments). This treatment, which reliably raises corticosterone levels (Friedman & Ader 1967) and the corticosterone response is slow to habituate (Hennessy & Lesine, 1977; Pfister & King 1976), maximizes the differential tumor growth rates observed (Weinberg & Emerman 1989). Five different novel environments were used: (1) a clear plastic container, 9 cm in diameter x 7 cm in height, (2) a polypropylene container, 12 x 10 x 4 cm, (3) a polyethylene container, 6 cm in diameter x 10 cm in height, (4) a cardboard box divided into compartments, 7 x 7 x 14 cm and, (5) a standard

rodent cage, 18 x 29 x 13 cm, empty of bedding, food, and water. Animals were exposed to one of the five environments (as ordered above) each day, 15 min/day, 5 d/week, between 0800 and 1200 h. Animals were not subjected to stress on the day of the experiments.

2.2 Methods for Preliminary Experiments

For the experiments described below, frozen SC115 tumor cells were used unless otherwise indicated.

2.2.1 Seeding Cells on Day 0 Versus Day 1

This experiment was designed to determine if tumor cells would grow better if given a one day recovery period after thawing and before seeding or if seeded immediately after thawing. We thought that a one day recovery period might allow the dead or dying cells to be easily separated from viable cells.

SC115 tumor cells were quickly thawed and washed twice with DMEM. Half of the cells were seeded immediately at 5×10^4 cells/cm^2 onto 35 mm collagen-coated tissue culture dishes in DMEM containing 2% dextran charcoal-treated fetal bovine serum (DCTFBS, also called basic medium, Appendix 4) with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M). The remaining cells were first plated overnight onto 10 cm non-tissue culture Petri dishes (Falcon) in 15 ml basic medium at 37° C, 95% air: 5% CO_2. The next day, the cell suspension was centrifuged and the supernatant decanted to eleminate non-viable cells. The cells were then seeded at 5×10^4 cells/cm^2 onto collagen-coated dishes. Media

were changed 48 h after cell seeding and thereafter on alternative days. On day 7 (after cell seeding), the cultures were terminated and viable cells, determined by trypan blue exclusion, were counted on a hemacytometer.

2.2.2 Seeding Density Experiment

This experiment was designed to choose the optimal seeding density for all cell culture studies in this thesis. SC115 tumor cells were quickly thawed and seeded at 2.5×10^4, 5×10^4, and 1×10^5 cells/cm^2 onto collagen-coated 16 mm wells in basic medium with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M). Media were changed every other day. On day 5, cultures were terminated. Viable cells, determined by trypan blue exclusion, were counted on a hemacytometer.

2.2.3 Selecting an Appropriate Serum-free Medium for the Growth of SC115 Tumor Cells in Primary Culture

This experiment was designed to find the appropriate serum-free medium for growing SC115 tumor cells. Three serum-free media were tested: serum-free-I (F12/DME/H-containing 0.1 % BSA, Appendix 5), which is the serum-free medium described in the literature for growing SC-3 cells (Nonomura *et al.* 1988; Omukai *et al.* 1987), serum-free-II (F12/DME/H-containing 0.1 % BSA, 0.1 µg/ml insulin, and 1.0 ng/ml cholera toxin, Appendix 5), and serum-free-III (F12/DME/H-containing 0.1 % BSA, 1.0 µg/ml insulin, and 10 ng/ml cholera toxin, Appendix 5) which are two serum-free media used for growing human mammary epithelial cells

in our laboratory (Emerman *et al.*, manuscipt in preparation). SC115 tumor cells were quickly thawed, washed twice with DMEM, and incubated in basic medium in non-tissue culture Petri dishes overnight. The cell suspension was centrifuged to separate viable cells from non-viable cells. Viable cells were seeded at 5×10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates and incubated in basic medium. On day 1, the serum-containing medium was removed and serum-free-I, serum-free-II, and serum-free-III media were added to the plates. The media were changed on day 3, 4, and 5. The cultures were terminated and cell numbers determined by the MTT assay. Cell growth was expressed as a percentage of the controls.

SC115 tumor cells grew optimally in serum-free-II medium (see Results Section). Therefore, the serum-free-II medium was used for all experiments (in this thesis serum-free medium always refers to serum-free-II medium).

2.2.4 Determining Time Points for Studing the Effects of Opioids on SC115 Tumor Cell Growth

In a series of preliminary experiments, cultures were terminated at different time points to identify the most appropriate time to measure the effects of opioids on growth. SC115 tumor cells were grown for 3 d, 5 d, and 7 d.

In the first set of experiments, SC115 tumor cells were cultured at 5×10^4 cells/cm^2 in basic medium with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M) on collagen-coated 96-well plates. Three opioids, ß-EP, CZ, and MS, at concentrations of 10^{-11}-10^{-7}

M were added to cultures on days 1 and 2; cultures were terminated on day 3 and growth determined by the MTT assay.

In the second set of experiments, SC115 tumor cells were cultured at 5 x 10^4 cells/cm^2 in basic medium with or without DHT (3.5 x 10^{-8} M) or HC (10^{-6} M) on collagen-coated 96-well plates. ß-EP, CZ, and MS at concentrations of 10^{-11}-10^{-7} M were added to cultures on days 2 and 4; cultures were terminated on day 5 and growth determined by the MTT assay.

In the third set of experiments, SC115 tumor cells were cultured at 5 x 10^4 cells/cm^2 in basic medium with or without DHT (3.5 x 10^{-8} M) or HC (10^{-6} M) on collagen-coated 35 mm plates. ß-EP, CZ, and MS at concentrations of 10^{-9}-10^{-7} M were added on days 2, 4, and 6. Cultures were terminated on day 7 and viable cell numbers determined by trypan blue exclusion and hemacytometer counts. Growth was expressed as a percentage of the controls.

2.3 Experiments

2.3.1 Effects of Steroid Hormones and Growth Factors on the Growth of SC115 Tumor Cells in Serum-containing Medium

SC115 tumor cells were quickly thawed, washed twice with DMEM, and incubated in basic medium in non-tissue culture Petri dishes overnight. The cell suspension was centrifuged to separate viable from non-viable cells. Viable cells were then seeded at 5 x 10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates and incubated in DMEM under one of the following 3 conditions: 1) DMEM plus 2% DCTFBS (basic medium); 2) basic medium plus one of

the following steroid hormones: DHT ranging from 1 ng/ml-1 μg/ml (3.5×10^{-10}-3.5×10^{-6} M), HC ranging from 3.6 ng/ml-3.6 μg/ml (10^{-9}-10^{-5} M), or E_2 ranging from 0.27 ng/ml-0.27 μg/ml (10^{-9} - 10^{-6} M); or 3) basic medium plus one of the following growth factors: bFGF or EGF at concentrations of 1, 10, and 100 ng/ml (5.7×10^{-11}-5.7×10^{-9} M and 1.65×10^{-10}-1.65×10^{-8} M, respectively). The media were changed on days 2, 3, and 4. On day 5, cultures were terminated and cell numbers determined by the MTT assay. Cell growth was expressed as a percentage of the controls.

2.3.2 Effects of Steroid Hormones, Growth factors, and Anti-growth Factors on the Growth of SC115 Tumor Cells in Serum-free Medium

SC115 tumor cells were quickly thawed, washed twice with DMEM, and incubated in basic medium in non-tissue culture Petri dishes overnight. The cell suspension was centrifuged to separate viable from non-viable cells. Viable cells were seeded at 5×10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates and incubated in basic medium. On the next day (day 1), the serum-containing medium was removed and the serum-free medium alone or serum-free medium containing one of the following was added to the plates: 1) steroid hormones: 0.01 μg/ml DHT (3.5×10^{-8} M) or 2.7 ng/ml E_2 (10^{-8} M); 2) growth factors: bFGF at concentrations of 1, 10, 100, 250, and 500 ng/ml or EGF at concentrations of 1, 10, 50, and 100 ng/ml; 3) bFGF at concentrations of 1, 10, 100, 250, and 500 ng/ml with 3.5×10^{-8} M DHT; 4) 400 μg/ml anti-bFGF

alone or with 3.5 x 10^{-8} M DHT or with 10 ng/ml bFGF; 5) control antibody alone or with 3.5 x 10^{-8} M DHT or with 10 ng/ml bFGF; 6) 1:100 dilution of anti-EGF alone or with 10^{-8} M E_2 or with 10 ng/ml EGF; and 7) control antibody alone or with 10^{-8} M E_2 or with 10 ng/ml EGF. The media were changed on day 4 and on day 6, cultures were terminated and cell numbers determined by the MTT assay.

2.3.3 Effects of Opioids on the Growth of SC115 Tumor Cells

SC115 tumor cells were quickly thawed, washed twice with DMEM, and incubated in basic medium in non-tissue culture Petri dishes overnight. Viable cells, collected by centrifugation, were seeded at 5 x 10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates and incubated in 5% FBS (containing endogenous physiological concentrations of steroid hormones) or basic medium with or without 0.01 μg/ml (3.5 x 10^{-8} M) DHT, 0.36 μg/ml (10^{-6} M) HC, or 10 ng/ml bFGF. Concentrations of steroid hormones and growth factor were those shown to be optimal for growth stimulation in the experiments above. Beginning on day 2 in culture, ß-EP, CZ, or MS were added with each medium change for the next 3 consecutive days at concentrations ranging from 10^{-11}-10^{-7} M. On day 5, cultures were terminated and cell numbers determined by the MTT assay. Cell growth was expressed as a percentage of the controls.

2.3.4 Effects of the Opioid Antagonist Naloxone on the Growth of SC115 Tumor Cells

SC115 tumor cells were quickly thawed, washed twice with 10 ml DMEM, and incubated in basic medium in non-tissue culture Petri dishes overnight. Viable cells, collected by centrifugation, were seeded at 5 x 10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates and incubated in basic medium with or without 0.01 µg/ml (3.5 x 10^{-8} M) DHT or 0.36 µg/ml (10^{-6} M) HC. Beginning on day 2 in culture, naloxone, an opioid antagonist, at concentrations of 10^{-8} and 10^{-6} M (3.6 ng/ml and 360 ng/ml) was added with or without either ß-EP (10^{-9} M) or MS (10^{-9} M) with each medium change for the next 3 d. On day 5, cultures were terminated and cell numbers determined by the MTT assay. Cell growth was expressed as a percentage of the controls.

2.3.5 Effects of Opioids on the Growth of SC115 Tumor Cells from the Animal-tumor Model

This study examined the effects of opioids on hormone responsiveness of SC115 tumor cells taken from mice in our experimental housing conditions. Animals were terminated at 3 wk post tumor-cell injection, when the faster growing tumors had reached a mass of 2-3 g. Tumors were removed from GI (largest tumors) and IG (smallest tumors) animals, and dissociated to single cell suspensions (as described in 2.1.2). Cells were seeded at 5 x 10^4 cells/cm^2 onto collagen-coated 96-well microtiter plates either with basic medium alone or with basic medium plus either 3.5 x 10^{-8} M DHT or 10^{-6} M HC. Cells were

incubated for 5 d at 37°C 5% CO_2. ß-EP and CZ ranging from 10^{-10}-10^{-7} M were added to half of the wells in each condition on days 2, 3, and 4 with medium changes. In another experiment, naloxone (10^{-10}, 10^{-8}, and 10^{-6} M) was also added alone or combined with 10^{-9} M ß-EP. On day 5, numbers of viable cells were determined by the MTT assay.

2.4 Statistical Analysis

Statistical analysis of the data was carried out using analyses of variance (ANOVAs) to examine dose-response relationships for steroid hormones, growth factors, and opioids. 1 way ANOVA was used for the factors of group or dose as appropriate for each experiment. 2 way ANOVA was used for the factors of group and dose for each experiment. Significant main effects or interactions in ANOVAs were further analyzed using Tukey's post-hoc tests.

III. Results

3.1 Results of Preliminary Experiments to Develop Procedures

3.1.1 Seeding Cells on Day 0 Versus Day 1

In this experiment, Shionogi mouse mammary carcinoma (SC115) cells were thawed and seeded at 5 x 10^4 cells/cm^2 onto collagen-coated tissue culture dishes with or without dihydrotestosterone (DHT, 3.5 x 10^{-8} M) or hydrocortisone (HC, 10^{-6} M). Seeding was done either immediately (day 0) or following pre-incubation with DMEM containing 2% dextran charcoal-treated fetal bovine serum (DCTFBS), hereafter called basic medium, in non-tissue culture Petri dishes for 24 h. Fig.2 shows that cells pre-incubated for 1 d had a higher growth rate in all media than cells seeded immediately after thawing. Therefore, pre-incubation of cells for 24 h prior to seeding was chosen as the standard method for all experiments using frozen SC115 tumor cells.

3.1.2 Seeding Density Experiment

SC115 tumor cells were seeded onto collagen-coated plates with basic medium, or medium containing DHT (3.5 x 10^{-8} M), or HC (10^{-6} M) to determine the optimal initial seeding density. Fig.3 shows the growth rates after 5 d in culture of cells seeded at 2.5 x 10^4, 5 x 10^4, and 1 x 10^5 cells/cm^2 in basic medium with or without DHT or HC. At seeding densities of 5 x 10^4 and 1 x 10^5 cells/cm^2, cell growth was optimally stimulated by DHT and HC. However, at a seeding density of 5 x 10^4 cells/cm^2, cultures became 75-80% confluent whereas at a seeding density of 1 x 10^5 cells/cm^2, cultures were 100% confluent in these media.

Fig.2 Seeding cells on day 0 versus day 1. Shionogi mouse mammary carcinoma (SC115) cells were grown for a total of 7 d. The growth of SC115 tumor cells seeded on day 1 was greater than that of cells seeded on day 0 in DMEM containing 2% dextran charcoal-treated fetal bovine serum (DCTFBS, also called basic medium) and in dihydrotestosterone (DHT; 3.5×10^{-8} M)-, and hydrocortisone (HC; 10^{-6} M)-containing media. Data represent the results of 2 replications. The control group consisted of cells grown in basic medium alone. Data are expressed as a percentage of the controls.

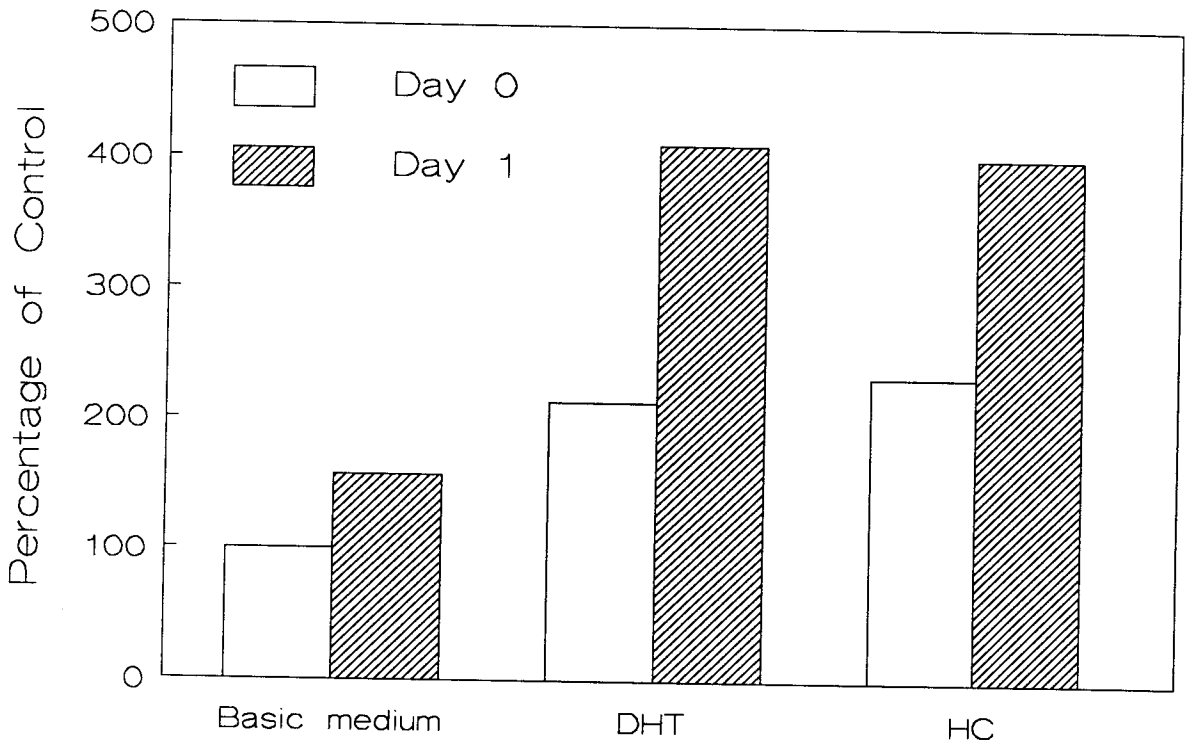
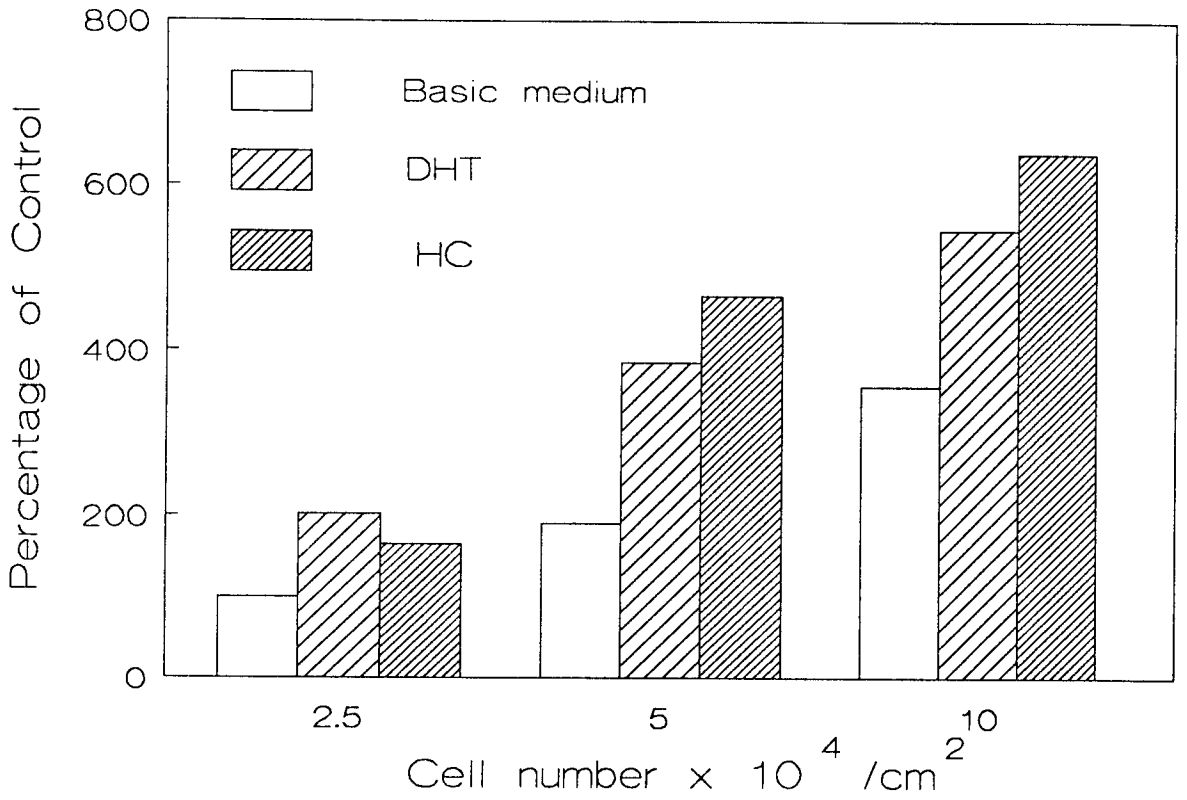

500
400
300
200
100
0
Percentage of Control
Day 0
Day 1
Basic medium
DHT
HC

Fig.3 Seeding density experiment. SC115 tumor cells, seeded with 2.5×10^4, 5×10^4, and 1×10^5 cells/cm^2, were grown for a total of 5 d. The growth of SC115 tumor cells was increased with increasing seeding densities whether in basic medium, or in DHT (3.5×10^{-8} M)-, or HC (10^{-6} M)-containing medium. Data represent the results of 2 replications. Error bars are too small to show. The control group consisted of cells grown in basic medium. Data are expressed as a percentage of the controls.

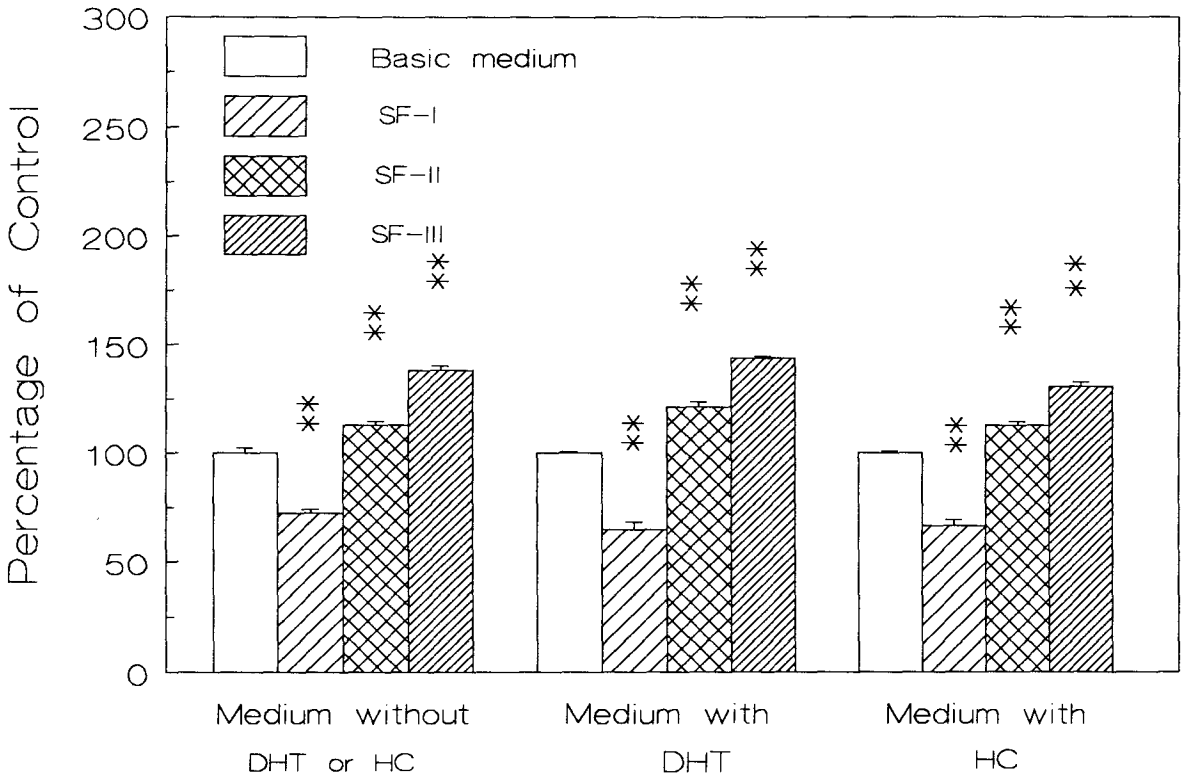

800
600
400
200
0
Percentage of Control
Basic medium
DHT
HC
2.5
5
10
Cell number x 10 4 /cm 2

Therefore, a seeding density of 5 x 10^4 cells/cm^2 was chosen as the standard density for all experiments, since cultures terminated after 5 d were still in the exponential growth phase.

3.1.3 Selecting an Appropriate Serum-free Medium for the Growth of SC115 Tumor Cells in Primary Culture

This experiment was designed to determine the appropriate serum-free medium for growing SC115 tumor cells in primary culture. Growth of SC115 tumor cells was examined in serum-free-I medium, serum-free-II medium, and serum-free-III medium with and without DHT (3.5 x 10^{-8} M) or HC (10^{-6} M). The ANOVA showed a main effect of treatment ($p<0.01$). Post-hoc tests showed that cell numbers were lower in serum-free-I medium than in basic medium, $p<0.01$, basic medium with DHT, $p<0.001$, and basic medium with HC, $p<0.001$ (Fig.4). In contrast, cell growth was significantly stimulated in serum-free-II and serum-free-III media compared to that in basic medium, p's<0.01, basic medium with DHT, p's<0.01, and basic medium with HC, p's<0.01. Furthermore, cell growth in serum-free-III medium was also significantly stimulated compared to serum-free-II medium in all 3 conditions, p's<0.01. Since the growth pattern of SC115 tumor cells in serum-free-II medium was closer to basic medium than that in either serum-free-I or serum-free-III medium, serum-free-II medium was chosen as the optimal serum-free medium for growing SC115 tumor cells (from now on, serum-free medium refers to serum-free-II medium).

Fig.4 Growth of SC115 tumor cells in serum-free (SF) medium. SC115 tumor cells were seeded onto collagen-coated 96-well plates (5×10^4 cells/cm^2) containing 100 ul basic medium. On the next day, medium was changed to basic medium with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M), or SF-I, SF-II, and SF-III media with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M). Cell growth was expressed as a percentage of the controls (basic medium with or without DHT or HC). Values represent means ($\pm$SEM) of 6 determinations. SC115 tumor cell growth was significantly inhibited in SF-I medium alone ($p<0.01$), or SF-I medium with DHT ($p<0.01$), or HC ($p<0.01$) compared to that in basic medium, but cell growth was significantly increased in both SF-II and SF-III media with either DHT ($p<0.001$) or HC ($p<0.001$) compared to that in basic medium. **, $p<0.01$ compared to controls.

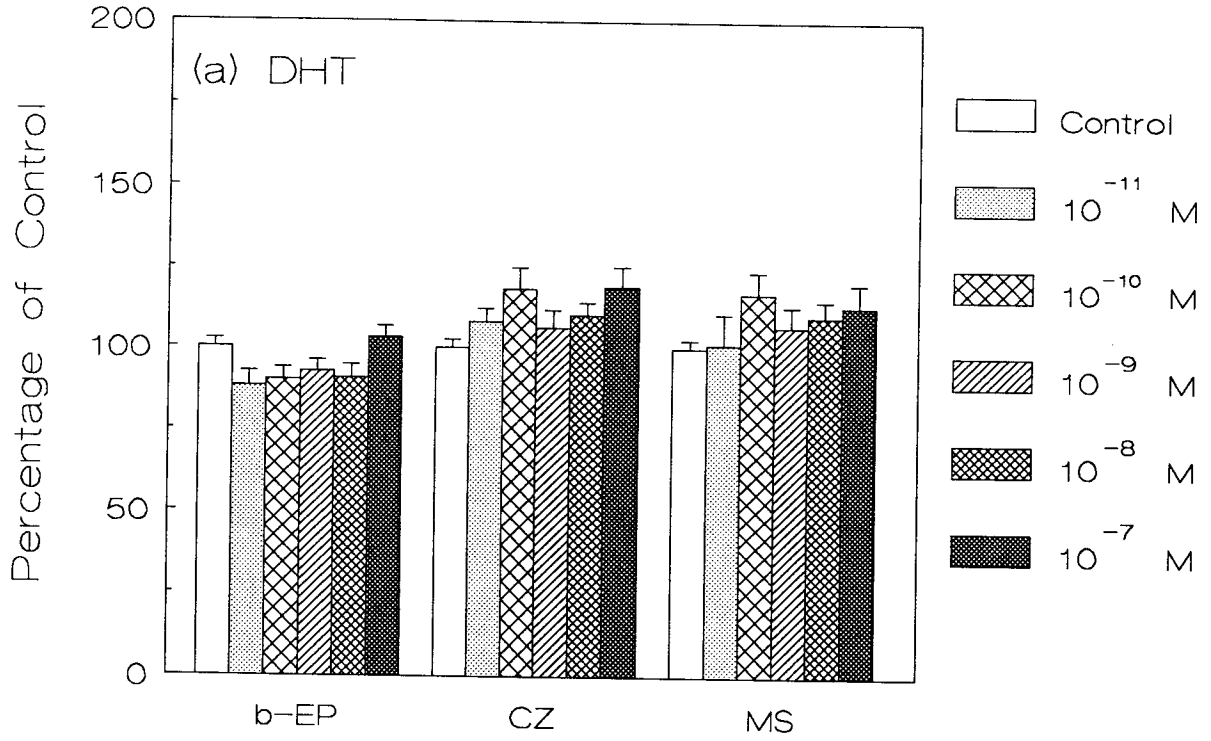

Percentage of Control
300
250
200
150
100
50
0
Basic medium
SF-I
SF-II
SF-III
Medium without DHT or HC
Medium with DHT
Medium with HC

3.1.4 Determining Time Points for Effects of Opioids on SC115 Tumor Cell Growth

In a series of preliminary experiments, cultures were terminated at different time points. In the first set of experiments, ß-endorphin (ß-EP), cyclazocine (CZ), and morphine sulfate (MS) at concentrations of 10^{-11}-10^{-7} M were added to cultures containing basic medium plus DHT (3.5 x 10^{-8} M) or HC (10^{-6} M) on days 1 and 2, and cultures were terminated on day 3 by the tetrazolium dye reduction (MTT) assay. As shown in Fig.5a, ß-EP, CZ, and MS at all concentrations examined had no significant effects on SC115 cell growth in DHT-containing medium (p's>0.10). Similarly, ß-EP and CZ had no effect on SC115 tumor cell growth in medium containing HC. However, the ANOVA indicated a main effect for treatment of MS, $p<0.01$. Post-hoc tests showed that MS at concentrations of 10^{-10}-10^{-8} M had a significant stimulatory effect on SC115 tumor cell growth in HC-containing medium compared to the control, p's<0.05, (Fig.5b).

In the second set of experiments, ß-EP, CZ, and MS were added to cultures containing basic medium plus DHT (3.5 x 10^{-8} M) or HC (10^{-6} M) on days 2 and 4, and cultures were terminated on day 5 and assayed by the MTT method. In medium containing DHT, SC115 tumor cell growth was stimulated by 10^{-7} M ß-EP, 10^{-10} M CZ, and 10^{-11}-10^{-10} M MS compared to the controls, p's<0.01, (Fig.6a). None of the remaining concentrations of the 3 opioids had any effect on cell growth (Fig.6a). In medium containing HC, post-hoc test indicated that 10^{-9}-10^{-8} M ß-EP and 10^{-10}-10^{-9} M CZ significantly inhibited cell growth compared to that in the

Fig.5 Effects of opioids on the growth of SC115 tumor cells cultured on collagen-coated dishes after 3 d in primary culture. SC115 tumor cells were cultured in basic medium with DHT (3.5×10^{-8} M) or HC (10^{-6} M). Three opioids, ß-endorphin (ß-EP), cyclazocine (CZ), and morphine sulfate (MS) at concentrations of 10^{-11}-10^{-7} M, were added with the medium changes. Cell growth was expressed as a percentage of the controls (medium without opioids). Values represent means ($\pm$SEM) of 11-19 determinations in 2-4 experiments. (a) ß-EP, CZ, and MS at all concentrations examined had no effects on SC115 cell growth in DHT-containing medium. (b) ß-EP and CZ at all concentrations examined had no effects on SC115 cell growth in HC-containing medium. MS at concentrations of 10^{-10}-10^{-8} M had a significant stimulatory effect on SC115 cell growth in HC-containing medium, p's<0.05. *, $p<0.05$, **, $p<0.01$ compared to controls.


(a) DHT
Percentage of Control
200
150
100
50
0
b-EP
CZ
MS
Control
10 -11 M
10 -10 M
10 -9 M
10 -8 M
10 -7 M

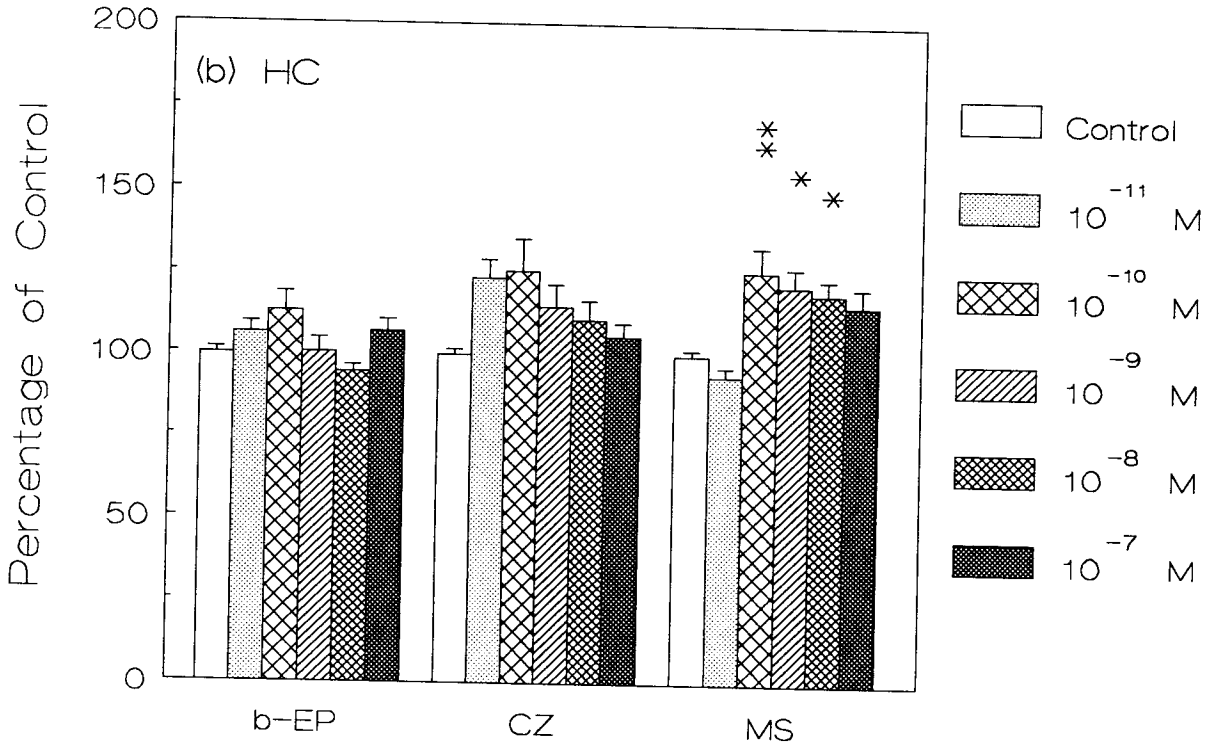
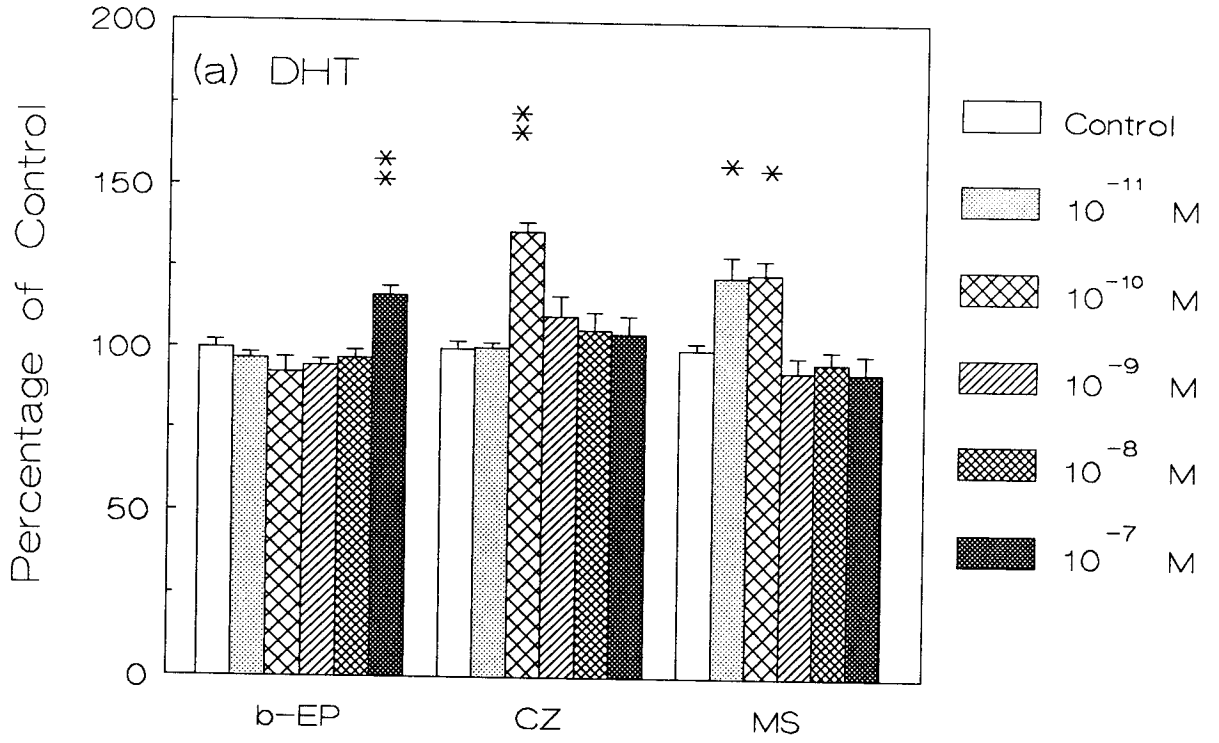
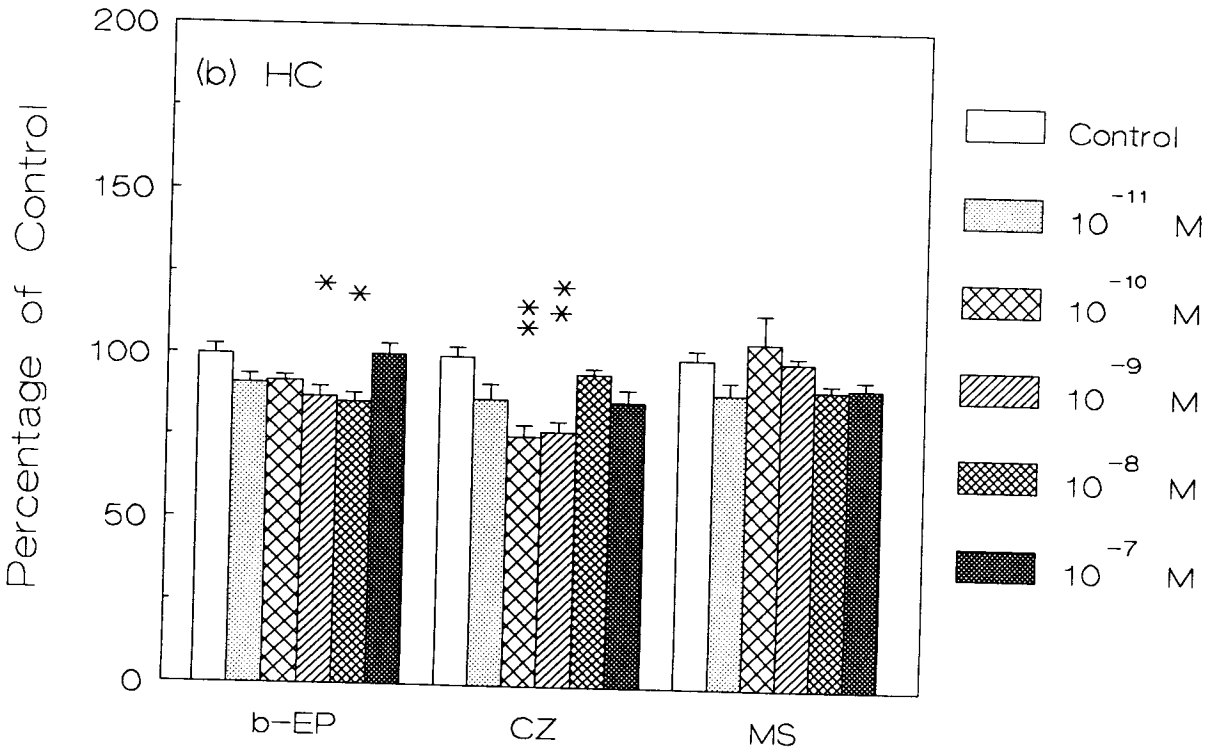

(b) HC
Percentage of Control
200
150
100
50
0
b-EP
CZ
MS
Control
10 -11 M
10 -10 M
10 -9 M
10 -8 M
10 -7 M

Fig.6 Effects of opioids on the growth of SC115 tumor cells cultured on collagen-coated dishes after 5 d in primary culture. SC115 tumor cells were cultured in basic medium with DHT (3.5×10^{-8} M) or HC (10^{-6} M). Three opioids, ß-EP, CZ, and MS at concentrations of 10^{-11}-10^{-7} M, were added with the medium changes. Cell growth was expressed as a percentage of the controls (medium wthout opioids). Values represent means (±SEM) of 6-26 determinations in 2-5 experiments. (a) ß-EP at a concentration of 10^{-7} M, CZ at a concentration of 10^{-10} M, and MS at concentrations of 10^{-11}-10^{-10} M significantly stimulated SC115 cell growth compared to the controls, p's<0.01, in DHT-containing medium. (b) ß-EP at concentrations of 10^{-9}-10^{-8} M and CZ at concentrations of 10^{-10}-10^{-9} M significantly inhibited SC115 cell growth compared to the controls, p's<0.05, in HC-containing medium. MS had no effect on SC115 cell growth in HC-containing medium. *, p<0.05, ** p<0.01 compared to controls.


(a) DHT
Percentage of Control
200
150
100
50
0
b-EP
CZ
MS
Control
10 -11 M
10 -10 M
10 -9 M
10 -8 M
10 -7 M

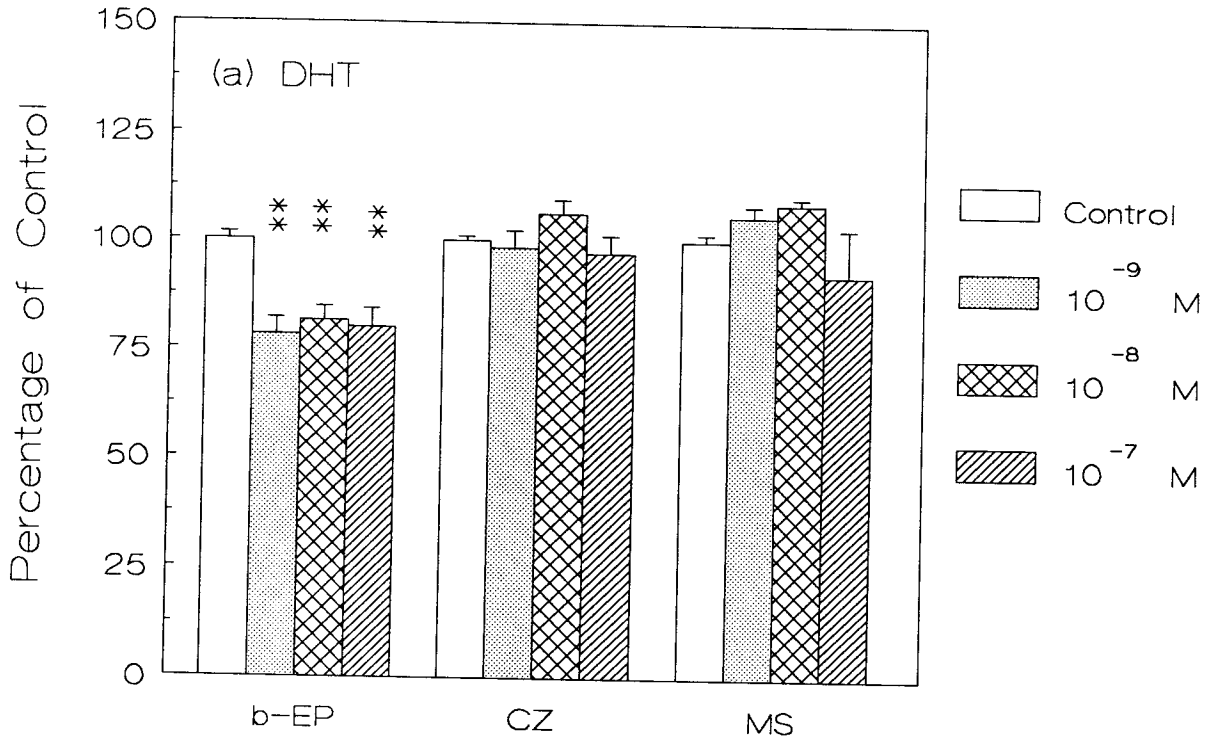

(b) HC
Percentage of Control
200
150
100
50
0
b-EP
CZ
MS
Control
10 -11 M
10 -10 M
10 -9 M
10 -8 M
10 -7 M

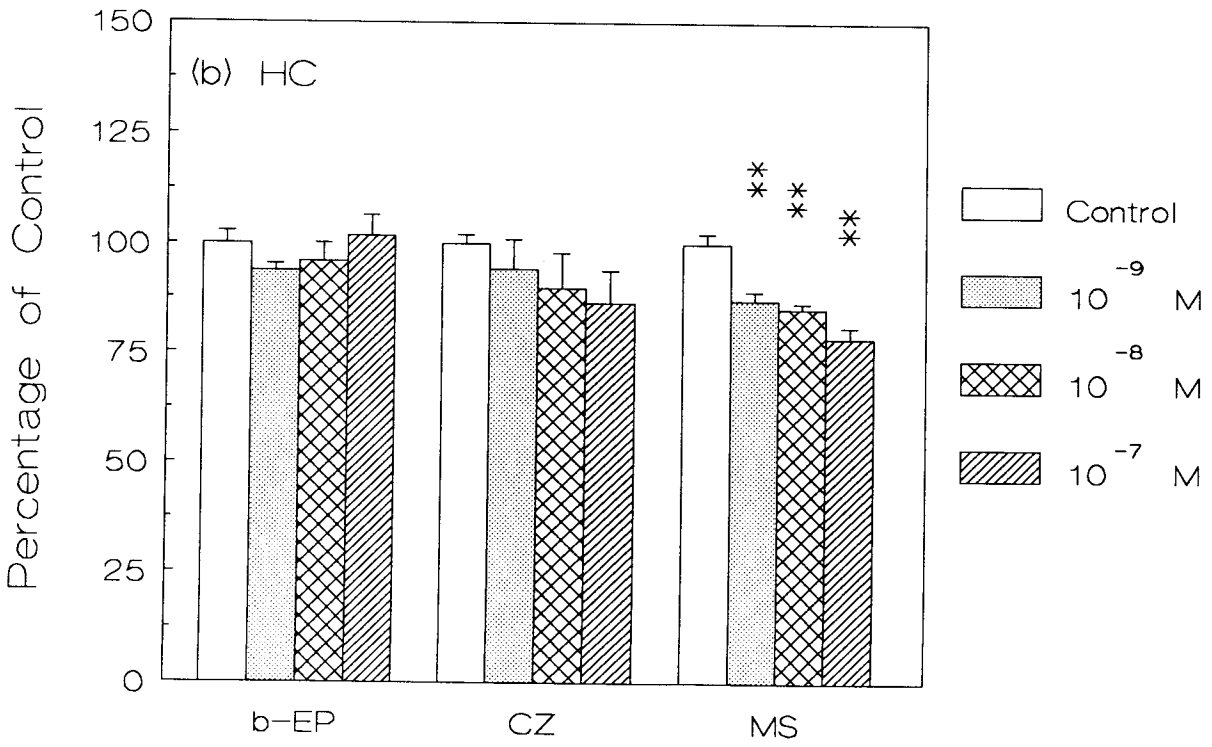
controls, p's<0.05, (Fig.6b). MS at all concentrations examined had no significant inhibitory effect on cell growth.

In the third set of experiments, SC115 tumor cells were grown for 7 d and ß-EP, CZ, and MS were added to cultures containing basic medium plus DHT (3.5×10^{-8} M) or HC (10^{-6} M). Media were changed on days 2, 4, and 6. As shown in Fig.7a, ß-EP at all concentrations examined (10^{-9}-10^{-7} M) significantly inhibited SC115 tumor cell growth in DHT-containing medium compared to that in the controls, p's<0.01. CZ and MS had no effects on cell growth in the DHT-containing medium (Fig.7a). On the other hand, statistical analyses revealed that ß-EP and CZ at all concentrations examined (10^{-9}-10^{-7} M) had no effects on SC115 tumor cell growth in medium containing HC. Post-hoc showed that MS (10^{-9}-10^{-7} M)) significantly inhibited SC115 tumor cell growth compared to that in control conditions (p's<0.01) in HC-containing medium (Fig.7b).

In these preliminary experiments designed to determine the time for the optimal effect of opioids on SC115 tumor cell growth, opioids were added every other day. The results of the preliminary experiments were variable. ß-EP had the most consistent inhibitory effect, whereas CZ and MS had variable effects, resulting in inhibition, no effect or slight stimulation. The reason for this is not clear. One possible explanation might be that the opioids are not stable in the solution. Thus, adding the opioids every other day in medium may not be sufficient. Therefore, in the experiments undertaken for this thesis, opioids were added to cultures daily with each

Fig.7 Effects of opioids on the growth of SC115 tumor cells cultured on collagen-coated dishes after 7 d in primary culture. SC115 tumor cells were cultured in basic medium with DHT (3.5 x 10^{-8} M) or HC (10^{-6} M). Three opioids, ß-EP, CZ, and MS at concentrations of 10^{-9}-10^{-7} M, were added with the medium changes. Cell growth was expressed as a percentage of the controls (medium wthout opioids). Values represent means (±SEM) of 6-14 determinations in 2-3 experiments. (a) ß-EP at all concentrations examined significantly inhibited SC115 cell growth in DHT-containing medium compared to that in the control, p's<0.01. CZ and MS had no effects on cell growth. (b) ß-EP and CZ had no effects on SC115 cell growth in HC-containing medium. MS at all concentrations examined significantly inhibited SC115 cell growth in HC-containing medium compared to that in the control, p<0.01. **, p<0.01 compared to controls.


(a) DHT
Percentage of Control
150
125
100
75
50
25
0
b-EP
CZ
MS
Control
10 -9 M
10 -8 M
10 -7 M


(b) HC
Percentage of Control
150
125
100
75
50
25
0
b-EP
CZ
MS
Control
10 -9 M
10 -8 M
10 -7 M

medium change. As described in the results below, more consistent inhibitory effects of the opioids were observed.

3.2 Experimental Results

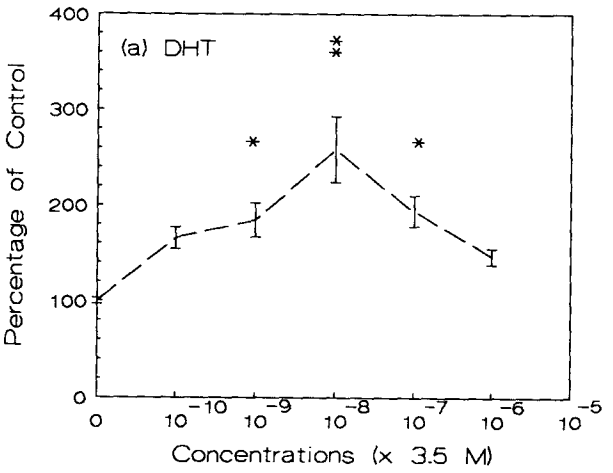
3.2.1 Effects of Steroid Hormones and Growth Factors on the Growth of SC115 Tumor Cells in Serum-containing Medium

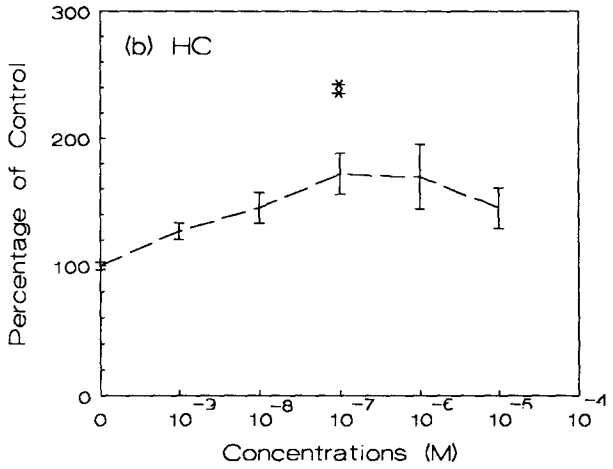
(1) Steroid Hormones

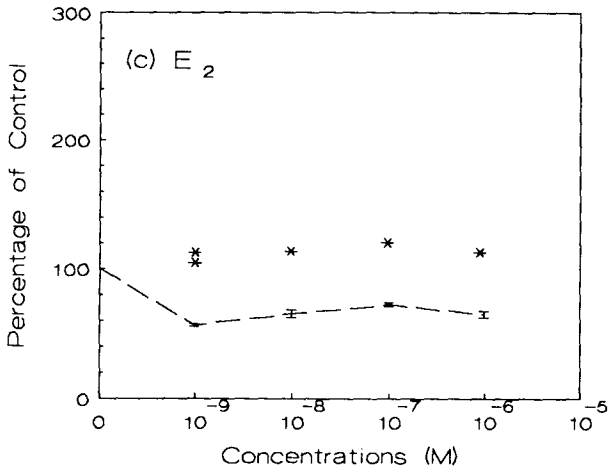
Effects of DHT (3.5×10^{-10}-3.5×10^{-6} M) and HC (10^{-9}-10^{-5} M) on the growth of SC115 tumor cells were examined. After 5 d in culture, the ANOVA revealed a significant dose-response curve for DHT, $p<0.001$ (Fig.8a). Post-hoc tests indicated that doses of 3.5×10^{-9}-3.5×10^{-7} M were significantly different from control (basic medium without hormones). Growth stimulation was maximal at the physiological concentration of DHT (3.5×10^{-8} M) and growth in response to this dose was significantly different from growth in response to doses of 3.5×10^{-10}, 3.5×10^{-9}, and 3.5×10^{-6} M, p's<0.05. There was also a significant main effect of dose for HC, $p<0.01$ (Fig.8b). Growth of SC115 cells at a dose of 10^{-7} M HC was significantly stimulated over the control level, $p<0.01$.

In contrast, statistical analyses showed that 17ß-estradiol (E_2), from physiological to pharmacological concentrations (10^{-9}-10^{-6} M), significantly inhibited SC115 tumor cell growth after 5 d in culture compared to that in basic medium without E_2, p's<0.001 (Fig.8c).

Fig.8 Effects of DHT, HC, or E_2 on the growth of primary cultures of SC115 tumor cells. Cells were cultured in basic medium plus or minus DHT, HC, or E_2 on collagen-coated 96-well tissue culture plates and cultures were terminated after 5 d. Cell growth, as determined by the MTT assay, was expressed as a percentage of the control cultures (basic medium without steroid hormones). Values (means $\pm$ SEM) with DHT or HC represent means of 29-30 determinations in 5 experiments; values with E_2 represent 6-12 determinations in 2 experiments. (a) SC115 cell growth was significantly stimulated by 3.5×10^{-9}-3.5×10^{-7} M DHT (p's<0.001) compared to that in the control. Growth stimulation was maximal at the physiological concentration of DHT (3.5×10^{-8} M) and growth in response to this dose was significantly different from growth in response to doses of 3.5×10^{-10}, 3.5×10^{-9}, and 3.5×10^{-6} M, p's<0.05. (b) SC115 cell growth was also significantly stimulated by 10^{-7} M HC compared to that in the control (p<0.01). (c) SC115 cell growth was significantly inhibited by 10^{-9}-10^{-6} M E_2 compared to that in the control (p's<0.001). *, p<0.05, **, p<0.01 compared to controls.


(a) DHT
Percentage of Control
400
300
200
100
0
0
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
Concentrations (x 3.5 M)


(b) HC
Percentage of Control
300
200
100
0
0
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
Concentrations (M)


(c) E 2
Percentage of Control
300
200
100
0
0
10 -9
10 -8
10 -7
10 -6
10 -5
Concentrations (M)

(2) Growth Factors

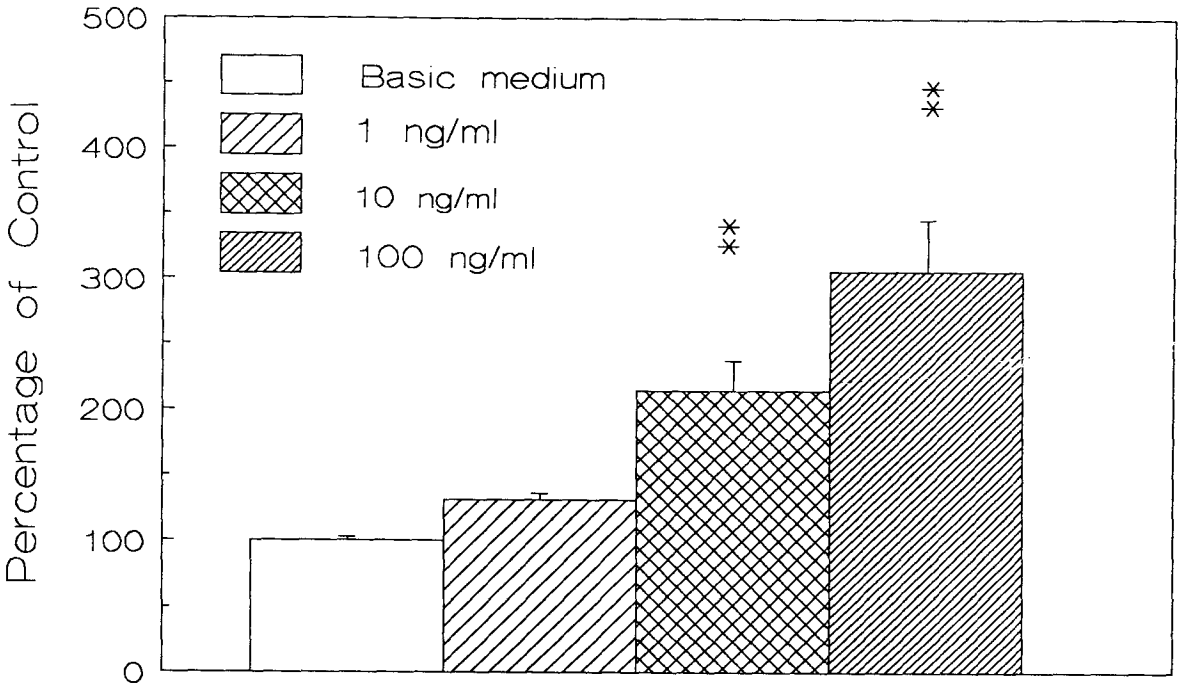
Literature has shown that growth stimulation of SC115 tumor cells by physiological doses of androgens may be mediated by a fibroblast growth factor (FGF)-like polypeptide via an autocrine or paracrine mechanism. Similary, growth stimulation of mammary epithelial cells by estrogens in the female may be mediated by epidermal growth factor (EGF) via an autocrine mechanism. Therefore, effects of bFGF and EGF, at concentrations of 1, 10, and 100 ng/ml, on SC115 tumor cell growth were investigated. The ANOVA revealed that bFGF significantly enhanced SC115 tumor cell growth in a dose-responsive manner in serum-containing medium, $p<0.001$ (Fig.9a). Significant stimulation over the control level occurred at the physiological concentration of 10 ng/ml, $p<0.01$, but maximal stimulation occurred at the pharmacological concentration (100 ng/ml), $p<0.05$. In contrast, EGF markedly inhibited SC115 tumor cell growth compared to the control at all concentrations tested, p's<0.01, (Fig.9b).

3.2.2 Effects of Steroid Hormones, Growth Factors, and Anti-growth Factors on the Growth of SC115 Tumor Cells in Serum-free Medium

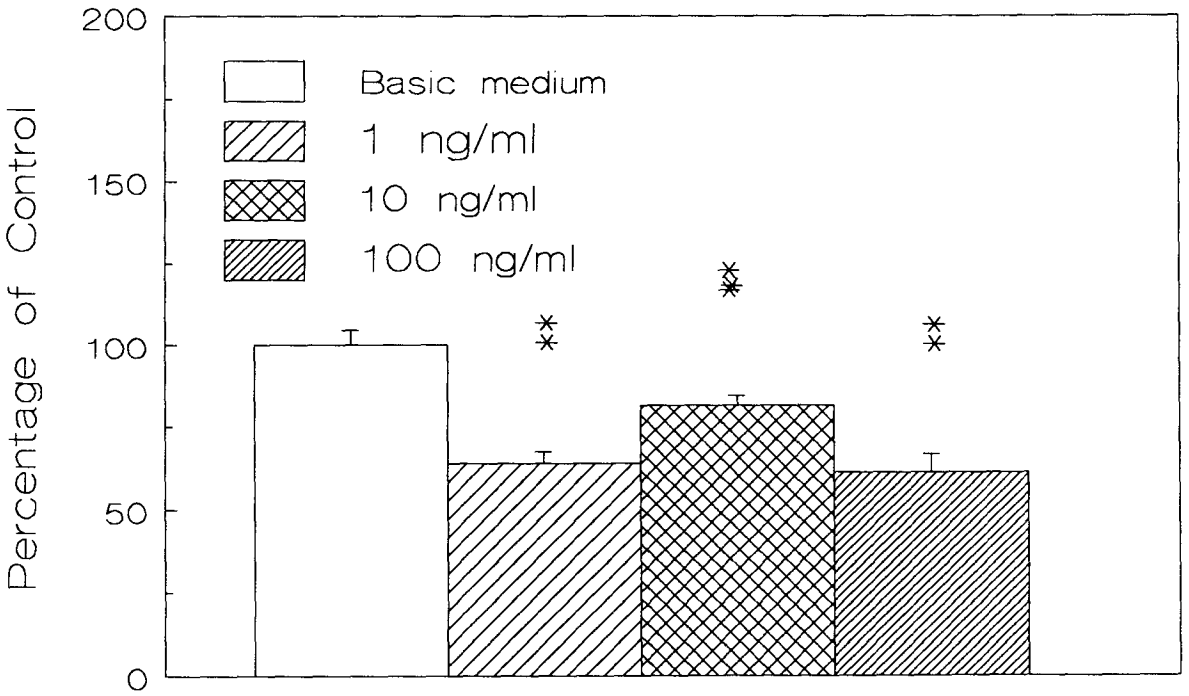
(1) Effects of DHT and bFGF on the Growth of SC115 Tumor Cells

The effects of DHT and bFGF on the growth of SC115 tumor cells in serum-free medium were studied. Fig.10a shows that DHT (3.5×10^{-8} M) alone significantly increased SC115 tumor cell growth ($p<0.01$) over the control levels. In addition, bFGF at all concentrations examined (1, 10, 100, 250, and 500 ng/ml)

Fig.9 Effects of bFGF and EGF on the growth of primary cultures of SC115 tumor cells. Cells were cultured as described in Fig.8. Cell growth was expressed as a percentage of the control cultures (basic medium without bFGF or EGF). Values represent the means (±SEM) of 12-18 determinations in 2-3 experiments. (a) SC115 cell growth was significantly stimulated by 1-100 ng/ml bFGF in a dose-responsive manner ($p<0.01$). Significant stimulation over the control level occurred at the physiological concentration of 10 ng/ml, $p<0.01$, but maximal stimulation occurred at the pharmacological concentration (100 ng/ml), $p<0.05$. (b) SC115 cell growth was significantly inhibited by 1-100 ng/ml EGF at all concentrations examined (p's<0.01). **, $p<0.01$ compared to controls.

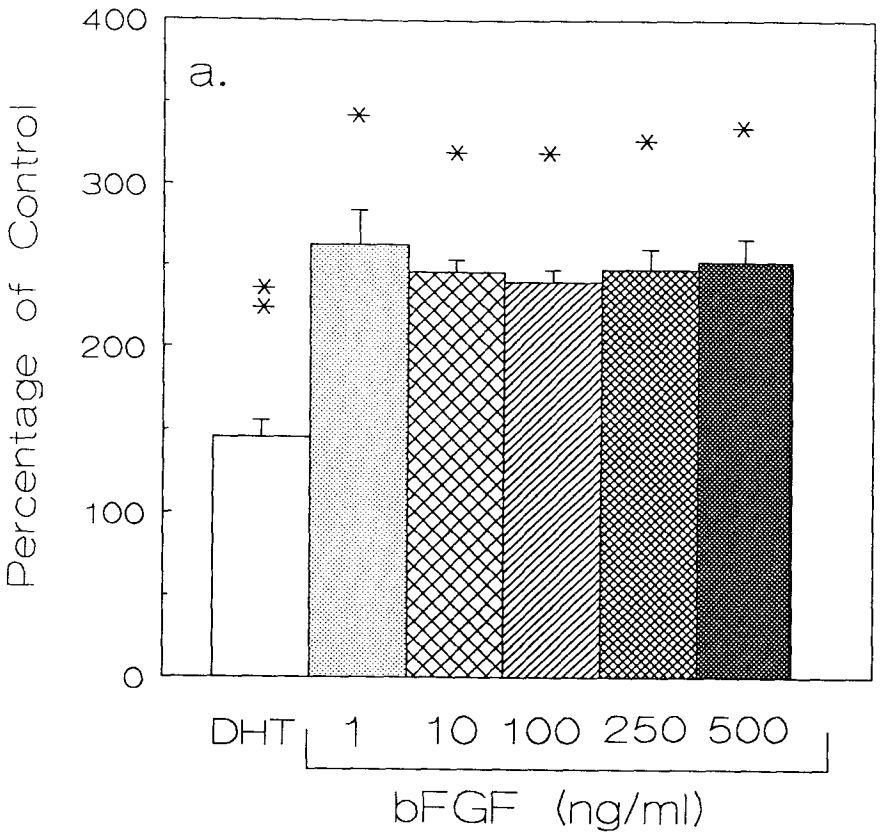


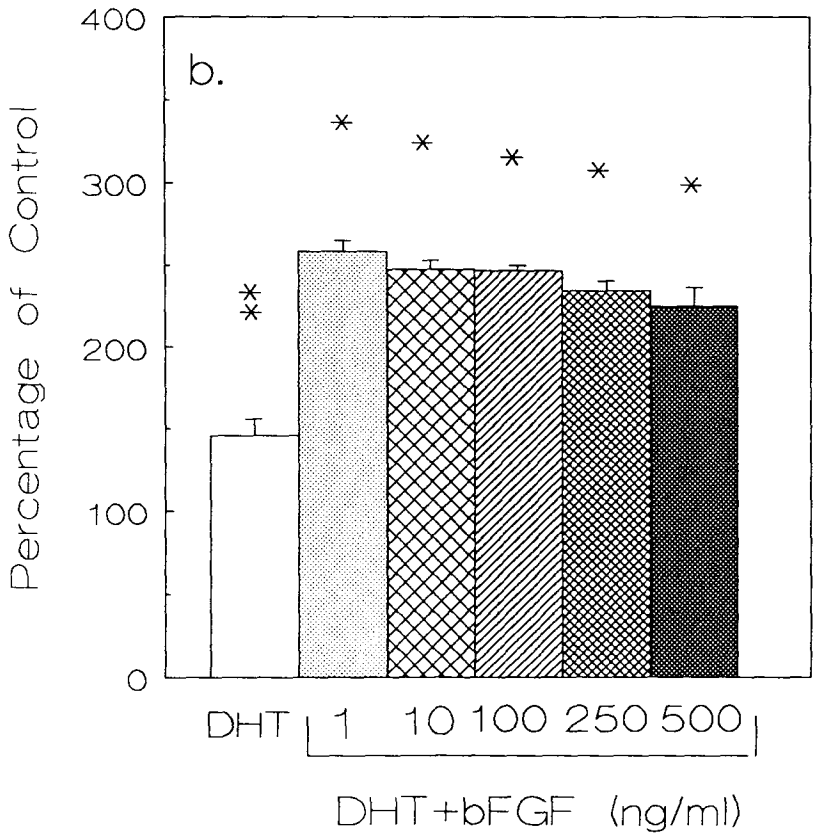
(a) FGF Concentrations



(b) EGF Concentrations

Fig.10 Effects of DHT, bFGF, or DHT (3.5×10^{-8} M) plus bFGF on the growth of primary cultures of SC115 tumor cells in serum-free medium. Cells were cultured on collagen-coated 96-well tissue culture plates in serum-free medium plus or minus DHT or bFGF. After 5 d, cultures were terminated and cell growth determined by the MTT assay. Cell growth was expressed as a percentage of the control cultures [serum-free medium without bFGF or DHT (100% growth) which is not shown in this graph]. Values represent the means ($\pm$SEM) of 4-8 determinations. (a) bFGF at concentrations of 1, 10, 100, 250, and 500 ng/ml and DHT (3.5×10^{-8} M) significantly stimulated SC115 cell growth compared to the control, p's<0.01. (b) bFGF at all concentrations examined when added to cultures containing DHT significantly stimulated SC115 cell growth compared to that in the control, p's<0.01. *, p<0.05, **, p<0.01 compared to controls.


a.
Percentage of Control
400
300
200
100
0
DHT
1
10
100
250
500
bFGF (ng/ml)

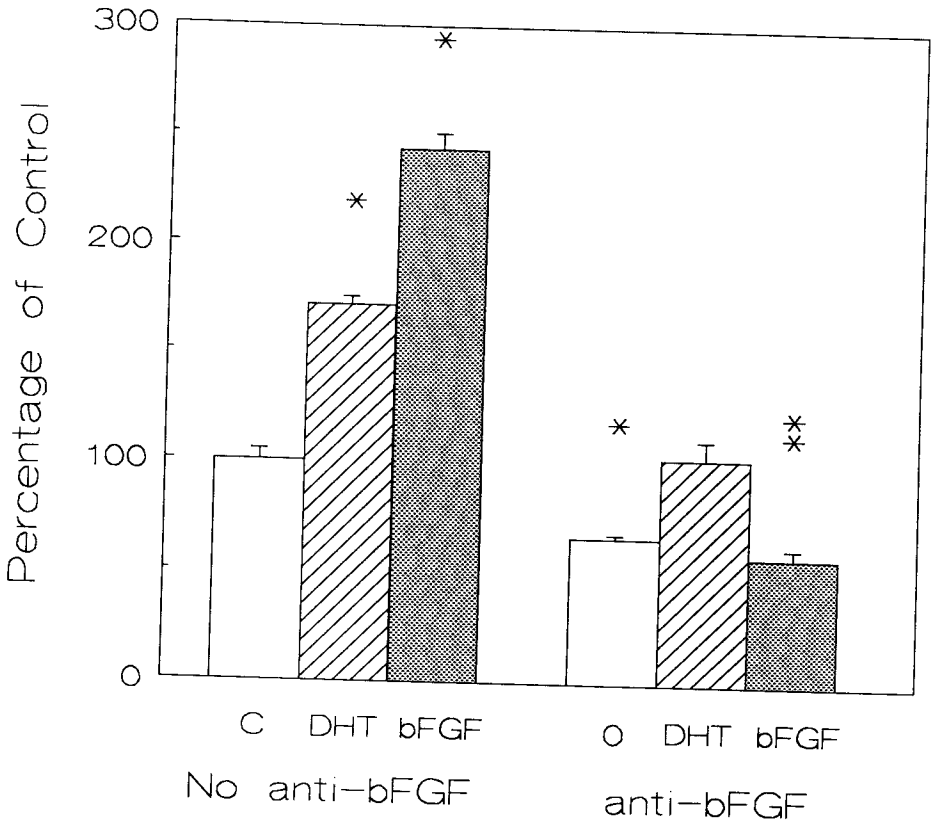

b.
Percentage of Control
400
300
200
100
0
DHT
1
10
100
250
500
DHT+bFGF (ng/ml)

significantly increased cell growth in serum-free medium, p's<0.05, to a level similar to that of the pharmacological concentration of bFGF in basic medium. Furthermore, the possibility that DHT and bFGF may have any synergestic effects on SC115 cell growth was also examined. Data indicated that bFGF at all concentrations, when added to the medium together with 3.5 x 10^{-8} M DHT, significantly increased SC115 cell growth compared to the control, p's<0.001, (Fig.10b). Fig. 10a and 10b indicates that the growth stimulation produced by the addition of both DHT and bFGF was not significantly greater than that produced by bFGF alone. Therefore, it appears that DHT and bFGF do not have synergestic effects on SC115 cell growth. All concentrations of bFGF alone or bFGF added with DHT had greater stimulatory effects on SC115 tumor cell growth than DHT alone, p's<0.01.

(2) Inhibitory Effects of Anti-bFGF Antibody on DHT- or bFGF-induced Growth of SC115 Tumor Cells in Serum-free Medium

The effects of anti-bFGF antibody on DHT- or bFGF-stimulated growth of SC115 tumor cells were examined in serum-free medium using the MTT assay. As shown in Fig.11, SC115 tumor cell growth was markedly and significantly inhibited by the addition of 400 ug/ml anti-bFGF antibody to medium containing DHT or bFGF (p's<0.01), whereas the same amount of control IgG had no significant inhibitory effects (data not shown). When anti-bFGF antibody was added to the medium containing DHT, it significantly suppressed SC115 tumor cell growth compared to that in DHT alone

Fig.11 Effects of anti-bFGF antibody on DHT- or bFGF-stimulated SC115 tumor cell growth in serum-free medium. Cells were cultured as described in Fig.10. Cell growth was expressed as a percentage of the control cultures (c; serum-free medium alone). Values represent the means (±SEM) of 4 determinations. Anti-bFGF antibody at a dose of 400 ug/ml significantly inhibited DHT (3.5×10^{-8} M)- or bFGF (10 ng/ml)-stimulated SC115 cell growth (p's<0.01) and also inhibited cell growth in control medium alone (sero; anti-bFGF in control medium alone), ($p<0.05$). *, $p<0.05$, **, $p<0.01$ compared to controls.


300
200
100
0
Percentage of Control
C DHT bFGF
O DHT bFGF
No anti-bFGF
anti-bFGF

(by 40%), $p<0,01$, and growth was suppressed to the control level. However, when anti-bFGF antibody was added together with bFGF, it significantly inhibited SC115 tumor cell growth compared to that in bFGF alone (by 70%), $p<0.01$, and growth was suppressed to below the control level. It should be noted, however, that anti-bEGF antibody also significantly inhibited growth in the absence of DHT and bFGF, albeit to a lesser degree (by 33%).

(3) Effects of E_2, EGF, and Anti-EGF Antibody on the Growth of SC115 Tumor Cells in Serum-free Medium

The ANOVA indicated a main effect of treatment for E_2. Post-hoc tests showed that after 5 d in culture, E_2 (10^{-8} M) significantly decreased SC115 tumor cell growth compared to the serum-free medium without E_2, $p<0.01$, (Fig.12). EGF at all concentrations examined (1, 10, 50, and 100 ng/ml) had no effect on SC115 tumor cell growth (Fig.12). In addition, after 5 d in culture, anti-EGF antibody at a concentration of 1:100 significantly inhibited SC115 tumor cell growth in serum-free medium alone and in serum-free medium containing E_2, or EGF (10 ng/ml), p's<0.01 (Fig.13).

3.2.3 Effects of Opioids on the Growth of SC115 Tumor Cells

Effects of the opioid agonists, ß-EP, CZ, and MS, on the growth of SC115 tumor cells were studied. SC115 tumor cells were cultured in basic medium with or without steroid hormones (DHT at 3.5×10^{-8} M or HC at 10^{-6} M), growth factor (bFGF at 10 ng/ml), or 5% FBS. Beginning on day 2 in culture, ß-EP, CZ, and MS were

Fig.12 Effects of EGF or E_2 on the growth of primary cultures of SC115 tumor cells in serum-free medium. Cells were cultured as described in Fig.10. Cell growth was expressed as a percentage of the control cultures (c; serum-free medium without EGF or E_2). Values represent the means ($\pm$SEM) of 4 determinations. E_2 (10^{-8} M) significantly decreased SC115 tumor cell growth compared to the serum-free medium without E_2, $p<0.01$. EGF at all concentrations examined (1, 10, 50, and 100 ng/ml) had no effect on SC115 tumor cell growth compared to the control. **, $p<0.01$ compsred to controls.

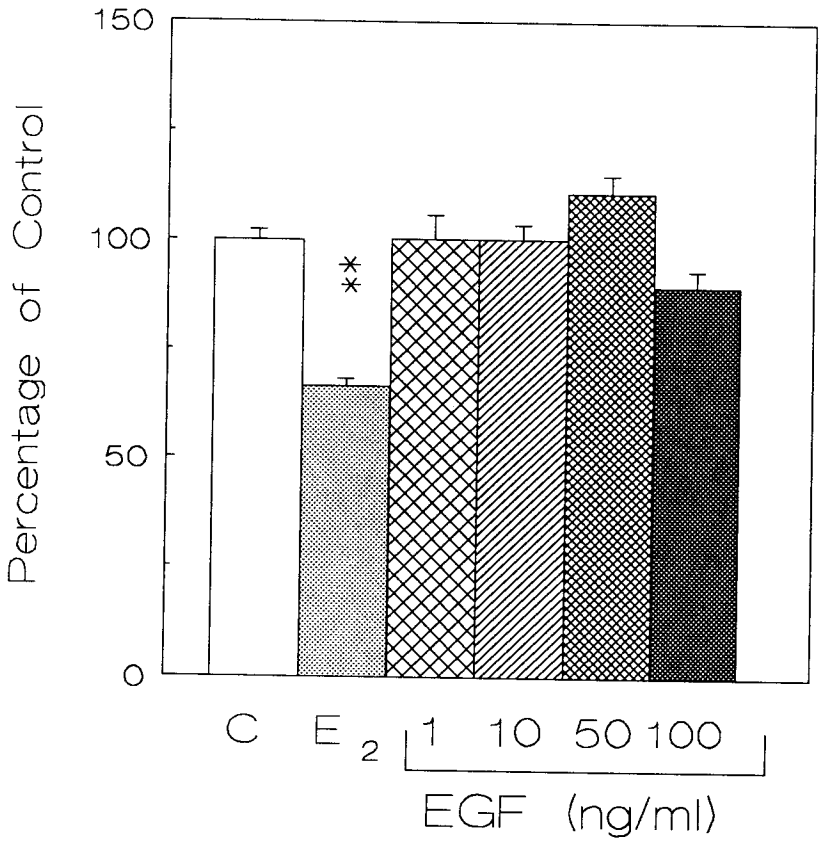
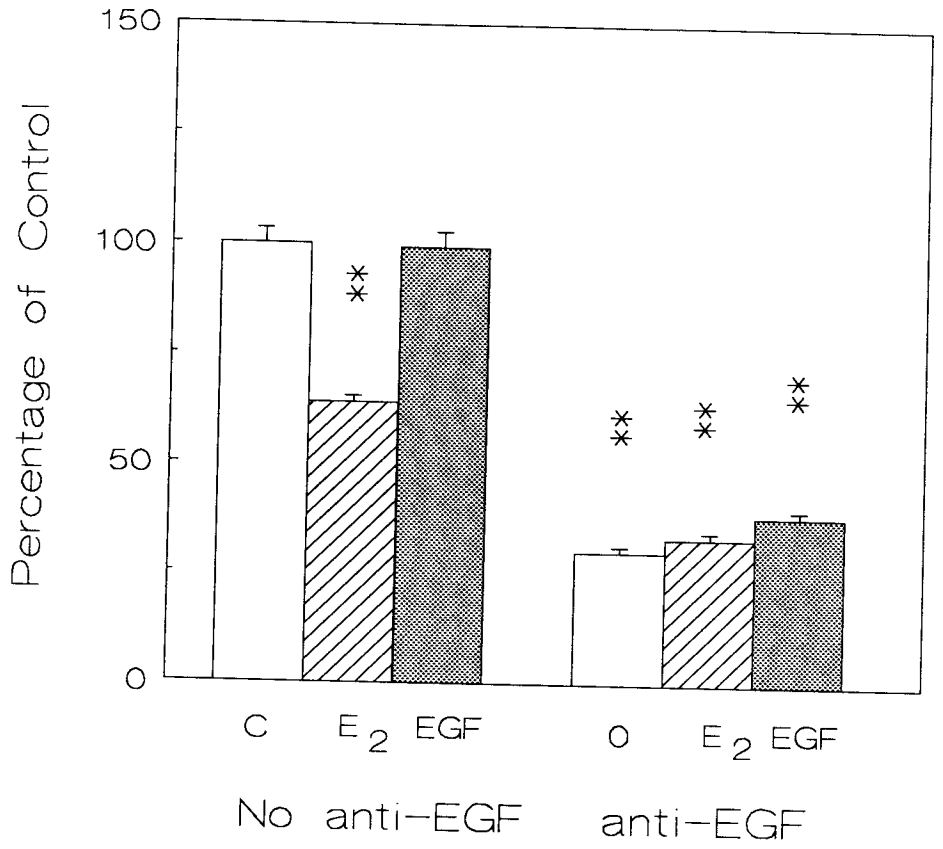

150
100
50
0
Percentage of Control
**
C
E 2
1
10
50
100
EGF (ng/ml)

Fig.13 Effects of anti-EGF antibody on SC115 tumor cell growth in serum-free medium with or without E_2 or EGF. Cells were cultured as described in Fig.10. Cell growth was expressed as a percentage of the control cultures (c; serum-free medium alone). Values represent the means ($\pm$SEM) of 4 determinations. Anti-EGF antibody at a concentration of 1:100 significantly inhibited SC115 tumor cell growth in serum-free medium alone (sero; anti-EGF antibody in serum-free medium alone), serum-free medium containing E_2, or serum-free medium containing EGF compared to that in the control, p's<0.01. **, p<0.01 compared to controls.


150
100
50
0
Percentage of Control
C
E2
EGF
0
E2
EGF
No anti-EGF
anti-EGF

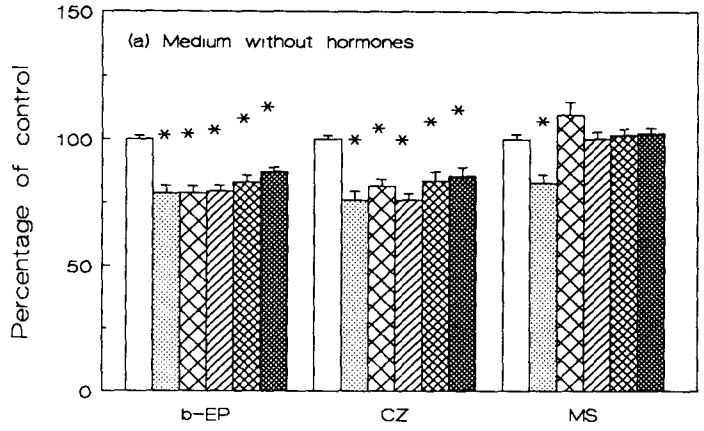
added with each medium change at concentrations ranging from 10^{-11}-10^{-7} M for 3 consecutive days. As shown in Fig.14a, growth of SC115 tumor cells cultured in the basic medium (without steroid hormones) was significantly decreased (approximately 20%) with the addition of ß-EP, $p<0.001$ and CZ, $p<0.001$ at all concentrations examined. MS had no effect on cell growth in this basic medium except at a concentration of 10^{-11} M where it had an inhibitory effect ($p<0.01$).

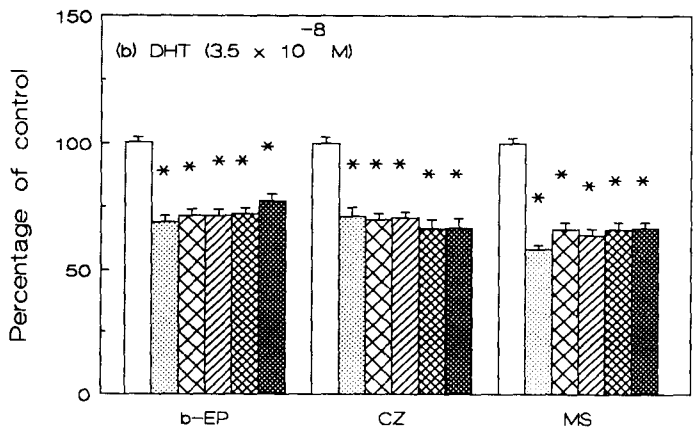
Growth of SC115 tumor cells in medium containing DHT (3.5 x 10^{-8} M) or HC (10^{-6} M) was significantly retarded (from 25%-40% and 10-35%, respectively) compared to the growth in the controls (no opioids) condition, p's<0.001 (Fig.14b and 14c, respectively). This was true for all 3 opioids at all concentrations examined. The ANOVAs revealed no dose-response relationships for ß-EP, CZ, or MS in medium containing DHT or HC.

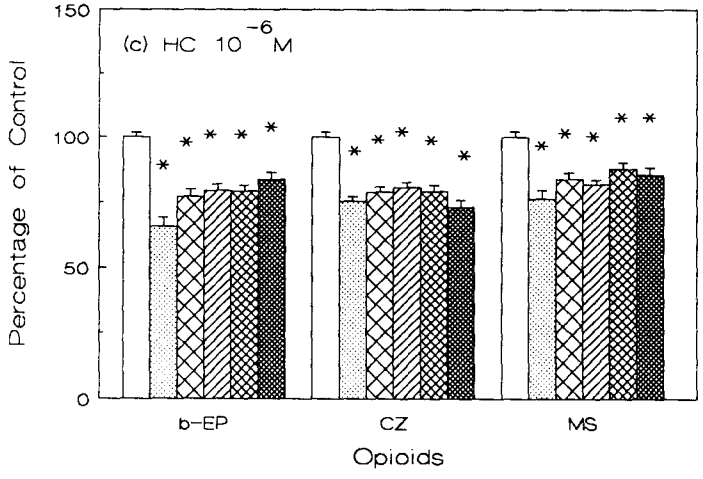
ß-EP, CZ, and MS (10^{-11}-10^{-7} M) were also added to SC115 tumor cells cultured in medium containing 5% FBS (which contains physiological concentrations of all the endogeneous steroid hormones). Statistical analyses indicated that all 3 opioids at all concentrations examined significantly inhibited SC115 tumor cell growth (by about 30-35%) after 5 d in culture, p's<0.001 (Fig.14d).

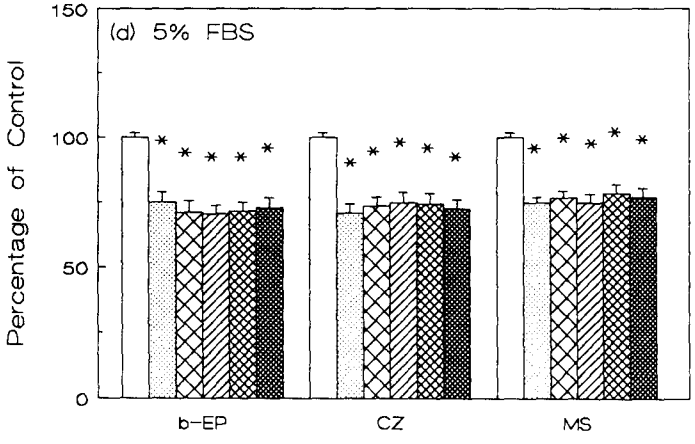
Finally, the effect of ß-EP on the growth of SC115 tumor cells in medium containing 10 ng/ml bFGF was also investigated. The ANOVA revealed a main effect of treatment. Post-hoc tests showed that ß-EP at concentrations of 10^{-10}-10^{-7} M, but not in concentrations lower than 10^{-10} M (10^{-14}, 10^{-13}, and 10^{-11} M),

Fig.14 Effects of ß-endorphin (ß-EP), cyclazocine (CZ), and morphine sulfate (MS) on the growth of SC115 tumor cells cultured in medium with or without DHT, HC, or 5% fetal bovine serum (FBS). Cells were cultured in basic medium without or with DHT (3.5×10^{-8} M), HC (10^{-6} M), or 5% FBS, and ß-EP, CZ, or MS, as described in Fig.8, and terminated after 5 d. Cell growth was expressed as a percentage of the control cultures (medium without opioids). Open bar represents control which is medium without opioids, while patterned bars represent medium containing opioids with doses of 10^{-11}-10^{-7} M going from left to right. Values represent the means ($\pm$SEM) of 18-30 determinations in 3-5 experiments. (a) SC115 tumor cell growth was significantly inhibited by 10^{-11}-10^{-7} M ß-EP (p's<0.001), and CZ (p's<0.001), and by 10^{-11} M MS (p<0,01) in basic medium. (b) SC115 tumor cell growth was significantly inhibited by all 3 opioids at all doses examined in medium containing DHT (p's<0.001). (c) SC115 tumor cell growth was significantly inhibited by all 3 opioids at all doses examined in medium containing HC (p's<0.001). (d) SC115 tumor cell growth was significantly inhibited by all 3 opioids at all doses examined in medium containing 5% FBS (p's<0.001). *, p<0.01 compared to controls.


(a) Medium without hormones
Percentage of control
150
100
50
0
b-EP
CZ
MS


(b) DHT (3.5 x 10 -8 M)
Percentage of control
150
100
50
0
b-EP
CZ
MS


(c) HC 10 -6 M
Percentage of Control
150
100
50
0
b-EP
CZ
MS
Opioids


(d) 5% FBS
Percentage of Control
150
100
50
0
b-EP
CZ
MS

significantly inhibited cell growth (by about 20%) compared to that in medium without ß-EP, p's<0.05 (Fig.15).

3.2.4 Effect of the Opioid Antagonist Naloxone on the Growth of SC115 Tumor Cells

In order to study the mechanisms of these opioid effects on SC115 tumor cell growth, a μ opioid receptor antagonist, naloxone (10^{-8} and 10^{-6} M), was added to SC115 tumor cells cultured together with ß-EP (10^{-9} M) or MS (10^{-9} M). The ANOVA indicated a significant effect of treatment on SC115 tumor cell growth in DHT-containing medium, $p<0.001$. Fig.16a illustrates that ß-EP significantly inhibited SC115 cell growth, $p<0.001$, and naloxone at concentrations of 10^{-8} and 10^{-6} M totally blocked the effect of ß-EP. MS also significantly inhibited SC115 tumor cell growth, $p<0.001$. Naloxone at a concentration of 10^{-8} M completely blocked the inhibitory effect of MS, and at a concentration of 10^{-6} M partially, $p<0.05$, blocked the inhibitory effect of MS in DHT-containing medium.

The ANOVA also indicated a significant effect of treatment on SC115 tumor cell growth in HC-containing medium, $p<0.01$. Fig.16b shows that both ß-EP and MS significantly inhibited SC115 tumor cell growth, p's<0.01, and naloxone at doses of 10^{-8} and 10^{-6} M totally blocked the inhibitory effect of ß-EP and MS in HC-containing medium.

A main effect of treatment indicated that naloxone alone also significantly inhibited the growth of SC115 tumor cells in basic medium, $p<0.01$, as well as in medium containing DHT,

Fig.15 Effects of ß-EP on the growth of SC115 tumor cells cultured for 5 d in medium containing bFGF (10 ng/ml). Cells were cultured as described in Fig.8. Cell growth was expressed as a percentage of the control cultures (medium without opioids). Values are the means (±SEM) of 12 determinations in 2 experiments. SC115 tumor cell growth was significantly inhibited by 10^{-10}-10^{-7} M ß-EP compared to that in the control (p's<0.01). **, p<0.01 compared to controls.

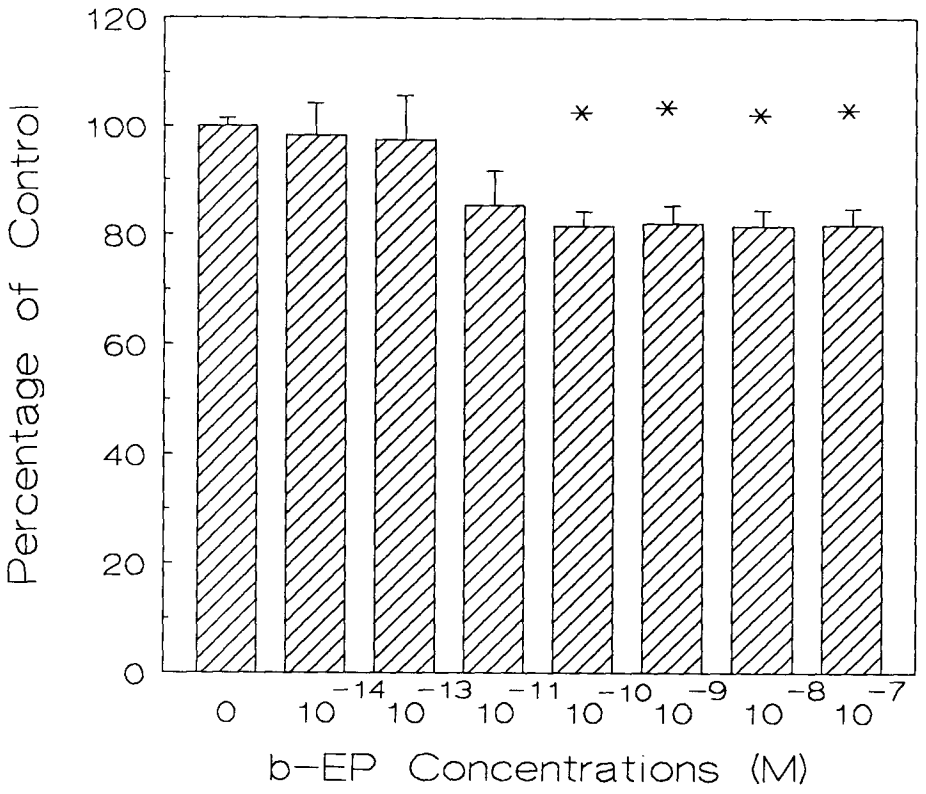
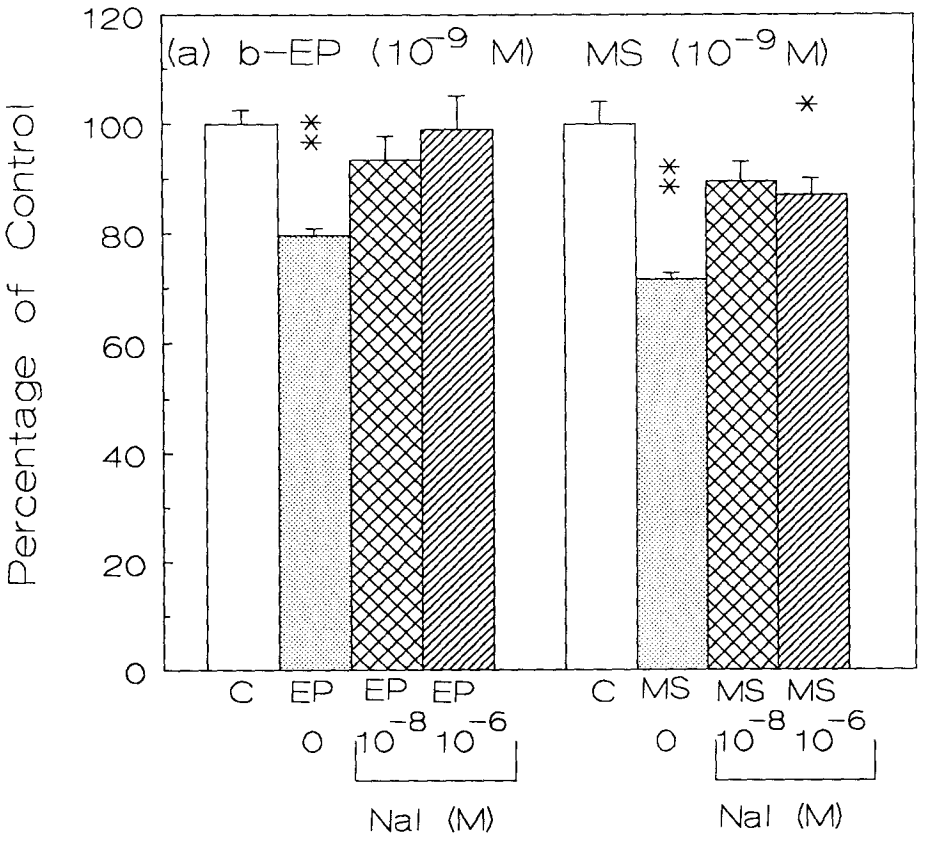
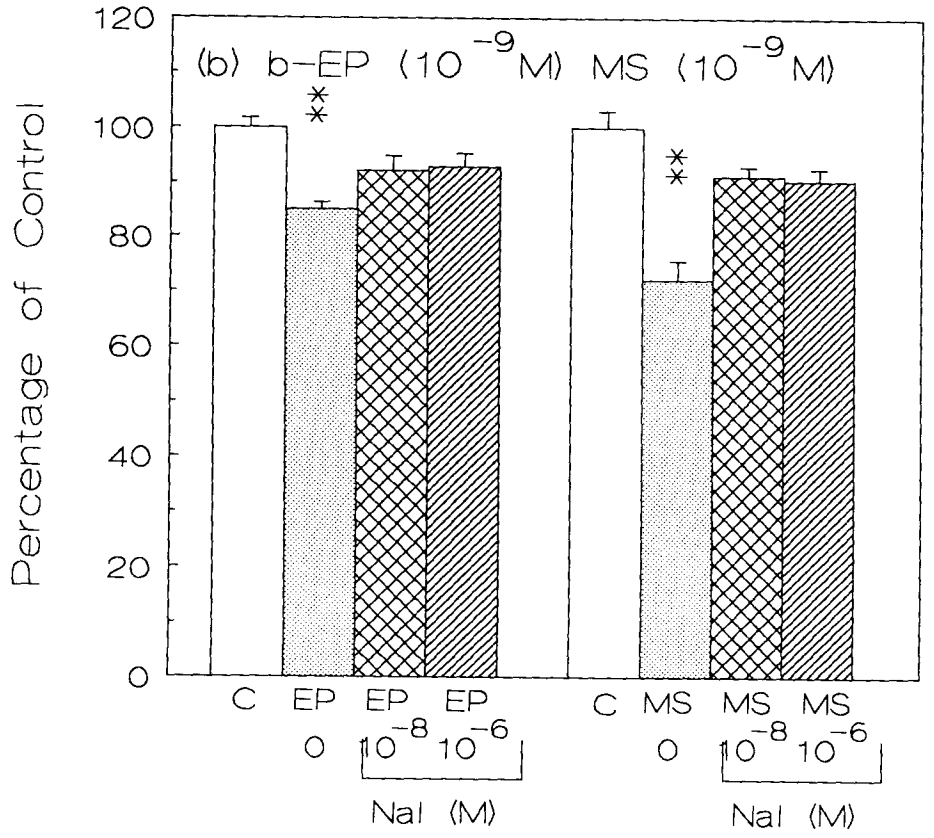

Percentage of Control
120
100
80
60
40
20
0
0
10 -14
10 -13
10 -11
10 -10
10 -9
10 -8
10 -7
b-EP Concentrations (M)
*
*
*
*

Fig.16 Effects of opioids and naloxone on the growth of primary cultures of SC115 tumor cells. Cells were cultured for 5 d in basic medium plus either DHT (3.5×10^{-8} M) or HC (10^{-6} M), and ß-EP (10^{-9} M) or MS (10^{-9} M) with or without naloxone (10^{-8} and 10^{-6} M) as described in Fig.8. Cell growth was expressed as a percentage of the control cultures (medium without opioids or naloxone). Values represent the means ($\pm$SEM) of 12-18 determinations in 2-3 experiments. (a) Naloxone at concentrations of 10^{-8} and 10^{-6} M totally blocked the inhibitory effect of ß-EP on the growth of SC115 tumor cells in DHT-containing medium. Naloxone at a concentration of 10^{-8} M totally blocked the inhibitory effect of MS on the growth of SC115 tumor cells in DHT-containing medium. Naloxone at a concentration of 10^{-6} M partially blocked the inhibitory effect of MS on the growth of SC115 tumor cells in DHT-containing medium, $p<0.05$. (b) Naloxone at concentrations of 10^{-8} and 10^{-6} M totally blocked the inhibitory effects of ß-EP or MS on the growth of SC115 cells in HC-containing medium. *, $p<0.05$, **, $p<0.01$ compared to controls.


(a) b-EP (10^{-9} M) MS (10^{-9} M)
Percentage of Control
120
100
80
60
40
20
0
C EP EP EP C MS MS MS
0 10^{-8} 10^{-6} 0 10^{-8} 10^{-6}
Nal (M) Nal (M)


(b) b-EP (10^{-9} M) MS (10^{-9} M)
Percentage of Control
120
100
80
60
40
20
0
C EP EP EP C MS MS MS
0 10^{-8} 10^{-6} 0 10^{-8} 10^{-6}
Nal (M) Nal (M)

$p<0.001$, but had no effect in medium containing HC, $p>0.10$, (Fig.17). Post-hoc tests showed that 10^{-6} M naloxone significantly inhibited SC115 tumor cell growth in basic medium by approximately 20%, $p<0.01$ (Fig.17a) and that 10^{-10}, 10^{-8}, and 10^{-6} M naloxone significantly inhibited cell growth in DHT-containing medium by approximately 10-20%, p's<0.05 (Fig.17b).

3.2.5 Effects of Opioids on the Growth of SC115 Tumor Cells from the Animal-tumor Model

(1) Effects of Steroid Hormones on the Growth of SC115 Tumor Cells from Mice in Experimental Housing Groups

This study examined hormone responsiveness of SC115 tumor cells taken from mice in our experimental housing conditions. Cells from mice in both IG (small tumors) and GI (large tumors) groups were significantly stimulated by both 3.5×10^{-8} M DHT ($p<0.01$) and 10^{-6} M HC ($p<0.01$) compared to that seen in basic medium as shown in Fig.18. There were no significant differences between the 2 groups in response to either DHT or HC, $p>0.10$. However, in both GI and IG groups, DHT had a significantly greater stimulatory effect on SC115 tumor cell growth than HC, p's<0.01.

(2) Effects of Opioids and Naloxone on the Growth of SC115 Tumor Cells from Mice in Experimental Housing Groups

This study examined the role of opioids in the differential tumor growth rates of SC115 cells from IG (small tumor) and GI (large tumor) animals. Addition of ß-EP to cells cultured in

Fig.17 Effect of naloxone alone on the growth of primary cultures of SC115 tumor cells. Cells were cultured for 5 d in basic medium with or without DHT (3.5×10^{-8} M) or HC (10^{-6} M) as described in Fig.8. Cell growth was expressed as a percentage of the control cultures (medium without naloxone). Values represent the means ($\pm$SEM) of 16-42 determinations in 3-7 experiments. (a) 10^{-6} M naloxone significantly inhibited SC115 cell growth in basic medium by approximately 20%, $p<0.01$. (b) 10^{-10}, 10^{-8}, and 10^{-6} M naloxone significantly inhibited cell growth in DHT-containing medium by approximately 10-20%, p's<0.05. (c) 10^{-12}-10^{-6} M naloxone had no effect on SC115 cell growth in HC-contining medium. *, $p<0.05$, **, $p<0.01$ compared to controls.

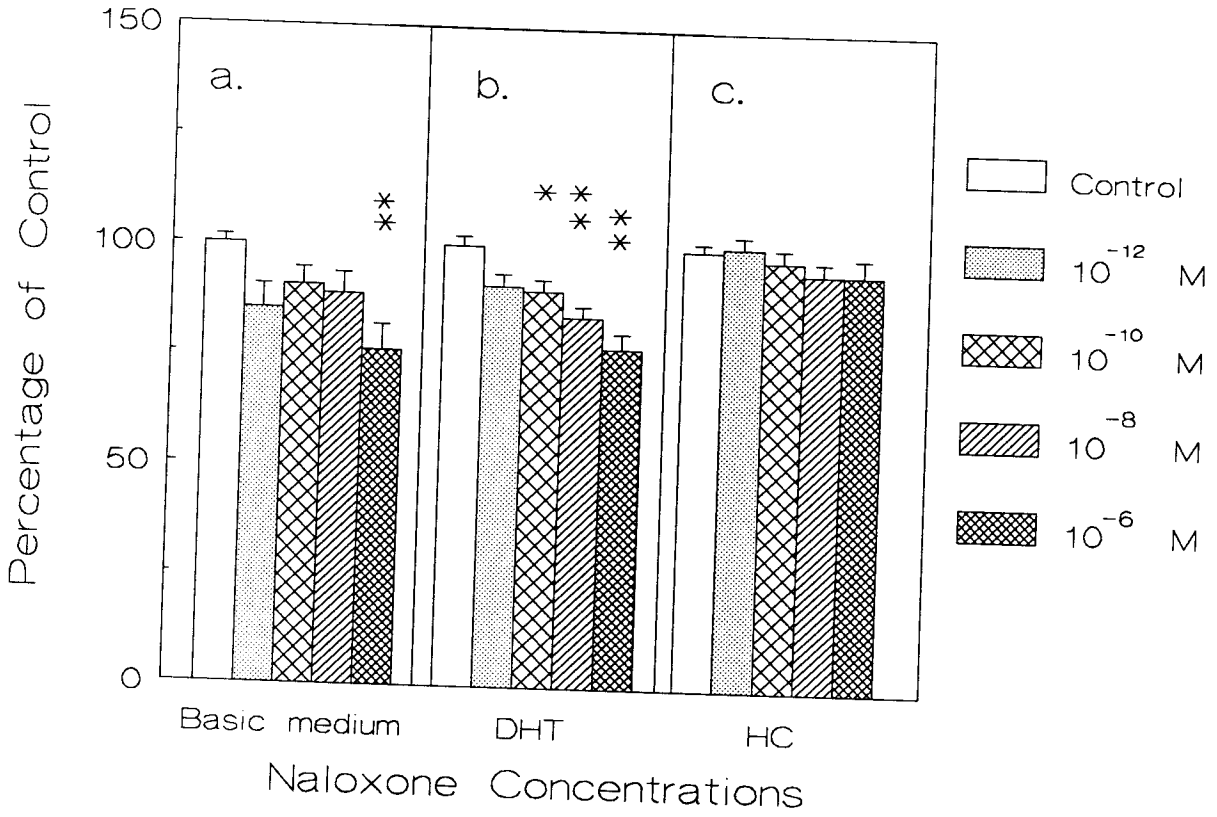
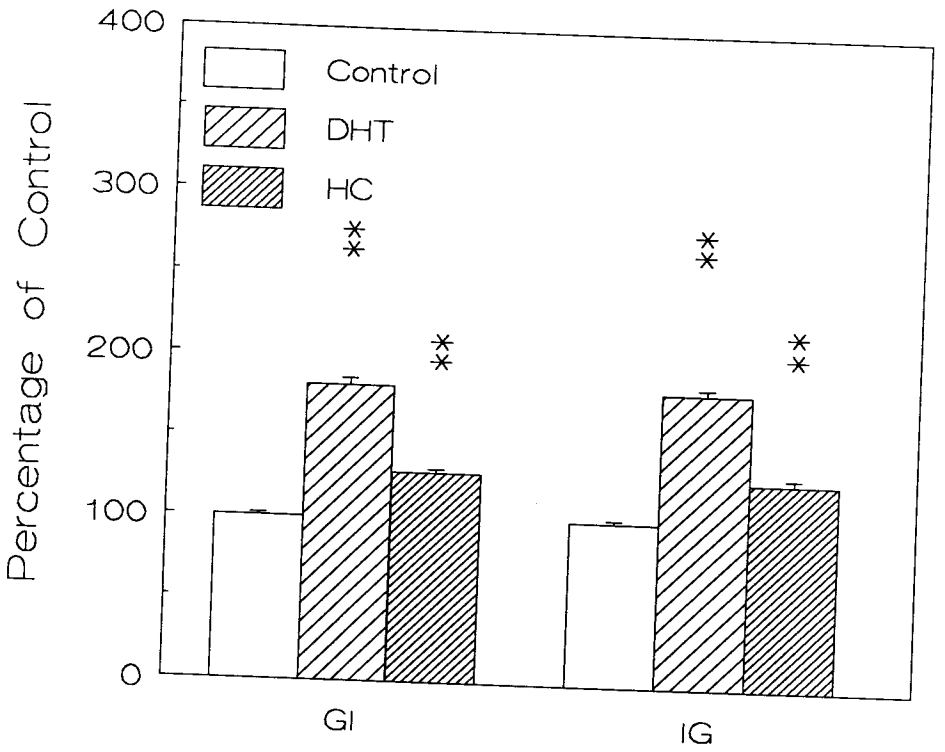

150
100
50
0
Percentage of Control
a.
b.
c.
Basic medium
DHT
HC
Naloxone Concentrations
Control
10 -12 M
10 -10 M
10 -8 M
10 -6 M

Fig.18 Effects of DHT and HC on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Values represent the means ($\pm$SEM) of 6 determinations. SC115 tumor cells from both GI and IG mice were significantly stimulated to the same degree by DHT (3.5×10^{-8} M, p's<0.01) and HC (10^{-6} M, p's<0.01). *, p<0.05 compared to controls.


400
300
200
100
0
Percentage of Control
Control
DHT
HC
GI
IG

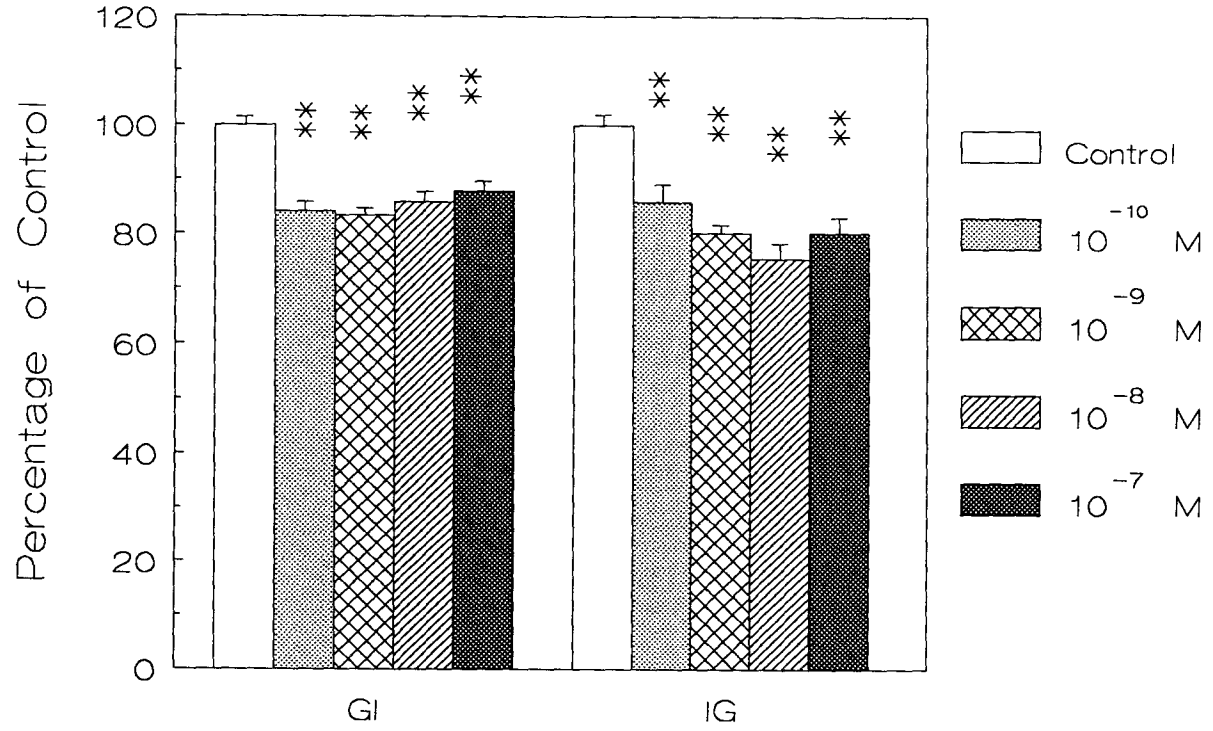
basic medium resulted in a significant main effect of group, $p<0.01$, as well as a group x dose interaction, $p<0.05$, (Fig.19a). Overall, tumor cells from IG mice showed a greater inhibition of growth with ß-EP than tumor cells from GI mice. All concentrations of ß-EP significantly inhibited SC115 tumor cell growth compared to the controls in both GI and IG groups, p's<0.01. In addition, 10^{-8} M ß-EP had a greater inhibitory effect than 10^{-10} M ß-EP on cells from IG mice, $p<0.05$.

With the addition of CZ, there was also a significant main effect of group, $p<0.05$, and a group x dose interaction, $p<0.001$, (Fig.19b). In contrast to the results for ß-EP, with CZ, tumor cells from GI mice showed a greater suppression than tumor cells from IG mice. All concentrations of CZ significantly inhibited SC115 tumor cell growth compared to the controls in both GI and IG groups, p's<0.001. In addition, 10^{-7} M CZ had a greater inhibitory effect than 10^{-9} M CZ on cells from IG mice, $p<0.05$.

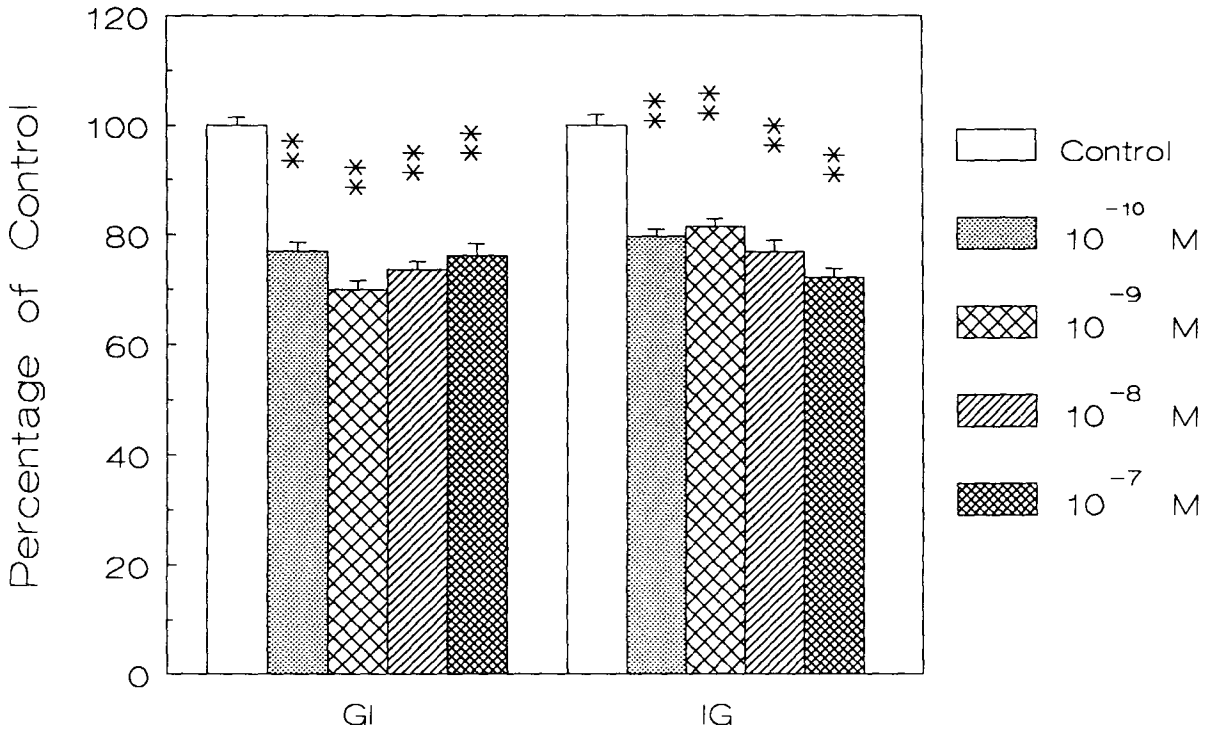
If ß-EP was added to medium containing DHT, there was a significant main effect of group, $p<0.001$, (Fig.20a). Tumor cells from GI mice showed a greater inhibition of growth with ß-EP than tumor cells from IG mice, $p<0.01$. All concentrations of ß-EP significantly inhibited SC115 tumor cell growth compared to the controls in both GI and IG groups, p'2s<0.01. No dose-response relationships were observed for either animal group.

If CZ was added to medium containing DHT, there was also a significant main effect of group, $p<0.001$, (Fig.20b). Tumor cells from GI mice showed a greater inhibition of growth with CZ than tumor cells from IG mice, $p<0.001$. All concentrations of CZ

Fig.19 Effects of ß-EP and CZ on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium. Cell growth was expressed as a percentage of the control cultures (medium without opioids). Values represent the means (±SEM) of 6 determinations. (a) SC115 tumor cell growth from both GI and IG mice was significantly inhibited by 10^{-10}-10^{-7} M ß-EP (p's<0.01). (b) SC115 tumor cell growth from both GI and IG mice was significantly inhibited by 10^{-10}-10^{-7} M CZ (p's<0.01). **, p<0.01 compared to controls.

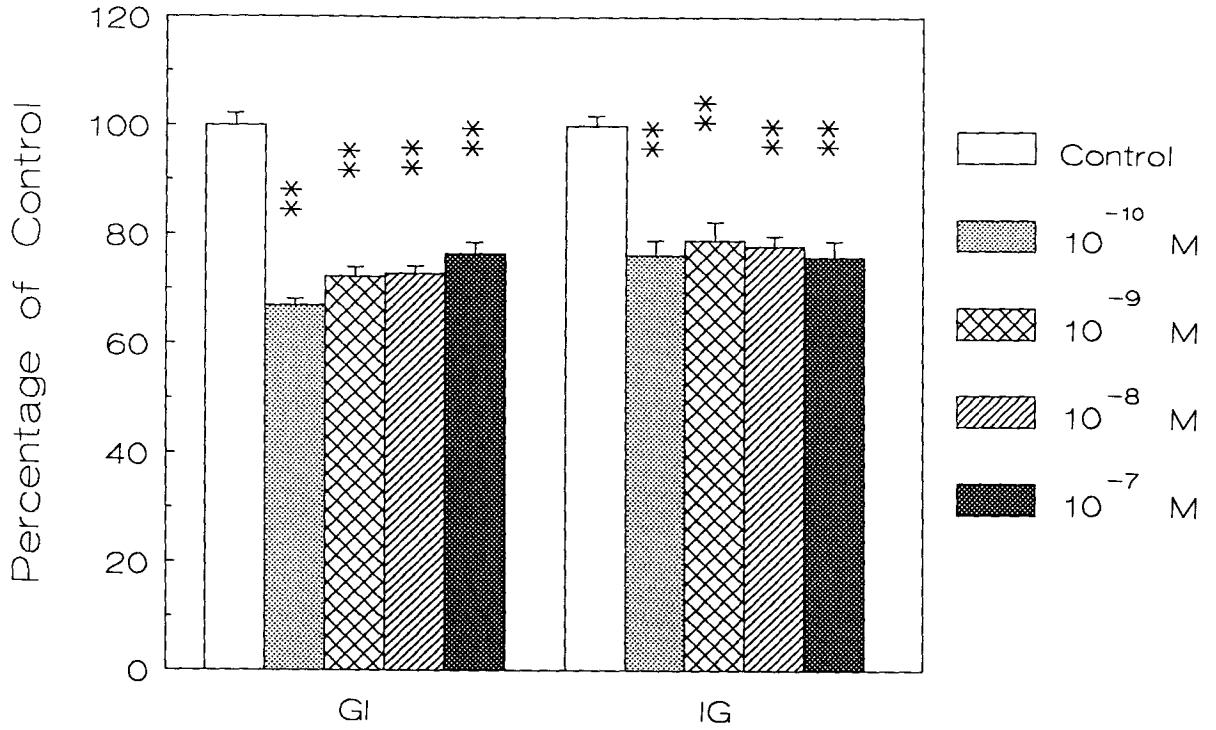

120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(a) b-EP

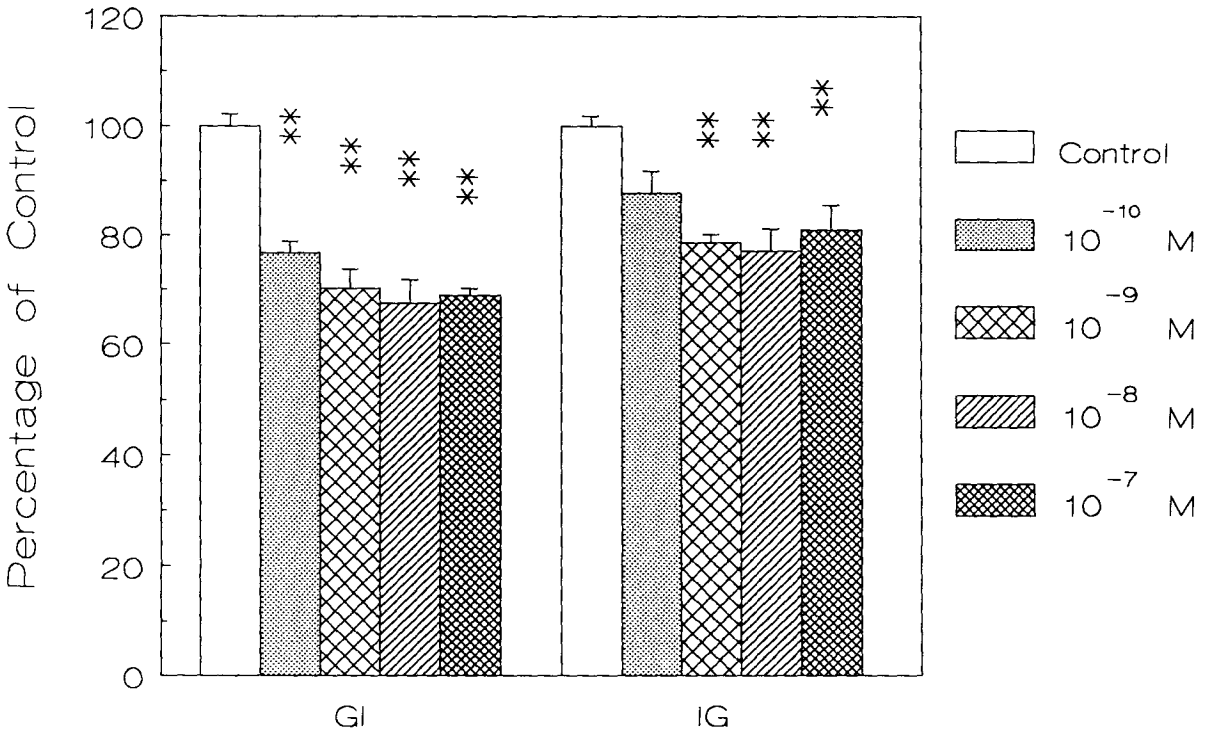

120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(b) CZ

Fig.20 Effects of ß-EP and CZ on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium containing DHT (3.5×10^{-8} M). Cell growth was expressed as a percentage of the control cultures (medium without opioids). Values represent the means ($\pm$SEM) of 6 determinations. (a) Growth of SC115 cells from both GI and IG mice was significantly inhibited by 10^{-10} - 10^{-7} M ß-EP (p's<0.01). (b) Growth of SC115 cells from GI mice was significantly inhibited by 10^{-10}-10^{-7} M CZ (p's<0.01), whereas growth of SC115 cells from IG mice was significantly inhibited by 10^{-9}-10^{-7} M CZ (p's<0.01). *, p<0.05, **, p<0.01 compared to controls.


120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(a) b-EP


120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(b) CZ

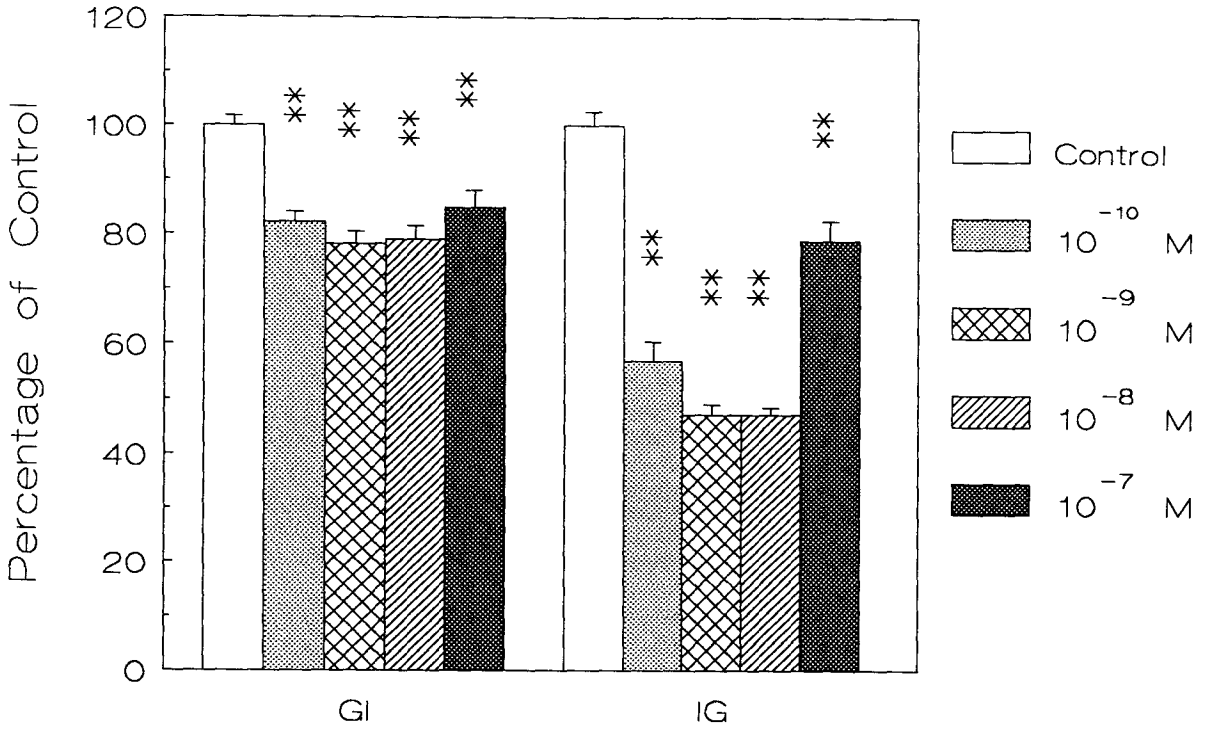
significantly inhibited SC115 tumor cell growth compared to the controls in GI mice, p's<0.001. CZ at concentrations of 10^{-9}-10^{-7} M significantly inhibited SC115 tumor cell growth compared to the controls in IG mice, p's<0.001. No dose-response relationships were observed for either animal group.

If ß-EP was added to medium containing HC, there was a significant main effect of group, p<0.001, as well as a group X dose interaction, p<0.001 (Fig.21a). Tumor cells from IG mice showed a greater inhibition of growth with ß-EP than tumor cells from GI mice. All concentrations of ß-EP significantly inhibited SC115 tumor cell growth compared to the controls in both GI and IG groups, p's<0.01. In addition, growth inhibition was greater with concentrations of 10^{-10}-10^{-8} M ß-EP than with a concentration of 10^{-7} M ß-EP on cells from IG mice, p's<0.001.

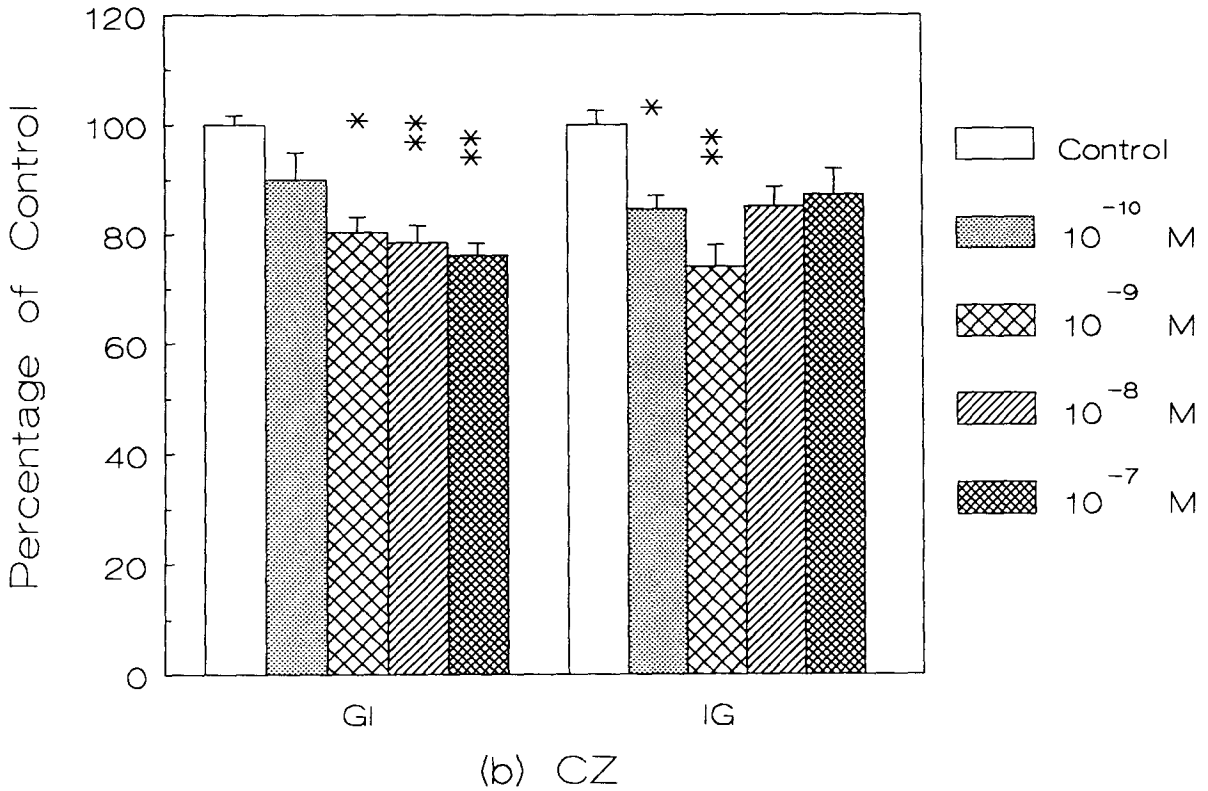
If CZ was added to medium containing HC, there was a significant group X dose interaction, p<0.05 (Fig.21b). In GI mice, CZ at concentrations of 10^{-9}-10^{-7} M significantly inhibited SC115 tumor cell growth, p's<0.01, whereas in IG mice, CZ at concentrations of 10^{-10}-10^{-9} M significantly inhibited SC115 tumor cell growth, p's<0.05, compared to that in control cultures. No dose-response relationships were observed for either animal group.

Additionally, naloxone at concentrations of 10^{-12}-10^{-6} M totally blocked the effects of 10^{-9} M ß-EP at all concentrations examined in the GI group both in basic medium and in HC-containing medium (Fig.22a and Fig.24a). However, naloxone at a concentration of 10^{-6} M had a slight but significant stimulatory

Fig.21 Effects of ß-EP and CZ on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium containing HC (10^{-6} M). Cell growth was expressed as a percentage of the control cultures (medium without opioids). Values represent the means ($\pm$SEM) of 6 determinations. (a) Growth of SC115 cells from both GI and IG mice was significantly inhibited by 10^{-10}-10^{-7} M ß-EP (p's<0.01). (b) Growth of SC115 cells from GI mice was significantly inhibited by 10^{-9}-10^{-7} M CZ (p's<0.01), whereas growth of SC115 cells from IG mice was significantly inhibited by 10^{-10}-10^{-9} M CZ (p's<0.01). *, p<0.05, **, p<0.01 compared to controls.

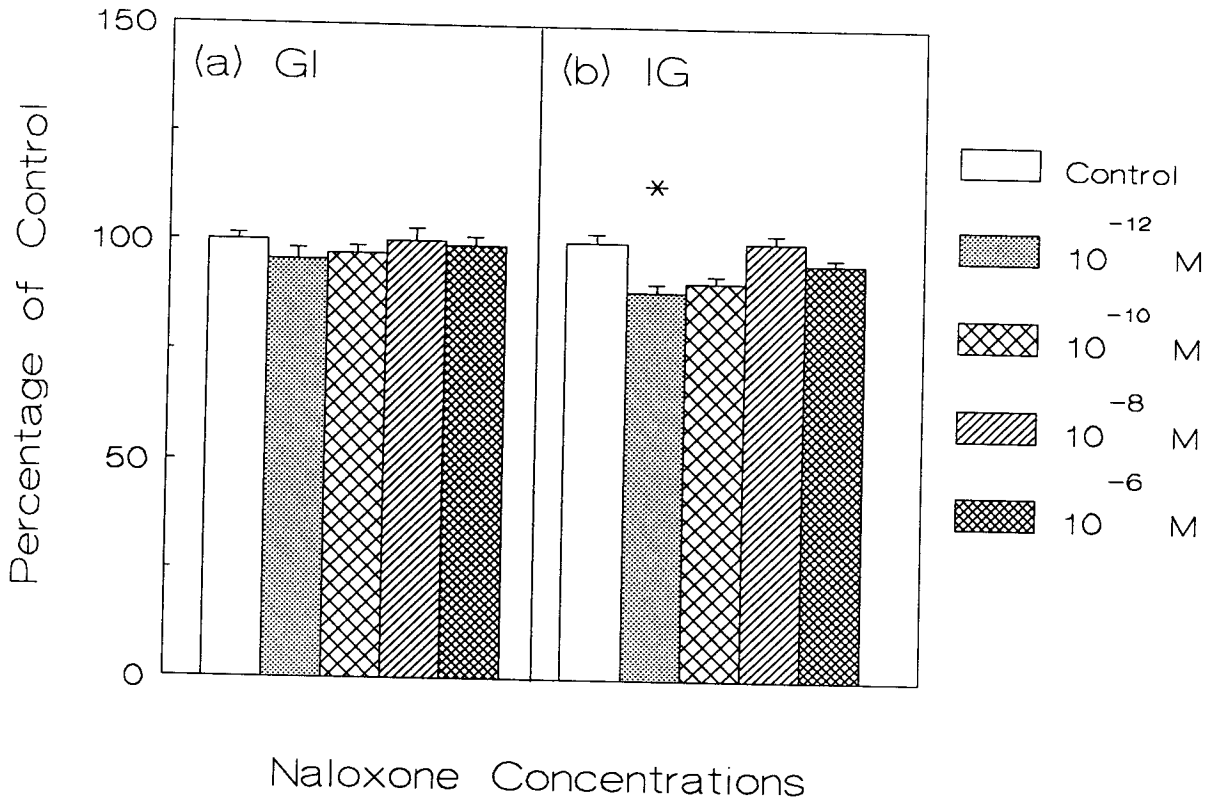

120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(a) b-EP


120
100
80
60
40
20
0
Percentage of Control
GI
IG
Control
10 -10 M
10 -9 M
10 -8 M
10 -7 M

(b) CZ

Fig.22 Effects of ß-EP plus naloxone in basic medium on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium with ß-EP (10^{-9} M) plus naloxone (10^{-12}-10^{-6} M). Cell growth was expressed as a percentage of the control cultures (medium without opioid or naloxone). Values represent the means (±SEM) of 6 determinations. (a) In GI mice, naloxone at concentrations of 10^{-12}-10^{-6} M totally blocked the inhibitory effect of ß-EP on SC115 cell growth compared to that in the control. (b) In IG mice, naloxone at a concentration of 10^{-12} M partially blocked the inhibitory effect of ß-EP on SC115 cell growth compared to that in the control ($p<0.01$), but naloxone at concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M totally blocked the inhibitory effect of ß-EP on cell growth. **, $p<0.01$ compared to controls.


150
100
50
0
(a) GI
(b) IG
*
Percentage of Control
Control
10 -12 M
10 -10 M
10 -8 M
10 -6 M
Naloxone Concentrations

effect on the growth of SC115 tumor cells from GI mice in medium containing DHT (3.5×10^{-8} M), p's<0.01 (Fig. 23a). In the IG group, naloxone, at a concentration of 10^{-12} M partially blocked the inhibitory effect of ß-EP (by about 92%) in basic medium, p<0.05 (Fig.22b) and at concentrations of 10^{-10}-10^{-6} M totally blocked the inhibitory effect of ß-EP, p's>0.10. Naloxone at all concentrations examined totally blocked the inhibitory effect of ß-EP in HC-containing medium in both GI and IG animals, p's>0.10 (Fig.24a and 24b) and at a concentration of 10^{-8} M partially blocked the inhibitory effect of ß-EP in DHT-containing medium (by almost 95%), p<0.01 (Fig.23b).

Naloxone alone had no effect on the growth of tumor cell from either GI or IG groups in basic medium or in medium containing DHT or HC, p>0.10 (Fig.25a and 25b, respectively).

Fig.23 Effects of ß-EP plus naloxone in DHT-containing medium on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium with ß-EP (10^{-9} M) plus naloxone (10^{-12}-10^{-6} M). Cell growth was expressed as a percentage of the control cultures (medium without opioid or naloxone). Values represent the means ($\pm$SEM) of 6 determinations. (a) In GI mice, naloxone at concentrations of 10^{-12}-10^{-8} M totally blocked the inhibitory effect of ß-EP on SC115 cell growth compared to that in the control whereas naloxone at a concentration of 10^{-6} M significantly increased cell growth ($p<0.01$). (b) In IG mice, naloxone at a concentration of 10^{-8} M partially blocked the inhibitory effect of ß-EP on cell growth compared to that in the control ($p<0.05$), but naloxone at concentrations of 10^{-12}, 10^{-10}, and 10^{-6} M totally blocked the inhibitory effect of ß-EP on cell growth. *, $p<0.05$, **, $p<0.01$ compared to controls.

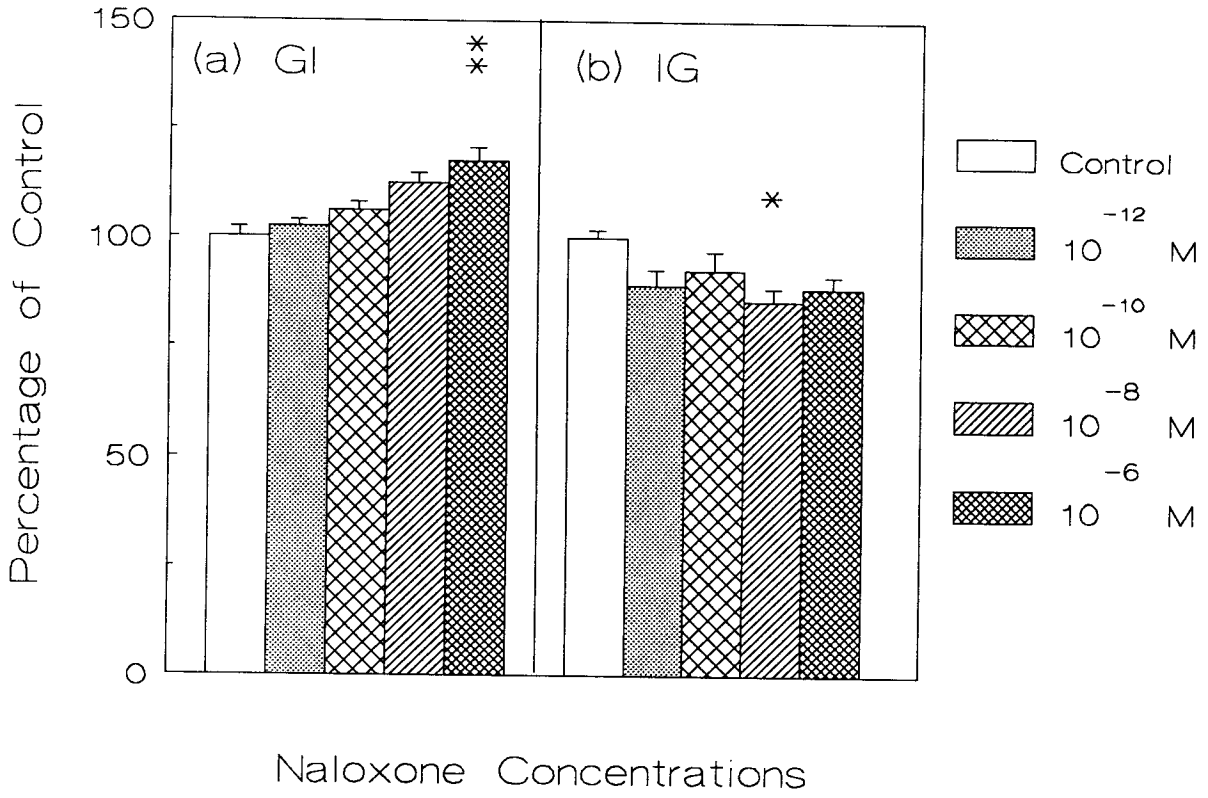

150
100
50
0
Percentage of Control
(a) GI
(b) IG
Control
10 -12 M
10 -10 M
10 -8 M
10 -6 M
Naloxone Concentrations

Fig.24 Effects of ß-EP plus naloxone in HC-containing medium on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium with ß-EP (10^{-9} M) plus naloxone (10^{-12}-10^{-6} M). Cell growth was expressed as a percentage of the control cultures (medium without opioid or naloxone). Values represent the means (±SEM) of 6 determinations. (a) In GI mice, naloxone at concentrations of 10^{-12}-10^{-6} M totally blocked the inhibitory effect of ß-EP on SC115 cell growth compared to that in the control. (b) In IG mice, naloxone at concentrations of 10^{-12}-10^{-6} M totally blocked the inhibitory effect of ß-EP on cell growth compared to that in the control.

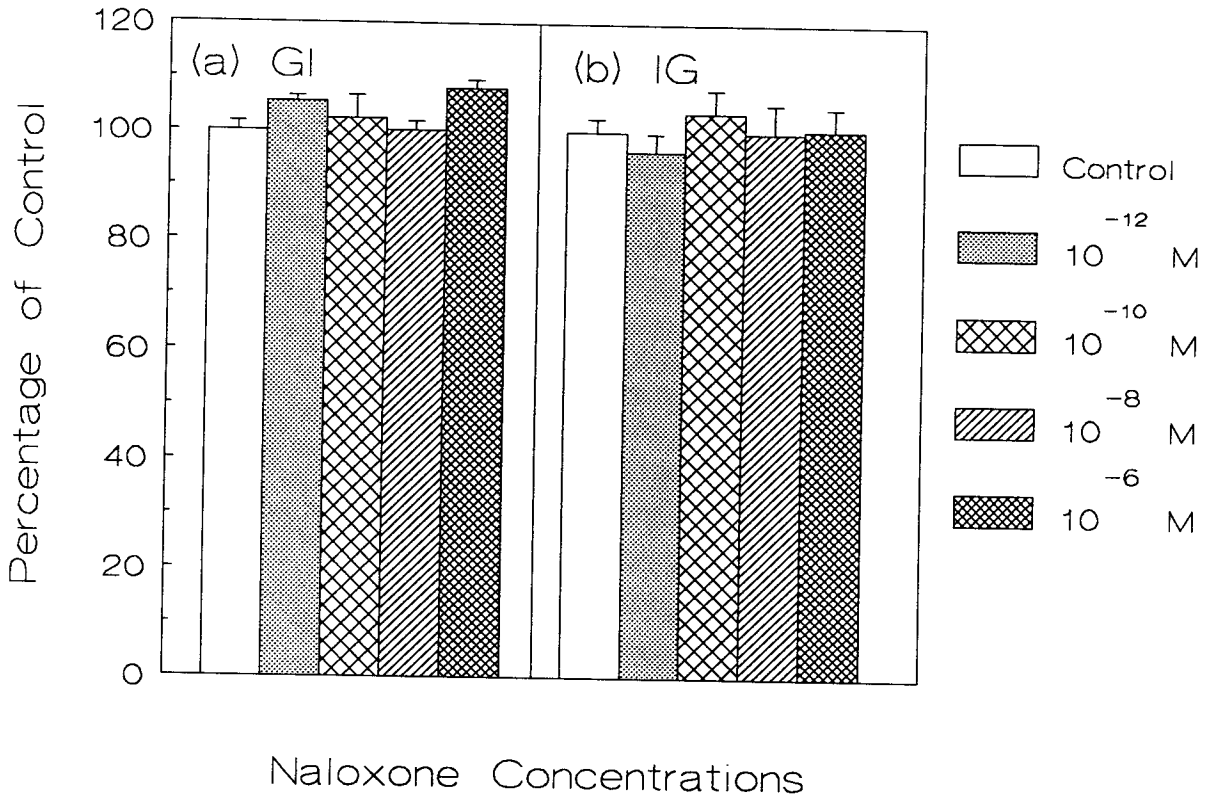
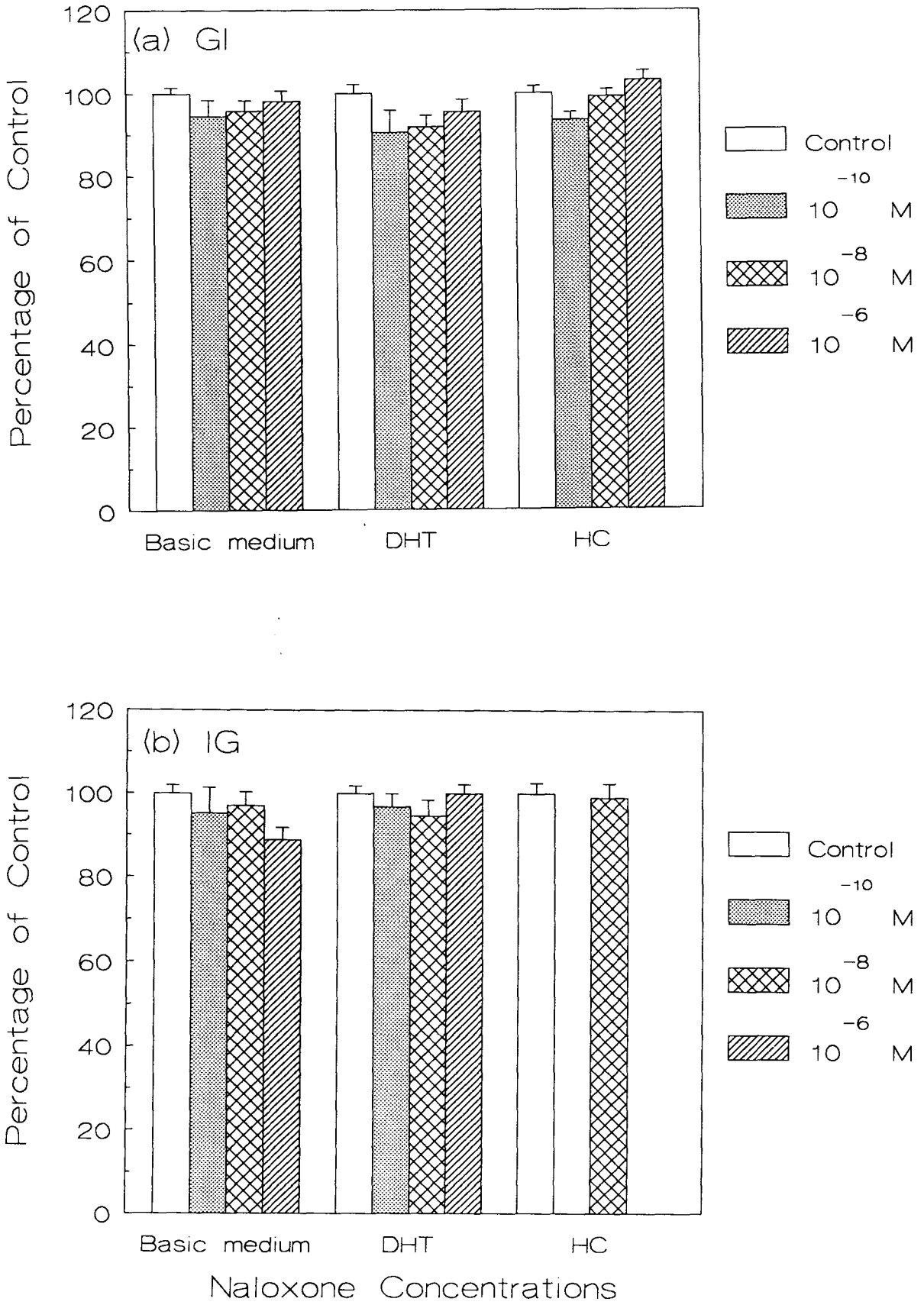

120
100
80
60
40
20
0
Percentage of Control
(a) GI
(b) IG
Control
10 -12 M
10 -10 M
10 -8 M
10 -6 M
Naloxone Concentrations

Fig.25 Effects of naloxone on the growth of primary cultures of SC115 tumor cells from mice in the experimental housing groups. Cells were cultured in basic medium or medium containing DHT (3.5×10^{-8} M) or HC (10^{-6} M). Cell growth was expressed as a percentage of the control cultures (medium without naloxone). Values represent the means ($\pm$SEM) of 6 determinations. (a) Naloxone at concentrations of 10^{-10}-10^{-6} M had no effect on the growth of SC115 tumor cells from GI mice. (b) Naloxone at concentrations of 10^{-10}-10^{-6} M had no effect on the growth of SC115 tumor cells from IG mice.


(a) GI
(b) IG
Percentage of Control
120
100
80
60
40
20
0
Basic medium
DHT
HC
Control
10 -10 M
10 -8 M
10 -6 M
Naloxone Concentrations

IV. Discussion and Conclusions

The present study investigated the effects of steroid hormones, growth factors, and opioid peptides on the growth of the androgen-responsive (AR) Shionogi mouse mammary carcinoma (SC115) in primary culture.

4.1 Culturing AR SC115 Tumor Cells on a Collagen Substrate

Mammary tumor cells cultured on conventional tissue culture plastic lose their tissue specific characteristics, making it difficult to extrapolate data from experiments *in vitro* to the *in vivo* situation. For example, Yates and King (1981) cultured a cell line originating from the AR SC115 tumor on plastic, and observed that changes in growth properties and morphology accompanied changes in hormone responsiveness which did not reflect tumor behavior *in vivo*. In contrast, it has been shown that normal mammary epithelial cells from several species retain their normal morphological and functional characteristics when grown on a collagen substrate (Emerman *et al.* 1990; Emerman & Pitelke 1977). Emerman and Worth (1985) demonstrated that the growth rates, morphological, and tumorigenic potential characterizing the AR SC115 tumor and its androgen-independent (AI) variant persist when cells from these tumors are cultured on collagen gels. Furthermore, a study of the interaction between hormones and chemotherapeutic agents on AR SC115 tumor cell survival demonstrated that results in the *in vitro* collagen gel system (Emerman 1988) parallel the results obtained in *in vivo*

studies (Emerman & Siemiatkowcki 1984). In the present study, AR SC115 tumor cells were cultured on collagen-coated plates in all experiments. Therefore, we believe that our *in vitro* results accurately reflect the *in vivo* situation.

4.2 Growth-stimulation of AR SC115 Tumor Cells by Steroid Hormones and Growth Factors

Previous data have shown that physiological concentrations of androgens and pharmacological concentrations of glucocorticoids significantly stimulate the proliferation of SC115 tumor cells *in vivo* (Bruchovsky & Meakin 1973; Hiraoka *et al.* 1987; Matsumoto *et al.* 1982; Watanabe *et al.* 1982) as well as the growth of the AR cell line, SC-3 (derived from the SC115 tumor) *in vitro* (Hiraoka *et al.* 1987; Omukai *et al.* 1982; Yates *et al.* 1980; Yates & King 1978). Data from the present experiments with SC115 tumor cells in primary culture confirm that dihydrotestosterone (DHT) ranging from 3.5×10^{-10} - 3.5×10^{-6} M and hydrocortisone (HC) ranging from 10^{-9}-10^{-5} M significantly increase growth of SC115 tumor cells. With DHT, a significant dose-response curve is observed. Maximal stimulation by DHT occurs with the physiological concentration (3.5×10^{-8} M) whereas maximal stimulation by HC occurs with a pharmacological concentration (10^{-7} M).

Our data also show that 17ß-estradiol (E_2), ranging from physiological to pharmacological concentrations (10^{-9}-10^{-6} M) inhibits the growth of SC115 tumor cells. Previous studies indicate that E_2 in concentrations even higher than 10^{-6} M has no

effect on SC-3 cell growth *in vitro* (Noguchi *et al.* 1987), but inhibits the growth-stimulating effect of testosterone on SC-3 cells in a dose-dependent manner in serum-supplemented medium (Noguchi *et al.* 1987). E_2 also shows competitive effects on [^{3}H]-testosterone binding to androgen receptors in SC-3 cells. Effects of estrogens on growth of the AR SC115 tumor *in vivo*, however, show conflicting results. Nohno *et al.* (1982) showed that when E_2 at a daily dose of 2 mg/kg is given to intact male mice bearing the original androgen-dependent SC115 tumor, tumor growth as well as weight gains of seminal vesicles and prostate gland are inhibited. Estrogens bind to the estrogen receptor (ER) but also bind to the androgen receptor which is likely responsible for their inhibitory effects on growth. In other studies, pharmacological concentrations of E_2 (160-400 μg/kg) were shown to stimulate this androgen-dependent SC115 tumor cell growth in castrated male mice in a dose-dependent manner (Noguchi *et al.* 1984; 1985a). E_2 can act synergistically with low doses of testosterone propionate (TP) to stimulate tumor growth (Noguchi *et al.* 1984). The stimulatory effect of estrogen on proliferation of SC115 tumor cells was shown to be mediated by ER, but not androgen receptor *in vivo*. The inhibitory effect of E_2 observed in our experiments may help to explain the fact that the original AR SC115 tumor grows more rapidly in males than in females. It is possible that E_2 may bind to the ER as well as to the androgen receptor (Nohno *et al.* 1982).

Recent studies have shown that growth stimulatory effects of sex steroids on cell proliferation are mediated by specific

polypeptide growth factors (Lippman *et al.* 1986). Data suggest that the effect of androgens on proliferation of SC115 tumor cells is mediated by a fibroblast growth factor (FGF)-like polypeptide in an autocrine fashion (Nakamura *et al.* 1989). Our results show that bFGF also enhances SC115 tumor cell growth in primary culture in a dose-dependent manner (by 3 fold) in serum-containing medium. Similarly, evidence suggests that E_2 stimulation of mammary epithelial cell growth in the female (Davidson & Lippman 1989) may be mediated by epidermal growth factor (EGF) through an autocrine mechanism (Bates *et al.* 1986; Dickson *et al.* 1986). In estrogen-responsive cancer cells, Lippman *et al.* (1986) have suggested that all stimulatory effects of estrogens on the growth of human breast cancer cells such as MCF-7 are mediated through transforming growth factor (TGF)-a or insulin-like growth factor-I (IGF-I) through an autocrine mechanism. Our data demonstrate that EGF inhibits cell growth at all concentrations examined in serum-containing medium. These results suggest that the inhibitory effect of estrogen on SC115 tumor growth may also be mediated by EGF.

In order to investigate the molecular mechanisms of tumor cell growth, however, a culture system using a serum-free medium is desirable, since serum contains various growth factors and unknown other factors. Other investigators (Nakamura *et al.* 1987; Nonomura *et al.* 1988) using a serum-free culture system (serum-free-I as defined in Methods), found that testosterone, high concentrations of dexamethasone (Dex), and bFGF significantly stimulate the growth of SC-3 cells. For our studies, we developed

a serum-free medium which was optimal for growing SC115 tumor cells in primary culture (*i.e.*, serum-free-II as defined in Methods). Consistent with previous studies, our data show that DHT (3.5×10^{-8} M) and bFGF (1-500 ng/ml) at all concentrations examined significantly stimulate SC115 tumor cell growth in primary culture in our serum-free culture medium. Furthermore, our data demonstrate a marked inhibitory effect of anti-bFGF antibody on bFGF- and androgen-induced growth of SC115 tumor cells. Therefore, the present study provides evidence suggesting that SC115 tumor cells may produce a FGF-like peptide(s) which mediates, at least partially, their androgen-induced growth. Anti-bFGF antibody also inhibited growth to a limited degree in the absence of DHT or bFGF. It is possible that bFGF is an autocrine growth factor for these cells that is produced in smaller quantities in the absence of DHT. In contrast, EGF at all concentrations examined (1-100 ng/ml) had no effect on the growth of SC115 tumor cells in serum-free medium whereas E_2 had a marked growth inhibitory effect on SC115 tumor cells in serum-free medium. So it is unlikely that the inhibitory effect of E_2 on the growth of SC115 tumor cells is mediated by EGF. In addition, anti-EGF antibody also had a significant inhibitory effect on the grwoth of SC115 tumor cells in serum-free medium, and this effect was the same in medium containing EGF, E_2, or in serum-free medium alone. The reason for the non-specific effect of anti-EGF on SC115 tumor cell growth remains to be investigated. This non-specific effect may be due to the fact that the concentration of anti-EGF antibody is too high, so it inhibits cell growth

nonspecifically. Alternatively, the antibody may bind to some unknown factors in the serum-free medium to inhibit the cell growth.

4.3. Growth-stimulation of AR SC115 Tumor Cells by Steroid Hormones is Inhibited by Opioid Agonists

Opioids and opioid peptides can modulate both normal and malignant cell growth (Lewis *et al.* 1983; Slotkin *et al.* 1980; Willson *et al.* 1976; Zagon & McLaughlin 1981a). The present study is the first to demonstrate that the opioid peptide system may be involved in modulating the growth of SC115 tumor cells in primary culture. Three opioids, ß-endorphin (ß-EP), cyclazocine (CZ), and morphine sulfate (MS), at the minimal dose of 10^{-11} M were shown to retard SC115 tumor cell growth in basic medium, as well as in DHT-, HC-, 5% FBS-, and bFGF-containing media. The opioids had a greater inhibitory effect in the presence of DHT than in its absence. Furthermore, the inhibitory effect of ß-EP occurred at the minimal dose of 10^{-10} M in bFGF-containing medium. No dose-response relationship was observed at doses of 10^{-11}-10^{-7} M for all 3 opioids in all medium conditions.

The various neuropharmacological effects of opioids and endogenous opioid peptides are known to occur through interaction with at least one of the 4 opioid receptor subtypes (δ, k, μ, and σ). Although multiple opioid receptor subtypes have not been characterized from SC115 tumor cells at this time, it is possible that opioid modulation of tumor growth may be mediated by peptide binding to opioid receptors. Multiple cellular receptors for

opioid peptides have been found in several other tumor lines, including neuroblastomas, the MCF-7 cell line, and small cell carcinomas of the lung (Kazmi & Mishra 1986; Maneckjee *et al.* 1990; Roth & Bachas 1986). The effects of opioids on tumor growth have been seen both *in vivo* (Lewis *et al.* 1983; Zagon & McLaughlin 1981a) and *in vitro* (Maneckjee *et al.* 1990; VonHoff & Forseth 1982; Zagon & McLaughlin 1981c). *In vitro*, opioids probably exert their inhibitory effects on tumor growth via direct binding to tumor cell receptors. This has been demonstrated in neuroblastoma (Zagon & McLaughlin 1981c) and in the human breast cancer MCF-7 cell line (Maneckjee *et al.* 1990). The effects of opioids on tumor growth *in vivo* may be stimulatory or inhibitory. Chronic administration of heroin effectively retards tumor growth and prolongs survival time in mice with transplanted neuroblastoma (Zagon & McLaughlin 1981a). Rats injected with a mammary ascites tumor and subjected to an inescapable footshock stress, which causes endogenous opioid release, manifest enhanced tumor growth indicated by decreased survival time and decreased percent survival (Lewis *et al.* 1983). Three possible mechanisms may underlie opioid effects on tumor growth *in vivo*. First, they may alter tumor growth through direct effect on opioid receptors present on tumor cells. Multiple opioid receptors have been characterized from several tumor cell lines, although further work needs to be done to characterize the opioid receptors in the SC115 tumor. Second, they may alter tumor growth through the release of hormones which can modulate tumor growth, such as prolactin and growth hormone. These two hormones

are known to have a significant impact on mammary tumor growth (Emerman *et al.* 1985; Shiu *et al.* 1987). Third, they may alter tumor growth indirectly through modulation of immune surveillance mechanisms. Opioids have been found to influence the functions of most of the major cell types within the immune system (Fischer 1988; Weber & Pert 1984). Opioids can decrease antibody production, depress mitogen-induced blastogenesis by both T and B lymphocytes, enhance or reduce CTL or NK cell activities, and modulate the production of lymphokines (Bocchini *et al.* 1983; Faith *et al.* 1984; Gilman *et al.* 1982; Gilmore & Weiner 1988; Johnson *et al.* 1982, Shavit *et al.* 1987). The final effect of opioids on tumor growth will be the net effect of these three influences.

Our data further demonstrate that when naloxone, a μ opioid receptor antagonist, is added to the culture medium, it totally or partially blocks the inhibitory effects of ß-EP and MS on tumor cell growth. Such reversal of opioid effects has been demonstrated in several other systems both *in vivo* (Zagon & McLaughlin 1981a) and *in vitro* (Maneckjee *et al.* 1990; Zagon & McLaughlin 1983), and may reflect both direct and indirect mechanisms of action of opioid peptides and their antagonists. The regression of growth of spontaneous and transplanted mammary tumors in C3H mice by intratumoral injections of naloxone suggests that the effect of this drug may be through a direct action against the tumor itself (Tsunashima *et al.* 1982) involving the opioid receptor system. In addition, the opioid antagonist naltrexone has been shown to have both positive and

negative effects on the growth of neuroblastomas in mice (Zagon & McLaughlin 1983). These apparently conflicting effects result from dose-dependent actions of the drug. Lower doses of naltrexone reduce tumor incidence and delay the appearance of tumors, whereas higher doses reduce the time before tumor appearance and decrease survival time compared to control, tumor bearing mice. In our system, naloxone alone also shows small but significant inhibitory effects on tumor cell growth in medium containing physiological doses of DHT and in basic medium, but not in medium containing HC. It is possible that if naloxone is added to medium together with opioids, it acts as an antagonist, but if added alone, it may act as a partial agonist (through opioid receptors). Alternatively, naloxone alone may act through independent channels (through different receptors) or may provoke a compensatory release of endorphins, and these endogenous compounds may in turn work in a fashion similar to that of opioids. Evidence has shown that some tumor lines secrete endogenous opioids, including the MCF-7 cell line and small lung carcinoma (Roth & Barchas 1986). Naloxone is also a potent inhibitor of prolactin secretion by the pituitary (Armstrong *et al.* 1988) and this hormone has been implicated in growth and maintenance of mammary carcinoma.

In these experiments, the inhibitory effect of opioids is greater in the DHT-stimulated medium than in the basic steroid hormone-free medium. Since the majority of human breast cancers are ER positive and hence believed to be hormonally responsive, this observation may have clinical significance. In fact, in the

MCF-7 cell line, the inhibitory effect of opioids is restricted to the hormonally responsive fraction (Maneckjee *et al.* 1990). This observation may also have relevence for the choice of endocrine therapy and/or chemotherapy. Evidence has already shown that concentrations of androgen suboptimal for growth increase the sensitivity of the AR SC115 tumor to chemotherapeutic agents whereas the condition with no androgens present is least sensitive to these agents (Emerman 1988). Whether or not these effects of opioids observed in culture translate to the *in vivo* system of solid tumor growth remains to be determined. However our data would suggest that a combination of endocrine ablation and administration of antineoplastic agents may not be beneficial to the clinical management of breast cancer.

4.4 Opioid Agonists Inhibit the Growth of AR SC115 Tumor Cells from Animals in the Experimental Conditions of Our Animal-tumor Model

The SC115 tumor is heterogenous, containing AR and AI cells (Emerman & Semiatkowski 1984; Emerman & Worth 1985). It has been demonstrated that growing SC115 tumor cells in an androgen deprived environment (in a female mouse or *in vitro*) results in the selection of AI cells (Darbre & King 1987; Emerman 1988). Therefore a study was undertaken to determine if selection for cells with greater (AR) or lesser (AI) hormone sensitivity may occur in animals in our different housing conditions resulting in differential tumor growth rates (Rowse *et al.* 1992). That is, we wanted to determine if tumors from GI mice (large tumors) have a

greater response to DHT or HC than tumors from IG mice (small tumors). The results of that study indicated that the differential tumor growth rates observed between animals from the GI and IG housing groups are not due to differential selection of AI versus AR tumor cells. Whereas *in vivo*, tumors in IG mice have a slower growth rate than tumors in GI mice, *in vitro*, cells from tumors of IG mice grow faster and are actually more responsive to DHT and HC than cells from tumors of GI mice. Data from our experiments replicate the findings of Rowse *et al.* (1992).

Furthermore, the possible role of opioid peptides in modulating the differential tumor growth rates of mice from the experimental housing groups was also examined. Tumors were taken from mice in GI and IG groups. Our data show that SC115 tumor cells from animals in both IG and GI groups are significantly inhibited by all 3 opioid peptides (ß-EP, CZ, and MS). Overall, ß-EP had a greater inhibitory effect on the growth of SC115 tumor cells from IG mice than on the growth of cells from GI mice in both basic medium and HC-containing medium. However, in DHT-containing medium, ß-EP had a greater inhibitory effect on the growth of SC115 tumor cells from GI mice than on the growth of cells from IG mice. CZ had a greater inhibitory effect on the growth of SC115 tumor cells from GI mice than on the growth of cells from IG mice in basic medium and DHT-containing medium, but CZ had the same inhibitory effect on the growth of cells from both groups in HC-containing medium. The inhibitory effect of ß-EP on tumor cell growth was totally blocked by the presence of naloxone in DHT- or HC-containing medium. Naloxone alone had no

effect on the growth of SC115 tumor cell from experimental mice.

As noted above, naloxone alone had a small but significant inhibitory effects on the growth of SC115 tumor cells from mice reared under our standard laboratory housing conditions (*i.e.* housed in groups of 3, not subjected to stress) in some medium conditions possibly due to naloxone's partial agonist effect. Naloxone had no effect on tumor cells from mice exposed to psychosocial stressors (different housing conditions). Stress may change the response of tumor cells to naloxone.

Evidence from previous work in our laboratory suggests that the differential tumor growth rates of mice from the different housing conditions in our animal-tumor model may be mediated by both endocrine and immune changes in these mice. Plasma levels of androgens appear to be particulary important (Rowse *et al.* 1993). Data from the present experiments now show that opioids are regulators of SC115 tumor cell growth *in vitro*. The relevance of changes in plasma and/or pituitary levels of ß-EP-like immunoreactivity in SC115 tumor growth rates of mice housed in the experimental housing conditions of our model remains to be determined.

4.5 Concluding Remarks

The experiments in this thesis were designed to investigate the effects of steroid hormones and growth factors on the growth of the AR SC115 mammary carcinoma in primary culture. A second objective of this thesis was to study the effects of opioid

peptides on the growth of the AR SC115 carcinoma. The major finding from this thesis are as follows:

1) DHT (3.5×10^{-10}-3.5×10^{-6} M) and HC (10^{-9}-10^{-5} M) significantly stimulate SC115 tumor cell growth in serum-containing medium. The stimulatory effect of DHT is dose-responsive. DHT (3.5×10^{-8} M) also stimulates SC115 tumor cell growth in serum-free medium. The maximal effect of DHT is at the physiological concentration (3.5×10^{-8} M) and that of HC is at a pharmacological concentration (10^{-7} M).
2) bFGF (1-100 ng/ml) significantly stimulates the AR SC115 tumor cell growth in serum-containing in a dose-response manner and in serum-free medium at all concentrations examined (1-500 ng/ml).
3) Anti-bFGF antibody significantly inhibits the DHT- and bFGF-induced SC115 tumor cell growth. AR SC115 tumor cells may produce a FGF-like polypeptide which partially mediates androgen-induced growth.
4) E_2, from physiological to pharmacological concentrations, significantly inhibits the AR SC115 tumor cell growth in serum-containing medium. In addition, E_2 (10^{-8} M) also inhibits SC115 tumor cell growth in serum-free medium.
5) EGF (1-100 ng/ml) significantly inhibits the AR SC115 tumor cell growth in serum-containing medium, but not in serum-free medium.
6) ß-EP, CZ, and MS (10^{-11}-10^{-7} M) significantly inhibit the growth of the AR SC115 tumor cells in medium containing DHT, HC, bFGF, or in medium containing 5% FBS at the minimal dose

of 10^{-11} M. Inhibition by ß-EP occurs at a minimal dose of 10^{-10} M in bFGF-containing medium. Inhibition of SC115 cell growth by ß-EP, CZ, and MS is maximal in DHT-containing medium.

7) Inhibitory effects of ß-EP (10^{-9} M) and MS (10^{-9} M) on the AR SC115 tumor cell growth are totally or partially blocked by an opioid antagonist, naloxone (10^{-8} and 10^{-6} M). Thus, multiple opioid receptors may be involved in the inhibitory effects of the opioids on the growth of SC115 tumor cells.
8) Naloxone (10^{-12}-10^{-6} M) alone also significantly inhibits SC115 tumor cell growth. Further experiments need to be done to characterize the possible mechanisms involved in this inhibitory effect of naloxone.
9) The AR SC115 tumor cells from both GI and IG animals are significantly stimulated by DHT (3.5×10^{-8} M) and HC (10^{-6} M). *In vitro*, cells from tumors of IG and GI mice are responsive to DHT and HC to the same degree. These data suggest that the slower growth rates of tumors in mice of the IG group result from alterations in the internal environment of the mice rather than from a decrease or increase in the tumor cell's ability to respond to hormones.
10) ß-EP and CZ (10^{-10}-10^{-7} M) significantly inhibit the growth of AR SC115 tumor cells from both GI and IG animals. The inhibitory effect of ß-EP (10^{-9} M) on the growth of SC115 tumor cells from both GI and IG animals is reversed by naloxone (10^{-12}-10^{-6} M).

In conclusion, we have demonstrated that the growth of AR SC115 tumor cells in primary culture is stimulated by physiological concentration to pharmacological concentrations of DHT, by pharmacological concentrations of HC, and by bFGF. These stimulatory effects are inhibited by opioid agonists. The opioid peptide systems may be involved in regulating endocrine control of the growth of the androgen-responsive Shionogi mouse mammary carcinoma *in vivo*. Opioid peptide systems may also play a role in the differential tumor growth rates of SC115 tumor cells from mice in the experimental housing groups of our animal-tumor model.

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VI. Appendices

1. Freezing Medium

Freezing mudium:	10 ml
DME:	5 ml
CS:	4.4 ml
DMSO:	0.6 ml

2. Collagenase/Hyaluronidase

Collagenase:	300 μ
Hyaluronidase:	100 μ
F12/DME/H:	100 ml

3. STV Solusion

STV:	1 Litre
NaCl:	8 gm
KCl:	0.4 gm
$NaHCO_3$:	0.35 gm
Glucose:	1 gm
Versene:	0.25 gm
Trypsin:	0.5 gm

Double distilled water to make to the volume

4. Dextran Charcoal-treated Fetal Bovine Serum

Serum:	100 ml
Charcoal:	10 mg/ml serum
Dextran:	1 mg/ml serum

Shake water bath for 2 h

Spin at 4000 RPM for 30 min at room temperature

Pre-filter (0.45 μ)

Sterile filter (0.22 μ)

5. Serum-free (SF) Medium

SF-I:	100 ml
F12/DME/H	100 ml
BSA	0.1%
SF-II:	100 ml
F12/DME/H	100 ml
Insulin	0.1 μg/ml
Cholera Toxin	1.0 ng/ml
BSA	0.1%
SF-III:	100 ml
F12/DME/H	100 ml
Insulin	1.0 μg/ml
Cholera Toxin	10 ng/ml
BSA	0.1%