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Regulation of Sex Steroid Formation by Interleukin-4 and Interleukin-6 in Breast Cancer Cells

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Sex steroids play a predominant role in the development and differentiation of normal mammary gland as well as in the regulation of hormone-sensitive breast cancer growth. There is evidence suggesting that local intracrine formation of sex steroids from inactive precursors secreted by the adrenals namely, dehydroepiandrosterone (DHEA) and 4-androstenedione (4-dione) play an important role in the regulation of growth and function of peripheral target tissues, including the breast. Moreover, human breast carcinomas are often infiltrated by stromal/immune cells secreting a wide spectra of cytokines. These might in turn regulate the activity of both immune and neoplastic cells. The present study was designed to examine the action of cytokines on 17β-hydroxysteroid dehydrogenase (17β-HSD) and 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) activities in human breast cancer cells. The various types of human 17β-HSD (five types) and 3β-HSD (two types), because of their tissue- and cell-specific expression and substrate specificity, provide each cell with necessary mechanisms to control the level of intracellular active androgens and estrogens. We first investigated the effect of exposure to IL-4 and IL-6 on reductive and oxidative 17β-HSD activities in both intact ZR-75-1 and T-47D human breast cancer cells. In ZR-75-1 cells, a 6 d exposure to IL-4 and IL-6 decreased E_2-induced cell proliferation, the half maximal inhibitory effect being exerted at 88 and 26 pM, respectively. In parallel, incubation with IL-4 and IL-6 increased oxidative 17β-HSD activity by 4.4- and 1.9-fold, respectively, this potent activity being observed at EC_{50} values of 22.8 and 11.3 pM, respectively. Simultaneously, reductive 17β-HSD activity leading to E_2 formation was decreased by 70 and 40% by IL-4 and IL-6, respectively. Moreover, IL-4 and IL-6 exerted the same regulatory effects on 17β-HSD activities when testosterone and 4-dione were used as substrates, thus strongly suggesting the expression of the type 2 17β-HSD ZR-75-1 cells. In contrast, in T-47D cells, IL-4 increased the formation of E_2, whereas IL-6 exerts no effect on this parameter. However, we found that T-47D cells failed to convert testosterone efficiently into 4-DIONE, thus suggesting that there is little or no expression of type 2 17β-HSD in this cell line. The present findings demonstrate that the potent regulatory effects of IL-4 and IL-6 on 17β-HSD activities depend on the cell-specific gene expression of various types of 17β-HSD enzymes. We have also studied the effect of cytokines on the regulation of the 3β-HSD expression in both ZR-75-1 and T-47D human breast cancer cells. Under basal culture conditions, there is no 3β-HSD activity detectable in these cells. However, exposure to IL-4 caused a rapid and potent induction of 3β-HSD activity, whereas IL-6 failed to induce 3β-HSD expression. Our data thus demonstrate that cytokines may play a crucial role in sex steroid biosynthesis from inactive adrenal precursors in human breast cancer cells. **© 1998 Elsevier Science Ltd. All rights reserved.**

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Abbreviations: 3β-hydroxysteroid dehydrogenase: 3β-HSD; 17β-hydroxysteroid dehydrogenase: 17β-HSD; 17β-estradiol: E_2; estrone: E_1; interleukin-4: IL-4; interleukin-6: IL-6; testosterone: TESTO; androstenedione: 4-DIONE; androst-5-ene-3β, 17β-diol: 5-DIOL; dehydroepiandrosterone: DHEA.

INTRODUCTION

Estrogens are well recognized to play a predominant role in regulating hormone-sensitive breast cancer cell growth [1–3]. Concentrations of the potent estrogen 17β-estradiol (E_2) are significantly higher in breast tumor tissue than in normal breast tissue [4–7], whereas the tumor-plasma E_2 ratio was much higher compared to that for estrone (E_1) [5, 6]. Moreover, a higher level of conversion of E_1 into E_2 compared with the reverse reaction was measured by direct uptake of labeled E_1 or E_2 in breast cancer tissue in postmenopausal women [8]. It is, therefore, quite possible that higher reductive 17β-HSD activity would contribute to greater levels of E_2 in tumor tissue by increasing the E_2/E_1 ratio, resulting in a progression of tumor growth. The 17β-hydroxysteroid dehydrogenase (17β-HSD) activity, therefore, exerts a key role in regulating the tissue concentration of active estrogens by the interconversion of E_1 and E_2 as well as that of dehydroepiandrosterone (DHEA) and androst-5-ene-3β, 17β-diol (5-DIOL), the latter being a relatively potent estrogen [9]. In many estrogen receptor-positive (ER+) human breast cancer cell lines (MCF-7, ZR-75-1, and T-47D), the reductive pathway leading to the formation of E_2 from E_1 is predominant [10–13], whereas in hormone-independent cell lines (MDA-MB-231 and MDA-MB-346), the oxidative pathway promoting inactivation of E_2 into E_1 predominates [10]. Numerous studies have reported the presence of multiple 17β-HSDs in human breast cancer cells [12, 15–18].

In general, the crucial role of 17β-HSD activities is performed by at least five different enzymes having individual cell-specific expression, substrate specificity, regulation mechanisms and reductive or oxidative catalytic activity and sharing a low sequence similarity [14]. The human type I 17β-HSD enzyme is a cytosolic protein that exists in a homodimeric form which catalyzes almost exclusively the interconversion of E_1 and E_2 [19–21]. The HSD17B1 gene which lies on chromosome 17q21 in close proximity to the BRCA1 locus, is contained within a genomic fragment of 3.3 kb and consists of six exons encoding a protein of 327 amino acids [22, 23]. Its X-ray structure was the first to be determined among mammalian steroidogenic enzymes [24–27]. The expression of this isoenzyme has been demonstrated in ZR-75-1 cells as well as in several other breast cancer cell lines and in human breast carcinoma [12, 15, 18, 22, 28–30]. The type II 17β-HSD is a NAD-dependent membrane-associated protein of 387 amino acids, which catalyzed the interconversion of E_2 into E_1, testosterone (TESTO) into 4-androstenedione (4-DIONE) and DHEA into 5-DIOL, and dihydrotestosterone (DHT) into androstanedione [31–33]. Because of the much higher intracellular bioavailability of NAD^+ in comparison to NADH, this enzyme is especially responsible for the oxidative pathway, thus being involved in the degradation of active estrogens and androgens [14]. The human type III 17β-HSD is a microsomal protein of 310 amino acids, predominantly expressed in the testis, that uses NADP(H) as cofactor and preferentially catalyzes the formation of TESTO from 4-DIONE, androstanedione into DHT and with a lesser affinity E_1 into E_2, because of the much higher bioavailability of NADPH [14, 34]. Thereafter, a fourth form of 17β-HSD has been cloned from human liver [35]. The type IV 17β-HSD is a NAD-dependent 736-amino acid protein, which is primarily responsible for the conversion of E_2 and 5-DIOL into E1 and DHEA, respectively [36]. This enzyme is expressed virtually in all tissues examined, including several breast cancer cell lines and is thus likely to play a role in the inactivation of estrogens in peripheral tissues. More recently, a human placental dehydrogenase that shares a high sequence identity with members of the 3α-HSD family but possessing a strong reductive 17β-HSD activity designated type V was cloned [14]. This NADP(H)-dependent enzyme, which is expressed in several peripheral target tissues, converts mainly 4-DIONE into TESTO, and to a lesser extent androstanedione into DHT [14]. Thus, the various types of human 17β-HSD, because of their tissue-specific expression and substrate and cofactor specificities, provide each peripheral cell with the necessary mechanisms to control the level of intracellular estrogens and androgens [37].

The membrane-bound NAD^+-dependent 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD), catalyzes the conversion of 5-ene-3β-hydroxysteroids into the corresponding 4-ene-3-ketosteroids. This activity is essential for the formation of all classes of steroids [38]. In addition, the enzymes of the 3β-HSD family also catalyze the formation and/or degradation of the 5α-androstanes and 5α-pregnanes, such as dihydrotestosterone (DHT) and dihydroprogesterone [38]. In humans and in the rhesus monkey, 3β-HSD activity is not only detectable in the adrenal cortex, gonads and placenta, but also in several peripheral tissues [38, 39]. Such a distribution of 3β-HSD expression suggests that these enzymes play a key role in the intracrine formation of sex steroids in peripheral target tissues. The human type I 3β-HSD is predominantly expressed in placenta and in peripheral tissues such as skin and mammary gland, whereas the type II is predominantly expressed in the adrenals and gonads [40]. This role of 3β-HSD isoenzymes is of major importance, especially in human and some other primates, since their adrenals secrete large amounts of the inactive steroid precursors DHEA and its sulfate (DHEA-S), which are converted into potent androgens and estrogens in peripheral tissues.

A large body of evidence suggests that breast tumors can modulate their hormonal environment.

Both endocrine and paracrine influences on the proliferation of human breast cancer cells are well recognized [41–43]. The presence in breast tumors of considerable numbers of tumor-associated macrophages and tumor-infiltrating lymphocytes secreting a wide spectra of cytokines also suggested a key role for these factors in neoplastic cell activity [44–46]. In support of this hypothesis, interleukin-1α (IL-1α), IL-4, and IL-6 and IL-13 inhibit the proliferation of ZR-75-1, T-47D and/or MCF-7 human breast cancer cells [47–57]. Furthermore, we have recently demonstrated that the expression of apolipoprotein D (apo-D) and/or gross cystic disease fluid protein-15 (GCDFP-15) is induced by IL-1α, IL-4 and IL-13 in ZR-75-1 cells [53, 55], while IL-6 inhibits the secretion of these two tumor markers [54]. Moreover, IL-6 also regulates 17β-estradiol (E_2) synthesis and degradation in MCF7 breast cancer cells [58, 59].

Because 17β-HSD and 3β-HSD activities represent critical sites of control and regulation of sex steroid formation and/or inactivation in human breast cancer cells, a better knowledge of the different mechanisms and factors regulating these activities would facilitate the understanding of breast cancer development and growth. The present study was thus designed to investigate the potential effects of IL-4 and IL-6 on 17β-HSD and 3β-HSD activities in ZR-75-1 and T-47D human breast cancer cell lines.

MATERIALS AND METHODS

Chemicals and radioactive steroids

17β-[6,7-^{3}H] estradiol (E_2) (specific activity, 40–50 Ci mmol^{-1}), [2,4,6,7-^{3}H] estrone (E_1) (80–100 Ci mmol^{-1}), [1β, 2β-^{3}H(N)] testosterone (TESTO) (41 Ci mmol^{-1}), and [1,2,6,7-^{3}H(N)] androst-4-ene3, 17-dione (4-DIONE) (85 Ci mmol^{-1}) were purchased from New England Corporation (Boston, MA). Unlabeled E_2 was obtained from Steraloids (Wilton, NH). IL-4 was kindly provided by Dr Nagabhushan and Dr Narula from Schering–Plough Research Institute (Kenilworth, NJ) (10 μg μl^{-1}). IL-6 was purchased from Boehringer Manheim Co. (Montréal, Québec) at a concentration of 1×10^6 IU ml^{-1} and specific activity of 2×10^8 IU mg^{-1}. Organic solvents were from BDH (Montréal, Québec).

Maintenance of stock cell cultures

All media and supplements for cell culture were obtained from Sigma Chemical Co. (St. Louis, MO), except for FBS (fetal bovine serum), which was purchased from Hyclone (Logan, UT). ZR-75-1 and T-47D human breast cancer cells were both obtained from the American Type Culture Collection (Rockville, MD), at passage 83 and 86, respectively. They were routinely grown in phenol red-free RPMI-1640 medium supplemented with 1 nM E_2, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 100 IU penicillin ml^{-1}, 50 μg streptomycin sulfate ml^{-1}, and 10% (vol/vol) FBS, as previously described [54, 55, 57]. Cells were used between passage 88 and 98 for ZR-75-1 and between passage 90 and 110 for T-47D. They were then subcultured once a week.

Studies of cell proliferation

Cells were harvested with 0.1% pancreatin (Sigma Chemical Co.) and resuspended in phenol red-free RPMI-1640 medium supplemented as above and 5% (vol/vol) FBS treated twice with dextran-coated charcoal. Cells were plated in Falcon 24-well plastic culture plates at a density of 10^4 cells/well and allowed to adhere for 72 h. Thereafter, the medium was replaced with fresh medium containing the indicated concentrations of steroids and/or interleukins. Cells were then incubated for the indicated times with medium changes every 48 h. Aliquots of IL-4 and IL-6 were thawed within 2 h of every medium change. At the end of the incubation period, cell number was determined by measurement of DNA content as previously described [57].

Determination of enzymatic activity in cell homogenates and in intact cells

For measurement of 17β-HSD activity, ZR-75-1 or T-47D cells were seeded in T-75 flasks at a density of 3×10^6 cells in hormone-depleted medium, supplemented as described above. After 72 h, cells were incubated for 6 d with either 140 pmol of IL-4 or 145 pmol of IL-6, with medium change every 48 h. On day 6, cells were collected using 0.1% pancreatin, resuspended in reaction buffer A (100 mM KH_2PO_4; 20% glycerol; 1% EDTA; pH 7.4) and submitted to 3 cycles of sonication prior to rapid freezing at −80°C. Protein concentration was determined in parallel using a micro-Bradford assay. For the enzymatic activity assay, 50 to 100 μg of total protein was incubated in the presence of 10 nM of the indicated [^{3}H] steroids in the reaction buffer A. All incubations were performed at 37°C. For kinetic studies, enzymatic reactions were performed for 60 min at 37°C using 50 μg of total protein homogenate in the same buffer A, containing 1 mM NAD^+ as cofactor.

Determination of 3β-HSD activity in cell homogenates was performed using the same procedure but using the buffer B (50 mM NaH_2PO_4, 20% glycerol, and 1% EDTA (pH 7.4)). Ten μg of total protein were incubated at 37°C in the presence of 10 nM of either [^{3}H]-DHEA or [^{3}H]-PREG 1 mM NAD^+ as cofactor.

To determine 3β-HSD or 17β-HSD steroid metabolizing activity in intact cells, 10^4 to 2×10^4 cells were plated and treated as described above for cell proliferation assay. Cells were treated for 6 d, with

medium change on day 2 and day 4. After a 6 d incubation period, cells were then incubted for 16 h with 1 ml of fresh medium containing 10 nM of the indicated [^{3}H]-steroid. After the incubation period medium was collected and extracted as described.

The reaction was then stopped by adding 5 vol of diethyleter to the reaction mixture, and radiolabeled metabolites were characterized as previously described [13, 15, 32, 39, 40]. Organic fractions were then evaporated to dryness under a stream of nitrogen and dissolved in dichloromethane prior to separation by thin-layer chromatography (TLC) using toluene:acetone (4:1, vol/vol) as the mobile phase. TLC plates were then analyzed using a digital autoradiograph (Berthold, Germany). *Km* and *Vmax* values were calculated by the Lineweaver–Burk method using ENZFITTER software.

Calculations and statistical analysis

Dose-response curves were calculated using a weighted iterative nonlinear least-squares regression [60]. Statistical significance was measured according to the multiple-range test of Duncan–Kramer. All results were expressed as means ± SEM of triplicate dishes. When SEM overlaps with the symbol used, only the symbol is illustrated.

RESULTS

Effects of IL-4 and IL-6 on basal and E_2-induced cell proliferation

ZR-75-1 cells were exposed for 10 d to increasing concentrations of IL-4 or IL-6 in the presence or absence of a maximally effective concentration of E_2 (1 nM). As illustrated in Fig. 1, basal cell proliferation was decreased by 23% and 27% following an exposure to a maximally effective concentration of IL-4 and IL-6, respectively. However, IL-4 and IL-6 decreased the amplitude of the mitogenic effect of E_2 by 56% and 71% respectively, the half-maximal inhibitory action (IC_{50}) being observed at 88 ± 44 pM and 26 ± 10 pM.

Effect of IL-6 on oxidative and reductive 17β-HSD activities

We next investigated the effect of a 10 d exposure to increasing concentrations of IL-6 on oxidative (E_2 into E_1) and reductive (E_1 into E_2) 17β-HSD activities in ZR-75-1 cells. As shown in Fig. 2(A), incubation with IL-6 increased oxidative 17β-HSD activity by 1.9-fold, the stimulatory effect being exerted at an EC_{50} value of 11.3 ± 6.2 pM. Simultaneously, incubation with IL-6 caused a 40% decrease in the reductive 17β-HSD pathway, the inhibitory action being observed at an IC_{50} value of 2.2 ± 0.2 pM (Fig. 2(B)).

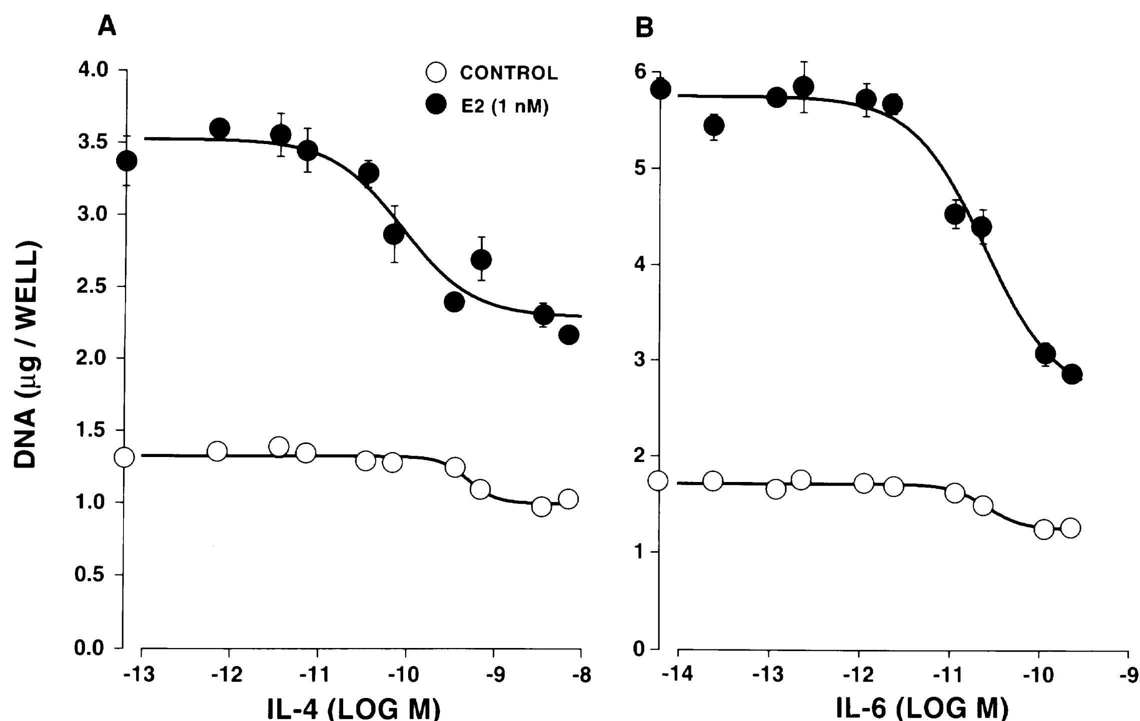

Fig. 1. Effect of increasing concentrations of IL-4 (A) and IL-6 (B) on ZR-75-1 human breast cancer cell proliferation. Three days after plating, cells were incubated for 10 d with the indicated concentrations of IL-4 or IL-6 in the presence or absence of 1 nM 17β-estradiol (E2). Media were changed every 2 d. At the end of incubation, cell proliferation was determined by measuring DNA content. Data are expressed as means ± SEM of triplicate dishes. When SEM overlaps with the symbol used, only the symbol is shown.

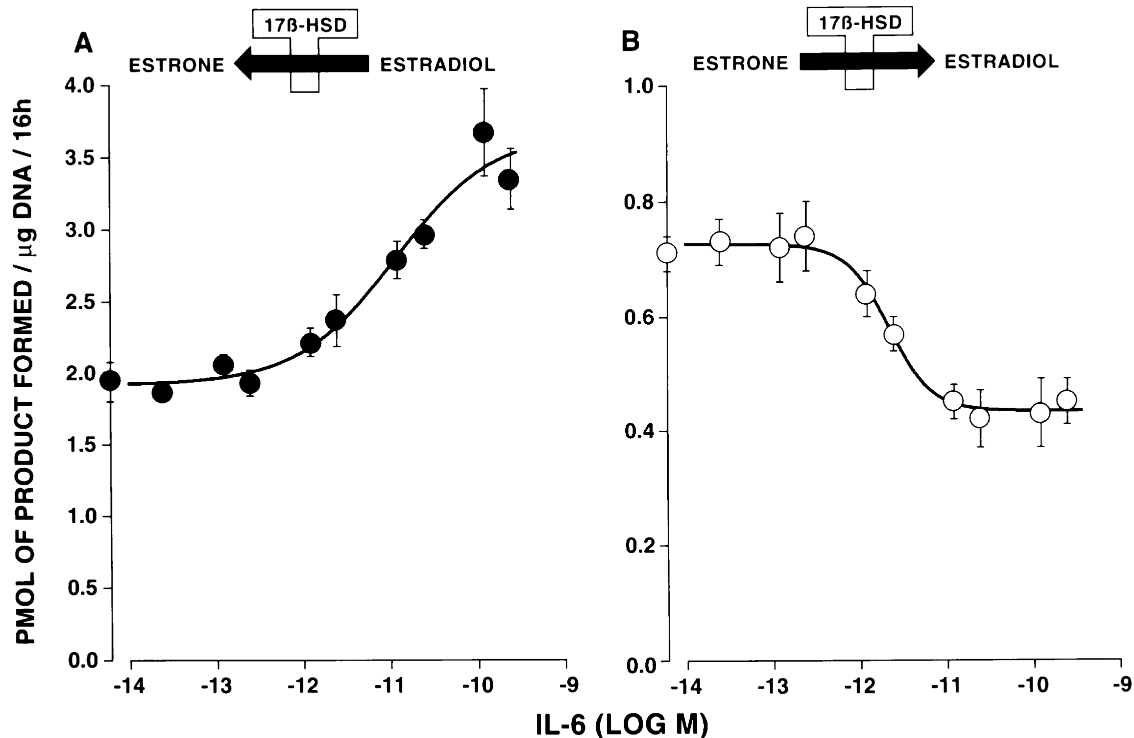

Fig. 2. Effect of increasing concentrations of IL-6 on the oxidative (A) and reductive (B) 17β-HSD activities in ZR-75-1 human breast cancer cells. Three days after plating, cells were incubated for 10 d with the indicated concentrations of IL-6, with changes of media every second day, prior to a 16 h incubation with 10 nM [^{3}H] E_2 (A) or [^{3}H3H] E_1 (B). Data are expressed as means ± SEM (n = 3).

Effect of IL-4 alone or in combination with IL-6 on 17β-HSD activity

We next studied the effect of a 10 d exposure to increasing concentrations of IL-4 in the presence or absence of a suboptimal effective concentration of IL-6 (75 pM). As illustrated in Fig. 3(A), exposure to IL-4 induced a maximal 4.4-fold increase in oxidative 17β-HSD activity, this simulatory effect being exerted at an EC_{50} value of 22.8 ± 4.8 pM. The combined effect of these two interleukins increased oxidative 17β-HSD activity by approximately 6.7-fold over basal values at an EC_{50} value of 13.8 ± 4.9 pM. In the same experiment, IL-4 induced a maximal 70% inhibition of reductive 17β-HSD activity at an IC_{50} value of 26.7 ± 3.2 pM. Furthermore, the inhibitory effect of IL-4 on the reductive 17β-HSD activity was also observed in the presence of IL-6, the inhibitory action being observed at an IC_{50} value of 19.6 ± 3.0 pM. These results thus indicate that IL-6 did not change the potency of IL-4 action on both oxidative or reductive 17β-HSD activities, but their effects are additive.

Comparison of IL-4 and IL-6 effects on 17β-HSD activities in ZR-75-1 and T-47D intact cells as well as in cell homogenates

ZR-75-1 and T-47D cells were incubated during 6 d with either 140 pM IL-4 or 145 pM IL-6 to compare their relative effects on both reductive and oxidative 17β-HSD activities. In ZR-75-1 cells, exposure to IL-4 or IL-6 decreased by 57% the reductive 17β-HSD activity, whereas such treatment increased by 1.7-fold and 1.9-fold the oxidative pathway (Fig. 4(A)). It can also be seen in Fig. 4(A), that when TESTO was used as substrate, IL-4 and IL-6 increased the oxidative 17β-HSD activity by 1.8-fold and 1.5-fold, respectively. However, it is of interest to note that the androgenic reductive 17β-HSD activity was almost undetectable in ZR-75-1 cells.

As shown in Panel B, when T-47D intact cells were incubated with these cytokines, a different enzymatic profile was observed. In contrast to the data obtained in ZR-75-1 cells, exposure to IL-4 increased by 3.8-fold the reductive 17β-HSD activity, whereas IL-6 failed to regulate this parameter. Moreover, there is no significant oxidative estrogenic 17β-HSD activity in T-47D cells. Neither is there any androgenic 17β-HSD activity in these cells, thus suggesting the absence of type II 17β-HSD expression.

Therafter, estrogenic and androgenic 17β-HSD activities were measured in cell homogenates prepared from ZR-75-1 and T-47D cells following a 6 d exposure to IL-4 or IL-6. As illustrated in Fig. 4(C), both oxidative (1.7-fold) and reductive (2.3-fold) estrogenic 17β-HSD activities were increased in IL-4-treated ZR-75-1 cells. It can also be seen in Fig. 4(C) that both the oxidative (1.4-fold) and reductive (2.4-fold) estrogenic 17β-HSD activities were increased in

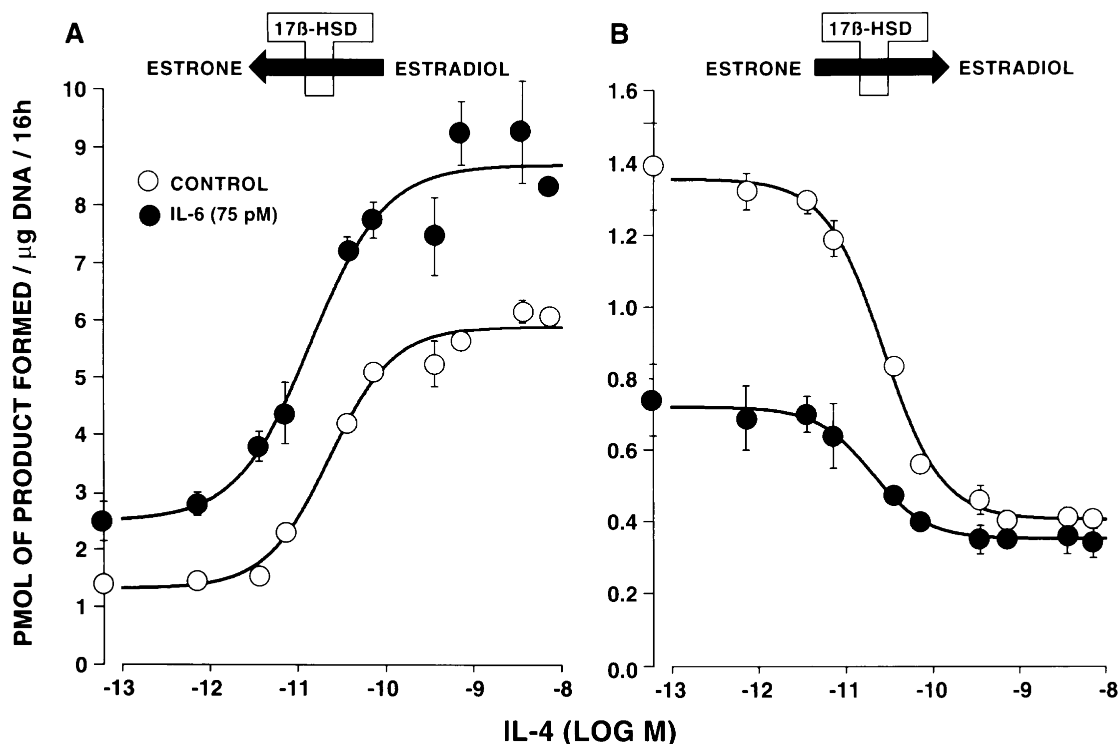

Fig. 3. Effect of increasing concentrations of IL-4 in the presence or absence of IL-6 on the oxidative (A) and reductive (B) 17β-HSD activities in ZR-75-1 human breast cancer cells. Three days after plating, cells were incubated for 10 d with the indicated concentrations of IL-4 in the presence or absence of 75 pM IL-6, with changes of media every 2 d, prior to a 16 h incubation with 10 nM [^{3}H] E_2 (A) or [^{3}H] E_1 (B). Data are expressed as means $\pm$ SEM (n = 3).

IL-6-treated ZR-75-1 cells. Closely similar data was obtained when using 4-DIONE or TESTO or as substrates.

Table 1 gives a summary of the kinetic properties of oxidative 17β-HSD activity using TESTO as substrate in cell homogenates prepared from ZR-75-1 cells incubated in the presence or absence of these cytokines. These results should be related almost exclusively to the activity of the type II 17β-HSD. IL-4 and IL-6 did not change significantly the affinity of 17β-HSD oxidative activity for TESTO as revealed by the lack of effect on the *Km* values while increasing the *Vmax* values by 4.5-fold and 2.7-fold, respectively.

In fact, in cell homogenates prepared from T-47D cells treated with IL-4, both reductive and oxidative 17β-HSD activities were increased by 3.3-fold and 2.5-fold, respectively (Fig. 4(D)). In support with the data obtained in T-47D intact cells, a treatment with IL-6 failed to modulate the 17β-HSD activity as measured in cell homogenates. It is noteworthy to mention that when 4-DIONE or TESTO were used as substrates, no 17β-HSD activity could be detected, even in the presence of an excess of cofactor, thus providing further evidence that type II 17β-HSD gene is not expressed in these cells.

Induction of 3β-HSD activity by IL-4 in T-47D and ZR-75-1 cells

Under basal growth conditions, no 3β-HSD activity can be detected in ZR-75-1 and T-47D cell lines. We first investigated the potential effect of a 6 d exposure to increasing concentrations of IL-4 on the 3β-HSD activity in T-47D intact cells. As illustrated in Fig. 5, exposure to IL-4 caused a potent induction of 3β-HSD activity, this marked stimulatory effect being observed at EC_{50} values of 130 $\pm$ 56 pM, 110 $\pm$ 57 pM, 78 $\pm$ 15 pM when using [^{3}H]-pregnenolone, [^{3}H]-DHEA or [^{3}H]-andros-5-ene-3β,17β-diol as substrates, respectively. Thereafter, we have measured the 3β-HSD activity in cell homogenates prepared from ZR-75-1 cells following 6 d of incubation in the presence or absence of IL-4 (140 pM) or IL-6 (145 pM), using either [^{3}H]-DHEA (A) or [^{3}H]-PREG (B) as substrate. As shown in Fig. 6, incubation with IL-4 induced 3β-HSD activity, whereas IL-6 exerted no effect on this parameter.

DISCUSSION

The present study shows for the first time that IL-4 and IL-6 regulate the 17β-HSD activity in ZR-75-1

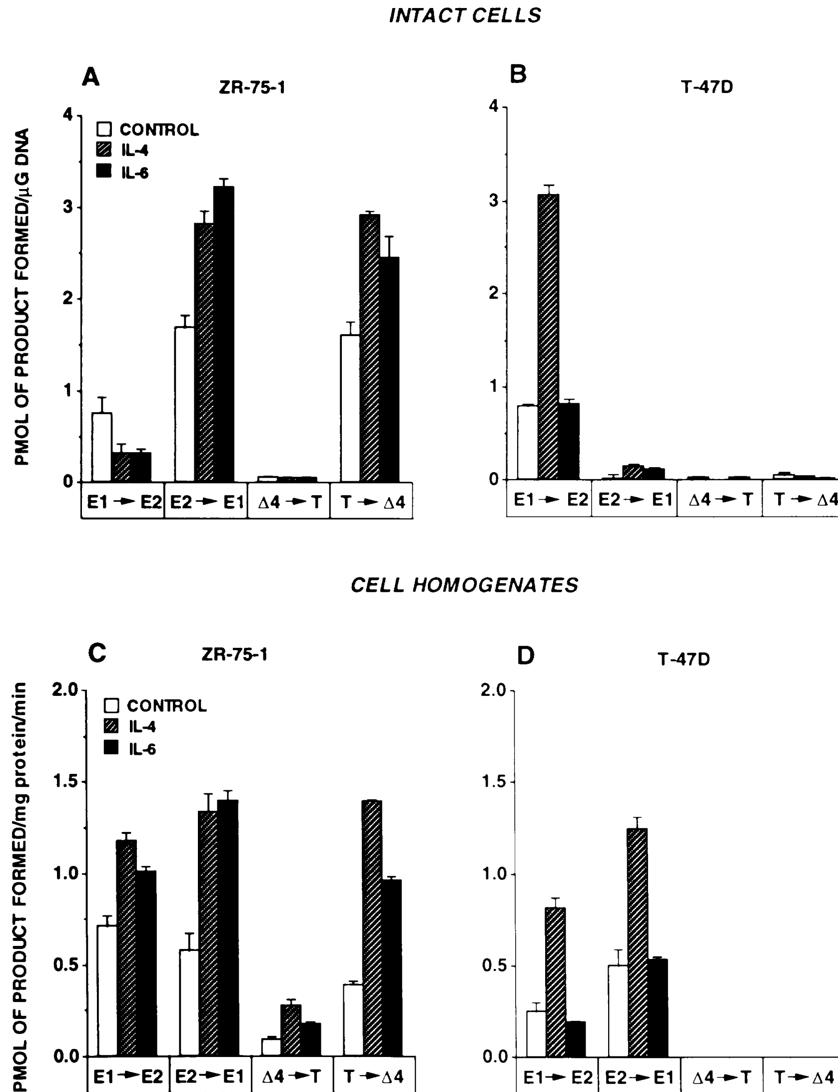

Fig. 4. Comparison of IL-4 and IL-6 effects on 17β-HSD activities in ZR-75-1 and T-47D intact cells as well as in cell homogenates. Three days after plating, cells were incubated for 6 d with either 140 pM of IL-4 or 145 pM of IL-6. ZR-75-1 (A) or T-47D (B) intact cells were then incubated for 16 h with 10 nM of the indicated [^{3}H]-steroids. Enzymatic assays in ZR-75-1 (C) or T-47D (D) cell homogenates were performed as described in Section 2 using 50 μg of total protein in the presence of 10 nM of the indicated [^{3}H]-steroids at 37°C, for 60 min. Data are expressed as means ± SEM (n = 3).

and T-47D human breast cancer cell lines, whereas IL-4, but not IL-6, may induce 3β-HSD activity in these two cell lines. In intact ZR-75-1 cells, both IL-4 and IL-6 increased the oxidative 17β-HSD activity while decreasing the reductive pathway. Such a regulatory effect in favor of the inactivation of active sex steroids was observed with both estrogenic and androgenic substrates. On the other hand, in T-47D cells, IL-6 failed to regulate the 17β-HSD activity, whereas IL-4 caused a marked increase in the reductive estrogenic 17β-HSD activity. The present study thus demonstrates that IL-4 and IL-6 may modulate not only the E_2-induced human breast cancer cell proliferation, but also the bioavailability of sex steroids in these cells by regulating their formation and/ or inactivation. This regulation being cell- and

Table 1. Kinetic properties of androgenic 17β-HSD activity in ZR-75-1 breast cancer cells

Treatment	K_m^{TESTO} (μM)	V_{max}^{TESTO} (nmol mg min)	Relative specificity V_{max}/K_m
Control	0.94 ± 0.19	0.040 ± 0.006	1.0
IL-4	0.92 ± 0.06	0.180 ± 0.074	4.6
IL-6	0.97 ± 0.08	0.109 ± 0.056	2.6

Enzymatic reactions were performed using 50 μg of total protein cell homogenate as described in Materials and Methods.

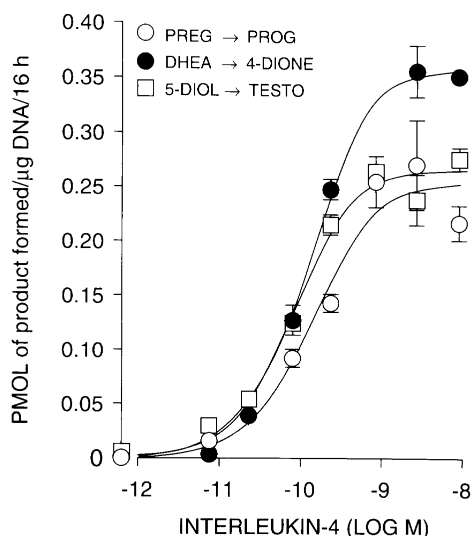

Fig. 5. Effect of IL-4 on 3β-HSD activity in breast cancer T-47D intact cells. Three days after plating, cells were incubated for 6 d with increasing concentrations of IL-4, and then incubated for 16 h with 10 nM of the indicated [^{3}H]-PREG, [^{3}H]-DHEA or [^{3}H]-5-DIOL. Data are expressed as means ± SEM (n = 3).

enzyme-specific. These cytokines may exert a finely tuned control on these enzymatic activities thus providing an additional mechanism by which the intracellular levels of estrogens and androgens is regulated in breast cancer cells.

The high potency of IL-4 and IL-6 to modulate 17β-HSD and 3β-HSD activities suggests that these effects are mediated through their respective high-affinity receptors, which possess dissociation constants (Kd) of 294 ± 54 pM in ZR-75-1 cells for the IL-4 receptor complex (IL-4R) and 10 pM for the IL-6 receptor complex (IL-6R) [61–63]. For IL-6, it appears that the intracellular signalling is mediated via an association of the unique IL-6 receptor α-chain and the signal transducer gp130, whereas IL-4 acts through an heteromultimeric receptor complex [63, 64]. The relevance of our data also pertains on the recent observation that gp130 and IL-6R are widely expressed in normal breast epithelial cells and ER$^+$ and ER$^-$ human breast cancer cell lines, whereas 96% and 80% of the 50 samples of malignant breast tissue tested expressed gp130 and IL-6R, respectively [56]. Moreover, in support with the biological role of IL-4 in normal and tumoral breast tissues, IL-4R-associated molecule gp220-MR6 is highly expressed in normal breast epithelial cells, while this protein is expressed at lower levels in malignant breast tissues [65, 66]. These authors suggested that gp200-MR6 molecule may be a useful marker in tumor grading and that the down-regulation of its expression may be one of the mechanisms through which breast cancer cells escape immune surveillance and may represent loss of a IL-4-mediated tumor suppression mechanism [65, 66]. In fact, the relevance of the potential action of IL-4 and IL-6 in normal and tumoral breast tissues is also supported by the findings that NK cells isolated directly from breast tumor site secrete more IL-4 than NK cells from peripheral blood of the same patients [46], the stimulation of IL-4 secretion by NK cells derived from healthy donors after exposure to conditioned medium by MCF-7 cells [46], and the detection IL-6 in breast cyst fluid and its secretion by breast tumor-derived fibroblasts, macrophages and breast tumor-infiltrating lymphocytes [67].

Our study also demonstrates that the IL-4 responsiveness of ZR-75-1 cells on the modulation of 17β-HSD activity is not significantly affected by a simultaneous exposure to IL-6, but their effects are rather

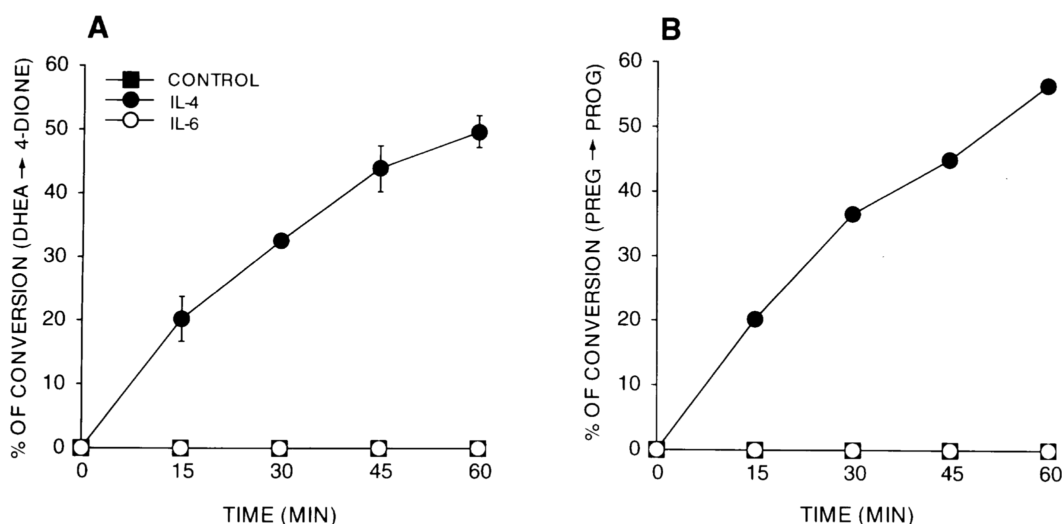

Fig. 6. IL-4 but not IL-6 induced 3β-HSD activity in ZR-75-1 cells After a 6 d incubation with either 140 pM of IL-4 or 145 pM of IL-6, cells were collected. Thereafter, 10 μg of total protein from cell homogenates were used for incubation in the presence of 10 nM of [^{3}H]-DHEA (A) or [^{3}H]-PREG (B) at 37°C, for 60 min. Results are presented in% of conversion of total free steroids. Data are expressed as means ± SEM (n = 3).

additive. This observation is in agreement with the current knowledge that the two cytokines exert their effects through different JAK/STAT transduction pathways [63, 64, 69, 70]. Indeed, IL-4 activates the STAT6, a member of a family of DNA-binding proteins designated Signal Transducers and Activators of Transcription, whereas the action of IL-6 is mainly mediated through an activation of STAT1 and STAT3. Recently, it has been suggested that STAT1 and possibly other members of the STAT family of signalling factors, including STAT3, are activated in breast cancer tissue [70].

In ZR-75-1 cells, it is tempting to suggest that the potent inhibitory effect of IL-4 and IL-6 on the E_2-induced cell proliferation (Fig. 1, [54, 55]) might be explained, at least in part, by their stimulatory effect on the oxidative 17β-HSD activity and the corresponding decrease in the reductive counterpart pathway. On the other hand, the situation found in MCF-7 cells differs markedly. It has been first reported that IL-6, which is present in conditioned medium from cultured breast tumor-derived fibroblasts, is one of the factors responsible for the ability of this medium to stimulate the estrogenic reductive 17β-HSD activity in MCF-7 cells [41, 42, 58]. Indeed, human breast fibroblasts can secrete high levels of IL-6 (10 μM) when being enriched from human breast tissue by 2–3 passages *in vitro* and thereafter incubated for 24 h with serum-free medium [58]. However, the IL-6 action on reductive 17β-HSD activity was not confirmed in another study, thus suggesting that other factors may also be present in conditioned medium and able to potentiate the stimulatory effect of IL-6 [68, 59]. In this regard, IL-6 increased the stimulatory effect of IL-1β and TNF-α on the estrogenic reductive 17β-HSD activity [59]. However, the absence of effect of IL-4 on 17β-HSD activity in MCF-7 cells (data not shown), whereas this cytokine has been reported to inhibit E_2-induced MCF-7 cell proliferation [71], is most likely due to the lack STAT6 expression [72], which factor is not believed to be required for IL-4-mediated mitogenesis [65, 72]. In fact, in T-47D cells IL-6 failed to modulate 17β-HSD activity, whereas IL-4 exerted a marked increase in the estrogenic reductive 17β-HSD activity. These findings taken together clearly indicate the heterogeneous pattern of IL-4 and IL-6 responsiveness on this parameter among ER+ breast cancer cell lines.

The data obtained in ZR-75-1 intact cells showing that both IL-4 and IL-6 increase the oxidative 17β-HSD activty when using E_2 or TESTO as substrates strongly suggest that these cytokine increase the type II 17β-HSD expression. This conclusion is based on the following observations: (i) the type II 17β-HSD is the only known enzyme being able to catalyze the conversion of TESTO into 4-DIONE in intact cells [14]; (ii) because of the much higher bioavailability of NAD^+ in comparison to NADH in intact cells, this enzyme is primarily responsible for the oxidative pathway, thus being involved in the inactivation of E_2 and TESTO [14]; (iii) when cell homogenates prepared from cells expressing the recombinant type II 17β-HSD enzyme are incubated with either estrogenic or androgenic substrates, in the presence of an excess of NAD^+ or NADH, the rate of the oxidative pathway is higher than that observed for the reductive reaction [14, 31]; (iv) exposure to IL-4 or IL-6 failed to affect the affinity of the enzyme for TESTO, but rather increased the rate of the enzymatic reaction, thus leading to higher relative specificity of the enzyme. However, the decrease observed in the conversion of E_1 into E_2, which is primarily catalyzed by the type I 17β-HSD isoenzyme, rather suggests that these cytokines reduced the expression of this latter isoenzyme. In fact, it cannot be rule out that a part of the effect of IL-4 and IL-6 on the estrogenic oxidative 17β-HSD activity results from a regulation of the type IV 17β-HSD activity, which is almost exclusively responsible for the conversion E_2 into E_1 while having no affinity for TESTO [35, 36].

Our findings in T-47D cells strongly suggest that IL-4 exerts a stimulatory effect on the type I 17β-HSD expression. This hypothesis is well supported by (i) its recognized predominant role in the conversion of E_1 into E_2 in intact cells [14], (ii) its lack of affinity for C19 substrates [14], and (iii) its reported gene expression in T-47D cells [18, 29, 30]. Moreover, the absence of significant 17β-HSD activity when using androgenic substrates strongly suggests that type II 17β-HSD is not expressed in these cells.

Finally, we have demonstrated that in contrast to IL-6, IL-4 induces 3β-HSD activity in both ZR-75-1 and T-47D cells. Our study thus shows for the first time that this enzymatic activity may be induced by an interleukin. Knowing that the aromatase is expressed in a large proportion of human breast carcinoma [28, 67], such a marked increase in the formation of its substrates 4-DIONE and TESTO, may well have a significant impact on the estrogen synthesis in breast tumors. However, it should be taken into consideration that androgens are well recognized to exert an antiproliferative action in breast cancer cells [53, 54, 73]. Thus, the IL-4-induced 3β-HSD activity would increase the intracellular levels of TESTO which in return would inhibit breast cancer cell growth. In support with this hypothesis DHEA exert a inhibitory effect on the development of ZR-75-1 human breast cancer cell xenografts in nude mice [74]. Further studies are in progress to determine the molecular mechanisms underlying the induction of 3β-HSD activty by IL-4 and to investigate if this cytokine may exert such stimulatory action in other normal and tumoral cell types.

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