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The Role and Proposed Mechanism by Which Oestradiol 17β-Hydroxysteroid Dehydrogenase Regulates Breast Tumour Oestrogen Concentrations

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Synthesis of the biologically active oestrogen, oestradiol, within breast tumours makes an important contribution to the high concentrations of oestrogens which are present in malignant breast tissues. In breast tumours, oestrone is preferentially converted to oestradiol by the Type I oestradiol 17β-hydroxysteroid dehydrogenase (E2DH). Several growth factors, such as insulin-like growth factor Type I, and cytokines, such as Tumour Necrosis Factor α (TNFα), have been shown to stimulate E2DH activity in MCF-7 breast cancer cells. As little is known about the regulation of Type I E2DH expression and activity in other breast cancer cell lines, the expression and activity of this enzyme was examined in other oestrogen receptor positive and also oestrogen receptor negative breast cancer cell lines. As it is possible that E2DH activity may be limited by co-factor availability, the effects of exogenous co-factors on enzyme activity in these cell lines was also investigated. For T47D and BT20 breast cancer cells, the addition of exogenous co-factors was found to enhance enzyme activity. TNFα, in addition to stimulating E2DH activity in MCF-7 cells, also increased activity in T47D and MDA-MB-231 cells, although to a lesser extent than in MCF-7 cells. An investigation of signalling pathways involved in the regulation of E2DH activity revealed that stimulation of both the protein kinase C (PKC) and PKA pathways may be involved in regulation of E2DH activity. As several growth factors and cytokines have now been found to be involved in regulating E2DH activity, the role that macrophages and lymphocytes have in supplying these factors and the mechanism by which these factors may stimulate tumour growth, is also reviewed.

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INTRODUCTION

Oestradiol is the most important mitogen which supports the growth of breast tumours and insight into the regulation of its synthesis in breast cancer and normal cells should help in the development of new therapies to inhibit its formation [1]. While initial investigations, in which plasma oestrogen concentrations were measured in women with or without breast cancer, revealed no consistent abnormality, there is now convincing evidence that oestradiol concentrations are significantly increased in malignant breast tissues [2–5].

The finding that tumour oestradiol concentrations are some 40–50-fold greater than in blood prompted investigation to examine the origins of tumour oestradiol. It is now generally accepted that while uptake from the circulation and binding to the oestrogen receptor (ER) makes a contribution to tumour oestrogen content, much of the oestrogen which is present in tumours is derived from *in situ* synthesis [6, 7].

The aromatase enzyme complex, which converts androstenedione to oestrone, and oestrone sulphatase, which hydrolyses oestrone sulphate to oestrone, are the main sources of tumour oestrone. Oestradiol 17β-hydroxysteroid dehydrogenase (E2DH) then converts oestrone to oestradiol. It was originally demonstrated, using an isotopic infusion technique, that the reductive direction of oestrogen metabolism predominates in

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breast tumours [8], and it is now apparent that this reductive activity is associated with the Type I E2DH [9, 10].

After detecting increased tumour oestradiol concentrations, it was found that E2DH activity was increased in tumour tissue compared with samples of normal tissue for which there was no histological evidence for the presence of malignant cells [3, 11]. This finding initially suggested that breast tumours may produce factors which could stimulate oestradiol synthesis and thus provide a favourable oestrogenic environment to support tumour growth. Support for this concept was obtained when it was found that there was a correlation between E2DH activity in tumours and non-involved tissues away from the tumour [12]. Another important finding, made by Beranek *et al.* [13], was that E2DH activity in tissues adjacent to tumours was related to tumour size. While these results provided the impetus to search for stimulatory factors produced by, or contained within, tumours which were able to regulate E2DH activity, the mechanism(s) responsible for these observations is only now being resolved.

The search to identify tumour-derived E2DH regulatory factors has involved a number of different strategies. These have included the culture of tumour-derived fibroblasts and an examination of the effect of conditioned medium (CM) collected from these cells on E2DH activity in MCF-7 cells [14], the preparation and bioassay of cytosol from normal and malignant breast tissues [15] and the assay of CM and cytosol for growth factors and cytokines [16].

Breast tumour cytosol was originally found to stimulate E2DH reductive activity, whilst cytosol from non-malignant tissue was without effect [15]. Insulin-like growth factors types I and II were identified in tumour cytosol and shown to preferentially stimulate E2DH reductive activity in breast cancer cells [17]. An albumin-like molecule was also isolated from tumour cytosol and some preparations of albumin were subsequently found to, not only stimulate E2DH activity, but also to greatly potentiate the ability of cytokines, such as interleukin-1β (IL-1β), to stimulate enzyme activity [18, 19]. It still remains to be resolved whether it is the albumin molecule itself, or a cytokine/growth factor/lipid bound to albumin which is responsible for its E2DH stimulatory effect. It is clear, however, that several other similar albumin-like molecules have now been isolated from endocrine tissues and that they all possess the ability to stimulate steroidogenesis [20].

CM collected from tumour-derived fibroblasts also markedly stimulated E2DH reductive activity in MCF-7 breast cancer cells. Preliminary purification of CM suggested that the stimulatory factor had a molecular weight in the 50 kDa region [14]. It was apparent from these early studies that while the partially purified factor could stimulate E2DH reductive activity, the stimulatory effect was associated with a marked inhibition of cell growth. It was postulated that this might have arisen from the co-purification of a growth inhibitory factor. IL-6 was subsequently identified in CM from tumour-derived fibroblasts and purported to be the E2DH stimulatory factor present in CM [21]. While there is no doubt that high IL-6 concentrations are present in CM, in at least two molecular forms, the E2DH stimulatory effect of IL-6 was not confirmed by others, using several different sources of this cytokine [19, 22]. So far the most potent E2DH stimulatory cytokine identified is Tumour Necrosis Factor α (TNFα), but as found for partially purified factors from tumour-derived fibroblast CM, this cytokine also inhibits cell proliferation [22]. In all these investigations the ability of the different factors to regulate E2DH oxidative activity was also examined, but no evidence for this was detected.

To examine the ability of some of the known E2DH reductive stimulatory factors to regulate Type I E2DH mRNA expression, RNA was isolated from T47D cells [23]. While an increase in expression of the Type I E2DH 1.3 kb mRNA was detected after these cells were treated with IL-1, TNFα or IGF-I, the most potent inducer of gene expression was found to be retinoic acid (RA). In parallel studies, RA was also shown to increase E2DH reductive activity, whilst having no effect on oxidative activity in T47D cells. As it had previously been reported that concentrations of co-factors may limit E2DH oxidative activity in these cells [24], the effect of adding exogenous co-factors on enzyme activity was also investigated. These results showed that while the addition of NAD^+ increased oxidative activity in RA treated cells, NADH did not further increase E2DH reductive activity. As an increase in E2DH reductive activity could, in the presence of its substrate oestrone, result in the synthesis of the potent mitogen oestradiol, the finding that RA stimulated E2DH reductive activity was surprising. It is well documented that RA has a marked inhibitory effect on breast cancer cell proliferation [25, 26]. As noted previously for the partially purified factor from CM and TNFα, the ability of RA to increase Type I E2DH 1.3 kb mRNA expression and its ability to increase enzyme activity, is again associated with a factor which inhibits cell proliferation. Thus a consistent trend is emerging, that any reduction in the proliferation of these breast cancer cells is associated with an increase in E2DH reductive activity. The only possible exception, so far, to this finding, is for IGF-I/II which, whilst both increasing E2DH activity, have variable stimulatory effects on cell proliferation [17].

To gain further insight into the regulation of E2DH we have compared the expression and activity of this enzyme in a number of different cell lines. The ability of TNFα to stimulate E2DH activity in cells other than MCF-7 was also examined, as well as the effects of exogenous co-factors on enzyme activity. The signalling pathways which may mediate cytokine stimulation of E2DH activity are also currently being

investigated. Lastly, as several growth factors, cytokines and proteins have now been identified, an hypothesis has been developed as to the likely origin of some of the stimulatory factors and how they may act in a concerted manner to regulate the conversion of oestrone to oestradiol, and thus tumour growth.

MATERIALS AND METHODS

Cell culture methods used for the maintenance of MCF-7 and T47D cells have been described previously [18, 22]. BT20 and MDA-MB-231 cells were routinely cultured in RPM1 1640 medium with 20 mM Hepes buffer (BT20) or medium 199 modified with Earle's salts and 20 mM Hepes buffer (MDA-MB-231). Medium was supplemented with non-essential amino acids, 2 mM L-glutamine, 10 mM sodium hydrogen carbonate and 5% (v/v) foetal calf serum (FCS) for MDA-MB-231 cells or 10% (v/v) for BT20 cells. Cells were grown to 50–75% confluence when the above medium was replaced with phenol red-free medium (MEM-AUTOPOW, buffered with 20 mM Hepes, pH 7.4) containing stripped FCS. TNFα was obtained from Bachem Ltd (Saffron Walden, U.K.) and added to cells in phenol red-free medium. Co-factors, and regulators of protein kinase C (PKC) and PKA activity were obtained from Sigma (Poole, U.K.) and included the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), staurosporine, calphostin C and forskolin.

E2DH mRNA expression

Analysis of E2DH mRNA expression in four different cell lines was compared in cells cultured under basal conditions or in the presence of RA (1 μM, Sigma). Cellular RNA was extracted from cells and used for Northern analysis as previously described [23]. A 1.2 kb E2DH Type I cDNA (obtained from Professor F. Labrie) was labelled using [α-^{32}P]deoxy-CTP (Amersham International, U.K.) and used as a probe to detect mRNA expression.

E2DH activity

E2DH reductive and oxidative activities in MCF-7 and T47D cells were measured as previously described [18, 22]. Activity in MDA-MB-231 cells was measured over a 4 h period after demonstrating that enzyme activity was linear with respect to time for up to 5 h. For BT20 cells, as previously found with T47D cells, the high level of activity in these cells required activity to be measured by carrying out a time-course assay for up to 20 min.

RESULTS AND DISCUSSION

Analysis of E2DH mRNA levels by different breast cancer cells revealed considerable differences in mRNA expression (Fig. 1). As previously observed, in T47D cells two main mRNA transcripts of 2.2 and 1.3 kb were detected with expression of the 1.3 kb mRNA being increased in cells treated with RA. In contrast, in MCF-7 and ZR-75-1 cells, these transcripts were not detectable under basal or RA stimulated conditions. In all cells an additional 0.6 kb mRNA transcript was detectable and in T47D cells under basal condition, and ZR-75-1 cells after treatment with RA, an additional 0.9 kb transcript was detected. The highest level of E2DH mRNA expression was detected in mRNA obtained from BT20 cells, confirming previous observations [27]. This transcript appeared to be about 1.3 kb in size, although it was difficult to obtain a more precise estimation of its size due to the intensity of expression. The addition of RA to BT20 cells did not appear to further enhance E2DH expression in these cells.

As found from the analysis of E2DH mRNA expression, marked differences in the reductive and oxidative activities were also detected in different breast cancer cell lines (Fig. 2). Differences in E2DH activity in MCF-7 and T47D cells, with the latter cells having greater reductive activity, reflect differences in mRNA

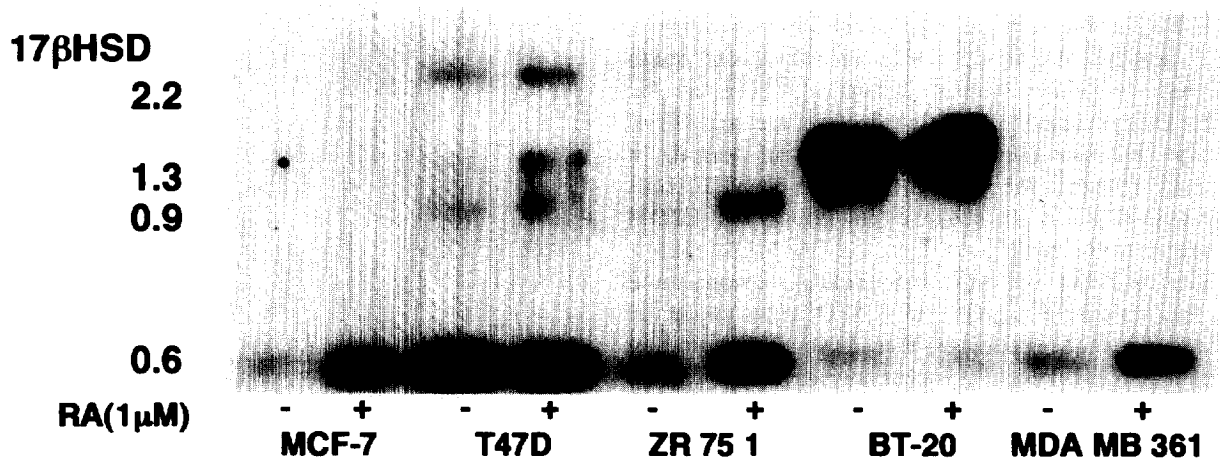

Fig. 1. Northern blot analysis of oestradiol 17β-hydroxysteroid dehydrogenase (17β-HSD) Type I mRNA expression in oestrogen receptor positive or negative breast cancer cell lines, in the absence (−) or presence (+) of all -trans -retinoic acid (RA, 1 μM).

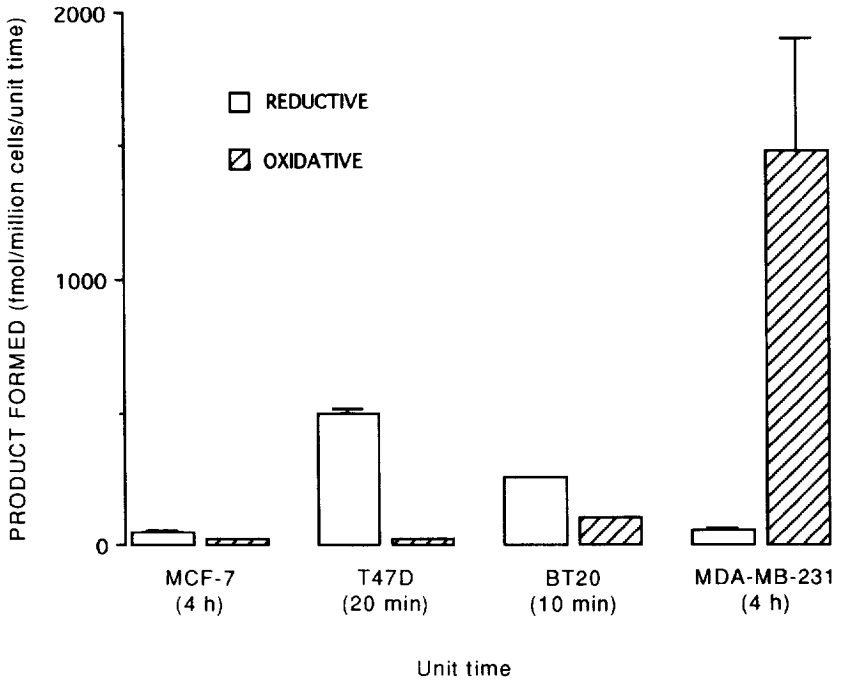

Fig. 2. Oestradiol 17β-hydroxysteroid dehydrogenase activity in oestrogen receptor positive or negative breast cancer cell lines. Activity was measured in both the reductive and oxidative directions.

expression. Similarly for BT20 cells, in which the highest E2DH Type I mRNA expression was detected, the high level of reductive activity resulted in activity being measured over a short period of time. However, in these cells, reductive activity predominated, reflecting the detection of a transcript in the 1.3 kb region with an E2DH Type I probe. For MDA cells, although so far, different sublines have been used for expression and activity studies, the failure to detect the Type I (1.3 kb transcript) in RNA isolated from these cells is consistent with the finding that the very high E2DH activity detected in these cells is mainly oxidative.

As TNFα has been found to be a potent stimulator of E2DH reductive activity in MCF-7 cells, its effect on the activity of this enzyme in other cell lines is currently under investigation. As shown in Fig. 3, while TNFα did increase E2DH reductive activity in T47D and MDA-MB-231 cells, its effect was much less than in MCF-7 cells. Whereas TNFα at the concentration tested had little effect on the growth of 3 of the cell lines over the 48 h treatment periods, it markedly reduced the growth ($>50\%$) of BT20 cells making it difficult to interpret its effects on E2DH activity in these cells.

Malet and her colleagues originally demonstrated that the oxidative activity of T47D cells could be influenced by concentrations of co-factor [24], a finding which was confirmed in a recent investigation [23]. In the present study the increase in oxidative activity in T47D cells resulting from the addition of NAD^+ or $NADP^+$ was confirmed (Fig. 4). A similar effect was noted for BT20 cells, in both reductive and oxidative directions, whereas the addition of exogenous co-factors was without effect in MCF-7 or MDA-MB-231 cells.

Cytokines, such as IL-1β and TNFα, are known to act by binding to cell surface receptors [28] and to transduce their effects, at least in part, via the PKC and PKA signalling pathways [29, 30]. The effects of specific stimulators of these pathways on E2DH reductive activity was therefore examined in MCF-7 cells. In addition, as it was postulated that the effects of TNFα might be mediated via the PKC pathway, it was

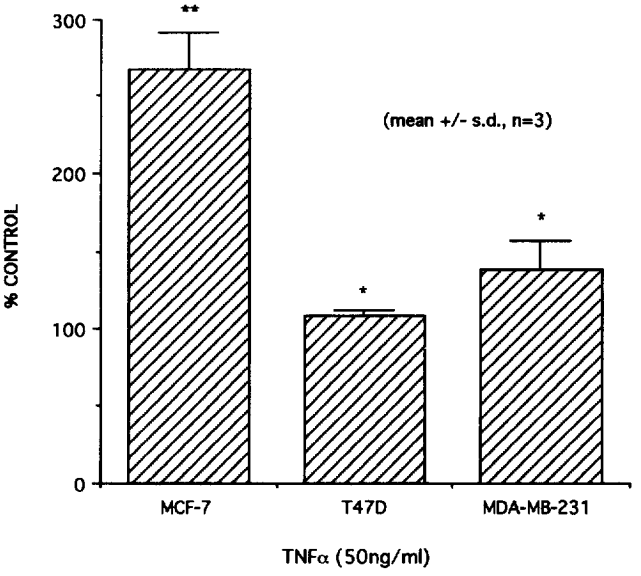

Fig. 3. Effect of Tumour Necrosis Factor α (TNFα) (50 ng/ml) on oestradiol 17β-hydroxysteroid dehydrogenase (reductive) activity in breast cancer cells.

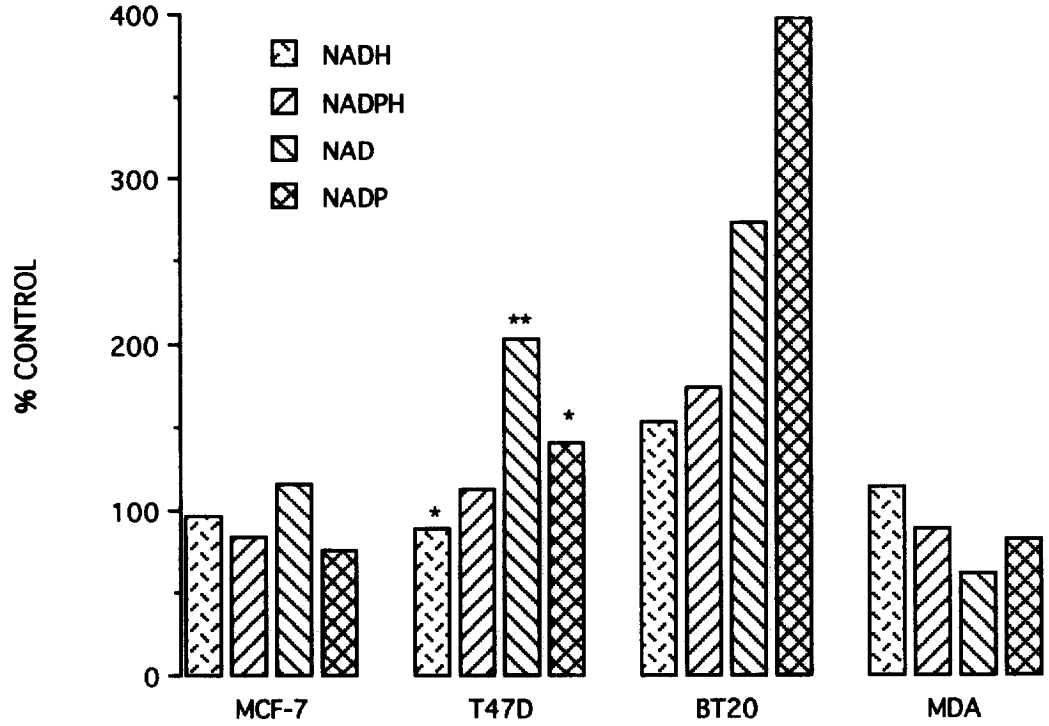

Fig. 4. Effect of co-factors on oestradiol 17β-hydroxysteroid dehydrogenase activities in MCF-7 breast cancer cells. NADH/NADPH (1 mM) were added to cells during the assay of reductive activity while $NAD^+/NADP^+$ (1 mM) were added to cells during the assay of oxidative activity.

intended to examine the effects of PKC inhibitors on the ability of TNFα to increase E2DH reductive activity. Both TPA and forskolin increased E2DH reductive activity after treatment for a 48 h period in a dose-dependent manner (Fig. 5). As previously noted, however, TPA markedly inhibited MCF-7 cell growth [31]. No significant effects of TPA on enzyme activity were observed after treatment of cells for 4 or 16 h. It

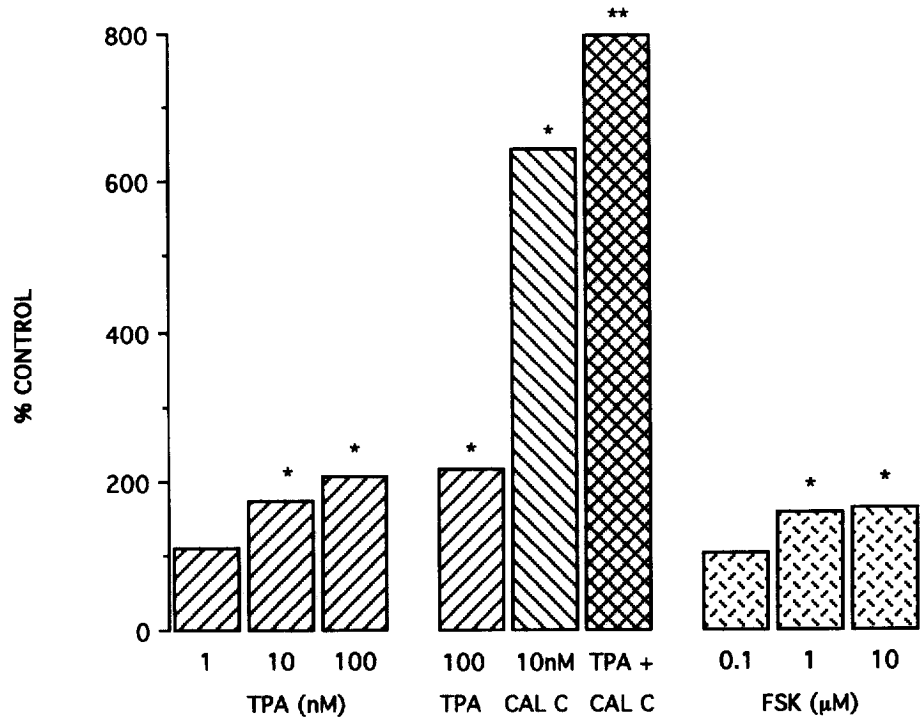

Fig. 5. Effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calphostin C (Cal C) or forskolin (FSK) on oestradiol 17β-hydroxysteroid dehydrogenase (reductive) activity in MCF-7 breast cancer cells.

is known that treatment of cells with TPA for 48 h down regulates, rather than stimulates, the activity of the PKC pathway [31]. In view of this finding it was not unexpected that the two inhibitors of PKC activity tested, staurosporine and calphostin C, also increased E2DH reductive activity (Fig. 5). However, this finding precluded attempts to examine the role of the PKC signalling pathway in mediating the stimulatory effects of TNFα on E2DH reductive activity. Both the PKC and PKA transduction pathways have previously been implicated in the regulation of E2DH Type I mRNA expression in the choriocarcinoma cell line JEG-3 [32].

Table 1. A comparison of the effects that factors which stimulate E2DH reductive activity have on cell growth

Factor	E2DH	Cell growth	Reference
IGF-I/II	+ +	+	[17]
TNFα	+ + +	− − −	[22]
TDF-CM	+ + +	− − −	[14]
IL-1	+ +	− −	[19]
TPA	+ +	− − −	[31, This paper]
aFGF	+	−	[38]
bFGF	+	−	[38]
HSA	+ + +	− − −	[18]
RA	+ +	− −	[23]

TDF-CM, tumour-derived fibroblast conditioned medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HSA, human serum albumin; RA, retinoic acid.

Current hypothesis of E2DH regulation

It is now apparent that the regulation of E2DH reductive activity is under multifactorial control. Cytokines are emerging as having a major role in regulating the activity of this enzyme, and it is therefore important to consider the possible origins of cytokines in relation to tumour development. In addition to examining the regulation of E2DH activity, similar studies have revealed that cytokines are also involved in the control of the two other enzymes which are involved in oestrogen synthesis in breast tumours, i.e. the aromatase and oestrone sulphatase [33, 34]. Some indication as to the possible origin of the stimulatory cytokines has been provided by results which have been obtained from clinical investigations. In the first of these, *in vitro* tumour aromatase activity was measured in samples obtained from patients before and after treatment with the aromatase inhibitor, 4-hydroxyandrostenedione (4-OHA) [35]. Whereas peripheral aromatase, as measured by an isotopic infusion technique, was almost completely suppressed by 4-OHA, tumour aromatase, as measured *in vitro*, increased markedly in 2/7 samples examined. This increase in tumour aromatase activity, in the presence of 4-OHA was associated with an increase in DNA polymerase activity, a marker of cell proliferation. This finding suggested, therefore, that the increase in aromatase activity in these two tumour samples resulted in the synthesis of sufficient oestrogen to exert a biological effect. In a second study, aromatase activity was measured in reduction mammoplasty tissue, obtained from a woman who had previously had breast augmentation involving a silicone injection which was not contained within a capsule. Aromatase activity in this tissue was considerably higher than any previously detected, and histological examination of the tissue revealed the presence of numerous infiltrating macrophages.

Macrophages, and other cells of the immune system such as lymphocytes, are known to produce many different cytokines. In view of the evidence indicating that up to 50% of the volume of breast tumours can be composed of macrophages [36], it seems likely that these cells may be the major source of E2DH stimulatory factors. It has been postulated that tumours may resemble wounds in their ability to attract cells of the immune system [37], but whereas upon healing wound tissue ceases to attract such cells, tumours continue to secrete chemo-attractant factors. This may result in the continued infiltration of macrophages and lymphocytes into tumours and the continued production of cytokines. If macrophages are responsible for the production of cytokines in tumours and adjacent tissues, then this could provide an explanation for the increased aromatase activity in biopsy samples obtained from women treated with 4-OHA. In these subjects the wound healing process would be in progress following removal of the initial biopsy sample. The ability of tumours to attract macrophages to tumours, and subsequent production of cytokines, could also account for one of the original observations which prompted the current research, i.e. the correlation between tumour size and E2DH activity in adjacent tissues [13].

Although it is now established that tumour cells do produce chemo-attractant factors which are responsible for recruiting macrophages and lymphocytes, it is difficult to understand why this leads to an increase in oestrogen synthesis, rather than to the elimination of tumour cells. A possible reason for this is suggested from an analysis of the effects that factors which have been identified as being able to stimulate E2DH reductive activity have on cell growth (Table 1). Apart from IGF-I and IGF-II, all the other E2DH stimulatory factors, markedly inhibit cell growth. These somewhat paradoxical findings have been difficult to understand, but it now appears possible that the increase in E2DH reductive activity caused by these factors, may result from some intracellular signalling mechanism that is stimulated when tumour cell proliferation is inhibited. Such a mechanism could provide sufficient oestradiol to overcome any anti-proliferative effects of cytokines or other factors. Given the molecular techniques which are now available, the identification of such a signal(s) should not prove too difficult and could lead to the development of novel forms of therapy to regulate oestrogen synthesis in women with breast cancer. Support for this concept, that cells of the immune system

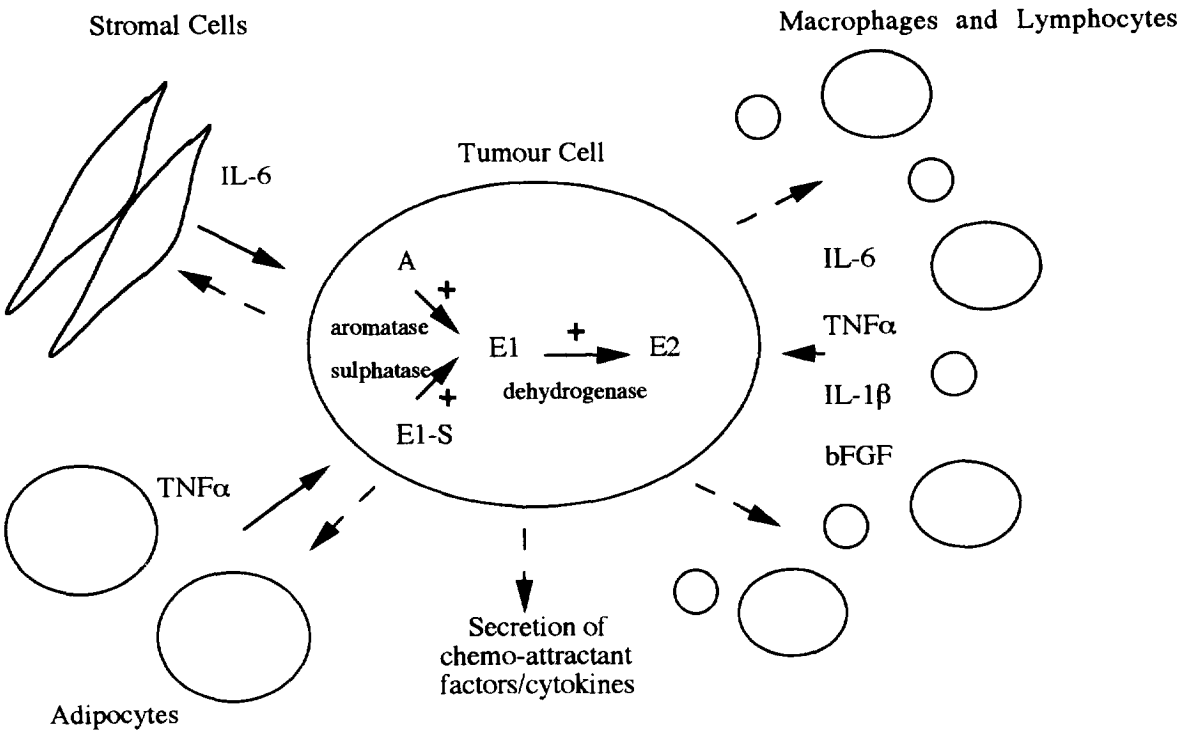

Fig. 6. Summary of an hypothesis for the regulation of oestradiol synthesis in breast cancer cells by cytokines and growth factors. Tumour cells secrete cytokines and chemo-attractant factors which attract lymphocytes and macrophages to the tumour site. In response to the secretion of cytokines by tumour cells, stromal cells and adipocytes may secrete cytokines, such as IL-6 and TNFα, which are known to stimulate aromatase, oestrone sulphatase and oestradiol dehydrogenase activities in breast cancer cells. Secretion of cytokines by lymphocytes and macrophages may also stimulate tumour oestrogen synthesis.

can produce factors which regulate E2DH reductive activity has already been obtained by showing that CM from macrophages and lymphocytes can markedly stimulate E2DH reductive activity [38]. Further studies are currently in progress to isolate macrophages and lymphocytes from breast tumours in order to confirm their ability to produce E2DH stimulatory factors.

The finding of a strong association between oncogene amplification and dense lymphocyte infiltration of tumours lends additional support to an important role of such cells in regulating tumour growth [39].

Having some years ago obtained evidence to suggest that breast tumours are producing or contain factors which are able to stimulate not only E2DH reductive activity, but also the activities of the aromatase and oestrone sulphatase enzymes, it is now possible to develop an hypothesis to suggest how such co-ordinated regulation might occur (Fig. 6). Tumour cells, by secreting chemo-attractant factors, attract cells of the immune system, including macrophages and lymphocytes. One of the functions of such cells is to produce cytokines which would normally be expected to inhibit or eliminate the proliferation of tumour cells. However, the cytokines produced, either directly or indirectly, increase oestrogen synthesis by the three enzyme complexes involved in tumour oestrogen synthesis and in the presence of the appropriate substrates, i.e. androstenedione, and oestrone sulphate, sufficient oestrone is produced for conversion to oestradiol. Production of a sufficient concentration of oestradiol would overcome any cytokine mediated inhibition of tumour cell growth. The high concentrations of oestradiol which have been detected in tumours would appear to support this potential mechanism.

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