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Hormonal Regulation of Proliferation and Transforming Growth Factors Gene Expression in Human Endometrial Adenocarcinoma Xenografts

Yuewen Gong,[1] Leigh C. Murphy[2] and Liam J. Murphy[1,3]*

Departments of [1]Physiology, [2]Biochemistry and [3]Internal Medicine, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

We have previously shown that estrogen and progestins regulate both cellular proliferation and transforming growth factor (TGF) expression in human endometrial adenocarcinoma cells *in vitro*. In the current study we examined the regulation of TGF-α and -β_1 expression in endometrial adenocarcinoma xenografts. Four human endometrial adenocarcinoma cell lines were inoculated into female BALB/c nude mice. Administration of 17β-estradiol (E2) increased tumor size in intact mice inoculated with Ishikawa, HEC-50 and HEC-1B cells but inhibited growth of HEC-1A xenografts. 4-Hydroxy tamoxifen (OH-Tam) had similar effects to E2 in animals carrying Ishikawa and HEC-1A cell xenografts but had no significant effect on growth of HEC-50 or HEC-1B xenografts. In intact mice inoculated with OH-Tam pellets and Ishikawa cells, the tumors were larger and had lower levels of TGF-α mRNA than in untreated or E2 treated mice. In mice carrying Ishikawa, HEC-50 and HEC-1B cell xenografts none of the hormones or agents tested altered TGF-β_1 mRNA levels. In contrast, both E2 and OH-Tam significantly increased xenografts TGF-β_1 mRNA levels in HEC-1A xenografts as well as significantly reduced tumor size. Medroxyprogesterone acetate (MPA) had no effect on tumor size of Ishikawa, HEC-1A and HEC-1B cell xenografts but significantly increased the size of HEC-50 xenografts. MPA significantly reduced TGF-α expression in Ishikawa cell xenografts but had no effect in the other cell xenografts. MPA had no effect on TGF-β_1 expression in any of the xenografts. These observations demonstrate a discordance between the hormonal effects on TGF expression and cellular proliferation and argue against a major role for the TGFs in regulation of human endometrial adenocarcinoma cell proliferation *in vivo*.

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INTRODUCTION

Estrogen has been shown to increase cell proliferation, progesterone receptor content, and alkaline phosphatase activity in some human endometrial adenocarcinomas [1–4]. The antiestrogen, 4-hydroxy tamoxifen (OH-Tam), has also been showed to stimulate proliferation of Ishikawa human endometrial carcinoma cells *in vitro* [5]. Recently, we have demonstrated that 17β-estradiol (E2), OH-Tam and medroxyprogesterone acetate (MPA) can modulate the expression of transforming growth factors (TGF-α and -β_1) and proliferation of human endometrial adenocarcinoma cells [6, 7]. Under estrogen-depleted culture conditions, E2 stimulated proliferation of Ishikawa cells, increased TGF-α mRNA levels and increased TGF-α secretion whereas OH-Tam, which also stimulated cell proliferation under these conditions, reduced TGF-α expression [6]. Both MPA and OH-Tam inhibited Ishikawa cell proliferation in estrogen replete medium and significantly reduced TGF-α expression [6, 7]. In addition, studies with antireceptor antibodies and antisense oligonucleotides suggested a potential autocrine involvement of TGF-α and -β_1 in the regulation of proliferation of Ishikawa and HEC-50 endometrial adenocarcinoma cells, respectively. Antiserum to the EGF-receptor was able to block cell proliferation when Ishikawa cells were grown in medium containing low concentrations of exogenous growth factors [8].

*Correspondence to L. J. Murphy, Department of Physiology.
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Table 1. Comparison of tumor formation and tumor growth in Ovex and intact mice

Cell line		Ovex	Intact
Ishikawa	Incidence	0/8	3/8[a]
	Tumor size	0	95.3 ± 6.3[b]
HEC-50	Incidence	6/6	8/8
	Tumor size	42.0 ± 8.7	63.1 ± 14.7
HEC-1A	Incidence	6/6	8/8
	Tumor size	74.6 ± 12.2	76.0 ± 4.5
HEC-1B	Incidence	8/8	6/6
	Tumor size	45.8 ± 7.0	45.5 ± 8.9

[a]The data represent the number of tumors per number of injections determined 2 months after tumor cell inoculation.

[b]The data represent mean ± SEM in square millimeters.

Proliferation of HEC-50 cells, which is enhanced by exogenous TGF-β_1, was specifically inhibited by antisense oligonucleotides which spanned the translation start site of TGF-β_1 [8].

Few studies reporting the hormonal effects on human endometrial cancer cell lines grown as xenografts exist. However, the studies so far reported indicate that there may be discordance between the hormonal responses observed *in vitro* and *in vivo* [9, 10]. We have previously reported the effects of estrogens, antiestrogens and progestins on endometrial cancer cell growth and growth factor expression *in vitro*. However, the effects of steroid hormones on tumor growth *in vivo* may be modulated by indirect effects on factors such as blood flow, angiogenesis, extracellular matrix in addition to effects on nutrition, immune responses and circulating hormones and growth factors. Here we report our investigation of the effects of steroid hormones on growth of four human endometrial adenocarcinoma xenografts and expression of TGF-α and -β_1 in these tumors.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's plus Ham's F-12 medium and all other cell culture medium ingredients were purchased from GIBCO Ltd (Burlington, Ontario). Pellets containing 2.5 mg of various steroid hormones or analogs were purchased from Innovative Research of America (Toledo, OH, U.S.A.). These pellets provide sustained release over 60 days. Female intact and ovex BALB/c athymic mice, 4–5 weeks old, were purchased from Life Science Inc. (St Petersburg, FL, U.S.A.).

Table 2. The effect of various hormones on tumor formation and tumor growth in intact athymic mice

	Ishikawa	HEC-50	HEC-1A	HEC-1B
Control	10/30	16/16	8/8	19/19
E2	12/12*	8/8	8/8	12/12
OH-Tam	16/16*	10/10	8/8	12/12
MPA	16/16*	10/10	8/8	8/8
DEX	6/6**	6/6	8/8	8/8
DHT	6/8	5/8	8/8	7/8

The data represent the number of tumors observed divided by the number of inoculations, 2 months after inoculation of cells.

*,**Represents $P < 0.001$ and $P < 0.025$, respectively for the difference between control and treated groups as determined by Chi-squared analysis.

Cell culture and cell growth in nude mice

The four human endometrial adenocarcinoma cell lines, Ishikawa, HEC-50, HEC-1A, and HEC-1B, were routinely passaged in basal medium (Dulbecco's

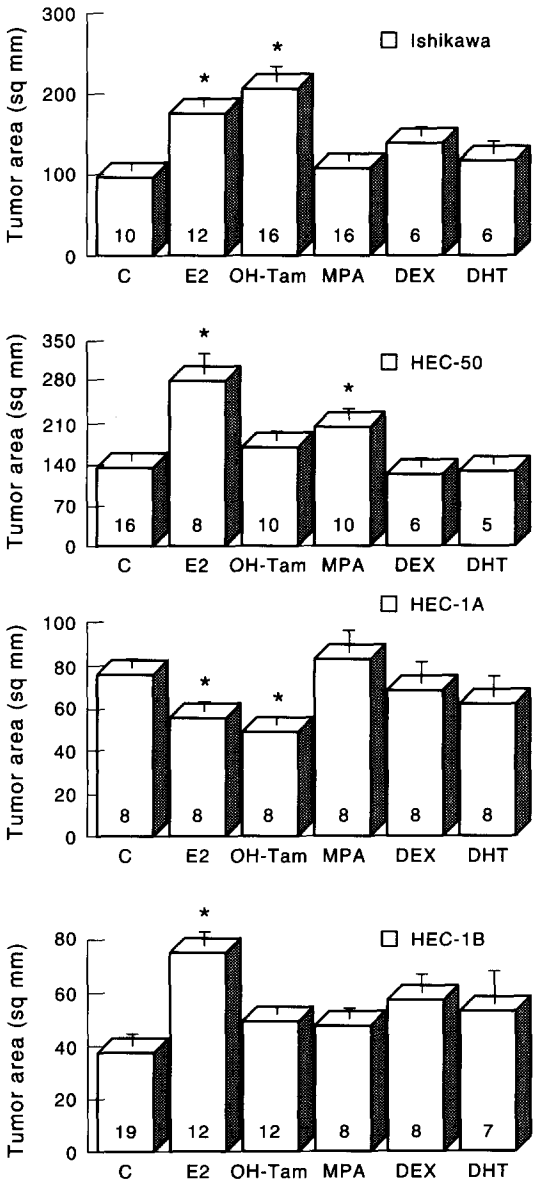

Fig. 1. Effect of 17β-estradiol (E2), 4-hydroxy tamoxifen (OH-Tam), medroxyprogesterone acetate (MPA), dexamethasone (DEX) and 5α-dihydrotestosterone (DHT) on tumor growth of the four human endometrial adenocarcinoma cell lives in athymic mice. The results represent tumor size after 8 weeks for Ishikawa and HEC-50 cells, and 4 weeks for HEC-1A and HEC-1B cells. Data are presented as the mean ± SEM. The number in the histogram represents the number of tumors. *Indicates $P < 0.05$ for the difference from control group.

modified Eagle's–Ham's F-12 medium, 1:1, 10 mM L-glutamine, 1% antibiotic–antimycotic solution and 5% fetal bovine serum). The sources and characteristics of these cell lines have been reported previously [6, 7, 11]. Of these cell lines, only Ishikawa cells have detectable estrogen receptors as determined by ligand binding and estrogen receptor mRNA was not detected in any of the other cell lines using reverse transcription polymerase chain amplification (Gong and Murphy, unpublished observations).

Female BALB/c nude mice were housed in laminar flow hoods. Ishikawa (8×10^6), HEC-50 (8×10^6), HEC-1A (1×10^7) and HEC-1B (2×10^6) cells suspended in the basal medium were inoculated subcutaneously into mammary fat pads of female BALB/c nude mice. Each mouse was inoculated at 2 sites. The hormone pellets were implanted subcutaneously in the back of animals 1 day after tumor cell inoculation. Tumors were measured using calipers at the indicated times and the mean cross-sectional area was calculated as length multiplied by the width. All procedures were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine, University of Manitoba.

RNA extraction and Northern blot analysis

Tumors were removed and rapidly frozen. RNA was isolated by the guanidinium thiocyanate–cesium chloride method and enriched for poly (A^+) RNA by one cycle of oligo(dT) cellulose chromatography [12].

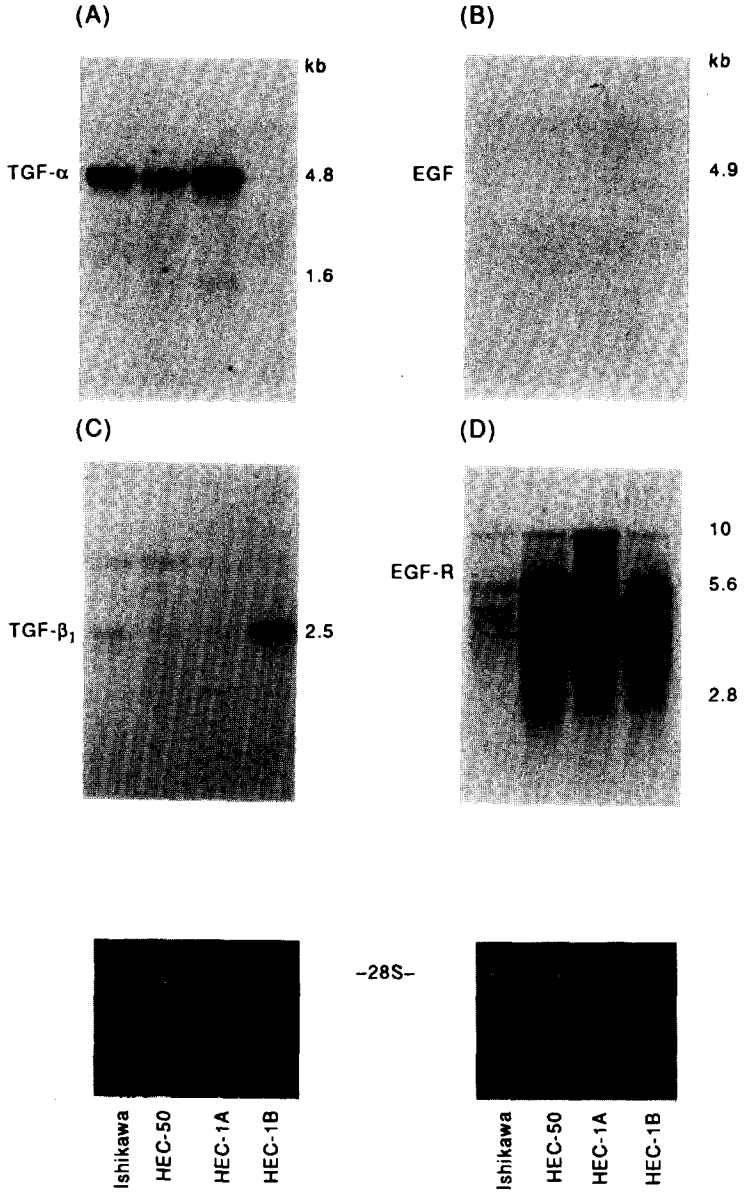

Fig. 2. TGF-α, TGF-β_1, EGF, and EGF-receptor mRNA levels in human endometrial adenocarcinoma cells grown in complete media *in vitro*. 10 μg of polyadenylated RNA was loaded on each lane. The Northern blots were hybridized with human TGF-α, TGF-β_1, EGF, and EGF-receptor cDNAs as indicated. The ethidium bromide stained gel are shown in the lower panel.

Poly(A$^+$) RNA, (10 μg) was analyzed by electrophoresis on 1% (w/v) agarose formaldehyde gel and then blotted onto nitrocellulose. In some experiments 4 μg poly(A$^+$) RNA was applied directly to nitrocellulose paper using a dot blot apparatus. The filters were prehybridized and hybridized with the human TGF-α and -β_1 cDNA probes radiolabeled with [^{32}P]dCTP by nick translation as described previously [6, 7]. Under the hybridization conditions used the human TGF-α and -β_1 cDNA probes do not cross hybridize with the mouse homologs. As a control for gel loading and transfer, filters were also hybridized with NB-29, a cDNA which encodes a constitutively expressed heat shock-like protein [13]. After autoradiography the hybridization signal was quantified by densitometry.

Statistical analysis

An analysis of variance and Dunnett's t-test were used to determine the statistical significance of differences between control and treatment groups. A

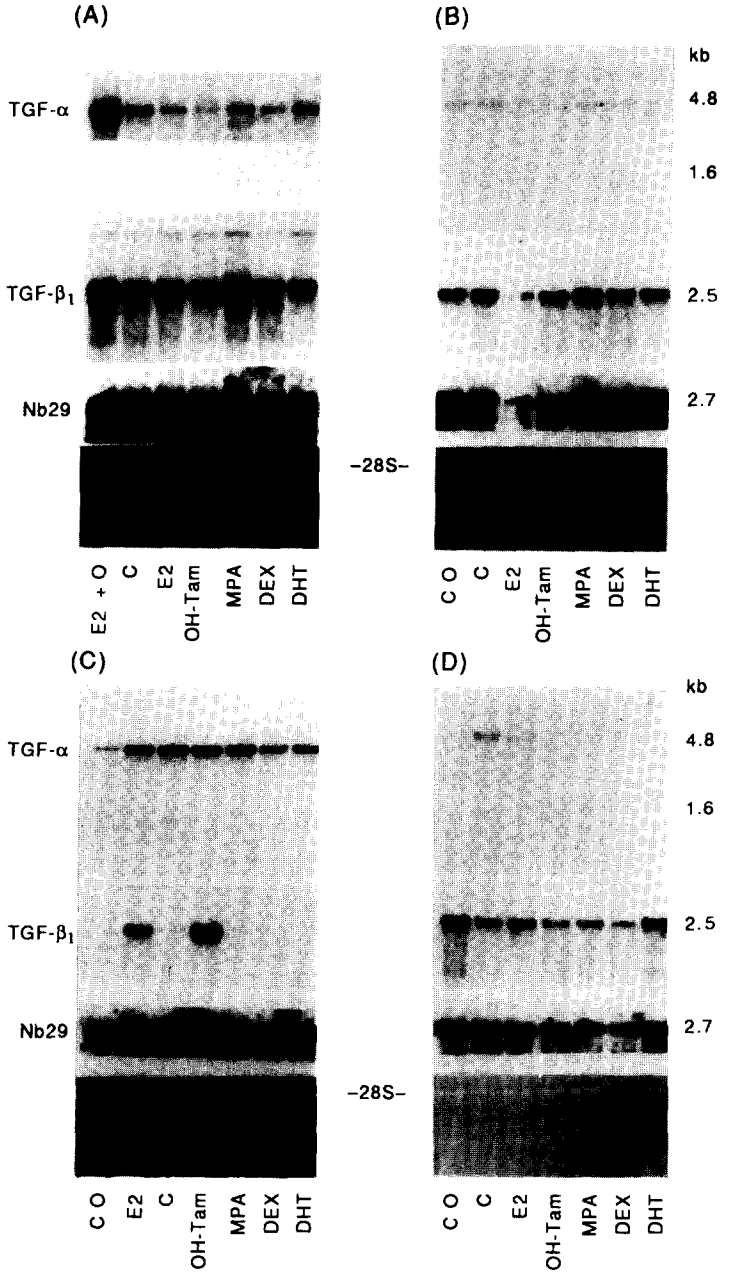

Fig. 3. Effect of 17β-estradiol (E2), 4-hydroxy tamoxifen (OH-Tam), medroxyprogesterone acetate (MPA), dexamethasone (DEX, and 5α-dihydrotestosterone (DHT) or TGF-α and -β_1 mRNA levels in tumors following inoculation with Ishikawa (A), HEC-50 (B), HEC-1A (C), and HEC-1B (D) cells. RNA from tumors in ovex with and without E2 pellets are indicated as E2 + O and C − O, respectively, whereas all other mice were intact. C indicates control mice which did not receive pellet implants. The upper panels show a representative Northern blot hybridized with the TGF-α and -β_1 cDNA probes. The lower panels show the same blots hybridized with Nb29 and the ethidium bromide stained gel as controls for gel loading.

Chi-squared analysis was used to determine the significance of differences in tumor incidences.

RESULTS

Effect of ovariectomy on human endometrial cancer tumorigenesis in athymic nude mice

Both intact and ovariectomized (ovex) mice were inoculated with each of the four cell lines so that the effects of endogenous ovarian steroids could be assessed. Inoculation of the four cells lines (Ishikawa, HEC-50, HEC-1A and HEC-1B) into intact mice resulted in tumor formation in all cases. Ishikawa cells did not form tumors in the ovex mice. However, in ovex mice implanted with E2 containing pellets, Ishikawa cell tumors were observed at 38% of the inoculation sites, 2 months after inoculations. The incidence of tumors and the tumor size were similar for HEC-50, HEC-1A and HEC-1B cell xenografts in ovex and intact mice (Table 1). These data suggest that tumorigenesis for these latter three cell lines is estrogen independent whereas Ishikawa cell tumorigenesis is estrogen dependent.

Effect of steroid hormones and antagonists on tumor formation and growth in athymic mice

The effects of various hormones and agents on tumor development and growth were examined in intact mice. In addition to E2, pellets containing OH-Tam, MPA, and dexamethasone (DEX) also significantly enhanced the frequency fo Ishikawa cell tumor formation (Table 2). None of these hormonal treatments reduced tumor formation in mice inoculated with the three other cell lines. With respect to tumor size, all four cell lines showed some response to E2. A significant increase in tumor size was seen for Ishikawa, HEC-50, and HEC-1B cell xenografts whereas a significant reduction in HEC-1A xenograft size was seen in intact nude mice implanted with E2 pellets (Fig. 1). OH-Tam had similar effects to E2 in animals inoculated with Ishikawa and HEC-1A cells but had no significant effect on the size of HEC-50 or HEC-1B xenografts. The size of HEC-50 cell xenografts was significantly increased in MPA treated mice compared to controls but MPA had no significant effect on any other cell line. DEX and 5α-dihydroestosterone (DHT) had no effect on tumor size in mice inoculated with any of the human endometrial adenocarcinoma cell lines (Fig. 1).

TGF-α and -β_1 expression in human endometrial cancer cells and xenografts

Expression of TGF-α, TGF-β_1 and epidermal growth factor (EGF)-receptor was detected in all four cell lines when grown *in vitro* (Fig. 2). Among these cell lines, TGF-α and EGF-receptor mRNA was most abundant in HEC-1A cells [Fig. 2(A and D)]. The expression of TGF-α was extremely low in HEC-1B cells. The highest levels of TGF-β_1 mRNA were observed in HEC-1B cells whereas the expression of TGF-β_1 was very low in HEC-1A cells. However using this technique EGF mRNA was not detectable in any of the four cell lines [Fig. 2(B)].

The abundance of TGF-α and -β_1 mRNA in xenografts that developed following inoculation with each of

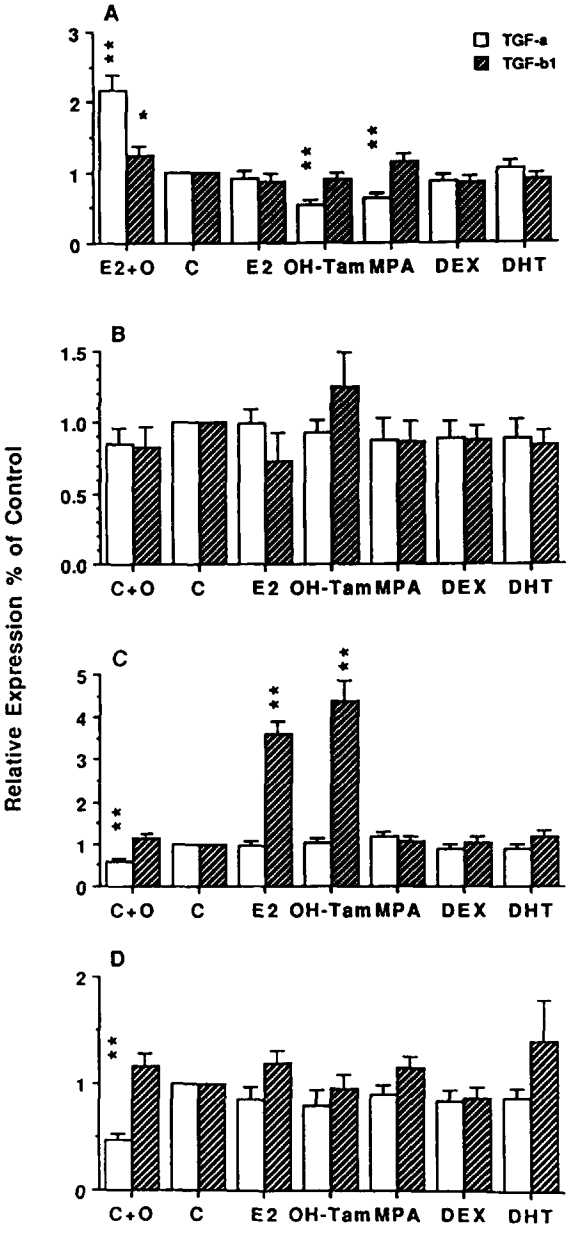

Fig. 4. Effect of 17β-estradiol (E2), 4-hydroxy tamoxifen (OH-Tam), methoxyprogesterone acetate (MPA), dexamethasone (DEX), 5α-dihydrotestosterone (DHT) on TGF-α and -β_1 mRNA accumulation in xenografts. Data were obtained by quantifying both Northern blots and dot blots and represent the mean ± SEM of 4 or 5 tumors per cell type. A, B, C, and D represent Ishikawa, HEC-50, HEC-1A, and HEC-1B cells, respectively. The significant differences between the treatment and control tumors in intact mice (C) are indicated as * and ** for $P < 0.05$ and $P < 0.01$, respectively. Data from tumors in ovex with and without E2 pellets are indicated as E2 + O and C + O, respectively.

the four human endometrial adenocarcinoma cell lines was examined. In ovex mice, E2 treatment increased TGF-α mRNA levels in Ishikawa cell xenografts more than 2-fold as compared to tumors in intact untreated mice. The same treatment also significantly increased the TGF-β_1 mRNA level (Figs 3 and 4). Since Ishikawa cells did not form tumors in untreated ovex mice, it was not possible to examine the effect of estrogen deficiency on TGF-α and -β_1 expression under these conditions. In the case of the other three cell lines, there is no difference in TGF-β_1 mRNA levels between ovex and intact mice. However, TGF-α mRNA levels were significantly reduced in HEC-1A and HEC-1B tumors in ovex compared to intact mice (Figs 3 and 4).

Effect of steroid hormones and antagonists on TGF-α and -β_1 expression in xenografts

In intact mice, both OH-Tam and MPA, agents which inhibit Ishikawa cell proliferation in estrogen replete medium *in vitro*, significantly reduced the TGF-α mRNA levels to 56 and 65% of untreated controls, respectively. MPA had no significant effect on TGF-α and -β_1 mRNA levels in any of the other cell types and DEX and DHT had no effect in any cell type (Fig. 4). TGF-β_1 mRNA was significantly more abundant in HEC-1A xenografts in mice implanted with E2 and OH-Tam pellets than in xenografts in untreated mice (Fig. 4).

DISCUSSION

We have previously investigated the effects of estrogens, antiestrogens and progestins on endometrial cancer cell growth and TGF-α and -β_1 expression *in vitro*. However, the effects of steroid hormones in normal uterine epithelium appears to involve an interaction with appropriate stromal tissue [14]. The effects of steroid hormones on tumor growth *in vivo* may also be modulated by stromal factors in addition to indirect effects of steroid hormones on factors such as blood flow angiogenesis, nutrition, immune responses and circulating hormones and growth factor concentrations. In this study, four human endometrial adenocarcinoma cell lines were grown as xenografts in athymic nude mice and the effects of different steroid hormones on tumor development, growth, TGF-α and -β_1 expression were examined. Although xenographs contain murine cells such as fibroblasts, vascular and lymphoid cells, the cDNA probes we used do not hybridize with the mouse homologs under the conditions used in this study. Thus, the hybridization signal identified represents human tumor TGF-α and -β_1 mRNA. The mammary fat pad was chosen as a convenient site for tumor cell inoculation since tumor formation could be monitored without the need to sacrifice the mouse. Furthermore, this site has been used by many investigators to examine steroid hormone responsive mammary xenografts.

Three of the endometrial adenocarcinoma cell lines. HEC-50, HEC-1A and HEC-1B formed tumors in both intact and ovex mice with similar frequencies. In our hands these three cell lines do not express estrogen receptor (Gong and Murphy, unpublished observations). Furthermore, *in vitro*, HEC-50 cells are unresponsive in terms of proliferation to both E2 and OH-Tam [6, 8]. However administration of E2 had significant effects on tumor size in mice inoculated with these three cell lines. In all mice except those inoculated with HEC-1A cells, xenograft size was increased by administration of E2. The Ishikawa cell line did not form tumors in ovex mice and was less tumorigenic than the other cell lines in intact mice. In mice inoculated with either Ishikawa or HEC-1A cells, OH-Tam had qualitatively similar effects to E2, whereas in mice carrying HEC-50 and HEC-1B tumors, OH-Tam was without an effect despite a significant effect of E2 on tumor size. This discordance may represent differences in the mechanisms underlying the E2 effect. In addition, to any direct effects E2 may have on the tumor cells, indirect host effects may also be important. E2 has been reported to have some effects on a number of parameters of both cell and humoral immunity and it is possible that these effects could be important in the observations reported here. Since implantation with hormone pellets was delayed for 24 h it is unlikely that the hormonal effects are exerted at the level of host receptivity.

Consistent with our previous *in vitro* findings, estrogen enhanced the growth of Ishikawa cell tumors and increased TGF-α mRNA levels in these tumors [6]. However, while OH-Tam was equally or even more potent in stimulating growth of Ishikawa cell tumors, OH-Tam treatment resulted in decreased TGF-α mRNA levels in the tumors. This discordance between growth stimulation and TGF-α expression was previously demonstrated *in vitro* [6]. Furthermore, E2 had effects on HEC-50, HEC-1A and HEC-1B tumor size but did not significantly alter TGF-α mRNA levels in these tumors in intact mice. Tumor TGF-α mRNA levels were significantly lower in ovex mice compared to intact mice inoculated with HEC-1A and HEC-1B cells indicating that ovarian steroids may have some role in the regulation of TGF-α expression in these cell lines.

MPA inhibits proliferation of both Ishikawa and HEC-50 cells *in vitro* [7]. However, no such growth inhibitory effect was observed *in vivo* [7]. MPA enhanced the frequency of tumor formation in cells inoculated with Ishikawa cells and increased the size of HEC-50 cell tumors. The latter response did not appear to be mediated via the glucocorticoid or androgen receptor since DEX and DHT were without effect on HEC-50 cells tumor size. Recent studies have suggested that under certain culture conditions progestins can stimulate proliferation of both breast

and endometrial adenocarcinoma cell lines [15, 16]. Furthermore, thymidine labeling studies have demonstrated that DNA synthesis in glandular epithelial cells in the basal endometrium is stimulated rather than inhibited by progesterone [17]. Consistent with the *in vitro* effects of MPA, this agent reduced TGF-α mRNA levels in Ishikawa cell tumors but in contrast to its growth inhibitory effect *in vitro*, MPA stimulated xenograft growth. Thus a discordance between the effects of MPA on TGF-α expression and growth was apparent in the xenografts.

In mice inoculated with HEC-1A cells, both E2 and OH-Tam resulted in a significant reduction in tumor size and also increased TGF-β_1 expression. Since TGF-β_1 when added to HEC-1A cells in culture results in growth inhibition [18], the E2 and OH-Tam induced growth inhibition of the xenografts may be partly explained by the enhanced TGF-β_1 expression. While E2-induced TGF-β_1 expression has also been demonstrated in human osteosarcoma cells [19], E2 results in a reduction in TGF-β_1 expression in Ishikawa cells and human breast cancer cells [6].

The experiments reported in the present study demonstrate the complexity of the effect of estrogen and progestins on endometrial tumor growth. Clearly both direct effects on the tumor cells themselves as well as indirect effects via the host are involved to varying degrees. Our data also emphasize the discordance between effects observed *in vitro* and those demonstrable *in vivo*. Further studies are required to determine the functional importance of indirect effects of steroid hormones on parameters such as nutrition, angiogenesis, blood flow, immunological responses, circulating growth factors and extracellular matrix on tumor growth. In addition it remains to be determined that the mammary fat pad accurately reflects the extracellular matrix present in the endometrium.

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