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Original Paper

Melanoma Cell-derived Factor Stimulation of Fibroblast Glycosaminoglycan Synthesis—The Role of Platelet-derived Growth Factor

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The hyaluronan-rich matrix surrounding many tumours may facilitate tumour growth, invasion and angiogenesis, with the majority of this hyaluronan apparently being synthesised by normal fibroblasts, stimulated to do so by tumour cell-derived factors. Melanoma cell-conditioned medium (CM) stimulates up to a 6-fold increase in fibroblast glycosaminoglycan (GAG) synthesis, with the active factors being present in tumour CM ultrafiltration fractions > 30 kDa and < 1 kDa. These fractions are poorly active individually, but when recombined, the activity is substantially greater than the additive effect. The objective of this study was to identify the factors present in the ultrafiltration fraction > 30 kDa that produce a greater than additive effect with the fraction < 1 kDa in stimulating the incorporation of ^{3}H glucosamine into fibroblast GAGs. A number of factors including basic fibroblast growth factor (bFGF), interleukin (IL)-1β, pleiotrophin, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) failed to stimulate any significant increase in GAG synthesis, but when added to the < 1 kDa tumour CM fraction, both PDGF and to a lesser extent, bFGF, exhibited potent stimulating activities. Neutralising antibodies to PDGF and bFGF added to the melanoma CM decreased the fibroblast GAG-stimulating activity by 29% and 40%, respectively, in C8161 melanoma CM and by 47% and 45%, respectively, in Hs294T melanoma CM. The activities of PDGF-AA and PDGF-BB isoforms were indistinguishable, suggesting the PDGF-α receptor plays a role in the GAG-stimulatory response. Western analysis following treatment with PDGF, bFGF or melanoma CM revealed banding patterns for PDGF and melanoma CM that were similar. Immunoprecipitation of the PDGF-α receptor revealed it to be phosphorylated in fibroblasts treated with PDGF and melanoma CM, but not with control fibroblast CM. These studies suggest that PDGF plays an important role in the GAG-stimulating activity of the melanoma CM, but requires the presence of an as yet unidentified novel low molecular weight factor for full activity. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: melanoma cell-derived factors, fibroblasts, glycosaminoglycans, hyaluronan, PDGF, bFGF
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INTRODUCTION

THE RELATIONSHIP between a tumour and normal host tissue has been well documented as playing an important role in tumour growth, migration, invasion and metastases. The interaction of many tumour cells with host fibroblasts stimulates the normal adult fibroblast to exhibit an elevated level of migratory behaviour [1] and increased synthesis of certain extracellular matrix (ECM) molecules including glycosaminoglycans (GAG) and in particular, hyaluronan. Many human tumours including melanoma, breast, prostate, pancreatic and colon carcinoma are associated with high levels of certain ECM molecules. The hyaluronan-rich matrix in carcinomas is associated with proliferation and invasion, and in many human breast carcinomas, higher levels of hyaluronan are found in tumoral compared with adjacent non-tumoral tissue, while the peripheral invasive areas contain the highest levels of hyaluronan [2]. Hyaluronan may also play a major

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role in facilitating tumour cell growth and migration, as hyaluronan synthesis is elevated during cell proliferation and may promote cell locomotion. Indeed, melanoma cells have been shown to express high levels of the hyaluronan receptor CD44 which is required for migration on hyaluronan substrates [3]. High levels of hyaluronan surrounding growing tumours may also affect angiogenesis, with hyaluronan oligosaccharides being potent stimulators of angiogenesis while high molecular weight hyaluronan is inhibitory to angiogenesis.

In murine B16F1 melanoma cells, elevated levels of cell surface hyaluronan expression correlate with metastatic potential, as measured by increased lung colonisation and larger tumours following tail vein injection [4]. However, the expression of hyaluronan is not common to all tumour cell types, but is frequently associated with host fibroblasts following stimulation by tumour-derived factors [5, 6]. Several tumour cell types have been shown to exhibit this paracrine effect on normal fibroblast cells including lung, breast, ovarian and colon carcinoma cells [7, 8]. It has been demonstrated that melanoma cell-conditioned medium (CM) [9] is a potent stimulator of fibroblast GAG synthesis, but the active factors within the CM have not yet been identified.

A large number of cytokines have been investigated for their ability to stimulate fibroblast GAG synthesis. Interferon-γ, tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) all mediate small increases in fibroblast GAG synthesis and in combinations stimulate overall additive or synergistic responses [10]. Interleukin-6 (IL-6) [11], oncostatin M (OSM) [12], platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [13] have also been shown to stimulate GAG synthesis in fibroblast cultures. Melanoma cells express RNA transcripts for a wide range of cytokines including IL-1β, IL-6, leukaemia inhibitory factor, IL-7, growth related protein-α, transforming growth factor-α, EGF, TGF-β, endothelial cell growth factor (ECGF), bFGF, nerve growth factor, stem cell growth factor, IL-8 and the p35 chain of IL-12. Only a limited number of these are expressed in normal melanocytes, of which many show very low levels of expression [14–16]. This suggests a possible relationship between the increased range and expression of growth factors produced by melanoma cells and the ability of many cytokines to stimulate GAG synthesis. There appears to be little consistency between the growth factor expression pattern of different human melanomas, thus ruling out the possibility of using this feature as a prognostic indicator [17].

We have already suggested that a number of melanoma-derived factors are involved in the stimulation of GAG synthesis in normal adult fibroblasts [8] and molecular weight fractionation of the melanoma cell CM has demonstrated low and high molecular weight fractions which are interdependent on one another for this effect.

The purpose of the present study was to identify which factors are involved in the activity of the high molecular weight fraction and to what extent their ability as stimulators of fibroblast GAG synthesis is dependent upon the presence of the low molecular weight fraction.

MATERIALS AND METHODS

Reagents

bFGF, IL-1β, pleiotrophin, PDGF, TGFβ, TNF-α, vascular endothelial growth factor (VEGF) and all growth factor antibodies were purchased from R&D Systems Europe Ltd (Abingdon, U.K.) while individual isoforms of PDGF were purchased from Genzyme Diagnostics (West Malling, Kent, U.K.). The antibody to phosphotyrosine was purchased from Sigma (Poole, U.K.). Eagle's minimal essential medium (EMEM), fetal calf serum (FCS), phosphate buffered saline (PBS; Dulbecco's A), and Nunclon tissue culture plastics were obtained from Life Technologies (Paisley, U.K.). Pronase E from *Streptomyces griseus* was from Sigma, while D-[6-^{3}H]-glucosamine (25.2 Ci/mmol) and enhanced chemiluminescence (ECL) reagents were from Amersham (Aylesbury, U.K.). All other reagents were of analytical grade.

Cell culture and preparation of conditioned medium

Human adult breast skin fibroblast cultures were initiated from explants obtained from reduction mammoplasty skin and were used between passages 5–10. Hs294T melanoma cells [18] were established from a lymph node metastasis of a 56-year-old male caucasian, while the C8161 melanoma cell line was established from an abdominal wall metastasis of a menopausal woman with recurrent malignant melanoma and has been characterised by Welch and associates [19]. All cell lines were routinely cultured in EMEM, supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air.

CM was prepared by washing almost confluent cultures twice with serum-free EMEM, incubating for 2 h in the presence of serum-free medium, and finally changing to fresh serum-free EMEM and incubating for a further 48 h. The CM was harvested and passed through a 0.45 μm filter, and stored at − 30°C.

Ultrafiltration

CM were passed through an Amicon ultrafiltration membrane with a molecular weight exclusion limit of 1 kDa at a pressure of 35 psi. Eighty per cent of each sample was passed through the membrane, effectively concentrating material greater than the molecular weight limit 5-fold. Samples passing through the membrane at < 1 kDa and samples concentrated on the membrane at > 1 kDa that were rediluted to a 1× concentration with MEM were tested for GAG-stimulating activity.

Heparin–Sepharose affinity chromatography

A heparin-Sepharose column (1×4 cm) (Pharmacia, Biotech, Uppsala, Sweden) was equilibrated with PBS prior to elution of CM or fractions thereof through the column at a flow rate of 0.25 ml/min. The column was then washed with 4 bed volumes of PBS before finally eluting any bound material with PBS containing 2 M NaCl. The desorbed fraction was desalted using an Amicon ultrafiltration membrane with a 1 kDa cut-off membrane (YM-1) by repeated filtering and dilution in EMEM.

Heat and trypsin sensitivity

The CM or individual fractions of the CM < 1 kDa or ≥ 1 kDa were subjected to heat treatment at 80°C for 10 min and 100°C for 2 min. The heat-treated samples were tested individually and after recombination on fibroblast cells for GAG stimulation. For testing the trypsin sensitivity, total CM and CM fractions were treated with 10 μg/ml trypsin and incubated at 37°C for 1 h after which trypsin inhibitor was added at 20 μg/ml. Control samples were treated with

premixed trypsin/trypsin inhibitor and incubated at 37°C for 1 h. All fractions, both trypsin-treated and controls were recombined in all possible combinations to measure the GAG stimulation.

Radiolabelling and isolation of GAGs

Confluent fibroblast cultures in 24-well multiwell tissue culture dishes were washed in serum-free EMEM and 0.6 ml of test media (conditioned media or MEM with or without the addition of growth factor) added and the fibroblasts incubated for 24 h in the presence of D-[6-^{3}H]-glucosamine (5 μCi/ml). The medium from each well was transferred to 1.5 ml Eppendorf tubes together with 2×0.1 ml PBS washes, and boiled for 5 min. Carrier GAGs (hyaluronan; 175 μg/ml and chondroitin-6-sulphate; 250 μg/ml) were added, and the samples treated with Pronase (0.5 mg/ml) for 24 h at 50°C, heat-inactivated, and GAGs precipitated by the addition of 1/5 volume of 10% cetylpyridinium chloride in 0.03 M NaCl, and incubated at 40°C for 30 min. The precipitated GAGs were collected by centrifugation (10 000 ***g***; 5 min) and the pellet washed twice with 0.1% cetylpyridinium chloride in 0.03 M NaCl. The GAG pellets were then dissolved in 1 ml of 2 M NaCl, and triplicate 0.1 ml samples taken for scintillation counting in 5 ml Packard Ultima-Flo AF scintillation fluid. The cells remaining in the multiwell dishes were detached by a 3-min exposure to 0.05% trypsin, 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free PBS, and counted using a Coulter counter. Total GAG synthesis is expressed on a per cell basis as the mean and standard deviation (S.D.) of triplicate wells.

Neutralising antibody studies

Specific neutralising antibodies to PDGF and bFGF and a number of other growth factors (IL-1β, TGF-β and TNF-α) were used to neutralise these growth factors from the melanoma cell CM. The anti-human PDGF antibody was a goat polyclonal antibody which can recognise all dimeric isoforms of human PDGF, i.e. PDGF-AA, PDGF-BB and PDGF-AB and was used at a concentration of 20 μg/ml with the ability to neutralise 10 ng/ml PDGF. Anti-human bFGF neutralising antibody, a goat polyclonal antibody raised against a recombinant human bFGF was used at 20 μg/ml, a concentration able to neutralise 0.25 ng/ml bFGF, while all other antibodies were tested at a range of concentrations. CM samples were pre-incubated with the specific growth factor antibodies for a period of 1 h before direct application to the GAG-stimulating assay, while control samples were incubated for 1 h in the presence of 20 μg/ml normal goat IgG.

ELISA assays

Levels of PDGF-AB and bFGF growth factors in the melanoma cell CM were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (Quantikine Human FGF basic and Quantikine human PDGF-AB both R&D Systems Europe Ltd). Assays were carried out according to the manufacturer's instructions.

Preparation of cell extracts for Western blotting

Breast fibroblasts were seeded in 25 cm^2 tissue culture flasks and grown to confluence in EMEM with the addition of 10% FCS then washed in serum-free EMEM for 3 h. The medium was then removed and the appropriate test medium prewarmed to 37°C, added, and the cells incubated at 37°C for 5 min. The cultures were then washed twice in ice-cold PBS with the addition of sodium orthovanadate (Na_2VO_4) at 1 mM followed by the addition of 0.6 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 1 mM Na_2VO_4, 2 mM EDTA plus a range of protease inhibitors) and the cells harvested by scraping, then centrifuged to remove cellular debris.

Western blotting and immunodetection

Sodium dodecyl sulphate (SDS), dithiothreitol (DTT) glycerol and bromophenol blue were added to the cell extracts and the protein samples denatured at 100°C for 2 min. Samples were then run out on 8% SDS/polyacrylamide gel electrophoresis (PAGE) gels, transferred on to nitrocellulose, blotted with an antibody against phosphotyrosine and finally detected using a horseradish peroxidase (HRP) linked anti-mouse IgG antibody followed by ECL.

Immunoprecipitation

Confluent fibroblast cells were treated with test medium for 5 min as before, then washed twice with ice-cold PBS containing 1 mM Na_2VO_4 before the addition of 1 ml of cold RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_2VO_4 and a selection of protease inhibitors). The cells were incubated on ice for 10 min then harvested using a cell harvester into an Eppendorf tube and cellular debris removed by centrifugation. Four micrograms of PDGF receptor α polyclonal antibody or normal goat IgG were added to 0.5 ml of each sample and incubated on ice for 2 h, after which 50 μl of prewashed protein G Sepharose beads were added and incubation continued for a further 4 h. Immunoprecipitations were then collected by centrifugation and washed 4 times with 1 ml samples of RIPA buffer, and finally the pellets were resuspended in 45 μl of 2× sample buffer (250 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 20 mM DTT and 0.1% bromophenol blue) to which an equal volume of distilled water was added. The samples were heated to 100°C for 2 min, centrifuged, and the supernatant run out on an 8% SDS/PAGE gel and immunoblotted for phosphotyrosine as before.

Statistical analysis

All values are shown as the means ± S.D. of three separate samples from a single representative experiment repeated at least three times. Differences between mean values were assessed for statistical significance by the two-tailed Student's *t*-test.

RESULTS

Conditioned media (CM) from two human melanoma cell lines, Hs294T and C8161 were tested on normal fibroblasts for their ability to stimulate GAG synthesis. As previously observed [8] both cell lines were able to stimulate a significant increase in the levels of fibroblast GAG synthesis over controls (data not shown). GAG synthesis was routinely measured using ^{3}H-glucosamine incorporation, although the stimulatory effect has also been measured in both cell lines using a direct uronic acid estimation and by measuring hyaluronan by a hyaluronan radiometric assay (Kabi Pharmacia Diagnostics, Milton Keynes, U.K.) [8].

When CM from each of the melanoma cell lines was passed through an ultrafiltration membrane with a molecular weight cut-off of 1 kDa, very low levels of GAG-stimulating activity were found in both the material passing through the

membrane and the material concentrated on the membrane which had been rediluted to its original volume with MEM before testing in the GAG assay. Full GAG-stimulating activity was only regained when the two fractions were recombined to their original concentrations (Figure 1). The GAG-stimulating activity of the high molecular weight fraction exhibited a limited amount of heat and trypsin sensitivity, while up to 50% of the activity was lost upon exposure to heparin-Sepharose, the activity being assessed following recombination with the low molecular weight fraction. These properties were not observed in the low molecular weight fraction (data not shown). Using these characteristics as a guide, a number of peptide growth factors were selected for examination of their ability to stimulate fibroblast GAG synthesis. bFGF, IL-1β, insulin, pleiotrophin, PDGF, TGF-β, TNF-α and vascular endothelial growth factor were all tested at a range of concentrations. However, only PDGF exhibited a significant effect on GAG synthesis although this only resulted in relatively small increases (Figure 2). This is in line with our observations of the fractionated tumour cell CM, in which the higher molecular weight fraction required the presence of the low molecular weight fraction for activity. These same growth factors were therefore tested for GAG-stimulating activity in the presence of the low molecular weight fraction (< 1 kDa) from melanoma cell CM. Of all the growth factors tested, only PDGF (a mixture of all isotypes at 5 ng/ml) and bFGF (10 ng/ml) produced a substantially greater than additive effect with the low molecular weight fraction (Figures 2 and 3) to stimulate fibroblast GAG synthesis.

In order to establish the role of PDGF and bFGF in the melanoma cell CM stimulation of fibroblast GAG synthesis, commercial ELISA techniques were utilised to detect the presence of PDGF and bFGF in the melanoma cell CM. In the C8161 CM, there was 1.7 ng/ml of PDGF-AB and 160 pg/ml of bFGF detected, and in the Hs294T CM, 1.6 ng/ml PDGF-AB and 12 pg/ml bFGF detected, while these levels were increased approximately 5-fold in the concentrated high molecular weight fraction. Neutralising antibodies were used against PDGF and bFGF in order to determine their role in stimulating GAG synthesis. A goat polyclonal antibody able to recognise all human isoforms of PDGF was added to the melanoma cell CM for 1 h before addition to the fibroblast cells. The CM of both the melanoma cell lines tested showed a significant loss in GAG-stimulating activity when compared with control samples treated with IgG alone (Figure 3a), suggesting melanoma-derived PDGF could play a role in the stimulation of GAGs

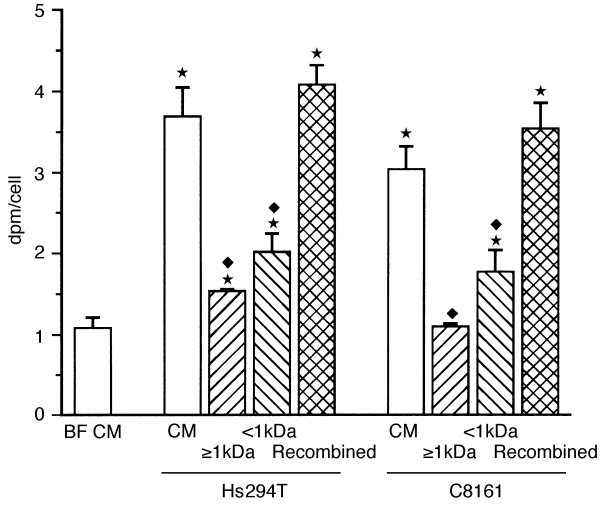

Figure 1. Effect of melanoma cell CM and low and high molecular weight fractions of melanoma cell CM on fibroblast GAG synthesis (dpm/cell). Breast fibroblasts were treated with breast fibroblast CM (BF CM), Hs294T or C8161 CM, ≥ 1 kDa or < 1 kDa CM fraction diluted to a 1× concentration with EMEM, or recombined ≥ 1 kDa and < 1 kDa fractions to the original concentrations. Each value represents the mean and S.D. of at least three separate samples. *Represents a significant difference from BF CM control and ◆ represents a significant difference from melanoma cell CM, using a two-sample *t*-test.

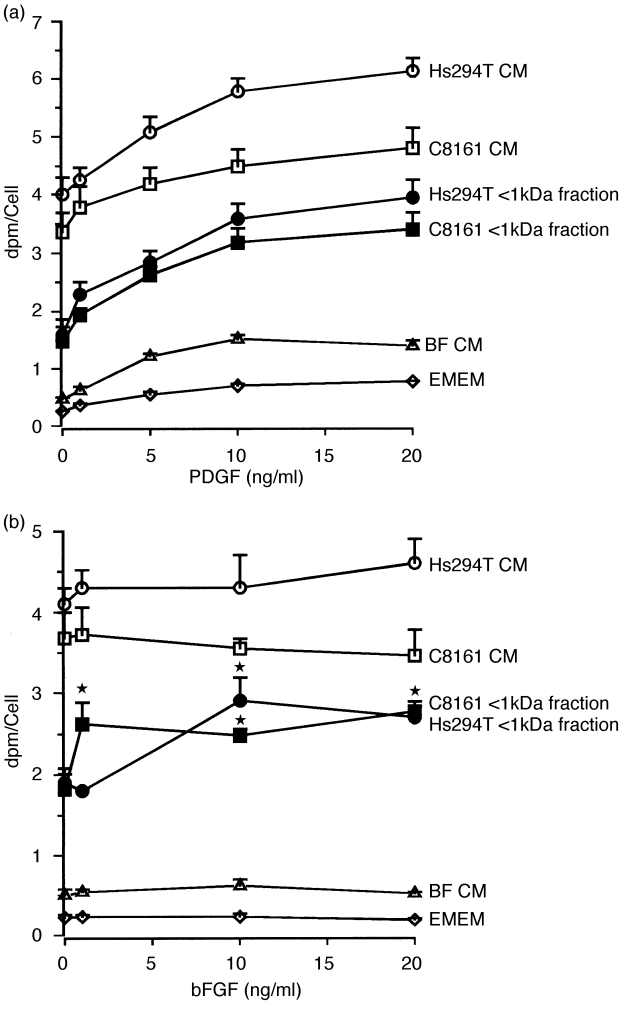

Figure 2. Dose response effects of: (a) PDGF (1, 5, 10 and 20 ng/ml)-treated; or (b) bFGF (1, 10 and 20 ng/ml)-treated CM on fibroblast GAG synthesis (dpm/cell). Breast fibroblast cells were treated with EMEM, breast fibroblast CM (BF CM), Hs294T CM, C8161 CM, Hs294T CM <1 kDa fraction, C8161CM <1 kDa fraction, with the addition of either PDGF or FGF. Each value represents the mean and S.D. of at least three separate samples. All concentrations of PDGF (1–20 ng/ml) gave a significant increase above appropriate CM control except C8161 and Hs294T CM, where a significant difference was observed at 5–20 ng/ml only, using a two-sample *t*-test. bFGF stimulated a significant increase in the fibroblast GAG synthesis above appropriate controls where marked with * using a two-sample *t*-test.

by melanoma-derived factors. The same procedure was repeated using a polyclonal antibody against bFGF and even although much lower concentrations of bFGF were detected in the melanoma cell CM, significant levels of GAG-stimulating activity was neutralised from the CM, suggesting bFGF is also playing a role in the stimulation of GAG synthesis (Figure 3b). Neutralising antibodies to IL-1β, TNF-α, and a pan-specific TGF-β antibody were also added to the melanoma CM at a range of concentrations but no decrease in the fibroblast GAG stimulation was found (data not shown).

The role played by PDGF and bFGF at the level of the fibroblast receptor was examined using Western blot analysis of tyrosine phosphorylation following exposure of fibroblasts to Hs294T complete CM, PDGF (10 ng/ml), bFGF (10 ng/ml), Hs294T CM < 1 kDa fraction, and control fibroblast CM. Solubilised cell extracts were run on SDS-PAGE gels and immunoblotted for phosphotyrosine. Exposure to Hs294T CM resulted in a similar phosphotyrosine pattern as that obtained with PDGF, but which was absent with the low molecular weight fraction. Exposure to bFGF resulted in no detectable effect on the phosphotyrosine banding pattern (Figure 4). These changes in band patterns were not due to uneven loading, as two constitutive bands at approximately

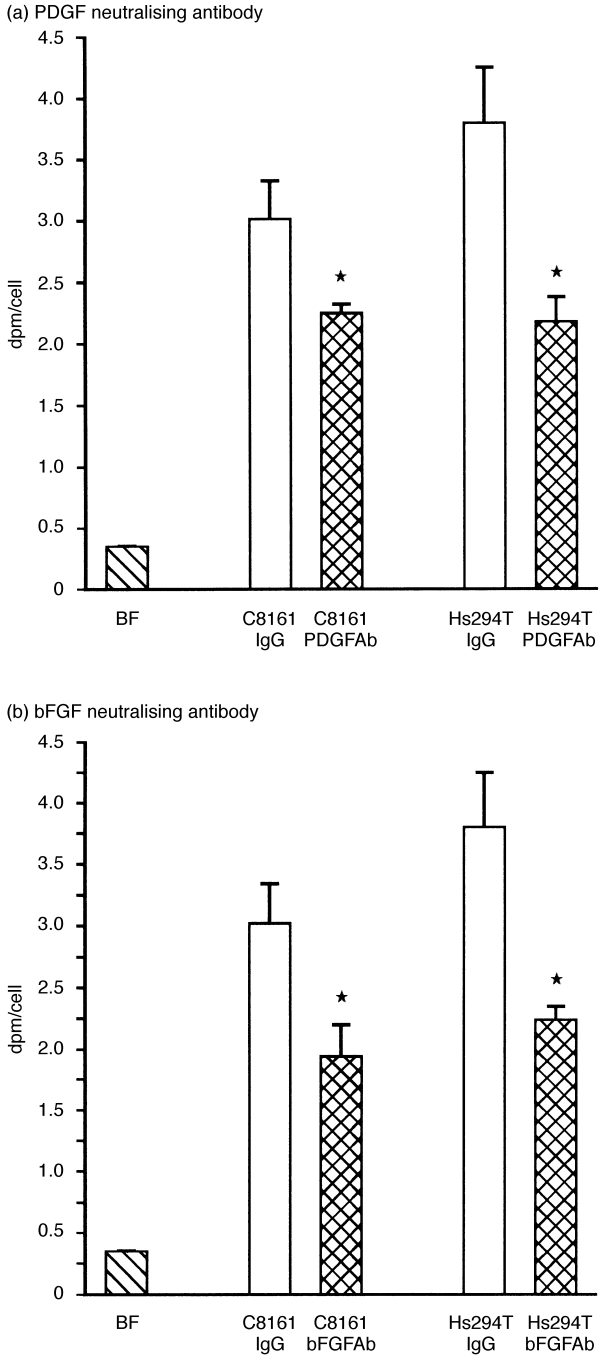

Figure 3. Effect of (a) PDGF neutralising antibody (polyclonal goat IgG, 20 μg/ml) or (b) bFGF neutralising antibody (polyclonal goat IgG, 20 μg/ml) on C8161 and Hs294T CM stimulation of fibroblast GAG synthesis. Normal goat IgG was utilised as a control. Each value represents the mean and standard deviation (S.D.) of at least three separate samples. *Represents a significant difference of the PDGF or bFGF Ab-treated sample against the IgG-treated control using a two-sample *t*-test at a *P* value <0.05.

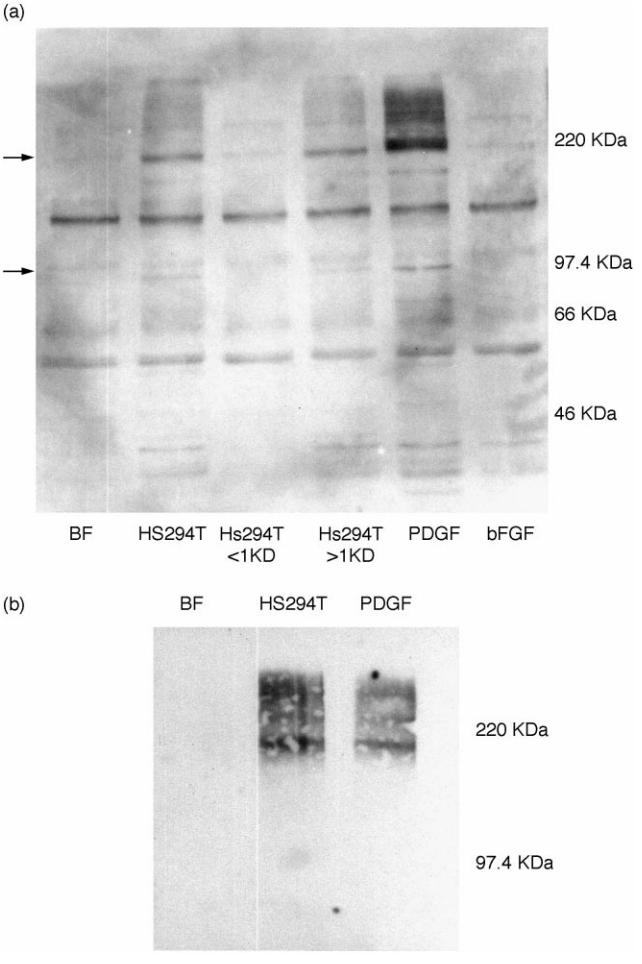

Figure 4. (a) Immunoblot for phosphotyrosine of SDS–PAGE of fibroblast extracts following exposure of cells to BF CM, Hs294T CM, Hs294T CM<1 kDa fraction, Hs294T CM≥1 kDa fraction, PDGF (10 ng/ml) and bFGF (10 ng/ml). Molecular weight markers are shown on the right of the blot, while arrows indicate phosphotyrosine bands unique to PDGF, bFGF or melanoma cell CM-treated fibroblasts. (b) Immunoblot for phosphotyrosine of immunoprecipitated PDGF receptor α run out on 8% PAGE. Samples are breast fibroblast extracts following exposure of cells to BF CM, Hs294T CM or PDGF (10 ng/ml).

130 and 57 kDa stained remarkably evenly in all samples and thus provided an ideal standard for comparison. To verify that the PDGF receptor was being phosphorylated by melanoma cell CM, PDGF receptor-α was immunoprecipitated from fibroblast cells after treatment with the control BF CM, Hs294T CM and PDGF (10 ng/ml) then run out on a Western gel and immunoblotted for phosphotyrosine. Phosphotyrosine was detected in Hs294T CM and PDGF-treated cells but not the breast fibroblast CM-treated controls (Figure 4b).

The isoforms of PDGF, PDGF-AA and PDGF-BB were added to the fibroblasts in the presence of Hs294T CM < 1 kDa fraction and GAG synthesis measured. Both isoforms mediated a significant stimulation of fibroblast synthesis at similar levels when applied to the fibroblast cells in the presence of the low molecular weight fraction (Figure 5), suggesting that PDGF receptor-α is able to transduce a GAG-stimulating signal in the presence of the low molecular weight factor.

DISCUSSION

The human melanoma cell lines Hs294T and C8161 release soluble factors which are potent stimulators of fibroblast GAG synthesis, with evidence for this being presented both here and in a previous report [8, 9]. Many other tumour cell types [5, 20] have shown evidence of a similar paracrine influence controlling ECM modulation and hence facilitating cell migration and possible tumour invasion. This close interdependent relationship between tumour and host cells is an important aspect of tumour progression, and may provide a target for the development of therapeutic interventions. We have, therefore, examined the relationship between the melanoma cell lines, Hs294T, C8161 and normal skin fibroblasts.

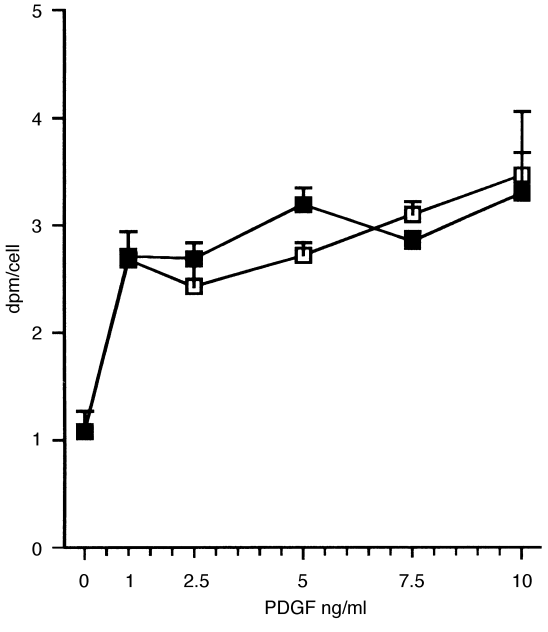

Figure 5. Dose–response for PDGF isoforms, PDGF-AA (□) and PDGF-BB (■) in the presence of Hs294T CM <1 kDa fraction. Each value represents the mean and standard deviation (S.D.) of at least three separate samples. All concentrations of PDGF-AA and PDGF-BB stimulated a significant increase in fibroblast GAG synthesis above the control Hs294T <1 kDa CM using a two-sample *t*-test.

At an early stage of the investigation it became clear no one single factor produced by the melanoma cells could be responsible for GAG stimulation in fibroblasts [8]. CM from Hs294T and C8161 melanoma cells can be split into two main fractions of activity < 1 kDa and ≥ 1 kDa, and although neither fraction produced a strong stimulatory response in fibroblasts, the recombined material stimulated a 4-fold increase in GAG synthesis over a 24-h exposure period. We have previously demonstrated that melanoma cell CM stimulates ^{3}H-glucosamine incorporation into fibroblast hyaluronan up to 9-fold, and sulphated GAGs 2.6-fold, while having no effect on sulphate incorporation, with the net effect of a reduction of polymer sulphation [9]. The hyaluronan synthesised in the presence of tumour cell CM also exhibited an increased molecular weight. Such changes in GAGs are frequently associated with cellular proliferation and migration.

CM from both the melanoma cell lines demonstrated a significant amount of active material binding to heparin which was ≥ 1 kDa, suggesting heparin-binding growth factors were playing a role in melanoma cell CM as fibroblast GAG stimulators. Amongst the heparin-binding growth factors, PDGF and bFGF are potential candidates for GAG-stimulating factors and were both shown to be produced by the melanoma cell lines in our assay. This study demonstrates that these two well characterised growth factors are involved in the stimulation of GAG production in fibroblasts by melanoma cell CM and are present in the high molecular weight fraction of the CM. Although PDGF and bFGF play a role in the overall activity of the CM, they remain dependent on the presence of the low molecular weight factor to stimulate GAG synthesis fully. While blocking antibody to bFGF reduced the GAG-stimulating activity of C8161 and Hs294T complete CM by 40 and 45%, respectively (Figure 3b), only modest increases in activity were observed when exogenous bFGF was added to the tumour cell CM low molecular weight fraction (Figure 2b). These apparently conflicting results are likely to be a result of bFGF interacting with other molecules in the complete CM to mediate its effects on fibroblast GAG synthesis. Such molecules/complexes would have been removed by ultrafiltration. Heparin/heparan sulphate is one molecule known to interact with bFGF and to potentiate its activity by facilitating binding to its high affinity receptor [21]. Both PDGF and bFGF have previously been found to increase hyaluronan synthesis in normal adult fibroblast cells and mesothelial cells [1, 22, 23] when added to cells in isolation using various culture conditions. We were able to rule out the involvement of a number of growth factors which we found to be inactive in the absence and presence of the melanoma cell CM low molecular weight fraction. A number of these growth factors had been previously reported to be active GAG stimulators including IL-1β [24] and TGF-β [22] and it is difficult to ascertain why such differences in growth factor effect are found, but the cell type used and the matrix molecules measured may account for such inconsistencies. An example of this is observed in the comparison between fetal and adult fibroblasts, where migration and hyaluronan synthesis in response to cytokines is very different [1].

For PDGF receptors to autophosphorylate and transduce signals, the PDGF receptors α and β must first dimerise either as homodimers or as a heterodimer. PDGF receptor-αα will bind all three isoforms of PDGF, whilst PDGF

receptor-αβ will bind PDGF-BB and PDGF-AB and PDGF receptor-ββ will bind only PDGF-BB. We have shown the PDGF-AA isoform is an equally potent fibroblast GAG stimulator as the PDGF-BB isoform when added to fibroblasts in the presence of the low molecular weight fraction of melanoma cell CM. Although we only measured PDGF-AB levels in the melanoma cell CM, there is evidence pointing to the expression of PDGF-AA and PDGF-BB isoforms of the growth factor in primary and metastatic melanoma [25]. PDGF-AA will only bind to the PDGF-receptor-α, which we also found was phosphorylated in the presence of melanoma cell CM, suggesting PDGF signalling through the PDGF receptor-α is an important factor in the melanoma/fibroblast relationship. This contrasts with much of the published literature which has found the PDGF-BB isoform to play the important role in GAG stimulation. In fetal lung fibroblasts [26], it was found that PDGF-BB but not PDGF-AA or PDGF-AB was able to stimulate GAG synthesis. When the signalling mechanisms involved in the activation of hyaluronan synthetase and hyaluronan synthesis in mesothelial cells and fibroblasts by PDGF were examined, it was found that PDGF-BB through tyrosine phosphorylation and activation of protein kinase C brought about full activity [22, 23]. The authors ruled out any role for cyclic AMP and protein kinase A (PKA) in PDGF-BB stimulation of hyaluronan synthetase.

Prostaglandin E_2 has been found to stimulate hyaluronan synthesis in rabbit pericardial mesothelial cells by a mechanism involving a cAMP-mediated protein kinase signal transduction process [27]. When we examined prostaglandin E_2 in our assay system, we were unable to find any GAG-stimulating activity in the breast fibroblast cells (data not shown) even with the addition of the high molecular weight fraction of the melanoma cell CM, thereby excluding this molecule as being involved. Many related molecules activating cAMP may be of particular relevance to the low molecular weight fraction which we suspect is acting through an independent pathway to mediate hyaluronan stimulation. The simultaneous activation of both pathways may result in the greater than additive response observed in the fibroblast cells.

In summary, we have demonstrated fibroblast GAG-stimulating activity present in the CM of two human melanoma cell lines can be divided into two interdependent fractions above and below a 1 kDa cut-off ultrafiltration membrane. Neutralising antibody studies strongly suggest the growth factors bFGF and PDGF are involved in the overall activity of the CM, and ELISA studies have found both to concentrate in the high molecular weight fraction as their size would predict. PDGF, and to a lesser extent, bFGF produced a strong GAG-stimulating response in normal adult fibroblasts when in the presence of the apparently novel low molecular weight factor present in the < 1 kDa melanoma cell CM fraction, yet are poor stimulators in isolation. The low molecular weight factor is non-heparin-binding and resistant to both trypsin and heat-treatment [8]. Phosphorylation of the fibroblast PDGF receptor-α was observed after the cells were stimulated with PDGF or melanoma cell CM, and activation of this receptor is involved in the GAG-stimulating activity of melanoma cell CM. The high molecular weight fraction of melanoma cell CM undoubtedly contains a number of active factors and we have demonstrated a major part of this activity to be due to bFGF and PDGF.

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