The regulation of aromatase activity in breast fibroblasts: the role of interleukin-6 and prostaglandin E\textsubscript{2}

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Abstract

Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and cytokines, such as interleukin-6 (IL-6) or tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) can regulate aromatase activity. In the present study we have compared their abilities to stimulate aromatase activity in fibroblasts derived from 'normal' breast adipose tissue proximal to a tumour or breast tumours. PGE\textsubscript{2}, TNF\(\alpha\) and IL-6 plus its soluble receptor (IL-6sR) all increased aromatase activity in these cells. Basal aromatase activity and the degree of aromatase stimulation by these factors were greater in fibroblasts derived from 'normal' breast tissue than from breast tumours. The ability of IL-6+IL-6sR to increase aromatase activity was only marginally reduced by the PG synthesis inhibitor, indomethacin, indicating that IL-6+IL-6sR does not appear to act via induction of PG synthesis. The ability of PGE\textsubscript{2} to stimulate aromatase activity in fibroblasts derived from 'normal' breast tissue was potentiated by IL-6sR suggesting that PGE\textsubscript{2} may act via induction of IL-6. This was confirmed by measurement of IL-6 in conditioned medium collected from these cells. A significant increase in IL-6 concentrations was detected in conditioned medium collected from cells treated with PGE\textsubscript{2}. It is concluded that in some fibroblasts PGE\textsubscript{2} may exert part of its regulatory effect on breast tissue aromatase activity via induction of IL-6.

Introduction

The aromatase enzyme complex catalyses the conversion of androgens to oestrogens. In postmenopausal women oestrogens are synthesized exclusively in peripheral tissues with adipose tissue being a major site in which conversion of androstenedione to oestrone occurs. Aromatase activity is also detectable in normal and malignant breast tissues (James \textit{et al.} 1987). \textit{In situ} synthesis of oestrone from androstenedione makes an important contribution to the high oestrogen content of some tumours (Reed \textit{et al.} 1989). There is also evidence that the oestrogen formed within tumours exerts a biological effect, i.e. stimulates cell proliferation (Lu \textit{et al.} 1996).

Several studies have now confirmed that aromatase activity (O'Neili \textit{et al.} 1988, Purohit \textit{et al.} 1995) or expression (Bulun \textit{et al.} 1993) is increased in the breast quadrant in which a tumour is located. It is possible, therefore, that tumours may arise in a region of the breast with high aromatase activity. Alternatively, breast tumours may secrete factors which stimulate aromatase activity in tissues proximal to the tumour.

The synthetic glucocorticoid, dexamethasone, in the presence of fetal calf serum (FCS) was originally shown to stimulate aromatase activity in cultured stromal cells derived from subcutaneous adipose tissue (Simpson \textit{et al.} 1981). Using fibroblasts derived from breast tumours or reduction mammoplasty tissue, the cytokines interleukin-1 (IL-1), IL-6 and tumour necrosis factor \(\alpha\) (TNF\(\alpha\)), in the presence of dexamethasone, but absence of FCS, were also identified as potent regulators of aromatase activity (Reed \textit{et al.} 1992, Macdiarmid \textit{et al.} 1994). Aromatase activity in fibroblasts derived from subcutaneous adipose tissue was also stimulated by IL-6 but only in the presence of its soluble receptor (IL-6sR) (Zhao \textit{et al.} 1995).

High levels of aromatase activity were subsequently detected in reduction mammoplasty tissue heavily infiltrated with macrophages. The demonstration that macrophage conditioned medium (CM), which contains high levels of IL-6, stimulated aromatase activity led to the
suggestion that IL-6 production by cells of the immune system, which can invade tumours, may be an important source of cytokines which regulate this activity in normal and malignant breast tissues (Purohit et al. 1995, Reed & Purohit 1997).

There is only one gene for the aromatase but its expression is regulated in a tissue-specific manner by the use of a number of different promoters (Simpson et al. 1994). In adipose tissue expression is regulated by promoter (P) L4 (Mahendroo et al. 1993). The 5'-upstream region of this promoter contains a glucocorticoid response element, and a GAS (IFNγ activating sequence) element which can bind transcription factors of the signal transducer and activator of transcription (STAT) family (Zhao et al. 1995). Cytokines, in the presence of glucocorticoids, regulate aromatase gene expression via the P1.4.

Recently, expression of aromatase transcripts has been examined in breast adipose tissue from cancer-free women and breast tumours and breast adipose tissue from women with breast cancer (Harada et al. 1993, Agarwal et al. 1996). In breast adipose tissue from tumour- and non-tumour-bearing quadrants there was a reduction in the level of aromatase P1.4 transcripts but an increase in P11 and P1.3 transcripts. Expression of the aromatase gene via P11 and P1.3 is regulated by cAMP (Mahendroo et al. 1993). Zhao and colleagues have obtained evidence that prostaglandin E2 (PGE2) may be the major factor regulating aromatase expression via P11 and P1.3 (Zhao et al. 1996).

As all three promoters appear to be used, albeit at different levels, in normal and malignant breast tissues regulation in vivo may depend on the relative concentrations of such factors as cytokines and PGE2 in breast tissues. In an attempt to obtain further information about the regulation of aromatase activity in breast tissues we have initiated a study to compare the abilities of a number of different promoters (Simpson et al. 1994). In adipose tissue expression is regulated by promoter (P) L4 (Mahendroo et al. 1993). The 5'-upstream region of this promoter contains a glucocorticoid response element, and a GAS (IFNγ activating sequence) element which can bind transcription factors of the signal transducer and activator of transcription (STAT) family (Zhao et al. 1995). Cytokines, in the presence of glucocorticoids, regulate aromatase gene expression via the P1.4.

Materials and methods

Samples of breast adipose tissue proximal to breast tumours (i.e. ‘normal’ breast tissue) and breast tumours

were collected from women undergoing lumpectomy for the removal of breast tumours after obtaining their informed consent. The study was approved by the hospital Ethics Committee.

Culture of fibroblasts

Resected tissues were minced with sterile scalpels and incubated in Eagles’ modified minimum essential medium (EMEM) for 18-24 h at 37 °C with collagenase (200 µg/ml). The dispersed cells were harvested by centrifugation and washed twice with medium to remove collagenase. Dispersed cells were seeded into 25 cm² culture flasks and allowed to attach. Cells were grown to confluence in EMEM containing Hepes buffer (20 mmol/l), 10% FCS and supplements (Reed et al. 1992). Cells were routinely passaged 2-3 times after which replicate 25 cm² culture flasks were seeded with fibroblasts and grown to confluency. The cells were washed once with phosphate-buffered saline and the medium was replaced with serum-free, phenol red-free EMEM for 24 h. Treatments were then added to this medium for 48 h, and included PGE2 (Sigma, Poole, Dorset, UK) IL-6 plus IL-6sR, IL-8, TNFα (R&D Systems Ltd, Abingdon, Oxon, UK) or indomethacin (Sigma).

Measurement of IL-6 concentration in fibroblast conditioned medium

To assess the ability of PGE2 to stimulate IL-6 production by fibroblasts, IL-6 concentrations in CM were measured by ELISA (R&D Systems Ltd). Intra- and interassay coefficients of variation for this assay were <10%. According to details provided with this assay, the IL-6 antibody used does not cross react with IL-11, oncostatin-M (OSM), leukaemia inhibitory factor (LIF) or the IL-6sR. Unless stated otherwise, all experimental treatments included dexamethasone (100 nM). Representative results are shown from at least two experiments.

Aromatase assay

Aromatase activity was measured in intact fibroblast monolayers using [1β-3H]Androstenedione (15-30 Ci/mmol, NEN-Du Pont, Stevenage, Herts, UK) over a 3-h period (Reed et al. 1992). The number of cells were measured by counting cell nuclei using a Coulter counter.

Statistics

The significance of differences in aromatase activity in treated and control cells was assessed using Student’s t-test.
Figure 1 Effect of PGE\(_2\), TNF\(\alpha\), IL-8 or IL-6 plus IL-6sR, in the presence of dexamethasone (100 nM), on aromatase activity in cultured fibroblasts derived from 'normal' breast adipose tissue proximal to a tumour. Values are given as means ± s.d., n=3; *P<0.05, ***P<0.001 compared with control.

Figure 2 Effect of PGE\(_2\), TNF\(\alpha\), IL-8 or IL-6 plus IL-6sR, in the presence of dexamethasone (100 nM), on aromatase activity in fibroblasts derived from breast tumour. Values are given as means ± s.d., n=3; *P<0.05, **P<0.01, ***P<0.001 compared with control.
Results and Discussion

The ability of PGE$_2$, TNF$\alpha$, IL-8 or IL-6 plus IL-6sR to stimulate aromatase activity was initially examined in fibroblasts derived from adipose tissue proximal to a breast tumour that was essentially ‘normal’ breast tissue for a postmenopausal woman (Fig. 1).

While IL-8 did increase aromatase activity marginally in these fibroblasts (10%), TNF$\alpha$ (8.2-fold) and IL-6 plus IL-6sR (20.1-fold) had a much greater effect. In contrast, although PGE$_2$ did increase aromatase activity (3.9-fold) it was lower than that resulting from either TNF$\alpha$ or IL-6 plus IL-6sR. The marked potentiation by IL-6sR of the ability of IL-6 to stimulate aromatase activity confirms previous observations (Singh et al. 1995, Zhao et al. 1995).

In a similar study, the effect of these cytokines or PGE$_2$ on aromatase activity was examined using fibroblasts derived from a breast tumour (Fig. 2). While the results obtained were in keeping with those obtained using fibroblasts derived from ‘normal’ breast tissue, there were important differences. First, the level of basal aromatase activity detected was considerably lower than that found for ‘normal’ fibroblasts. Secondly, while TNF$\alpha$, IL-6 plus IL-6sR and PGE$_2$ all significantly stimulated aromatase activity, the extent to which they did so was considerably lower than that detected for fibroblasts derived from ‘normal’ breast tissue.

In a further study, the ability of PGE$_2$ or IL-6 plus IL-6sR to stimulate aromatase activity was examined in ‘normal’ fibroblasts and tumour-derived fibroblasts obtained from the same subject. These experiments were carried out in the absence or presence of dexamethasone. As shown in Fig. 3, while IL-6 plus IL-6sR could stimulate aromatase activity in both types of fibroblasts in the absence of dexamethasone, this combination stimulated aromatase activity to the greatest degree in ‘normal’ fibroblasts in the presence of dexamethasone. The ability of IL-6 plus IL-6sR to stimulate aromatase activity in the absence of dexamethasone suggests that these factors may stimulate production of PGE$_2$ or other cytokines. This theory is currently under further investigation.

The ‘normal’ fibroblasts used in these investigations were derived from adipose tissue adjacent to a breast tumour which has previously been reported to possess an increase in aromatase transcripts for PII and PL3, although transcripts for PL4 are still present at a reduced level (Harada et al. 1993, Agarwal et al. 1996). It was reasoned, therefore, that if PII and PL3 were controlling aromatase expression in these cells, IL-6 and IL-6sR might be acting via induction of PGE$_2$. To examine this possibility, ‘normal’ fibroblasts were treated with IL-6+IL-6sR on
their own, or in the presence of the PGE\textsubscript{2} synthesis inhibitor, indomethacin (Fig. 4). Indomethacin alone, at 1 or 10 \(\mu\text{M}\), had little effect on aromatase activity in these cells while, as previously found, IL-6+IL-6sR markedly increased aromatase activity. The addition of IL-6+IL-6sR in the presence of indomethacin resulted in a small (16\%) reduction in the ability of this cytokine and its soluble receptor to stimulate aromatase activity. These results therefore appear to support a major role for IL-6+IL-6sR in regulating aromatase in these fibroblasts. As a small reduction in aromatase activity was detected when IL-6+IL-6sR plus indomethacin were used in combination to treat cells, this could suggest that IL-6+IL-6sR can stimulate PGE\textsubscript{2} synthesis, although only to a small degree. However, there is evidence that indomethacin can also inhibit IL-6 secretion in some cells (Ogle\textit{ et al.} 1994).

There is also evidence that PGE\textsubscript{2} may in fact be the major factor responsible for IL-6 secretion by macrophages (Hinson \textit{et al.} 1996) and fibroblasts (Zhang \textit{et al.} 1988).

The possibility that PGE\textsubscript{2} or cAMP may increase aromatase activity in these fibroblasts via induction of IL-6 was therefore considered. For this, cells were cultured in the absence or presence of dexamethasone, with PGE\textsubscript{2} or PGE\textsubscript{2} plus IL-6sR but no exogenous IL-6. It was reasoned that, if PGE\textsubscript{2} acted directly via stimulation of cAMP production, then it should stimulate activity in the absence of dexamethasone. If PGE\textsubscript{2} was acting indirectly via IL-6 induction then PGE\textsubscript{2} should only stimulate activity in the presence of dexamethasone. Furthermore, if PGE\textsubscript{2} acted via induction of IL-6 then the ability of PGE\textsubscript{2} to stimulate aromatase should be potentiated by IL-6sR.

As shown in Fig. 5, results from this experiment supported the hypothesis that in these fibroblasts, which were derived from ‘normal’ breast tissue, PGE\textsubscript{2} is stimulating aromatase activity via induction of IL-6. In these fibroblasts, in the absence of dexamethasone, PGE\textsubscript{2} was without effect indicating that stimulation via the cAMP, PII and PI3 pathway is inactive under the culture conditions used. In contrast, in the presence of dexamethasone aromatase activity was increased and the ability of PGE\textsubscript{2} to stimulate activity was potentiated by IL-6sR.

To confirm that PGE\textsubscript{2} did increase IL-6 production by these fibroblasts a preliminary study was undertaken to measure IL-6 concentrations in CM collected from these cells. It has previously been shown that IL-6 is secreted by breast tissue-derived fibroblasts, although in a number of different forms (Duncan \textit{et al.} 1994). Significant concentrations of IL-6 were detectable in CM collected from control cells with levels being significantly increased in PGE\textsubscript{2}-treated cells (Fig. 6). However, dexamethasone

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Effect of IL-6 (50 ng/ml) plus IL-6sR (100 ng/ml) in the presence of dexamethasone (100 nM) with or without the prostaglandin synthesis inhibitor, indomethacin (Ind) on aromatase activity in cultured fibroblasts. Values are given as means ± s.d., n=3; a \(P<0.01\), b \(P<0.001\) vs controls; c \(P<0.01\), d \(P<0.001\) vs cells treated with IL-6+IL-6sR.}
\end{figure}
Figure 5 Effect of PGE$_2$ (10 µM) on aromatase activity in fibroblasts, in the absence (−) or presence (+) of dexamethasone (100 nM), or IL-6sR (100 ng/ml). Values are given as means ± s.d., n=3; $^a$P<0.001 vs controls+; $^b$P<0.001 vs PGE$_2$+dexamethasone.

Figure 6 Concentrations of IL-6 in condition medium collected from fibroblasts in the absence (−) or presence (+) of dexamethasone (100 nM), PGE$_2$ (10 µM) or PGE$_2$ plus IL-6sR (100 ng/ml). Values are given as means ± s.d.; $^a$P<0.001 vs control−; $^b$P<0.001 vs control+; $^c$P<0.01 vs PGE$_2$−; $^d$P<0.001 vs PGE$_2$+.
did appear to have an inhibitory effect on the ability of PGE2 to stimulate IL-6 production in these cells. In cells treated with PGE2 plus IL-6sR, IL-6 concentrations in CM were significantly higher than cells only treated with PGE2 in the absence or presence of dexamethasone. There is evidence that IL-6sR itself may stimulate IL-6 production and this may account for the higher concentration of IL-6 measured in CM from cells treated with PGE2 plus IL-6sR (Cichy et al. 1996).

Results from this study have shown that using fibroblasts derived from ‘normal’ breast adipose tissue proximal to a tumour, IL-6 plus IL-6sR has a greater stimulatory effect on aromatase activity than PGE2, although PGE2 itself can increase activity. While indomethacin, which inhibits PGE2 synthesis, resulted in a small reduction in the ability of IL-6 plus IL-6sR to stimulate aromatase activity, it is possible that this effect is mediated by the compound inhibiting IL-6 production (Ogle et al. 1994).

The most important observation to arise from this study is that in some fibroblasts PGE2 appears to stimulate aromatase activity via induction of IL-6. This finding has important implications for understanding the in vivo regulation of aromatase activity in breast tumours. While aromatase PI.4, PI.3 and PII-specific transcripts are all expressed in breast tumours and adipose tissue proximal to tumours, there is an increase in levels of PI and PI.3 transcripts (Agarwal et al. 1996). If PGE2 enhances the local production of IL-6 in breast tumours and adjacent tissue, then the expression of PI.4 aromatase transcripts, even at a relatively low level, may be sufficient to ensure that this cytokine has an important role in regulating breast tumour aromatase activity. It is evident, however, that consistent with the different promoters expressed in ‘normal’ and malignant breast tissues, different factors can act to regulate aromatase activity in both types of fibroblasts.

Production of IL-6 by macrophages can be inhibited by dexamethasone (Akira et al. 1993). At the concentration of dexamethasone used for this study, production by fibroblasts was not completely inhibited. However, the stimulation of IL-6 production by PGE2 was lower in glucocorticoid-treated cells. In addition to the production of PGE2 by breast fibroblasts (Schrey & Patel 1995) tumour-associated macrophages are also likely to be an important source of this prostaglandin. Thus, in

![Diagram of cytokine production by T-lymphocytes, fibroblasts, and macrophages](attachment:diagram.png)

**Figure 7** Summary of possible mechanism by which cytokine production by Th lymphocytes and macrophages is regulated. The balance of dehydroepiandrosterone (DHA) to cortisol (F) is thought to regulate the progression of Th cells to either a Th1 or Th2 phenotype. PGE2 may have a crucial role in regulating IL-6 production by macrophages and possibly fibroblasts (MCP-1, macrophage chemoattractant protein-1). A, androstenedione; E1, oestrone.
combination with IL-6 produced by tumour fibroblasts and macrophages, the local production of PGE2 may, by stimulating IL-6 production, amplify the ability of this cytokine to stimulate aromatase activity.

It has previously been postulated that production of IL-6 (a Th-2 type cytokine) by T-helper (Th) cells may depend on the balance of the adrenal androgen, dehydroepiandrosterone, to that of the glucocorticoid, cortisol (Daynes et al. 1990, Rook et al. 1994). Lymphocytes can infiltrate breast tumours and may be an important source of the cytokines that are available to regulate oestradiol synthesis within breast tumours (Reed et al. 1995).

The regulation of cytokine production by macrophages within the tumour environment, however, remains to be resolved. In view of the results obtained in the present study and those of other investigations (Zhang et al. 1988; Hinson et al. 1996) it is possible that PGE2 may in fact be a major regulator of macrophage IL-6 production. Thus, the secretion of PGE2 by tumour cells and fibroblasts may provoke the production of IL-6 by tumour-associated macrophages. This, in turn, could enhance not only aromatase activity but also that of other enzymes involved in oestrogen synthesis, e.g. oestrone sulphatase, oestradiol 17β-hydroxysteroid dehydrogenase (Reed & Purohit 1997). A summary of such a possible mechanism is illustrated in Fig. 7.

There is epidemiological evidence that women using nonsteroidal anti-inflammatory drugs (NSAIDs) for a prolonged period may have a chemo-preventative potential against the development of breast cancer (Harris et al. 1996). This finding has been interpreted as support for a role for prostaglandins in regulating breast tumour aromatase activity (Zhao et al. 1996). However, in view of the results obtained in the present investigations, it is also possible that any beneficial effect of NSAIDs could result from a reduction in PGE2-mediated production of IL-6. It has also recently been shown that NSAIDs inhibit phenolsulphotransferase activity (Harris et al. 1998). As many carcinogens require sulphating before being active, it is possible that NSAIDS may also act by preventing carcinogen activation.

References


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