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The Interaction of Cytokines in Regulating Oestradiol 17β-Hydroxysteroid Dehydrogenase Activity in MCF-7 Cells

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Oestradiol 17β-hydroxysteroid dehydrogenase (E_2DH) has a pivotal role in the regulation of oestradiol (E_2) concentrations in normal and malignant breast tissues. Previous studies have suggested that a number of cytokines can stimulate E_2DH activity to increase the conversion of oestrone (E_1) to E_2. In this investigation we have examined the effect of TNFα, interleukin-1β (IL-1β) and IL-6 on E_2DH activity in MCF-7 breast cancer cells. These cytokines may be produced by breast tumours and their presence in conditioned medium (CM) from tumour-derived fibroblasts was also measured to assess their possible contribution to its E_2DH stimulatory activity. Treatment of MCF-7 cells with IL-1β and TNFα (5 ng/ml) significantly increased ($P < 0.001$) reductive E_2DH (red-E_2DH, the conversion of E_1 to E_2) activity. In contrast, IL-6 at a concentration of 100 ng/ml produced little, if any, stimulation of reductive activity. Combinations of all three cytokines acted synergistically to stimulate red-E_2DH activity. No cytokine, either alone or in combination, affected oxidative ($E_2 \rightarrow E_1$) activity. Significant concentrations of IL-6 and IL-1β were detected in CM, but the stimulation of red-E_2DH activity was much greater than that which could be explained by their levels alone. It is concluded that these cytokines may play an important role in regulating E_2DH activity in breast cancer cells and may act synergistically *in vivo* to enhance the formation of E_2 in breast tumours.

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INTRODUCTION

Oestradiol (E_2) has a well established role in the etiology of breast cancer. It is reversibly formed via oestradiol 17β-hydroxysteroid dehydrogenase (E_2DH) from the biologically inactive oestrogen, oestrone (E_1). The importance of E_2DH activity in breast tumour development and growth is indicated by the raised levels of E_2 [1] and the increased conversion of $[^3H]E_1$ to $[^3H]E_2$ [2] in breast tumours when compared to normal breast tissue. Moreover, E_2DH activity has been shown to correlate both with tumour size in tissues adjacent to the tumour [3] and with tumour E_2DH activity in tissue some 2–5 cm removed from the tumour site [4].

Taken together, these studies suggest that factors present in breast malignancies are able to regulate E_2DH activity not only in the tumour itself, but also in the surrounding "normal" tissue. This could involve autocrine, paracrine and endocrine mechanisms. Several putative factors have been identified with the ability to differentially regulate the two opposing activities of E_2DH. Insulin-like growth factor-I (IGF-I) and IGF-II [5], interleukin-1β (IL-1β) [6], IL-6 [7] and human serum albumin (HSA) [8] have all been shown to stimulate reductive E_2DH activity (the conversion of E_1 to E_2, red-E_2DH), whilst having no effect on the inactivating oxidation of E_2 (oxid-E_2DH) in MCF-7 breast cancer cells, thereby promoting an oestrogenic environment.

In this study, we have further examined the roles of cytokines in breast cancer. TNFα, IL-1β and IL-6 share many biological actions and frequently act together in a synergistic manner [9]. Their antiproliferative effects on breast cancer cell lines are well documented [10–12]. We have therefore studied their action on E_2DH activity in MCF-7 breast cancer cells. Since fibroblasts have been shown to synthesize all three cytokines [13–15], we have also investigated the

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possibility of a paracrine effect on E_2DH activity by treating MCF-7 cells with conditioned medium (CM) obtained from breast tumour-derived fibroblasts.

EXPERIMENTAL

Materials

The MCF-7 cell line was originally supplied by Dr M. Lippman (National Cancer Institute, Bethesda, MD, U.S.A.). Plasticware was obtained from Falcon (Marathon Laboratory Supplies, London, England), foetal calf serum from J. Bio (1, rue de Terre Neuve, 91967 Les Ulis Cedex, France), antibiotics from Sigma (Poole, Dorset, England) and all media and other supplements from ICN Biomedicals Ltd (High Wycombe, Bucks., England). Recombinant cytokines were purchased from Bachem (Saffron Walden, Essex, England). IL-6 was also kindly provided by Dr B. Rafferty (National Institute for Biological Standards and Control, Potters Bar, Herts., England). All isotopes and a further supply of IL-6 were obtained from Amersham (Aylesbury, Bucks., England). Cross-reaction of other cytokines in ^{125}I radioimmunoassay systems was $<0.001\%$, and intra- and inter-coefficients of variation were $<10\%$.

Cell culture

MCF-7 cells were routinely cultured in Minimum Essential Medium Eagle modified with Earle's salts and 20 mM Hepes buffer (MEM), 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), and is hereafter referred to as GM. Cells were grown to a minimum confluence of 30%, then treated with cytokines for 48 h. Treatments were prepared in MEM-AUTOPOW in order to eliminate the oestrogenic effects of phenol red. Supplements were added as for GM with the exception that FCS was replaced by serum (5% v/v) stripped of steroids with dextran coated charcoal (SFCS) [16].

Treatments were added to sets of six flasks, to enable measurement of reductive and oxidative E_2DH activity in parallel.

Preparation of CM

Fibroblasts were prepared and cultured from malignant breast tissue as outlined in [17].

CM was obtained by adding serum-free MEM to log phase fibroblasts (10 ml/10^6 cells) for 24 h. The first preparation was discarded to minimize the risk of contaminating plasma proteins. CM was then stored at $-20°C$ until used.

E_2DH assay [17]

Intact monolayers were washed with phosphate buffered saline and then incubated with 2 nM [2,4,6,7-^3H]E_1 (80–100 Ci/mmol) or [6,7-^3H]E_2 (40–60 Ci/mmol) (for the measurement of reductive or oxidative E_2DH, respectively) for 4 h at 37°C. Substrate was prepared in MEM-AUTOPOW containing all supplements except for serum (2.5 ml/flask). Blanks were run in parallel. On completion of the assay, 2 ml of medium were removed to test tubes containing 5000 cpm [^{14}C]E_2 (reductive) or [^{14}C]E_1 (oxidative) to monitor procedural losses. Steroids were extracted from the medium with 4 ml diethyl ether and then taken to dryness. E_1 and E_2 were separated by thin layer chromatography using a dichloromethane–ethyl acetate (4:1, v/v) solvent system. Product and recovery radioactivity were determined by liquid scintillation counting. Cell number was obtained by release of nuclei, determined by a Coulter counter and results were expressed as fmol product formed/4 h/10^6 cells (mean $\pm$ SD, $n = 3$). Statistical significances were determined by Student's t-test.

RESULTS

Dose–response assays were performed with TNFα, IL-1β and IL-6 at concentrations ranging from 1 to 100 ng/ml in MCF-7 cells. TNFα stimulated red-E_2DH activity in a dose-related manner [Fig. 1(a)]. A degree of inter-experimental variation in this effect was observed, at 5 ng/ml, TNFα raised red-E_2DH by 0–22% and at 100 ng/ml by 15–1076% [but at this concentration, stimulation of $199 \pm 58\%$ (mean $\pm$ SD) was achieved in 5 of 8 experiments]. The threshold at which a response was first detected was therefore variable in different experiments, but stimulation was always obtained at concentrations of 50 ng/ml and above. Some variation in the response to other cytokines was also obtained. IL-1β stimulated red-E_2DH from 0–58% at 5 ng/ml, and from 27–96% at 100 ng/ml. A representative result is shown in Fig. 1(b). IL-6 failed to have a marked effect on reductive activity when obtained from either the National Institute for Biological Standards and Control or from Amersham International [Fig. 1(c)]. IL-6 from Bachem also produced no, or at the most, a very limited increase (at 100 ng/ml only) in red-E_2DH activity [up to 27%, Fig. 2(a and b)]. None of the cytokines altered oxid-E_2DH activity.

Combination of any two of these cytokines resulted in synergistic stimulation of red-E_2DH activity [Fig. 2(a)]. Moreover, the combination of all three cytokines could significantly enhance this activity even at concentrations where each cytokine alone produced no effect [Fig. 2(b)]. No effect on oxid-E_2DH activity was found, even when all three cytokines were used together at concentrations of 100 ng/ml.

TNFα at concentrations of 50 and 100 ng/ml decreased cell numbers by 0–23 and 12–37%, respectively, when compared with controls. IL-1β and IL-6 also had slight growth inhibitory effects, but these were not significant at $P < 0.05$. Cell number was reduced in

an additive manner by combination of TNFα with IL-1β and/or IL-6.

CM from tumour-derived fibroblasts was found to stimulate red-E_2DH activity in MCF-7 cells. Immunoreactive IL-6 and IL-1β, but not TNFα, was detected in this medium. In order to characterize the stimulatory factor(s) present, CM was diluted 1:10 in Tris buffer (50 mM, pH 7.0) and applied to a DEAE cellulose anion ion-exchange column. The retained material was then eluted with a NaCl gradient (0–1 M) and each fraction assayed for its ability to stimulate red-E_2DH activity [Fig. 3(a)]. The fractions eluted with 150, 200 and 250 mM NaCl stimulated activity to the greatest degree (by 776, 951 and 918%, respectively) and immunoreactive IL-6 levels in these fractions were also higher than in other fractions (3.7, 4.8 and 0.8 ng/ml, respectively). IL-1β was present in some fractions and corresponded to another, smaller peak in stimulatory activity of the CM (fractions 500–1000 mM, where IL-1β levels ranged from 272–442 pg/ml).

To enable further characterization of the red-E_2DH stimulatory factor(s), aliquots of the 150–250 mM fractions were pooled and applied to a G-100 molecular sieve column. Fractions were again assayed for their ability to enhance red-E_2DH activity [Fig. 3(b)]. Stimulation ranged from 0 (fraction 12) to 404% (fraction 14) and peaks of stimulation corresponded to fractions containing factors of four different sizes. Radioimmunoassay for IL-6 in these fractions revealed the presence of two species, one with the monomeric molecular weight for the cytokine (present at 0.9 ng/ml), the other with a molecular weight greater than

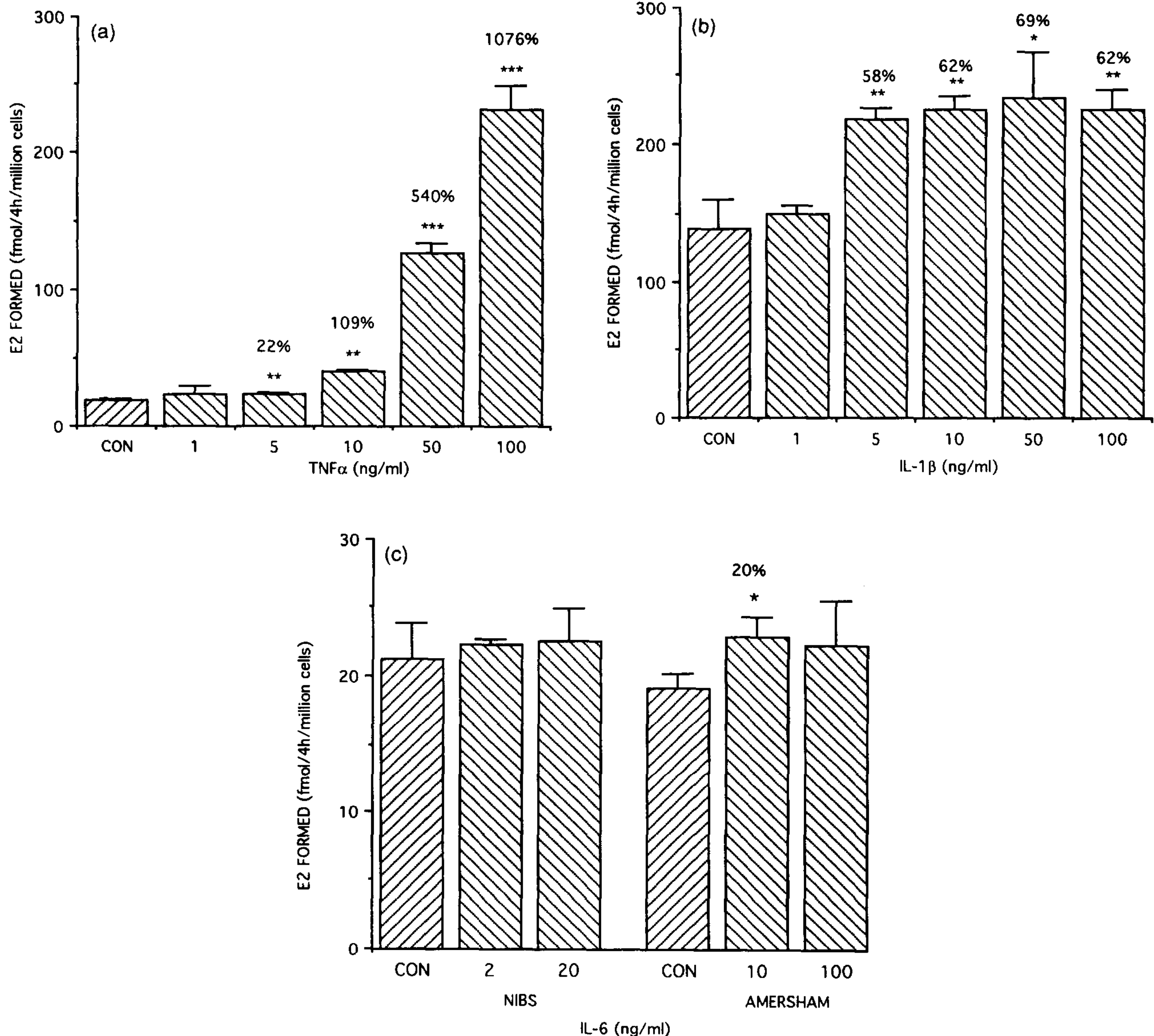

Fig. 1. Dose–response assays for cytokine stimulation of red-E_2DH activity in MCF-7 cells. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$. Concentrations = ng/ml. (a) TNFα; (b) IL-1β; and (c) IL-6.

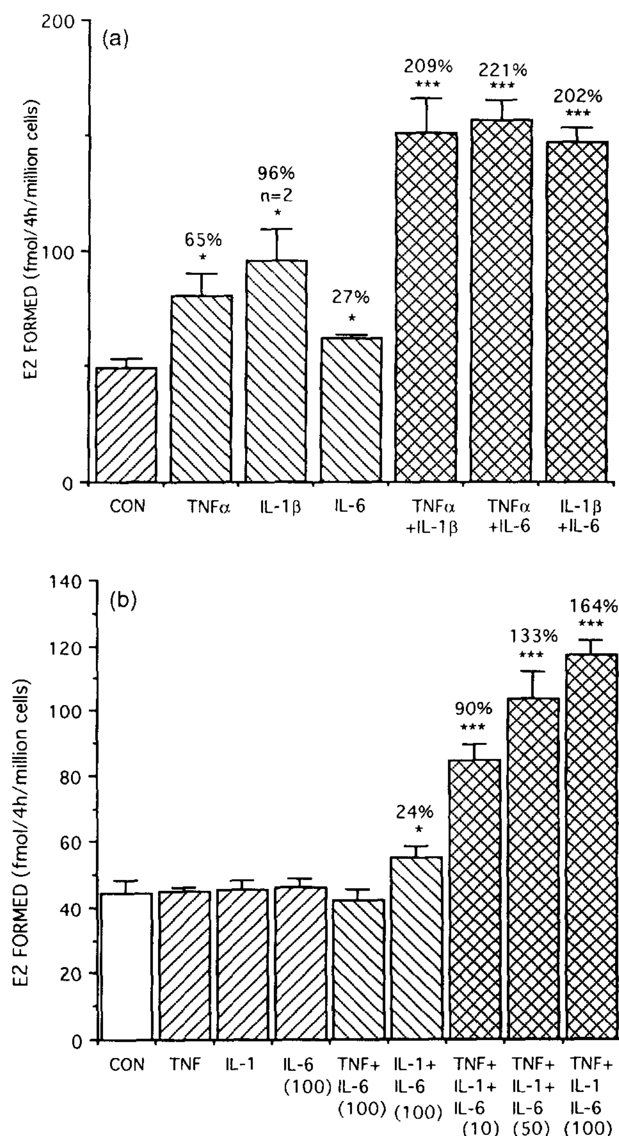

Fig. 2. Synergistic stimulation of red-E_2DH activity in MCF-7 cells by TNFα, IL-1β and IL-6. *$P < 0.05$, * < 0.001. Concentration used (ng/ml): (a) TNFα = 10; IL-1β = 100; IL-6 = 100 and (b) TNFα = 20; IL-1β = 20; IL-6 = 10, 50 and 100.**

200 kDa (0.7 ng/ml). Both of these peaks corresponded to peaks in red-E_2DH stimulation. The fractions were also assayed for IL-1β and TNFα immunoreactivity, but none was detected.

DISCUSSION

These studies to examine the regulation of E_2DH activity in MCF-7 cells have revealed that TNFα, IL-1β and IL-6 are able to stimulate the reductive activity of this enzyme. However, there was considerable variation in the extent of stimulation, with TNFα and IL-1β increasing activity by up to 1044 and 96%, respectively, while the effect of IL-6 was minimal (0–27%) at 100 ng/ml. In all of the combinations tested, these cytokines were able to act synergistically to further enhance red-E_2DH activity. Additive or synergisitic effects of these cytokines have been shown in many systems. This could be explained either by their use of different signal transduction pathways, or by the induction by TNFα, IL-1β and IL-6 of their own or each other's production and/or that of their receptors. Further studies are currently in progress to examine the mechanism(s) responsible for the interactions we have shown.

Although rIL-6 alone resulted in minimal, if any, stimulation of red-E_2DH activity in MCF-7 cells, we were able to measure significant IL-6 concentrations in fractionated CM. In addition to testing the Bachem source, rIL-6 was obtained from Amersham International and the National Institute for Biological Standards and Control. IL-6 from these sources had no significant effect on red-E_2DH activity. Similar results were obtained whether IL-6 treatments were prepared in phenol red-containing medium with FCS, phenol

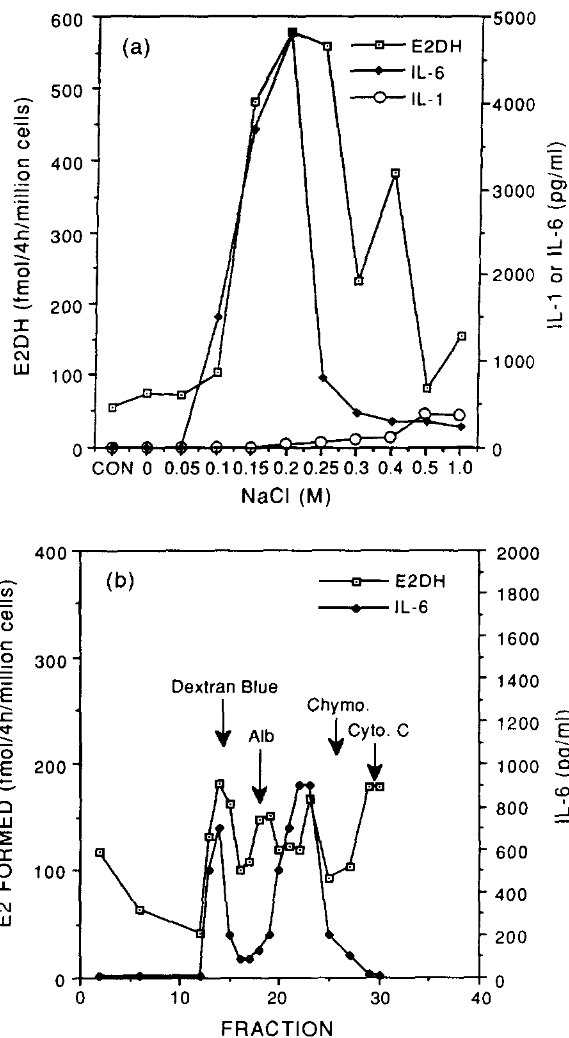

Fig. 3. Red-E_2DH stimulatory activity and cytokine concentrations in fractionated CM. (a) DEAE cellulose anion ion-exchange fractions. (b) G-100 molecular sieve fractions. Control (red-E_2DH activity) = 37 fmol/4 h/million cells.

red-free medium with SFCS or in phenol red-free, serum-free medium.

These results, whilst confirming that IL-6 is present in tumour fibroblast CM, suggest that this cytokine alone has little effect on red-E_2DH activity. This finding is in contrast to a previous report suggesting that IL-6 is the major factor in CM responsible for stimulating red-E_2DH activity [7]. In that report, however, whilst CM containing IL-6 at a concentration of 2.4 ng/ml resulted in a 250% stimulation of enzyme activity, rIL-6 from NIBSC at the much higher concentration of 80 ng/ml was only able to stimulate activity by 150%. The present study showed similar stimulation by CM with red-E_2DH activity stimulated almost 1000-fold by fractions in which the concentration of IL-6 was <5 ng/ml. It is therefore apparent that other cytokines produced by fibroblasts contribute to the marked stimulation achieved by exposing MCF-7 cells to CM.

Fractionation of CM by molecular sieve chromatography revealed two peaks of IL-6 immunoreactive species, one with the monomeric molecular weight for this cytokine and the other with a molecular weight in excess of 200 kDa. Matsuda *et al.* [18] have previously shown that α_2-macroglobulin is a carrier protein for IL-6 and that even when bound to this protein, full activity is preserved. It is possible that the high molecular weight form of IL-6 present in CM represents the cytokine bound to α_2-macroglobulin, but further studies are required to confirm this finding. IL-1β was also present in the CM and may contribute to its stimulatory effect on red-E_2DH activity. TNFα was not detected in CM but it is possible that this cytokine is present in a protein-bound or multimeric form not recognized by the antisera used in our cytokine assay.

Oxidative E_2DH activity was not affected by the cytokines tested. Although it has been reported that a unique gene encodes E_2DH [19], it would appear that several isoforms of the enzyme exist in breast tissues [20], and this may explain the differential stimulation of activity. Alternatively, co-factor levels for the enzyme may be limiting and the effects we have shown may be the result of altered NAD(P)/NAD(P)H ratios or levels.

The possibility of paracrine roles for TNFα, IL-1β and IL-6 in breast cancer is therefore evident. Whilst we have shown both IL-1β and IL-6 to be produced by breast tumour-derived fibroblasts, adipocytes (which are present in large numbers in the post-menopausal breast where breast cancer is most prevalent) have recently been reported to produce TNFα [21]. Autocrine roles for the cytokines may also be important since epithelial cells are able to produce all three cytokines [22–24].

A range of cytokines have now been implicated in the regulation of E_2DH activity in the breast. They, of course, do not operate in isolation and we have shown using only three such peptides that interactions between them may produce much larger responses than the individual cytokines. It is likely that *in vivo*, such factors act synergistically to enhance red-E_2DH activity and such a mechanism may account for the high concentrations of oestradiol which are detected in breast tumours.

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