Cell Surface-bound Collagenase-1 and Focal Substrate Degradation Stimulate the Rear Release of Motile Vascular Smooth Muscle Cells*§

Shaohua Li‡, Lawrence H. Chow, and J. Geoffrey Pickering§

From the John P. Robarts Research Institute (Vascular Biology Group), London Health Science Centre, Departments of Medicine (Cardiology), Medical Biophysics, and Biochemistry, University of Western Ontario, London, Ontario N6A 5K8, Canada

To migrate in the vessel wall, smooth muscle cells (SMCs) must contend with abundant type I collagen. We investigated the mechanisms used by human SMCs to efficiently migrate on type I collagen, following stimulation with fibroblast growth factor-2 (FGF-2). FGF-2-stimulated migration was inhibited by a hydroxamic acid inhibitor of matrix metalloproteinases and by a neutralizing anti-collagenase-1 antibody. Moreover, migration speed of SMCs plated on mutant collagenase-resistant type I collagen was not increased by FGF-2. Time-lapse video analysis of unstimulated SMCs migrating on collagen revealed discrete phases of leading edge membrane extension and rear retraction, the latter often after rupture of an elongated tail. FGF-2 stimulation yielded a more synchronous, gliding motion with a collagenase-1-mediated decrease in tail rippling. Surface labeling of SMCs with biotin followed by immunoprecipitation revealed that a proportion of active collagenase-1, expressed in response to FGF-2, was bound to the plasma membrane. Pericellular collagen substrate cleavage was verified by immunostaining for neoepitopes generated by collagenase-1 action and was localized to discrete zones beneath the cell tail and the leading edge. These results identify a novel mechanism by which SMC migration on collagen is enhanced, whereby rear release from the substrate is orchestrated by the localized actions of membrane-bound collagenase-1.

Migration of vascular smooth muscle cells (SMCs) is an important feature of arterial diseases such as atherosclerosis (1). In response to signals within the diseased vessel wall, SMCs translocate from the arterial media to the intima, or from one region in the intima to another, where they accumulate and elaborate extracellular matrix (ECM). These processes can lead to narrowing of the vascular lumen and organ ischemia.

In order to migrate in the vessel wall, SMCs must contend with the dense ECM. There is good evidence that SMC migration and vascular lesion formation are accompanied by expression of ECM-degrading enzymes, including matrix metalloproteinases (MMPs, matrixins) (2–6). Furthermore, SMC migration in the artery wall has been inhibited by infusion of a broad spectrum MMP inhibitor (5) and delivery of tissue inhibitor of metalloproteinase-2 (7). These data thus support a role for ECM proteolysis in SMC translocation, although it is not yet established which ECM components need to be degraded.

Fibrillar type I collagen is a dominant ECM protein of both the normal and diseased artery wall (8, 9). Unlike many ECM constituents, type I collagen is susceptible to degradation by only a few enzymes. Of these, collagenase-1 (MMP-1), which has been detected in human atherosclerotic lesions (2–4), appears to be the principal SMC-derived collagenolytic enzyme, although collagenase-3 (MMP-13) (10) and cathepsin K (11, 12) may also participate. Collagenase-I degrades type I collagen by cleaving a single site in the triple helical domain to yield a 3/4 N-terminal fragment and 1/4 C-terminal fragment. At physiologic temperature these fragments denature into gelatin. The extent to which this cascade is required for vascular SMC motility is unknown. However, there are at least two potential reasons why collagenase-1 action might facilitate movement of SMCs through the arterial wall. First, degradation of type I collagen may remove structural barriers to movement. Second, collagenase-1-mediated conversion of collagen to gelatin may yield a substrate that is more permissive to motility, as suggested by studies of keratinocytes (13).

Basic fibroblast growth factor (called FGF-2) is a documented mediator of vascular lesion formation (14). It is one of few growth factors shown to stimulate SMC migration both in vitro and in vivo (15–17). As well, FGF-2 can potentiate directed migration to platelet-derived growth factor-BB (PDGF-BB), another important mediator of vascular growth and repair (16, 18). Importantly, the extent to which FGF-2 stimulates SMC migration varies depending on the ECM substrate. We observed that for cells on fibronectin the effect was modest. However, on type I collagen FGF-2 almost doubled SMC migration speed (16). This enhanced SMC migration on type I collagen was dependent on α1β1 integrin, expression of which was also increased by FGF-2. Thus, an interplay between FGF-2, the vascular SMC, and type I collagen appears to regulate SMC motility. In this context, it is noteworthy that FGF-2...
is also one of few growth factors that can induce the expression of collagenase-1 by vascular SMCs (19).

At the single cell level, migration involves a coordinated but asymmetric pattern of movement. Cell translocation is initiated by a leading front of lamellipodial and/or filopodial extensions, some of which stably attach to the ECM substrate. Traction forces generated by myosin-based contraction then advance the cell body. Release of the rear of the cell from the substrate then completes the cycle and enables forward translocation. In cells with relatively high migration speeds, these phases are smoothly coordinated such that the cells assume a gliding motion. In slower moving cells, such as fibroblasts and SMCs, the phases of movement are more discontinuous, notably because the kinetics of rear release are slower than those of forward extension. In these cell types, as the cell front protrudes the cell rear elongates and forms a tail, which is under increasing tension until ultimately it releases from the substrate and retracts into the cell body (20). Tail release from the substrate can thus be rate-limiting for cell migration. The mechanisms that regulate rear release are unclear, but two general modes have been described. The first mode involves a reduction in the strength with which the cell rear attaches to the substrate. This feature has been documented in relatively fast moving cells. In neutrophils migrating on vitronectin, for example, there is a selective reduction in affinity of adhesion between \( \alpha \beta_3 \) integrin and the ECM at the rear of the cell, with subsequent shuttling of \( \alpha \beta_3 \) integrins to the front (21). The second mode of rear release involves rupturing of components of the adherent cell tail. This process has been identified in slower moving cells, in particular fibroblasts, where a persistently high strength of substrate adhesion at the cell rear delays the completion of the migration cycle. In this circumstance, ripping of \( \beta_1 \) integrin microaggregates at the cell rear can occur, leaving a proportion of integrins on the substrate (22, 23). Other studies have documented ripping of the plasma membrane itself, such that membrane-bound remnants of the tail, visible by light microscopy, are left behind (20).

In this study, we investigated the mechanisms used by human vascular SMCs to migrate on type I collagen under basal conditions, where migration is relatively slow, and following stimulation with FGF-2, in which migration speed is substantially faster. We found that chemical MMP inhibitors and a neutralizing anti-collagenase-1 antibody abrogated FGF-2-stimulated migration on type I collagen, and that SMCs plated on mutant collagenase-resistant type I collagen did not accelerate their migration speed in response to FGF-2. Using digital time-lapse video analysis, we established that ripping of elongated tails, which was prominent under basal conditions, was replaced by smoother, collagenase-1-dependent retraction of shorter tails, following FGF-2 stimulation. Furthermore, we established that a proportion of active collagenase-1 expressed in response to FGF-2 was bound to the surface of plasma membrane, and that collagen substrate cleavage by FGF-2-stimulated SMCs was spatially localized to a zone beneath the rear of the cell. These results identify a novel mechanism by which SMC migration on collagen is enhanced, whereby rear release from the substrate is orchestrated by the localized actions of membrane-bound collagenase-1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary cultures of human vascular SMCs were established by explant outgrowth of unused segments of internal thoracic artery retrieved at the time of coronary artery bypass surgery (24, 25). The identity of SMCs was confirmed morphologically and by positive immunostaining with a monoclonal antibody (mAb) against human smooth muscle \( \alpha \)-actin (SM \( \alpha \)-actin, 1A4, Dako). Cells were used between the 6th and 12th passage. The HITB5 human SMC line, also derived from internal thoracic artery, was also employed (26). Cells were grown in M199 supplemented with 10% fetal bovine serum and antibiotics.

**SMC Migration Assays**—Chemotaxis of SMCs was measured using a microchemotaxis chamber, as described previously (16). Briefly, SMCs were liberated with trypsin-EDTA, and 12,500 cells in M199 with 1% bovine serum albumin were added to the upper wells of the chamber. The lower wells were filled with normal mouse IgG (nm IgG, 50 \( \mu \)g/ml) or mouse anti-human collagenase-1 mAb (VI3 IA6, 50 \( \mu \)g/ml). PDGF-BB (10 ng/ml) was in the lower well. *, \( p < 0.01 \) versus control incubated with normal mouse IgG.
ture dishes precoated with 100 μg/ml type I collagen and positioned on the stage of an inverted microscope (Axiovert S100, Zeiss). Cells were incubated in M199 with Hanks' salts and 25 mmol/liter HEPES to maintain physiological pH in room air. Ambient temperature was maintained at 37 °C using a temperature control cell (BC-100, 20/20 Technology, Inc.), and evaporation/condensation was prevented by applying a stream of humidified warm (37 °C) air across the culture lid. Migration was monitored over an 8-h period using a CCD camera (XC-75CE, Sony), and images were digitally acquired. The location of cell centroids at hourly intervals was tracked off-line (Northern Eclipse, Empix Imaging Inc.), and migration speed was determined as the sum of hourly distances divided by the total time. The proportion of cells that demonstrated nonlethal rupturing of a portion of the tail from the remainder of the advancing cell over the 8-h recording period was also determined.

Assessment of Collagenase-1 expression in Human Vascular SMCs—SMCs at near-confluence were incubated in M199 with 1% fetal bovine serum for 24 h and then incubated with 25 ng/ml FGF-2 for the designated time. Culture supernatant was harvested, and cells were dissociated by incubating with 0.53 mmol/liter EDTA in PBS for 20 min. After washing twice in cold PBS, the cells were disrupted in a solution of 10 mmol/liter Tris, pH 7.4, 1% SDS, 0.1 mmol/liter EDTA, 0.1 mmol/liter phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml leupeptin. DNA in the samples was sheared by passing the cell lysate through a 27-gauge needle, and insoluble material was removed by centrifugation (12,000 rpm at 4 °C) for 30 min. The protein concentration in the supernatant was measured by Bio-Rad DC protein assay kit. Equal amount of proteins were resolved by 12% SDS-PAGE and electrophotically transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, Bedford, MA) for Western blot analysis. The membranes were blocked overnight at 4 °C and then incubated with anti-collagenase-1 antibody (Mb-1, Oncogene Sciences). Bound primary antibody was detected with horseradish peroxidase-conjugated goat anti-mouse IgG F(ab')2, and blots were developed with ECL detection reagents (Roche Molecular Biochemicals).

**FIG. 3.** Effect of anti-collagenase-1 neutralizing antibody on FGF-2-stimulated random migration of SMCs on type I collagen. SMCs were plated at 1000 cells/cm² on 35-mm dishes precoated with 100 μg/ml type I collagen. Cells were treated with 25 ng/ml FGF-2 or vehicle for 48 h. Random migration path (A) and speed (B) were quantified by digital time-lapse video microscopy for 8 h in the presence of 50 μg/ml mouse anti-human collagenase-1 neutralizing antibody or normal mouse IgG. *, p < 0.01 versus control incubated with normal mouse IgG (nm IgG); #, p < 0.01 versus FGF-2-pretreated cells incubated with normal mouse IgG.
Collagenase-1 expression was also evaluated by gelatin zymography. Culture media and cell lysates were prepared as above but without addition of protease inhibitors. Proteins were separated under nonreducing conditions on 12% polyacrylamide gels embedded with 1 mg/ml gelatin (Sigma). Gels were washed in 2.5% Triton X-100 and incubated at 37 °C for 48 h in buffer containing 50 mmol/liter Tris (pH 8.0), 5 mmol/liter CaCl2, and 40 mmol/liter NaNO3 and were stained with Coomassie Brilliant Blue (Sigma). MMP activity is manifest as clear lysis zones against a blue background. To confirm that MMP action was the cause of substrate degradation, duplicate gels were prepared in which zones against a blue background. To confirm that MMP action was the cause of substrate degradation, duplicate gels were prepared in which

**Analysis of SMC-associated Collagenase-1—**Association of collagenase-1 with the plasma membrane was assessed by surface labeling with biotin and immunoprecipitation. Control and FGF-2-treated SMCs were incubated with 0.5 mg/ml sulfo-NHS-biotin (Pierce) in PBS, pH 7.4, for 20 min on ice. After washing with cold PBS, cells were treated with gl y c i n e ( 0.1 mol/liter in PBS, pH 7.8) to block residual NHS groups. Cells were washed twice in cold PBS (pH 7.4) and disrupted in Nonidet P-40 lysis buffer (50 mmol/liter Tris, pH 7.5, 150 mmol/liter NaCl, 1% Nonidet P-40, 1% lV/liter EDTA, 1% lV/liter PMSF, 10 μg/ml leupeptin). Equal amounts of protein lysates were precleared with protein G-plus-agarose bead conjugate (Santa Cruz Biotechnology, Inc.) and then incubated with 1 μg of anti-collagenase-1 mAb (Oncogene Science) at 4 °C overnight with protein G-plus-agarose conjugate at 4 °C and the resulting complex washed four times in radiimunprecipitation assay buffer (50 mmol/liter Tris, pH 7.5, 150 mmol/liter NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l liter PMSF, 10 μg/ml leupeptin). Immunoprecipitates were analyzed by SDS-PAGE under reducing condition, and proteins were electrothoretically transferred to PVDF membranes. To verify induction of collagenase-1 expression, blots were probed with anti-collagenase-1 antibody and developed with horseradish peroxidase-conjugated goat anti-mouse IgG F(ab′)2 and chemiluminescence detection reagents (ECL, Roche Molecular Biochemicals), and exposed on Kodak O-Mat Blue XB-1 film. To determine if collagenase-1 was associated with the cell surface, blots were probed directly with avidin-horseradish peroxidase (Sigma). To confirm that cytoplasmic proteins were not labeled by the biotinylation procedure, parallel immunoprecipitation using anti-SM α-actin antibody was performed with blots probed for either SM α-actin or biotin.

**Assessment of Local Type I Collagen Substrate Degradation by Human SMCs—**Human vascular SMCs were incubated with 25 ng/ml FGF-2 or vehicle for 48 h and then plated on glass coverslips precoated with type I collagen (100 μg/ml) and cultured overnight. Cells and substrate were washed twice with PBS and fixed with 4% paraformaldehyde in PBS. After blocking nonspecific binding sites with 10% normal rabbit serum, culture materials were incubated with R1822, a rabbit anti-human 1/4 3/4 collagen fragment polyclonal antibody (kindly provided by Dr. A. R. Poole, Shriners Hospital, Montreal, Quebec, Canada). This antibody detects carboxyl-terminal (COL2–3/4C(short)) and amino-terminal (COL2–1/4N1) neoepitopes generated by collagenase-1 action. The mutation constitutes a substitution of 3 amino acids at the collagenase cleavage site of the collagen chain (30, 31). As shown in Fig. 4, SMCs migrating on collagenase-resistant mutant mouse type I collagen manifest a 1.5-fold increase in migration speed over 8 h was quantified by digital time-lapse video microscopy. * p < 0.01 versus control.

**RESULTS**

**FGF-2-stimulated SMC Migration on Collagen Requires Collagenase-1 Activity—**SMCs stimulated to migrate in the artery wall must utilize mechanisms to interact with the abundant type I collagen. We previously found that FGF-2 enhanced the migration of SMCs on collagen and can potentiate directed migration of cells on this substrate to PDGF-BB (16). In addition, FGF-2 induced the expression of collagenase-1 by SMCs (19). To determine if FGF-2-stimulated SMC migration on collagen required the action of MMPs, SMCs were treated with FGF-2 for 48 h and migration toward PDGF-BB was studied in the presence of the hydroxamic acid-containing MMP inhibitor, CP-138,521 (kindly provided by Pfizer, Groton, CT). This compound inhibits MMPs, nonselectively by ligating the zinc cation in the active site via its hydroxamic acid group. As shown in Fig. 1, the enhanced SMC chemotaxis to PDGF-BB afforded by FGF-2 was abrogated by CP-138,521. Similar results were seen with the MMP inhibitors phenanthroline and EDTA (data not shown).

To determine if FGF-2-enhanced chemotaxis on type I collagen was mediated specifically by collagenase-1, FGF-2-pre-treated or untreated SMCs were added to the upper well of chemotaxis chamber together with anti-human collagenase-1 neutralizing antibody (V13 IA6, kindly provided Dr. L. J. Winters, University of Alabama at Birmingham) (29) or normal mouse IgG. As shown in Fig. 2, FGF-2-stimulated SMC chemotaxis to PDGF was completely blocked by V13 IA6. This antibody had no significant effect on motility of SMCs not stimulated with FGF-2. To quantify SMC migration speed and migration path on type I collagen, digital time-lapse video microscopy was performed. SMCs were plated on culture dishes precoated with 100 μg/ml type I collagen and were incubated with FGF-2 or vehicle for 48 h. Time-lapse recording was carried out for 8 h in the presence of collagenase-1 neutralizing antibody or control normal mouse IgG. As shown in Fig. 3A, the migration path of SMCs treated with FGF-2 and control IgG was greater than that of cells incubated with control IgG alone, with a corresponding increase in migration speed (Fig. 3B). In contrast, the collagenase-1 neutralizing antibody reduced the migration speed to the control level (Fig. 3B), corresponding to a restrained migration path (Fig. 3A).

**FGF-2 Stimulation Does Not Enhance Migration of SMCs on Collagenase-resistant Type I Collagen**—Collagenase-1 cleaves a single site in the collagen triple helix, which, in turn, yields a denatured substrate that has the potential to interact with the cell in a manner distinct from that of native collagen. To corroborate the findings using the anti-collagenase-1 antibody and to directly determine if collagen proteolysis per se was required for the optimized SMC motility afforded by FGF-2, we studied the migration of SMCs plated on collagenase-resistant mutant collagen. This substrate was prepared from tails of mice bearing a targeted mutation at collagenase-1 cleavage site in the triple helical domain of type I collagen. Cells were treated with 25 ng/ml FGF-2 or vehicle for 48 h and plated onto mutant or wild-type mouse tail collagen, and migration speed over 8 h was quantified by digital time-lapse video microscopy. * p < 0.01 versus control.
human SMCs derived from internal thoracic artery, and in a human SMC clone (HITB5) derived from the same source (26), prominent tails are a consistent feature of migrating cells. In addition, ripping of a fragment of trailing plasma membrane, visible on Hoffman modulated contrast microscopy, was frequently observed just prior to retraction of the cell rear and advancement of the cell body. Occasionally, these remnants autonomously spread and crawled for short time. Fig. 5A shows time-lapse-acquired photomicrographs of control and FGF-2-treated HITB5 SMCs migrating on type I collagen (movies are available as supplemental material to the online version of this article). Compared with control cells, FGF-2-treated cells migrated more quickly, and many of the cells could be seen to migrate in a smooth, gliding motion. This was in striking contrast to the discrete extension and retraction events seen in the control SMCs. The mean tail length (distance from rearmost tip of the tail to rear edge of the cell nucleus) of FGF-2-treated SMCs was significantly shorter than that of control cells (124 ± 11 μm versus 226 ± 14 μm, p < 0.01, n = 26 cells per group, measured at the start of the recording period). As well, the proportion of SMCs that displayed tail ripping over the 8-h recording period was significantly lower in the FGF-2-treated cultures (4.6 ± 0.9% versus 17.4 ± 2.8%, p < 0.01) (Fig. 5B). Pooled data from control and FGF-2-treated SMCs established an inverse correlation between cell migration speed and tail ripping (r = −0.8, p < 0.01). Importantly, when FGF-2-treated SMCs were incubated with anti-collagenase-1 antibody, the gliding motion reverted to the more halting pattern of translocation. As well, mean tail length was not significantly different from control, unstimulated SMCs, and the frequency of tail-ripping likewise returned to baseline (Fig. 5, A and B).

A Proportion of FGF-2-induced Collagenase-1 Is Bound to the SMC Plasma Membrane—To maintain structural integrity of the vessel wall, the proteolytic actions of collagenases must be spatially confined. In recent years, soluble ECM proteinases, including MMP-2 and MMP-9, have been found to be bound to the plasma membrane, implicating a mechanism for localizing ECM proteolysis to the immediate pericellular environment (32, 33). As a first step is determining if collagenase-1 could bind to the SMC surface, we compared the expression profiles of collagenase-1 in cell homogenates and culture media. Cell lysates were prepared after gently releasing the cells from the substrate with EDTA to avoid including substrate-bound col-
lagenase-1 in the preparation. As shown in Fig. 6A, FGF-2 induced a time-dependent accumulation of collagenase-1 in the culture media, as assessed by Western blot analysis. Collagenase-1 was also detected in the SMC lysate preparation, and this fraction of the expressed collagenase-1 also increased in response to FGF-2. A strong increase in cell-associated collagenase-1 expression was clearly evident at 6 h and remained at a relatively constant level for up to 72 h. In comparison, collagenase-1 in the media was faintly detectable at 12 and 24 h, and strongly detectable only by 48 h. The time course of expression of cell-associated collagenase-1 closely paralleled the increase in migration speed in response to FGF-2 (data not shown). Interestingly, collagenase-1 detected in the media fraction was approximately 57 kDa in size, indicative of a proenzyme form, whereas that detected in the cell lysate preparation was approximately 42 kDa in size, consistent with an active form. To corroborate this difference, we performed gelatin zymography. As shown in Fig. 6B, zymography confirmed the presence of SMC-associated collagenase-1 after FGF-2 stimulation. Moreover, whereas the substrate degradation signal generated by collagenase-1 in the media appeared as a ~57/52-kDa doublet, that from the cell lysate was a smaller protein (~42 kDa) suggesting activation.

To determine if any of the collagenase-1 associated with the cell was bound to the outer surface of plasma membrane, we labeled the cell surface proteins with biotin and then immunoprecipitated the cell lysates using an anti-collagenase-1 antibody. The collagenase-1 immunoprecipitate was then run on SDS-PAGE, and the transfer was probed with horseradish peroxidase-conjugated avidin (avidin-HRP). As shown in Fig. 7, immunoprecipitated collagenase-1 could be detected by avidin-HRP, establishing it to be biotinylated, and thus a constituent of the cell surface. To ensure that cytoplasmic proteins were not labeled by the biotinylation procedure, lysates were immunoprecipitated with an anti-SM α-actin antibody and probed with avidin-HRP. No signal was detected under these circumstances.

Type I Collagen Substrate Degradation Is Asymmetrically Localized Beneath the Motile SMCs—To identify sites of type I collagen substrate degradation by FGF-2-stimulated SMCs, cultures were immunostained using a rabbit anti-human 1/3 type I collagen fragment polyclonal antibody. This antibody identifies carboxyl-terminal and amino-terminal neoepitopes generated by cleavage of native human type I (and II) collagen by collagenase-1 (28). As illustrated in Fig. 8, collagen degradation fragments were identified in a discrete region of substrate beneath the extreme rear of cells stimulated with FGF-2 (Fig. 8, B and D). The adjacent substrate beneath the cell body consistently showed little or no evidence for 1/4 or 3/4 collagen fragments. In some cells, collagen degradation fragments were also found in a zone beneath the leading lamellipodial extensions (Fig. 8, C and D). No collagen substrate degradation signal was detected in SMCs incubated without FGF-2. In addition, the degradation signal was abrogated when FGF-2-treated cultures were co-incubated with the anti-collagenase-1 neutralizing antibody (data not shown).

**DISCUSSION**

These studies identify a novel means by which the trailing aspect of motile SMCs releases from the substrate, thereby completing the cell migration cycle. By analyzing SMCs migrating on type I collagen in the presence or absence of FGF-2, we ascertained that site-specific degradation of the collagen substrate promoted detachment of the cell rear from the substrate and retraction into the cell body. As a consequence, there was greater synchrony between forward protrusion and rear retraction and migration speed was increased. Furthermore, we determined that this regionalized editing of the collagen substrate was orchestrated by the stimulated expression of collagenase-1, which was bound to the SMC plasma membrane.

The strength of integrin-mediated cell adhesion to the substrate is a major determinant of cell migration speed. At very low or very high levels of adhesion strength, migration is impeded, whereas maximum migration speeds occur at an inter-
mediate adhesion strength (22, 34). Factors that determine adhesion strength include integrin expression level, substrate ligand level, and integrin-ligand binding affinity (34). It has been shown previously that $\alpha_2\beta_1$ integrin, which binds native type I collagen with high affinity (35), is abundantly expressed by human SMCs in culture (16, 36). We previously found that expression of $\alpha_2\beta_1$ integrin on the SMC surface is increased even further upon stimulation of SMCs with FGF-2 (16). However, this FGF-2-enhanced expression of $\alpha_2\beta_1$ integrin was associated with increased, not decreased, migration on type I collagen (16). In the present study, an FGF-2-mediated increase in SMC migration was seen even when the collagen substrate level was high (100 $\mu$g/ml), a condition that would also be expected to favor increased adhesion strength. This seemingly paradoxical response, whereby migration speed on type I collagen was increased despite high levels of both substrate ligand and adhesion receptor, is at least partly explained by the current finding that FGF-2 stimulates the expression of collagenase-1 and the remodeling of the collagen substrate. The primacy of collagenase-1 in mediating the increased motility of FGF-2-treated SMCs was demonstrated using a hydroxamate inhibitor of MMP activity, an anti-collagenase-1 neutralizing antibody, and by plating SMCs on type I collagen from mice bearing a targeted mutation of the $\text{col1A1}$ gene, rendering the molecule resistant to collagenase-1-mediated cleavage.

Pilcher et al. (13) have proposed a paradigm that collagen degradation by keratinocytes may not only “clear a path” for migration, but also generate a substrate with adhesive properties favorable to migration (13). The current finding that FGF-2 stimulated the migration of SMCs on wild-type collagen but not on collagenase-1-resistant collagen establishes a role for collagen triple helix cleavage in SMC movement, and suggests that potentially subtle collagen editing may enhance SMC motility in the vessel wall, independent of an effect on structural barriers. The stimulation of motility by the remodeled substrate may involve a number of mechanisms. Collagenase-1 cleaves the collagen triple helix about 3/4 along the length from the N terminus and the resulting, thermally unstable fragments denature into gelatin. Denatured collagen fragments not only reduce their binding capacity for $\alpha_2\beta_1$ integrin (37) but can manifest cryptic RGD sites which may serve as ligands for $\beta_3$ integrins on the cell surface (38, 39). Because the strength of
binding between gelatin and α5β1 integrin is less than that of collagen binding to α5β1 integrin, motility may be favored over stationary adhesion. Recently, degraded collagen fragments were also found to stimulate calpain-mediated cleavage of components of the focal adhesion complex, including pp125Fak, with an associated reduction in SMC attachment (40). Calpain-mediated remodeling of the focal adhesion complex may thus be another pathway by which type I collagen cleavage functionally loosens the SMC from its substrate.

The increased SMC migration speed afforded by collagen proteolysis was accompanied by a striking shift in the morphological movement profile of individual SMCs. Instead of the staccato-like pattern of forward protrusion and rear retraction, FGF-2-stimulated SMCs displayed greater synchrony between front and back, yielding a smoother pattern of translocation. Dominant in this process was the behavior of the cell rear. Untreated human SMCs migrating on type I collagen displayed long tails that infrequently retracted into the cell body despite lamellipodial activity at the cell front, effectively pausing the migration cycle. Often, the tails retracted into the cell body only after a frank rupture event, such that a cell remnant was left behind the crawling cell. Sometimes the separated remnant autonomously spread and crawled for short time, indicating that the remnants contained not only adhesion molecules but also actin cytoskeletal machinery. This seemingly inefficient loss of cellular constituents by motile cells has previously been observed in randomly migrating fibroblasts (20, 22, 41) and appears to be a feature of relatively slow moving cells with strong attachment to the substrate. The current findings indicate, however, that the phenomenon of tail ripping is not an invariant feature of a given cell type on a given substrate, but is modifiable by external signals, including ones that lead to substrate degradation. SMCs treated with FGF-2 had shorter tails than untreated SMCs, and the tails typically did not need to rupture in order to retract into the cell body. Importantly, collagenase-1 neutralizing antibody completely abrogated these effects of FGF-2.

Immunodetection of collagen degradation products in FGF-2-treated cultures revealed that proteolysis of the collagen substrate was regionalized. Evidence for 3/4 1/4 cleavage of the triple helix domain of type I collagen was found beneath the cell rear but not beneath the cell body. This finding implicates a targeting process for collagenase action and suggests a regulated linkage between localized collagen turnover and the need to release the trailing edge of the cell from the substrate. Interestingly, we also identified discrete zones of collagen degradation beneath one or more lamellipodial extensions. Although the current studies are not centered around the behavior of the leading cell edge, we speculate that collagenase-1 actions at this region of the cell may both clear a path for movement and generate a local substrate that is favorable to lamellipodial activity. In this context, it is noteworthy that degraded collagen can stimulate remodeling of focal contacts (40), and this process at the front of a migrating cell may be critical to sustaining the protrusive membrane activity (42).

Biotinylation of cell surface proteins followed by immunoprecipitation with anti-collagenase-1 antibody identified that at least some of the cell-associated collagenase-1 was on the surface of plasma membrane. The molecular size of SMC-associated collagenase-1 was consistent with an active form, processed from its zymogen precursor. This contrasted with the collagenase-1 identified in the media fraction of FGF-2-stimulated cultures, which contained latent species. Our finding of active collagenase-1 bound to the plasma membrane would be consistent with a physiologically efficient means of ensuring that motile SMCs confine collagen degradation to only those fibers that are in close approximation to the cell, and indeed to only those subregions of the substrate in which proteolysis of collagen is likely to favor motility of that cell. Restricting ECM proteolysis to the immediate pericellular environment via binding between α5β1 integrin in angiogenic blood vessels (32, 43), as part of a membrane-tethered ternary complex with MT-MMP-1 and tissue inhibitor of metalloproteinase-2 (44), and in association with β1 integrin on the surface of invasive tumor cells via interaction with procollagen (45). Recently, MMP-9 has been localized to the hyaluronan receptor CD44 on the plasma membrane (33). It seems unlikely that collagenase-1 was bound nonspecifically to the cell surface because, as noted, only collagenase-1 in its active form was found on the cell surface, and the actions of cell-associated collagenase-1 were localized to discrete regions beneath the cell. In addition, cell-associated collagenase-1 could not be displaced with albumin (10 mg/mL, data not shown). The identity of molecule(s) interacting with collagenase-1 on the plasma membrane is currently under investigation.

During vascular remodeling, migrating SMCs must contend with the ubiquitous type I collagen. Degradation of type I collagen, however, must be exquisitely controlled because the hemodynamic loads on the vessel wall are considerable and the consequences for vessel rupture can be fatal. The current results identify a novel mechanism by which SMC migration on collagen is enhanced by pericellular collagen proteolysis, whereby rear release from the substrate is orchestrated by the localized action of membrane-bound collagenase-1.

Acknowledgments—We thank Dr. Jack Windsor (University of Alabama at Birmingham) for the anti-human collagenase-1 neutralizing antibody (VIII IA6); Dr. Robin Poole (Shriners Hospital, Montreal, Quebec, Canada) for the anti-human 1/4 3/4 collagen fragment antibody; Dr. Lawrence Reiter (Pfizer, Groton, CT) for the MMP inhibitor, CP-138,521; Drs. David Holdsworth and Maria Drangova (John P. Roberts Research Institute, London, Ontario, Canada) for assistance generating the movies; and Drs. Richard Novick, Douglas Boyd, and Alan Menkis (London Health Sciences Center, London, Ontario, Canada) for providing the artery fragments.

REFERENCES

1. Ross, R. (1993) Nature 362, 801–809
2. Nikkari, S. T., O’Brien, K. D., Ferguson, M., Hatsuokami, T., Welgus, H. G., Alpers, A. W. (1995) Circulation 92, 1393–1398
3. Galis, Z. S., Sukhova, G. K., Lark, M. W., and Libby, P. (1994) J. Clin. Invest. 94, 2493–2503
4. Nikkari, S. T., Geczy, R. L., Hatsuokami, T., Ferguson, M., Forough, R., Alpers, E., and Cloves, A. W. (1996) Am. J. Pathol. 148, 777–783
5. Bendeck, M. P., Irvin, C., and Reidy, M. A. (1996) Circ. Res. 78, 38–43
6. Strauss, B. H., Robinson, R., Batchelor, W. B., Chisholm, R. J., Ravi, G., Natarajan, M. R., Logan, R. A., Mehta, S. B., Levy, D. E., Eirin, A. M., and Keeley, P. W. (1996) Circ. Res. 79, 541–550
7. Cheng, L., Mantile, G., Pauly, R., Nater, C., Falcì, A., Monticone, R., Bilato, C., Glushband, Y. A., Crow, M. T., Stevels-Stevenson, W., and Capogrossi, M. C. (1998) Circulation 98, 2195–2201
8. Barnes, M. J. (1985) Collagen Relat. Res. 5, 65–97
9. Pickering, J. F., Ford, C. M., and Chow, L. H. (1996) Am. J. Cardiol. 78, 633–637
10. Sukhova, G. K., Schonbeck, U., Rakkin, E., Schoen, F. J., Poole, A. R.,Billinghurst, R. C., and Libby, P. (1999) Circulation 99, 2503–2509
11. Garnero, P., Borel, O., Byrjalsen, I., Ferreras, M., Drake, F. H., McMeneny, M. S., Foged, N. T., Delmas, P. D., and Delaforge, J. M. (1998) J. Biol. Chem. 273, 32347–32352
12. Sukhova, G. K., Shi, P. G., Simon, D. I., Chapman, H. A., and Libby, P. (1998) J. Clin. Invest. 102, 576–583
13. Pickler, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., and Parks, W. C. (1997) J. Cell Biol. 139, 1445–1457
14. Lindner, V., and Reidy, M. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 88, 3737–3743
15. Bilato, C., Pauly, R. R., Melillo, G., Monticone, R., Gorelick-Feldman, D., Glushband, Y. A., Sollet, S. J., Zeman, B., Lakatta, E. G., and Crow, M. T. (1995) J Clin. Invest. 96, 1905–1915
16. Pickering, J. G., Uniyal, S., Ford, C., Chau, T., Laurin, M. A., Chow, L. H., Ellis, C. G., Fish, J., and Chan, B. M. C. (1997) Circ. Res. 80, 627–637
17. Jackson, C. L., and Reidy, M. A. (1993) Am. J. Pathol. 143, 1024–1031
18. Ferns, G. A. A., Raines, E. W., Sprugel, K., Motani, A. S., Reidy, M. A., and Ross, R. (1991) Science 253, 1129–1132
19. Pickering, J. G., Ford, C. M., Tang, B., and Chow, L. H. (1997) Arterioscler.
Thromb. Vasc. Biol. 17, 475–482
20. Chen, W. T. (1981) J. Cell Biol. 90, 187–200
21. Lawson, M. A., and Maxfield, F. R. (1995) Nature 377, 75–79
22. Regen, C. M., and Horwitz, A. F. (1992) J. Cell Biol. 119, 1347–1359
23. Palecek, S. P., Schmidt, C. E., Lauffenburger, D. A., and Horwitz, A. F. (1996) J. Cell Sci. 109, 941–952
24. Pickering, J. G., Weir, L., Rosenfield, K., Stetz, J., Jekanowski, J., and Isner, J. M. (1992) J. Am. Coll. Cardiol. 20, 1430–1439
25. Pickering, J. G., Bacha, P., Weir, L., Jekanowski, J., Nichols, J. C., and Isner, J. M. (1995) J. Clin. Invest. 91, 724–729
26. Li, S., Sims, S., Jiao, Y., Chow, L. H., and Pickering, J. G. (1999) Circ. Res. 85, 338–348
27. Rocnik, E. F., Chan, B. M., and Pickering, J. G. (1998) J. Clin. Investig. 101, 1889–1898
28. Billinghurst, R. C., Dahlberg, L., Ionescu, M., Reiner, A., Bourne, R., Rorabeck, C., Mitchell, P., Hamber, J., Diekmann, O., Tschesche, H., Chen, J., Van Wart, H., and Bhown, A. S., Birkedal-Hansen, H. (1998) Biochemistry 27, 6751–6758
29. Birkedal-Hansen, B., Moore, W. G., Taylor, R. E., Bhown, A. S., and Birkedal-Hansen, H. (1988) Biochemistry 27, 6751–6758
30. Wu, H., Liu, X., and Jaenisch, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2819–2823
31. Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R. (1995) J. Cell Biol. 130, 227–237
32. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A. (1996) Cell 85, 683–693
33. Yu, Q., and Stamenkovic, I. (1999) Genes Dev. 13, 35–48
34. Palecek, S. P., Lotfus, J. C., Ginsberg, M. H., Lauffenburger, D. A., and Horwitz, A. F. (1997) Nature 385, 537–540
35. Staatz, W. D., Rajpara, S. M., Wayner, E. A., Carter, W. G., and Santoro, S. A. (1989) J. Cell Biol. 108, 1917–1924
36. Lee, R. T., Berditchevsky, F., Cheng, G. C., and Hemler, M. E. (1995) Circ. Res. 76, 209–214
37. Messent, A. J., Tuckwell, D. S., Knauper, V., Humphries, M. J., Murphy, G., and Gavrilovic, J. (1998) J. Cell Sci. 111, 1127–1135
38. Davis, G. E. (1992) Biochim. Cell Biol. 182, 1025–1031
39. Montgomery, A. M., Reisfeld, R. A., and Cheresh, D. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8856–8860
40. Carragher, N. O., Levkau, B., Ross, R., and Raines, E. W. (1999) J. Cell Biol. 147, 619–630
41. Bard, J. B., and Hay, E. D. (1975) J. Cell Biol. 67, 400–418
42. Horwitz, A. R., and Parsons, J. T. (1999) Science 286, 1102–3
43. Brooks, P. C., Silletti, S., von Schalscha, T. L., Friedlander, M., and Cheresh, D. A. (1998) Cell 92, 391–400
44. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) J. Biol. Chem. 270, 5331–5338
45. Steffen, B., Bigg, H. F., and Overall, C. M. (1998) J. Biol. Chem. 273, 20622–20628