Breast cancer cell-derived EMMPRIN stimulates fibroblast MMP2 release through a phospholipase A₂ and 5-lipoxygenase catalyzed pathway

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Metalloproteinases (MMP) produced by both cancer and normal stromal fibroblast cells play a critical role in the metastatic spread of tumours, however little is known of the regulation of their release. In this report we demonstrate that breast cancer cells in culture release apparently full length soluble EMMPRIN that promotes the release of pro-MMP2 from fibroblasts. The generation of MMP2 is mediated by activation of phospholipase A₂ and 5-lipoxygenase. These results suggest that the production of soluble EMMPRIN, phospholipase A₂ and 5-lipoxygenase activities are sites for potential therapeutic intervention.

Oncogene (2002) 21, 5765–5772. doi:10.1038/sj.onc.1205702

Keywords: EMMPRIN; MMP2; phospholipase A₂; 5-lipoxygenase; breast cancer cell; fibroblast

Introduction

The metastatic spread of tumours requires the extravasation of cancer cells from the primary tumour and their intravasation into a secondary site. Metastasis is dependent upon the degradation of the extracellular matrix that surrounds both the tumour and the secondary site. The degradative process is catalyzed by zinc-dependent endopeptidases: the metalloproteinases (MMPs). Greater than 20 human MMPs have been identified and classified according to domain structure into collagenases, gelatinases, stromelysins, membrane-type and others (Kleiner and Stetler-Stevenson, 1999). The gelatinases, MMP2 and MMP9, have been particularly implicated in tumour invasion and metastasis formation. The primary substrate for these enzymes is type IV collagen, a major component of the basement membrane, which represents a substantial barrier to tumour cell metastasis. Most MMPs are

secreted as zymogens and require activation through cleavage of a pro-domain sequence located at the amino-terminus of the protein. Complex regulatory mechanisms control MMP activity, including the proteolytic activation of catalytic activity and direct inhibition by the tissue inhibitors of MMPs (TIMPs). It is the balance between free activated MMP and TIMP concentrations that determines net proteolytic activity. However, the TIMPs also play a role in the activation of some MMPs. TIMP2 has been shown to interact directly with the membrane type MMPs (MT-MMPs) and MMP2, promoting its activation in a focused, cell-surface mediated fashion. In this way the membrane bound MT-MMP and TIMP2 act as a receptor for MMP2 (Butler *et al.*, 1998).

MMP expression is elevated during normal physiological processes, such as tissue remodelling during growth phases, following injury and in disease, e.g. chronic inflammatory processes and malignancy. The role of MMPs in malignancy is highlighted by the number of tumours reported to contain elevated MMP levels including breast, prostatic, gastric, ovarian, pancreatic and colorectal cancers (Bassett *et al.*, 1990; D'Errico *et al.*, 1991; Nomura *et al.*, 1995; Zeng *et al.*, 1999). This has led to great interest in the development of MMP inhibitors as potential chemo-preventative agents, since inhibiting the metalloproteinase activity would be anticipated to prevent metastasis. Additionally, the MMPs can have a tumour-promoting role, as suggested from MMP3 knock-out studies in breast carcinogenesis (Sternlicht *et al.*, 1999). Thus an understanding of the mechanisms involved in the increased induction of MMPs is essential.

It was initially thought that the malignant tumour was responsible for the production of MMPs. However, it is apparent that a major source of MMP activity is from surrounding stromal fibroblasts. Fibroblast secreted pro-MMP2 is activated at the tumour cell surface thereby facilitating tissue invasion (Ward *et al.*, 1994). Quiescent fibroblasts generally produce low amounts of MMPs, thus they must be influenced by cell–cell contact, or by a soluble factor to produce the elevated MMP level detected in tumours. This hypothesis is supported by the identification of CD147 (EMMPRIN) as a tumour cell surface glycoprotein is capable of stimulating the production of MMP1, MMP2 and MMP3 from fibroblasts (Ellis *et*

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Received 18 September 2001; revised 9 May 2002; accepted 20 May 2002

al., 1989; Guo *et al.*, 1997; Nabeshima *et al.*, 1991). Thus we have examined the ability of breast cancer cells in culture to produce soluble factors that stimulate MMP2 production by a human breast stromal fibroblast cell line. Additionally, we have investigated the mechanism whereby these factors stimulate MMP2 release.

Results

A number of breast cancer cells in culture release limited amounts of MMP2, in contrast the inactive pro-MMP9 is constitutively generated. This study focuses on these cell lines. Since active MMP2 is capable of catalyzing the cleavage and activation of MMP9, its generation must be stimulated in order to promote matrix degradation. Fibroblast cells can release MMP2 and therefore we examined the interaction between breast cancer epithelial cells and primary breast stromal fibroblasts. Five breast cancer cell lines (MTSV, T47D, MDA-MB231, MDA-MB-435 and MDA-MB-436) with increasing invasive capacity and spindle shaped morphology were cultured in serum-free medium for 24 h and the conditioned medium harvested. Incubation of KS1.2 fibroblasts in this medium for 24 h induced increased release of MMP2 when compared to cells incubated in serum free unconditioned medium. The MMP2 released by the fibroblasts was the higher molecular weight pro form; the active form was not generated. Whilst experiments were performed upon each conditioned cell medium, that from MDA-MB231 cells was examined in detail (but see Figure 5). Stimulation of MMP2 release by MDA-MB231 cell conditioned medium from the fibroblasts was detectable within 1 h (Figure 1a). The basal release of pro-MMP2 by fibroblasts incubated in unconditioned serum-free medium was only apparent after 3 h. MMP2 release was detected by both zymography and Western blotting with an anti-MMP2 antibody (Figure 1a,b).

Incubation of the fibroblasts with cyclohexamide, but not actinomycin D, suppressed the release of MMP2 both after 1 and 3 h (Figure 1b) after addition of MDA-MB231 cell conditioned medium; cyclohexamide also suppressed the basal secretion. The lack of effect of the transcription inhibitor upon MMP2 release was confirmed by RT-PCR experiments, which showed no increase in MMP2 mRNA level in response to MDA-MB231 cell conditioned medium (data not shown).

Tumour cells secrete a number of growth factors with autocrine and/or paracrine functions e.g. TGF α , occupation of the cognate fibroblast cell surface receptors by such factors could signal an increase in synthesis and secretion of MMP2. Suramin, which displaces polypeptide growth factors from their receptors and is a lysophosphatidic acid receptor antagonist, had no effect upon the ability of MDA-MB231 cell conditioned medium to stimulate MMP2 secretion by fibroblasts (data not shown). Experiments

were performed to characterize the stimulatory factor(s) in the conditioned medium. Figure 2 shows that the factor(s) is greater than 10 kDa in size (compare lanes 2, 7 and 8), trichloroacetic acid precipitable (lanes 5 and 6) and is not extractable with diethyl ether (lane 11). Figure 2 also shows that the factor is heat stable (lanes 9 and 10) and partitions equally between chloroform and methanol (lanes 3 and 4) and is most likely a hydrophobic protein.

To identify the stimulatory factor, conditioned medium was fractionated by anion exchange chromatography and the fractions tested for their ability to promote pro-MMP2 generation by the fibroblasts. Figure 3a shows that fractions eluted by salt concentrations of 160–200 mM (fractions 9, 10, 11 and 12, lanes h and i) were capable of stimulating fibroblast MMP2 release. The zymogram also demonstrates that the MMP9 present in the MDA MB231 conditioned media co-eluted with the activating factor(s). The active fractions were pooled and subjected to gel filtration on a Superose 12 column. Figure 3b shows that the MMP2-inducing activity eluted in fractions 18

Figure 1 Stimulated release of MMP2 by KS1.2 fibroblasts. (a) Fibroblasts were treated with MDA-MB231 conditioned media (+) or control serum free media (–) for the times indicated. Media was removed, concentrated and MMP2 detected by Western blotting. (b) Fibroblasts were pre-treated with cycloheximide (CHX) or actinomycin D (AD) (at 10 μ g/ml) for 10 min prior to stimulation with MDA-MB231 conditioned media for either 1 or 3 h. Forty μ l media was used for gelatin zymography to detect MMP activity

Figure 2 The nature of the stimulatory factor in MDA-MB231 conditioned medium. MDA-MB-231 conditioned media was subjected to a variety of treatments prior to stimulation of fibroblasts for 1 h. Following stimulation, 40 μ l of media was used for zymogram analysis (left hand panel) or 30 μ l for Western analysis (right hand panel). Fibroblasts were treated with: (1) serum free DMEM, (2) MDA-MB-231 conditioned media, or conditioned media after treatment; (3) Chloroform phase or, (4) Methanol phase of chloroform/methanol extraction; (5) Supernatant or, (6) Resuspended pellet of TCA precipitation; (7) Filtrate or, (8) Dialysate of 10 kDa filtration; (9) Heat treatment of 56°C 30 min or, (10) Heat treatment of 100°C 5 min; (11) Conditioned media after ether extraction

and 19 had a retention time 1–2 min slower than BSA. Zymographic analysis showed that MMP9 was absent from fractions 18 and 19 suggesting that MMP9 is not the activating factor.

EMMPRIN (CD147) is a 58 kDa glycoprotein located on the surface of tumour cells capable of stimulating the expression of MMPs in fibroblasts (Biswas *et al.*, 1995), presumably by binding to its

cognate receptor on the stromal fibroblasts. Thus we examined the gel filtration fractions for the presence of EMMPRIN by Western blotting. Figure 4a shows that EMMPRIN is present in the MDA-MB231 conditioned medium and in fractions 18 and 19 from the gel filtration column, as indicated by the presence of a 58 kDa band. Blotting of the conditioned medium with two distinct antibodies, one recognizing a C-terminal and the other an N-terminal epitope, confirmed the presence of apparently full length EMMPRIN (Figure 4b). It has been suggested that EMMPRIN is a membrane protein and is not secreted and therefore apparently soluble EMMPRIN may actually be present in shed vesicles. Thus the conditioned medium was acidified to pH5 with 0.5 M citrate and centrifuged at 436 000 *g* to sediment vesicular material and the

Figure 3 Partial purification of the MMP2 inducing serum factor. (a) MDA-MB-231 conditioned media was fractionated by anion exchange chromatography on a MonoQ column. Fibroblasts were treated for 1 h with fractions diluted 1:20 in DMEM, and MMP2 secretion was analysed by gelatin zymography. Treatments were (a) DMEM, (b) run through, (c) MDA-MB-231 conditioned media, or fractions: (d) 1+2, (e) 3+4, (f) 5+6, (g) 7+8, (h) 9+10, (i) 11+12, (j) 13+14. (b) The active fractions 9+10+11+12 from the anion exchange chromatography were pooled and fractionated by gel filtration using a Superose 12 column, 50 μ l fractions were eluted and fraction collection was initiated at 0.5 ml (thus fraction number 18 equates to 1.4 ml volume on the chromatogram). BSA=retention time for bovine serum albumin (66 kDa). CA=retention time for carbonic anhydrase (14 kDa). Fibroblasts were treated with all fractions with absorbance at 280 nm, and MMP2 secretion was analysed by gelatin zymography

Figure 4 Detection of EMMPRIN. (a) The fractions from the Superose 12 column (Figure 3) were separated by SDS-PAGE and probed with an anti-EMMPRIN antibody. (b) 1.5 ml MDA MB.231 (MDA) and fibroblast (C) conditioned media was concentrated to 60 μ l and analysed by Western blotting using antibodies against the N- or C-terminus of EMMPRIN. (c) Two ml conditioned media was centrifuged in a TLA 100.2 rotor (Beckman) at 436 000 *g* for 15 min, i.e. 6.54×10^6 *g*. The supernatant was removed and the pellet resuspended in 2 ml DMEM. Fibroblast cells were treated with: (1) DMEM, (2) MDA-MB-231 conditioned media, (3) Supernatant, (4) Pellet. MMP2 secretion was analysed by zymography, and EMMPRIN by Western blotting. (d) EMMPRIN immunodepletion of MDA MD.231 conditioned media was carried out as described in Materials and methods. Fibroblasts were treated with: (1) DMEM, (2) MDA-MB.231 conditioned media, (3) α -EMMPRIN IP supernatant, (4) bead IP supernatant. MMP2 secretion was analysed by zymogram and EMMPRIN content by Western blotting: (2) MDA-MB-231 conditioned media, (3) α -EMMPRIN IP supernatant, (4) bead IP supernatant were concentrated by centrifuge filtration for analysis

supernatant and pellet were examined by Western blotting. Following this treatment EMMPRIN was only detected in the soluble fraction (Figure 4c). Passage of conditioned medium through a 0.2 μ m filter did not diminish its pro-MMP2 releasing ability (data not shown). To determine if EMMPRIN was the sole activating factor the conditioned medium was incubated with an anti-EMMPRIN antibody bound to protein A beads for 2 h at 4°C. The beads were isolated by centrifugation and the ability of the immunodepleted medium and the bound fraction to stimulate pro-MMP2 release examined. Figure 4d shows that the conditioned medium was depleted of EMMPRIN by this procedure and that the immunodepleted medium was unable to activate MMP2 release.

The presence of EMMPRIN in the serum free conditioned medium from the other breast cell lines was also examined. Each line produced EMMPRIN, however the greatest levels were consistently found in that medium conditioned by MTSV, T47D and MDA-MB231 cells. Immunodepletion experiments showed that removal of the EMMPRIN suppressed the ability of these conditioned media to stimulate MMP2 release by the fibroblasts (Figure 5).

Having demonstrated that EMMPRIN was probably the factor that stimulated MMP2 secretion from fibroblasts in response to MDA-MB-231 conditioned medium we examined the mechanisms. It has been suggested that the receptor that mediates the effects of EMMPRIN in fibroblasts is a fibroblast-associated KS1.2 cells would be expected to contain some EMMPRIN. Lane 1 in Figure 6 shows that a small but detectable amount of EMMPRIN is found in KS1.2 cells. Two bands can be seen at approximately 66 and 55 kDa. A comparison to an EMMPRIN blot

Figure 5 Anti-EMMPRIN immunodepletion of conditioned medium from five breast cancer cell lines. Conditioned media was prepared from MDA-MB-231, MDA-MB-435, MDA-MB-436, MTSV and T47D breast cancer cell lines. Immunodepletion with the anti-EMMPRIN antibody was as described in the Materials and methods. Gelatin zymography was performed upon medium from KS1.2 cells which had been incubated with serum free medium (C), the conditioned serum free medium (CM) or EMMPRIN-immunodepleted (ID) conditioned serum free medium from each breast cell line

of MDA-MB231 lysates (lane 4) shows that the fibroblasts contain far less EMMPRIN than the tumour cells, none was found in fibroblast conditioned medium. Furthermore, Figure 6 also shows that the EMMPRIN secreted from the MDA-MB-231 cells (lane 5) is similar in size to the cellular protein. By taking into account the amount of material loaded on to the gel from the lysates and the amount of material loaded from concentrated conditioned medium we estimate that around 2–3% of EMMPRIN is released into the medium.

The EMMPRIN activated signalling pathways were examined by incubating fibroblasts with defined inhibitors whilst stimulating the cells with MDA-MB231 conditioned media. Under these conditions non-specific or cytotoxic effects were not observed. Figure 7a shows that inhibition of PI-3-kinase (LY294002), the Raf-MAP kinase pathway (PD98059), p38 (SB203580) or the phospholipase D

Figure 6 Detection of EMMPRIN in MDA-MB-231 and KS 1.2 cell lysates and MDA-MB-231 conditioned medium. KS1.2 and MDA-MB-231 cells (80% confluent) were incubated for 24 h in serum free medium. The conditioned medium was collected and concentrated and diluted with SDS-PAGE sample buffer. The cells were washed and harvested in 1 ml of SDS-PAGE sample buffer. Following electrophoresis, EMMPRIN was detected by Western blotting using a combination of the N and C terminal directed antibodies. The molecular weight of proteins are indicated on the side of the figure in kiloDaltons (kDa). Lane 1, 10 μ l of KS1.2 lysate; Lane 2, 5 μ l of KS 1.2 lysate; Lane 3, 2 μ l of KS 1.2 lysate; Lane 4, 2 μ l of MDA-MB-231 lysate (0.2% of total). Lane 5, MDA-MB-231 conditioned medium, equivalent of 2 ml, which was 20% of the total

Figure 7 Inhibitor analysis of the EMMPRIN-stimulated pathway in fibroblasts. (a) Fibroblasts were pre-incubated with inhibitor for 10 min, followed by treatment in either DMEM + inhibitor or MDA-MB-231 conditioned media + inhibitor for 1 h. MMP2 secretion was analysed by Western blotting or gelatin zymography. Inhibitors used: A. AACOCF₃ (10 μ M), B. LY294002 (20 μ M), C. SB203580 (5 μ M), D. PD98059 (10 μ M), E. cycloheximide (20 μ g/ml), F. LY294002 (20 μ M), G. butan-1-ol (0.3% v/v). (b) Fibroblasts were pre-incubated with AACOCF₃ or BEL (at the concentrations indicated) for 10 min, followed by treatment in either DMEM + inhibitor or MDA-MB-231 conditioned media + inhibitor for 1 h. MMP2 secretion was analysed by Western blotting

pathway (butan-1-ol) was without effect upon MMP2 release. In contrast inhibition of phospholipase A₂ (AACOCF₃) had a partial effect. A role for phospholipase A₂ (PLA₂) was further suggested by the inhibition of MMP2 release by bromoenol lactone (BEL) which is more selective for the Ca²⁺-independent iPLA₂ enzymes (Figure 7b). To determine if the conditioned medium was stimulating PLA₂ activity, cells were radiolabelled with [³H]arachidonate prior to addition of the conditioned medium. Stimulated release of [³H]arachidonate from the radiolabelled cells was observed after 2 min (Figure 8a) and a sustained elevation of [³H]arachidonate was detected in cell lysates after less than 60 s (Figure 8b). EMMPRIN mediated the activation of PLA₂ by the conditioned medium since the EMMPRIN-containing Superose 12 fractions that were able to induce pro-MMP2 release also stimulated release of [³H]arachidonate. In contrast the EMMPRIN-free fractions were without effect (Figure 8c). Arachidonate is the precursor of eicosanoid biosynthesis, being a substrate for both the lipoxygenase and cyclooxygenase pathways. Indomethacin, a cyclooxygenase inhibitor was without effect, whereas two lipoxygenase inhibitors, NDGA and curcumin both inhibited MMP2 release (Figure 9a). It was not possible by HPLC separation to identify which lipoxygenase product was responsible for MMP2 release; however it was partly mimicked by 5-hydroxyeicosanetetraenoic acid (5HETE), which also potentiated conditioned medium-stimulated release (Figure 9b).

Discussion

It has often been suggested that molecules expressed on the tumour surface or released from tumour cells have direct effects upon the surrounding stroma. In this paper we demonstrate that breast tumour cells in culture release soluble EMMPRIN that induces MMP2 release from fibroblasts. Further we show that the EMMPRIN effects are mediated through the activation of a phospholipase A₂/5-lipoxygenase pathway in the fibroblasts. These results add further support to the proposal of (Zucker *et al.*, 2001) that EMMPRIN plays an important role in breast cancer progression by increasing MMP synthesis.

EMMPRIN was initially characterized as a tumour cell surface protein (Ellis *et al.*, 1989; Guo *et al.*, 1997; Nabeshima *et al.*, 1991). Two extracellular immunoglobulin domains, a putative transmembrane domain and an apparent cytoplasmic domain have been suggested by sequence analysis. EMMPRIN's reported molecular weight is variable due to different degrees of glycosylation of the native 32 kDa protein, however it is generally observed to have a M_r of 58 kDa (Biswas *et al.*, 1995). These analyses lead to the suggestion that EMMPRIN is a transmembrane glycoprotein and that its effects upon adjacent cells are a consequence of cell-to-cell contact. The cell surface localization of EMMPRIN has been confirmed by immunofluores-

Figure 8 Stimulation of arachidonate release in fibroblasts by EMMPRIN. Fibroblasts pre-labelled with [³H]arachidonate (1 μ Ci/ml) were washed with DMEM and treated as Ctrl (DMEM) or MDA (MDA-MB-231 conditioned media) for the times indicated. Following treatment, the media (a) was transferred to scintillation vials, the cells (b) extracted into chloroform and radioactivity was determined in each fraction by liquid scintillation counting. The experiments shown are one representative of three. (c) [³H]-arachidonate-labelled fibroblasts were stimulated with MDA-MB-231 conditioned media, Superose 12 fractions 24+25 (F1: EMMPRIN negative) and fractions 18+19 (F2: EMMPRIN positive) for 2 min. The media was aspirated, and the lysate harvested in 0.5 ml methanol:acetic acid (100:1.5 v/v) and [³H]-arachidonate levels determined

cence staining of cells and tissue sections (Caudroy *et al.*, 1999), though intracellular vesicular staining can also be observed. Consequently, the identification of

Figure 9 EMMPRIN utilizes lipoxygenase pathways to stimulate MMP2 generation. (a) Fibroblasts were pre-incubated with inhibitor (at the indicated concentrations for curcumin, or 20 μ M NDGA) for 10 min, followed by treatment in either DMEM + inhibitor or MDA-MB-231 conditioned media + inhibitor for 1 h. MMP2 secretion was analysed by Western blot, probing membranes with α -MMP2, or by gelatin zymography. (b) Treatment of fibroblasts with 5-HETE was performed in the presence or absence of MDA-MB-231 conditioned media (+: 1 nM; ++: 1 μ M)

EMMPRIN as the factor released from the breast cell that induced the secretion of MMP2 from fibroblasts was unexpected. One possible explanation for the release was that the extracellular portion of EMMPRIN is cleaved and thus released from the cells. However this explanation is probably not correct since firstly, antibodies that recognize epitopes located in both N- and C-terminal regions detected the glycoprotein in the medium, secondly the EMMPRIN extracted from the conditioned medium was found to be hydrophobic in nature and thirdly the molecular weight of the cell associated and secreted EMMPRIN were very similar to the anticipated 58 kDa.

The EMMPRIN detected in the breast cell conditioned medium was not vesicular since it could not be sedimented by centrifugation at either pH 7.4 or 5.0. Thus it would appear that the panel of breast cancer cells each secrete or release EMMPRIN. Whilst the predicted sequence suggests that EMMPRIN is a transmembrane molecule, there is actually no definitive evidence for this, indeed one of the earliest identifications of EMMPRIN was in the culture medium of LX-1 carcinoma cells (DeCastro *et al.*, 1996). Affinity purification of EMMPRIN from several cell lysates has also been reported which would not generally be expected of a transmembrane protein. It is not clear if the secretion of EMMPRIN is regulated, or if it is released from the surface of the cells, however immunofluorescence detects EMMPRIN both within a vesicular compartment as well as on the surface of breast and colon cancer cells (data not shown).

There are, therefore, two mechanisms whereby the glycoprotein can induce metalloproteinase release from the stromal fibroblasts, either by direct cell-cell contact, or as a consequence of paracrine release. Only 2–3% of the EMMPRIN was estimated to be found in the medium but this fraction may well be biologically important as it would be able to leave the site of synthesis and stimulate MMP2 synthesis in stromal

fibroblasts that are not in contact with tumour cells. However, despite the potential for remote metalloproteinase release if a freely soluble inducer diffuses away from the tumour cell, the MMP2 is released as the inactive proenzyme which will then require activation by MT1-MMP/TIMP on the surface of the tumour cell (Butler *et al.*, 1998). This cross-talk mechanism could point to maximal release of MMP2 from the surrounding stroma, but activation of the metalloproteinase only at the leading edge of the metastatic tumour.

Whilst a receptor for EMMPRIN remains to be identified, it has recently been suggested that EMMPRIN activates cells by homophilic interaction (Sun and Hemler, 2001). The presence of EMMPRIN in lysates from the fibroblast cells employed here is consistent with this proposal but does not confirm that fibroblast derived EMMPRIN was acting as a receptor in this system. EMMPRIN has been shown to interact with cell surface integrins on HT1080 cells (Berditch-evski *et al.*, 1997), thus if this is also the case on fibroblasts, signalling may proceed through the regulation of integrins which could activate PLA₂ through interaction with small GTPases. The inhibitor studies shown in Figure 7 strongly suggest that EMMPRIN activates fibroblast phospholipase A₂ with subsequent metabolism of the arachidonate by 5-lipoxygenase to generate an active 5-HETE. That this is the mechanism of EMMPRIN action is emphasized by the ability of the EMMPRIN containing fractions from the Superose 12 column to stimulate an increase in free [³H]arachidonate.

The inhibitor selectivity suggested that iPLA₂ rather than cPLA₂ was involved in EMMPRIN-stimulated MMP2 secretion. Whilst initially surprising, this is in keeping with a lack of effect of inhibitors of p38 MAP kinase and the Raf/MAP kinase pathway, since these kinases have been shown to regulate cPLA₂ activity by phosphorylation (Balsinde and Dennis, 1996). iPLA₂ was initially characterized as an enzyme involved in phospholipid remodelling, however recent reports have pointed to a signalling role (Winstead *et al.*, 2000). Whilst [³H]arachidonate was released from the stimulated cells it was apparent that a greater amount remained cell associated implying intracellular function and/or utilization. This suggested the involvement of an arachidonate metabolite. Two structurally distinct lipoxygenase inhibitors NDGA and curcumin inhibited the release of MMP2, in contrast a cyclooxygenase inhibitor, indomethacin, was without effect. Since curcumin is a selective 5-lipoxygenase inhibitor (Huang *et al.*, 1997; Light *et al.*, 1997) we added 5-HETE to the cells. 5-HETE partially mimicked the stimulation of MMP2 release by conditioned medium and was able to potentiate release when added to conditioned medium. Whilst 5-HETE can cross the plasma membrane it is probable that it is functioning intracellularly since an organic extract prepared from the medium incubating the stimulated cells was unable to induce MMP2 release from fresh fibroblasts (data not shown).

EMMPRIN stimulated MMP1 mRNA expression in fibroblasts in a p38-dependent manner (Lim *et al.*, 1998), thus the glycoprotein may utilize distinct signalling pathways in the regulation of different metalloproteinases. In addition, it is probable that two distinct pathways are being stimulated by EMMPRIN in the KS1.2 cells since there appear to be two phases of MMP2 release, one apparent within 1 h whilst the second is slower but more sustained.

The importance of arachidonic acid metabolism in cancer has been emphasized with the development of cyclooxygenase inhibitors as potential chemotherapeutics. Additionally a number of reports have demonstrated elevated PLA₂, cyclooxygenase and lipoxygenase activities in a range of human cancers. The data in this paper point to a novel role for arachidonic acid metabolism in tumour progression acting through the lipoxygenase rather than cyclooxygenase pathways in mediating the biochemical effects of tumour-stromal interaction. Curcumin has been suggested to have both chemotherapeutic and chemopreventative roles. Indeed, it has been shown to prevent FGF-induced MMP2 production in cultured rabbit corneal cells (Mohan *et al.*, 2000) suggesting that the EMMPRIN-induced pathway of MMP2 generation may be common to other stimuli. Increasing emphasis is being placed on the search for mechanistically novel anticancer agents, particularly those that could inhibit invasion and metastasis. Matrix metalloproteinase inhibitors have entered extensive clinical trial programmes and there is some evidence of benefit in gastric cancer. The implication of PLA₂ and 5-lipoxygenase catalyzed pathways in the release of MMP2 opens up new therapeutic pathways. 5-lipoxygenase inhibitors have entered clinical trial and have been found to be well-tolerated, effective enzyme inhibitors and to display limited clinical efficacy in treatment of advanced pancreatic cancer (Ferry *et al.*, 2000). There is potential synergy in combinations of inhibitors of MMP2 and 5-lipoxygenase which merit development as anti-metastatic agents.

Materials and methods

Cell lines

The MDA-MB231, 435 and 436 cells were cultured in L15 medium containing 10% FCS and penicillin and streptomycin, the T47D and KS1.2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FCS and penicillin and streptomycin and the MTSV cells were grown in DMEM containing 10 mM HEPES, insulin and hydrocortisone plus 10% FCS and antibiotics. Conditioned media was collected from cells that were approximately 80% confluent, after washing in PBS the cells were incubated in serum free medium for 24 h, the collected medium was centrifuged for 5 min at 1500 g to remove cellular material.

Cell stimulation and MMP analysis

Fibroblasts were grown to 80% confluence washed three times in PBS and then conditioned media added together with

stimulants/inhibitors as described. At the stated time the medium was removed and subjected to electrophoresis for zymography or Western blotting. Zymography gels (Novex) contained 1 mg/ml gelatin and after electrophoresis were washed 2.5% Triton X-100 and low-salt collagenase buffer (0.05 M Tris pH 7.6, 0.2 M NaCl, 3.75 mM CaCl₂, 2% Brij-35) followed by incubation in low-salt collagenase buffer at 37°C for 6–16 h. Gels were stained with 0.5% Coomassie R250 (in 30% methanol, 10% acetic acid v/v) and destained in 30% methanol, 10% acetic acid to obtain optimum contrast. Western blotting used mouse monoclonal antibodies to MMP2 (Calbiochem) and anti-N-term EMMPRIN (a gift from Dr F Berditchevski, Institute for Cancer Studies, Birmingham University, UK) whilst a goat anti C-terminal EMMPRIN antibody was from SantaCruz, detection was by ECL (Amersham).

Fractionation of conditioned media

Thirty ml of MDA-MB-231 conditioned media was diluted in buffer A (25 mM Tris, pH 8.0) (1:3 by volume) and applied to an equilibrated MonoQ anion exchange column (HR 5 × 5) at 1 ml/min. Bound material was eluted with a linear gradient of Buffer B in buffer A (Buffer B = Buffer A plus 0.5 M NaCl) over 25 ml and 1 ml fractions were collected in glass tubes. Fractions generated from MonoQ anion exchange were assayed to identify activity. One hundred and fifty µl of active fractions were pooled and concentrated by microcentrifuge filtration to 100 µl total volume. Gel filtration was performed on a Superose 12 column (3.2 × 300 mm) at a flow rate of 0.05 ml/min, and 50 µl fractions were eluted isocratically with buffer (250 mM NaCl, 25 mM Tris, pH 8.0).

Immunodepletion of conditioned media

Mouse IgG-conjugated protein A beads were mixed with α-EMMPRIN culture supernatant at a final dilution of 1:100 in 1 ml PBS, and incubated on an end-to-end mixer at 4°C for 2 h. The beads were pelleted and washed at 4°C with 8 × 1 ml PBS. The beads were resuspended in 1.5 ml-conditioned media, and samples were incubated on an end-to-end mixer at 4°C for 2 h. The beads were collected by centrifugation and the supernatant was treated as immunodepleted conditioned media, as assessed by Western blotting. Negative control immunoprecipitations omitted the primary antibody step.

Phospholipase A₂ and arachidonate metabolism studies

KS1.2 cells were labelled for 24 h in DMEM containing 0.1% FCS and 1 µCi/ml [³H]arachidonic acid. Cells were stimulated for the times indicated and the media was transferred to glass vials containing 300 µl ice-cold methanol:acetic acid (100:1.5 v/v) for determining levels of free [³H]arachidonate. The cells were scraped into ice-cold methanol:acetic acid (100:1.5 v/v), transferred to a glass vial containing chloroform and 4 µg arachidonic acid. The phases were separated by the addition of chloroform and H₂O, the organic phase was dried and separated by thin layer chromatography using hexane/diethyl ether/acetic acid (70:30:2 v/v), the arachidonic acid containing spots were identified with iodine, excised and radioactivity determined by scintillation counting. To determine the release of arachidonate metabolites the media was acidified to pH 3.5 with HCl and loaded onto a C18 Sep-Pak reverse-phase mini-column, organic material was eluted with ethyl acetate. Samples were then separated by HPLC on a reverse-phase C18 column (5 µm, 2.1 × 250 mm, Spherisorb ODS2). A linear gradient of water/methanol/acetonitrile/acetic acid (45:30:25:0.05, v/v; pH 5.7) changing to 100%

methanol in 40 min with a flow rate of 0.5 ml/min was used to elute the eicosanoids with detection at 235 and 270 nm. One-minute fractions were collected in scintillation vials and radioactivity determined by scintillation counting.

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Acknowledgements

This work was supported by Cancer Research UK and The Wellcome Trust, MN Hodgkin was a Beit Memorial Research Fellow.