Pergamon

0960-0760(95)00087-9

J. Steroid Biochem. Molec. Biol. Vol. 53, No. 1–6, pp. 413–420, 1995

0960-0760/95 $9.50 + 0.00

The Role of Cytokines and Sulphatase Inhibitors in Regulating Oestrogen Synthesis in Breast Tumours

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Synthesis of oestrogens within breast tissues makes an important contribution to the high concentrations of oestradiol which are found in breast tumours. The activities of the enzymes involved in oestrogen synthesis, i.e. the aromatase, oestradiol dehydrogenase (E2DH) and oestrone sulphatase (E1-STS), can be stimulated by several growth factors and cytokines. As it is possible that some of these factors may be derived from cells of the immune system (macrophages and lymphocytes), the effects of basic fibroblast growth factor (bFGF) and interleukin-2 (IL-2), which are produced by these cells, on E2DH activity was examined in MCF-7 cells. Treatment of these cells with bFGF resulted in a dose-dependent increase in E2DH reductive activity whereas IL-2 was inactive at the concentration tested. To obtain further evidence that factors produced by macrophages and lymphocytes can modulate the activities of enzymes involved in oestrogen synthesis, conditioned medium was collected from these cells and found to stimulate both E1-STS and E2DH activities. In addition to understanding the control of oestrogen synthesis in breast tumours an inhibitor to block the synthesis of oestrone via the oestrone sulphatase pathway was developed. Oestrone-3-*O*-sulphamate (EMATE) is a potent, irreversible, inhibitor of E1-STS. A single dose of EMATE (10 mg/kg) inhibited tissue E1-STS activity in rats by more than 95% for up to 7 days, indicating that this compound may have considerable therapeutic potential for the treatment of breast cancer. Evidence is also reviewed that another steroid sulphatase, dehydroepiandrosterone sulphate sulphatase, may have a crucial role in regulating cytokine production and that this may indirectly control tumour oestrogen synthesis.

J. Steroid Biochem. Molec. Biol., Vol. 53, No. 1–6, pp. 413–420, 1995

INTRODUCTION

The high concentrations of the biologically active oestrogen, oestradiol, which are found in breast tumours has stimulated research into both the regulation of the enzymes involved in oestrogen synthesis in breast tumours and the development of potent inhibitors to block oestrogen synthesis. Three main enzyme complexes are involved in oestrogen synthesis in breast tumours, the aromatase which converts androstenedione to oestrone, oestradiol dehydrogenase (E2DH), which in breast tumours preferentially transforms oestrone to the biologically active oestrogen, oestradiol, and oestrone sulphatase (E1-STS) which hydrolyses the large amounts of oestrone sulphate (E1S) formed from oestrone, back to oestrone [1].

During the last few years, evidence has been obtained indicating that breast tumours produce factors which are able to enhance the activities of the enzymes involved in oestrogen synthesis, to promote an oestrogenic environment favouring tumour growth [2]. The first evidence suggesting that tumours might influence enzyme activity was the finding of a correlation between tumour size and E2DH activity in tissues adjacent to tumours [3]. Subsequently, breast tumours were shown to preferentially reduce oestrone to the

Proceedings of the IX International Congress on Hormonal Steroids, Dallas, Texas, U.S.A., 24–29 September 1994.
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biologically active oestrogen, oestradiol [4]. This contrasts with E2DH activity in other endocrine dependent tissues such as the endometrium, where oestradiol is inactivated by conversion to oestrone [5]. Tumour location also influences aromatase activity in breast tissue quadrants bearing a tumour [6] and there is some recent evidence that tumour location may also influence E1-STS activity [7]. These findings resulted in an intensive search to identify factors which can stimulate the activities of enzymes involved in oestrogen synthesis in breast cancer cells and breast tumours. Several such factors have now been identified including the growth factors insulin-like growth factor-type I (IGF-I) and IGF-II, the cytokines interleukin-1β (IL-1β), IL-6 and tumour necrosis factor α (TNFα), and an albuminoid-like molecule [8–12]. Although several factors, and in particular cytokines, have now been shown to stimulate the activities of these enzymes, as will be discussed later, it is only now possible to begin to understand the complex role that cytokines may have in regulating the functioning of these enzymes.

With regards to the development of enzyme inhibitors for use in the treatment of breast cancer, so far most effort has been directed towards identifying aromatase inhibitors. This has resulted in the synthesis of a number of steroidal and non-steroidal inhibitors, some of which are currently undergoing clinical evaluation. However, although all oestrone is formed via the aromatase pathway, much of the oestrone synthesized is converted to oestrone sulphate which can act as a large reservoir for the formation of oestrone by the action of E1-STS [13]. The finding that blood oestrogen concentrations only decrease by about 50% during treatment with the aromatase inhibitor, 4-hydroxyandrostenedione, prompted the development of an E1-STS inhibitor [14]. It was reasoned that the continued presence of oestrone and oestradiol in the plasma of women, in whom aromatase activity was effectively inhibited, might result from the hydrolysis of the relatively high E1S concentrations which remain in blood [13]. A number of E1-STS inhibitors have now been synthesized, some of which are active *in vivo*.

In this paper recent progress in identifying growth factors and cytokines which can regulate oestrogen synthesis in breast tumours is reviewed. As it now seems possible that some of the cytokines involved in regulating enzyme activity in breast tumours may originate from cells of the immune system (i.e. macrophages and lymphocytes) the effects on enzyme activity of members of the fibroblast growth factor family and IL-2, which are secreted by these cells, were examined [15]. Recent advances in developing potent inhibitors of E1-STS are also reviewed. Finally, the possibility is considered that a related sulphatase, dehydroepiandrosterone sulphatase (DHA-STS), may have a crucial role in regulating the production of cytokines, and hence the synthesis of oestrogens in breast tumours.

MATERIALS AND METHODS

Regulation of oestrogen synthesis by cytokines and growth factors

Cell culture. The effects of cytokines and growth factors on E2DH reductive activity (i.e. oestrone to oestradiol conversion) was examined using intact monolayers of MCF-7 breast cancer cells. These cells were cultured in Minimum Essential Medium, Eagle modified with Earle's salts and 20 mM Hepes buffer (MEM). Medium was supplemented with essential nutrients and 5% (v/v) foetal calf serum. Cells were grown in this medium until they were 40–50% confluent when treatments were added in phenol-red free medium (MEM-AUTOPOW) containing 5% (v/v) charcoal stripped foetal calf serum. IL-2, acidic FGF (aFGF) and basic FGF (bFGF) were obtained from Bachem, Saffron Walden, Essex, U.K.

E2DH assay. Intact monolayers of MCF-7 cells were washed with phosphate buffered saline (PBS) and incubated with 2 nM [^{3}H]oestrone (80–100 Ci/mmol, Amersham International, Amersham, Bucks, U.K.) [12].

Preparation of macrophage and lymphocyte conditioned medium. To examine possible effects of cytokines produced by cells of the immune system on enzyme activity, conditioned medium (CM) was prepared from macrophages and lymphocytes. Blood (30 ml) was taken from normal male and female subjects and plasma containing the white blood cells obtained by the addition of 2% dextran in saline and centrifugation. After the addition of 0.83% ammonium chloride to remove contaminating red blood cells, the cells were pelleted by centrifugation, resuspended in phenol-red free MEM (10 ml) and transferred to a tissue culture flask. Cells were incubated at 37°C for 4 h, during which time some cells (mainly macrophages) adhered to the flask allowing separation from the non-adherent (mainly lymphocyte) cells. Conditioned medium was collected from LPS (10 μg/ml)-stimulated adherent and non-adherent cells over a 48 h period.

E1-STS activity. The effect of macrophage and lymphocyte CM on E1-STS activity was examined using MCF-7 cells. Intact cell monolayers were incubated for 20 h at 37°C with 2 nM [^{3}H]E1S (4×10^5 d.p.m., N.E.N. Du-pont, U.K.) in serum-free medium [16].

Inhibition of E1-STS activity

Oestrone-3-*O*-sulphamate (EMATE) has emerged as the lead compound in the development of a potent E1-STS inhibitor [17] (Fig. 1). Investigations were carried out *in vivo* to determine the extent and duration of inhibition of E1-STS activity by EMATE in rats. For this, EMATE was administered to rats (250–300 g) by either the oral or subcutaneous routes. Tissues, including liver, ovaries, brain, adrenals and uterus were obtained and homogenized in PBS (pH 7.4, 50 mM)

and nuclei and cell debris removed by centrifugation (4°C, 2000 *g*, 20 min). The protein concentration of the resulting supernatant was measured and aliquots used for the assay of E1-STS activity [18]. Briefly, [^{3}H]E1S was adjusted to 20 μm with unlabelled substrate (Sigma, U.K.) and after incubation for 30 min, the product formed was isolated by toluene extraction, using [4-^{14}C]oestrone (7×10^3 d.p.m., Amersham International, U.K.) to monitor procedural losses.

RESULTS AND DISCUSSION

Regulation of oestrogen synthesis by cytokines and growth factors

The possibility that some of the cytokines and growth factors which have been found to stimulate the activities of enzymes involved in oestrogen synthesis may be derived from cells of the immune system led to an investigation of the effects of members of the FGF family on E2DH activity. There are several members of the FGF family which can interact with a number of different FGF-receptors. Basic FGF lacks a secretion signal peptide and is not normally exported by cells, but has recently been shown to be secreted by T lymphocytes [15]. Since up to 50% of a breast tumour can be composed of macrophages and lymphocytes [19], it is likely that the growth factors and cytokines which can be secreted by these cells may have an important role in regulating breast tumour oestrogen synthesis.

The effect of aFGF, which is not produced by macrophages or lymphocytes, was initially examined for its effect on E2DH activity. When tested in the range of 1–500 ng/ml it did produce a small, but significant, increase in E2DH reductive activity at the higher concentrations tested (Fig. 2). It has been reported previously [8, 20] that the effects of growth factors can be potentiated by human serum albumin (HSA). Preliminary evidence was obtained that the stimulatory effect of aFGF could also be potentiated by HSA (Fig. 2). Over the 48 h period for which cells were treated with aFGF no effect on the growth of MCF-7 cells was detected.

In contrast to the small effect of aFGF, treatment of

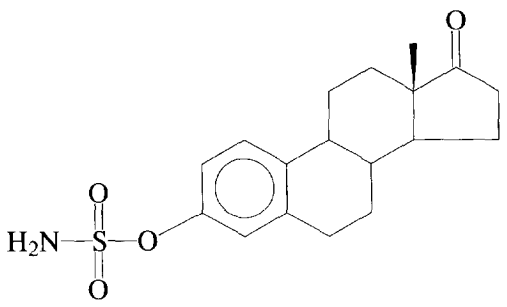

Fig. 1. Structure of oestrone sulphatase inhibitor, oestrone-3-*O*-sulphamate.

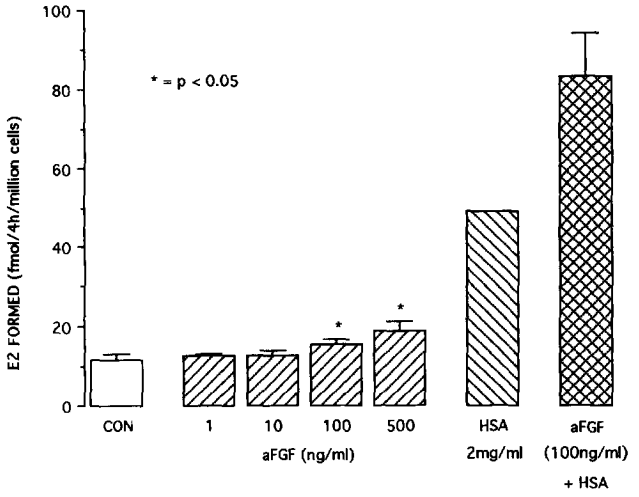

Fig. 2. Effect of acidic fibroblast growth factor (aFGF) on E2DH reductive activity in MCF-7 breast cancer cells. The effect of human serum albumin (HSA) alone or in combination with aFGF on E2DH activity is also shown.

cells with bFGF, which is secreted by cells of the immune system, resulted in a marked, dose-dependent stimulation of E2DH activity [Fig. 3(A)]. The stimulatory effect of bFGF was similar to that of IGF-I [also shown in Fig. 3(A)] which has previously been found to stimulate E2DH activity [8]. Some indication was also obtained that the effect of bFGF on E2DH activity could also be potentiated by HSA [Fig. 3(A)]. In this experiment MCF-7 cells were treated with IGF-I or bFGF for 48 h, but even at the highest concentrations tested, IGF-I had little effect on cell growth whereas bFGF significantly reduced growth at some of the concentrations tested [Fig. 3(B)].

Basic FGF, which is produced by macrophages and T-lymphocytes, is therefore another growth factor which can modulate the activity of the enzymes involved in oestrogen synthesis in breast cancer cells. This growth factor also stimulates E1-STS activity in MCF-7 cells [21]. Additionally, experiments were carried out to examine the effect of another lymphocyte-derived cytokine, IL-2, on E2DH activity. When tested alone at 20 ng/ml it had little effect on E2DH reductive activity but when used in combination with TNFα (20 ng/ml) a small, but significant, increase in activity was detected (Fig. 4).

In order to obtain more direct evidence that cytokines and growth factors produced by cells of the immune system may have a role in regulating breast tumour oestrogen synthesis, a series of experiments were carried out to examine the effect of CM collected from these cells on enzyme activity. A representative result showing the effect of macrophage and lymphocyte CM on E1-STS activity is illustrated in Fig. 5. CM from both adherent (mainly macrophages) and non-adherent (mainly lymphocytes) cells stimulated E1-STS activity.

At the dilution of CM used (1–10% v/v) there was little effect on cell growth. CMs from adherent and non-adherent cells also significantly stimulated E2DH reductive activity but had no effect on oxidative activity (Fig. 6). While further research is in progress to isolate and characterize the growth factors and cytokines produced by macrophages and lymphocytes, the results obtained, so far, do support the hypothesis that the secretion of such factors by these cells within breast tumours may have an important effect on tumour oestrogen synthesis.

Inhibition of E1-STS activity

The importance of the oestrone sulphatase pathway as a source of oestrogen to support tumour growth [22] and the failure of aromatase inhibitors to completely reduce blood oestrogen concentrations stimulated research to find a potent E1-STS inhibitor. Several E1-STS inhibitors have now been developed including a number of indole derivatives [23], oestrone-3-sulphonyl chloride [24] and oestrone-3-methylthiophosphonate [18]. However, for these compounds concentrations of 10 μM or greater were required to achieve significant inhibition. Recently a series of steroid sulphamate derivatives were synthesized and of these oestrone-3-*O*-sulphamate (EMATE, Fig. 1) was most potent [17]. EMATE inhibits E1-STS in a concentration- and time-dependent manner, i.e. acts as an irreversible inhibitor [17].

Initial studies confirmed that EMATE is active *in vivo* in rats when administered by the oral or subcutaneous routes [25]. As EMATE is an irreversible inhibitor of E1-STS, the extent and duration of its ability to inhibit enzyme activity *in vivo* was examined. For this EMATE (10 mg/kg, s.c.) was administered to

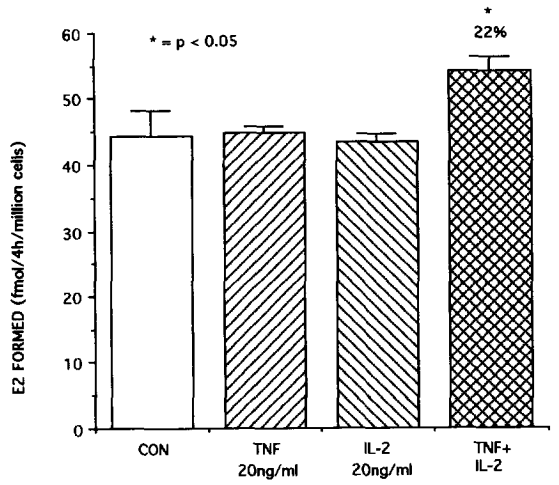

Fig. 4. Effect of interleukin-2 (IL-2) and transforming growth factor α(TGFα) alone, or in combination, on E2DH reductive activity in MCF-7 breast cancer cells.

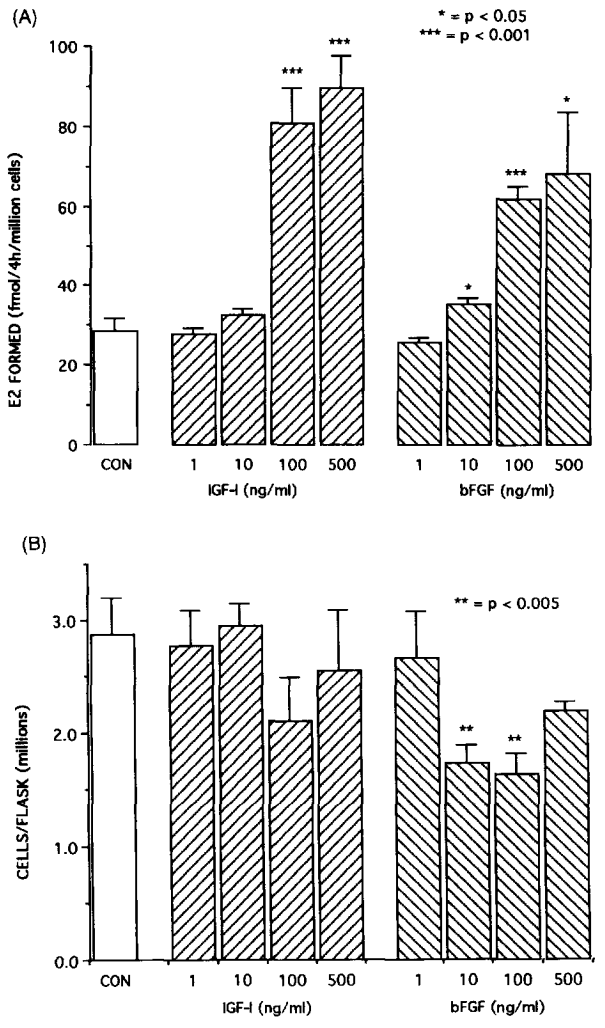

Fig. 3. Effect of insulin-like growth factor-type I (IGF-I) and basic fibroblast growth factor (bFGF) on E2DH reductive activity in MCF-7 breast cancer cells (A). The effect of human serum albumin (HSA) alone or in combination with bFGF is also shown (B).

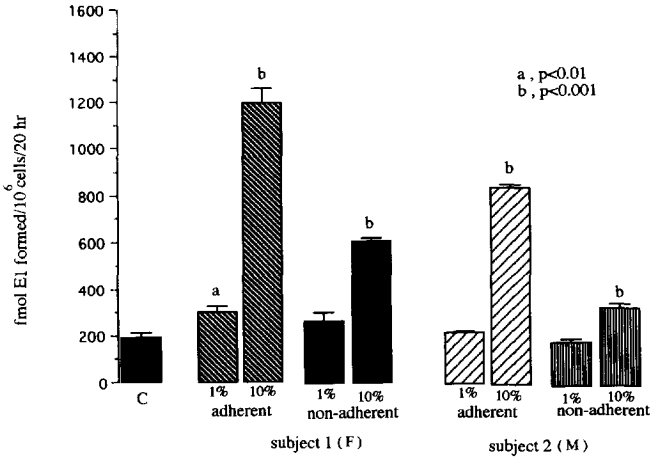

Fig. 5. Effect of conditioned medium collected from adherent (mainly macrophages) and non-adherent (mainly lymphocytes) cells on oestrone sulphatase activity in MCF-7 breast cancer cells. Adherent and non-adherent cells were prepared from blood samples obtained from female (F) and male (M) subjects.

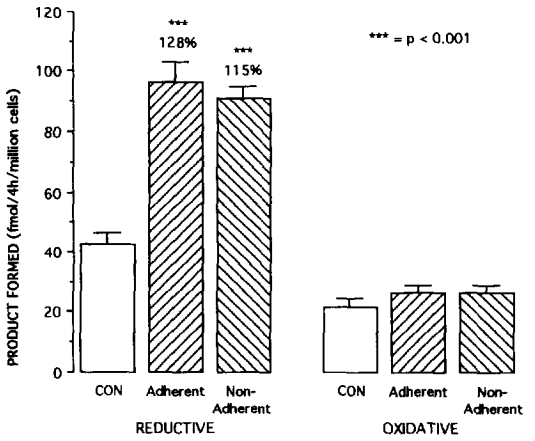

Fig. 6. Effect of conditioned medium (10% v/v) collected from adherent (mainly macrophages) and non-adherent (mainly lymphocytes) cells on E2DH reductive and oxidative activities in MCF-7 breast cancer cells.

rats for 10 days after which groups of animals were killed (using an approved procedure) 1, 5, 10 and 15 days after the last injection. As shown in Table 1, inhibition of E1-STS activity was still greater than 90% 10 days after administration of the last dose of EMATE. Even after 15 days, liver E1-STS activity had only recovered by about 10% with slightly higher recoveries occurring in uterine, ovarian and adrenal tissues.

To further examine the duration of inhibition of E1-STS achieved by EMATE, rats were injected with a single dose (10 mg/kg, s.c.) of the inhibitor. E1-STS activity, in several tissues examined was still inhibited by more than 95% 1 week after injection of a single dose. Further studies are in progress to determine the full length of time for which a single dose of EMATE can inhibit E1-STS activity. It is already apparent that EMATE is a potent inhibitor with a long duration of action. These results suggest that EMATE will only need to be administered on a once or twice weekly basis to achieve complete inhibition of E1-STS when used for the treatment of breast cancer.

Table 1. *Inhibition of oestrone sulphatase activity by oestrone 3-O-sulphamate*

	Oestrone sulphatase activity (% inhibition compared with controls)			
Tissue	Day 1	Day 5	Day 10	Day 15
Liver	99.9 ± 0.1	99.8 ± 0.1	99.3 ± 0.1	87.0 ± 2.75
Brain	98.6 ± 0.5	98.0 ± 0.5	97.2 ± 1.4	78.8 ± 8.1
Adrenal	99.6 ± 0.1	99.1 ± 0.2	98.5 ± 0.5	78.3 ± 8.6
Ovary	98.1 ± 0.6	98.6 ± 0.2	98.8 ± 2.7	69.8 ± 12.1
Uterus	99.5 ± 0.1	99.1 ± 0.3	98.1 ± 0.9	70.8 ± 6.1

The inhibitor (10 mg/kg, s.c.) was injected daily for 10 days and oestrone sulphatase measured in tissues 1, 5, 10 and 15 days after the last injection (mean ± SD, $n = 3$).

The role of steroid sulphatase in regulating cytokine and breast tumour oestrogen synthesis

As previously discussed the findings that breast tumours influenced aromatase and E2DH activities in adjacent tissues resulted in the isolation and identification of a number of cytokines and growth factors which can stimulate the activities of these enzymes. In particular IL-6 appears to have an important role in regulating aromatase activity [9, 26] and possibly in association with TNFα, in regulating E2DH activity [11, 12]. Natural and synthetic glucocorticoids have previously been implicated in regulating aromatase activity [27, 28], but, while dexamethasone is a potent inducer of *in vitro* aromatase activity, it is without effect *in vivo* [29, 30].

When MacDonald and Siiteri first demonstrated the presence of peripheral aromatase activity they made the important observation that activity was positively correlated with age [31]. While the reason for the increase in aromatase activity which is detected in older subjects is not yet clear, it is relevant that concentrations of IL-6 are higher in serum from elderly subjects and show a positive correlation with age [32, 33]. Serum IL-6 concentrations are also increased in aged mice [33]. The knowledge that dehydroepiandrosterone sulphate (DHA-S) blood concentrations decrease with ageing recently led Daynes and his colleagues to examine the effect of treating aged mice with DHA-S. These investigations revealed that the elevated serum IL-6 concentrations, which are found in aged mice, could be corrected by chronic or acute administration of DHA or DHA-S [33]. *In vitro*, DHA, but not DHA-S, could suppress the release of specific cytokines from cultured mononuclear cells [34]. As most DHA is present in blood as the sulphate conjugate, this finding suggests that DHA-sulphatase (DHA-STS) may have a key role in regulating the immune response.

Many of the cytokines produced within the body are secreted by T-lymphocytes. While T cells are genetically programmed to produce a wide range of cytokines it is now evident that there are two types of T cells, Th1 and Th2, each of which secretes a characteristic set of cytokines [35]. The cytokine, IL-2, for example, is secreted exclusively by Th1 cells, while IL-6 is secreted by Th2 cells. IL-6 is also produced by other cells within the body (e.g. macrophages and fibroblasts) but little is known about the control of its secretion by these cells.

The microenvironment within which T cells reside, i.e. mainly lymphoid tissue, but also possibly breast tumours, is a major factor in determining whether T cells progress to the Th1 or Th2 type [36]. *In vivo* DHA-S can be converted to DHA in lymphoid (and breast tumour) tissues [36]. If sufficient DHA-S, and DHA-STS activity, are present then T cells progress to Th1 cells and secrete a characteristic cytokine profile

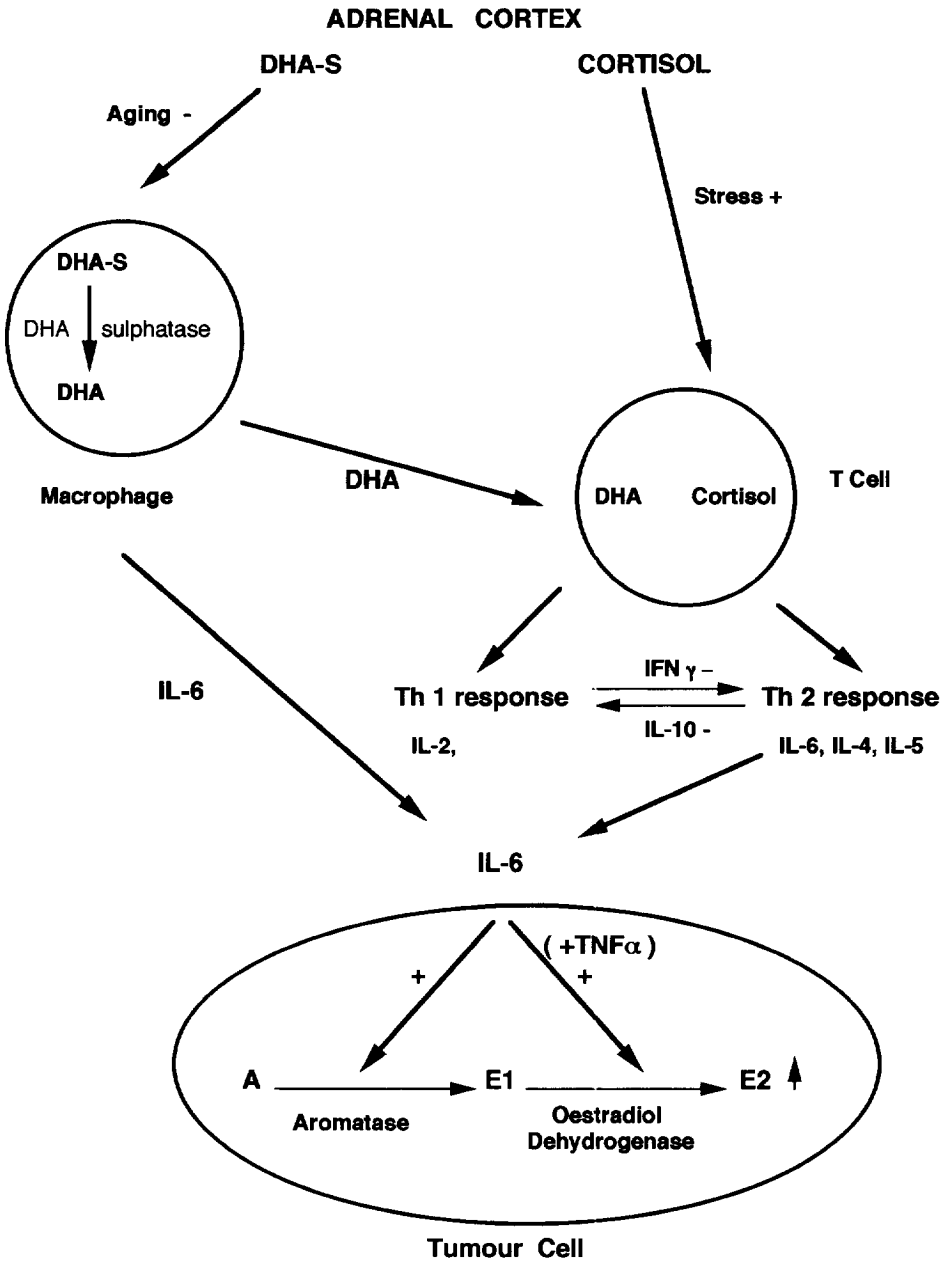

Fig. 7. Summary of possible mechanism by which dehydroepiandrosterone sulphate (DHA-S) and DHA-S-sulphatase may regulate T cell and macrophage cytokine production and indirectly regulate breast tumour oestrogen synthesis.

including IL-2 [37]. However, in the absence of sufficient DHA-S (or DHA-STS activity) glucocorticoids have an important role in allowing T cells to progress to the Th2 cell type, inducing the secretion of a different cytokine profile, which includes IL-6. It is becoming evident, therefore that DHA-STS in lymphoid tissue has a crucial role in determining which cytokines are secreted by T cells. As DHA-STS is also present in most breast tumours, and up to 50% of tumour volume may be composed of macrophages and lymphocytes, it is likely that local secretion of cytokines from these cells may be an important factor in regulating oestrogen synthesis in breast tumours. Although the role of IL-6 in regulating aromatase and E2DH activities has been extensively studied, it is likely that some of the other cytokines produced by Th1 and Th2 cells or macrophages may also have an important role in regulating oestrogenic enzyme activity. Further support for the role of different cytokines secreted by Th1 or Th2 cells is provided by the result obtained in the present study, showing that IL-2, a Th1 derived cytokine, has no effect on E2DH reductive activity in MCF-7 breast cancer cells. A summary of the potential role of cytokines produced by T cells and macrophages in regulating oestrogenic enzyme activity in breast tumours is shown in Fig. 7.

The regulation of peripheral and breast tumour oestrogen synthesis by IL-6

As previously discussed the correlation found between serum IL-6 concentrations and age may account for the increase in aromatase activity which is found in older subjects. As macrophages and lymphocytes congregate around and within breast tumours, the local release of IL-6 from these cells could account for the influence of tumour location on aromatase and E2DH activities in adjacent normal breast tissues. If IL-6 is the major factor involved in regulating *in vivo* aromatase activity then this might also explain the discrepancy, as previously noted, in the ability of glucocorticoids to stimulate *in vitro*, but not *in vivo*, aromatase activity. Dexamethasone can only induce aromatase activity *in vitro*, in the presence of foetal calf serum. As dexamethasone induces IL-6 receptor (IL-6-R) expression, it is possible that this glucocorticoid acts *in vitro* to increase the number of IL-6-receptors on cells [38], thereby increasing the ability of IL-6, which may be present in foetal calf serum, to stimulate aromatase activity. However, while glucocorticoids act to increase IL-6-R expression, they act *in vivo* to inhibit IL-6 gene expression [39], possibly as part of a feedback mechanism to limit the response to stress and infection. Inhibition of IL-6 gene expression by glucocorticoids *in vivo* could therefore account for their inability to stimulate *in vivo* aromatase activity.

CONCLUSIONS

The search for factors which are able to stimulate the activities of enzymes involved in oestrogen synthesis in breast cancer cells has now identified a number of growth factors and cytokines which markedly enhance enzyme activity. As reviewed in this paper DHA-STS in lymphoid (and possibly breast tumour) tissue has an important role in regulating cytokine secretion by T cells (and possibly macrophages) and this may be a key step in regulating oestrogen synthesis in breast tumours. Almost 30 years ago it was found that the excretion of DHA and its metabolites was reduced in women who developed breast cancer [40]. DHA has a well-documented anti-cancer effect in a number of animal models [41, 42]. It is tempting to speculate that DHA acts to enhance the progression of T to Th1 cells and thus the secretion of cytokines, such as IL-2, which may help to prevent tumour development. It is possible, therefore, that DHA may have an important role in cancer chemoprevention, given the current controversy surrounding the administration of tamoxifen to women with a high risk of breast cancer. The development of potent inhibitors of E1-STS, which are due to enter clinical trials within the next 12 months, should confirm whether their use, either alone or in association with an aromatase inhibitor, can improve the response rate for women with breast cancer.

Acknowledgements—This research was supported by Imperial College Exploitation Ltd., and the Cancer Research Campaign. B. V. L. Potter is a Lister Institute Professor.

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