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Prostaglandin E_2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators

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Summary Malignant human breast tumours contain high levels of prostaglandin E_2 (PGE_2). However, the mechanisms controlling PGE_2 production in breast cancer are unknown. This *in vitro* study investigates the capacity for PGE_2 synthesis and metabolism in several human breast cancer cell lines and early passage human breast fibroblasts and seeks to identify potential regulatory factors which may control these pathways. Basal PGE_2 production rose up to 30-fold in breast fibroblast lines on addition of exogenous arachidonic acid (10 μM), whereas no such changes were observed in six out of seven cancer cell lines, with the exception of modest increases in MDA-MB-231 cells. Interleukin 1β (IL-1β) also induced PGE_2 production in breast fibroblasts in the presence of excess substrate, consistent with cyclo-oxygenase induction by the cytokine. Under these conditions only Hs578T cells and MDA-MB-231 cells demonstrated large increases in PGE_2 in response to IL-1β or phorbol ester; no such responses were seen in MCF-7, T47-D, ZR-75-1, BT-20 or CLF-90-1 cells. In the absence of added arachidonate, bradykinin (BK) and endothelin-1 (ET-1), potentiated PGE_2 production in IL-1β-treated fibroblasts, possibly by mobilising endogenous substrate. PGE_2 also stimulated ET-1 production by breast cancer cells. In co-cultures with T47-D cells both basal and stimulated PGE_2 production by breast fibroblasts was greatly reduced. This appeared to be due to metabolic inactivation by the cancer cell since T47-D cells readily converted PGE_2 to 15-keto-PGE_2. This apparent 15-hydroxy-PG dehydrogenase activity was stimulated by TPA and inhibited by cycloheximide. In conclusion, breast fibroblasts, particularly under the influence of inflammatory mediators, provide a potentially rich source for PGE_2 production in breast tumours, whereas significant contributions from the epithelial tumour component may be restricted to cancer cells exhibiting an invasive phenotype. Metabolic inactivation by the cancer cells may also play an important role in the regulation of breast tumour PGE_2 levels.

Keywords: Breast cancer; prostaglandin; endothelin; interleukin-1β, bradykinin

Elevated levels of prostaglandin E_2 (PGE_2) have been widely reported in malignant human breast tumours (Bennett, 1979; Rolland *et al.*, 1980; Karmali *et al.*, 1983) as well as experimental murine mammary tumour models (Tan *et al.*, 1974). Furthermore, these high levels are often associated with tumours exhibiting high metastatic potential (Rolland *et al.*, 1980; Karmali *et al.*, 1983). Similarly, *in vitro* studies with tissue explants or primary cultures of human breast tumour cells have also demonstrated increases in PGE_2 production from malignant tissue compared with benign or normal (Watson and Chuah, 1985; 1992). Although the pathophysiological significance of this correlation in the context of a role for PGE_2 in breast cancer growth and progression is unclear, several studies with murine mammary tumour models indicate that PGE_2 may indeed play a multifunctional role in controlling growth, metastasis and host immune responses (Foecking *et al.*, 1982; Fulton and Heppner 1985; Fulton *et al.*, 1991). PGE_2 has been shown to be mitogenic for normal human and murine mammary epithelial cells (Bandyopadhyay *et al.*, 1987; Balakrishnan *et al.*, 1989) but inhibits both human breast cancer cell growth (Fentiman *et al.*, 1984) and DNA synthesis in human breast fibroblasts (Patel and Schrey, 1992). Both stromal (Fernig *et al.*, 1990) and epithelial (Bandyopadhyay *et al.*, 1987) murine mammary cells have been implicated as potential sources for this eicosanoid.

Notwithstanding the considerable evidence supporting a role for PGE_2 production in breast tumour biology, neither the nature of the cell types involved nor the paracrine factors controlling PGE_2 levels in human breast tumour tissue are known. In the present study we sought to address these questions by monitoring PGE_2 production in several human breast cancer cell lines and breast stromal fibroblasts in response to various putative agonists of both phospholipase A_2 and cyclo-oxygenase, the two rate-limiting enzymes in PG synthesis.

Under the combined influence of the inflammatory mediators IL-1β and BK breast fibroblasts represent a potentially rich source of PGE_2. In contrast, under the various conditions employed we were unable to detect any PGE_2 production in the majority of breast cancer cell lines investigated, with the exception of two hormone-independent lines (MDA-MB-231 and Hs578T). Net PGE_2 production by breast fibroblasts was greatly reduced in co-cultures with breast cancer cells (T47-D or MCF-7). This appeared to be due to metabolic inactivation by PG dehydrogenase present in the cancer cells.

Materials and methods

[5,6,8,11,12,14,15(n)-^{3}H] PGE_2 160 Ci mmol^{-1} was obtained from Amersham International UK. Anti-PGE_2 methyl oxime was a gift from Dr RW Kelly, Edinburgh, UK. Antibodies to endothelin 1 (ET-1) were obtained from Alan O'Shea and Terry Jowett, University College London, UK. 15-Keto PGE_2 was obtained from Cascade Biochem, Reading, UK. Arachidonate and all other PG metabolites as well as bradykinin (BK), des-Arg^{9}BK, ET-1 and 12-*O*-tetradecanoylphorbol (TPA) were purchased from Sigma (UK). IL-1β was obtained from Bachem (UK). All culture media and cell culutre products were obtained from Flow Laboratories (UK). All other chemicals and reagents were of analytical grade and were obtained from Fison (UK).

Human breast cancer cell lines

T47-D, MDA-MD-231 and Hs578T cell lines were obtained from the National Cell Bank at Porton Down, UK. MCF-7, ZR-75-1 and BT-20 cells were respectively obtained from Dr M Lippman (Georgetown University, Washington DC,

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USA), Dr M O'Hare (Haddow Laboratories, Sutton, UK) and Dr J Taylor-Papadimitriou (Imperial Cancer Research Fund Laboratories, London, UK). CLF-90-1 cells were established in our own laboratory from tissue fragments of a primary ductal infiltrating carcinoma of the breast and display a typical epithelial morphology in culture. CLF-90-1 cells are oestrogen and progesterone receptor-positive and exhibit growth stimulation and growth inhibition in response to oestradiol and medroxyprogesterone acetate respectively. All these cell lines were maintained as previously described (Patel and Schrey, 1990) in Eagle's minimal essential medium (EMEM) supplemented with glutamine (2 mM) and bicarbonate (4.5 mM) containing 20 mM Hepes (pH 7.4) and 5% fetal calf serum (FCS) with the exception of BT-20 which was grown in 10% FCS.

Human breast fibroblasts

Human breast fibroblasts (HBFs) were prepared and maintained in culture as previously described (Patel and Schrey, 1992). Normal tissue was obtained from three women undergoing reductive mammoplasty. Following tissue disaggregation with collagenase, primary cultures grown to confluence were subcultured in EMEM containing the above supplements with 10% FCS. The three cell lines obtained (HBF-10,-11,-12) exhibited typical fibroblast-like morphology at confluence forming whorls and parallel bundles of spindle-shaped cells. Cells with epithelial morphology were absent. Experiments with these breast fibroblast cultures employed cells between passages 3 and 7.

For studies monitoring PGE_2 production in either fibroblasts or cancer cell lines, cells were grown to 70% confluence in either 12- or 24-well plates (containing 1 ml or 0.5 ml medium respectively), serum-starved for 4 h, and incubated for 18 h in the presence or absence of IL-1β and/or arachidonic acid. The next day the cells were incubated in fresh media for various times with different agents (see Figure legends for details). Unless otherwise stated studies employing cancer cell lines monitored PGE_2 production during the initial 18 h period. PGE_2 release into the medium was measured by radioimmunoassay (see below).

Co-culture studies

In some experiments comparative studies were conducted to monitor PGE_2 production in co-cultures of breast fibroblasts and T47-D cells and separate parallel cultures of fibroblasts and T47-D cells alone. Fibroblasts grown to 50% confluence in 12-well plates with EMEM containing 10% FCS were seeded with T47-D cells (5×10^5 cells) and cultured for 18 h. Separate cultures comprising appropriate numbers of either T47-D cells or fibroblasts alone were also established. All cultures were serum-starved for 4 h and incubated for a further 18 h in the presence or absence of IL-1β (1 ng ml^{-1}) in EMEM containing 10 μM arachidonate. Instead of being selected for co-culture some fibroblasts were incubated with T47-D cell-conditioned medium throughout this protocol. This conditioned medium was obtained from T47-D cells incubated overnight under exactly the same conditions of cancer cell density and media composition employed in the co-culture study. PGE_2 release into the media after this 18 h incubation was measured by radioimmunoassay. PGE_2 was undetectable in T47-D cell conditioned medium.

In studies employing breast cancer cell lines, cell numbers were determined by releasing and counting cell nuclei in a Coulter counter as previously described (Patel and Schrey, 1990). This technique was readily applicable to co-culture studies since fibroblasts remained attached and resistant to the Zaponin (Coulter Electronic, Luton, UK) treatment conditions employed to release cancer cell nuclei. Fibroblast content per well was quantitated by measuring cellular protein using the method of Lowry *et al.* (1951). However, no differential measurements of either cancer cell number or fibroblast content were apparent in any incubations over the relatively short time span of the experiments irrespective of the different conditions employed. Hence, PGE_2 production has been consistently expressed as ng ml^{-1} incubation medium.

PGE_2 measurement radioimmunoassay

All experiments monitoring PGE_2 production by fibroblasts and cancer cell lines were performed in serum-free EMEM. Release of PGE_2 into the culture media was measured using a specific radioimmunoassay as previously described (Kelly *et al.*, 1986). The antisera employed was raised to the methyl oxime of PGE_2. At the end of each experiment aliquots of the culture media were derivatised at room temperature overnight with equal volumes of methyloximating reagent (Kelly *et al.*, 1986). No agents employed in this study interfered with the assay at the concentrations used. The sensitivity of the assay is 15 pg ml^{-1}. The percentage cross-reactivity of the antibody with various derivatised arachidonate metabolites has been determined as: PGE_2 100; 15-keto PGE_2 0.25; $PGF_{2\alpha}$ <0.02; PGB_2 0.2; PGD_2 0.02; TXB_2 <0.02; 6-keto $PGF_{1\alpha}$ 0.02. The mean intra-assay coefficient of variation was 6.2%. All samples within the same experimental group were measured in the same assay.

Endothelin immunoradiometric assay

The production of ET-1 by MCF-7 cells was measured using a specific solid phase-based sandwich assay in 96-well microtitre plates as previously described (Patel and Schrey, 1995). The sensitivity of the assay is 0.5 pmol l^{-1} with percentage cross-reactivities for ET-1, big ET-1, ET-2 and ET-3 being 100, 0.01, 100 and 50 respectively.

[^{3}H]PGE_2 metabolism

T47-D cells grown to 70% confluence in 12-well plates were serum starved for 4 h and then incubated for 24 h in 1 ml medium with or without TPA (10 nM) or cycloheximide (1 μM). During the last 4 h of this 24 h period [^{3}H]PGE_2 (0.1 μCi) was added to the cells at a final concentration of 1 μM. Incubations were terminated and PGE_2 metabolites were extracted, chromatographed and the radioactivity profile determined. The position of various PGE_2 metabolites including 15-keto-PGE_2 was determined with various standards and R_F values obtained agreed with those previously reported for this TLC system (Mitchell *et al.*, 1993). Control parallel incubations were always performed with [^{3}H]PGE_2 in the absence of cells and the corresponding blanks for each area of the plate were subtracted from the appropriate product bands.

Expression of data

All values are presented as the means ± s.d. from individual representative experiments each performed in triplicate unless otherwise stated. Statistical evaluation of the data when comparing control groups with treatment groups was by the Student's *t*-test.

Results

PGE_2 production in different human mammary cell lines

PGE_2 production was monitored in ten different human breast mammary cell lines: three stromal fibroblast lines (HBF-10,-11,-12); four hormone-dependent cancer cell lines (MCF-7, T47-D, ZR-75-1 and CLF-90-1) and three hormone-independent lines (BT-20, MDA-MB-231 and Hs578T). Under optimal conditions in the presence of 10 μM arachidonate, basal PGE_2 production increased up to 30-fold in breast fibroblasts, whereas with the exception of MDA-MB-231 cells, no changes in PGE_2 production were observed in the presence of exogenous substrate in any breast cancer cell lines (Table I). IL-1β appeared to be a potent inducer of

Table I PGE_2 production in different human mammary cell lines

Cell line	PGE_2 production, pg ml^{-1} Basal	AA	IL-1β	IL-1β + AA	TPA + AA
HBF-10	45 ± 13[a]	1380 ± 182*	344 ± 20*	3441 ± 132**	ND[b]
HBF-11	22 ± 4	358 ± 14*	485 ± 63*	2760 ± 208**	ND
HBF-12	23 ± 9	763 ± 42*	674 ± 100*	4515 ± 510	ND
MCF-7, T47-D,	<15	<15	<15	<15	<15
ZR-75-1, BT-20,	<15	<15	<15	<15	<15
CLF-90-1	<15	<15	<15	<15	<15
MDA-MB-231	31 ± 13	162 ± 20*	22 ± 2	236 ± 12*	966 ± 204*
Hs578T	25 ± 8	44 ± 2	27 ± 4	610 ± 114*	4410 ± 531*

PGE_2 production by fibroblasts (HBF 10–12) and cancer cell lines was measured by radioimmunoassay following treatment of the cells with or without arachidonic acid (AA, 10 μM), IL-1β (1 ng ml^{-1}) or TPA (10 nM) as described in Materials and methods, [a]Mean ± s.d., $n = 3$. [b]ND, not determined. *$P < 0.001$ compared with basal; **$P < 0.001$ for synergistic response.

cyclo-oxygenase in both breast fibroblasts and the carcinosarcoma line Hs578T, since synergism was clearly observed with IL-1β in the presence of arachidonate (Table I). No cytokine-inducible PGE_2 production was detectable in any other breast cancer cell lines. The phorbol ester TPA, a known inducer of cyclo-oxygenase in various epithelial cell lines, caused large increases in PGE_2 in MDA-MB-231 and Hs578T cells but was without effect in any other breast cancer cell line (Table I). In a separate study TPA also enhanced PGE_2 production in breast fibroblasts in the presence of arachidonate, the respective values for arachidonate alone, TPA alone and arachidonate plus TPA being: 357 ± 33, 58 ± 7 and 3799 ± 604 pg ml^{-1} (mean ± s.d., $n = 3$). Cell lines unresponsive to IL-1β or TPA (e.g. MCF-7, T47-D) with regard to PGE_2 production exhibited evidence of functional receptors for these agonists as demonstrated by increases in ET-1 production in response to both agonists (MP Schrey and KV Patel, unpublished results). Cyclic AMP (cAMP) elevation is essential for IL-1β-stimulated PGE_2 production in certain cell types (Burch and Connor, 1992). However, PGE_2 production by either MCF-7 cells or T47-D cells remained unresponsive to IL-1β in the presence of cAMP elevating agonists such as cholera toxin or forskolin. PGE_2 synthesis was also undetectable in MCF-7 and T47-D cells incubated with either oestradiol or epidermal growth factor (MP Schrey and KV Patel, unpublished results). Transient pretreatment of breast fibroblasts with indomethacin reduced basal PGE_2 production in the presence of 10 μM arachidonate but did not prevent subsequent induction of PGE_2 production by IL-1β (Figure 1). Previous studies in various cell types including fibroblasts have shown IL-1β induction of cyclo-oxygenase 2 (COX-2) activity and PGE_2 production to be inhibited by glucocorticoids (Raz *et al.*, 1990; Crofford *et al.*, 1994). In the present study, dexamethasone treatment also inhibited IL-1β-induced PGE_2 production in breast fibroblasts (Figure 1).

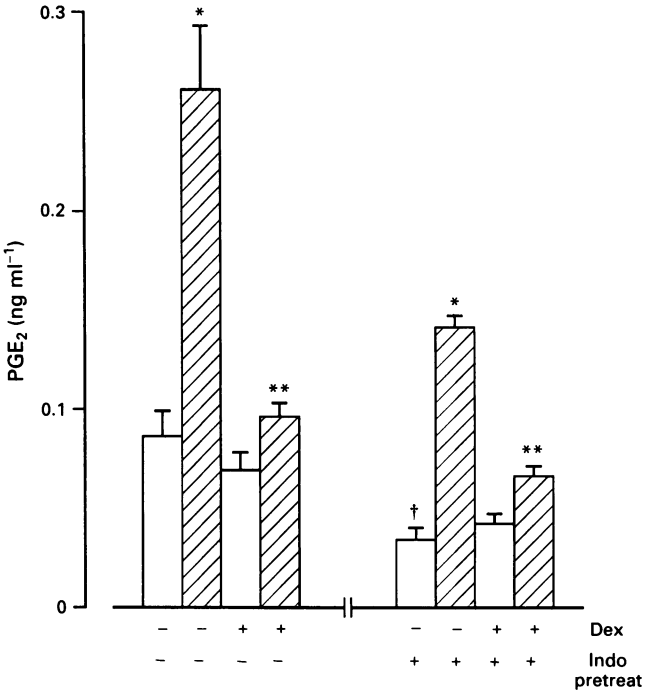

Figure 1 Effect of dexamethasone and indomethacin pretreatment on IL-1β-stimulated PGE_2 production in breast fibroblasts. HBF-10 cells were pretreated for 30 min with or without indomethacin, (Indo; 10 μM), washed twice and incubated for 18 h in medium containing arachidonic acid (10 μM) in the absence (□) or presence (▨) of IL-1β (1 ng ml^{-1}) and/or dexamethasone (Dex; 1 μM). PGE_2 production was then measured. All values represent means ± s.d., $n = 3$. *$P < 0.01$ for stimulation compared with appropriate untreated and indomethacin-pretreated controls; **$P < 0.001$ for inhibition of IL-1β responses by dexamethasone. †$P < 0.01$ for inhibition compared with untreated controls.

Synergistic interactions between IL-1β and inflammatory peptides during fibroblast PGE_2 production

In the absence of exogenous arachidonate, optimal IL-1β-induced PGE_2 production in breast fibroblasts was greatly enhanced in the presence of BK (Figure 2a). Similarly, synergism in IL-1β pretreated fibroblasts was also observed in response to the B_1 BK-receptor agonist des-Arg^9-BK or ET-1 (Figure 2b). Apparent cyclo-oxygenase activity as measured by PGE_2 production in the presence of 10 μM arachidonate was unaffected by BK, des-Arg^9-BK or ET-1, whereas PGE_2 synthesis in IL-1β-treated fibroblasts was enhanced 5.7-fold in the presence of exogenous substrate (Figure 2b).

Apparent inhibition of PGE_2 production in stromal/epithelial co-cultures

In view of the heterogenous nature of primary cell or tissue cultures previously employed to monitor PGE_2 production in human breast tumours, we have also examined the effect of stromal/epithelial co-cultures on PGE_2 production. The effect of overnight co-culture with T47-D cells on basal and IL-1β-stimulated PGE_2 production by fibroblasts is shown in Figure 3a. PGE_2 production in T47-D cells alone is undetectable (compare Table I), whereas basal and IL-1β-stimulated production by breast fibroblasts was inhibited by 83% and 42% respectively when co-cultured with T47-D cells (Figure 3a). The lack of effect of T47-D cell-conditioned medium on fibroblast PGE_2 production compared with that observed in co-cultures is shown in Figure 3b. An inhibitory effect of T47-D cells *per se* on both basal and IL-1β-stimulated PGE_2 production in fibroblasts is clearly evident (Figure 3b). Basal fibroblast PGE_2 production was similarly inhibited in co-cultures with T47-D cells or MCF-7 cells (Figure 3c). To

investigate a potential metabolic capacity of breast cancer cells to modulate net PGE_2 production in co-culture, the recovery of exogenous PGE_2 added to T47-D cells was monitored. A temporal decline in exogenous PGE_2 recovered from T47-D cell cultures was observed (Figure 3 insert). This decreased recovery of exogenous PGE_2 appeared to be due to the T47-D cells themselves rather than soluble factors elaborated by the cells as evidenced by differential PGE_2 recovery in the presence or absence of T47-D cells *vs* T47-D cell-conditioned medium (Figure 3d).

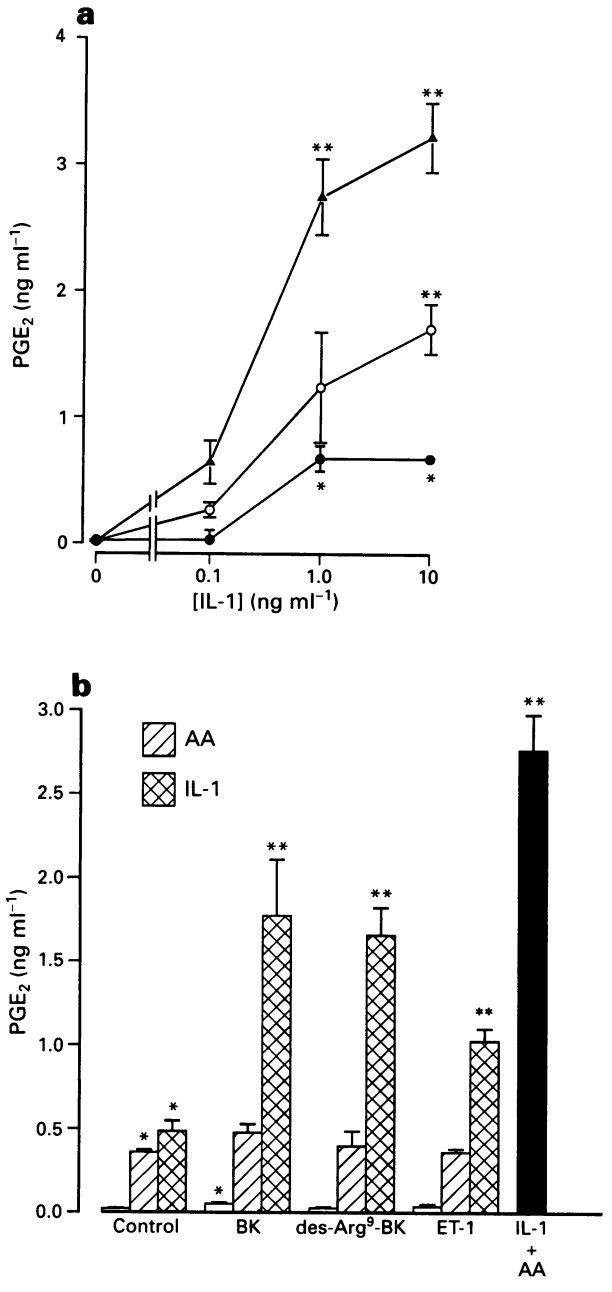

Figure 2 Effect of IL-1β pretreatment on breast fibroblast PGE_2 production in response to BK, des-Arg^{9}BK, ET-1 and arachidonic acid, AA. (**a**) Fibroblasts (HBF-12) were pretreated with different concentrations of IL-1β for 18 h and PGE_2 production was measured in a subsequent 4 h incubation in the absence (●) or presence of BK, 0.01 μM (○) and 1 μM (▲). (**b**) Fibroblasts (HBF-10) were similarly pretreated with (▨) or without (□) IL-1β (1 ng ml^{-1}) before incubation in the presence or absence of BK (1 μM), des-Arg^{9}BK (1 μM) and/or AA (10 μM) (▨). Some fibroblasts were pretreated with IL-1β and then incubated with AA alone (■). All values represent means ± s.d., $n = 3$. *$P<0.01$ for stimulation compared with basal control values. **$P<0.001$ for potentiation of responses in the presence of IL-1β.

PGE_2 metabolism by T47-D cells

To further investigate the metabolic fate of PGE_2 in the presence of breast cancer cells, T47-D cells were incubated with [^{3}H]PGE_2. Following extraction and TLC the major metabolite co-eluted with 15-keto PGE_2, with no conversion to $PGF_{2\alpha}$ being detectable (Figure 4a). These data indicate that the predominant metabolic pathway for PGE_2 in T47-D cells involves the enzyme 15-hydroxy PG dehydrogenase. In similar parallel studies with MDA-MB-231 cells or human breast fibroblasts we were unable to detect significant metabolism of PGE_2 (MP Schrey and KV Patel, unpublished observations). Recent studies in human promyelocytic leukaemia (HL-60) cells report a rapid turnover of this PG dehydrogenase, continued enzyme synthesis being necessary to maintain activity (Xun *et al.*, 1991). Furthermore, *de novo* enzyme synthesis and activity was stimulated in these cells by protein kinase C (PKC)-activating phorbol esters (Xun *et al.*, 1991). In the present study, when T47-D cells were pretreated with TPA or cycloheximide, basal metabolic conversion of PGE_2 to 15-keto PGE_2 + PGE metabolite (PGEM) was increased by 77% or inhibited by 93% respectively (Figure 4b).

PGE_2 stimulates ET-1 production in breast cancer cells

Several human breast cancer cell lines including MCF-7 produce ET-1 which may exercise a paracrine mitogenic influence on neighbouring breast stroma (Schrey *et al.*, 1992*a*). Another novel paracrine action of ET-1 is the stimulation of PGE_2 production by breast fibroblasts as described above (see Figure 2b). To investigate the potential for a bidirectional paracrine cross-talk between epithelial and stromal cells, the effect of PGE_2 on ET-1 production by MCF-7 cells was also assessed. During a 24 h incubation, basal ET-1 production by MCF-7 cells was increased from 30.1 ± 0.3 to 70.9 ± 6.9 fmol 10^{-6} cells ($P<0.001$) in the presence of PGE_2 (1 μM).

Discussion

Several studies have reported elevated levels of PGE_2 in malignant human breast tumour tissue (Bennett, 1979; Rolland *et al.*, 1980; Karmali *et al.*, 1983; Watson and Chuah, 1985, 1992). However, the cell types involved and the regulatory mechanisms controlling PGE_2 levels in breast tumours are unknown. A major limitation of *in vitro* studies employing breast tumour tissue or even primary cell cultures derived from tumour tissue is the presence of non-malignant cells which may obscure the true capacity of PG synthesis by the cancer cells. Indeed, the stromal fibroblast component may represent a significant contribution to tumour cell mass given the marked desmoplasia often associated with breast cancer. The availability of established human breast cancer cell lines and early passage human breast fibroblasts provide simplified experimental models which permit the study of PG production under various culture conditions.

Two important criteria essential for the control of optimal PGE_2 production are the regulation of substrate availability and the capacity to metabolise free arachidonate to PGE_2. Furthermore, phospholipase A_2 and cyclo-oxygenase, the two rate-limiting enzymes involved, are themselves subject to regulation by a variety of paracrine factors. In this respect it is desirable to know if the activity of these enzymes is constitutively expressed in breast cancer cells and fibroblasts and whether a potential exists for their induction and/or activation.

In the present study apparent constitutive cyclo-oxygenase activity, as measured by PGE_2 production in the presence of exogenous substrate was only detectable in one (MDA-MB-231) out of seven breast cancer cell lines investigated. In contrast, under the same conditions all breast fibroblast lines consistently exhibited a high capacity for PGE_2 production. Although such arachidonate metabolism is often characteristic of mammalian fibroblasts derived from various tissues this

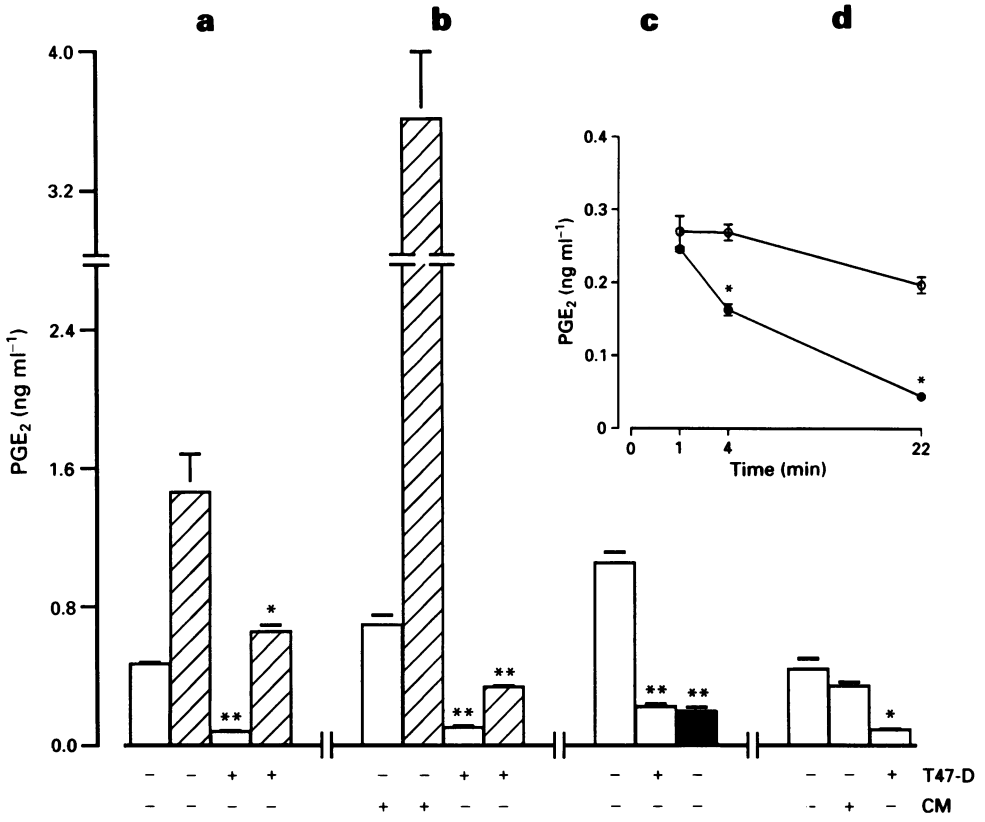

Figure 3 Effect of T-47D breast cancer cells on breast fibroblast-derived PGE_2 and recovery of exogenous PGE_2. (**a**) Comparative effect of PGE_2 production in fibroblasts alone and in co-culture with T-47D cells. (**b**) Comparative effect of fibroblast PGE_2 production in T-47D cell-conditioned medium (CM) *vs* co-culture with T-47D cells. (**c**) Comparative effect of T-47D and MCF-7 cells (shaded bar) on fibroblast PGE_2 production. (**d**) Comparative effect of T-47D cells and T-47D conditioned medium on recovery of exogenous PGE_2 after 18 h. Inset, recovery of exogenous PGE_2 after 1,4 and 22 h incubation in the absence (○) or presence (●) of T47-D cells. For experimental details on co-culture and CM studies refer to Materials and methods. In (**a**) , (**b**) and (**c**) cells were incubated without (□) or with (▨) IL-1β (1 ng ml^{-1}) for 18 h. The shaded bar in (**c**) represents co-culture with MCF-7 cells. Recovery of immunoreactive PGE_2 from T47D cell cultures was measured after addition of exogenous PGE_2 at 0.5 ng ml^{-1} in (**d**) and 0.25 ng ml^{-1} in inset. $^*P<0.01$ and $^{**}P<0.001$ for reduction of basal and IL-1β fibroblast responses by T-47D or MCF-7 cells in (**a**), (**b**) and (**c**). $^*P<0.01$ for reduced recovery of exogenous PGE_2 in the presence of T-47D cells in (**d**) and inset.

is to our knowledge the first report of PGE_2 production in human breast stromal cells. Recently an inducible form of cyclo-oxygenase, COX-2, has been described whose activity and expression is enhanced by IL-1β and phorbol ester and inhibited by dexamethasone (Crofford *et al.*, 1994). Despite possessing receptors for IL-1β (Paciotti and Tamarkin, 1988) the majority of the cancer cell lines remained unresponsive to the cytokine, with the exception of Hs578T cells. This cell line also displayed a potent induction of PGE_2 production in response to the phorbol ester TPA as did MDA-MB-231 cells. The pathophysiological significance of this differential ability of PGE_2 production in breast cancer cell lines is unknown. Nonetheless, it is perhaps relevant to note that these hormone-independent cell lines (MDA-MD-231 and Hs578T) both exhibit an invasive phenotype (Thompson *et al.*, 1992). It has also been shown that MDA-MA-231 cells possess an activated c-Ki-*ras* gene (Kozma *et al.*, 1987) and Hs578T cells contain an activated H-*ras* gene (Kraus *et al.*, 1984). Since *ras*-transformed cells have been reported to express elevated cyclo-oxygenase activity and raised basal PGE_2 production (Gorman *et al.*, 1991) a possible role for this proto-oncogene in the regulation of breast cancer cell PGE_2 production seems warranted. The apparent disparity between the high breast tumour tissue PGE_2 levels previously reported and the inability of most human breast cancer cell lines to synthesise PGE_2 remains a paradox. However, this finding is not exceptional since previous studies have also described dramatic differences in PGE_2 production between cell lines and primary tumours from specific tissues, including those derived from human lung, colon, prostate and ovarian tumours (Hubbard *et al.*, 1988). Again despite the fact that these tumours are also characterised by elevated PGE_2 levels (Karim and Rao, 1976), the majority of these cell lines with the exception of certain non-small-cell carcinomas of the lung, produced little or no detectable PGE_2 (Hubbard *et al.*, 1988; 1989). Nonetheless, it is possible that, despite the high exogenous arachidonate concentrations used in the present study, sequestration of substrate via transacylation of phospholipid esters may predominate in some breast cancer cells and prevent metabolism via cyclo-oxygenase. Alternatively, rapid metabolic inactivation of any PGE_2 formed by the cancer cells may also limit its detection. Whether the duration of passage affects the capacity of some cell lines to synthesise PG is unknown. However, CLF-90-1 cells, from an early-passage hormone-dependent breast cancer cell line recently established in our laboratory also fail to synthesise PGE_2. Notwithstanding our present findings, caution must of course be exercised when interpreting and extrapolating any such study to tumour cells *in vivo*.

Interleukin 1β also caused large increases in breast fibroblast PGE_2 production in the absence and presence of exogenous substrate. Hence in addition to cyclo-oxygenase induction, the cytokine may also increase phospholipase A_2 activity as described in other cell types (Lyons-Giordano *et al.*, 1993). In the absence of added arachidonate, the fibroblast PGE_2 response to IL-1β was greatly enhanced by BK and ET-1, both of which are known to stimulate phospholipase A_2 in several cell types. Since cyclo-oxygenase activity in breast fibroblasts appeared to be unaltered by these peptides, this synergism between peptide and cytokine may be partly mediated by the concerted activation and induction of phospholipase A_2 and cyclo-oxygenase respec-

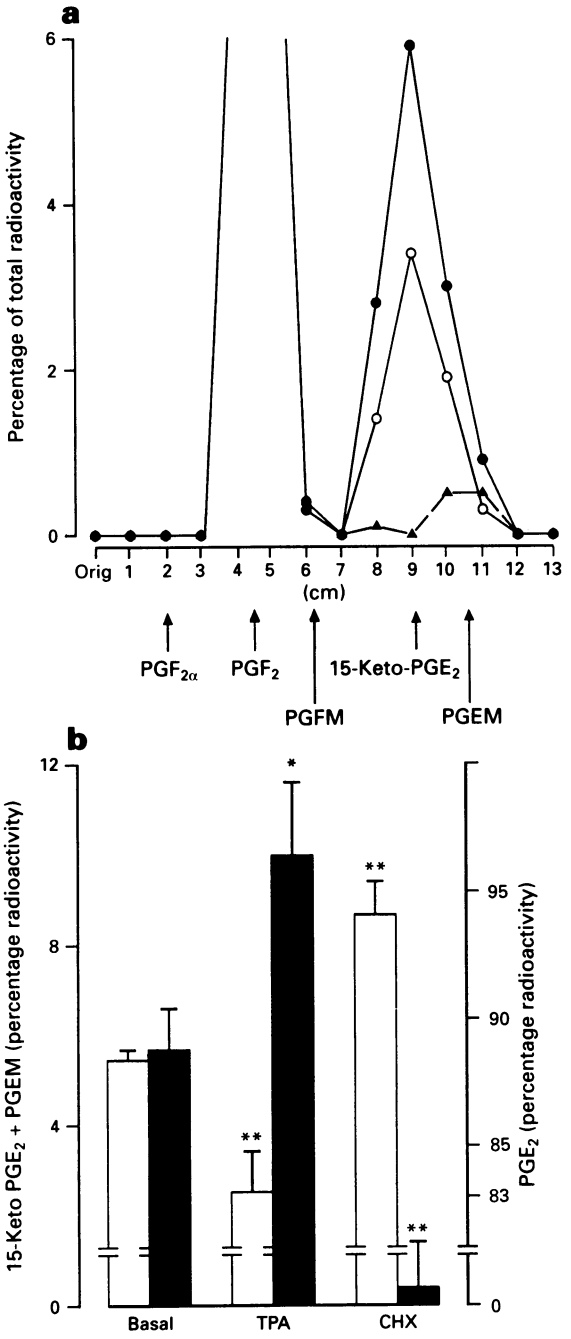

Figure 4 Effect of phorbol ester and cycloheximide on PGE_2 metabolism by T-47D breast cancer cells. T-47D cells were incubated for 24 h with or without TPA (10 nM) or cycloheximide (CHX; 1 μM) and the metabolism of [^{3}H]PGE_2 (1 μM) was then monitored during the last 4 h period. Incorporation of radiolabel into various product bands was then determined following extraction and TLC (see Materials and methods). (**a**) Shows typical radioactivity profiles from single incubations of control (○), TPA (●) and cycloheximide (▲) treated cells. (**b**) Shows means ± s.d. values ($n = 3$) for the sum of the product bands 15-keto PGE_2 and PGEM (■) as well as the PGE_2 (□) substrate band. $^*P<0.02$. $^{**}P<0.01$ for significant changes in relation to basal values.

tively. Similar synergistic interactions between these inflammatory mediators have been described previously for the regulation of PG synthesis in decidual (Schrey *et al.*, 1992*b*; Schrey and Hare, 1992), gingival (Lerner and Modeer, 1991) and synovial fibroblasts (Bathon *et al.*, 1989). The observed inhibition of IL-1β action in breast fibroblasts by dexamethasone would also be characteristic of and consistent with a role for COX-2 induction by the cytokine in these cells as recently described during IL-1β-stimulated PG production in other cell types (Crofford *et al.*, 1994).

The significance of this stromal cell PGE_2 production in response to inflammatory kinins and cytokines with regard to breast tumour biology is at present uncertain. Nonetheless IL-1β-mediated increases in matrix–metalloproteinase activity in the tumour microenvironment may be subject to negative regulation by PGE_2 (Case *et al.*, 1990; Mackay *et al.*, 1992). We have previously proposed a mediatory autocrine role for PGE_2 in the inhibition of breast fibroblast growth by BK (Patel and Schrey, 1992). Interestingly, in this regard BK has been reported to inhibit the *in vivo* growth of spontaneous mammary tumours and sarcomas in mice (Mashiba and Matsunaga, 1985) and SV-40-induced fibrosarcomas in hamsters (Koppelmann *et al.*, 1978). Stromal PGE_2 may also exert an inhibitory paracrine influence on breast cancer cell growth as evidenced by its anti-proliferative action on MCF-7 cells (Fentiman *et al.*, 1984).

Notwithstanding an equivocal role for inflammatory mediators such as IL-1β and BK in breast cancer, tumour stroma generation exhibits many parallels with the processes involved in inflammation, connective tissue remodelling and wound repair, including infiltration of cytokine-producing host immune cells (Whalen, 1990). Furthermore, a number of studies indicate a local activation of the kallikrien–kinin cascade in tumours from several different cancers (Maeda *et al.*, 1988; Karlsrud *et al.*, 1991; Matsumura *et al.*, 1991).

A role for ET-1 as a paracrine mitogen for breast stromal tissue has been recently implicated (Baley *et al.*, 1990; Schrey *et al.*, 1992*a*). This action may partially account for the extensive desmoplasia seen in breast tumours (Yamashita *et al.*, 1991, 1992). In the present study we have presented evidence for a novel potential paracrine cross-talk between epithelial and stromal breast tumour components involving ET-1 and PGE_2. Thus, ET-1 stimulates PGE_2 production by breast fibroblasts and PGE_2 stimulates ET-1 production by breast cancer cells. Whether such a positive feedback loop might contribute to the elevated levels of PGE_2 and ET-1 seen in breast tumours and to the control of tumour stroma generation remains speculative.

In an attempt to investigate a potential paracrine control of breast stromal cell PG production, co-cultures of T-47D (or MCF-7) cells and breast fibroblasts were employed. Under these co-culture conditions both basal and IL-1β-stimulated stromal cell PGE_2 production appeared to be inhibited. Experiments indicating the lack of an inhibitory effect of T47-D cell-conditioned media on fibroblast PGE_2 and a reduced recovery of exogenous PGE_2 from T47-D cell cultures are consistent with metabolic inactivation of PGE_2 by the breast cancer cells. This interpretation was further supported by the metabolic capacity of T47-D cells to readily convert PGE_2 to 15-keto-PGE_2. 15-Hydroxy-PG dehydrogenase, which catalyses this conversion, is considered to be the key enzyme controlling the biological inactivation of prostaglandins. Although this enzyme appears to be ubiquitous in mammalian tissues, the specific cell types responsible for prostaglandin catabolism are uncertain. Clearly, the regulation of this enzyme may also play an important role in determining breast tumour tissue PGE_2 levels. The production of 15-keto-PGE_2 in T47-D cells was stimulated by the phorbol ester TPA and inhibited by cycloheximide treatment. In accordance with a recent study using the leukaemia HL-60 cell line to investigate 15-hydroxy PG dehydrogenase regulation (Xun *et al.*, 1991) our observations would be consistent with an induction of the enzyme in T47-D cells by TPA. Similarily, previous studies have also indicated a loss of enzyme activity following cycloheximide treatment consistent with a rapid turnover of the dehydrogenase in lung, kidney (Blackwell *et al.*, 1975) and HL-60 cells (Xun *et al.*, 1991). Since maintenance of dehydrogenase activity appears to require continued *de novo* enzyme synthesis (Xun *et al.*, 1991) a susceptibility to hormonal control might be expected. This is indeed the case with regard to the steroidal regulation of this PG dehydrogenase in the uterine decidua, where progestins appear to induce the enzyme (Alam *et al.*, 1976) and anti-progestins are particularly effective in suppressing enzyme activity and elevating *in situ* PG concentrations

(Cheng *et al.*, 1993). The potential for similar hormonal regulation of this enzyme in breast tissue requires investigation. Indeed, PGE_2 levels in DMBA-induced rat mammary tumours increase following ovariectomy (Foecking *et al.*, 1982) and prostaglandins have also been implicated in mastalgia associated with premenstrual syndrome (Budoff, 1986).

Despite the association of elevated PGE_2 with a malignant metastatic phenotype, the value of breast tumour PGE_2 levels as a prognostic indicator remains uncertain. Although initial studies suggested a poor prognosis in patients with high PG levels (Bennett *et al.*, 1977) others have shown either no relationship to established prognostic indices (Wilson *et al.*, 1980; Watson *et al.*, 1987) or a correlation with favourable prognostic factors (Karmali *et al.*, 1983; Watson and Chuah, 1985; Fulton *et al.*, 1982). These disparate findings could be attributed to various factors including: differences in the techniques employed; substrate availability; and tissue handling as well as the labile nature of both tissue PGE_2 levels *per se* and the rapid turnover of the enzymes regulating PGE_2 production and metabolism. Interestingly, a recent study measuring immunoreactive membrane-associated phospholipase A_2 in breast tissue reported high expression in malignant *vs* benign or normal tissue (Yamashita *et al.*, 1993). Whether this overexpression of phospholipase A_2 in malignant breast tissue is instrumental in causing elevated PGE_2 levels in these tumours remains to be established.

In summary, this study identifies the breast stromal fibroblast as a potential source for PGE_2 production in breast tumours. Putative paracrine factors which may regulate this arachidonate metabolism include cytokines and peptides such as IL-1β, kinins and endothelin. We are currently engaged on a comparative study to determine whether fibroblasts derived from normal or malignant breast tissue exhibit any differential capacity for PGE_2 synthesis. Since the capacity for PGE_2 production in breast cancer cells appears to be restricted to oestrogen receptor-negative cell lines with an invasive phenotype, whether the cancer cells *per se* contribute towards prostaglandin synthesis may depend on the specific histological class of tumour. Finally, metabolic inactivation by the cancer cells may also play an important regulatory role in the control of breast tumour PGE_2 levels.

Abbreviations

HBF, human breast fibroblast; PG, prostaglandin; PGEM, prostaglandin E metabolite; IL-1β, interleukin 1β; BK, bradykinin; ET-1, endothelin-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; COX-2, cyclo-oxygenase 2; TLC, thin layer chromatography; EMEM, Eagle's minimal essential medium; FCS, fetal calf serum.

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