Placental growth factor (PIGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement

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BACKGROUND: During metastasis, cancer cells migrate away from the primary tumour and invade the circulatory system and distal tissues. The stimulatory effect of growth factors has been implicated in the migration process. Placental growth factor (PIGF), expressed by 30–50% of primary breast cancers, stimulates measurable breast cancer cell motility in vitro within 3 h. This implies that PIGF activates intracellular signalling kinases and cytoskeletal remodelling necessary for cellular migration. The PIGF-mediated motility is prevented by an Flt-1-antagonising peptide, BP-1, and anti-PIGF antibody. The purpose of this study was to determine the intracellular effects of PIGF and the inhibiting peptide, BP-1.

METHODS: Anti-PIGF receptor (anti-Flt-1) antibody and inhibitors of intracellular kinases were used for analysis of PIGF-delivered intracellular signals that result in motility. The effects of PIGF and BP-1 on kinase activation, intermediate filament (IF) protein stability, and the actin cytoskeleton were determined by immunohistochemistry, cellular migration assays, and immunoblots.

RESULTS: Placental growth factor stimulated phosphorylation of extracellular-regulated kinase (ERK)1/2 (pERK) in breast cancer cell lines that also increased motility. In the presence of PIGF, BP-1 decreased cellular motility, reversed ERK1/2 phosphorylation, and decreased nuclear and peripheral pERK1/2. ERK1/2 kinases are associated with rearrangements of the actin and IF components of the cellular cytoskeleton. The PIGF caused rearrangements of the actin cytoskeleton, which were blocked by BP-1. The PIGF also stabilised cytokeratin 19 and vimentin expression in MDA-MB-231 human breast cancer cells in the absence of de novo transcription and translation.

CONCLUSIONS: The PIGF activates ERK1/2 kinases, which are associated with cellular motility, in breast cancer cells. Several of these activating events are blocked by BP-1, which may explain its anti-tumour activity.

Keywords: PIGF; breast cancer; motility; BP-1 peptide; Flt-1; IF protein

A growing body of clinical and experimental evidence implicates placental growth factor (PIGF) in pathologies, such as abnormal blood vessel formation and cancer progression (DePrimo et al, 2007; Ho et al, 2007; Fischer et al, 2007, 2008), including breast cancer (Parr et al, 2005). The PIGF is a member of the vascular endothelial growth factor (VEGF) family first isolated from the placenta, but later found to be normally expressed during wound healing and in the thyroid (Li et al, 2006; Maes et al, 2006; Kagawa et al, 2009). In cancer cells, PIGF mediates a number of cellular activities that promote metastasis, including attraction of endothelial cells for establishment of a blood supply, enhanced invasiveness, and cellular movement (Taylor et al, 2003; Casalou et al, 2007; Taylor and Goldenberg, 2007). In all 30–50% of human breast cancers express constitutive PIGF and its expression can be induced de novo in other PIGF-negative tumour cells that survive radioimmunotherapy (Taylor et al, 2002; Taylor and Goldenberg, 2007).

Alteration of growth factor receptor type or frequency, such as HER2/neu in breast cancer, is associated with poor prognosis or metastasis. The three known PIGF receptors are the receptor tyrosine kinase (RTK) Flt-1 (also designated as VEGFR1), and the glycosylphosphatidylinositol-anchored co-receptors, neuropilin-1 and -2 (NRP-1, NRP-2). Flt-1 is ectopically expressed by breast cancer tissue, blood vessels, and breast cancer cell lines, in contrast to normal breast tissue, in which NRP-1 is present and Flt-1 is absent or near background (Starzec et al, 2006; Taylor and Goldenberg, 2007). Therefore, it is possible that Flt-1 transmits PIGF signals, including those that lead to enhanced motility, in breast cancer.

Cellular motility is essential in the metastatic process, which requires cellular migration away from the primary tumour, invasion of surrounding tissue, entrance into the circulation, and then migration into a distant organ or tissue. It is not surprising then that alterations in cytoskeletal components responsible for motility mark the progression from a normal to neoplastic and then to a metastatic phenotype (Brotherick et al, 1998). The cytoskeleton is composed of three elements, actin microfilaments, microtubules, and intermediate...
filaments (IFs). Some pro-metastatic growth factors, such as epidermal growth factor, cause actin microfilaments to disassemble stress fibres, restructure focal adhesion complexes, and form actin-containing lamellipodia and filopodia for migration (Ronstrand and Heldin, 2001; Harper et al., 2007). The PlGF, as it stimulates cellular migration, may also be a pro-metastatic growth factor.

The IFs, which include cytoskeletons (CKs) and vimentin, are structural components that allow cells and tissue to endure stress, but IFs also participate in other cellular functions (Helfand et al., 2004; Oriolo et al., 2007). These proteins vary according to cell type and cellular differentiation stage in normal tissue, and are often dysregulated in primary cancers and cancer cell lines. The specific intracellular consequences of altered IF expression are uncertain, but it has been shown that co-expression of certain CKs and vimentin augments carcinoma cell motility (Domagala et al., 1990; Chu et al., 1996; Hendrix et al., 1997; Thomas et al., 1999), and this was demonstrated more specifically in a recent report by Schoumacher et al. (2010). CK8, CK18, and CK19 are expressed by normal breast tissue, but often CK19 predominates in the progression to malignancy, and its expression with vimentin, a mesenchymal IF, which is not normally expressed by epithelium, is indicative of poor outcome (Brotherick et al., 1998). This may be due to its role in promoting cellular mechanical stability, its participation in cellular movement, and its recently established role in stabilising invadopodia of metastasising cells (Eckes et al., 1998; Schoumacher et al., 2010).

The changes in cytoskeletal microfilament and IF necessary for migration or metastasis of cancer cells can be mediated through activation of a number of intracellular pathways (Tsuganezawa et al., 2002). Common intracellular targets of activated RTKs are the extracellular-regulated kinases (ERKs) and phosphotidylinositol-3-kinase (PI3K).

In a previous report, we investigated PlGF/Flt1 inhibition using the PlGF/Flt-1-expressing MDA-MB-231 xenograft metastatic model, and the PlGF receptor-inhibiting peptide, BP-1. Treatment with BP-1 was sufficient to inhibit the formation of pulmonary metastases in mice implanted with MDA-MB-231 (Taylor and Goldenberg, 2007). Using tissue microarrays it was also observed that 30–50% of primary breast cancers express PlGF, Flt-1, or both, whereas normal breast tissue expressed neither. Expression of Flt-1 was not confined to the blood vessels, but was also on tumour cells, and on breast tumour cell lines. This suggested that PlGF may not only serve as an angiogenic factor, which it is, but may also directly affect tumour cells expressing Flt-1. Evidence for the effect of PlGF on tumours is also offered by clinical data documenting that PlGF expression by human tumours, including breast cancers, is predictive of poor clinical outcome, which is characterised by aggressive disease, post-treatment recurrence, and metastases (Chen et al., 2004; Parr et al., 2005; Wei et al., 2005; Ho et al., 2007; Escudero et al., 2010).

This paper documents the results of in vitro analyses to determine how PlGF promotes cellular motility. To do this, the activation of several kinases by PlGF was investigated. The other goal of this study was to determine how the peptide, BP-1, which demonstrates anti-motility activity and its recently established role in stabilising invadopodia of metastasising cells (Eckes et al., 1998; Schoumacher et al., 2010).

**MATERIALS AND METHODS**

**Cell lines and treatments**

Cell lines were from the American Type Culture Collection (Manassas, VA, USA). Treatment of cells with BP-1 (1 μM) and PlGF (1 nM) was previously reported (Taylor and Goldenberg, 2007). The concentration of inhibitors was actinomycin D (ActD, de novo transcription), 1 or 10 μg ml⁻¹ (migration assay), 1 μg ml⁻¹ (immuno-blots); cycloheximide (CHX), 3 μg ml⁻¹ (de novo translation); PD98059 (PD98) (MEK pathway), 0.5–1 μM; LY294002, 2 μM (PI3K pathway) (all from Calbiochem, La Jolla, CA, USA), wortmannin (non-specific PI3K inhibitor), 5 nM (Sigma, St Louis, MO, USA).

**Migration assay**

Spontaneous migration (wound) assays were performed as previously described (Ilic et al., 1995; Mitra et al., 2005; Wong et al., 2005; Taylor and Goldenberg, 2007) using cells treated with PlGF, BP-1, inhibitor, or with antibody (1 μg ml⁻¹). Cells were treated with PlGF either simultaneously with, or 15 min before inhibitors, as indicated. At the indicated time points up to 3 h, coverslips were stained, mounted, and examined microscopically by two blinded researchers. Five to ten ×100 fields per slide were evaluated by counting cells separated from the wound edges and that appeared to be migrating. In two instances, results were normalised by a constant factor (e.g., two-fold) to diminish inter-experimental variation. The results are reported as the average number of cells migrating into the wound per ×100 field ± s.d. from 5 to 10 fields per coverslip; one to two coverslips per treatment per experiment, from two to five experiments, depending on the treatment.

**Immunoblots**

Immunoblot analysis was performed using standard methods (Chu et al., 1996). In all, 10 μg of protein per lane was loaded on gels; the primary antibody concentration was 1–5 μg ml⁻¹ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, or Cell Signaling Technology, Inc). (Beverly, MA, USA, http://www.cellsignal.com); biotinylated secondary antibody, 0.1–2 μg ml⁻¹ (Santa Cruz Biotechnology, Inc. or Sigma), along with a half-standard dilution of an avidin–biotin–HRP complex (Vector Laboratories, Inc., Burlingame, CA, USA). Blots were developed in Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed on Kodak X-AR film (Sigma). Blots were then stripped (Immunopure IgG Elution Buffer, Pierce), and re-probed for actin or another marker.

Immunoblot signal was quantified by scanning densitometry (Un-Scan-It Automated Digitizing System Software, Silk Scientific Corp., Orem, UT, USA), using the total pixel density. Relative expression of each protein is presented as the densitometry units, or calculated as the ratio of signal density to actin density, or compared to untreated controls.

**Immunofluorescence, immunohistochemistry, and detection of actin filament disruption**

Depolymerisation of the actin cytoskeleton was determined according to the method described by several groups (Arthur and Burridge, 2001; Hirshman et al., 2001, 2005; Barros and Marshall, 2005). Cells on coverslips were treated as indicated, fixed in 3.7% methanol-free formaldehyde (in PBS) for 15 min, followed by permeabilisation in 0.5% Triton X-100 (T-X-100) in PBS for 5 min, and blocked in 1% BSA in 0.1% T-X-100/PBS for 15 min. Cells were then labelled with 1 U FITC-labeled phallolidin (1 μg ml⁻¹) (Sigma), or labelled with both FITC-phallolidin and AlexaFluor594-DNase I (AF-DNase I) together corrects for differences in cell size or number per image, and thus aids in the quantification of changes in the polymerised actin fibres (Chan et al., 1998; Hirshman et al., 2001). Isoproterenol, which measurably depolymerised the actin
cytoskeleton after 1 min (2 and 20 μm) (MDA-MB-231), was the
depolymerisation control. Multiple views containing > 20 cells per × 400 microscopic field were captured at room temperature with Microfire software (Olympus America, Center Valley, PA, USA) with the exposure time, image-intensity gain, and enhancement held constant throughout to minimise intra- and inter-experimental variation. For each actin form, the number of pixels of a given fluorescence intensity was determined using Photoshop software (Adobe Systems, San Jose, CA, USA) at intensity values given fluorescence intensity was determined using Photoshop

Separate samples were permeabilised, blocked, and stained with antibodies (e.g., p-ERK1/2) at a concentration of 1 μg ml⁻¹. Primary antibody binding was detected by HRP-, FITC-, or PE-labeled secondary antibodies. The location of phosphorylated ERK (pERK) was determined for nuclear staining by counting cells with darkly stained nuclei in six to ten × 400 microscope fields at the wound edges vs total number of cells (average number of cells per treatment: 406 ± 11). Blue counterstained nuclei were considered negative. Nuclei with intermediate staining were counted, did not vary substantially between samples, and so are not included in the analysis. Cells were considered positive for pERK in the periphery if 40% of the cellular border was moderately to heavily positive.

For both bright field and fluorescent detection, mounted coverslips were examined at ×100 and ×400 with an Olympus BH-2 microscope (Olympus America, C2 40 objective, NA 0.70), and captured digitally with an Olympus U-PMTVC camera using Microfire software (Olympus America).

Statistics

Values are expressed as the mean ± s.d. or s.e.m. to summarise results. One-way analysis of variance or Student’s t-test was used to determine the P-values. P < 0.05 was considered significant.

RESULTS

Immediate migration response is independent of de novo mRNA or protein synthesis

We reported previously that MDA-MB-231 human breast cancer cells incubated with exogenous PlGF at a concentration of 1 nM attained significantly (analysis of variance) increased invasive potential (transwell) and motility (wound). MDA-MB-231 showed consistent and significantly increased motility of 1.5- to 2-fold within 3 h after ‘wounding’ the cell monolayer. On the other hand, invasion was measurable at a later time point (20 h) for MDA-MB-231, and the two other model cell lines, MCF-7 and MDA-MB-468. Similar to MDA-MB-231, MCF-7 responded to PlGF with increased invasiveness in 24 h, but MDA-MB-468 was unresponsive at all time points (Taylor and Goldenberg, 2007).

As the purpose of this study was to document the immediate effect of PlGF on kinase activation within 1–3 h of exposure, spontaneous motility assays (wound) with MDA-MB-231 were used because of the rapid and measurable kinetics of PlGF-stimulated migration, and because this cell line is tumorigenic and metastatic in mice. Similar to 30–60% of primary breast cancers, MDA-MB-231 also expresses the PlGF receptor, Flt-1. In addition, it expresses pERK when used alone or in the presence of PlGF (not shown, MDA-MB-231). PD98 also inhibited PlGF-stimulated phosphorylation when used alone or in the presence of PlGF (Figure 1A shows representative immunoblots). On the other hand, MDA-MB-468 displayed decreased pERK after 1 h (Figure 1A). The ERK1/2 kinases are the sole substrates of mitogen-activated protein kinase kinase (MAPKK or MEK); therefore, the MEK-specific inhibitor, PD98059, which are not substrates of MEK. PD98 (50 μM) treatment ablated ERK phosphorylation when used alone or in the presence of PlGF (not shown, MDA-MB-231). PD98 also inhibited PlGF-stimulated phosphorylation when used alone or in the presence of PlGF (Figure 1B). These results suggest a relationship between PlGF, increased motility, and the activity of the MEK/ERK1/2 pathway.

The PI3K cell surface receptors, Flt-1, and the glycosylphosphatidylinositol-linked co-receptor, NRP-1, are differentially expressed in normal breast tissue vs breast cancer. Soluble Flt-1, anti-PIGF antibody, and the Flt-1-antagonising peptide, BP-1, inhibited PlGF-stimulated cellular movement (Taylor and Goldenberg, 2007). These findings, taken together with the ablation of cellular motility by ERK1/2 inhibition (PD98) shown in Table 1, suggest that ERK1/2 and Flt-1 participate in PlGF-driven breast cancer cell movement.

The specificity of ERK activation by PlGF was further demonstrated when lysates from anti-PIGF or anti-Flt-1 antibody-treated

| Treatment                  | Average number of migrating cells per × 100 field ± s.d. |
|----------------------------|---------------------------------------------------------|
| Untreated                  | 4.3 ± 2.5                                               |
| PlGF (1 nM)                | 7.4 ± 2.8a                                              |
| PD98059 (50 μM) + PlGF     | 4.0 ± 2.3                                              |
| LY294002 (2 μM) + PlGF    | 7.5 ± 3.3a                                              |
| LY294002 + PD98059         | 7.6 ± 2.6a                                              |
| Untreated                  | 8.5 ± 3.9                                               |
| PlGF (1 nM)                | 11.2 ± 4.5a                                             |
| Actinomycin D (10 μg ml⁻¹) | 7.5 ± 4.0                                               |
| Actinomycin D + PD98       | 9.5 ± 4.1a                                              |
| Cycloheximide (3 μg ml⁻¹)  | 7.1 ± 4.8                                               |
| Cycloheximide + PD98       | 9.9 ± 5.3a                                              |

Table 1

PIGF-stimulated cellular motility is independent of de novo mRNA and protein synthesis, and inhibition of MEK/ERK pathway prevents PIGF-stimulated migration.
cells were probed for pERK by western blot analysis. Addition of either antibody inhibited ERK phosphorylation (Figure 1A), a result that further demonstrates the specificity of the PlGF-Flt-1-mediated phosphorylation of ERK.

Active ERK kinases translocate to the nucleus and to the cytoskeleton. As determined by immunohistochemistry, intranuclear pERK was abundant, even in untreated MDA-MB-231 cells, with 72–80% of nuclei staining heavily. Treatment of cells with the BP-1 peptide, combined with PlGF, significantly decreased the frequency of positive nuclei to 62 ± 5% (P < 0.001) (Figure 1B). The pERK was also found in a dotted pattern at the cellular periphery (see Materials and Methods) and in the perinuclear region. The frequency of cells staining for peripheral pERK differed among treatment groups with PlGF almost doubling vs untreated). Treatment with BP-1 prevented the PlGF-stimulated migration of breast cancer cells.

**IF expression by breast cancer cell lines**

As PlGF-stimulated cellular motility and ERK activation were prevented by BP-1 and anti-Flt-1 antibody, it was concluded that PlGF may exert its effect on the cytoskeleton at least partially through pERK. ERK kinases interact with both the actin and the IFs of the cytoskeleton, including vimentin. Changes in IF type and quantity are associated with breast cancer aggressiveness and invasion. Normal breast epithelium does not express the IFs of the cytoskeleton, including vimentin. Changes in IF type and quantity are associated with breast cancer aggressiveness and invasion. Normal breast epithelium does not express the mesenchymal IF protein, vimentin. Phosphorylated ERKs1/2 bind vimentin, stabilising ERK in its phosphorylated form, which in turn leads to rearrangement of the vimentin network (Perlson et al., 2006; Henson and Vincent, 2008).

To investigate the relationship of ERK1/2, PlGF, and IF expression in cellular motility, the relative amounts of vimentin and the CKs 8/18 and 19 were first assessed for three breast cancer cell lines (MDA-MB-231, -468, and MCF-7). Results are shown in Figure 2A. MDA-MB-231 expresses abundant vimentin, but it was undetectable in MDA-MB-468 and MCF-7 even after long exposures (Figure 2A). The immunoblots showed that MDA-MB-231 expresses abundant vimentin, but it was undetectable in MDA-MB-468 and MCF-7 even after long exposures (Figure 2A). The immunoblots showed that MDA-MB-231 expresses abundant vimentin, but it was undetectable in MDA-MB-468 and MCF-7 even after long exposures (Figure 2A). The immunoblots showed that MDA-MB-231 expresses abundant vimentin, but it was undetectable in MDA-MB-468 and MCF-7 even after long exposures (Figure 2A).

The MCF-7 CK8/18 to actin ratio was slightly increased over the CK19 ratio, but both were abundantly expressed (3.9 ± 0.6 vs 2.9 ± 0.32). On the other hand, MDA-MB-231, an aggressive cell line that forms metastases from subcutaneous...
xenograft tumours, expressed six-fold greater CK19 than CK8/18 (Figure 2A). MDA-MB-468, also tumourigenic but non-metastasising, expressed high levels of the CKs but no vimentin.

Alterations in vimentin expression vs PlGF

To assess whether PlGF would alter vimentin expression, MDA-MB-231 was stimulated with PlGF for 1–3 h, and the relative amount of vimentin was determined by immunoblot. Increased vimentin expression of 1.9- to 4-fold compared with the untreated control was detected at the 1-h time point (Figure 2B, P < 0.02). Addition of the peptide BP-1 to the PlGF-containing cultures resulted in a reversal of the PlGF-stimulated increase at 3 h (1.07 of untreated control) (Figure 2B).

To investigate this early effect of PlGF on vimentin expression mediated by de novo mRNA or protein synthesis, MDA-MB-231 cells were incubated with sub-toxic doses of ActD and CHX (1 and 3 μg/ml, respectively) to inhibit mRNA and protein synthesis, respectively. Lysates were assessed for vimentin at 30 min, 1 h, and 3 h. In these experiments incubation of MDA-MB-231 with ActD resulted in declining vimentin over 3 h. The CHX treatment, on the other hand, caused a 50% decrease in vimentin in 1 h, but after 1 h the amount of vimentin stabilised and did not decline significantly thereafter. As shown on the graph in Figure 2C, it appears that both de novo mRNA transcription and translation are involved in vimentin production, but in the absence of translation, a mechanism for stabilisation of vimentin is activated that prevents or slows its loss.

In further experiments investigating the influence of PlGF on vimentin expression, MDA-MB-231 cells were pre-incubated with PlGF for 15 min before addition of ActD or CHX. This resulted in persistence of vimentin, even in the presence of ActD or CHX (Figure 2C). Thus, it is probable that PlGF activates mechanisms that promote the stability of vimentin independently of mRNA or protein synthesis.

PlGF mediates stability of specific IF CKs

Just as expression of mesenchymal vimentin is associated with aggressive breast cancer, changes in the pattern of CK expression are also considered indicative of breast cancer aggressiveness and motility, and co-expression of CK19 and vimentin is indicative of poor clinical outcome (Brotherick et al, 1998; Buhler and Schaller, 2005). The effect of PlGF on CK expression was assayed, and it was found that PlGF did not influence the abundance of CK8/18 (not shown). However, after incubation for 1 h with PlGF, CK19 increased 1.5-fold over untreated controls (P = 0.05) (MDA-MB-231, four experiments). Treatment with BP-1 measurably attenuated CK19 expression in PlGF-treated samples by 2 h (average 0.58 ± 0.32 of PlGF-only, two experiments), suggesting that autocrine PlGF signalling, which was blocked by the peptide, may have a role in the maintenance of CK19 expression. A control peptide did not affect CK19 expression (not shown).

The antagonistic effect of BP-1 on CK19 expression could be due to interruption of PlGF-Fit-1-mediated stabilisation of CK19 protein. To further investigate the influence of PlGF on the stability of CK19, cells were treated with either ActD or CHX at the sub-toxic doses described above. Cumulative results are shown in Figure 2A and Figure 2B shows representative blots. In the CHX-treated samples, CK19 diminished to 50% of initial levels by 30 min, remained at that level at the 1-h time point, after which it declined to very low levels by 3 h (as shown in Figure 3A). The ActD treatment also brought about a steady decrease in CK19 (Figure 3A). These results suggest that CK19 expression is regulated, in part, by transcription and translation.

In separate experiments, MDA-MB-231 was pre-treated with PlGF for 15 min before adding ActD or CHX. Pre-treatment prevented the decrease in CK19 seen with single-agent treatment, such that the CK19 levels remained higher than the untreated controls (Figure 3A). Thus, the effect of PlGF on CK19 seems to be independent of de novo transcription or translation, and may be related to the rate of degradation of CK19.

PlGF stimulates rearrangement of the actin cytoskeleton

The microscopic appearance of PlGF-treated breast cancer cells stained for F-actin differed from untreated cells by the appearance of increased actin-rich punctate structures on the edges of the cells (not shown). This observation may be due to the re-organisation of the actin cytoskeleton as the cell becomes motile, and which has been documented for other growth factors and carcinomas, for example, epidermal growth factor (Chan et al, 1998; Harper et al, 2007). The state of the actin cytoskeleton was quantified by determination of the F-actin (filamentous, polymerised) to G-actin (non-polymerised) ratio using phalloidin and DNase I, which stain F-actin and G-actin, respectively, as described in Materials and methods. The ratio of F-actin to G-actin decreased to less than half of untreated controls (Figure 4). In contrast to the effect of PlGF alone, addition of the inhibitory peptide, BP-1, to PlGF-treated cultures prevented lowering of the F-actin to G-actin ratio associated with PlGF.
of inhibitors on cellular motility, a pro-metastatic behaviour, was analysed. The results show that PGF-driven breast tumour cell motility include the activation of ERK1/2 kinases, stabilisation of IF proteins, and re-organisation of the actin cytoskeleton. The PGF specificity of these results was shown by their ablation with anti-Flt-1 antibody or BP-1.

The ERKs are activated by a number of different factors, including c-Src, the G-protein-linked kinase Raf, and MEK (Barros and Marshall, 2005; Chen et al., 2006). Increased pERK stimulated by PGF was reversed by treatment with BP-1 or a MEK-specific inhibitor. Ablation of MEK not only prevented pERK formation, but it also prevented PGF-stimulated cellular migration. Thus, as the only known substrates of MEK are the ERK1/2 kinases, it can be concluded that PGF-stimulated motility depends on activation of the ERKs by MEK. This does not eliminate other potential ERK activators, and preliminary data (not shown) suggest that Raf participates in PGF effects as well.

While this study was in progress, another report investigated PGF-mediated migration of leukaemia cells (Casalou et al., 2007). Our findings are in agreement with this report, in that we found no evidence of Akt involvement in PGF-mediated breast cancer cell movement. However, unlike Casalou et al, little evidence of p38 MAPK involvement in PGF-mediated breast cancer cell movement was found because movement was abrogated by inhibition of MEK, which does not activate p38 MAPK. Therefore, it is unlikely that p38 MAPK contributes substantially to the movement of breast cancer cells in the presence of PGF. Another point of difference was the finding that VEGF had little to no effect on breast cancer cell movement (Taylor and Goldenberg, 2007). This contrasts with the results obtained by Casalou et al for leukaemia cells. These differences may be due to different effects of VEGF and PGF on cancers of epithelial origin, such as breast cancer, in contrast to cancers of hematopoietic origin, where the function of VEGF and PGF may be redundant.

When activated, the ERK kinases localise to the nucleus or to focal adhesions where they are associated with motility by interaction with cytoskeletal substrates, including IF proteins, the actin cytoskeleton, and myosin light chain kinase (Zheng and Guan, 1994; Schlaepfer et al., 1998; Arthur and Burridge, 2001; Pawlak and Helfman, 2002; Yin et al., 2005). This study presented evidence that PGF promoted translocation of pERK1/2 to the cellular periphery, and so there it may function to increase motility.

Although CK18 and CK19 are expressed by normal breast epithelium, their expression is often dysregulated in breast cancers (Ferrero et al., 1990; Boecker et al., 2002). Aggressive MDA-MB-231 displays a pattern of predominant CK19 with little or no CK18, whereas this is not the case for MCF-7 or MDA-MB-468, which both produce abundant CK18 (and CK19). Human breast cancers expressing high levels of CK18 (without vimentin), similar to MCF-7 or MDA-MB-468, are less likely to recur. In addition, MDA-MB-231 transfected with CK18 obtains an epithelial phenotype, reduces aggressiveness in tumour growth, and loses vimentin expression (Buhler and Schaller, 2005). On the basis of the results of this study, in which CK19 and vimentin expression rose on PGF stimulation, it is possible that RTK activation, such as Flt-1, stimulates co-expression of CK19 and vimentin.

Likewise, vimentin, a mesenchymal IF protein not expressed by normal breast cells, is associated with poorer clinical outcomes and resistance to apoptosis and drugs (Yuan et al., 1997; Stathopoulou et al., 2002; Willipinski-Stapelfeldt et al., 2005), as well as increased metastasis and invasion (Hartig et al., 1998; Tolstonog et al., 2001; Singh et al., 2003; Hu et al., 2004; Schoumacher et al., 2010). McInroy and Maatta (2007) showed that the aggressiveness of MDA-MB-231 depends on vimentin expression, because its ablation impaired migration and invasion. Our data show that disrupting PGF or Flt-1 signalling with antibody or BP-1 depressed vimentin, CK19 expression, and motility.

**DISCUSSION**

The purpose of this study was to analyse the early events triggered by PGF in the responsive breast cancer cell, and elucidate how the peptide, BP-1, works in inhibiting cellular motility in vitro, and in preventing metastasis formation in xenograft models of breast cancer (Taylor and Goldenberg, 2007). To carry out this, the effect
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