Enzymatic processing of collagen IV by MMP-2 (gelatinase A) affects neutrophil migration and it is modulated by extracatalytic domains

SUSANNA MONACO,[1,2] VALENTINA SPARANO,[1] MAGDA GIOIA,[1,2] DIEGO SBARDELLA,[1] DONATO DI PIERRO,[1] STEFANO MARINI,[1] AND MASSIMO COLETTA[1,2]

[1]Department of Experimental Medicine and Biochemical Sciences, University of Roma Tor Vergata, I-00133 Roma, Italy
[2]InterUniversity Consortium for the Research on the Metal Chemistry in Biological Systems (CIRCMSB), I-70100 Bari, Italy

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Abstract

Proteolytic degradation of basement membrane influences the cell behavior during important processes, such as inflammations, tumorigenesis, angiogenesis, and allergic diseases. In this study, we have investigated the action of gelatinase A (MMP-2) on collagen IV, the major constituent of the basement membrane. We have compared quantitatively its action on the soluble forms of collagen IV extracted with or without pepsin (from human placenta and from Engelbreth–Holm–Swarm [EHS] murine sarcoma, respectively). The catalytic efficiency of MMP-2 is dramatically reduced in the case of the EHS murine sarcoma with respect to the human placenta, probably due to the much tighter packing of the network which renders very slow the speed of the rate-limiting step. We have also enquired on the role of MMP-2 domains in processing collagen IV. Addition of the isolated collagen binding domain, corresponding to the fibronectin-like domain of whole MMP-2, greatly in hibits the cleavage process, demonstrating that MMP-2 interacts with collagen type IV preferentially through its fibronectin-like domain. Conversely, the removal of the hemopexin-like domain, using only the catalytic domain of MMP-2, has only a limited effect on the catalytic efficiency toward collagen IV, indicating that the missing domain does not have great relevance for the overall mechanism. Finally, we have investigated the effect of MMP-2 proteolytic activity ex vivo. MMP-2 action negatively affects the neutrophils' migration across type IV coated membranes and this is likely related to the production of lower molecular weight fragments that impair the cellular migration.

Keywords: endopeptidase/gelatinase A; collagen IV; fragmentation; kinetics; cell migration

Gelatinases (MMP-2, MMP-9), like the other matrix metalloproteinases (MMPs), are a class of Ca^{+2}- and Zn^{+2}-dependent endopeptidases characterized by a Zn^{+2} atom on the active site that is coordinated by three histidyl residues. They show a multidomain structural organization, usually made by a propeptide domain (which is removed upon enzyme activation), the catalytic domain, and a hemopexin-like domain, which are connected by a hinge domain (Sternlicht and Werb 2001). In addition, the two matrix metalloproteinases MMP-2 and MMP-9 possess a unique collagen binding domain (CBD), called the fibronectin-like domain, inserted on the catalytic domain and consisting of

Reprint requests to: Massimo Coletta, Department of Experimental Medicine and Biochemical Sciences, University of Roma Tor Vergata, Via Montpellier 1, I-00133, Roma, Italy; e-mail: coletta@seneca.uniroma2.it; fax: +39-06-72596353.

Abbreviations: EHS, Engelbreth–Holm–Swarm murine sarcoma; rCBD, recombinant collagen binding domain from MMP-2; cdMMP-2, catalytic domain of MMP-2; LPS, lipopolysaccharide; OPD, orthophenilendiamine.

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three 58–amino acid fibronectin type II-like modules (Allan et al. 1995). Furthermore, the membrane-bound MMPs have an intramembrane portion with a short cytoplasmatic tail. These ancillary exosites play an important role in the biochemistry of different substrates' recognition, and a great amount of information has been built up in the past years to elucidate the modulation mechanism of this crucial aspect (Marini et al. 2000; Patterson et al. 2001; Gioia et al. 2002; Overall 2002; Chung et al. 2004; Tam et al. 2004; Xu et al. 2005).

Like other MMPs, gelatinases cleave most of the major macromolecules of the extracellular matrix (i.e., collagen types IV, V, VII, and X; elastin; and others) and activate growth factors and chemokines, thus participating in normal tissue homeostasis and different kind of pathologies, such as arthritis (Dean et al. 1989; Somerville et al. 2003). In particular, it has been shown that MMP-2 and MMP-9 are involved in the so-called ''angiogenic switch,'' a process consisting of the induction of a new vasculature during tumor dissemination and wound healing (Bergers et al. 2000; Fang et al. 2000). In fact, during the degradation of type IV collagen, a major component of the vascular basement membrane, gelatinases lead to VEGF release and the consequent migration and proliferation of the endothelial cells in the provisional matrix in order to create the new blood vessels (Kalluri 2003). Moreover, processing of type IV collagen gives rise to the release of fragments located in the collagenous domain that are able to behave as epitopes, since they can be bound by circulating IgA antibodies. This is the case of recurrent Goodpasture's disease secondary to an autoreactive IgA antibody (Borza et al. 2005).

Collagen type IV represents ~50% of all basement membrane proteins, and it is also termed ''network-forming collagen'' for its capacity to self-assemble into organized networks. In fact, unlike fibrillar collagens of types I, II, III, and V, type IV collagen forms a network structure and it is found crucial for basement membrane stability and assembly, as demonstrated in murine Engelbreth–Holm–Swarm sarcoma (EHS) (Kühn et al. 1981; Timpl et al. 1981). Type IV collagen in mammals is derived from six genetically distinct α-chain polypeptides (serially numbered from α-1 to α-6), with similar domain structures, which share a 50%–70% homology at the amino acid level; the different chains differ from one another in length, being distinct from those forming fibrillar collagens for imperfections in the triple helix of the collagenous domain. The α-chains can be separated into three domains: an amino-terminal 7S domain, a middle triple-helical domain, and a carboxy-terminal globular noncollagenous domain (called NC1). The NC1 domain is considered important for the assembly of the trimeric structure, which is made by three α-chains associated through their NC1 terminal domain to form a trimer called protomer, and in this respect a role of driving force can be proposed for the α-2 chains (Khoshnoodi et al. 2006). The protomer is formed by three α-chains, usually two α-1 chains and one α-2 chain, whose primary structures indicate an apparent molecular weight of ~185 and 170 kDa, respectively (Hostikka and Tryggvason 1987; Soininen et al. 1987). Further, two protomers of type IV collagen associate via NC1 trimers to give an NC1 hexamer, and finally four hexamers interact at the glycosylated diamino-terminal 7S region to form tetramers. This structure is the nucleus of the type IV collagen scaffold, which assembles in a suprastructure with laminin polymers during basement membrane formation (Kalluri 2003) and it begins to unfold only at relatively high temperatures, namely, over 40°C (Dölz et al. 1988). The degradation of type IV collagen is a very important process, since it occurs during several physiological and pathological processes involving the basement membrane, such as embryonic development or tumorigenesis (Ortega and Werb 2002), producing the exposure of cryptic sites important for angiogenesis (Xu et al. 2001). However, it has been also shown recently that cleavage of collagen IV, and particularly of its noncollagenous domains, brings about the production of lower molecular weight fragments, which inhibit angiogenesis and tumor growth (Petitclerc et al. 2000; Roth et al. 2005), envisaging the possibility of multiple actions with opposite effects connected to the enzymatic processing of collagen IV by MMPs.

Cell migration (and in particular neutrophil migration) across the basement membrane is a relevant process (in which collagen IV is certainly involved) during the acute phases of inflammation and allergic diseases (Tani et al. 2001). Such a phenomenon has been heavily associated to the proteolytic activity of MMP-9 and elastase, which are the main components of neutrophil secretion able to cleave collagen IV (Delclaux et al. 1996). However, they cannot be the only proteolytic enzymes involved in the migration across the basement membrane, since mice knockout for MMP-9 display as well a migration capability (Betsuyaku et al. 1999).

Therefore, since previous papers have already shown the capability of MMP-2 to process type IV collagen from different sources (Mackay et al. 1990; Eble et al. 1996) and the fragment originating from collagen IV degradation has been shown to play a relevant role in the modulation of several processes, such as migration and apoptosis (Ortega and Werb 2002), we decided to investigate the possible auxiliary role of MMP-2 on neutrophil migration. In this article we associate this role to a quantitative analysis of the catalytic efficiency of MMP-2 on type IV collagen from commercial human placenta (pretreated with pepsin), as well as on native type IV collagen from EHS, in order to characterize some aspects of the mechanism by which gelatinase A proteolytically processes collagen IV. These two types of collagen IV sources have been selected because they are the most commonly employed in the literature and they are representative of a type IV collagen

partially digested (i.e., that from human placenta) and of a substantially intact collagen IV (i.e., that from murine EHS sarcoma). This parallel approach, never carried out before, allows us to fully characterize for the first time the catalytic parameters of MMP-2 for different subunits of both type IV collagens. It is very important to note that this overall analysis (from the molecular and cellular standpoint) allows us to relate the activity of MMP-2 on collagen IV to its negative role on the migration of neutrophils across a membrane coated with both sources of type IV collagen. This behavior is likely related to the production of lower molecular weight fragments, which impair the migration process (Petitclerc et al. 2000; Roth et al. 2005), and such an investigation represents a first attempt to obtain a deeper insight into a functionally relevant mechanism, in spite of the unavoidable limitations to the extrapolation to in vivo conditions.

Results

Intact MMP-2 and its catalytic domain alone (i.e., lacking the hemopexin-like domain) display a closely similar electrophoretic pattern for the degradation of type IV collagen from human placenta, acting on the same species but with different catalytic parameters (see below). In Figure 1A we report a typical electrophoretic pattern at

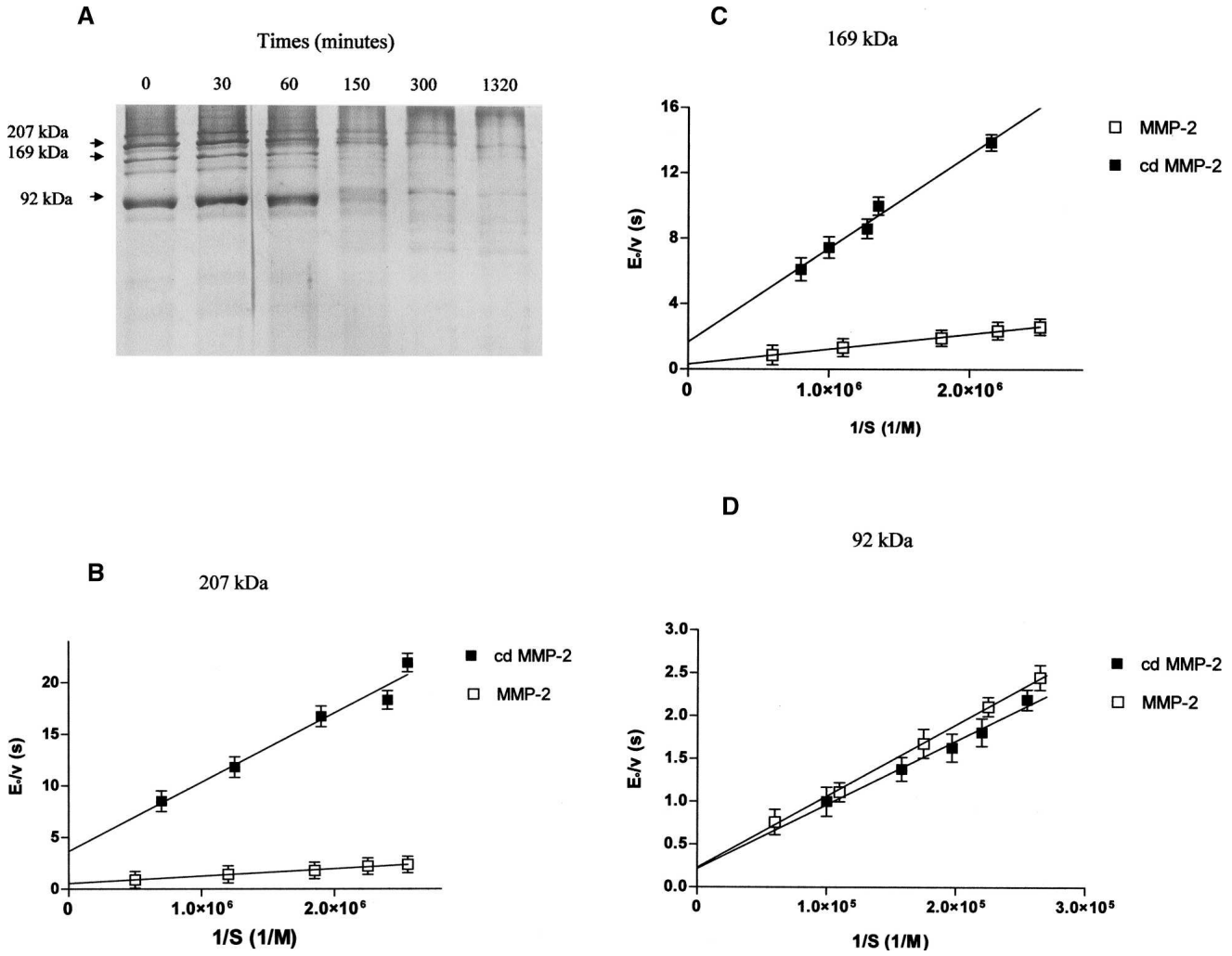

Figure 1. Enzymatic processing of collagen IV from human placenta by MMP-2 and double-reciprocal plots of the enzymatic processing of different chains by whole MMP-2 and by the catalytic domain of MMP-2. (*A*) SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta by the catalytic domain of MMP-2 as a function of time (0, 30, 60, 150, 300, and 1320 min) at 37°C and pH 7.3. Fragments under investigation are indicated by their molecular mass: 207, 169, and 92 kDa. (*B*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 207-kDa fragment. (*C*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 169-kDa fragment. (*D*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 92-kDa fragment. (For further details, see text.)

37°C and at pH 7.3, where it is possible to observe that only three species are progressively cleaved by MMP-2—namely, those characterized by molecular masses of 207, 169, and 92 kDa, respectively—whereas the other species seem not to be affected by the cleavage event.

Considering that the structure of type IV of collagen is very complex, it is not so easy to identify the different species involved in the final structure. For the three species enzymatically processed by MMP-2, the 92-kDa species turns out to be a fragment of the α-2 chain (P. van den Steen and G. Opdenakker, pers. comm.), resulting from the pepsin digestion and likely not involved in the triple helix and partially unwound. On the other hand, the 207- and the 169-kDa species are likely referable to the intact α1-chain and α2-chain, respectively, on the basis of sequence-based mass determination (Hostikka and Tryggvason 1987; Soininen et al. 1987).

It is important to underline that the first cleavage step of the enzymatic processing appears to follow the Michaelis–Menten mechanism for all three species, as from the linearity of the reciprocal plot for the velocity versus substrate concentration (see Fig. 1B–D).

Degradation of collagen type IV from human placenta by whole MMP-2

The first degradation step was characterized by catalytic parameters reported in Table 1, where intact MMP-2 turns out to cleave the two putative α-1 (207 kDa) and α-2 chains (169 kDa) with a similar catalytic efficiency (as represented by k_{cat}/K_m), while the processing of the 92-kDa species takes place with an efficiency lower by 1 order of magnitude. However, if we dissect the overall enzymatic activity, splitting the contribution arising from the substrate recognition (as grossly referable to K_m) from the speed of the rate-limiting step (i.e., k_{cat}), the difference between the mechanisms by which whole MMP-2 processes the three species becomes even more drastic (see Table 1). Thus, MMP-2 shows a very high affinity for the recognition sites on the two putative α-1 and α-2 chains (as from the very low values of K_m; see Table 1), but an ~10-fold lower affinity for the 92-kDa form, leaving almost unchanged the speed of the rate-limiting step for the proteolytic cleavage (see Table 1). As a whole, these data suggest a much more dense structure for the α-1 and α-2 chains (with more specific and structured recognition sites), but a more disordered conformation for the 92-kDa species, which brings about the partial loosening of the recognition site with a decreased affinity for MMP-2 (see Table 1). It must be also stressed that our kinetic analysis has been limited to the first cleavage step of the three components of collagen IV, since this is the only process that follows the Michaelis–Menten approximation and that can be analyzed quantitatively. Fragments of lower molecular weight indeed are formed, but their kinetic building up cannot be followed appropriately with our detection methods.

Degradation of collagen type IV from human placenta by cdMMP-2

In the case of the catalytic domain of MMP-2, the lack of the hemopexin-like domain leads to a much lower value for k_{cat}/K_m, with respect to the whole enzyme for the two putative chains (by at least 1 order of magnitude), whereas no significant effect is observed for the catalytic efficiency toward the 92-kDa species (see Table 1). However, when we focus on values of k_{cat} and K_m some peculiar differences are observed for the various species with respect to the whole enzyme. Thus, for both chains the lower catalytic efficiency appears to be almost only due to a decreased k_{cat} (see Table 1). In the case of the 92-kDa species, the closely similar catalytic efficiency by cdMMP-2 with respect to the whole enzyme indeed reflects similar catalytic parameters (see Table 1).

Degradation of native collagen type IV from murine EHS sarcoma by whole MMP-2

The enzymatic processing of the native collagen type IV by whole MMP-2 is shown in Figure 2A at physiological

Table 1. *Catalytic parameters for the enzymatic processing at 37°C and pH 7.3 of collagen type IV from human placenta by the whole MMP-2 and by the catalytic domain of MMP-2*

	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (M)
207 kDa			
Whole MMP-2	1.4 (±0.2) × 10^6	1.8 (±0.3)	1.2 (±0.2) × 10^{-6}
Catalytic domain MMP-2	1.5 (±0.2) × 10^5	0.3 (±0.1)	2.0 (±0.3) × 10^{-6}
169 kDa			
Whole MMP-2	1.1 (±0.2) × 10^6	3.0 (±0.5)	2.7 (±0.4) × 10^{-6}
Catalytic domain MMP-2	1.7 (±0.3) × 10^5	0.6 (±0.2)	3.5 (±0.6) × 10^{-6}
92 kDa			
Whole MMP-2	1.2 (±0.2) × 10^5	4.6 (±0.7)	3.9 (±0.6) × 10^{-5}
Catalytic domain MMP-2	1.4 (±0.3) × 10^5	4.6 (±0.8)	3.4 (±0.7) × 10^{-5}

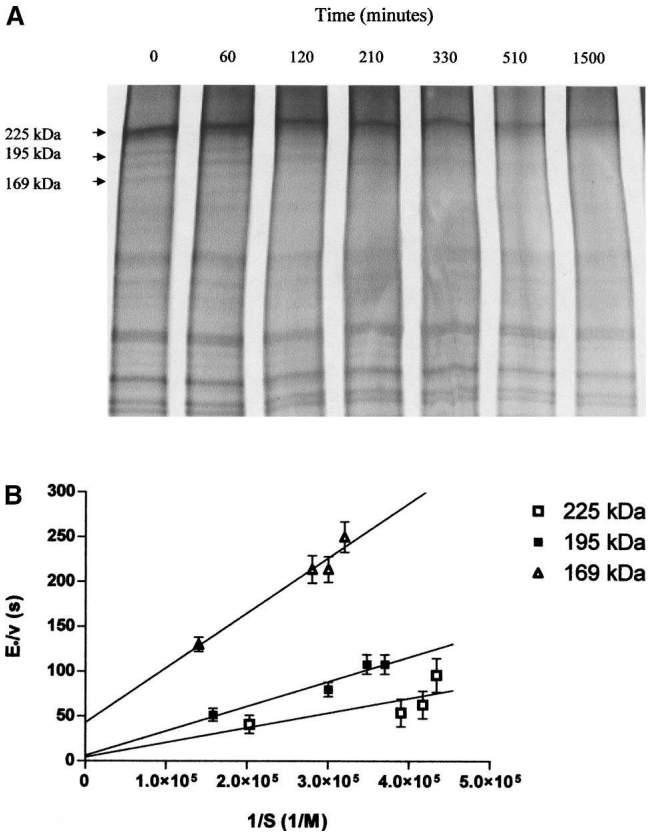

Figure 2. Enzymatic processing of native collagen IV by whole MMP-2 and double-reciprocal plots of different chains. (*A*) SDS-PAGE electrophoresis of the processing of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma by whole MMP-2 as a function of time (0, 60, 120, 210, 330, 510, and 1500 min) at 37°C and pH 7.3. Fragments under investigation are indicated by their molecular mass: 225, 195, and 169 kDa. (*B*) Lineweaver–Burk plots of the processing by whole MMP-2 of different fragments of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma, namely those characterized by 225 kDa (□), 195 kDa (■), and 169 kDa (Δ) molecular mass. Catalytic parameters are reported in Table 2. (For further details, see text.)

temperature and pH as a function of the incubation time with the enzyme. The electrophoretic pattern shows a much larger number of species than in collagen type IV from human placenta (see Fig. 1A), probably due to the higher complexity of the native collagen type IV from the murine EHS sarcoma, as also reported by others (Mackay et al. 1990).

However, also in this case, only three species (namely, those corresponding to a 225-, 195-, and 169-kDa molecular mass, respectively) appear to be cleaved to a significant amount by whole MMP-2, while the other species with a lower molecular weight appear intact over the time range of the kinetic observation.

Also in the case of native collagen type IV, we observe for the first cleavage step a behavior compatible with the Michaelis–Menten mechanism (see Fig. 2B), and the catalytic parameters of the enzymatic processing are reported in Table 2. As is immediately obvious from data in Table 2, for all three species the catalytic cleavage efficiency by whole MMP-2 is drastically lower than that for species from collagen type IV from human placenta (see Fig. 1A; Table 1). In particular, for all three species the substantially reduced enzymatic activity seems mostly attributable to a dramatic decrease of k_{cat} values and only to a lesser extent to an increase for K_m, suggesting that the likely higher structural complexity of this type of collagen IV renders all the network much less susceptible to cleavage, even though the interaction of whole MMP-2 with native collagen type IV remains characterized by a relatively high affinity (see Table 2).

Increasing the temperature to 42°C brings about a partial unfolding of collagen IV (Dölz et al. 1988), and indeed we observe a marked enhancement of the enzymatic activity of MMP-2 (see Fig. 3). It is remarkable that the increase of k_{cat}/K_m (i.e., 1.2×10^4 $M^{-1}sec^{-1}$ at 42°C vs. 5.0×10^3 $M^{-1}sec^{-1}$ at 37°C; see Table 2) greatly exceeds what is expected on the basis of the activation enthalpy for MMP-2 (Fasciglione et al. 2000). It clearly suggests that an important contribution to this enhancement stems from the partial unfolding of the collagen IV network, supporting the idea that the lower catalytic efficiency toward native type IV collagen must be related to the tight assembly of the network.

Role of MMP-2 in the migration of neutrophils across a type IV collagen–coating membrane

Figure 4 shows the extent of migration of neutrophils across a type IV collagen coating in the absence of any agent (column 1), in the presence of only LPS (as a chemoattractant factor) at a final concentration of 0.5 μg/mL (column 2), in the presence of Ilomastat (a wide range inhibitor of MMPs) at a final concentration of 100 μM (column 3), in the presence of both 0.5 μg/mL LPS and of active MMP-2 at different concentrations (columns 4–7), and in the presence of both 0.5 μg/mL LPS and the digestion products of 1 μM collagen IV by 40 nM MMP-2

Table 2. *Catalytic parameters for the enzymatic processing at 37°C and pH 7.3 of native collagen type IV (EHS) by whole MMP-2*

	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (M)
225 kDa			
MMP-2	5.0 (±0.4) × 10^3	0.1 (±0.07)	1.9 (±0.20) × 10^{-4}
195 kDa			
MMP-2	3.3 (±0.2) × 10^3	0.2 (±0.01)	5.0 (±0.4) × 10^{-5}
169 kDa			
MMP-2	1.7 (±0.1) × 10^3	0.02 (±0.001)	1.0 (±0.1) × 10^{-5}

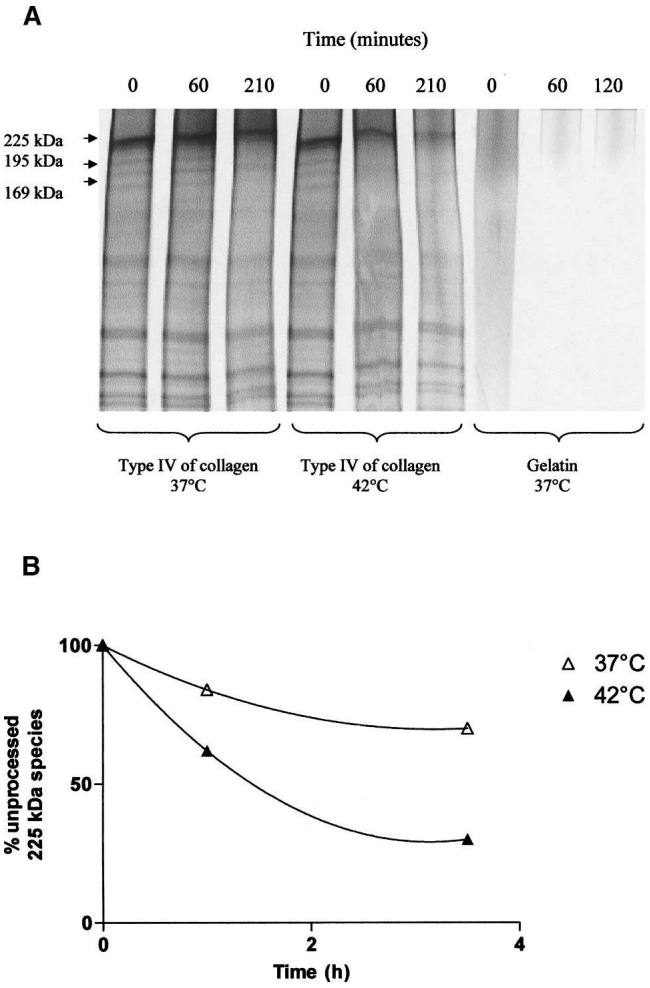

Figure 3. Effect of temperature on the enzymatic degradation of native type IV collagen by intact MMP-2. (*A*) SDS-PAGE electrophoresis of the processing of (1) native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma by whole MMP-2 after 0, 60, or 210 min, respectively, of enzyme incubation at 37°C (lanes *1–3*) and at 42°C (lanes *4–6*) at pH 7.3; and (2) gelatin after 0, 60 and 120 min of enzyme incubation at 37°C (lanes *7–9*). Fragments under investigation are indicated by their molecular mass: 225, 195, and 169 kDa. (*B*) Time dependence of the 225-kDa species degradation at 37°C (Δ) and 42°C (▲) by MMP-2. The ordinate refers to percentage of unprocessed species (%), while the evolution time is expressed in hours (h). The continuous lines are simply drawn through the points.

(column 8). It is important to underline that neutrophil extracts do not contain appreciable amounts of MMP-2 activity, as from the gelatin zymographic analysis (see inset in Fig. 4), but only MMP-8 collagenase activity and MMP-9 gelatinase activity. As a matter of fact, data reported in columns 2 and 3 of Figure 4 indicate that (1) gelatinase activity is absolutely required for the neutrophil migration, as from the marked inhibitory effect exerted by Ilomastat (which is an inhibitor of MMPs; see Bendeck et al. 1996); and (2) Ilomastat, at concentrations used, does not inhibit MMPs production and it does not exert any toxic activity on neutrophils. The complete inhibition of MMPs produced during experiments in the presence of Ilomastat was confirmed by fluorimetric experiments performed using cell culture supernatant (data not shown). It should be underlined that Ilomastat inhibition is not irreversible, since during sample incubation with SDS the inhibitor is removed from MMPs that in turn become able to degrade gelatine during zymography (see in Fig. 4 the gelatine zymography corresponding to column 3). However, even more interesting is the effect observed if we add active MMP-2, since these data seem to support the idea that the presence of increasing amounts of active MMP-2 is depressing the migration of neutrophils across the type IV collagen–coated membrane (which is an oversimplified model for a basement membrane). The mechanism underlying such behavior can be explained by the production of lower molecular weight fragments of collagen IV, consequent to the cleavage by MMP-2, which impairs the migration of neutrophils. This hypothesis finds support in data reported in column 8 of Figure 4, where the LPS-enhanced neutrophil migration (see Fig. 4, column 2) is dramatically inhibited by the addition of digestion products of collagen IV by MMP-2.

Similar results have been obtained in the case of membrane coated with native type IV collagen from EHS murine sarcoma, even though in this case all processes are

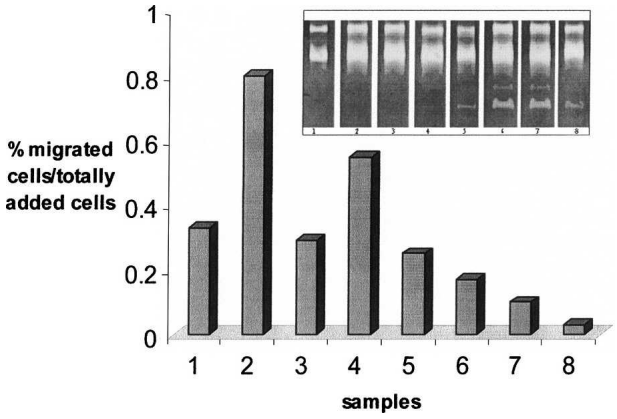

Figure 4. Extent of neutrophil migration across a barrier of collagen IV. Neutrophil chemotaxis through empty Transwell clots prepared with the indicated type IV collagen concentration was measured as described in Materials and Methods. The number of cells migrated into the lower chamber containing control buffer (1); in the presence of 0.5 μg/mL LPS (2); in the presence of 0.5 μg/mL LPS, 1 nM MMP-2, and 100 μM Ilomastat (3); in the presence of 0.5 μg/mL LPS and of active MMP-2 at different concentration (namely 1 nM in 4, 3 nM in 5, 10 nM in 6, and 40 nM in 7); and in the presence of 0.5 μg/mL LPS and the product of collagen IV digestion by 5 nM MMP-2 (8) was determined using an LDH assay as described. The results are presented as a percentage of migrated cells vs. total cells. (*Inset*) Zymography of the neutrophil supernatant. Lanes refer to the analysis of the supernatant for the conditions reported in the corresponding bars of the main figure. Pro-MMP-9 (pMMP9), MMP-9 (MMP9), and MMP-2 (MMP2) are indicated on the *right* side of the inset. (For further details, see text.)

much less evident because of the greater difficulty of neutrophils to migrate across this membrane coating (data not shown).

Role of the fibronectin-like domain of MMP-2 on the processing of collagen type IV from human placenta by whole MMP-2

The SDS-PAGE electrophoretic pattern in Figure 5 shows the role of rCBD during the enzymatic processing of collagen type IV from human placenta by whole MMP-2. At the same incubation time it appears evident as the presence of rCBD (to a final concentration of 50 μM) mostly inhibits the proteolytic activity of MMP-2 on all three species of collagen type IV susceptible to cleavage. It clearly demonstrates that the affinity of rCBD is comparable to that of whole MMP-2, suggesting that the actual binding site for MMP-2 on all three species of collagen type IV from human placenta takes place through the fibronectin-like domain of MMP-2.

Discussion

It is well known that the macromolecular organization and the biomechanical stability of basement membrane is mainly determined by the network of type IV collagen (Kühn 1994); therefore, the mechanism of its proteolytic processing is of the utmost importance for a better comprehension of the tumor cells spreading across the basement membrane (Stetler-Stevenson et al. 1993) as well as of most remodeling and repair processes. Previous studies have shown that both collagen type IV from human placenta and native type IV collagen from EHS can be processed by MMP-2 (Eble et al. 1996), even though this process is sensibly temperature-dependent, being more efficient at 37°C than at lower temperatures (Mackay et al. 1990), probably because of some loosening on the tightness of the network and of the triple-helical assembly upon temperature rise (Dölz et al. 1988). Such a statement is further demonstrated in this article, where the catalytic efficiency (i.e., k_{cat}/K_m) is increased upon raising the temperature from 37°C to 42°C (see Fig. 3), well beyond what is expected on the basis of the activation enthalpy for MMP-2 activity (Fasciglione et al. 2000).

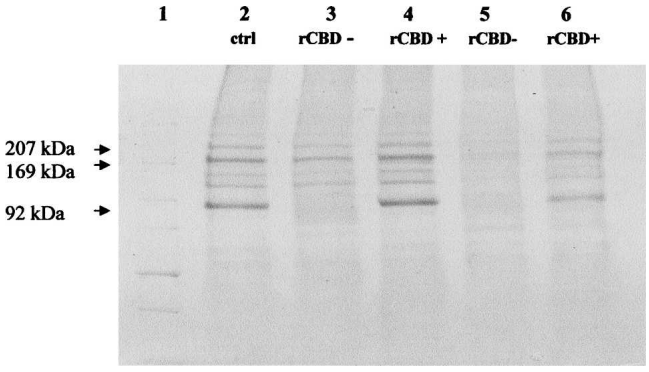

Figure 5. Effect of the presence of collagen binding domain of MMP-2 on the enzymatic processing of collagen IV from human placenta by whole MMP-2. SDS-PAGE electrophoresis of the processing of type IV collagen from human placenta by whole MMP-2 in the absence (lanes *3*,*5*) and in the presence of 50 μM rCBD (lanes *4*,*6*) at two different intervals after exposure to whole MMP-2, namely, 30 min (lanes *3*,*4*) and 45 min (lanes *5*,*6*). Lane *1* corresponds to molecular weight markers, and lane *2* is intact collagen type IV from human placenta. (For further details, see text.)

However, no attempt has been carried out up to now to correlate the integrity of collagen IV (and of other components of the basement membrane) with the capability of cells to migrate across the basement membrane for different physiological and pathological processes, such as inflammatory response, angiogenesis, tumor dissemination, and others. This correlation can only be made by associating the detailed mechanism by which a MMP is cleaving and fragmenting different chains of collagen IV (and of other components), its molecular control and modulation by environmental conditions, and the effect of these events on the cellular migration.

In this article we present clear-cut evidence that gelatinase activity by MMP-9 indeed is important for the migration of neutrophils across a barrier represented by type IV collagen and that inhibition of its activity (upon addition of Ilomastat, a MMP inhibitor; see Bendeck et al. 1996) leads to a great depression of neutrophil migration (see column 3 of Fig. 4). However, this observation does not represent an absolute novelty, since previous papers suggested the importance of this role (Delclaux et al. 1996). On the other hand, a partially unexpected and very novel result is represented by the observation that MMP-2 plays a strong negative action for this process (see Fig. 4). Therefore, also on the basis of this functional cellular evidence, we have deemed it of the utmost importance to carry out a more quantitative kinetic analysis of this process by MMP-2 on collagen type IV from human placenta at 37°C and at pH 7.3, comparing it with the behavior of the catalytic domain of MMP-2, in order to characterize also the role of the hemopexin-like domain in the recognition and cleavage of collagen IV. The experiments have been carried out under nondenaturing conditions (since the temperature of 37°C is not denaturing collagen IV triple-helix, but only loosening it; see Dölz et al. 1988), even though the electrophoretic analysis is undertaken under denaturing conditions, so as to record the degradation of individual polypeptides. We focused our observations on three types of polypeptides, namely, those corresponding to a molecular mass of 207, 169, and 92 kDa in collagen type IV from human placenta; the first two are likely corresponding to the α-1 and α-2 chains, in view of the sequence mass determination (Hostikka and Tryggvason 1987;

Soininen et al. 1987), whereas the third one is probably a peptide of the α-2 chain (P. van den Steen and G. Opdenakker, pers. comm.), resulting from the extraction treatment by pepsin with a possible unwound gelatine-like structure, as suggested by others (Kajimura et al. 2004).

The results obtained (see Fig. 1; Table 1) clearly indicate that the hemopexin-like domain does not play any essential role in the recognition process, whereas it turns out to be fairly important for determining the speed of the rate-limiting first cleavage event. This role is reminiscent of what has been reported for the processing of fibrillar collagen I by MMP-2 (Patterson et al. 2001), where also the role of the hemopexin-like domain seems more important for the cleavage process than for the substrate recognition. Therefore, data reported in Table 1 seem to indicate that a similar role is played by the hemopexin-like domain of MMP-2 in the processing of the triple-helical portion of collagen type IV from human placenta, as represented by the α-1 and α-2 chains (see above).

If we compare the catalytic parameters for the processing of the α-1 and α-2 chains (characterized by 207 kDa and 169 kDa, respectively) by whole MMP-2, a closely similar behavior is observed not only for the catalytic efficiency (as from k_{cat}/K_m; see Table 1) but also (within a factor of two) for the recognition mechanism and the rate-limiting cleavage event (i.e., K_m and k_{cat}; see Table 1), clearly suggesting a very similar proteolytic process by MMP-2 on the two chains of the triple-helical portion. However, an interesting result from Table 1 concerns the much-reduced catalytic efficiency of MMP-2 on the fragment of the α-2 chain, characterized by a molecular mass of 92 kDa, with a likely gelatine-like structural conformation. This feature seems mostly due to a more difficult recognition mechanism for both intact MMP-2 and its catalytic domain, as if the triple-helical arrangement (present in the other fragments, corresponding to the α-1 and α-2 chains) might favor the interaction between MMP-2 and the substrate. This difficulty is only partially compensated (only in the case of the catalytic domain; see Table 1) by a much faster rate-limiting step kinetic constant (possibly related to the much more unwound and unstructured conformation of the peptide), resulting in a similar value of k_{cat} for the intact MMP-2 and its catalytic domain (see Table 1).

As a whole, these results indicate that the hemopexin-like domain of MMP-2 is playing a very minor role in the substrate recognition process (as from the similar K_m values between intact MMP-2 and its catalytic domain; see Table 1). On the other hand, an effect linked to the presence or not of the hemopexin-like domain is observed for the k_{cat} of the two intact chains and it suggests that the hemopexin-like domain might play some role in the partial unwinding of the substrate when it is involved in the triple-helical arrangement. This statement seems supported by the evidence that no effect due to the lack of the hemopexin-like domain is observed for k_{cat} in the case of the 92-kDa fragment, which is likely not assembled in the triple helix and it is probably partially unwound (Kajimura et al. 2004).

We have also carried out an investigation of the proteolytic processing by whole MMP-2 of native collagen type IV from murine EHS sarcoma, which has been reported to be partially cleaved at 37°C by gelatinase A (Mackay et al. 1990; Eble et al. 1996). In this case, we have followed the enzymatic processing of fragments corresponding to 225 kDa, 195 kDa, and 169 kDa, respectively, which appear to be the only ones to be processed to a significant extent by MMP-2. It is very likely that these fragments refer to three types of chains, one of which (i.e., the 169-kDa fragment) has the same molecular mass as the α-2 chain, and the 195-kDa fragment has a molecular mass very close to that reported for the α-1 chain (Hostikka and Tryggvason 1987; Soininen et al. 1987). Catalytic parameters for the proteolytic cleavage of these three fragments by whole MMP-2 are reported in Table 2. It comes out very clear that the enzymatic efficiency is drastically lower than for the collagen type IV from human placenta (see Table 1) by ~3 orders of magnitude, especially if we compare the fragment that seems identical in the two types of collagen IV (i.e., the 169-kDa fragment). A closer comparison for the catalytic parameters of this fragment indicates that the low proteolytic efficiency (i.e., k_{cat}/K_m) is mostly due to a very slow rate constant for the cleavage rate-limiting step (i.e., k_{cat}), which is ~100-fold lower, and also in part to a 10-fold decrease for the substrate affinity (as from the higher K_m; see Tables 1, 2). The catalytic efficiency was even more reduced for the catalytic domain, impairing the possibility to obtain reliable catalytic parameters.

In order to have deeper information on the determinants of the recognition process, we have compared the fragmentation of collagen type IV from human placenta by whole MMP-2 in the presence and in the absence of the rCBD domain, which has been reported to simulate the interaction of the fibronectin-like domain of MMP-2 with both collagen I and collagen IV (Steffensen et al. 2002; Tam et al. 2004; Xu et al. 2005). The results clearly indicate a marked inhibition of the proteolytic processing (see Fig. 4) by rCBD, suggesting that indeed the whole MMP-2 interacts with the two chains of collagen type IV from human placenta through its fibronectin-like domain, as shown for collagen I (Tam et al. 2004).

As a whole, from these data it comes out that the complexity of the structural assembly of collagen type IV indeed represents a limitation for the proteolytic action of MMP-2 (and likely of other MMPs), and this conclusion seems strengthened by the effect of raising the temperature (see Fig. 3), which greatly exceeds the effect

expected on the basis of the activation energy for MMP-2 activity (see Fasciglione et al. 2000), implying an important role deriving also from the partial unfolding of collagen IV (Dölz et al. 1988). This reduced enzymatic activity for the tight assembly of native collagen IV turns out to be referable to a reduced rate for the cleavage itself of the chains (which is dramatically slowed down in the native form from murine EHS sarcoma with respect to that from human placenta; see Tables 1, 2), whereas the recognition process (i.e., K_m) for the formation of the ES complex does not appear particularly compromised by the tight network formed by different chains. This feature, which turns out to be true only for the putative α-1 and α-2 chains of the type IV collagen network, suggests a possible direct role for MMP-2 in the modulation of the endoproteolytic process of the glomerular basement membrane responsible for the pathogenesis of the Alport's syndrome (Hudson et al. 2003). Thus, while from one side the action of MMP-2 on the chains of collagen IV allows the exposure of cryptic sites, which enhance the susceptibility of the enzymatic processing by other proteases (Xu et al. 2001), gelatinase A seems also responsible for a negative effect on neutrophil migration thanks to the production of anti-angiogenic fragmentation products (see Fig. 3). This observation clearly indicates the complexity of effects associated to the activity of MMP-2 (and probably of other MMPs), raising the question of whether a simple inhibitory effect on this activity can be advantageous for the control of pathological effects associated to it.

Materials and methods

Materials

Type IV collagen from human placenta (Sigma Chemical Co.) was dissolved in 0.1 M acetic acid at room temperature to a final concentration of 1 mg/mL. The suspension was centrifuged for 1 h at 10,000*g*, and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford (1976), employing soybean trypsin inhibitor as a reference.

Native type IV collagen from EHS (Sigma Chemical Co.) was dissolved in 0.1 M acetic acid at room temperature to a final concentration of 0.75 mg/mL. The suspension was centrifuged for 1 h at 10,000*g*, and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford (1976), employing soybean trypsin inhibitor as a reference.

MMP-2 recombinant proenzyme was either of commercial origin (R&D System) or a generous gift of Dr. Chris Overall (University of British Columbia); no significant functional difference has been detected between the two preparations after activation. The isolated purified MMP-2 was activated by incubating 0.1 mL of a 0.1 μg/mL progelatinase solution with *p*-aminophenyl mercuric acid (i.e., APMA) (Sigma Chemical Co) at 37°C for 30 min.

The catalytic domain (cdMMP-2) of human recombinant MMP-2 (Biomol International), containing the fibronectin-like domain, was dissolved in a solution of 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.3.

The recombinant fibronectin-like domain (rCBD) of MMP-2 was a generous gift of Dr. Chris Overall (University of British Columbia) and its identity was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Xu et al. 2004) after running a small amount of rCBD on a small column of gelatin Sepharose (Sigma Chemical Co.) (Laemmli 1970).

The quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 was purchased from Calbiochem.

Lipopolysaccharide W *Escherichia coli* LPS was purchased from DIFCO Laboratories. Orthophenilendiamine (OPD) was purchased from Sigma Chemical Co. GM6001 (Ilomastat) was purchased from BioMol.

Activity assay

The active amount of the enzyme (MMP-2) was determined by gelatin zymography and by the fluorimetric assay, as described by others (Knight et al. 1992), following the progressive decrease of hydrolysis of the quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 upon addition of Batimastat (BB-94), a peptidomimetic inhibitor (kindly provided by British Biotech Pharmaceutical), which stoichiometrically inhibits MMPs.

Kinetics of digestion of collagen type IV from human placenta and of native collagen type from murine EHS sarcoma by whole MMP-2 and cdMMP-2

For substrate fragmentation kinetics, activated whole MMP-2 was added to collagen type IV solutions from human placenta or from murine EHS sarcoma, at a final concentration of 10 pM, while the catalytic domain of MMP-2 was added to collagen solutions at a final concentration of 15–20 pM. The kinetics was carried out in 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.3, employing different concentrations of collagen type IV (spanning between 1 μM and 4 μM). Kinetics was carried out keeping the mixtures at 37°C (or at 42°C) and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to −80°C until used.

The aliquots in reducing sample buffer were separated on 4%–15% gradient SDS-PAGE gels, which were stained using 0.5% Coomassie Blue and destained in 10% acetic acid and 40% methanol until substrate bands were clearly visible or employing a Silver Staining solution. The broad spectrum protein markers (Bio Rad) were used as molecular weight standards.

Human neutrophils isolation

Neutrophils were isolated from healthy donors volunteers. Whole blood was diluted (blood–PBS1× ratio 1:4) and stratified on Ficoll separating solution, then centrifugated at 1400 rpm for 30 min at 4°C. Plasma and mononuclear cells were then removed by aspiration, and the red blood cells were lysed by adding 18 mL of ice-cold water for 30 sec; thereafter 2 mL of PBS 10× were added and suspension was centrifuged (1600 rpm for 5 min at 4°C) in order to remove RBC membrane debris and hemoglobin.

The neutrophil-containing pellet was then washed twice at 1600 rpm for 5 min at 4°C and resuspended in RPMI 1640

(EuroClone) medium supplemented with BSA (0.2%) to a final concentration of 4×10^6 cells/mL.

Viability of purified cells was measured by Trypan blue dye exclusion count, and it was found always >95%.

Chemotaxis assay

Human neutrophil chemotaxis assay was measured in Falcon 24-well plates containing Transwell inserts with 3.0 μm pore diameter (Roberto Da Costa et al. 2003). Transwell inserts were coated overnight with type IV collagen from human placenta (Sigma Chemical Co.) or from murine EHS sarcoma previously dissolved in 0.1 M acetic acid at room temperature and diluted with 1× PBS to a final concentration of 10 μg/mL.

Lower chambers contained 500 mL of RPMI 1640 medium supplemented with 0.5% BSA, to which we have added (for different experiments):

LPS to a final concentration of 500 ng/mL as a positive control;
LPS 500 ng/mL and exogenous MMP-2 at different concentrations spanning between 1 and 40 nM;
LPS 500 ng/mL, exogenous MMP-2 to a final concentration of 1 nM and Ilomastat (a powerful inhibitor of MMPs, see Bendeck et al. 1996) to a final concentration of 100 μM;
LPS 500 ng/mL and the digestion product (250 μl) of collagen IV by 5 nM MMP-2. In this experiment, type IV collagen (20 μg/mL) was digested for 6 h with MMP-2 to obtain 50% degradation measured in SDS-PAGE; after degradation, MMP-2 was stoichiometrically inhibited with Ilomastat, a synthetic MMPs inhibitor, as confirmed by fluorimetric assay. This experiment was performed on uncoated Transwell in order to directly measure the effect of type IV collagen digested fragments on MMP-independent LPS-mediated chemiotaxis.

In the upper compartment 250 μL of neutrophils (10^6 cells/well) were added.

Cells were allowed to transmigrate for 4 h at 37°C in a humidified 5% CO_2 incubator (Hanson and Quinn 2002).

To evaluate the transmigrated neutrophils, the Transwell inserts were removed after washing the lower part of the membrane and the bottom solution was collected and centrifuged; cell pellets were then suspended in a solution containing Triton X-100 to a final concentration of 0.5%, OPD 600 μg/mL, and H_2O_2 6 μl/mL in 1× PBS.

Diluted concentrations of neutrophils, from 10^6 cells/well to 6×10^4 cells/well were used to obtain a standard curve.

The oxidation of OPD performed by myeloperoxidase contained in neutrophils was stopped by adding 40 μL of 2 M HCl; the reading was performed at 492 nm in a spectrophotometer (Jasco V-530). Any experiment was performed in triplicate at least with two different blood samples from different donors.

Role of the fibronectin-like domain of MMP-2 (rCBD) on the processing of collagen type IV from human placenta by whole MMP-2

The fibronectin-like rCBD domain of MMP-2 (at a final concentration of 50 μM) was incubated with collagen type IV from human placenta (1 mg/mL) for 30 min at 37°C to allow the interaction to occur. After this time period, MMP-2 was added to the mixture and the solution was put for another 30 min at 37°C. At the same time, three additional control solutions were prepared, namely, (1) collagen type IV alone, (2) collagen type IV incubated for the same time with MMP-2 at 37°C, and (3) a solution of collagen type IV with 50 μM rCBD but without MMP-2.

Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to −80°C until used. The different samples were visualized by SDS-PAGE electrophoresis.

Kinetic analysis

Electrophoretic spots, corresponding to different aliquots at different time intervals, were analyzed by a laser densitometer (LKB 2202 UltraScan) and their intensity was calibrated (in order to obtain concentration values) using standard substrate solutions. For the different species, the substrate disappearance rates were derived at each concentration of type IV collagen employed.

The measurement of the initial velocity was referred to a time period of 1 h for collagen type IV from human placenta and 3 h from native collagen type IV over which <10% of the substrate was degraded during the assay, and in any case the analysis was limited to the time interval over which linearity of the rate was observed. It ensured a steady-state condition for the first cleavage step, and it was a prerequisite for the subsequent analysis step. The verification for the applicability of the Michaelis–Menten approximation to the first cleavage step and the determination of the catalytic parameters were undertaken by a global analysis of experimental data according to both the Lineweaver–Burk equation

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}} \quad (1)$$

and the Eadie–Hofstee equation

$$\frac{v}{E_0} = k_{cat} - \frac{v}{E_0 \cdot [S]} \cdot K_m \quad (2)$$

where E_0 is the total enzyme concentration, v is the actual rate (expressed as **M**/s), K_m is the Michaelis–Menten equilibrium constant (expressed as **M**), k_{cat} is the rate-limiting step kinetic constant (expressed as sec^{-1}), and $[S]$ is the substrate concentration.

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References

Allan, J.A., Docherty, A.J., Barker, P.J., Huskisson, N.S., Reynolds, J.J., and Murphy, G. 1995. Binding of gelatinases A and B to type-I collagen and other matrix components. *Biochem. J.* **309:** 299–306.

Bendeck, M.P., Irvin, C., and Reidy, M.A. 1996. Inhibition of matrix metalloproteinase activity inhibits smooth muscle cell migration but not neointimal thickening after arterial injury. *Circ. Res.* **78:** 38–43.

Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., et al. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol.* **2:** 737–744.

Betsuyaku, T., Shipley, J.M., Liu, Z., and Senior, R.M. 1999. Neutrophil emigration in the lungs, peritoneum and skin does not require gelatinase B. *Am. J. Respir. Cell Mol. Biol.* **20:** 1303–1309.

Borza, D.B., Chedid, M.F., Colon, S., Lager, D.J., Leung, N., and Fervenza, F.C. 2005. Recurrent Goodpasture's disease secondary to a monoclonal IgA1-κ antibody autoreactive with the α1/α2 chains of type IV of collagen. *Am. J. Kidney Dis.* **45:** 397–406.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

Chung, L., Dinakarpandian, D., Yoshida, N., Lauer-Fields, J.L., Fields, G.B., Visse, R., and Nagase, H. 2004. Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. *EMBO J.* **23:** 3020–3030.

Dean, D.D., Martel-Pelletier, J.P., Howell, D.S., and Woessner Jr., J.F. 1989. Evidence of metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J. Clin. Invest.* **84:** 678–685.

Delclaux, C., Delacourt, C., D'Ortho, M.P., Boyer, V., Lafuma, C., and Harf, A. 1996. Role of gelatinase B and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am. J. Respir. Cell Mol. Biol.* **14:** 288–295.

Dölz, R., Engel, J., and Kühn, K. 1988. Folding of collagen IV. *Eur. J. Biochem.* **178:** 357–366.

Eble, J.A., Ries, A., Lichy, A., Mann, K., Stanton, H., Gavrilovic, J., Murphy, G., and Kühn, K. 1996. The recognition sites of the integrins α1β1 and α2β1 within collagen IV are protected against gelatinase A attack in the native protein. *J. Biol. Chem.* **271:** 30964–30970.

Fang, J., Shing, Y., Wiedereschain, D., Yan, L., Butterfield, C., Jackson, G., Harper, J., Tamvakopoulos, G., and Moses, M.A. 2000. Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. *Proc. Natl. Acad. Sci.* **97:** 3884–3889.

Fasciglione, G.F., Marini, S., D'Alessio, S., Politi, V., and Coletta, M. 2000. pH- and temperature dependence of functional modulation in metalloproteinases. A comparison between neutrophil collagenase and gelatinases A and B. *Biophys. J.* **79:** 2138–2149.

Gioia, M., Fasciglione, G.F., Marini, S., D'Alessio, S., De Sanctis, G., Diekmann, O., Pieper, M., Politi, V., Tschesche, H., and Coletta, M. 2002. Modulation of the catalytic activity of neutrophil collagenase MMP-8 on bovine collagen I. Role of the activation cleavage and of the hemopexin-like domain. *J. Biol. Chem.* **277:** 23123–23130.

Hanson, A.J. and Quinn, M.T. 2002. Effect of fibrin sealant composition on human neutrophil chemotaxis. *J. Biomed. Mater. Res.* **61:** 474–481.

Hostikka, S.L.A. and Tryggvason, K. 1987. Extensive structural differences between genes for the α 1 and α 2 chains of type IV collagen despite conservation of coding sequences. *FEBS Lett.* **224:** 297–305.

Hudson, B.G., Tryggvason, K., Sundaramoorthy, M., and Neilson, E.G. 2003. Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N. Engl. J. Med.* **348:** 2543–2556.

Kajimura, D., Takahashi, S., Yoshikawa, K., Hattori, S., Sado, Y., Imamura, Y., and Hayashi, T. 2004. Non-helical type IV collagen polypeptides in human placenta. *Biochem. Biophys. Res. Commun.* **314:** 11–16.

Kalluri, R. 2003. Basement membrane: Structure, assembly and role in tumour angiogenesis. *Nat. Rev. Cancer* **3:** 422–433.

Khoshnoodi, J., Sigmundsson, K., Cartailler, J.-P., Bondar, O., Sundaramoorthy, M., and Hudson, B.G. 2006. Mechanism of chain selection in the assembly of collagen IV. A prominent role for the α2 chain. *J. Biol. Chem.* **281:** 6058–6069.

Knight, C.G., Willenbrock, F., and Murphy, G. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* **296:** 263–266.

Kühn, K. 1994. Basement membrane (type IV) collagen. *Matrix Biol.* **14:** 439–445.

Kühn, K., Wiedemann, H., Timpl, E., Risteli, J., Dieringer, H., and Voss, T. 1981. Macromolecular structure of basement membrane collagens. *FEBS Lett.* **125:** 123–128.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Mackay, A.R., Hartzler, J.L., Pelina, M.D., and Thorgeirsson, U.P. 1990. Studies on the ability of 65-kDa and 92-kDa tumor cell gelatinases to degrade type IV collagen. *J. Biol. Chem.* **265:** 21929–21934.

Marini, S., Fasciglione, G.F., De Sanctis, G., D'Alessio, S., Politi, V., and Coletta, M. 2000. Cleavage of bovine collagen I by neutrophil collagenase MMP-8. Effect of pH on the catalytic properties as compared to synthetic substrates. *J. Biol. Chem.* **275:** 18657–18663.

Ortega, N. and Werb, Z. 2002. New functional roles for non-collagenous domains of basement membrane collagens. *J. Cell Sci.* **115:** 4201–4214.

Overall, C.M. 2002. Molecular determinants of metalloproteinase substrate specificity: Matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol. Biotechnol.* **22:** 51–86.

Patterson, M.L., Atkinson, S.J., Knäuper, V., and Murphy, G. 2001. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett.* **503:** 158–162.

Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., Sarras Jr., M.P., Hudson, B.G., and Brooks, P.C. 2000. New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth *in vivo*. *J. Biol. Chem.* **275:** 8051–8061.

Roberto Da Costa, R.P., Carvalho, H., Agricola, R., Alpoim-Moreira, J., Martins, C., and Ferreira-Dias, G. 2003. Peripheral blood neutrophil function and lymphocytes subpopulation in cycline mares. *Reprod. Domest. Anim.* **38:** 464–469.

Roth, J.M., Akalu, A., Zelmanovich, A., Policarpio, D., Ng, B., MacDonald, S., Formenti, S., Liebes, L., and Brooks, P.C. 2005. Recombinant α2(IV)NC1 domain inhibits tumor cell-extracellular matrix interactions, induces cellular senescence and inhibits tumor growth *in vivo*. *Am. J. Pathol.* **166:** 901–911.

Soininen, R., Haka-Risku, T., Prockop, D.J., and Tryggvason, K. 1987. Complete primary structure of the α 1-chain of human basement membrane (type IV) collagen. *FEBS Lett.* **225:** 188–194.

Somerville, R.P., Oblander, S.A., and Apte, S.S. 2003. Matrix metalloproteinases: Old dogs with new tricks. *Genome Biol.* **4:** 216.

Steffensen, B., Xu, X., Martin, P.A., and Zardeneta, G. 2002. Human fibronectin and MMP-2 collagen binding domains compete for collagen binding sites and modify cellular activation of MMP-2. *Matrix Biol.* **21:** 399–414.

Sternlicht, M.D. and Werb, Z. 2001. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* **17:** 463–516.

Stetler-Stevenson, W.G., Aznavoorian, S., and Liotta, L.A. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.* **9:** 541–573.

Tam, E.M., Moore, T.R., Butler, G.S., and Overall, C.M. 2004. Characterization of the distinct collagen binding, helicase and cleavage mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): The differential roles of the MMP hemopexin c domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities. *J. Biol. Chem.* **279:** 43336–43344.

Tani, K., Ogushi, F., Shimizu, T., and Sone, S. 2001. Protease-induced leukocyte chemotaxis and activation: Roles in host defense and inflammation. *J. Med. Invest.* **48:** 133–141.

Timpl, R., Wiedemann, H., van Delden, V., Furthmayr, H., and Kühn, K. 1981. A network model for the organization of type IV of collagen molecules in basement membranes. *Eur. J. Biochem.* **12:** 203–211.

Xu, J., Rodriguez, D., Petitclerc, E., Kim, J.J., Hangai, M., Yuen, S.M., Davis, G.E., and Brooks, P.C. 2001. Proteolytic exposure of a cryptic site within collagen IV is required for angiogenesis and tumor growth in vivo. *J. Cell Biol.* **154:** 1069–1079.

Xu, X., Wang, Y., Lauer-Fields, J.L., Fields, G.B., and Steffensen, B. 2004. Contributions of the MMP-2 collagen binding domain to gelatin cleavage substrate binding domain is required for hydrolysis of gelatin but not short peptides. *Matrix Biol.* **23:** 171–181.

Xu, X., Chen, Z., Wang, Y., Yamada, Y., and Steffensen, B. 2005. Functional basis for the overlap in ligand interactions and substrate specificities of matrix metalloproteinases-9 and -2. *Biochem. J.* **392:** 127–134.