teinases and asking if proteinases serve additional functions in the disease process. We have long held elastin degradation to be essential for the development of emphysema, but definitive evidence is lacking and the involvement of other ECM proteins is intriguing. However, while collagen is lost from the alveolar space, it appears to accumulate in the small airways. Understanding the complexities of matrix turnover is essential. Finally, when the damage has been done, how do we go about repairing emphysematous lung tissue? Understanding the mechanisms of normal lung development is a reasonable place to start.

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Role of newly synthesized fibronectin in vascular smooth muscle cell migration on matrix-metalloproteinase-degraded collagen

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Abstract

The migration of vascular smooth muscle cells (VSMC) is known to be a key process in the development of a number of vascular lesions, although the precise mechanisms involved have still to be elucidated. In the present study, the production of endogenous fibronectins by VSMC migrating across intact and matrix-metalloproteinase-degraded collagen type I has been explored. Cellular fibronectin seems to play a role in the enhanced migration seen when VSMC are exposed to degraded collagen and platelet-derived growth factor-BB. VSMC were found to synthesize both exon IIIA-containing fibronectin (which predominated) and exon IIIB-containing fibronectin. When these cells were exposed to substrates consisting of recombinant exon IIIA- or exon IIIB-containing fibronectin, rates of mi-
gra
tion were not elevated above those seen with undegraded collagen. Endogenous fibronectin production may thus be necessary, but not sufficient, for VSMC migration over degraded collagenous substrates.

**Introduction**

During the formation of an atherosclerotic plaque, vascular smooth muscle cells (VSMC) lose their contractile phenotype and acquire a synthetic and migratory phenotype [1]. This new phenotype is believed to be generated by one or more stimuli resulting from cellular interaction with growth factors and with extracellular matrix (ECM) modified by matrix metalloproteinases (MMPs) [1,2]. We have shown previously that migration of human VSMC was significantly enhanced when cells were exposed to platelet-derived growth factor-BB (PDGF-BB) and plated on the 3/4 collagen fragment, generated by MMP-13 (collagenase-3) digestion of collagen type I [3]. In this system, VSMC migration was mediated by the α5β3 integrin receptor and the tyrosine kinase activity of the PDGF receptor-β.

**In vivo** studies have shown that, during the formation of an atherosclerotic plaque, there is increased production of collagenases [4], growth factors [5], specific integrin receptors, including α5β3 and α5β1 [6], and newly synthesized ECM proteins, such as collagen type I [7] and fibronectin [8]. Fibronectin is a large glycoprotein that is present in plasma and the ECM of most tissues. It plays a role in cellular events including differentiation, development, wound healing, adhesion and cell migration. Most of the heterogeneity in fibronectin involves the alternative splicing of at least three exons, exon IIIB (EIIIB; EDB), EIIIA (EDA) and EV (Variable or CS-1), during post-transcriptional processing [9]. The V region can undergo multiple splice variations involving complex exon subdivision, depending on species and cell type. However, EIIIA and EIIIB can be either entirely present or absent in the mature fibronectin mRNA as result of exon skipping. In plasma fibronectin, both EIIIA and EIIIB are skipped (B−A− isofom); the fibronectin containing both exons (B+A+) is called mesenchymal or onco-foetal fibronectin [10]. It has been shown that mesenchymal fibronectin is produced by cells during the development of blood vessels. In the adult organism the main isofom of fibronectin produced contains EIIIA [11]. In the process of neointimal thickening and during atherosclerotic plaque formation, increased de-

position of fibronectin has been observed [8]. In particular, there is a significant increase in the detection of EIIIA during neointimal formation, at both the mRNA and protein levels, and a small increase in EIIIB expression [11].

In the present study we investigated the role of fibronectin and its different isoforms in VSMC migration. In our system, *de novo* deposition of newly synthesized fibronectin by VSMC increases when the cells interact with the 3/4 collagen fragment generated by MMP-13-dependent degradation, in the presence of PDGF-BB. Furthermore, we analysed the possible role that this newly produced cellular fibronectin might have in cell migration.

**Materials and methods**

Untreated and PDGF-BB-treated (for 24 h) human umbilical artery VSMC were plated on tissue culture dishes coated with 2 μg/ml collagen type I or 3/4 collagen fragment, generated by degrading collagen type I with recombinant MMP-13 (collagenase-3) as described previously [3,12].

To analyse the production of newly synthesized fibronectin, untreated and PDGF-BB-treated VSMC plated on collagen type I and 3/4 collagen fragment were washed thoroughly with serum-free medium and then serum-starved for 2 days. After lysis and SDS/PAGE separation, Western blot analysis was performed using an antibody against plasma fibronectin [Sigma; affinity-purified rabbit anti-(human fibronectin)] which recognizes all fibronectin isoforms. In order to confirm that fibronectin produced by VSMC was derived only from the cells themselves, Western blot analysis of native type I collagen and the 3/4 collagen fragment with this antibody revealed that no contaminating fibronectin was present (results not shown). Immunofluorescence was performed as described previously [3] using specific mouse anti-(human EIIIA) antibodies (clone DH1 [17]; from ICN) and anti-(human EIIIB) antibodies (clone BC-1, [18]; generously donated by Professor L. Zardi, University of Genova, Italy).

For analysis of the potential role of fibronectin in the migration of VSMC on intact collagen type I and the 3/4 collagen fragment, an antibody to cellular fibronectins (Sigma; clone FN3E2) was added to the cultures once the VSMC had adhered to the substrate. Migration was investigated using time-lapse video microscopy over a 15 h period, as described previously [3]. The migration of untreated and PDGF-BB-treated VSMC on
different fibronectin isoforms was analysed by coating tissue culture dishes with 10 μg/ml chicken recombinant fibronectins containing or not EIIIA and/or EIIIB. For simplicity, the four different combination of isotypes were called B+A+, B+A-, B−A+ and B−A−, where the plus (+) sign indicates the spliced-in exon, and the minus sign (−) indicates that the exon has been spliced out. These recombinant proteins were produced using the Bac-to-Bac insect cell culture system for production of recombinant proteins [19]. Although these were chicken recombinant proteins, they were appropriate for this study, since there is an extremely high degree of sequence conservation between species [13]. Cell migration was then performed and analysed as described previously [3].

Results

In this study we present evidence that the exposure of PDGF-BB-treated VSMC to 3/4 collagen fragment results in increased production of endogenous fibronectin. Western blot analysis of cell lysates revealed that, although fibronectin production by VSMC was observed under all conditions, the greatest fibronectin production was seen when PDGF-BB-treated VSMC were plated on the 3/4 collagen fragment (Figure 1, upper panel). The nature of the type of fibronectin deposited was analysed by immunofluorescence (Figure 1, lower panels). Untreated VSMC plated on intact collagen type I produced fibronectin containing EIIIA (Figure 1a) and EIIIB (Figure 1b) localized over the region of the cell body. PDGF-BB treatment did not affect the presence of EIIIA (Figure 1e), but EIIIB production was reduced (Figure 1f). EIIIA-containing fibronectin was detected in untreated VSMC plated on 3/4 collagen fragment (Figure 1c). These cells were positive for EIIIB (Figure 1d), and therefore untreated VSMC plated on 3/4 collagen fragment produced fibronectins containing EIIIA and/or EIIIB (B+A−, B−A+ and/or B+A+), which were detected mainly over the cell body. PDGF-

![Figure 1](https://example.com/fibronectin_production.png)

**Figure 1**

Fibronectin production by VSMC

Upper panel: Western blot with anti-fibronectin antibodies of lysates from VSMC exposed to substrates and PDGF-BB as indicated; Col I, collagen I; 3/4 frag, 3/4 collagen fragment. Lower panels: immunolocalization of fibronectin in VSMC with isoform-specific antibodies. VSMC were plated on intact type I collagen (a, b, e, f) or the 3/4 collagen fragment (c, d, g, h) and either untreated (a, b, c, d) or PDGF-BB-treated (e, f, g, h). VSMC were stained with antibodies specific for EIIIA (a, e, c, g) or EIIIB (b, f, d, h), as described in the Methods section. Magnification × 240.
BB-treated VSMC produced EIIIA-containing fibronectin that was deposited over the cell body and in the leading and trailing edge of the cells (Figure 1g).

**Figure 2**
Results from time-lapse videomicroscopy of VSMC migrating over intact type I collagen (upper panel) or the 3/4 collagen fragment (lower panel)

Images were captured over 15 h and migration rates were determined by individual cell tracking. Where indicated, antibodies to fibronectin (FN) or an isotype control (IgM) were added after VSMC had adhered to the substrate. Each bar represents the mean±S.E.M. for at least 10 cells.

A role for newly synthesized fibronectin in cell migration was demonstrated using an antibody specific for cell-produced fibronectin. Following inclusion of this antibody, no effect was observed on the migration of untreated or PDGF-BB-treated VSMC on collagen type I, or on the migration of untreated VSMC on the 3/4 collagen fragment, in comparison with the antibody isotype control (Figure 2). However, significant inhibition of cell migration was observed in comparison with the control isotype antibody (23.26 ± 2.43 μm/h) when the anti-(cellular fibronectin) antibody was added to PDGF-BB-treated VSMC cultures on the 3/4 collagen fragment (9.4 ± 2.3 μm/h; P < 0.05; Figure 2, asterisk). Endogenously produced fibronectin may therefore play a role in regulating the migration of PDGF-BB-treated VSMC on degraded ECM.

To dissect the role of specific fibronectin isoforms, the migration of untreated and PDGF-BB-treated VSMC was analysed using plates coated with recombinant fibronectin isoforms containing or not EIIIA and EIIIB (Figure 3). The absolute level of migration on all four fibronectin isoforms was comparable with that on intact collagen I, and never reached that observed on the 3/4 collagen fragment, regardless of the presence or absence of PDGF-BB. In addition, as shown in Figure 3, the migration of untreated and PDGF-BB-treated VSMC on the different cellular isoforms B+A+ (mesenchymal isotype), B+A− (cartilage isotype) and B−A+ was not significantly different from cell migration on the plasma isotype (B−A−). Therefore the interaction of VSMC with the various fibronectin isoforms alone was not sufficient to modulate their migration.

**Discussion**

In this work we have demonstrated that PDGF-BB stimulation of VSMC combined with exposure to the 3/4 collagen fragment results in increased production of fibronectin. Untreated VSMC seemed to produce predominantly the B+A+ isoform of fibronectin, whereas PDGF-BB-treated VSMC produced fibronectin containing predominantly EIIIA (B−A+ isoform), regardless of the substrate. These data suggest a role for de novo ECM produced by highly motile cells. A number of attempts have been made to analyse the isoforms of the fibronectin deposited in atherosclerotic lesions, mainly at the level of mRNA analysis. Our in vitro results seem to be consistent with the in vivo situation, where fibronectin...
isoform B–A+ seems to be the predominant isoform produced in atherosclerotic lesions [11,14]. Therefore our system appears to mimic some of the in vivo events occurring during the formation of an atherosclerotic lesion. In vivo, a small quantity of B+ fibronectin was detected in atheromas [11]. To analyse the role of newly synthesized fibronectin in cell migration, our initial approach was to include an antibody targeting specifically cellular fibronectin in cell migration assays. Only migration of PDGF-BB-treated VSMC on the 3/4 collagen fragment was affected by the sequestering action of the cellular anti-fibronectin antibody, resulting in significant inhibition of migration, suggesting a role for newly deposited fibronectin in the migration of PDGF-BB-treated VSMC on this substrate.

It is interesting to speculate that there might be a link between the interaction of integrin with the substrate, the activity of the growth factor receptor resulting in new production and the deposition of ECM molecules in order to regulate cell migration. An earlier study showed that PDGF-BB enhanced fibronectin production in rat aortic VSMC in culture [20]. The migration of PDGF-BB-treated VSMC on the 3/4 collagen fragment was blocked not only by anti-αvβ3 antibodies [3], but also by anti-αv (3.67±0.62 μm/h in comparison with the control 35.13±4.64 μm/h; P<0.05) and anti-β1 antibodies [3]. It is of interest to note that, in the rat carotid artery, there is a correlation between the expression of α5β1 integrin and fibronectin deposition at the injury site, and that β1 integrin plays a role in VSMC fibronectin assembly [21]. However, adhesion of VSMC to the 3/4 collagen fragment depended only on the cell interaction with αvβ3 integrin, and not on αv integrin (results not shown). These results are in keeping with our observations that αvβ3 integrin can bind to the 3/4 fragment [12] and has a role in VSMC adhesion and migration [3]. However, our data suggest that the αv and β1 integrins may also be important players in VSMC fibronectin assembly, and may thus play a role in migration.

As shown in Figure 1 (upper panel), PDGF-BB exposure and cell interaction with the 3/4 fragment led to increased production of fibronectin. And an anti-(cellular fibronectin) antibody blocked VSMC migration under these conditions. Since this antibody does not discriminate between the EIIIB and EIIIA isoforms of fibronectin, we took an additional approach to try to determine the contributions of these fibronectins to VSMC migration. The migration of untreated or PDGF-BB-treated VSMC on B+A+, B+A− and B−A+ fibronectin isoforms did not show significant differences compared with migration over the isoform equivalent to plasma fibronectin (B−A−) or with migration of PDGF-BB-treated cells on native type I collagen. At this stage it is thus not possible to assign a direct role for specific fibronectin isoforms in the regulation of VSMC migration.

In conclusion, the PDGF-BB-induced migration of VSMC on the 3/4 collagen fragment depends not only on the integrin–substrate interaction and growth factor receptor activation, but also on the interaction with newly synthesized fibronectin. These data suggest a role for newly synthesized fibronectin in the development of the atherosclerotic plaque in vivo, as de novo ECM production is a typical wound-healing response.

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Modulation of extracellular matrix using adenovirus vectors

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Abstract

Metabolism of the extracellular matrix (ECM) is a complex process that becomes disregulated in disease states characterized by chronic inflammation of joints, as is seen in rheumatoid arthritis or fibrosis of the lung. The participation of certain cytokines in this process is generally accepted (transforming growth factor-β induces fibrosis), while the roles of other cytokines are less clear. Oncostatin M (OSM) is a member of the interleukin-6/leukaemia inhibitory factor (or gp130) cytokine family, and its participation in inflammation and the regulation of ECM metabolism is supported by a number of activities identified in vitro, including regulation of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinases-1. Local overexpression of transforming growth factor-β has been shown to be fibrogenic in mouse lung, whereas local OSM overexpression via intra-articular administration has been shown to induce a pannus-like inflammatory response in the synovium of mouse knee joints. Here we examine the effects of OSM in the context of those of transforming growth factor-β using an established adenovirus vector that expresses mOSM (AdmOSM). We administered the virus intra-nasally into Balb/C mice to achieve high expression of OSM in the lung, and examined the effects at various time points. AdmOSM resulted in a vigorous inflammatory response by day 7 which was characterized by an elevation of neutrophil and mononuclear cell numbers and a marked increase in collagen deposition. These data support the use of such systems to study the ECM in vivo, and indicate a potential role for OSM in inflammatory responses that can modulate steady-state ECM deposition in Balb/C mice.

Introduction

Our investigations have focused on the role of gp130 cytokines, also referred to as the interleukin-6 (IL-6)/leukaemia inhibitory factor (LIF) cytokine family, in inflammation, and particularly the effects of these ligands on connective tissue cells, including fibroblasts, epithelial and endothelial cells. The gp130 cytokines [IL-6, LIF, oncostatin M (OSM), IL-11, ciliary neurotropic factor, cardiotrophin-1 (CT-1) and the more recently identified neurotrophin-1/B-cell-stimulating factor 3 (NNT-1/BSF-3)] utilize receptor complexes that include the common signal-transducing receptor subunit gp130, which probably contributes to their biological redundancy [1-5]. In addition, there is evidence of differential signalling and in vitro activities for the individual cytokines and receptor complexes [6].

Our results from studies in human and mouse cells in vitro have shown a dominant ability of OSM (among the gp130 family) to regulate cell responses in connective tissue cell types.

Human OSM, originally characterized by its ability to inhibit the growth of the A375 melanoma cell line [7,8], stimulates the production of acute-phase proteins and of the low-density lipoprotein receptor by hepatocytes [9,10], induces the gene expression of several immediate-early genes, including egr-1 and c-fos, regulates chemokine expression, induces tissue inhibitor of metalloproteinases-1 (TIMP-1) and participates in the regulation of matrix metalloproteinase-1.

Key words: fibrosis, inflammation, lung, oncostatin M, TIMP-1.

Abbreviations used: CT-1, cardiotrophin-1; ECM, extracellular matrix; EGF, epidermal growth factor; IL-6 (etc.), interleukin-6 (etc.); LIF, leukaemia inhibitory factor; MMP, matrix metalloproteinase; OSM, oncostatin M; mOSM, mouse OSM; AdmOSM, adenovirus vector expressing mOSM; p.f.u., plaque-forming units; STAT, signal transduction and activators of transcription; TIMP, tissue inhibitor of metalloproteinases.

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