Inhibition of IL-6+IL-6 soluble receptor-stimulated aromatase activity by the IL-6 antagonist, Sant 7, in breast tissue-derived fibroblasts

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Interleukin 6 (IL-6) and its soluble receptor (IL-6sR) can markedly stimulate aromatase activity in cultured fibroblasts derived from normal or malignant breast tissues. IL-6 acts by binding to a low-affinity membrane-spanning receptor (IL-6R), which must associate with a high-affinity receptor (gp130) for signal transduction to occur. Sant 7 is a mutated form of IL-6 that can bind to the IL-6R, but inhibits its ability to interact with the gp130 signal transducing protein. In this study, we have used Sant 7 to examine its ability to inhibit IL-6+IL-6 soluble receptor (IL-6sR)-stimulated aromatase activity in breast tissue-derived fibroblasts. As previously observed, IL-6+IL-6sR markedly stimulated aromatase activity (7.7–20.8-fold) in fibroblasts derived from reduction mammoplasty tissue, tissue proximal to tumours and breast tumours. Sant 7 inhibited basal aromatase activity in some fibroblasts by 25–30% that had a high basal activity, but almost completely blocked the ability of IL-6+IL-6sR to stimulate aromatase activity. The IC50 for the inhibition of IL-6+IL-6sR-stimulated aromatase activity by Sant 7 was 60 ng ml–1. A comparison of the effects of prostaglandin E2 (PGE2), which can also regulate aromatase activity, and IL-6+IL-6sR revealed a greater degree of aromatase stimulation by IL-6+IL-6sR. Sant 7, however, inhibited PGE2-stimulated aromatase activity by 70% suggesting that PGE2 acts, in part, by stimulating IL-6 production. Much of the IL-6 and IL-6sR available to stimulate breast tumour aromatase activity may originate from infiltrating macrophages and lymphocytes. The ability to block aromatase stimulation by these factors may offer a novel therapeutic strategy for reducing oestrogen synthesis in breast tumours.

Keywords: breast cancer; aromatase; cytokines; interleukin 6; prostaglandin E2; Sant 7

A number of potent aromatase inhibitors have now been introduced for use in postmenopausal women with hormone-dependent breast tumours (Miller, 1999). While their development represents an important advance in the therapies available for the treatment of women with breast cancer, the complete and partial response rates, when used as second-line therapy, remain relatively low (10–20%) with the time to tumour progression being relatively short (3–6 months) (Santen and Harvey, 1999). However, in a recent trial into the adjuvant use of an aromatase inhibitor vs tamoxifen, alone or in combination, disease-free survival was significantly longer for subjects receiving aromatase inhibitor therapy (ATAC Trialist Group, 2002). In addition, a number of adverse side effects, including gastrointestinal problems, dizziness and nausea, are associated with the use of some of the inhibitors (Buzdar et al, 1997). The use of aromatase inhibitors in postmenopausal women with breast cancer has also been reported to have an unfavourable effect on the serum lipid profile (Elisaf et al, 2001). There is, therefore, a need to develop new methods of inhibiting aromatase activity that may act specifically within the breast but spare other oestrogen-sensitive tissues.

The aromatase enzyme complex, which converts androstenedione to oestrone, has a pivotal role in controlling oestrogen synthesis in peripheral tissues in postmenopausal women. The enzyme is present not only in adipose tissue but also in normal and malignant breast tissues (James et al, 1993). Previous studies have revealed that the expression of the aromatase gene is regulated in a tissue-specific manner by the use of a number of different promoters (Mahendroo et al, 1993; Zhao et al, 1995a,b). Cytokines in the presence of glucocorticoids regulate gene expression via PI4. The 5’-upstream region of this promoter contains a glucocorticoid response element and a GAS (IFNg, activating sequence) element, which can bind transcription factors of the signal transducer and activation of transcription (STAT) family (Zhao et al, 1995a,b).
Cells were routinely passaged two to three times after which replicate 25 cm² culture flasks were seeded and grown to 70–80% confluency. The medium was replaced with 2% charcoal-stripped FCS, phenol red-free EMEM and treatments were added in this medium for 48 h in the presence of dexamethasone (100 nM) and included: IL-6+IL-6R (50 and 100 ng ml⁻¹, R&D Systems Ltd, Abingdon, Oxon, UK) and PGE₂ (10 μM, Sigma, Poole, Dorset, UK).

Sant 7
The IL-6R superantagonist Sant 7 was obtained from Sigma-Tau (Rome, Italy) and synthesised as previously described (Salvati et al., 1995). Sant 7 was dissolved in culture medium before adding to cells.

Aromatase assay
Aromatase activity was measured in intact fibroblast monolayers using (1β-³H) androstenedione (15–30 Ci mmol⁻¹, NEN- Du Pont, Stevenage, Herts, UK) over a 3–20 h period (Reed et al., 1992). Briefly, fibroblast monolayers were washed once with Earle’s balanced salt solution EM (2.5 ml) unless stated otherwise. To each flask ³H androstenedione (0.25 μCi) was added to give a final substrate concentration of 3–4 nM. Fibroblast monolayers were incubated with substrate for 3–20 h at 37 °C depending on the basal aromatase activity in the cells. Flasks containing no cells were also incubated with substrate and serum-free medium as assay blanks. After incubation, an aliquot of medium (2 ml) was removed from each flask and aliquots were extracted twice with diethyl ether (5 ml), which was discarded. The remaining aqueous phase was treated with an equal volume of a solution containing charcoal (5.0%) and dextran (0.5%), centrifuged and an aliquot of the supernatant (1 ml) was taken to determine its radioactive content by liquid scintillation spectrometry. It has previously been established that aromatase activity, as measured in fibroblasts, is linear with respect to time for up to 24 h (Macdiarmid et al., 1994).

Statistics
The significance of differences in aromatase activity in treated and control cells was assessed using Student’s t-test. Representative example of results are shown for experiments that were repeated 2–3 times.

RESULTS
The ability of Sant 7 to inhibit cytokine-stimulated aromatase activity was initially examined in fibroblasts derived from breast adipose tissue of a subject undergoing reduction mammoplasty (Figure 1). In these fibroblasts, IL-6 alone, (at 50 ng ml⁻¹) increased aromatase activity by 27%. The addition of IL-6R in combination with IL-6, however, markedly potentiated its ability to stimulate aromatase activity (7.7-fold compared with controls). Sant 7 caused a significant (P<0.05) decrease in basal aromatase activity and IL-6-stimulated activity (P<0.001). Sant 7 was able to almost completely block the ability of IL-6+IL-6R to stimulate aromatase activity. In a further series of experiments, the ability of Sant 7 to block cytokine-stimulated aromatase activity in fibroblasts derived from tissue proximal to a tumour (proximal fibroblasts) and also the tumour (tumour fibroblasts) from the same subject was examined (Figures 2A and B). In the presence of dexamethasone, basal aromatase activity was 10 times higher in proximal fibroblasts than in tumour fibroblasts. The extent to which IL-6+IL-6R-stimulated aromatase was also greater in proximal fibroblasts (20.8-fold) than in tumour fibroblasts (7.9-fold). No apparent differences were detected in the cellular homogeneity or viability of fibroblasts derived from tumour or...
The ability of PGE2 to stimulate aromatase activity in proximal and tumour fibroblasts was considerably greater by 521 and 103%, respectively (Figures 5A and B). The extent of stimulation by IL-6+IL-6sR was considerably greater seen in the absence or presence of dexamethasone. However, the responses to IL-6, IL-6sR or Sant 7, alone or in combination was of this glucocorticoid (Figure 4). As Sant 7 effectively blocks stimulation of aromatase activity by the induction of IL-6 (Singh et al., 1993). There is evidence, however, that PGE2 may act, in part, to bind to the IL-6R in an irreversible manner. The results obtained from these studies have confirmed previous findings that IL-6+IL-6sR can markedly stimulate aromatase activity in breast tissue-derived fibroblasts (Singh et al., 1995; Zhao et al., 1995a,b). The potentiation by IL-6sR of the IL-6 stimulation of aromatase activity presumably results from an increase in the interaction of the IL-6–IL-6sR complex with the gp130 signal-transduction protein. In some cell systems the proximal breast tissues that might explain the marked differences in basal and IL-6+IL-6sR-stimulated aromatase activity in these different fibroblasts. In both types of fibroblasts Sant 7 completely blocked IL-6+IL-6sR stimulation of aromatase activity. Preincubation of proximal fibroblasts with Sant 7, prior to the addition of IL-6+IL-6sR, did not increase its ability to block aromatase stimulation (Figure 2A). A dose–response study was carried out using proximal fibroblasts. The IC50 was calculated as the concentration of Sant 7 that inhibited IL-6+IL-6sR-stimulated aromatase activity by 50% and was 60 ng ml⁻¹ (Figure 3).

To examine if dexamethasone was an absolute requirement for the ability of Sant 7 to block IL-6+IL-6sR-induced aromatase activity, an experiment was carried out in the absence or presence of this glucocorticoid (Figure 4). As shown, a similar pattern of responses to IL-6, IL-6sR or Sant 7, alone or in combination was seen in the absence or presence of dexamethasone. However, the extent of simulation by IL-6+IL-6sR was considerably greater (772%) in its presence than in its absence (252%). Sant 7 did inhibit the IL-6+IL-6sR-induced aromatase activity in the absence of dexamethasone.

The regulation of aromatase gene expression is complex and controlled by factors such as PGE2 and IL-6 (Mahendroor et al., 1993). There is evidence, however, that PGE2 may act, in part, to stimulate aromatase activity by the induction of IL-6 (Singh et al., 1999). As Sant 7 effectively blocks stimulation of aromatase activity by IL-6+IL-6sR, it was used to obtain further insight into its regulation by IL-6 or PGE2. In proximal and tumour fibroblasts, derived from the same subject, PGE2 stimulated aromatase activity by 521 and 103%, respectively (Figures 5A and B). The combination of IL-6+IL-6sR was considerably more potent at stimulating activity in proximal and tumour fibroblasts than PGE2, by 9.6– and 7.1-fold, respectively. In these fibroblasts Sant 7 itself inhibited basal aromatase activity by 30%. Sant 7 reduced the ability of PGE2 to stimulate aromatase activity in proximal and tumour fibroblasts by 69 and 75%, respectively. In these experiments the ability of Sant 7 to act in a reversible or irreversible manner was also examined (Figures 4A and B). Fibroblasts were preincubated for 12 h with Sant 7, after which it was removed from the cells by washing with phosphate-buffered saline. Subsequent addition of IL-6+IL-6sR showed that they were able to stimulate aromatase activity indicating that Sant 7 did not bind to the IL-6R in an irreversible manner.

**DISCUSSION**

The results obtained from these studies have confirmed previous findings that IL-6+IL-6sR can markedly stimulate aromatase activity in breast tissue-derived fibroblasts (Singh et al., 1995; Zhao et al., 1995a,b). The potentiation by IL-6sR of the IL-6 stimulation of aromatase activity presumably results from an increase in the interaction of the IL-6–IL-6sR complex with the gp130 signal-transduction protein. In some cell systems the
combination of IL-6+dexamethasone can markedly upregulate the expression of gp130 mRNA (Schoollink et al, 1992). In a previous study, the ability of Sant 7 to inhibit the proliferation of multiple myeloma cells was found to be dependent upon the presence of dexamethasone and retinonic acid (Honemann et al, 2001). This was not the case in the present study where Sant 7 was able to inhibit IL-6+IL-6sR-induced aromatase activity in the absence of glucocorticoid. The ability of IL-6+IL-6sR to stimulate aromatase activity was almost completely blocked by Sant 7 in all the fibroblasts examined. Sant 7 is a mutated form of IL-6 that binds to the IL-6R with an increased affinity that results in an inactive configuration of the receptor (Demartis et al, 1996). In addition to blocking cytokine-stimulated aromatase activity, Sant 7 also blocked IL-6R-stimulated aromatase activity in some fibroblasts that had a relatively high basal activity, by up to 30%. It has previously been shown that breast tissue-derived fibroblasts can secrete IL-6.
The finding that Sant 7 can reduce basal aromatase activity in these cells suggests that the IL-6 they produce is able to act in an autocrine/paracrine manner to increase aromatase activity.

In related studies into the control of aromatase activity, the ability of a number of 10–16 amino-acid peptides to inhibit IL-6+IL-6sr-stimulated aromatase activity was previously examined (Parish et al, 2001). The 16 amino-acid peptide, AROHIB, at 10 μM inhibited the ability of these cytokines to stimulate aromatase activity by 65%. AROHIB is therefore a less potent inhibitor of IL-6+IL-6sr-stimulated aromatase activity than Sant 7. Furthermore, to be effective it was necessary to preincubate cells with AROHIB prior to the addition of IL-6+IL-6sr. For Sant 7 no preincubation period was found to be necessary. Sant 7, however, does not bind to the IL-6R in an irreversible manner as preincubation of fibroblasts followed by washing with phosphate-buffered saline restored the ability of IL-6+IL-6sr to stimulate aromatase activity.

There is now good evidence that malignant fibroblasts produce IL-6 and IL-6sr and that tumour infiltrating macrophages and lymphocytes may also be an important source of factors that can stimulate oestrogen synthesis in breast tumours (Purohit et al, 1995; Singh et al, 1997). If IL-6 and IL-6sr, derived from these cells, are important regulators of aromatase activity, then the use of Sant 7 may offer a means of selectively blocking aromatase stimulation within the breast. Although small molecule-based aromatase inhibitors are being used for breast cancer therapy they can only be used in postmenopausal women. Their use in premenopausal women results in increased gonadotrophin production that overcomes the aromatase blockade. Thus, the ability to inhibit cytokine-stimulated aromatase activity in breast tissues of premenopausal women, either in the preventive or therapeutic setting, could be an important option for the use of Sant 7.

In addition to cytokines stimulating aromatase activity, PGE2 has also been implicated in the control of this enzyme (Zhao et al, 1996). However, determining the extent of regulation of aromatase activity by PGE2 in fibroblasts is complicated by the finding that PGE2, or factors that can increase intracellular CaMP levels, can stimulate IL-6 secretion by cells (Zhang et al, 1988; Hinson et al, 1996). Sant 7 was therefore employed in an attempt to determine whether PGE2 acts to stimulate aromatase activity by induction of IL-6. It was reasoned that if PGE2 is acting by the induction of IL-6, then Sant 7 should block, or reduce, its ability to stimulate aromatase activity. It has previously been shown that the ability of PGE2 to stimulate aromatase activity in breast tissue-derived fibroblasts is associated with a significant increase in IL-6 production by these cells (Singh et al, 1997). As consistently observed in previous studies, the ability of PGE2 to stimulate aromatase activity in normal and malignant fibroblasts (60% and 100%, respectively) was considerably lower than that achieved with IL-6+IL-6sr (960 and 710%, respectively). Sant 7 reduced the PGE2 stimulation of aromatase activity by 69 and 75% in proximal and tumour fibroblasts, respectively. As Sant 7 only blocks IL-6-stimulated activity, this important finding indicates that a major part of the ability of PGE2 to stimulate aromatase activity results from its effect on IL-6 production.

IL-6 is a pleiotropic cytokine that has a number of important physiological functions (Van Snick, 1990). Excess production is associated with a number of pathological conditions including multiple myeloma (Ludwig et al, 1991). IL-6 is also known to have a role in regulating androgen receptor expression in an androgen-independent manner (Chen et al, 2000). As a result it may be involved in making prostate tumours resistant to endocrine therapy (Lin et al, 2001). By blocking the action of IL-6, Sant 7 has been shown to potentiate the sensitivity of the hormone-dependent prostate carcinoma cell line PC-3 to the cytotoxic effects of etoposide and cisplatin (Borsellino et al, 1999). The development of IL-6R superantagonists should allow the role of IL-6 in hormone-dependent and -independent conditions, such as breast and prostate cancer, to be elucidated and may lead to their use as novel therapeutic options for their treatment.

REFERENCES

Agarwal V, Buhun SE, Leitch M, Rohrich R, Simpson ER (1996) Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients. J Clin Endocrinol Metabol 81: 3843 – 3849

ATAC Trialist Group (2002) Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with breast cancer: first results of the ATAC randomised trial. Lancet 359: 2131 – 2139

Borsellino N, Bonavida B, Ciliberto G, Toniatti C, Travali S, D’Alessandro N (1999) Blocking signalling through the gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumour cells to etoposide and cisplatin-mediated cytotoxicity. Cancer 85: 134 – 144

Buzdar AU, Jones SE, Vogel CL, Worster J, Plourde P, Webster A (1997) Aromatase inhibition in breast cancer: first results of a randomized trial. Breast Cancer 7: 730 – 739

Chen T, Wang LH, Farrar WL (2000) Interleukin 6 activates androgen receptor-mediated gene expression through a signal transducer and activator of transcription 3-dependent pathway in LNCap prostate cancer cells. Cancer Res 60: 2132 – 2135

Demartis A, Bernassola F, Savino R, Melino G, Ciliberto G (1996) Interleukin 6 receptor superantagonists are potent inducers of human multiple myeloma cell death. Cancer Res 56: 4213 – 4218

Elisaf MS, Bairakrati ET, Nicolaides C, Kakkad R, Tzallas CS, Katsaraki A, Pavlides NA (2001) Effect of letrozole on the lipid profile in postmenopausal women with breast cancer. J Clin Endocrinol Metab 86: 1510 – 1513

Grodin JM, Siiteri PK, MacDonald PC (1973) Sources of estrogen production in postmenopausal women. J Clin Endocrinol Metabol 36: 207 – 214

Harada N, Utsumi T, Takagi Y (1993) Tissue-specific expression of the human aromatase cytochrome P450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc Natl Acad Sci USA 90: 11312 – 11316

Hemsell DL, Grodin JM, Brenner PF, Sitteri PK, MacDonald PC (1974) Plasma precursors of estrogen II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. J Clin Endocrinol Metabol 38: 476 – 479

Hinson RM, Williams JA, Shacter E (1996) Elevated interleukin-6 is induced by prostaglandin E2 in a murine model of inflammation: possible role of cyclooxygenase 2. Proc Natl Acad Sci USA 93: 4885 – 4890

Hornemann D, Chatterjee M, Savino R, Bomment R, Gramatzki M, Dorken B, Bargou RC (2001) The IL-6 receptor antagonist Sant 7 overcomes bone marrow stromal cell-mediated drug resistance of multiple myeloma cells. Int J Cancer 93: 675 – 680

James VHT, McNeill JM, Lai LC, Newton CJ, Ghilchik MW, Reed MJ (1987) Aromatase activity in normal breast and breast tumour tissues: in vivo and in vitro studies. Steroids 50: 269 – 279

Kishimoto T, Akira S, Nagarzaki M, Taga R (1995) Interleukin-6 family of cytokines and gp130. Blood 86: 1243 – 1254

Klein B, Wijdenes J, Zhang X-G, Jourdan M, Boiron J-M, Brochier J, Liatard J, Merlin L, Clement C, Mourel-Fournier B, Zhao-Liang L, Mannoni P, Sany J, Bailleul R (1991) Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukaemia. Blood 79: 1198 – 1204

Lin D-L, Whitney MC, Yao Z, Keller ET (2001) Interleukin-6 induces androgen responsiveness in prostate cancer cells through up-regulation of androgen receptor expression. Clin Cancer Res 7: 1773 – 1781

Ludwig H, Nacbmard BM, Fritz E, Krainer M, Huber H (1991) Interleukin-6 is a prognostic factor in multiple myeloma. Blood 77: 2794 – 2795
