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Collagen degradation and platelet-derived growth factor stimulate the migration of vascular smooth muscle cells

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SUMMARY

Cell migration is a key event in many biological processes and depends on signals from both extracellular matrix and soluble motogenic factors. During atherosclerotic plaque development, vascular smooth muscle cells migrate from the tunica media to the intima through a basement membrane and interstitial collagenous matrix and proliferate to form a neointima. Matrix metalloproteinases have previously been implicated in neointimal formation and in this study smooth muscle cell adhesion and migration on degraded collagen have been evaluated. Vascular smooth muscle cells adhered to native intact collagen type I and to its first degradation by-product, 3/4 fragment (generated by collagenase-3 cleavage), unwound at 35°C to mimic physiological conditions. PDGF-BB pre-treatment induced a fourfold stimulation of smooth muscle cell motility on the collagen 3/4 fragment whereas no increase in smooth muscle cell motility on collagen type I was observed. Cell migration on collagen type I was mediated by α2 integrin, whereas PDGF-BB-stimulated migration on the 3/4 collagen fragment was dependent on αvβ3 integrin. αvβ3 integrin was organised in clusters concentrated at the leading and trailing edges of the cells and was only expressed when cells were exposed to the 3/4 collagen fragment. Tyrphostin A9, an inhibitor of PDGF receptor-β tyrosine kinase activity, resulted in complete abolition of migration of PDGF-BB treated cells on collagen type I and 3/4 fragment. These results strongly support the hypothesis that the cellular migratory response to soluble motogens can be regulated by proteolytic modification of the extracellular matrix.

Key words: Smooth muscle cell, Cell migration, Collagen degradation, PDGF-BB, αvβ3 integrin

INTRODUCTION

Cell migration is an essential feature of normal development and wound healing, and in pathological conditions such as following cardiovascular damage and tumour invasion. It is widely accepted that growth factors inducing a migratory phenotype (motogenic factors) and cell interaction with the extracellular matrix (ECM) are involved in cell translocation. Although matrix metalloproteinases (MMPs) have been implicated in cell migration in a number of systems, the mechanisms involved have not as yet been determined.

During the formation of an atherosclerotic lesion, vascular smooth muscle cells (VSMC) are believed to receive a stimulus responsible for their transition from a contractile to a synthetic phenotype. As a consequence, VSMC migrate from the media to the intima and proliferate giving rise to neointimal formation (Newby and George, 1993; Newby, 1994; George, 1998). Multiple stimuli are believed to be responsible for VSMC proliferation and migration including growth factors produced by platelets, macrophages, VSMC and endothelial cells, together with proteinases capable of degrading the ECM (Newby and George, 1993; Newby, 1994; George, 1998). For instance, it is widely established that platelet-derived growth factor (PDGF)-BB is upregulated at the lesion site (Barrett et al., 1984; Wilcox et al., 1988) and stimulates VSMC migration and invasion in vitro (Engel and Ryan, 1997; Lindahl et al., 1999) and neointimal formation in vivo (Jawien et al., 1992).

Much evidence now indicates that effects of growth factors may often be dependent on ligation of ECM ligands by cell surface integrins (Miyamoto et al., 1995; Schneller et al., 1997; Cybulsky et al., 1997), and it has been postulated that proteolytic modification of ECM may result in altered responses to growth factors (Schneller et al., 1997). In addition, modified ECM can be generated by the action of matrix metalloproteinases (MMPs), which can then influence cell migration (Giannelli et al., 1997; reviewed in Murphy and Gavrilovic, 1999). The association of MMPs with cardiovascular disease progression has received considerable attention in recent years (reviewed in George, 1998). MMPs have been localised in atherosclerotic lesions (Bendeck et al., 1996) and their activity has been demonstrated by in situ zymography in vulnerable regions of atherosclerotic plaques (Galis et al., 1994) as well as in VSMC within the neointima of organ cultures of saphenous vein (Kranzhofer et al., 1999). It has been shown that MMPs-2 and -9 (gelatinases A and B) are induced after balloon angioplasty in rat (Bendeck et al., 1994). Membrane-type 1 MMP expression was elevated in VSMC after arterial injury in rat (Shofuda et al., 1998) and

after balloon angioplasty in rabbit aorta (Wang and Keiser, 1998). Studies with an antibody to a neo-epitope generated following collagen degradation showed the presence of increased collagenolysis by interstitial collagenases 1 and 3, predominantly synthesised by macrophages in human atheroma (Sukhova et al., 1999). PDGF-BB stimulation of aortic SMC migration seems to be dependent on MMP action (Kenagy et al., 1997). PDGF-BB has been shown to induce the production of MMP-1 (collagenase-1; Yanagi et al., 1991; Yang et al., 1998) as well as interstitial collagens by smooth muscle cells (Amento et al., 1991).

It is thus conceivable that the ECM surrounding VSMC is degraded and following exposure to growth factors these cells are able to respond, migrate and generate a neointima. In this study we have exposed VSMC to such an environment in order to test the hypothesis that a degraded collagen and/or exposure to a growth factor may modulate VSMC adhesion and migration. Triple helical collagen type I is degraded by interstitial collagenases at the specific site between amino acid residues 775 and 776 (Highberger et al., 1979), generating two fragments, one 3/4 and the other 1/4 of the original native molecule length (Gross and Nagai, 1965). These triple-helical fragments melt below 37°C, becoming monomers (Stark and Kuhn, 1968). The elegant experiments of Pilcher et al. (1997) strongly support the idea that collagenase cleavage of type I collagen is an essential element for keratinocyte migration. These authors showed that collagen isolated from mice lacking the major collagenase cleavage site is not permissive for cell migration. The aim of our study was to investigate the interactions between VSMC and intact and degraded type I collagen after exposure to PDGF-BB. We focused on the migratory capability of VSMC in response to PDGF-BB exposure, and analysed the integrin pattern of expression of VSMC integrin receptors on intact or degraded collagen type I. The identification of signalling pathways indicating cross-talk between integrin receptors and PDGFR-β was also analyzed using a tyrosine kinase activity inhibitor, tyrphostin A9.

MATERIALS AND METHODS

Cell culture

Vascular smooth muscle cells (VSMC) derived from human umbilical artery were purchased at passage 3 from Clonetics (Walkersville, MD, USA) and maintained in Ham's F12 medium containing 10% foetal calf serum (FCS, Globepharm, Esger, UK), 2 mM glutamine (Gibco BRL), 30 μg/ml endothelial cell growth supplement (Sigma, St Louis, MO, USA), 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml sodium selenite (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). The cells were used up to passage number 7.

Prior to growth factor treatment, VSMC were starved overnight in serum-free Ham's F12 medium containing 0.1% BSA. Where indicated cells were then incubated with 10 ng/ml PDGF-BB (R&D Systems, Minneapolis, MN, USA) for 24 hours prior to experiment.

Degradation of collagen type I and purification of the 3/4 fragment

Collagen type I was isolated from rat skin as previously described (Cawston and Barret, 1979) and dialyzed into 100 mM Tris-buffered saline, pH 7.4 (TBS) containing 10 mM $CaCl_2$. The 3/4 fragment was generated using 7.2 μg collagenase 3 (Knäuper et al., 1996) per mg of collagen type I and purified by means of subsequent ammonium sulphate precipitation steps, as previously described (Messent et al., 1998). In most experiments intact collagen type I and the 3/4 fragment were used following incubation for 15 hours at 35°C, in order to mimic physiological conditions. Under these conditions, collagen type I maintains its triple helical configuration, while the 3/4 fragment unwinds (Messent et al., 1998). Where indicated native collagen or 3/4 fragment were coated at 25°C prior to cell plating in order to prevent unfolding of the 3/4 fragment (Messent et al., 1998).

Cell adhesion assays

Cell adhesion assays were performed essentially as described previously (Messent et al., 1998). Briefly, tissue culture 96-well plates (Nunclon Delta, Gibco BRL) were coated with indicated concentrations of collagen type I or 3/4 fragment for 1 hour at room temperature or overnight at 4°C. After washing with phosphate-buffered saline (PBS), the plates were blocked with heat-denatured 1% BSA in PBS for 1 hour at room temperature and after extensive washing with serum-free Ham's F12, 5×10^4 VSMC were plated and incubated for 40 minutes at 37°C. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and stained with 1% Methylene Blue in 10 mM sodium borate buffer for 30 minutes at room temperature. The plates were washed extensively with tap water, the cell-bound dye was released with a solution of 50% ethanol and 0.1% HCl, and absorbance (OD) at 630 nm was estimated.

Where indicated collagen type I and 3/4 fragment were also pre-incubated at 35°C overnight. In this case plates were coated at 37°C for 1 hour and blocked with warm BSA for 1 hour at 37°C and the procedure followed as above.

Cell adhesion inhibition assays

Tissue culture 96-well plates were treated as described above. A fixed concentration of 2 μg/ml collagen type I or 3/4 fragment was used. VSMC were used at the same density as above. After trypsinization, untreated or PDGF-BB treated cells were pre-incubated with 10 μg/ml blocking or control antibodies for 10 minutes at 37°C, then plated for 40 minutes at 37°C. The plates were washed, fixed and stained as described for the adhesion assay. Antibodies were mouse anti-human monoclonal anti-α2 integrin (clone 6F1, a kind gift of Dr B. Coller, Mount Sinai Medical Center, New York, NY, USA; Coller et al., 1989), mouse anti-human monoclonal anti-αvβ3 integrin complex (clone LM609, Chemicon, Temecula, CA, USA; Wayner et al., 1991) and rat anti-human monoclonal anti-β1 integrin (clone mAb 13, a generous gift of Dr K. Yamada, NIH, Bethesda, MD, USA; Akiyama et al., 1989). A mouse IgG1 (Dako, Glostup, Denmark) was used as an isotype control antibody for 6F1 and LM609. A rat IgG2a,k (Pharmingen, San Diego, CA, USA) was used as an isotype control antibody for mAb 13. As a negative control, VSMC were incubated with 2 mM EDTA. The positive control consisted of the cells plated on the substrate alone.

Immunocytochemical analyses

Cytoplasmic actin was analysed by means of fluorescein-conjugated phalloidin staining in untreated (not shown) or PDGF-BB treated VSMC plated on 8-well glass-chamber slides (Nalge Nunc International) coated with 10 μg/ml substrate, as described above. Briefly, the cells were fixed for 5 minutes with a fresh solution of PBS containing 2% paraformaldehyde, 100 mM sucrose and 4.5 mM $CaCl_2$, permeabilised with 0.1% Triton X-100 in PBS for 5 minutes at room temperature and washed extensively with PBS. VSMC were incubated with 33 U/ml fluorescein-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA) for 30 minutes at room temperature, then washed again. The slides were then mounted using a fluorescence preservative medium (Citifluor, Agar Scientific, Stansted, UK) and the cells viewed under an epifluorescence microscope (Eclipse 800, Nikon, UK). Immunocytochemical analysis was performed to localise the cytoskeletal molecule vinculin, a marker for focal adhesions, using

a monoclonal mouse anti-human antibody (Upstate Biotechnology, Lake Placid, NY, USA; Asijee et al., 1990). Integrin expression was analysed using a monoclonal mouse anti-human anti-αvβ3 antibody (LM609) and a monoclonal mouse anti-human anti-α2 (6F1). A Texas Red-conjugated donkey anti-mouse (Jackson Laboratories, Cambridge, UK) was used as secondary antibody. Briefly, VSMC were plated, fixed and permeabilised as described above. After PBS washes, the cells were incubated for 1 hour at room temperature with 10 μg/ml primary antibodies diluted in PBS containing 5% donkey normal serum. The cells were washed with PBS and 1:200 dilution of secondary antibodies in PBS were added and the cells incubated for 1 hour at room temperature. The slides underwent a last washing step in PBS, were mounted in Citifluor and viewed as above. Sometimes, fluorescein-conjugated phalloidin was used as counterstaining. Cells were photographed on Kodak Tmax 400 film.

Cell migration assays

Tissue culture 4-well plates (Nunclon Delta, Gibco BRL) were coated with 2 μg/ml collagen type I or 3/4 fragment after 35°C incubation, as described above. PDGF-BB treated or untreated VSMC were plated at the concentration of 7500 cells/ml/well using L15 air-buffered medium (Sigma) supplemented with glutamine, antibiotics and 0.1% BSA. The cells were left attaching in a humidified chamber at 37°C for 3 hours, then their random ambulatory movement was recorded using computerised time-lapse video microscopy (Nikon, UK): the images were acquired every 5 minutes by a close-circuit video-camera (JVC) for 15 hours and stored in a computer file. After manual tracking of 10-20 cells per movie, the computer software system, Lucia 32G/Magic 4.11 (Nikon, UK) was used to calculate the cell translocation distance, in order to quantify cell migration, expressed as μm/hour. All the migration experiments were performed in triplicate.

Cell migration inhibition assays

Cell migration plates were prepared and VSMC were plated as described above. After a 3 hour adhesion step, 5 μg/ml blocking antibodies were added to the cell medium and time-lapse image acquisition was performed, as described above, for the ensuing 12 hours. Anti-α2 (6F1) and anti-αvβ3 (LM609) integrin blocking antibodies were used. Mouse IgG1 was used as an isotype antibody control at the same concentration as the blocking antibodies.

In order to analyse the role of PDGFR-β activity on cell motility, 1 μM of a PDGFR-β tyrosine kinase activity inhibitor, Tyrphostin A9 (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) was added to the cells prior to time-lapse acquisition. In control experiments, 1 μM Tyrphostin A1, an analogue molecule of Tyrphostin A9 lacking tyrosine kinase blocking activity, and 1 μl DMSO, used to dissolve the tyrphostins, were added.

Statistical analyses

Statistical significance was assessed using one-way analysis of variance (ANOVA); $P<0.05$ was considered statistically significant.

RESULTS

The VSMC used in these studies made low levels of MMPs 2 and 9 and TIMPs (detected by gelatin zymography and reverse zymography, respectively) or collagenolytic MMPs (assessed by [^{14}C]collagen activity assay and ELISA). MMPs and TIMPs were not apparently modulated under the conditions used in these experiments (results not shown). As a result these cells were suitable candidates for an approach where the effect of exogenous degradation of collagen on cell migration could be assessed, as VSMC motile behaviour on degraded ECM was unknown. Therefore, the aim of our study was to analyse the adhesive and migratory properties of primary VSMC on collagen type I and on its first degradation by-product, the 3/4 fragment, generated after collagenase 3 digestion. Prior to use, collagen type I and the 3/4 fragment were incubated overnight at 35°C and then the coating process was performed at 37°C in order to mimic physiological body temperature.

Adhesion of VSMC on collagen type I and 3/4 fragment

The adhesion properties of VSMC on intact collagen I and on 3/4 fragment are shown in Fig. 1. VSMC showed a dose-dependent adhesion on collagen type I and on the 3/4 fragment (Fig. 1A). At high coating concentrations (i. e. 1-10 μg/ml) of substrate, VSMC adhesion on the 3/4 fragment was lower than on collagen type I. Moreover, VSMC binding to collagen type

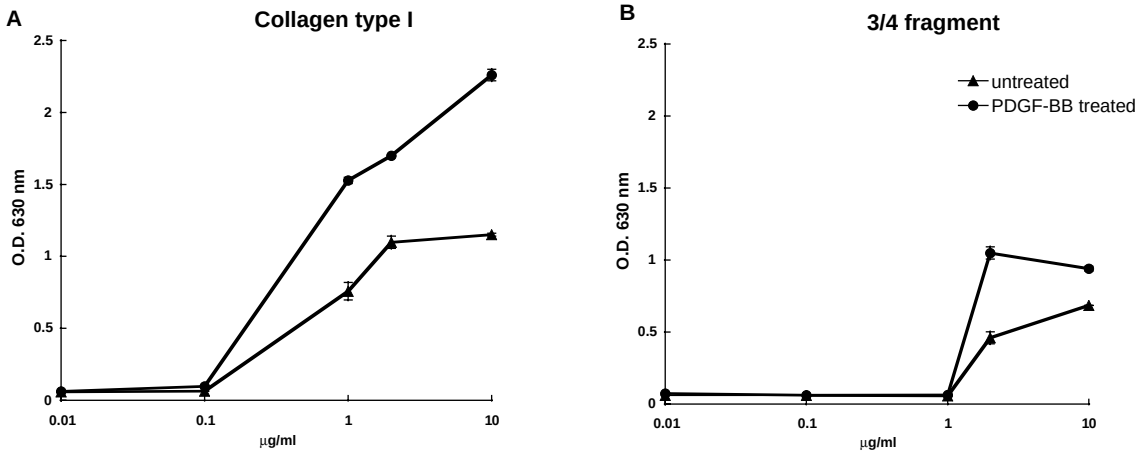

Fig. 1. VSMC adhesion to collagen type I and 3/4 collagen fragment. For details of the assay, see Materials and Methods. (A) Untreated VSMC adhere to collagen type I in a dose-dependent manner, reaching a plateau at 2 μg/ml. PDGF-BB pre-treated VSMC also adhere to collagen type I in a dose-dependent manner. (B) Cell adhesion on 3/4 fragment reached a plateau at 2 μg/ml. PDGF-BB pre-treated VSMC adherence to the 3/4 fragment is also dose-dependent. Cell adhesion was estimated by absorbance at 630 nm.

I and 3/4 fragment reached a plateau at 2 μg/ml. PDGF-BB pre-treatment improved significantly adhesion of VSMC on 2 and 10 μg/ml of collagen I and on 3/4 fragment (Fig. 1A,B). It is noteworthy that pretreatment of VSMC with PDGF-BB also resulted in an apparently lower level of cell adhesion to the 3/4 fragment in comparison to collagen type I.

Analysis of integrin localisation in VSMC

Integrin receptor localisation on the surface of PDGF-BB treated VSMC when plated on collagen type I or on 3/4 fragment was analysed by means of fluorescent immunocytochemical staining. It was not possible to localise αvβ3 integrin receptor in PDGF-BB treated VSMC when plated on collagen type I (Fig. 2A). However, the αvβ3 integrin receptor was detected in treated cells on 3/4 fragment (Fig. 2B): the majority of αvβ3 integrin positive staining is concentrated at the leading (Fig. 2B, arrows) and trailing edges of the cell (Fig. 2 B, arrowheads).

When plated on collagen type I, PDGF-BB treated VSMC also showed robust staining for α2 integrin localised at the cell edges and body (Fig. 2C), as expected. Interestingly, PDGF-BB treated VSMC also showed positive detection of α2 integrin when plated on 3/4 fragment (Fig. 2D), where the staining was mainly found at the cell edges (Fig. 2D, arrows).

A marker for focal adhesions, vinculin, was immunocytochemically detected in PDGF-BB treated VSMC that showed robust focal adhesion organisation, when plated on collagen type I (Fig. 2E) and on 3/4 fragment (Fig. 2F). Vinculin staining was found in cells on both substrates to be

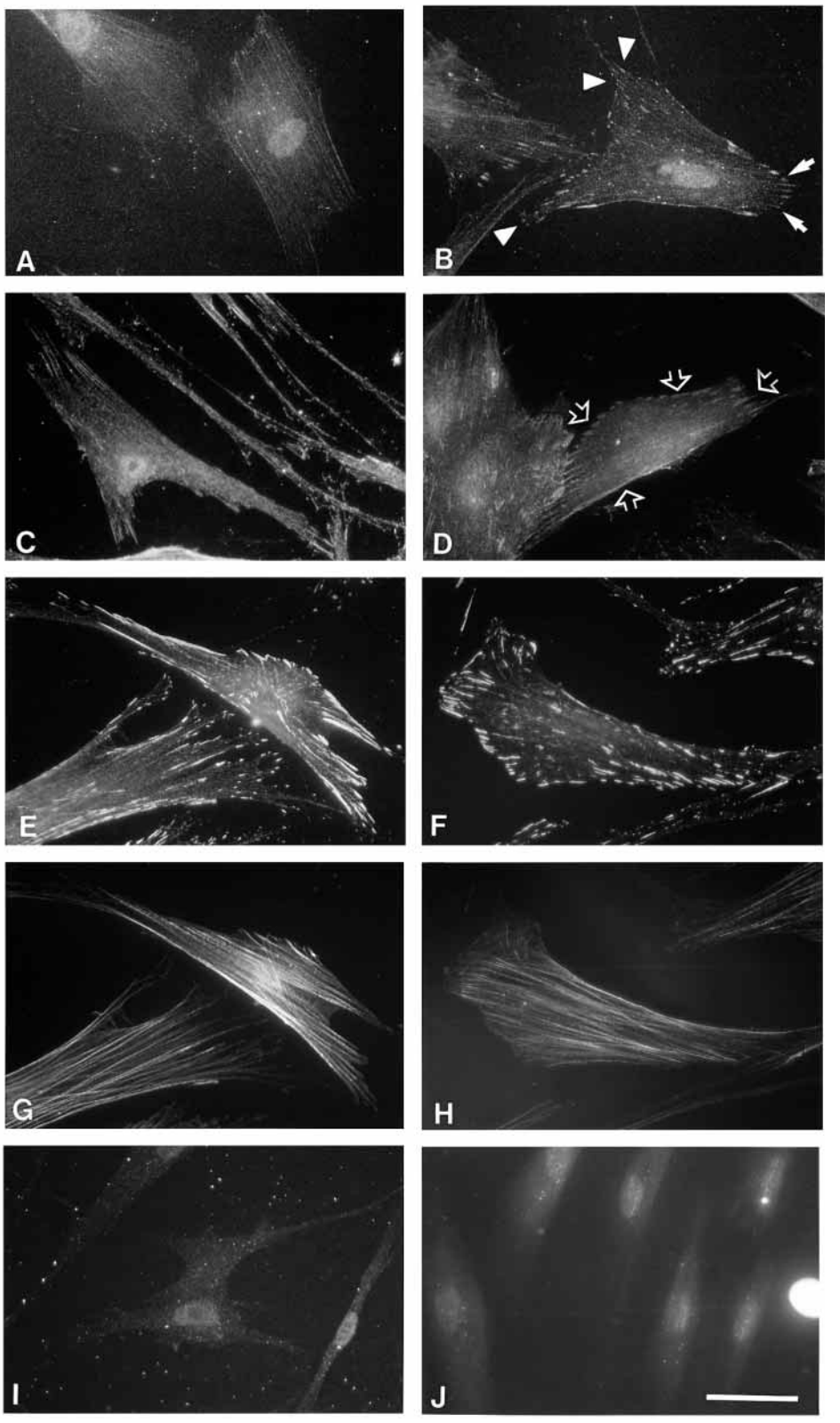

Fig. 2. Fluorescent immunocytochemical localisation of integrin receptors in PDGF-BB treated VSMC adhesion to collagen type I (A-E) and 3/4 fragment (B-J). (A,B) αvβ3 immunolocalization using mouse anti-human LM609 monoclonal antibody and a Texas Red-conjugated donkey anti-mouse secondary antibody. (C,D) α2 immunolocalization using mouse anti-human 6F1 monoclonal antibody and the same secondary antibody as in A. (E,F) Focal adhesion detection by means of vinculin immunolocalization using a mouse anti-human primary monoclonal antibody and the same secondary antibody as in A. (G,H) Same cells as in E and F, counterstained with fluorescein-conjugated phalloidin in order to analyze the actin cytoskeletal fibers. (I,J) Antibody negative control. Arrows in B, αvβ3 clusters in leading edge of PDGF-BB treated VSMC on 3/4 fragment. Arrowheads in B, αvβ3 clusters in trailing edge of treated cells on 3/4 fragment. Empty arrows in D, α2 clusters on edge of a treated cell plated on 3/4 fragment. Bar, 50 μm.

localised in 'clusters' at the cell edges and cell body. The same cells were counterstained with fluorescent conjugated phalloidin in order to locate cytoplasmic actin stress fibres. PDGF-BB treated VSMC had robust actin stress fibres organised in parallel filaments, regardless of the substrate (Fig. 2G,H). It is noteworthy that, comparing vinculin and phalloidin staining, focal adhesions were located at the end of the cytoplasmic stress fibres (compare Fig. 2E with G for collagen type I, and F with H for 3/4 fragment). Moreover, in PDGF-BB treated VSMC plated on 3/4 fragment, a comparison between αvβ3 integrin staining (Fig. 2B) and phalloidin staining (not shown) indicated that αvβ3 integrin is mainly located at the far end of the cytoplasmic actin stress fibres. Finally, from Fig. 2 it is interesting to observe that the gross morphology of PDGF-BB treated VSMC plated on collagen type I differs from that of cells plated on 3/4 fragment. Cells plated on intact collagen have several protrusions randomly emerging around the cell periphery. In contrast, treated VSMC plated on 3/4 fragment seemed to exhibit a directional organisation of their cytoskeleton and focal adhesions and cell body shape, where it is possible to recognise a leading and a trailing edge, therefore resulting in a more elongated phenotype.

Immunolocalisation of αvβ3, α2 and vinculin, and phalloidin staining was also performed in untreated VSMC. Integrin αvβ3 was detected only in untreated VSMC plated on 3/4 fragment, while integrin α2 was detected in cells plated on collagen I and on 3/4 fragment. Cytoplasmic actin stress fibres and vinculin staining did not show any difference compared to PDGF-BB treated cells (data not shown).

Analysis of cell-substrate binding via integrins

In order to understand which integrins are involved in the adhesion of VSMC on collagen type I or on the 3/4 fragment, a range of monoclonal antibodies was used to try to inhibit VSMC adhesion to the substrate. In Fig. 3A, it is shown that adhesion of PDGF-BB treated VSMC on 2 μg/ml collagen type I was reduced by 50% with function-blocking anti-integrin α2 antibodies (6F1, Coller et al., 1989). This inhibitory effect was not so efficient without PDGF-BB treatment, being only about 25% inhibition. The adhesion of VSMC on collagen type I was completely inhibited by functional-blocking anti-integrin β1 antibodies (mAb 13, Akiyama et al., 1989), regardless of the cell treatment (Fig. 3A). Function-blocking antibodies against integrin αvβ3 (LM609, Wayner et al., 1991) did not inhibit VSMC adhesion on collagen type I, nor did the mouse and rat isotype control antibodies. Complete inhibition of cell adhesion was obtained in the presence of EDTA. These results suggest that β1 integrin(s) mediate binding of untreated VSMC to collagen I and α2β1 is mainly responsible for PDGF-BB treated VSMC adhesion on collagen type I.

Anti-αvβ3 integrin antibodies (LM609) showed 70% inhibition of VSMC adhesion on 3/4 fragment compared to the substrate (Fig. 3B). Anti-α2 integrin antibodies (6F1) inhibited VSMC adhesion to 3/4 fragment by 50% compared to the substrate (Fig. 3B). The most efficient inhibitory effect was obtained with functional blocking antibodies against β1 integrin (mAb 13), that showed total inhibition of cell adhesion. Mouse and rat isotype antibody controls did not inhibit cell adhesion to the substrate, while EDTA completely abolished cell adhesion. Similar inhibition was observed with or without PDGF-BB pretreatment of VSMC (Fig. 3B). Therefore, the data reported in Fig. 3B suggest that VSMC use αvβ3 and α2β1 integrin receptors to adhere to the 3/4 fragment.

VSMC migration on collagen type I and 3/4 fragment

VSMC migration on collagen type I and on 3/4 fragment was recorded using computerised video-microscopy and the random ambulatory path of the cells was quantified in μm/hour.

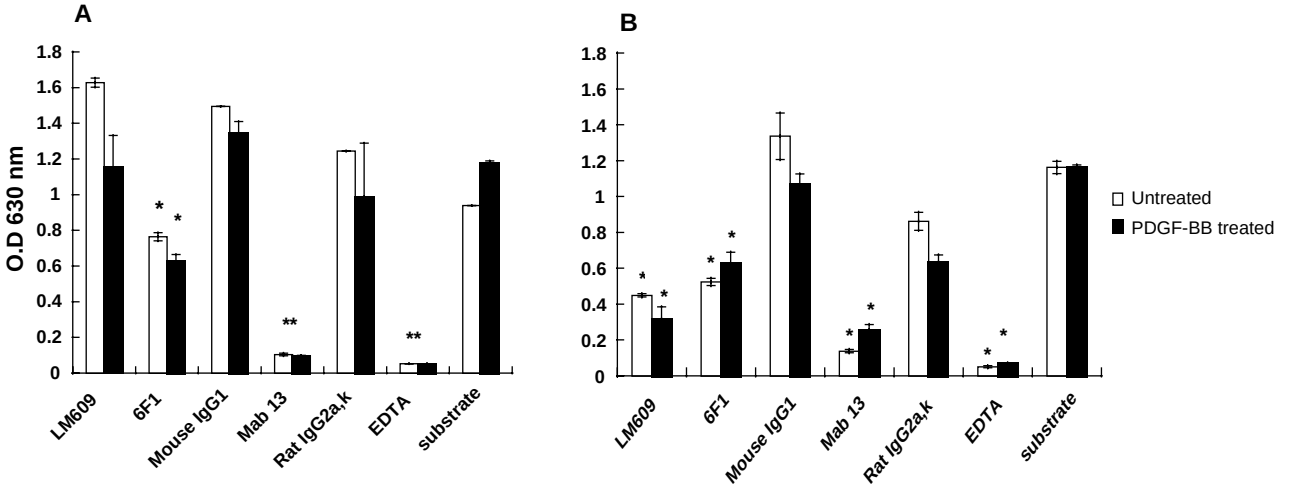

Fig. 3. Cell-adhesion inhibition studies. Open bars indicate untreated cells, shaded bars indicate PDGF-BB pre-treated cells. (A) Inhibition assay of untreated and PDGF-BB pre-treated VSMC adhesion on collagen type I. Only 6F1 (anti-α2 integrin antibody) and mAb 13 (anti-β1 integrin antibody) inhibited cell adhesion on collagen type I, while LM609 (anti-αvβ3 integrin antibody) did not. Mouse IgG1 and Rat IgG2a,k isotype controls did not have inhibitory activity. EDTA completely abolished cell adhesion. (B) Inhibition assay of untreated and PDGF-BB treated VSMC adhesion on 3/4 fragment. LM609 and 6F1 showed a 50% inhibitory effect on cell adhesion on 3/4 fragment when compared to cell adhesion to the substrate only or the mouse IgG1 isotype control. mAb 13 also inhibited cell adhesion. Values are means ± s.e.m. (n=3). Asterisks, values significantly different from controls (substrate) P<0.05.

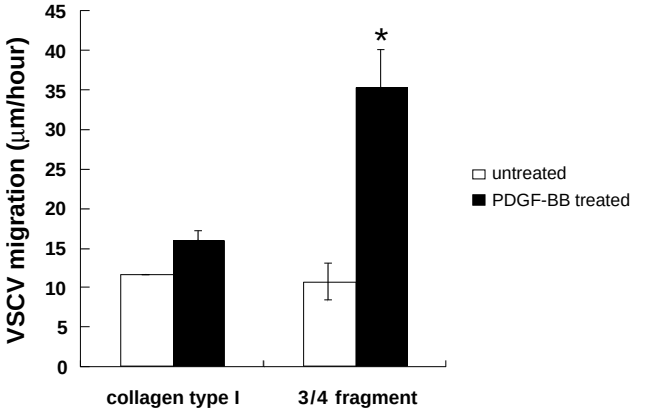

Fig . 4. Quantification of VSMC migration on collagen type I and 3/4 fragment. No statistically significant difference was found between untreated and PDGF-BB treated VSMC migration on collagen type I. Untreated VSMC migration on 3/4 fragment was comparable to cell migration on collagen type I. However, PDGF-BB pre-treatment induced a significant fourfold increment of VSMC migration on 3/4 fragment (asterisks, $P<0.05$). Each bar represents the mean ± s.e.m. of three independent time-lapse films, with 15 cells tracked per film.

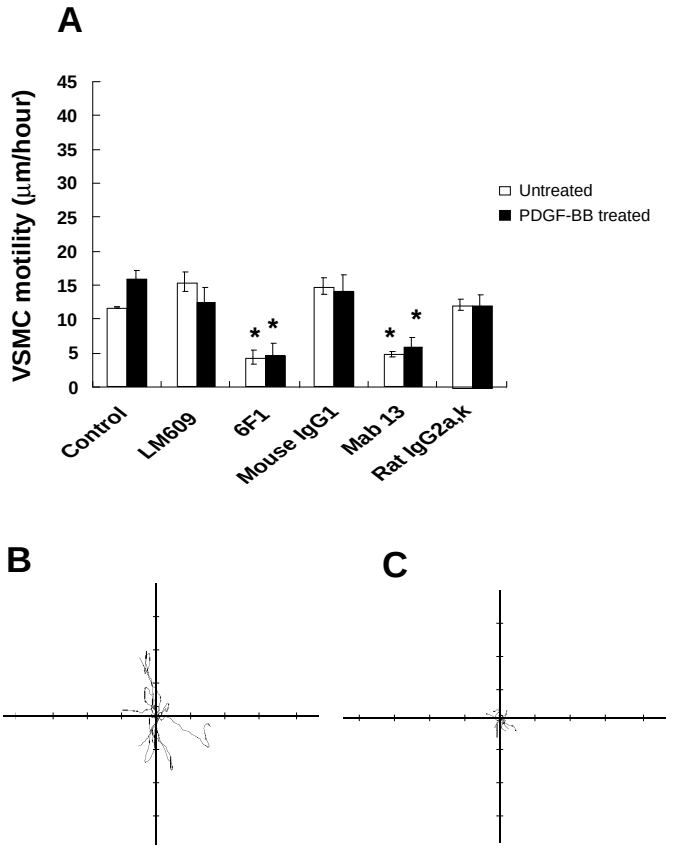

Fig. 5. Inhibition of untreated and PDGF-BB pre-treated VSMC motility on collagen type I. (A) 6F1 inhibited cell motility on collagen type I, while LM609 did not. Mouse IgG1 isotype control did not have an effect on cell motility. Each bar represents the mean ± s.e.m. of three independent time-lapse films, with 15 cells tracked per film. Asterisks, $P<0.05$. (B) Representative experiment of PDGF-BB treated VSMC migratory tracks on collagen type I. (C) Representative experiment of the inhibitory effect of 6F1 on migration tracking of PDGF-BB treated VSMC on collagen type I. In B and C, each scale division represents 100 μm.

VSMC plated on collagen type I moved at a speed of 11.46±0 μm/hour (Fig. 4). PDGF-BB pre-treatment improved the cells' speed on intact collagen, but not in a statistically significant manner ($P>0.05$). Moreover, VSMC plated on 3/4 fragment moved at 10.53±2.33 μm/hour, that is, at a similar rate to the cells plated on intact collagen type I. However, in marked contrast to cells on native type I collagen, PDGF-BB pre-treatment induced a significant fourfold increase in VSMC motility on 3/4 fragment (35.13±4.64 μm/hour; $P<0.05$; Fig. 4). This was a very specific phenomenon due the unwound nature of the 3/4 fragment and also to the action of PDGF-BB. In fact, when the 3/4 fragment was left in its native form (that is, not pre-incubated at 35°C and plates coated at 25°C), VSMC migration in the presence of PDGF-BB was significantly lower (18.02±1.17 μm/hour; $P<0.05$) than that observed on the unfolded 3/4 fragment (35.13±4.64 μm/hour).

Analysis of integrins involved in cell migration

The integrin(s) involved in VSMC migration on intact collagen type I or the 3/4 fragment were identified using function-blocking antibodies. Only antibodies against α2 integrin (6F1) inhibited VSMC migration on collagen type I in untreated or in PDGF-BB treated cells (Fig. 5A). Antibodies against αvβ3 (LM609) did not inhibit VSMC motility on collagen type I in untreated and PDGF-BB treated cells (Fig. 5A). Therefore, these data suggest that α2 integrin plays an important role in VSMC motility on collagen type I. Fig. 5B shows a representative time-lapse video tracking of PDGF-BB treated VSMC random ambulatory migration on collagen type I. This migration was effectively inhibited by 6F1 antibody, as shown in Fig. 5C.

PDGF-BB treated VSMC migration on the 3/4 fragment was significantly (fivefold) inhibited by incubation with anti-αvβ3 integrin antibodies (LM609; Fig. 6A). Importantly the cells were observed to be viable in the presence of this antibody, in as much as the cells attempted to send out processes that were quickly withdrawn throughout the image-capturing period. Addition of a blocking anti-α2 integrin antibody seemed to inhibit VSMC motility, but this result was not statistically significant and the cells were observed to translocate. In untreated cells, VSMC migration on 3/4 fragment was not inhibited by functional blocking antibodies against αvβ3 (LM609) or against α2 integrin (6F1; Fig. 6A). Therefore, αvβ3 plays an important role in PDGF-BB-treated VSMC migration on 3/4 fragment.

Inhibition experiments performed in untreated and PDGF-BB treated VSMC using a blocking monoclonal antibody against β1 integrin (mAb 13) showed complete abolition of cell motility regardless of the substrate or the cell treatment (data not shown). In this case the cells were observed to round up in the presence of the antibody, and may thus be a reflection of inhibition of adhesion.

Role of PDGFR-β in VSMC motility

Tyrphostin A9, a tyrosine kinase inhibitor that can inhibit the activity of PDGFR-β, did not influence VSMC motility on

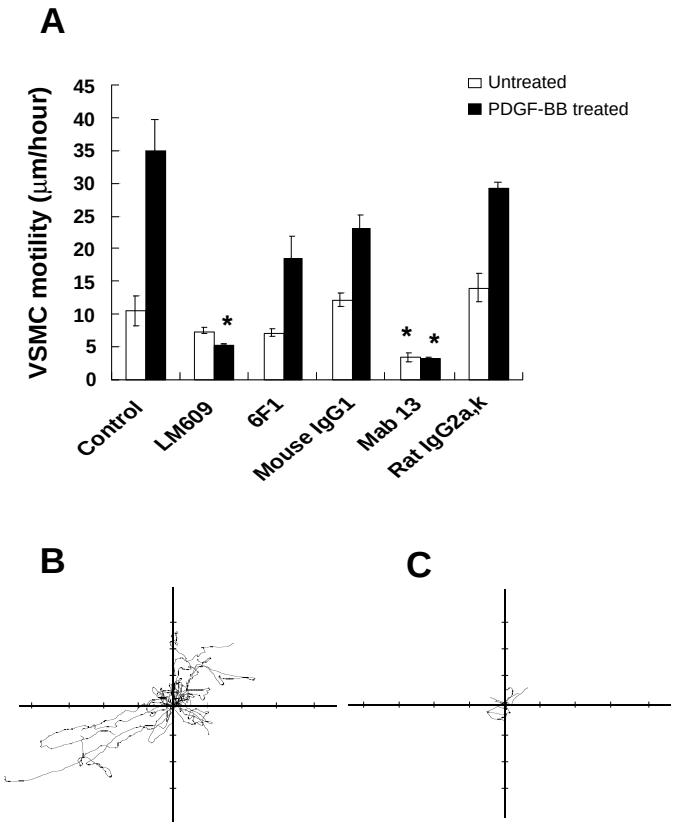

Fig. 6. Inhibition of untreated and PDGF-BB pre-treated VSMC motility on 3/4 fragment by integrin antibodies. (A) Untreated VSMC (white bars) migration was not significantly inhibited by LM609 (anti-αvβ3 antibody) or by 6F1 (anti-α2 integrin antibody). PDGF-BB pre-treated VSMC migration (black bars) was significantly abolished by LM609 compared to the mouse IgG1 isotype control. 6F1 did not show significant inhibitory effect compared to the control and the mouse IgG1 isotype control. Each bar represents the mean ± s.e.m. of three independent time-lapse films, with 15 cells tracked per film. Asterisk, $P<0.05$.
(B) Representative experiment of PDGF-BB pre-treated VSMC migratory tracks on 3/4 fragment. (C) Representative experiment of migration tracks of PDGF-BB pre-treated VSMC on 3/4 fragment in the presence of LM609. In B and C each scale division represents 100 μm.

collagen type I and 3/4 fragment in untreated cells (Fig. 7). On the other hand, PDGF-BB-induced cell migration on collagen type I was inhibited by tyrphostin A9 compared with the control tyrphostin A1. Moreover, compared to the control, tyrphostin A9 induced a fivefold inhibition of PDGF-BB treated VSMC motility on 3/4 fragment. Tyrphostin A1 and DMSO did not inhibit PDGF-BB treated VSMC migration on 3/4 fragment. Therefore, the activity of the PDGFR-β played a major role in the migration of VSMC on collagen type I and on 3/4 fragment.

DISCUSSION

Cell-ECM interactions are important players in regulating cell differentiation, proliferation and death (Werb, 1997). It is widely established that cells interact with ECM components mainly via integrin receptors consisting of α and β subunits, which define the specificity for a particular ECM component (Briesewitz et al., 1995; Kim and Yamada, 1997; Yamada et al., 1995). In the current study we have focused on the mechanisms involved which may induce migration in VSMC, a process relevant to initial neointimal formation and to restenosis after balloon angioplasty. However, it is also important to recognise that in certain circumstances VSMC can also be involved in the repair process where de novo synthesis of specific ECM components plays an important role in the maintenance of a stabilised atherosclerotic plaque.

In this study we have focused on the relations between VSMC interactions with intact collagen type I and its degraded form, the 3/4 fragment, together with the role of PDGF-BB pre-treatment on VSMC adhesion and migration.

Cell adhesion and integrin localisation

Cell adhesion on collagen type I was more efficient than on the 3/4 fragment and cell adhesion was also significantly enhanced on both the substrates by PDGF-BB pre-treatment. PDGF-BB has previously been shown to elevate binding of VSMC to native type I collagen and a suggestion has been made that this is due to increased binding avidity of the integrin receptors to the substrate (Seki et al., 1996). This may also be a possibility in the case of VSMC binding to the 3/4 fragment in the presence of PDGF-BB, since immunocytochemical analysis did not show a qualitative increase of αvβ3 or α2β1 integrins under these conditions. Taken together, the immunocytochemical detection and the cell-adhesion inhibition data show that α2β1 integrins mediated untreated and PDGF-BB treated VSMC adhesion to collagen type I, as previously shown (Pickering et al., 1997; Carragher et al., 1999). However, in our experiments, since the anti-α2 integrin antibody (6F1) partially blocked adhesion to native type I collagen, and anti-β1 integrin completely abolished adhesion to this substrate, it is possible that a further receptor is involved. VSMC are able to express α1β1 and α2β1 integrin receptors and both integrin pairs have been shown to mediate adhesion to collagen type I (Skinner et al., 1994). Further studies will reveal whether α1β1 integrins are expressed by the cells in the current study and their involvement in adhesion.

It was of great interest that it was possible to detect αvβ3 integrin by immunocytochemistry in these cells following exposure to a relevant ligand, the 3/4 fragment. Further studies are required to elucidate the mechanism underlying this observation, but it may reflect an upregulation in the synthesis of αvβ3 integrin or altered trafficking of this receptor. Moreover, it is likely that 3/4 fragment and PDGF-BB exposure may induce a reorganization of the αvβ3 integrin complexes in robust focal adhesions from a random cellular distribution. This has also been shown to be true in other systems (Trusolino et al., 1998). This substrate-dependent localisation may explain the fact that αvβ3 has been reported to be detected in VSMC in some studies (Brown et al., 1994) but not others (Skinner et al., 1994). It is interesting to speculate that this organisation and the more polarised morphology (leading and trailing edge; see Results), reflects the enhanced migration of VSMC when exposed to degraded ECM and growth factor.

In a similar manner a rat VSMC line, A10, was recently shown to switch from β1 to β3 integrin expression when

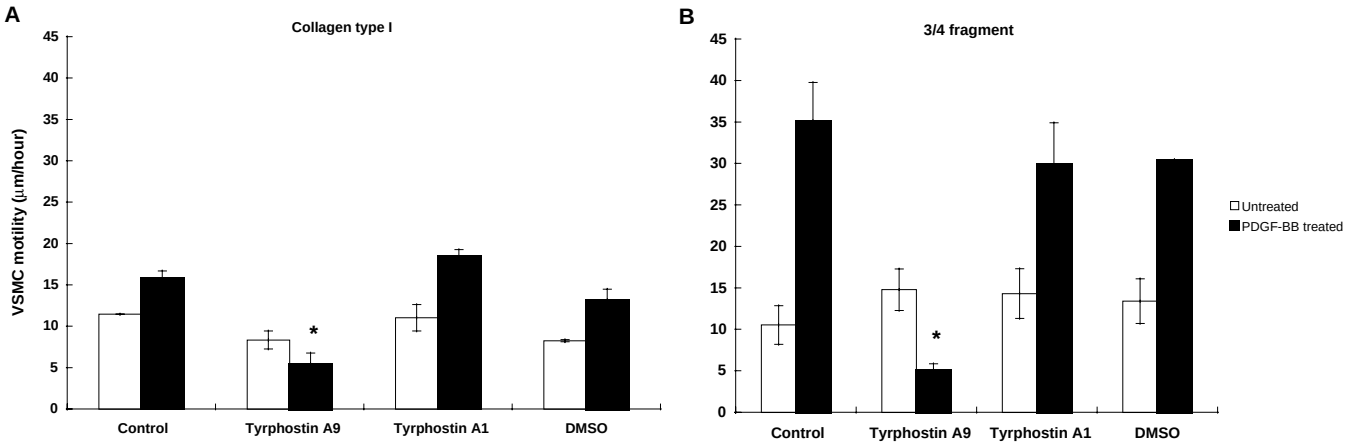

Fig. 7. Effect of inhibition of tyrosine kinase activity by tyrphostin A9 on untreated (white bars) and PDGF-BB pre-treated (black bars) VSMC motility. (A) Untreated VSMC migration on collagen type I was not inhibited by tyrphostin A9 (TyrPA9). Tyrphostin A9 significantly inhibited PDGF-BB treated VSMC migration on collagen type I. The inactive analogue, tyrphostin A1 (TyrPA1), did not inhibit cell motility. (B) Untreated VSMC migration on 3/4 fragment was not inhibited by tyrphostin A9, but significant inhibition of PDGF-BB treated VSMC migration was obtained with tyrphostin A9. Each bar represents the mean ± s.e.m. of three independent time-lapse films, with 15 cells tracked per film. Asterisks, $P<0.05$.

interacting with heat-denatured collagen type I (Jones et al., 1999). Our cell adhesion studies further showed that VSMC used mainly αvβ3 integrins to adhere to 3/4 fragment, which is in agreement with previous studies of cellular binding to collagenase-degraded collagen (Davis, 1992; Messent et al., 1998). It is of interest to note that αvβ3 expression is upregulated in VSMC in atherosclerotic lesions through an unknown mechanism (Corjay et al., 1999; Hoshiga et al., 1995). VSMC organised robust clusters of focal adhesions all over the cell surface when plated on the 3/4 fragment, resembling those seen on intact type I collagen. It is interesting to note that Pickering and colleagues (1997) observed a disassembly of actin stress fibres following treatment of VSMC with FGF-2, another growth factor that is motogenic for these cells. In our studies we did not observe any effect on the actin cytoskeleton following treatment with PDGF-BB, regardless of the ECM substrate. The recent paper of Carragher et al. (1999) indicates that addition of soluble bacterial collagenase-generated collagen fragments to VSMC cultured on type I collagen results in cell rounding within 5 minutes and the degradation of pp125 focal adhesion kinase ($pp125^{FAK}$), which could be blocked by antibodies to α2 and αv integrins. It should be noted that in our experiments we have exposed VSMC to substrate-bound collagen or 3/4 fragment, whereas Carragher et al. presented bacterial collagenase-generated fragments in solution, to the apical surface of VSMC.

Taken together, our data suggest that VSMC respond to an injury by changing interactions with the ECM via modulation of integrin receptors.

Cell migration

Several integrin pairs have been previously associated with cell migration, including α2β1 for movement through type I collagen (Skinner et al., 1994). Generally, it has been shown that β3 integrins rather than β1 are responsible for cell-ECM interactions after injury in VSMC (Slepian et al., 1997). VSMC adhesion to and contraction of fibrin clots were regulated by β1 integrin (Yee et al., 1997) and αv and β1 integrins played a role in chick smooth muscle contractility when interacting with vitronectin (Dahm and Bowers, 1998). Growth factor effects on cell migration have previously been implicated, including FGF-2 (Pickering et al., 1997). PDGF-BB altered VSMC relations with fibronectin by altering ECM-integrin interactions (Fujio et al., 1993).

VSMC migrated at low but consistent rates over type I collagen, and the migration was not significantly stimulated by pre-treatment with PDGF-BB. Untreated and PDGF-BB treated VSMC motility on intact collagen type I was inhibited by anti-α2 integrin antibodies, as previously reported in a chemotaxis assay (Skinner et al., 1994). Noteworthy from our data is that PDGF-BB pre-treatment enhanced VSMC motility on the 3/4 fragment fourfold compared with the untreated cells. It was previously reported that NIH3T3 and endothelial cell migration through vitronectin is increased by PDGF-BB in an αvβ3-dependent manner (Woodard et al., 1998), and PDGF stimulated keratinocyte migration through collagen gel (Andresen et al., 1997). Moreover, PDGF-BB was a potent chemoattractant for VSMC in vitro (Jawien et al., 1992; Benzakour et al., 1995). In our system, migration of PDGF-BB-treated VSMC on the 3/4 fragment was entirely dependent on αvβ3 integrin, since only LM609 (anti-αvβ3 antibody) significantly inhibited cell migration. Previous studies have implicated αvβ3 integrin in the migration of VSMC through a vitronectin matrix in vitro (Brown et al., 1994). In vivo αvβ3 integrin has been strongly implicated in smooth muscle activity since previous studies have shown that neointimal hyperplasia was blocked by LM609 (Choi et al., 1994; van der Zee et al., 1998). We cannot at this stage rule out the possibility that enhanced migration is observed because of the rapid induction of an ECM component that promotes VSMC migration. Indeed Jones et al. (1997, 1999) have reported that exposure of a VSMC cell line to heat-denatured collagen results in increased tenascin-C expression through a β3 integrin-mediated mechanism, in this case resulting in enhanced proliferation. In our time-lapse experiments it was clear that very few VSMC underwent cell division and no increase in cell proliferation

was observed in experiments where cells had been pre-treated with PDGF-BB. Taken together, cell-adhesion inhibition data and cell-motility inhibition data show that αvβ3 plays a role in both VSMC adhesion and motility of PDGF-BB treated VSMC on 3/4 fragment.

Growth factor/integrin synergy

It is noteworthy that under the conditions of this study, ECM degradation alone is not sufficient to induce enhanced VSMC migration. The stimulus raised by cell interaction with degraded ECM had to be accompanied by growth factor stimulation. In fact, our data show that VSMC motility is dependent also on protein tyrosine kinase activity, probably of the PDGF receptor β (PDGFR-β). Tyrphostin A9 inhibits tyrosine kinase activity and has been shown to inhibit PDGF-induced mitogenic activity (Bryckaert et al., 1992) and neointimal formation after balloon angioplasty (Golomb et al., 1996). Tyrphostin A9 abolished PDGF-BB treated VSMC migration on 3/4 fragment and on collagen type I. It has been previously shown that in NIH-3T3 cells PDGF not only induced activation of PDGFR-β but also its association with αvβ3 as well as cell proliferation and migration through a vitronectin substrate (Schneller et al., 1997; Woodard et al., 1998). From our data it is not clear whether PDGF-BB is exerting its effect through re-organisation of αvβ3 integrins and/or signalling cross-talk. It is possible that both effects may be implicated and further studies will unravel the mechanisms involved. It has been reported that in the ECV 304 human endothelial cell line and in the EGF-receptor transfected NIH3T3 cells there is a functional interaction between EGF receptor and β1 integrins, resulting in both enhanced cell survival and cell cycle progression (Moro et al., 1998). In this case the authors indicate that ECM ligation itself was insufficient to drive cell proliferation but that entry into the cell cycle was promoted by prior integrin activation of the EGF receptor. This co-operation was also shown in a mouse cell line lacking β1 integrin, GD25, which shows poor chemotactic migration towards PDGF. When these cells are rescued by transfection with the β1A integrin subunit they are then able again to respond to the chemotactic action of PDGF (Fassler et al., 1995).

In conclusion, the data reported here strongly indicate that ECM degradation can play a critical role in the control of cell migration. The resulting synergistic interaction of modified matrix and motogenic factor supports the idea that a multi-factorial approach is essential in the design of therapies for the treatment of disease where cell migration is a major feature.

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