17β-estradiol, Progesterone, and Dihydrotestosterone Suppress the Growth of Human Melanoma by Inhibiting Interleukin-8 Production

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We studied the effects of 17β-estradiol, progesterone, and dihydrotestosterone on *in vitro* growth of human metastatic melanoma. Each sex hormone inhibited the growth of melanoma receptor-dependently; 17β-estradiol inhibited ^{3}H-thymidine uptake of estrogen receptor-positive WM266-4 and NM26, but not that of the receptor-negative HS15. Progesterone inhibited ^{3}H-thymidine uptake of progesterone receptor-positive WM266-4 and HS15, but not that of the receptor-negative NM26. Dihydrotestosterone inhibited ^{3}H-thymidine uptake of androgen receptor-positive HS15 and NM26, but not that of the receptor-negative WM266-4. The growth inhibition by each hormone was counteracted by the respective hormone receptor antagonist. The combination of more than two hormones neither gave additive nor synergistic growth inhibition. The growth inhibition by each sex hormone was counteracted by interleukin-8 but not by the other growth factors. Each sex hormone reduced the constitutive interleukin-8 secretion and mRNA levels in the respective receptor-positive melanoma but not in the receptor-negative melanoma. Transient transfection showed that each sex hormone inhibited the constitutive chloramphenicol acetyltransferase expression driven by interleukin-8 promoter in the respective receptor-positive melanoma but not in the receptor-negative melanoma. Transfection with a series of 5′-deleted interleukin-8 promoter/chloramphenicol acetyltransferase reporter constructs demonstrated that the sequences between −98 and −63 bp on interleukin-8 promoter may be involved in the transcriptional repression. These data suggest that 17β-estradiol, progesterone, and dihydrotestosterone suppress the growth of melanoma by inhibiting interleukin-8 production in a receptor-dependent manner. *Key words: receptor/sex hormone/transcription. J Invest Dermatol 117:274–283, 2001*

It has long been suggested that sex hormones modulate the growth of malignant melanoma. Mutually conflicting data are reported, however; melanomas seemed to metastasize more slowly in women than in men (Shaw *et al*, 1978), and survival after metastasis was longer in women than in men (Rampen, 1980). These data indicate the growth inhibition by estrogen or progesterone and/or growth stimulation by androgens on melanoma. In contrast, for stage II melanoma, a lower survival rate was observed in pregnant women than in nonpregnant women with melanoma (Shiu *et al*, 1976). Early studies also reported the occurrence, rapid enlargement, or frequent metastasis of melanoma during pregnancy (Pack and Scharnagel, 1951). These reports suggest the growth stimulation by estrogen and/or progesterone on melanoma; however, it is still controversial if pregnancy affects the growth of melanoma. Holly (1986) summarized the etiologic studies and reported that 10 of 11 studies showed no survival difference between women with melanoma associated with pregnancy and those with no association, indicating few deleterious effects of pregnancy on the survival. Wong *et al* (1989), Mackie *et al* (1991), and Driscoll *et al* (1993) also suggested that pregnancy may have no effects on the outcome of patients with melanoma.

Various experimental results for the hormonal growth regulation on melanoma are confusing and inconclusive; estrogen *in vivo* inhibited the growth of hamster HM-1 melanoma (Schleicher *et al*, 1987) or human melanoma UISO-MEL-2 (Feucht *et al*, 1988) grafted in athymic mice. Melanoma B16 grew more slowly in normal female mice than oophorectomized female or male mice (Proctor *et al*, 1976). These data support the inhibitory effect of estrogen on the *in vivo* growth of melanoma. Some data denied the growth-regulatory effect of estrogen, however; estrogen did not alter the *in vitro* growth of S91 mouse melanoma B line cells or human melanoma cell lines UISO-MEL-1, 2, and 4 (Cobb and McGrath, 1974; Feucht *et al*, 1988), and there were no differences in the growth rate of B16 between pregnant and nonpregnant female mice (Proctor *et al*, 1976). On the other hand, several studies suggest the growth-stimulatory effects of estrogen; estrogen enhanced *in vivo* growth and lung metastasis of B16 in mice in a concentration-dependent manner (Lopez *et al*, 1978). Melanoma grew larger in normal female hamsters than in oophorectomized females (Rosenberg *et al*, 1963). Thus the hormonal growth regulation on melanoma seems highly complicated and may involve a variety of elements in melanoma itself and tumor-surrounding or distant tissues or organs. One of such elements is the

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Abbreviations: AR, androgen receptor; bFGF, basic fibroblast growth factor; CAT, chloramphenicol acetyl transferase; DHT, dihydrotestosterone; E2, 17 β-estradiol; ER, estrogen receptor; PR, progesterone receptor; GRO, growth-regulated protein; PDGF, platelet-derived growth factor.

presence or absence of the respective hormone receptors. The incidence of estrogen receptor (ER) is reported to be 9–79% (Walker *et al*, 1987; Cohen *et al*, 1990) and that of progesterone receptor (PR) is 21–44% (Neifeld and Lippman, 1980; Karakousis *et al*, 1980), and that of androgen receptor (AR) is 15–17% (Neifeld and Lippman, 1980) in melanoma by radiolabeled ligand binding assays using dextran-coated charcoal. Owing to the high false positivity of the binding assays (Cohen *et al*, 1990), however, different methods have been recently used for the detection of hormone receptors; western blotting (Swami *et al*, 2000), immunohistochemical methods (Cohen *et al*, 1990; Duncan *et al*, 1994), or enzyme immunoassays (Kuenen-Boumeester *et al*, 1996). Even with the improved methods, however, different papers still report different results on the incidence of hormone receptor, possibly because different laboratories may use different anti-hormone receptor antibodies, or because the presence or absence of hormone receptor may depend on tumor location in the whole body or tumor activation status. Ferno *et al* (1987) reported that ER was positive in 56% of metastatic melanoma by enzyme immunoassay, whereas Cohen *et al* (1990) showed that ER was negative in all 33 primary and metastatic melanoma by immunohistochemistry.

Previous studies support that metastatic melanoma constitutively produces a variety of growth factors and thus regulates its own growth in an autocrine manner (Herlyn, 1990). The mRNA or protein expression in melanoma is reported for basic fibroblast growth factor (bFGF), interleukin (IL) -8, melanocyte growth stimulatory activity/growth-regulated protein (GRO) -α platelet-derived growth factor (PDGF), etc. (Herlyn, 1990), and the pattern of the expression is heterogeneous among various melanoma cells. It is thus hypothesized that sex hormones may upregulate or downregulate the autocrine production of growth factors in certain melanoma cells and thus regulate their growth.

In this study, we aimed to examine the growth-regulatory effects of sex hormones, 17β-estradiol (E2), progesterone, and dihydrotestosterone (DHT) in human metastatic melanoma cell lines. We further examined the mechanism for the growth-regulatory effects of these sex hormones, focusing on their effects on autocrine production of growth factors.

MATERIALS AND METHODS

Cell lines and maintenance Metastatic melanoma cell line WM266-4 obtained from a female patient was purchased from Dainippon Pharmaceutical (Osaka, Japan). HS15 and NM26 were removed from metastatic lymph nodes of male patients, and were established by a monolayer system as described (Carey *et al*, 1976; Baker *et al*, 1986). Briefly, biopsy specimens were dissected free of adherent normal tissues, and finely minced. The resulting cell suspensions were washed, resuspendend in culture medium, then inoculated into 35 mm diameter dishes, cultured at 37°C in a humidified atmosphere of 5% CO_2, and fed twice weekly. Confluent culture was trypsinized, and expanded into larger dishes or flasks. The subculture was performed once a week. Human breast cancer MCF-7 cells were purchased from Dainippon. These cell lines were cultured in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco-BRL), 1% nonessential amino acids, 1 mM sodium pyruvate (ICN Biomedicals, Aurora, OH), and 100 U per ml penicillin G, 100 μg per ml streptomycin, 0.25 μg per ml amphotericin B (Gibco-BRL). Human prostate cancer LNCaP cells were purchased from Dainippon, and were maintained in RPMI 1640 (GIBCO-BRL) supplemented with 10% fetal bovine serum.

Reagents E2, 17α-estradiol, progesterone, pregnenolone, DHT, and β-dihydrotestosterone were purchased from Sigma (St Louis, MO). ICI 182,780, bicalutamide were obtained from Zeneca Pharmaceuticals (Macclesfield, U.K.). RU486 was from Schering AG (Berlin, Germany). These agents were dissolved in ethanol as 10 mM stock solution and were kept in the dark until used. Recombinant human IL-8 was from Sigma. Recombinant human GRO-α was from Pepro Tech EC (London, U.K.). Mouse IgG monoclonal anti-human IL-8 antibody was from BioSource International (Camarillo, CA), and was specific to natural and recombinant human IL-8, with no cross-reactivity to human or mouse GRO-α, GRO-β, or GRO-γ. Human recombinant PDGF-AB was from Carbiochem-Novabiochem Corp. (San Diego CA). Recombinant human IL-6 was from Boehringer Mannheim (Indianapolis, IN). Recombinant human bFGF was from Becton Dickinson (San Jose, CA). Control mouse IgG was from Dako Corp. (Carpinteria, CA).

Hormone-binding studies Melanoma cells were harvested and lyzed. The cytosolic fractions were separated, and used for radiolabeled ligand binding assays as described (Feucht *et al*, 1988; Pottratz *et al*, 1994). The protein concentration of cytosolic preparation was determined using a Bio-Rad protein assay kit (Hercules, CA). Aliquots of the fractions were incubated at 4°C for 3 h with 0.05–1.0 nM ^{3}H-E2 (Amersham Corp., Arlington Heights, IL), ^{3}H-R5020 (New England Nuclear, Cambridge, MA), or ^{3}H-R1881 (New England Nuclear) for ER, PR, or AR measurements, respectively. One hundred-fold excess of nonradioactive hormone was used to correct for nonspecific binding. Bound and free hormones were separated using dextran-coated charcoal, and specific binding was determined as described (Feucht *et al*, 1988). Binding constants, K_d and B_{max}, were calculated according to the method of Scatchard (Scatchard, 1949).

Western blot Aliquots (50 μg protein) of the extracts from melanoma, MCF-7, and LNCaP cells were resolved by sodium dodecyl sulfate–polyacrylamide (8%) gel electrophoresis under nonreducing conditions. The proteins were electrotransferred on to nitrocellulose membranes as described (Zhu *et al*, 1999; Swami *et al*, 2000). After blocking with 5% nonfat dry milk, membranes were probed for 2 h with 10 μg per sheet primary antibodies: mouse monoclonal IgG1 anti-ERα C-314 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal IgG anti-PR H-190 (Santa Cruz Biotechnology), or rabbit polyclonal IgG anti-AR N-20 (Santa Cruz Biotechnology). The blots were washed and incubated for 60 min with second antibodies: peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Pierce, Rockford, IL). After three washes, immunoreactive bands were detected using an enhanced chemiluminescence kit (Amersham).

Proliferation assays WM266-4, HS15, and NM26 cells were plated at 5×10^3 cells per well in triplicate in flat-bottomed 96-well plates in 100 μl of culture medium and adhered for 18 h. The medium was discarded and the plates were washed with phosphate-buffered saline three times, then incubated with sex hormones and/or recombinant cytokines at the indicated concentrations in 100 μl per well of serum-free, phenol red-free Dulbecco's modified Eagle medium for 20 h. Then 0.5 μCi per well of ^{3}H-thymidine (Amersham) was added and the cells were incubated for an additional 4 h prior to harvest. The incorporation of ^{3}H-thymidine was assayed by liquid scintillation.

Measurement of cytokines To measure the secretion of cytokines, melanoma cells were plated in triplicate at 5×10^4 per well in 24-well plates, adhered overnight, washed, and incubated with sex hormones in 1 ml per well of phenol red-free, serum-free medium for 24 h, and the culture supernatants were harvested and stored at –70°C until used. The activity of IL-6 and IL-8 in the supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Biosource), that of GRO-α and bFGF was measured by ELISA kits (R&D Systems, Minneapolis, MN), and that of PDGF-AB was measured by ELISA kit (Genzyme Techne, Minneapolis, MN), according to the manufacturers' instructions. The sensitivity of the assay for IL-6, IL-8, GRO-α, bFGF, or PDGF-AB was 2, 5, 10, 3, or 8.4 pg per ml, respectively.

For the analysis of cytokine mRNA expression, northern blot was performed as described (Zachariae *et al*, 1991). Melanoma cells were incubated with sex hormones as above for 8 h, and were harvested. Total RNA was extracted from the harvested cells by guanidium isothiocyanate method (Ultraspec, Houston, TX). RNA (25 μg) was electrophoresed in a 1% agarose gel and was transferred to nitrocellulose membranes. Blots were hybridized with a ^{32}P-labeled cDNA probe for human IL-8, which was 0.45 kb *Eco*RI–*Eco*RI fragment (Mukaida *et al*, 1989). To control for differences in RNA sample loading and transfer, the blots were also hybridized with a ^{32}P-labeled, 1.8 kb *Hin*dIII–*Hin*dIII fragment of the gene encoding human β-actin. The membranes were exposed to X-ray films (Hyperfilm MP; Amersham) for 17 h at –80°C. Autoradiograms were scanned using a Molecular Dynamics computing densitometer (model 300 A; Molecular Devices, Menlo Park, CA). IL-8 mRNA levels were normalized to those of β-actin.

Plasmids and transfection pCAT3-basic vector carrying two SV40 poly(A) signals, one downstream of the chloramphenicol acetyl transferase (CAT) reporter gene, and the other upstream of the multicloning site was purchased from Promega (Madison, WI). The *Hinc*II–*Hin*dIII fragment of the genomic IL-8 DNA, which spans nucleotides –546 to +44 bp relative

to the transcriptional start site (Mukaida *et al*, 1989), was subcloned into PUC19, treated with appropriate restriction endonucleases and further subcloned into pCAT3-basic vector (Dorn and Derse, 1988; Mukaida *et al*, 1989). cDNA encoding human ER (1.8 kb) (Green *et al*, 1986), PR (4.4 kb) (Kastner *et al*, 1990), and AR (3.2kb) (Brinkmann *et al*, 1989) were subcloned into *Eco*RI site of pSG5 (Stratagene, La Jolla, CA), downstream of T7 promoter as described (Green *et al*, 1988) to produce expression plasmids for ER, PR, and AR, respectively. The carboxyl-terminal-truncated mutant plasmids for ER, PR, and AR were generated by cutting the wild-type plasmids with restriction enzymes, inserting oligonucleotides with translation stop codons, and religating the vectors as described (Kastner *et al*, 1990; Simental *et al*, 1991; Stein and Yang, 1995). Transfection of melanoma cells was carried out by the calcium coprecipitation method using CellPhect transfection kit (Amersham) according to the manufacturer's instruction. Briefly, 1 d before the experiment, confluent cultures of melanoma cells were trypsinized, and cells were seeded at 1×10^6 cells per 100 mm diameter dish and incubated overnight at 37°C. The cultures were replenished with fresh medium and kept at 37°C for 4 h before transfection. The cells were incubated for an additional 4 h with the DNA-calcium precipitate containing 10 μg pIL-8 CAT. In some experiments, the cells were cotransfected with either 5 μg of empty vector pSG5 or 5 μg of wild-type or mutant hormone receptor expression plasmid together with pIL-8 CAT. The cultures were then glycerol shocked and replenished with fresh medium. After 3 h, the cells were trypsinized and subdivided into 24-well plates at 5×10^4 cells per well and incubated in 1 ml per well of culture

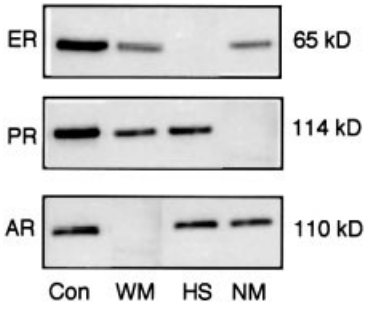

Figure 1. Western blot analysis for ER, PR, and AR in melanoma cells. The lysates from melanoma WM266-4, HS15, or NM26 were analyzed for the expression of hormone receptors. The ER, PR, and AR were identified as 65 kDa, 114 kDa, and 110 kDa band, respectively. The positive controls were the lysates from human breast cancer MCF-7 cells for ER and PR, and those from prostate cancer LNCaP cells for AR.

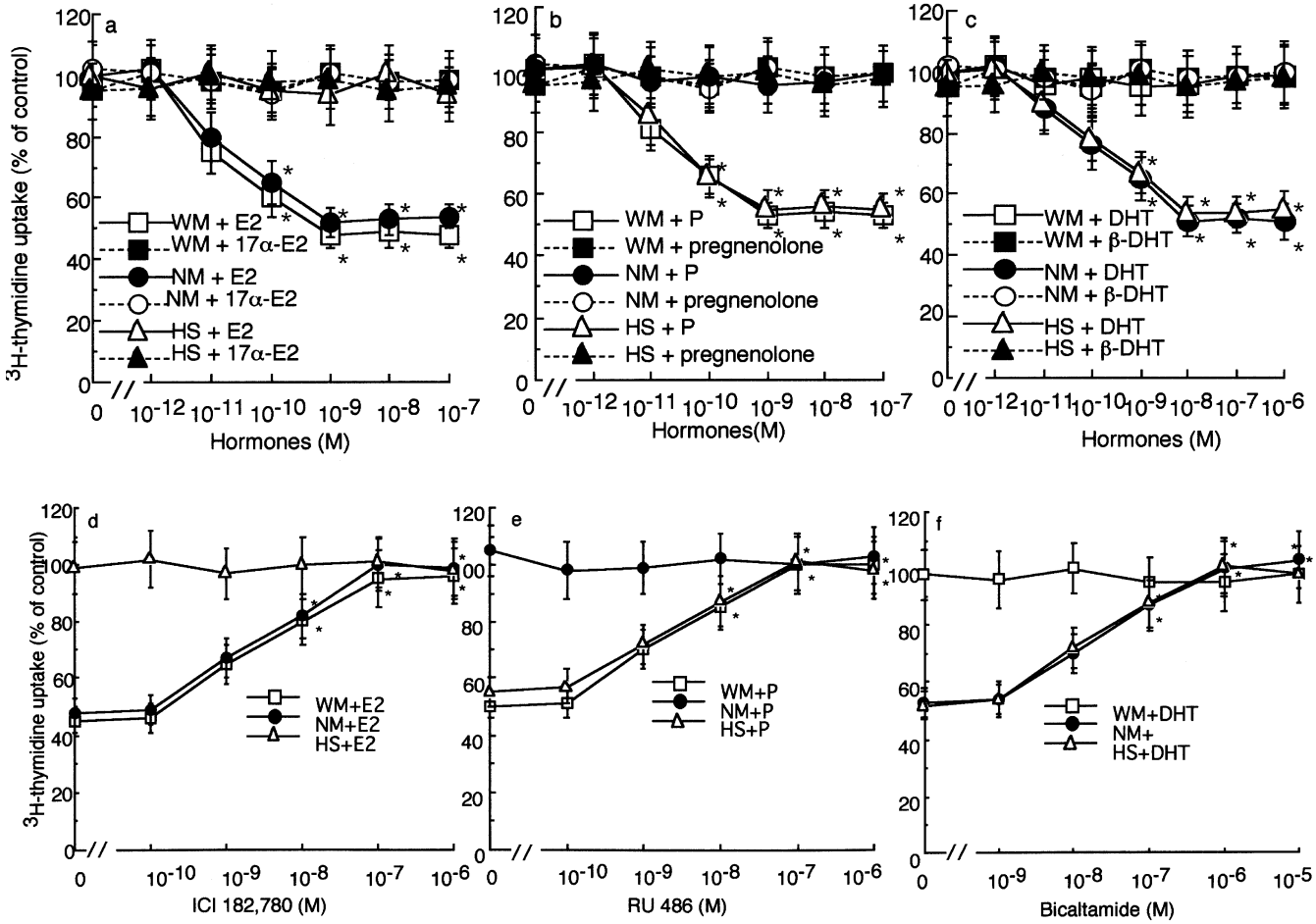

Figure 2. The inhibitory effects of E2, progesterone, and DHT on the proliferation of melanoma cell lines. (*a, d*) E2, (*b, e*) progesterone, (*c, f*) DHT. WM266-4, HS15, and NM26 cells were incubated with E2, progesterone, or DHT at the indicated concentrations and were pulsed with ^{3}H-thymidine before harvesting as described in *Materials and Methods*. The data are shown as percentage *vs* ^{3}H-thymidine uptake of control cultures with medium alone. In parallel experiments, 17α-estradiol (*a*), pregnenolone (*b*), or β-dihydrotestosterone (*c*) was added to the melanoma cells, and ^{3}H-thymidine uptake was analyzed. In (*d–f*), melanoma cells were treated with E2 (10^{-9} M) in the presence or absence of indicated concentrations of ICI 182,780, or treated with progesterone (10^{-9} M) in the presence or absence of indicated concentrations of RU486, or treated with DHT (10^{-8} M) in the presence or absence of indicated concentrations of bicaltamide. Results represent the mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control cultures with medium alone, by analysis of variance with Dunnet's multiple comparison test in (*a–c*). *$p < 0.05$ *vs* cultures with E2, progesterone, or DHT alone, by analysis of variance with Dunnet's multiple comparison test in (*d–f*). The values of ^{3}H-thymidine uptake in control cultures with medium alone were mean ± SEM (n = 4) 18,536 ± 1523, 15,231 ± 1324, and 10,324 ± 1094 cpm for WM266-4, HS15, and NM26 cells, respectively.

Table I. The presence of ER, PR, and AR in metastatic melanoma cell lines[a]

	ER		PR		AR	
	K_d (nM)	B_{max} (fmol per mg)	K_d (nM)	B_{max} (fmol per mg)	K_d (nM)	B_{max} (fmol per mg)
WM 266-4	0.3 ± 60.1[b]	5.4 ± 40.6	0.4 ± 60.1	6.5 ± 10.7	ND[c]	ND[c]
HS15	ND	ND	0.4 ± 0.1	4.8 ± 0.5	1.2 ± 0.3	4.7 ± 0.5
NM26	0.3 ± 0.1	3.1 ± 0.5	ND	ND	1.0 ± 0.2	5.5 ± 0.6

[a]The presence of ER, PR, or AR was examined by specific radioligand binding assays, and binding constants were calculated as described in *Materials and Methods*.
[b]Results are expressed as mean ± SEM of four separate experiments.
[c]ND, not detected (no significant ligand binding).

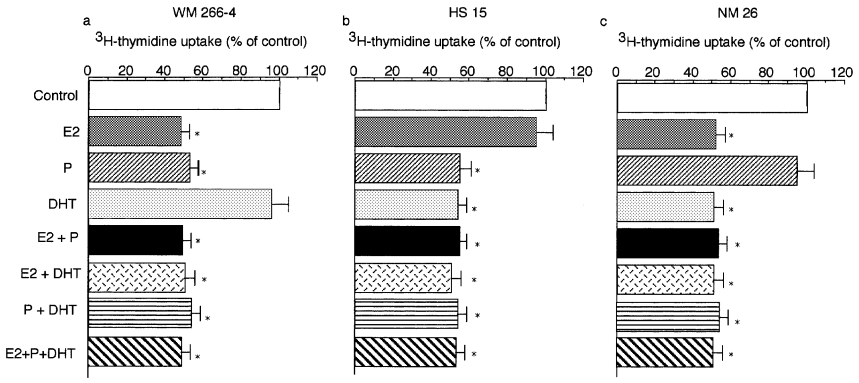

Figure 3. The inhibitory effects by combination of E2, progesterone, or DHT on the proliferation of melanoma cell lines. WM266-4 (*a*), HS-15 (*b*), or NM26 (*c*) cells were cultured with the indicated combination of E2 (10^{-9} M), progesterone (10^{-9} M), or DHT (10^{-8} M) before examining ^{3}H-thymidine uptake. The data are shown as percentage *vs* ^{3}H-thymidine uptake of control cultures with medium alone. Results represent the mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control cultures, by analysis of variance with Scheffe's multiple comparison test. The values of ^{3}H-thymidine uptake in control cultures were mean ± SEM (n = 4) 19,236 ± 1523, 15,231 ± 1224, and 10,354 ± 1194 cpm for WM266-4, HS15, and NM26 cells, respectively.

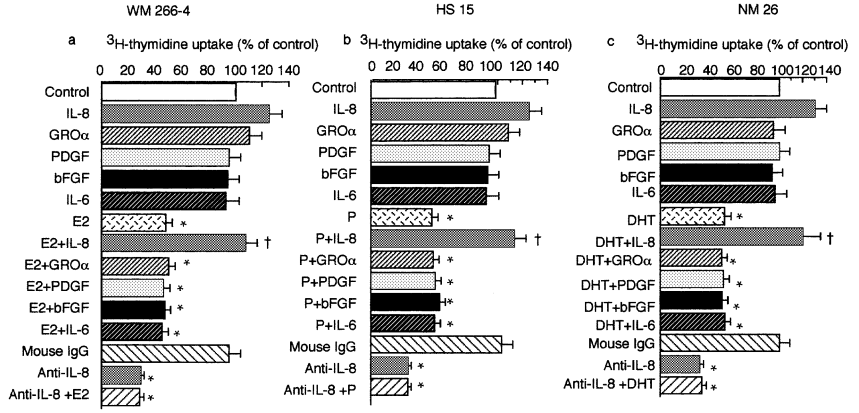

Figure 4. Cytokine-induced recovery from the inhibitory effects of E2, progesterone, or DHT on the proliferation of melanoma cells. WM266-4 (*a*), HS15(*b*), or NM26 cells (*c*) were cultured in the presence or absence of E2 (10^{-9} M) (*a*), progesterone (10^{-9} M) (*b*), or DHT (10^{-8} M) (*c*) with or without various cytokines (each 5 ng per ml) before examining ^{3}H-thymidine uptake. In some experiments, the culture of melanoma was performed in the presence of mouse anti-IL-8 antibody or control mouse IgG (each 10 μg per ml). The data are shown as percentage *vs* ^{3}H-thymidine uptake of control cultures with medium alone. Results represent the mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control cultures and †$p < 0.05$ *vs* cultures of E2, progesterone, or DHT alone, by analysis of variance with Scheffe's multiple comparison test. The values of ^{3}H-thymidine uptake in control cultures were mean ± SEM (n = 4) 18,289 ± 1626, 15,253 ± 1329, and 11,385 ± 1096 cpm for WM266-4, HS15, and NM26 cells, respectively.

medium overnight. This procedure eliminates differences in transfection efficiency as the same construct is used for the transfection of separate cultures. Then the medium was discarded and the cells were washed with phosphate-buffered saline, and maintained in serum-free, phenol red-free medium in the presence or absence of sex hormones at indicated concentrations. After 24 h, the cells were harvested and lyzed by three freeze/thaw cycles. The cell lysate was centrifuged and supernatant was assayed for CAT expression by CAT-ELISA (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. Total protein amount was measured by a Bio-Rad protein assay kit. The CAT expression was presented as picograms of CAT enzyme synthesized per microgram of total protein. pCAT3-control vector (Promega) containing SV40 early promoter and enhancer sequences was used as a positive control, and promoterless pCAT3-basic vector was used as a negative control. The expression of wild-type or mutant hormone receptors was analyzed by Western blot using cell lysate and specific antibodies. The hormone-binding activity of the expressed receptors was analyzed by radiolabeled ligand binding assays.

Statistical analyses One-way analysis of variance with Dunnet's multiple comparison test was used for the data in **Fig 2**. One-way analysis of variance with Scheffe's multiple comparison test was used for the data in **Figs 3–5**, **7**, and **8**, and **Table II**.

RESULTS

ER, PR, and AR expression in melanoma cells First, the presence of sex hormone receptors was analyzed in three different metastatic melanoma cell lines. As examined by binding assays, WM266-4 expressed ER and PR, but not AR (**Table I**). HS15

Table II. The effects of E2, progesterone, or DHT on the secretion of cytokines other than IL-8 in melanoma cells[a]

	GROα (ng per ml)			PDGF-AB (ng per ml)			bFGF (ng per ml)			IL-6 (ng per ml)		
Conditions	WM	HS	NM	WM	HS	NM	WM	HS	NM	WM	HS	NM
Control	8.0[b]	2.0	6.0	0.4	0.9	0.5	0.6	0.5	0.3	0.3	0.4	0.6
E2 (10–9 M)	8.1	2.0	5.8	0.5	0.9	0.6	0.5	0.4	0.3	0.4	0.4	0.5
p (10–9 M)	8.1	2.1	5.8	0.5	0.8	0.6	0.6	0.5	0.4	0.3	0.5	0.5
DHT (10^{-8} M)	7.8	2.0	5.8	0.5	1.0	0.5	0.5	0.4	0.3	0.3	0.4	0.6

[a]WM266-4, HS15, and NM26 cells were plated, washed, and incubated with E2, progesterone, or DHT at indicated concentrations as described in *Materials and Methods*. The cytokine secretion in the culture supernatants was analyzed by ELISA.
[b]The results are the mean of four separate experiments and SEM are less than 8% of the means.

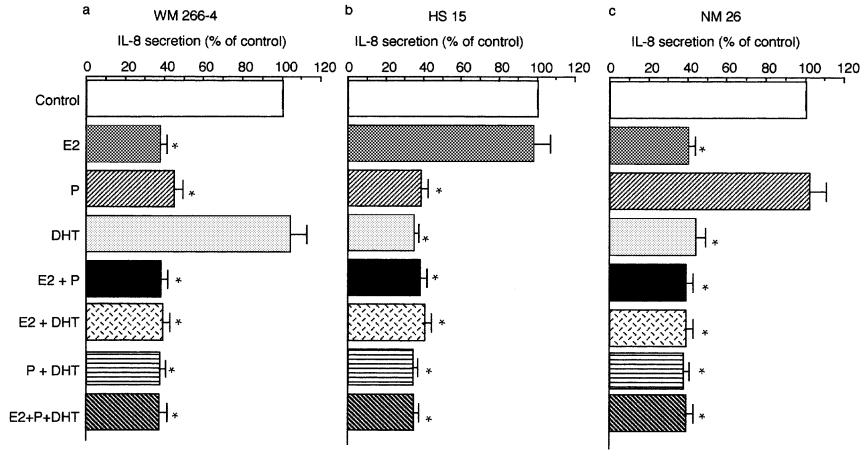

Figure 5. The inhibitory effects by combination of E2, progesterone, and/or DHT on the proliferation of melanoma cell lines. WM266-4 (*a*), HS-15 (*b*), or NM26 cells (*c*) were cultured with the indicated combination of E2 (10^{-9} M), progesterone (10^{-9} M), and/or DHT (10^{-8} M) for 24 h, and IL-8 secretion in culture supernatants was analyzed by ELISA. The data are shown as percentage *vs* IL-8 secretion in control cultures with medium alone. Results represent the mean ± SEM of four separate experiments. *$p < 0.05$ *vs* control cultures, by analysis of variance with Scheffe's multiple comparison test. The amounts of IL-8 secretion in control cultures were mean ± SEM ($n = 4$) 8.1 ± 1.2, 7.6 ± 0.9, and 5.2 ± 0.6 ng per ml for WM266-4, HS15, and NM26 cells, respectively.

expressed PR and AR, but not ER. NM26 expressed ER and AR, but not PR. Thus the presence or absence of each hormone receptor differed among cell lines; however, the affinity of ER, PR, or AR as expressed as a K_d value, was similar between each receptor-positive cell line. The results in western blot (**Fig 1**) were consistent with those in binding assays; a 65 kDa band for ER protein was detected in WM266-4 and NM26 but not in HS15. A 114 kDa band for PR was detected in WM266-4 and HS15, and not in NM26. A 110 kDa band for AR was detected in HS15 and NM26, but not in WM266-4. We next examined if each hormone may alter the growth of melanoma cell lines in a receptor-dependent manner.

The inhibitory effects of E2, progesterone, and DHT on the growth of melanoma cells As shown in **Fig 2**(***a***), E2 inhibited ^{3}H-thymidine uptake of ER-positive WM266-4 and NM26 in a concentration-dependent manner, but not that of ER-negative HS15; percentage inhibition *vs* control by 10^{-9} M of E2 was 52%, 48%, and 2% in WM266-4, NM26, and HS15, respectively. E2 stereoisomer 17α-estradiol, known to be biologically inactive as an estrogen, did not alter the growth of any melanoma cell lines. In addition, ER antagonist ICI 182,780 counteracted the growth-inhibitory effect of E2 in both ER-positive WM266-4 and NM26 cells in a concentration-dependent manner, but did not alter the growth of ER-negative HS15 treated with E2 (**Fig 2*d***). These results suggest that E2 may inhibit the growth of melanoma cells dependently on the binding to ER. Progesterone also inhibited ^{3}H-thymidine uptake of PR-positive WM266-4 and HS15 but not that of PR-negative NM26 (**Fig 2*b***); percentage inhibition by 10^{-9} M of progesterone was 47%, 45%, and 3% in WM266-4, HS15, and NM26, respectively, and the growth-inhibitory effect was not manifested by the progesterone stereoisomer, pregnenolone. The PR antagonist RU486 counteracted the growth inhibition by progesterone in both WM266-4 and HS15 cells in a concentration-dependent manner (**Fig 2*e***). DHT also inhibited ^{3}H-thymidine uptake of AR-positive NM26 and HS15 in a concentration-dependent manner, but not that of AR-negative WM266-4; percentage inhibition by 10^{-8} M of DHT was 49%, 46%, and 2% in NM26, HS15, and WM266-4, respectively, and the growth-inhibitory effect was not mediated by DHT stereoisomer, β-dihydrotestosterone. The AR antagonist bicaltamide counteracted the growth inhibition by DHT in both HS15 and NM26 cells in a concentration-dependent manner (**Fig 2*f***). As compared with the other two hormones, the dose–response curve for DHT shifted to the right by 1 order, which may reflect the lower affinity of AR, i.e., larger K_d value (**Table I**). As 10^{-9} M for E2 and progesterone, and 10^{-8} M for DHT appeared to be the optimal concentrations for growth inhibition, respectively, these concentrations were used in further experiments.

We next examined if the combination of different hormones may give additive or synergistic inhibition on the growth of melanoma cells. In ER and PR-positive and AR-negative WM266-4 (**Fig 3*a***), the inhibitory effect by combination of E2 and progesterone was not higher than that of either hormone alone. The addition of DHT did not alter the inhibitory effect of E2 or progesterone alone, or that of E2 plus progesterone. Similar results were obtained in HS15 and NM26 (**Fig 3*b***, ***c***). Thus the combination of more than two different hormones neither gave additive nor synergistic inhibition, indicating that the mechanism for the growth inhibition may be common to E2, progesterone, and DHT, and that the response of melanoma may be saturated by an optimal concentration of either hormone.

The counteracting effects of IL-8 on growth inhibition by E2, progesterone, and DHT As metastatic melanoma cells constitutively produce various growth factors and autonomously

proliferate through such growth factors, it is indicated that the growth-inhibitory effects of E2, progesterone, and DHT may be mediated by inhibiting the autocrine growth factor production in melanoma. To identify the cytokine(s) that may be involved in the growth inhibition by sex hormones, various cytokines reported as autocrine growth factors of melanoma (Herlyn, 1990) are added to the melanoma cells cultured with hormones, and their influence on E2, progesterone, or DHT-induced growth inhibition was examined. Among various cytokines, only IL-8 overcame the growth inhibition by E2 in WM266-4 (**Fig 4*a***). When endogenous IL-8 activity was neutralized by anti-IL-8 antibody, the ^{3}H-thymidine uptake of WM266-4 was reduced by 70% compared with control, indicating that IL-8 may act as an autocrine growth factor in WM266-4. In the presence of this antibody, E2 did not further reduce the ^{3}H-thymidine uptake of WM266-4. Similar results were obtained in NM26 cultured with E2 (data not shown). These results suggest that the growth-inhibitory effect of E2 may be mediated via IL-8 in WM266-4 and NM26. As shown in **Fig 4(*b*, *c*)**, IL-8 overcame the growth inhibition by progesterone in HS15 and that by DHT in NM26. Anti-IL-8 antibody reduced the ^{3}H-thymidine uptake of both cell lines, suggesting that IL-8 may act as an autocrine growth factor in both cell lines. When endogenous IL-8 was neutralized by anti-IL-8 antibody, the growth-inhibitory effects of progesterone and DHT were not detected in HS15 and NM26, respectively. Similar results were obtained in WM266-4 cultured with progesterone and in HS15 cultured with DHT (data not shown). These results suggest that IL-8 may be involved in the growth-inhibitory effects of progesterone in HS15 and WM266-4 and those of DHT in NM26 and HS15. We then examined if E2, progesterone, or DHT may alter IL-8 production in the three different melanoma cell lines.

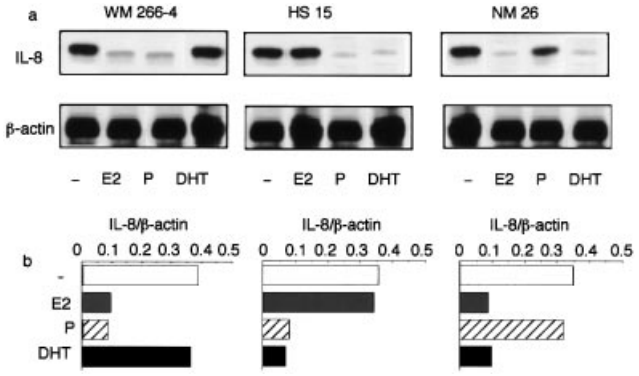

Figure 6. Northern blot analysis for the effects of E2, progesterone, or DHT on IL-8 mRNA expression in melanoma cells. WM266-4, HS15, or NM26 cells were cultured with E2 (10^{-9} M), progesterone (10^{-9} M), or DHT (10^{-8} M) for 8 h. Cells were then harvested and total RNA (25 μg) from each sample was subjected to northern blot analysis and probed for IL-8 and β-actin mRNA (*a*) and the intensity of the bands were analyzed by densitometry, and the intensity ratio was calculated (*b*). The data are representative of four different experiments.

The effects of sex hormones on IL-8 production in melanoma WM266-4, HS15, and NM26 constitutively secreted large amounts of IL-8 (5.2–8.1 ng by 5×10^4 cells). E2 reduced IL-8 secretion in ER-positive WM266-4 and NM26 by 62.5% and by 60% compared with controls, respectively, whereas it did not alter that in ER-negative HS15 (**Fig 5**). Progesterone also reduced IL-8 secretion in PR-positive WM266-4 and HS15 by 56.3% and by 61.6%, respectively, and did not affect that in PR-negative NM26. DHT also reduced IL-8 secretion in AR-positive HS15 and NM26 by 65.3% and by 56%, respectively, and did not alter that in AR-negative WM266-4. In parallel with the growth inhibition, anti-estrogen ICI 182,780 counteracted the E2-induced inhibition of IL-8 production in WM266-4 and NM26 cells (data not shown), and similar results were obtained for anti-progesterone RU486 on progesterone-induced inhibition, and anti-androgen bicaltamide on DHT-induced inhibition of IL-8 production (data not shown). Thus E2, progesterone, or DHT inhibited IL-8 secretion in melanoma cells in a respective receptor-dependent manner. In each melanoma cell line, the combination of more than two different sex hormones neither gave additive nor synergistic inhibition on the IL-8 secretion, which correlated with the hormone-induced inhibition of ^{3}H-thymidine uptake (**Fig 3**). Though WM266-4, HS15, and NM26 constitutively secreted large amounts of GRO-α, none of E2, progesterone, and DHT altered the secretion of GRO-α in any cell lines. The secretion of PDGF-AB, bFGF, and IL-6 were lower than that of IL-8 or GRO-α, and were not affected by any hormones in any cell lines (**Table II**).

We then analyzed if IL-8 mRNA expression in melanoma cells may be altered by E2, progesterone, or DHT. As analyzed by northern blot (**Fig 6*a*, *b***), WM266-4, HS15, and NM26 constitutively expressed IL-8 mRNA, and the expression was suppressed by sex hormones in a respective receptor-dependent manner; E2 reduced steady-state IL-8 mRNA levels of ER-positive

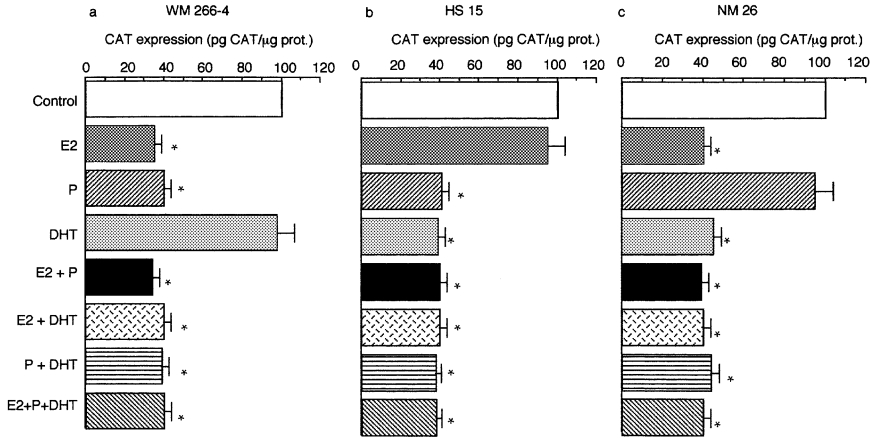

Figure 7. The inhibitory effects of E2, progesterone, or DHT on IL-8 promoter activities in melanoma cells. WM266-4 (*a*), HS15 (*b*), and NM26 cells (*c*) were transiently transfected with IL-8 promoter CAT reporter constructs and cultured with indicated combinations of E2 (10^{-9} M), progesterone (10^{-9} M) or DHT (10^{-8} M). The IL-8 promoter activity was assessed by CAT expression of the cell lysate. The data are shown as percentage *vs* CAT expression in control cultures with medium alone. Results represent the mean ± SEM of four separate experiments. *p < 0.05 *vs* control cultures, by analysis of variance with Scheffe's multiple comparison test. The CAT expression in control cultures was mean ± SEM (n = 4) 32.4 ± 3.5, 30.6 ± 2.9, and 20.3 ± 1.8 pg CAT per μg protein for WM266-4, HS15, and NM26, respectively. The CAT expression in pCAT3-control vector was 130 ± 11, 151 ± 14, and 145 ± 13 pg CAT per μg protein for WM266-4, HS15, and NM26 cells, respectively. The CAT expression by promoterless pCAT3-basic vector was less than detectable level in all three cell lines.

WM266-4 and NM26 and did not affect that of ER-negative HS15. Progesterone reduced IL-8 mRNA levels of PR-positive WM266-4 and HS15, but did not alter that of PR-negative NM26. DHT reduced IL-8 mRNA levels of AR-positive HS15 and NM26, and did not affect that of AR-negative WM266-4. Thus the inhibitory effects of E2, progesterone, and DHT on IL-8 mRNA expression closely correlated with those on IL-8 protein secretion (**Fig 5**), suggesting the pretranslational inhibition. We then examined if E2, progesterone, and DHT may exert their effects at the transcriptional level by analyzing the effects of the hormones on the activity of IL-8 promoter.

The effects of E2, progesterone, and DHT on CAT expression driven by IL-8 promoter WM266-4, HS15, and NM26 were transiently transfected with plasmid containing human IL-8 promoter driving CAT reporter gene, and the promoter activity was assessed by the expression of CAT enzyme. Though it is known that IL-8 promoter activity is upregulated by IL-1 or phorbol 12-myristate 13-acetate (Mukaida *et al*, 1989), the melanoma cell lines showed considerable levels of CAT expression driven by IL-8 promoter (20.3–32.4 pg CAT per μg protein) even in unstimulated state. As shown in **Fig 7**, the constitutive activity of IL-8 promoter in each melanoma cell line was inhibited by E2, progesterone, or DHT in a respective receptor-dependent manner; E2 reduced IL-8 promoter activity of ER-positive WM266-4 (**Fig 7*a***) and NM26 (**Fig 7*c***) by 65% and by 60% compared with controls, respectively, and did not alter that of ER-negative HS15 (**Fig 7*b***). Progesterone reduced IL-8 promoter activity of PR-positive WM266-4 and HS15 by 61% and by 60%, respectively, but did not affect that of PR-negative NM26. DHT reduced IL-8 promoter activity of AR-positive HS15 and NM26 by 62% and by 58%, respectively, and did not affect that of AR-negative WM266-4. The combination of more than two different sex hormones neither gave additive nor synergistic inhibition on IL-8 promoter activity in each melanoma cell line, which was consistent with the sex hormone-induced inhibition of IL-8 secretion (**Fig 5**). These results suggest that E2, progesterone, and DHT may inhibit IL-8 production at the transcriptional level in a respective receptor-dependent manner. To confirm that the hormone-induced transcriptional inhibition may be mediated via the respective hormone receptor, we transiently cotransfected ER, PR, or AR-negative melanoma with the respective receptor expression plasmid together with IL-8/CAT construct, and examined if E2, progesterone, or DHT may inhibit the IL-8 promoter activity in the respective receptor-transfected cells. Transient transfection of AR-negative WM266-4 with wild-type AR vector resulted in the measurable ^{3}H-R1881 binding; K_d = 1.1 nM and B_{max} = 17.3 fmol protein per mg. On the other hand, WM266-4 transfected with hormone-binding domain-deleted mutant AR (**Fig 8*g***) did not show significant binding to ^{3}H-R1881. By western blotting, the expression of wild-type and mutant AR was confirmed as 114 kDa and 87 kDa band, respectively (**Fig 8*d***). The IL-8 promoter activity in wild-type AR-transfected WM266-4 was not reduced compared with empty vector pSG5-transfected cells (**Fig 8*a***), suggesting that the overexpression of wild-type AR itself may not inhibit IL-8 promoter activity. In the wild-type AR-transfected WM266-4 (**Fig 8*a***), however, DHT reduced IL-8 promoter activity by 71.4% compared with control cultured with medium alone whereas in WM266-4 transfected with hormone-binding domain-deleted mutant AR, DHT did not reduce the IL-8 promoter activity. Thus wild-type AR, transfected into melanoma, could repress IL-8 promoter activity in the presence of DHT, and the repression required the binding of DHT to AR. Similarly, transient transfection of ER-negative HS15 with wild-type ER resulted in the measurable ^{3}H-E2 binding; K_d = 0.4 nM and B_{max} = 15.1 fmol per mg protein. On the other hand, HS15 transfected with hormone-binding domain-truncated mutant ER (**Fig 8*h***) did not show significant binding to ^{3}H-E2. By western blotting, the expression of wild-type and mutant ER was confirmed as 66 kDa and 37 kDa band, respectively (**Fig 8*e***). In wild-type ER-transfected HS15 (**Fig 8*b***), E2 inhibited IL-8 promoter activity by 63.6% compared with control, but did not alter that in HS15 transfected with hormone-binding domain-deleted mutant ER, indicating that E2-induced transrepression was dependent on E2-binding to ER. Transient transfection of PR-negative NM26 with wild-type PR resulted in the measurable ^{3}H-R5020 binding; K_d = 0.45 nM and B_{max} = 16.5 fmol protein per mg. On the other hand, NM26 transfected with hormone-binding domain-truncated mutant PR (**Fig 8*i***) did not show significant binding to ^{3}H-R5020. By western blotting, the expression of wild-type and mutant PR was confirmed as 110 kDa and 79 kDa band, respectively (**Fig 8*f***). Progesterone inhibited IL-8 promoter activity by 68.2% compared with control in the wild-type PR-transfected NM26, but did not affect that in NM26 transfected with hormone-binding domain-truncated mutant PR, indicating that progesterone-induced transrepression was dependent on

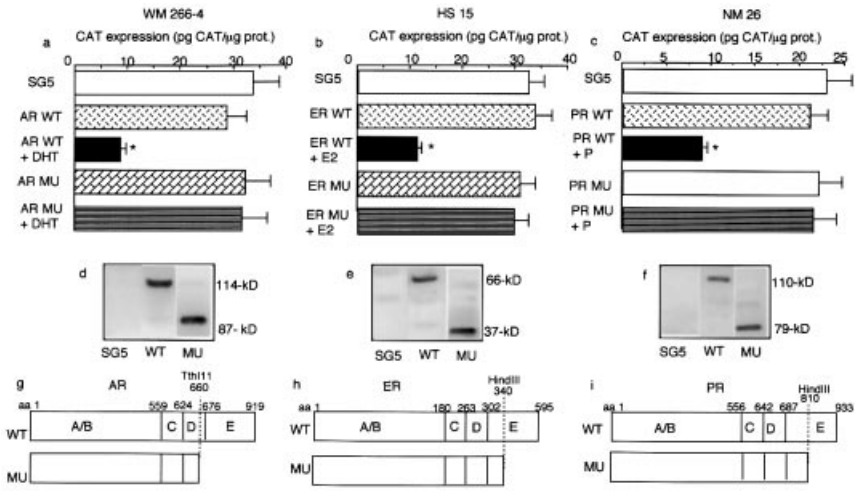

Figure 8. The inhibition by E2, progesterone, or DHT on IL-8 promoter activity in wild-type or mutant ER, PR, or AR-transfected melanoma cells. WM266-4 (*a*), HS15 (*b*), or NM26 (*c*) cells were cotransfected with IL-8 promoter CAT reporter construct and either empty vector pSG5 or wild type (WT) or hormone-binding domain-deleted mutant (MU) AR (*a*), ER (*b*), or PR (*c*) expression vector, and incubated with or without DHT 10^{-8} M (*a*), E2 10^{-9} M (*b*), or progesterone 10^{-9} M (*c*). The IL-8 promoter activity was assessed by CAT expression of the cell lysate. Results represent the mean ± SEM of four separate experiments. *p < 0.05 *vs* wild-type receptor-transfected cells cultured with medium alone, by analysis of variance with Scheffe's multiple comparison test. The transfected wild-type and mutant AR (*d*), ER (*e*), and PR (*f*) were verified for expression by western blot. The structure of wild-type and mutant receptors is presented in (*g–i*). Regions A/B, C, D, and E represent the amino-terminal, DNA-binding, hinge, and the hormone-binding domains, respectively. The number of amino acid residues and restriction enzyme-cut sites are indicated.

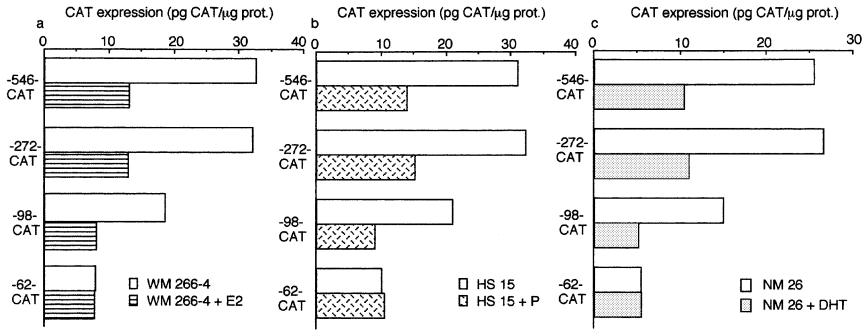

Figure 9. The influence of 5′-deletion on E2, progesterone, or DHT-induced inhibition of IL-8 promoter activity. WM266-4 (*a*), HS15 (*b*), or NM26 cells (*c*) were transfected with CAT reporter vectors into which various 5′-deleted IL-8 promoters were cloned. After transfection, the cells were incubated with or without E2 (10^{-9} M) (*a*), progesterone (10^{-9} M) (*b*), or DHT (10^{-8} M) (*c*) for 24 h. CAT expression was then analyzed.

progesterone binding to PR. (**Fig 8*c***). These results suggest that the transcriptional repression by E2, progesterone, and DHT may be specifically mediated by binding to the respective receptor.

To characterize the DNA sequences involved in hormone-induced transcriptional repression on the IL8 gene, we used CAT expression plasmids linked to serially 5′-deleted IL-8 promoters, and compared the effects of hormones on the activities of the deleted promoters. In transfected WM266-4 cells, the basal promoter activity of –272 CAT (containing –272 to +44 bp of IL-8 promoter) was not different from that of –546 CAT, and both were repressed by E2 (**Fig 9*a***). The basal promoter activity of – 98 CAT was reduced as compared with that of –546 or –272 CAT, indicating that the sequences between –272 to –99 bp may partially direct the constitutive promoter activity. The promoter activity of –98 CAT, however, was still repressed by E2; percentage inhibition by E2 was 61% and 61.5% in –272 and –98 CAT-transfected cells, respectively. These results indicate that the sequences up to –98 bp may be dispensable for the transcriptional repression by E2. When WM266-4 was transfected with –62 CAT, the basal promoter activity was further reduced, and was not at all repressed by E2. These results suggest that the sequences between –98 and –63 bp may partially direct the constitutive IL-8 transcription and may be mainly involved in the E2-induced transcriptional repression. Similar results were obtained in the 5′-deleted IL-8/CAT-transfected and E2-incubated NM26 cells (data not shown). In the 5′-deleted IL-8/CAT-transfected and progesterone-treated HS15 (**Fig 9*b***) or DHT-treated NM26 (**Fig 9*c***), the deletion up to –98 bp of IL-8 promoter preserved the repression by progesterone or DHT, respectively, whereas deletion up to –62 bp resulted in the complete loss of the repression. Similar results were obtained in the 5′-deleted IL-8/CAT-transfected and progesterone-treated WM266-4 or DHT-treated HS15 (data not shown). These results suggest that the sequences between –98 and –63 bp are mainly involved in the hormone-induced repression of IL-8 transcription in melanoma.

DISCUSSION

This study demonstrated that E2, progesterone, and DHT inhibited the growth of metastatic melanoma WM266-4, HS15, and NM26 by inhibiting IL-8 transcription. The inhibitory effect of each hormone was mediated via the respective receptor. The growth of the other melanoma cell lines was also receptor-dependently inhibited by sex hormones via the suppression of IL-8 production; among melanoma TO 23, HO 14, and YN 115 established in our laboratory, the presence of ER and E2-induced growth inhibition via IL-8 were only seen in TO 23, but not in the others. The presence of PR and progesterone-induced growth inhibition via IL-8 were only seen in HO 14 and not in the others. The presence of AR and DHT-induced growth inhibition via IL-8 were only seen in YN 115 and not in the others (data not shown). The previous study also reported that progesterone inhibited basal and IL-1-induced IL-8 mRNA expression in rat uterine fibroblasts (Ito *et al*, 1994); however, it is unknown how E2, progesterone, or DHT may suppress IL-8 transcription. It is likely that the three different hormones may exert their effects by a common mechanism as the combination of more than two hormones gave neither synergistic nor additive effects. One possible mechanism is that each hormone-bound receptor may inhibit the transcriptional activity and/or DNA binding of transcriptional activators on the IL-8 promoter. It is known that several transcriptional activators co-operate with one another through protein–protein interaction to activate the IL-8 promoter (Roebuck, 1999). The sequences from –98 to –63 bp appeared to direct the constitutive IL-8 transcription in melanoma, and may be the main target for hormone-induced transcriptional repression. This region includes adjacent elements for C/EBP (–94 to –81) and NF-κB (–80 to –70). It is reported that NF-κB subunit p65 and C/EBPβ form a ternary complex with this region of the IL-8 promoter, which results in synergistic transcriptional activation (Kunsch *et al*, 1994). It is thus hypothesized that each sex hormone/receptor complex may interact with NF-κB and/or C/EBP family proteins, and alter their conformation, which may repress their DNA-binding and/or the cooperative transcriptional activity on IL-8 promoter. The previous studies reported the direct protein–protein interaction of ER with NF-κB p65 and with C/EBPβ(Stein and Yang, 1995). PR and AR directly interacted with NF-κB p65 and repressed its transcriptional activity on several gene promoters (Kalkhoven *et al*, 1996; McKay and Cidlowski, 1999). E2 and DHT also inhibited the transcriptional activity of NF-κB via IkBα (Keller *et al*, 1996; Sun *et al*, 1998) IkBα is constitutively associated with NF-κB in cytosol and inhibits its translocation to the nucleus where DNA binding occurs. E2 and DHT repressed the degradation of IkBα, and maintained the IkBα protein level, and thus inhibited the transcriptional activity of NF-κB. As dexamethasone and 1α,25-dihydroxyvitamin D_3 suppressed IL-8 transcription by inhibiting the DNA binding of NF-κB in a glioblastoma cell line (Mukaida *et al*, 1994; Harant *et al*, 1997), a similar mechanism is implicated for E2, progesterone, or DHT. DNA-binding activity and mRNA levels of NF-κB subunits p65 and p50 are constitutively enhanced in melanomas compared with normal melanocytes (Meyskens *et al*, 1999), which may be related to the constitutively high level of IL-8 production in melanoma. Taken together, it is hypothesized that NF-κB may be one of the main targets for the sex hormone-induced transcriptional repression. It is also likely that C/EBP may be the target. C/EBP is known to mainly mediate the constitutive IL-8 transcription without any stimuli (Wu *et al*, 1997). DHT-bound AR also suppressed the C/EBP-mediated transcription on rat dehydroepiandrosterone sulfotransferase promoter (Song *et al*, 1998). In the present study, the deletion of – 272 to – 99 bp on IL-8 promoter greatly reduced the constitutive promoter activity (**Fig 9**). As this region contains AP-1 element (–126 to –120) (Mukaida *et al*, 1989), AP-1 may partially confer the constitutive IL-8 transcription in melanoma. A previous study also reported that the constitutive DNA-binding activity of AP-1 was higher in melanomas than in melanocytes (Meyskens *et al*, 1999). It is known that AP-1 as well as C/EBP physically interacts with NF-κB and synergistically activates IL-8 transcription (Stein *et al*, 1993; Roebuck, 1999). AP-1, however, may not be involved in hormone-induced transcriptional repression as the repression was not abolished by the deletion of the region containing –126 to –120 (**Fig 9**).

In addition to the inhibition of transcriptional activators, sex hormones may promote the synthesis and/or activity of repressor(s) for IL-8 transcription. C/EBP site (–94 to –81) on IL-8 promoter overlaps with an element for Oct-1 (–91 to –84) (Roebuck, 1999), and the binding of Oct-1 displaces C/EBP from this element and thus represses the constitutive IL-8 transcription (Wu *et al*, 1997). Thus sex hormones may promote such transcriptional repression by Oct-1. On the other hand, Oct-1 acts as a transcriptional activator on mouse mammary tumor virus promoter (Prefontaine *et al*, 1999). It is known that progesterone/PR complex binds to the hormone response element on this promoter, and facilitates the binding of Oct-1 to its consensus element, and thus promotes its transcriptional activation (Prefontaine *et al*, 1999). There have been no reports, however, that the hormone-bound receptor may enhance the repressor activity of Oct-1, and this possibility should further be investigated. Another possible mechanism is that the sex hormone-bound receptor may directly interact with the IL-8 promoter and thus displace certain transcriptional activator(s) from the respective element(s). This possibility is, however, rather unlikely as the sequences responsible for transcriptional repression (–98 to –63) contain no consensus hormone response elements. Previous studies also reported that E2-bound ER repressed IL-6 promoter activity (Pottratz *et al*, 1994) and DHT-bound AR repressed rat dehydroepiandrosterone sulfotransferase promoter activity (Song *et al*, 1998), both without direct interaction with DNA. IL-8 promoter contains the binding site for PR and AR at –330 to –325 (Mukaida *et al*, 1989); however, this element did not appear to mediate the transcriptional repression by sex hormones as the repression was not abolished by the deletion of this element-containing region (–546 to –273) (**Fig 9**). It is also speculated that the other undefined transcriptional activators or repressors may be the target for sex hormones. Further studies should elucidate the target molecule(s) and the precise mechanism for transcriptional repression by sex hormones on the IL-8 promoter.

In this study, WM266-4, HS15, and NM26 appeared to proliferate by an autocrine loop of IL-8 as their growth was greatly inhibited by the anti-IL-8 antibody (**Fig 4**). Previous reports also confirmed the role of IL-8 as an autocrine growth factor of melanoma, and the correlation of its expression with metastatic potential (Schadendorf *et al*, 1993; Andrew *et al*, 1995; Luca *et al*, 1997). Certain melanomas, however, may grow independently from IL-8. E2, progesterone, or DHT may enhance the growth of certain melanomas by promoting the production of growth factors different from IL-8, such as transforming growth factor-β, the production of which is enhanced by DHT (Pederson *et al*, 1999). Thus further studies should elucidate the hormonal effects on the IL-8-independent melanomas.

This study shows that these sex hormones can be used for the treatment of patients with melanoma, and that the therapeutic efficiency may depend on the respective receptors. The combination of sex hormones with other chemotherapeutic agents may prevent postoperational recurrence or metastasis of melanoma. This study, however, was performed on the established melanoma cell lines and thus the results may be valid only in the *in vitro* limited settings. Similar examination should be extended into primary tumor cell cultures from freshly biopsied material as the hormonal effects on such material may be more close to those *in vivo*. To date, several clinical trials have been done on advanced melanomas by hypophysectomy or the administration of testosterone, ethinylestradiol (Bodenham and Hale, 1972), 6α-methylpregn-4-ene,3,11,20-trione (Johnson *et al*, 1966), medroxyprogesterone acetate (Baretta *et al*, 1979), or recently tamoxifen (Neifeld, 1996; Chapman *et al*, 1999; Creagan *et al*, 1999; Propper *et al*, 2000); however, only limited subsets of patients were responsive to hormonal therapy, and the response did not correlate with the presence of hormone receptor in their melanoma cells (Linder and Borden, 1997; Karakosis *et al*, 1980; Neifeld and Lippman, 1980; Neifeld, 1996). These indicate nonreceptor-mediated hormonal effects or the effects via alternate receptors other than conventional intracellular counterparts and/or indirect growth regulation via the other hormone-responding cells. It is reported that E2 disrupts the cytoplasmic microtubule network and thus inhibited the growth of both ER-positive and -negative human breast cancer cell lines, indicating the receptor-independent effect (Aizu-Yokota *et al*, 1994). The recent studies also suggest that membrane-associated receptors, structurally similar to (Papas *et al*, 1995) or different from conventional intracellular counterparts (Bression *et al*, 1986), may exist and mediate some rapid, nontranscriptional actions such as calcium influx (Blackmore *et al*, 1990) or the activation of tyrosine kinase (Migliaccio *et al*, 1996). In addition to the high-affinity receptors, different hormone-binding sites are identified; type II estrogen binding sites exhibit lower affinity but higher capacity for estrogen compared with ER, and are detected in several human melanoma cells, and may be involved in the *in vitro* hormonal growth regulation on those cells (Piantelli *et al*, 1995). The unconventional receptor-mediated hormonal effects *in vivo* should further be clarified.

Melanoma-derived growth factors act on adjacent stromal cells such as endothelial cells, macrophages, fibroblasts, or lymphocytes, whereas these cells in turn produce various factors that regulate tumor growth, angiogenesis, adhesion, motility, or metastasis (Shih and Herlyn, 1993; Laza-Molnar *et al*, 2000). Sex hormones may alter the autocrine and/or paracrine production of angiogenic factors, such as vasculoendothelial growth factor (Hyder *et al*, 1996). Sex hormones may also alter the expression of adhesion molecules (Lupetti *et al*, 1996), or activity of proteolytic enzymes in melanoma (Herlyn, 1990; Lazar-Molnar *et al*, 2000). Sex hormones also affect the host immune responses to melanoma by natural killer cells (Hanna and Schneider, 1983) or macrophages (Ackermann *et al*, 1986). We are now studying the *in vivo* effects of sex hormones on the invasion and metastasis of presently used melanomas in athymic nude mice. Further studies should also elucidate the growth-regulatory effects of sex hormones on the coculture of melanoma with endothelial cells, macrophages, or fibroblasts *in vitro*.

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