Matriplysin 1 Influences Colon Carcinoma Cell Migration by Cleavage of the Laminin-5 β3 Chain

Lionel Remy,1,3 Cécile Trespeuch,1,3 Sophie Bachy,2,3 Jean-Yves Scoazec,1,3 and Patricia Rousselle2,3

1Institut National de la Sante et de la Recherche Medicale, U 45/IFR62; 2IFR 128 BioSciences Lyon-Gerland, Institut de Biologie et Chimie des Protéines, Centre National de la Recherche Scientifique UMR 5086; and 3Université Claude Bernard Lyon I, Lyon, France

Abstract
Matriplysin 1 [matrix metalloproteinase 7 (MMP7)] is one of the most important metalloproteinases expressed in human tissues. This enzyme is generally not expressed by normal differentiated epithelial colon cells, but has been shown to be up-regulated in human colon adenomas and adenocarcinomas. Little is known about the role of MMP7 in cell invasion and its involvement in proteolytic processes. By searching the ligands of MMP7 in the colonic carcinoma cells HT29, we identified laminin-5/laminin-332 (LN5) as a specific target for MMP7 enzymatic activity. LN5, composed of α3, β3, and γ2 chains, is an important component of epithelial basement membranes where it induces firm adhesion and hemidesmosome formation. In this study, we show that MMP7 and LN5 are coexpressed in HT29 cells as well as in HT29 xenograft tumors and human colorectal adenocarcinomas. We provide evidence that human LN5 is a ligand for MMP7 and that a specific cleavage occurs in its β3 chain. Videomicroscopic analysis of HT29 cells plated on LN5 substrates reveals that the MMP7-processed LN5 significantly enhances cell motility. Moreover, the delayed migration of HT29 cells obtained after specific inhibition of MMP7 reinforces the hypothesis supporting its involvement in cell migration. Altogether, our results show that MMP7 is likely to play a crucial role in the regulation of carcinoma cell migration by targeting specific proteolytic processing of the LN5 β3 chain. (Cancer Res 2006; 66(23): 11228-37)

Introduction
One of the most critical steps in cancer progression is the escape of malignant cells from the tumor to invade the peritumoral stroma and form distant metastasis. In carcinomas, the basement membrane, a specialized form of extracellular matrix (ECM) that separates the tumor from the stroma and acts as a mechanical barrier against cancer cell invasion, must first be degraded to allow these cells to migrate. This degradation results from more or less extensive proteolysis by various kinds of proteases, namely those of the matrix metalloproteinase (MMP) family, also called matrixins, which, in humans, consist of 24 zinc-dependent endopeptidases. A typical MMP consists of a propeptide of ~80 amino acids, a catalytic domain of ~170 amino acids, a linker peptide called the “hinge region,” and a hemopexin domain of ~200 amino acids (1).

Matriplysin-1 (MMP7), which lacks the hemopexin domain, is one of the most important member of this family and is mostly expressed in the cells of gastrointestinal, breast, and lung carcinomas (2). It has been shown to play important roles in the regulation of several biochemical processes, such as the activation-degradation of various proteins such as pro-α-defensin, Fas-ligand, pro-tumor necrosis factor α, and E-cadherin (1). Otherwise, MMP7 is known for its role on ECM degradation. It has a broad substrate specificity against most ECM components (3) and correlates in colon carcinoma with distant and lymph node metastasis (4, 5).

One of the main components of epithelial basement membranes is the laminin isofrom laminin-5/laminin-332 (LN5; refs. 6, 7). This trimeric molecule formed by the association of the α3, β3, and γ2 chains is known for its role in promoting cell migration, notably those with malignant characteristics, and may act as a ligand for invasive carcinoma cells (8). Neoexpression of LN5 has also been associated with the proliferating activity of carcinoma cells. Moreover, this expression is often located in invasive areas of carcinomas (9). For example, in vivo observations have reported the overexpression of LN5 in budding cells, which then escape from the tumor as part of the metastatic cascade (10). Little is known about the mechanism by which LN5 plays a role in cell invasion but numerous earlier studies have shown that limited proteolytic processing of specific LN5 subunits alter protein structure and thereby modulates its functions, e.g., in cell motility (11).

Specific cleavage of the LN5 γ2 chain by several members of the MMP family has been proposed to favor cell migration (12), among which the cleavage of the γ2 chain by both MMP2 and membrane type-1 (MT1)-MMP (13). These findings were recently challenged by a study showing that the γ2 chain in LN5 of human origin was insensitive to the MMP2 activity (14). For example, in vivo observations have reported the overexpression of LN5 in budding cells, which then escape from the tumor as part of the metastatic cascade (10). Little is known about the mechanism by which LN5 plays a role in cell invasion but numerous earlier studies have shown that limited proteolytic processing of specific LN5 subunits alter protein structure and thereby modulates its functions, e.g., in cell motility (11).

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edges of colonies. We further showed that incubation of MMP7 with human LN5 produced a 90 kDa fragment and we characterized the nature of this fragment as a β3 chain product. We designed HT29 cell migration experiments on MMP7-cleaved LN5 and provided evidence that this cleavage led to enhancement of migratory behavior. Finally, we examined the expression of MMP7 and LN5 in HT29 cell xenografts and in human colonic adenocarcinomas.

Figure 1. Distribution of LN5 and MMP7 in HT29 cells analyzed by confocal microscopy. A to D, cultured HT29 cells were fixed, permeabilized, and processed for immunofluorescence analysis. The distribution of LN5 was studied using the anti-α3β2γ2 pAb L132 (A, red) and that of MMP7 with mAb ID2 (A, green). The distributions of the γ2 (B, red), β3 (C, green), and α3 (D, green) chains of LN5 were studied using the anti-γ2 pAb G2, the anti-β3 mAb 6F12 mAb, and the anti-α3 mAb BM165, respectively. The MMP7 stainings (A-D) were detected with the mAb ID2 (A, B, green) and the pAb 8118 (C, D, red). A to D, nuclei (blue) with the MMP7 stainings. Views of entire HT29 colonies. Note that LN5 is expressed in the colonies, whereas intense MMP7 staining is seen exclusively in cells at the periphery. Superimposed staining shows that MMP7 colocalizes with LN5 (yellow) in cells localized at the edges of colonies in location where cells divide and migrate. Bars, 20 μm.
Materials and Methods

Cell culture and antibodies. The human colon carcinoma cell line HT29, established by Fogh and Trempe (16), was cultured in DMEM supplemented with 2 mmol/L glutamine, 10% FCS, and a cocktail of antibiotics. The characterization of the LN5 antibodies, polyclonal antibody (pAb) L132 (anti-α3β3 chains), monoclonal antibody (mAb) 6F12 (anti-β3 chain), and mAb BM165 (anti-α3 chain) was described elsewhere (6, 17). The pAb PAB-G2 antibody was raised against a peptide corresponding to the amino-terminal extremity of the γ2 chain. The mAb D4B5 (anti-γ2 chain) was from Chemicon (Euromedex, Souffelweyersheim, France) and mAb kalinin B1 (against the carboxyl-terminal extremity of the β3 chain) was from BD Biosciences (Le Pont-de-Clai, France). The rabbit anti-MMP7 pAb 8118, the anti-MMP7 mAb ID2, and the anti-MMP7 mAb 3322 (clone 176-5F12) against the peptide YSLFP, R78-82 were from Chemicon.

Purification of human LN5 and cleavage of LN5 by MMP7. Mature human LN5 was purified as previously described (6, 18). Recombinant expressed human MMP7 and human MMP2 (Chemicon) were used. One microgram of purified LN5 was mixed with 0.5, 0.7, or 1 μg of either activated MMP7 or MMP2 for 3 hours at 37°C in 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 10 mmol/L CaCl2 (final volume of 20 μL). Concentrations of enzymes were 25, 35, and 50 μg/mL, respectively. After incubation, the samples were prepared for electrophoresis in a 7.5% SDS-PAGE gel under reducing conditions. Proteins were transferred to nitrocellulose followed by immunodetection with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Science, Boston, MA).

Analytic methods. For amino-terminal amino acid sequencing, 5 μg LN5 were digested with 2 μg MMP7 as described above, and the resulting protein was subjected to electrophoresis in a 7.5% SDS-PAGE gel under reducing conditions. Proteins were electrotransferred onto polyvinylidene difluoride membrane (Problott, Applied Biosystems, Courtaboeuf, France) for 2 hours at 60 V in 10 mmol/L CAPS, 5% methanol (pH 11), then the band of interest was excised after brief staining with 0.1% Coomassie brilliant blue in 1% acetic acid (Sigma, Saint-Quentin Fallavier, France). Amino acid sequence analysis was done by automated Edman degradation using an Applied Biosystems 473A protein sequencer.

Tumor xenografting. HT29 cells (10⁶), suspended in 100 μL PBS, were grafted onto 10 Wistar newborn rats (IFCA CREDO, Lyon, France) through a ventral s.c. injection. The rats were immunosuppressed by dorsal s.c. injections of 50 μg LN5 from keratinocyte culture medium with 10% FCS in PBS. After washing, sections were incubated 45 minutes in 0.5% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.1% Triton X-100 for 3 minutes, and rinsed with PBS. Cells were incubated with the anti-LN5 antibodies, washed in PBS, and incubated an additional 45 minutes with the anti-MMP7 antibodies (either mAb ID2 or pAb 8118). Cy3- and FITC-conjugated antibodies (Jackson, Beckman Coulter, Paris, France) were applied together for 30 minutes. Staining of the nuclei was done with a 15-minute incubation with a 0.5 μg/mL Hoechst stain solution (Sigma Immunochimicals, Dutscher, Brumath, France). Slides were observed with an Axioscan Zeiss microscope coupled to a CoolSnap FX Camera (Roper Scientific, Effry, France).

HT29 cells were grown on coverslips. After washing, cells were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.1% Triton X-100 for 3 minutes, and rinsed with PBS. Cells were incubated with the anti-LN5 antibodies, washed in PBS, and incubated an additional 45 minutes with the anti-MMP7 antibodies (same as above). Alexa Fluor 488 and Alexa Fluor 546 antibodies were applied for 30 minutes. A 5-minute incubation with Syto 59 red allowed staining of nuclei. Cells were observed by laser scanning confocal microscopy (Zeiss LSM 510).

Protein detection by Western blotting. For LN5 and MMP7 detection, medium samples were prepared for electrophoresis in a 7.5% SDS-PAGE gel under reducing conditions. Proteins were transferred to nitrocellulose, followed by immunodetection with enhanced chemiluminescence (ECL). For MMP7 detection, nitrocellulose membranes were incubated for 1 hour at room temperature with primary antibodies, followed by biotinylated species-specific antibodies, washed, and incubated with peroxidase-streptavidin-biotin complexes (Amersham Pharmacia Biotech, Saclay, France). Detection was assessed by ECL.

Reverse-transcription PCR. Briefly, total RNA was extracted from cultured cells with TRIzol and were reverse-transcribed. cDNAs were amplified by PCR with previously published primers (20). β-Actin was amplified as a reference gene. PCR gels (2% agarose stained with ethidium bromide) were digitized with the Image System (Quantum Appligene, Pleasanton, CA).

Wound-healing assay. Wound-healing assays were done using cultured cells. After confluence, a wound was made with a razor blade and the culture medium was replaced. The cells were allowed to heal for 10 days at 37°C. All experiments were performed in triplicate.

Figure 2. A, SDS-PAGE and Western blot analysis of LN5 from HT29 cell culture medium. Immunoblot analysis of LN5 from HT29 cell culture medium compared with LN5 from keratinocyte culture medium. One-microliter aliquots of conditioned HT29 culture medium (lanes 2, 4, 6, and 8) were precipitated with trichloroacetic acid, and 1 μg of purified LN5 from keratinocyte culture medium (lanes 1, 3, 5, and 7) was analyzed by 7.5% SDS-PAGE under reducing conditions. Immunoblot analysis was performed with the anti-α3 mAb BM165 (lanes 1 and 2), the anti-β3 mAb 6F12 (lanes 3 and 4), the anti-γ2 mAb D4B5 (lanes 5 and 6), and the anti-α3β3γ2 pAb L132 (lanes 7 and 8). Molecular masses of the LN5 subunits and markers are annotated. B, evidence for MMP7 gene expression in HT29 cells. Total HT29 cell RNA was isolated, and MMP7 mRNA was analyzed by RT-PCR. Amplified PCR fragments were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide (lane 1). Molecular markers (lane 2) and amplification of the reporter gene β-actin (lane 3). Representative of three separate experiments performed in triplicate. C, Western blotting of HT29 culture supernatant using the anti-MMP7 pAb 8118. Two forms of MMP7 are secreted into the medium: the proform (28 kDa, lane 2) and the mature form (19 kDa, lane 3). Recombinant MMP7 was used as a positive control (lane 1).
Figure 3. SDS-PAGE and Western blot analysis of MMP7-treated human LN5 in comparison with intact human LN5. A, purified human LN5 (1 μg) was incubated with 0.5 (lanes 4 and 7), 0.7 (lanes 5 and 8), or 1 μg (lanes 6 and 9) of either activated MMP7 (lanes 4, 5, and 6) or MMP2 (lanes 7, 8, and 9) for 3 hours at 37°C as indicated (final volume of 20 μL). Final concentrations of enzymes were 25 μg/mL (lanes 4 and 7), 35 μg/mL (lanes 5 and 8), and 50 μg/mL (lanes 6 and 9). Controls LN5 were incubated with the corresponding amount of digestion buffer in which the enzyme was omitted (lanes 1, 2, and 3). After incubation, the samples were subjected to electrophoresis in a 7.5% SDS-PAGE gel under reducing conditions. Proteins were transferred to nitrocellulose followed by immunodetection with the pAb L132 against the three chains of LN5. *, presence of an additional band around 90 kDa in the lanes corresponding to the MMP7-digested LN5. Left, migration positions of molecular weight markers.

B, immunoblot analysis of MMP7-cleaved LN5 with the mAb against the h3 chain. Purified human LN5 (1 μg) was incubated with 0.5 μg MMP7 under the same conditions as above (25 μg/mL, lane 2) or without (lane 1). The resulting products were analyzed by SDS-PAGE followed by immunoblotting with the anti-h3 chain mAb "antikalinin." Note that the 90 kDa band, detected by the anti-h3 mAb, is present only in the MMP7-digested LN5 (lane 2). Arrow, amino-terminal sequence of this 90 kDa h3 chain fragment, as described below.

C, identification of the processed h3 chain and of its cleavage site by amino-terminal sequence analysis. Amino acid sequence analysis of the 90 kDa band was performed by automated Edman degradation after Coomassie blue staining. The cleavage site is located at position Ala515-Ile516.

MMP7 Cleavage of the β3 Chain in Laminin-5
Figure 4. MMP7-cleaved LN5 provided a better substratum for migration than intact LN5. HT29 cells were plated onto intact LN5 or MMP7-cleaved LN5, and cell migration was recorded by time-lapse videomicroscopy at 5-minute intervals over a period of 18 hours in serum-free medium. A, plots of cells on MMP7-cleaved LN5 and intact LN5 showing 12-hour interval positions for 30 cells in one field oriented so that cell origins are x(0), y(0). Distance migrated in micrometers is indicated on the plots (P < 0.001). B, phase-contrast microscopic images at different time points and representative of the behavior of cells plated on intact LN5 and MMP7-cleaved LN5. Note the presence of cellular protrusions on cells adhering to both intact and MMP7-cleaved LN5. Note that the cells plated on MMP7-cleaved LN5 display cellular extensions oriented toward the direction of movement. Bar, 10 μm.
37°C in the presence of the MMP7 function neutralizing mAb 3322. A dish was fixed every day with 2% paraformaldehyde in PBS. The average extent of wound closure was quantified by multiple measurements of the width of the wound. Twenty-five measurements of two separate trials were made for all conditions. Ki67 labeling was done at each point to verify that the presence of tumor cells beyond the wound line had resulted from migration rather than proliferation processes.

Time-lapse videomicroscopy, Permanox Lab-tek chamber slides (Nalge Nunc International/Dutscher, Dutscher, Brumath, France) were coated overnight with LNs- or MMP7-cleaved LNs at 4°C. After saturation with 1% bovine serum albumin, cultured HT29 cells suspended in DMEM containing 5% CO2 using an Axiosvert 100 M Zeiss microscope equipped with a CoolSnap Fx Camera (Roper Scientific). In each experiment and on each substrate, we analyzed movements of 100 cells using MetaView (Universal Imaging Corporation, Molecular Device, Roper Scientific). The rate of movement (μm/h) was calculated and P values were determined.

Results

LN5 and MMP7 expression in the colon carcinoma HT29 cells. To assess whether LN5 and MMP7 are both expressed in HT29 cells and to compare their localization, we analyzed the distribution of LN5 and MMP7 by confocal microscopy (Fig. 1). Double labeling of LN5 (α3, β3, and γ2 chains) and MMP7 (Fig. 1A) revealed that, whereas LN5 was expressed in the entire colony, an intense expression of MMP7 was seen in cells located in the periphery of the colony. The superposition of both the LN5 and MMP7 staining revealed an obvious area of colocalization in and around the peripheral cells of the colony. To verify whether all LN5 subunits were expressed in a comparable pattern, similar experiments were done using antibodies against the γ2 chain (Fig. 1B), the β3 chain (Fig. 1C), or the α3 chain (Fig. 1D) of LN5. In these experiments, the detection of MMP7 was assessed concomitantly with either monoclonal (Fig. 1A and B) or polyclonal antibodies (Fig. 1C and D). In a manner comparable with the results obtained with staining of the entire LN5, each of the three LN5 subunits was expressed in all cells with increased intensity at the colony periphery. The staining of MMP7 was, as expected, identical to that described in Fig. 1A. Again, the superposition of the staining of each of the three LN5 subunits and that of MMP7 revealed an apparent peripheral ring-shaped area of colocalization identical to that described above. These data show that the three LN5 subunits α3, β3, and γ2 colocalize with MMP7 in these areas. Taken together, these data suggest that because all LN5 subunits were expressed in a similar pattern, the entire heterotrimeric LN5 is expressed in close proximity to MMP7 in specific areas of cultured HT29 cells. Most interestingly, as the cell clusters enlarge, only the peripheral cells express MMP7, suggesting that the cells at the interior of the cluster are no longer synthesizing this antigen. A similar LN5 pattern was previously described in cultured normal human keratinocytes in which only growing and migrating cells at colony peripheries were intensively expressing LN5 (6). As LN5 was expressed by HT29 cells, we next analyzed the molecular composition of the LN5 produced by these cells in comparison with LN5 purified from keratinocyte cultures (Fig. 2A). As previously documented (21), and as shown in our electrophoresis analysis followed by Western blotting detection using antibodies specific for each of the three LN5 chains, the LN5 produced by HT29 cells is identical to the human LN5 isolated from the culture medium of normal keratinocytes (Fig. 2A).

The expression level of MMP7 in HT29 cells was further evaluated at both RNA and protein levels. Total RNA was isolated and analyzed by reverse transcription-PCR (RT-PCR; Fig. 2B). Based on the nature of the primers, the expected size of 393 bp of the amplicons revealed the presence of the transcripts of MMP7 in HT29 cells. Western blot analysis of the MMP7 protein in the conditioned medium of HT29 cells revealed that a large amount of MMP7 was present in its 28 kDa precursor form, whereas the 19 kDa mature form was present in lower amount.

MMP7 induces a specific cleavage in the β3 chain of LNs. As both LN5 and MMP7 were coexpressed in HT29 cells, we analyzed whether LN5 could serve as a substrate for this enzyme and whether a proteolytic effect could be observed (Fig. 3). For this, 1 μg purified human LN5 was incubated with amounts of human MMP7 varying from 0.5 to 1 μg for 3 hours at 37°C. As MMP2 was shown to process the γ2 chain in LN5 of rat origin (13), but seemed to be inefficient with human LN5 (14), human MMP2 was used under the same conditions as MMP7 in our assay. After incubation, the products were analyzed by electrophoresis on 7.5% SDS-PAGE under reducing conditions, followed by Western blot analysis with the anti-LN5 pAb L132 (Fig. 3A). As expected, the pAb L132 detected the three chains of LN5 (α3, 165 kDa; β3, 140 kDa; γ2, 155 and 105 kDa) in the control lanes (lanes 1, 2, and 3), in which LN5 was incubated with the corresponding amounts of digestion buffer but lacking the enzymes. Although these bands were also

Figure 5. Wound-healing assays were performed using cells cultured on plastic Petri dishes. After culture confluence, a wound was made with a razor blade on the cell sheet. Cells were allowed to heal for 10 days at 37°C in the presence of a MMP7 function–neutralizing mAb (mAb 3322; Chemicon). Each day, a dish was fixed and the average extent of the wound closure was quantified by multiple measurements of the width of the wound on micrografs. Twenty-five measurements of two separate trials were made for all conditions. Ki67 labeling was performed at each point to verify that the presence of tumor cells beyond the wound line resulted from a migration process and not only from a proliferation process (not shown). A, curve of the average extent of the wound closure obtained from 25 measurements of the width of the wound space for each condition. B, wound width on day 10 of culture. Anti-MMP7 mAb; MMP7 blocking mAb 3322 (clone 176-5F12).
detected in the samples in which LN5 was preincubated with either MMP7 (lanes 4, 5, and 6) or MMP2 (lanes 7, 8, and 9), an additional band of 90 kDa was detected in the samples corresponding to the MMP7-digested LN5 only (lanes 4, 5, and 6, asterisks). Moreover, this 90 kDa band was equivalent in the three samples corresponding to the three concentrations of MMP7. This band was absent in the LN5 control samples (lanes 1, 2, and 3) and in the samples containing the LN5 preincubated with MMP2 (lanes 7, 8, and 9). To reveal the identity of this LN5-MMP7 proteolytic product, Western blot experiments were conducted with several mAbs directed against the three chains of LN5 (data not shown). A mAb directed against the carboxyl-terminal extremity of the \( \beta3 \) chain recognized specifically the 90 kDa band (Fig. 3B, lane 2) as well as the intact form of the \( \beta3 \) chain (140 kDa). These results suggest that the 90 kDa fragment could arise from the \( \beta3 \) chain cleavage and represent a portion corresponding to its carboxyl-terminal extremity. To verify this hypothesis and further check the identity of the 90 kDa fragment cleavage site, the amino acid sequence analysis of the 90 kDa fragment was done by automated Edman degradation. For this purpose, 5 \( \mu \)g human LN5 was digested with 2 \( \mu \)g MMP7, and the resulting protein was subjected to electrophoretic migration in a 7.5% SDS-PAGE, electrotransferred to an adequate membrane, and stained with Coomassie brilliant blue. The amino-terminal sequence of the 90 kDa band was determined to be IRQCPDRTY, which starts at the Ile\(^{316} \) of the laminin \( \beta3 \) chain and confirmed the identity of the fragment as the carboxyl-terminal portion of the \( \beta3 \) chain.

MMP7-cleaved LN5 enhances HT29 cell motility. As proteolytic cleavages in LN5 subunits are suspected to play a role in the control of cell motility, we assessed the migratory behavior of HT29 cells on MMP7-cleaved LN5 and control LN5 directly by monitoring cell migration for 16 hours with a time-lapse video recorder. Representative tracks of HT29 plated on MMP7-cleaved LN5 and LN5 rates are shown in Fig. 4. Individual cell tracks clearly indicate marked differences in migratory behavior on the two substrates. On LN5, the HT29 cells remained stationary or migrated over short distances, whereas on MMP7-cleaved LN5, the majority of cells were highly motile, displaying both directional and random movements (Fig. 4A). Quantitative analysis of the migration assay showed that the cells plated on MMP7-treated LN5 migrated over longer distances with a mean speed of 28 \( \mu \)m/h, whereas HT29 cells plated on LN5 moved around over shorter and less linear distances with speeds ranging from 0 to 15 \( \mu \)m/h. On representative images, the presence of cellular protrusions in cells adhered to both intact and MMP7-cleaved LN5 should be noted. Although cells plated on LN5 displayed cellular extensions all over the cell periphery, the cells plated on MMP7-cleaved LN5 displayed cellular extensions oriented solely toward the direction of movement. HT29 cells are known to express the three LN5 subunits and have previously been shown to assemble, synthesize, and deposit the resulting heterotrimeric LN5 in their surrounding ECM (21–23). Furthermore, because these cells express MMP7, we verified the level of involvement of MMP7 expression in a wound assay closure. To test the effect of MMP7, we assessed the effect of the anti-MMP7 antibody, which inhibits the activity of MMP7, in an HT29 wound assay. Scrape wounds were introduced into confluent HT29 cultures and then allowed to heal for 10 days in the presence, or absence, of the appropriate antibody. Wounds in cell populations incubated without antibody migrated progressively within the experimental frame to heal over time (Fig. 5B). When the anti-MMP7 antibody was added to the wounded cultures, healing was totally blocked at day 5 (Fig. 5A). This result reinforces the statement that MMP7 plays a major role in HT29 cell migration, and strengthens our observation that MMP7 is intensively expressed by growing and migrating HT29 cells.

Distribution of MMP7 and LN5 in HT29 cell xenografts and in human colon adenocarcinomas. To evaluate whether the LN5 \( \beta3 \) chain cleavage by MMP7 is relevant to \textit{in vivo} tumorigenesis, we analyzed the expression of both antigens in HT29 xenografts. For this purpose, s.c. tumors were generated by xenografting HT29 cells in 10 immunosuppressed newborn rats. The tumors were excised and processed for histologic analysis (Fig. 6A) and immunolabeling of LN5 (Fig. 6C), or its \( \beta3 \) chain (Fig. 6D), and MMP7 (Fig. 6C and D). As shown in Fig. 6A, histologic analysis revealed numerous differentiated tumor cell clusters surrounded by abundant s.c. rat stroma. The analysis of LN5 in similar tissue sections (Fig. 6C and D) revealed an intense and disorganized extracellular staining not resembling basement membranes. The labeling of MMP7 (Fig. 6C and D) was restricted to cells and their close environment. The superimposed staining revealed that some tumor cells expressed both LN5, or its \( \beta3 \) chain, and MMP7. In some cases, the staining appeared punctuated and peripheral (Fig. 6D), suggesting that cleavage could occur in the cell membrane area.

We next analyzed the expression of LN5 and MMP7 in eight human colorectal adenocarcinomas. Five of these revealed coexpression for LN5 and MMP7 and representative images of one case are presented in Fig. 6B to F. Histologic analysis revealed a disorganized tissue in which remaining epithelial structures were embedded (Fig. 6B). The absence of tissue architecture was also revealed by the LN5 or its \( \beta3 \) chain detections (Fig. 6D and F), which appeared intense and chaotic at both the intracellular and extracellular levels. The staining of rare cells with MMP7 was exclusively cell associated. Most interestingly, as revealed by the superimposed staining, all MMP7-positive cells were also LN5-positive cells. Normal tissue

Figure 6. Localization of LN5 and MMP7 in xenografts and in colorectal adenocarcinomas. A, C, D, HT29 cells (10\(^6\)) were s.c. grafted onto 10 newborn rats immunosuppressed by s.c. injections of 50 \( \mu \)l antithymocyte serum at days 0, 2, 7, and 14 after grafting. One tumor of each of the above xenografts was excised and processed for histologic analysis (Fig. 6A) and immunohistochemical analysis (C and D). The expression of LN5 (C, red) and of the \( \beta3 \) chain (D, green) was studied with pAb L132 and mAb 6F12, respectively. Staining of MMP7 was performed either with the mAb ID2 (C, green) or pAb 8118 (D, red). Nuclei (blue) are shown with the red and D, green channel. (A) Control LN5 control staining. (B, E, F, red) Immunohistochemical analysis of LN5 and MMP7 in colorectal adenocarcinomas. Hematoxylin-safran staining (B) and immunohistochemistry of LN5 and MMP7 (E, F) using serial section of a colorectal adenocarcinoma. The expression of LN5 (E, red) and of the \( \beta3 \) chain (F, green) were studied with pAb L132 and mAb 6F12, respectively. Staining of MMP7 was performed either with the mAb ID2 (E, green) or pAb 8118 (F, red). Nuclei (blue) with the LN5 staining. E and F, superimposed staining show that, in some cells, LN5 colocalizes with MMP7. Note that tumor cells strongly expressed LN5 (E) and its \( \beta3 \) chain (F) concomitantly with MMP7. The staining was intense and regular (magnified regions in F). E and F, images are representative of five of the eight colorectal adenocarcinomas analyzed, which revealed LN5/MMP7 colocalization. Bars, 50 \( \mu \)m (A-B) and 10 \( \mu \)m (D-F).
was analyzed in each case and did not show a similar costaining pattern (data not shown).

Discussion

Proteolytic degradation of ECM by metalloproteinases is now considered as an essential event in tumor invasion and metastasis (24). Matrilysin-1 (MMP7), a member of the metalloproteinase family that plays an important role in cancer progression (5, 25), is locally expressed in cells at the base of intestinal crypts but not by normal differentiated colon epithelial cells (26). Interestingly, it is up-regulated in human colon adenomas and mostly in adenocarcinomas. In addition to its role in tumor development (27), the expression of MMP7 correlates significantly with nodal or distant metastasis in colorectal carcinomas (4, 5). This MMP has a very broad spectrum of proteolytic action and is the only one with matriylsin-2 (MMP26) to be synthesized and secreted exclusively by the tumor cells. MMP7 is capable of degrading various ECM components such as fibronectin, collagen IV, laminin-111, elastin, entactin, and cartilage proteoglycan aggregates, as well as other proteins such as casein and insulin. It is known to cleave and activate the proforms of other MMPs such as pro-MMP2 and pro-MMP9 (27, 28). In addition, it has a proteolytic action on adhesion molecules such as E-cadherin, which link epithelial cells to each other (29) and integrins such as β4 (30) that link epithelial cells to LN5, a component of epithelial basement membranes, which has a very determining role in the promotion of cell migration, including that of carcinoma cells.

Previous studies have reported the proteolytic action of some MMPs, such as MMP2 (13) and MT1-MMP (31), on the γ2 chain of LN5, leading to the migration of tumor cells. Our immunohistochemical analysis conducted with the colon carcinoma cells HT29 revealed a coexpression of the three LN5 subunits with MMP7 exclusively, in and around cells, localized at the periphery of growing colonies, suggesting a coexpression of these antigens in dividing and/or migrating cells. Potential interactions between MMP7 and LN5 have not been fully studied, and only a few reports have thus far been reported. Salmela et al. (32) showed that the MMP7 mRNA and LN5 γ2 chain partly colocalized at the invasive border of Barrett’s esophageal adenocarcinoma. A coexpression of MMP7 and of the LN5 γ2 chain was described in colorectal carcinomas (33). Here, we report that MMP7 and the β3 chain in LN5 colocalize in HT29 cell xenografts and in human colorectal adenocarcinomas. Little is known about the role of LN5 in cell invasion; however, a specific cleavage of the γ2 chain by several MMPs has been proposed to play a role in favoring tumor cell migration. Indeed, Pirilä et al. (12) showed a cleavage of the LN5 γ2 chain by MMP3, MMP12, MMP13, and MMP20, but not by MMP7. Based on the fact that MMP7 up-regulates MMP2 and other metalloproteinases, the observations of Salmela et al. (32) and Masaki et al. (33) may be explained by the cleavage of the γ2 chain by metalloproteinases other than MMP7.

A specific and direct interaction between MMP7 and LN5 has never been shown before. Interestingly, Akimoto et al. (34) have often observed the coexpression of β3 and γ2 chains in tumor cells budding from the tumor nest at the invasive front of colorectal carcinomas, which was also observed by Sordat et al. (22), who considers that the γ2β3 expression marks the transition from a stationary to an invading phenotype. The mechanism of the intracellular accumulation of γ2β3 in budding cells remains unknown. Thus, these observations strongly suggest that LN5 γ2 and β3 chains are both important in the invasiveness of cancer cells and encouraged us to investigate a possible relation between MMP7 and LN5. Therefore, by searching for the ligand of MMP7 in colon carcinomas, we found an interesting connection with LN5. In vitro incubation of human purified LN5 with recombinant MMP7 specifically produced a 90 kDa band that did not show in the MMP2-digested human LN5 control. Using an anti-β3 mAb, we showed that this band was a part of the 140 kDa β3 chain. Finally, the amino-terminal sequence determination of the 90 kDa MMP7 generated fragment revealed its identity as a β3 chain portion. This is a novel and important finding where we show that MMP7 specifically cleaves LN5 in only one location without degrading the entire molecule. The cleavage occurred rapidly and required physiologic concentrations of MMP7. We provide the MMP7 cleavage site in the β3 chain as being located at position Ala315. To our knowledge, our study reports the identification of the first MMP7 cleavage site in a laminin and reinforces the hypothesis of a crucial role for MMP7 in biological events, such as cell migration and tumor invasion. Moreover, we provide evidence that the colon carcinoma HT29 cells plated on MMP7-cleaved LN5 migrated over longer distances with a mean speed of 28 μm/h compared with HT29 cells plated on control LN5, which moved around over shorter distances with speeds ranging from 0 to 15 μm/h. Most interestingly, the stable transfection and expression of the β3 chain in prostate carcinoma cells increased their spreading and migration properties (35). The fact that the overexpressed β3 chain was not found to assemble into a LN5 heterotrimer and that the overexpressing cells developed higher tumor growth capacity after injection in severe combined immunodeficient mice (35) suggests that a specific sequence within the free β3 chain may become available and responsible for cellular interactions and signal transduction. This is further strengthened by a study showing that the up-regulation of an NH2 terminus–truncated β3 isoform in Ewing family tumors may favor the observed oncogenic phenotype (36). Together, these results suggest that the MMP7 cleavage of the β3 chain may allow a partial dissociation of the LN5 heterotrimer followed by a possible release/exposure of an active fragment within the β3 subunit. Moreover, as the β3 chain interacts with the NC1 domain of collagen VII (37, 38) to promote cohesion of anchoring complexes, it is also conceivable that its cleavage could dissociate this interaction and favor the disassembly of anchoring structures.

Our findings are in agreement with recent studies showing that the MT1-MMP cleavage of the β3 chain in LN5 also enhanced human prostate carcinoma cell migration (15). A β3 chain–derived 80 kDa fragment was also found in the culture medium of normal keratinocytes (38). Although the released 80 kDa fragments were shown to belong to the carboxyl-terminal portion of the β3 chain (15), the cleavage sites remain unknown, raising the question whether these are different or identical to that of MMP7. Further studies will be dedicated to solve this question.

The role of MMP7 in tumor progression remains unclear. Our data, showing that a MMP7 function-blocking antibody was able to significantly delay the closure of a wound made in confluent HT29 cell layer, strongly suggests that MMP7 plays a role in cell migration. Altogether, our results show that MMP7 is likely to play a crucial role in the regulation of carcinoma cell migration by targeting specific proteolytic processing of the LN5 γ2β3 chain. Such results bring new evidence to the importance of relations between MMP and ECM components in cancer progression and give specific roles to MMP7 and LN5 interactions in the invasive process.
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References


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Lionel Remy, Cécile Trespeuch, Sophie Bachy, et al.


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