Matrix Metalloproteinase-9 Silencing by RNA Interference Triggers the Migratory-adhesive Switch in Ewing’s Sarcoma Cells*

Josiane Sancéau, Sandrine Truchetet, and Brigitte Bauvois‡

From the Unité 365 INSERM, Institut Curie, 75248 Paris cedex 05, France and §USM503 MNHN, UMR 8646 CNRS-MNHN, U565 INSERM, Museum National d’Histoire Naturelle, 75005 Paris, France

Enhanced expression of (pro)matrix metalloproteinase-9 (MMP-9) is associated with human tumor invasion and/or metastasis. COH cells derived from a highly invasive and metastatic Ewing’s sarcoma constitutively express proMMP-9. Transfection of a double stranded RNA that targets the MMP-9 mRNA to COH cells depleted the corresponding mRNA and protein as demonstrated by reverse transcriptase-PCR, enzyme-linked immunosorbent assay, and gelatin zymography. proMMP-9 extinction resulted in the following: (i) decreased spreading on extracellular matrix (fibronectin, laminin, collagen IV)-coated surfaces, (ii) inhibition of migration toward fibronectin, and (iii) induced aggregation, which was specifically disrupted by a function-blocking E-cadherin antibody. MMP-9 knockdown concurrently resulted in increased levels of surface E-cadherin, redistribution at the plasma membrane of β-catenin, and its physical association with E-cadherin. Moreover, induction of E-cadherin-mediated adhesion was associated with RhoA activation and changes in paxillin cytoskeleton. Finally, an inhibitor of gelatinolytic activity of pro-MMP9 did not reduce COH cell migration confirming that the enzymatic property of COH MMP-9 was not required for migration toward fibronectin. Overall, our observations define a novel critical role for proMMP-9 in providing a cellular switch between stationary and migratory cell phases.

Invasion and metastasis of tumor cells is a multiple process that depends on uncontrolled interactions between adjacent cells and/or cells and their extracellular environment (1, 2). These interactions are mediated directly by specific adhesion receptors and indirectly by extracellular proteins that mediate degradation of the extracellular matrix (ECM).¹

Many of the relevant proteases belong to the matrix metalloproteinases (MMPs), which are a family of related zinc-containing proteases that have the ability to degrade ECM (3). One member of the MMP family, MMP-9 (gelatinase B, 92 kDa) is capable of degrading type I, IV, V, VII, and XI collagens and laminin (1, 4). Such proteolytic ability suggests that MMP-9 ultimately regulates cell migration, tumor growth, and angiogenesis (1, 2, 4). MMP-9 is overexpressed in many human solid and hematological malignancies (2, 5–7). MMP-9 promoter activity is induced coincidently with invasion during tumor progression (8, 9). Further, in vitro overexpression of MMP-9 confers a metastatic phenotype (10–12). Conversely, selective suppression of MMP-9 by antisense gene transfer impairs in vitro cell migration of glioma cells (13, 14) and osteoclast-like cells (15). Different families of adhesion receptors are likely to play a role in directing cell motility and include integrins, CAMs, and cadherins (16, 17). Cadherins function by connecting cells to each other by homophilic interactions, in which they bind selectively to identical cadherin types. A cytoplasmic protein termed β-catenin interacts directly with the cadherin cytoplasmic domain and indirectly with the cytoskeleton via α-catenin, which interacts with actin and α-actinin (18). Signal transduction pathways from cadherin to RhoA, Rac1, and Cdc42 have been identified recently (19, 20).

Compiled studies indicate a clear relationship between loss of cadherin expression and increased invasiveness in tumor cells (21–23). Moreover, various carcinoma are shown to exhibit an inverse relationship between MMP-9 and E-cadherin expression (24–31). However, the molecular links between these divergent profiles of expression for MMP-9 and E-cadherin remain unknown.

Ewing’s sarcoma (ES) is a malignant childhood bone and soft tissue tumor known to be highly aggressive and invasive (32). ES is characterized by a specific recurrent balanced chromosomal translocation t(11;22) (q24;q12) (33). Previous studies from our laboratory (34–36) have demonstrated the inhibitory action of interferons on ES cell growth and MMP-9 expression. In the present investigation, we sought to determine whether MMP-9 extinction could affect ES cell behavior. Because recent reports (37–39) have demonstrated the utility of gene silencing by siRNA in mammalian cells, we used a ds-RNA to interfere with the expression of MMP-9 gene. Our findings support the conclusion that MMP-9 constitutes a trigger for the switch between adhesive and migratory states of ES cells through β-catenin/RhoA/paxillin signaling pathways in a manner independent of its enzyme activity.

MATERIALS AND METHODS

Reagents—Goat F(ab)2 fragment anti-mouse and anti-rat fluorescein-conjugated Ig, irrelevant rat isotype IgG2a, mouse (m) IgG1, IgG2a, IgG2b, and monoclonal antibodies (mAbs) specific for β1 (4B4, mlgG1), α5 (HP-2B6, mlgG1), αv (G9, mlgG1), αv (HP2/1, mlgG1), α5 (SAM1, mlgG2a), αv (GoH3, rlG2a), αv (AMF7, mlgG1), ICAM-1 (8H10, mlgG1), E-cadherin (67A4, mlgG1), and CD44 (LAZ21ALL, 36537

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1 The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; CAM, cellular adhesion molecule; ICAM-1, intercellular adhesion molecule 1; VCAM, vascular cell adhesion molecule; NCAM, neural cell adhesion molecule; ES, Ewing’s sarcoma; ds, double stranded; m, mouse; Ab, antibody; mAb, monoclonal antibody; pTyr, phosphotyrosine; FCS, fetal calf serum; TRITC, tetramethylrhodamine isothiocyanate; RT, reverse transcriptase; PBS, phosphate-buffered saline; VEGF, vascular epidermal growth factor; RNAi, RNA-mediated interference; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter; Fn, fibronectin; Ln, Laminin; Col, Collagen IV; BSA, bovine serum albumin; snc, negative control si-RNA.
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CT-3 (Tebu, France). ILK (65.1.9, mIgG2b), PYK2 (clone 11, mIgG1), and FAK (clone 77, mIgG1) Abs were from BD PerkinElmer Life Sciences. M-PERTM-mammalian protein extraction sciences. Renaissance Enhanced Luminol Reagent Plus was from Amersham Biosciences. Human collagen type IV was from BD Bio-nin (clone 2H4A7, mIgG1), and integrin pure goat anti-mouse IgG and TRITC-conjugated affinity pure goat bit polyclonal) Ab was from Cell Signaling Technology. Anti-actin (clone EA-53, mIgG1), talin

RT-PCR—RNA extraction from Ewing cells and subsequent cDNA synthesis were conducted as described previously (34). MMP-9 cDNA (296 bp) was amplified using the sense primer 5'-GGA GAC CATG ACG ACC AAT CTC-3' and the antisense primer 5'-TCC AGG TGA TGT GGT CGT-3' according to published sequences (42). β2-Integrin cDNA was amplified using the sense primer 5'-GTT AAT GGG AAC AAC GAG GTG-3' and the antisense 5'-ACA ATA AAA CGA TAA AAG GCA T-3' according to published sequences (43). VEGF cDNA was amplified using the sense primer 5'-ACA TCT TCT AGG ACG ACC CTG ATG AG-3' and the antisense 5'-GCA TTC ACA TTT GTT GCG T-3' according to published sequences (44). 18S ribosomal RNA was used as an internal control (QuantumRNA™ 18 S Internal Standard; Ambion Inc). The PCR products were visualized by electrophoresis in a 1.6% agarose gel containing 0.2 μg/ml ethidium bromide. The NIH Image 1.44 β11 software was used for the analysis.

Flow Cytometry—Intact cells were immunostained as described previously (40). Clones used were β1 (4B4), α1 (HP-2B6), α2 (G9), α5 (HP2/1), αα (SAM1), αβ (G0H3), ICAM-1 (84H10), VCAM-1 (BBI-V1), NCAM (ERIC-1), CD44 (LA2Z21A1L), α5 (P1B5), and E-cadherin (67A4). Analysis was performed in a FACS flow cytometer analyzer (BD Biosciences). Values are given as percentages of positive cells and relative intensity of fluorescence, which is an indication of the level of expression.

Adhesion Assays—Twenty-four-well flat bottomed microtiter plates (Nunc) were coated with fibronectin (Fn) or Laminin (Lm) or Collagen IV (Col) with PBS as a blank. Cells (4 x 10^5 cells per well) were added to each substrate-coated well at 37 °C for 1 h. After washing, adherent cells were trypsinized and quantitated using a cell Coulter Counter channelizer 256 (the diameters of living migrated cells ranged from 7 to 14 μm). Results from triplicates (mean ± S.D.) were expressed as relative cell adhesion (number of attached cells/total number of cells x 100). Specific adhesion to substrates was determined by subtracting the nonspecific attachment of cells to BSA-coated surfaces from cell attachment to coated surfaces. Morphology of attached cells was assessed by staining with the Hemacolor kit from Merck and subsequent light microscope examination.

Immunofluorescence Staining and Confocal Microscopy—Cells were
washed twice with cold PBS and once with PBS containing 1% BSA before low speed cytocentrifugation to polylysine-treated slides (O. Köhler GmbH & Co., Freiburg, Germany). Cells were fixed in ice-cold methanol for 7 min and then washed twice with PBS. After 60 min of blocking in PBS containing 3% BSA at room temperature, cells were incubated in the same buffer with a polyclonal E-cadherin Ab (H108; 5 μg/ml), a monoclonal β-catenin Ab (clone 2A4H7; 1 μg/ml), or an isotype mlgG1 (1 μg/ml) for overnight at 4 °C. The preparations were washed three times for 15 min with PBS/0.05% Tween 20 and one wash with PBS, followed by an incubation for 90 min at room temperature with a fluorescein isothiocyanate-conjugated anti-mouse Ab or a TRITC-conjugated anti-rabbit Abboth diluted 1/1000 in PBS/BSA. Preparations were then washed three times with PBS/0.05% Tween 20 and once in PBS. Slides were mounted in Vectashield (47). Labeled samples were further analyzed by confocal microscopy on an Nikon microscope equipped with the Bio-Rad Laser-Sharp MRC-1024 confocal laser scanning software, using a Nikon Fluor ×100 oil-immersion objective and the 488- and 568-nm excitation wavelengths of the laser (47). The fluorescence of β-catenin and E-cadherin was analyzed concurrently in the same cell samples.

RESULTS

Efficient Extinction of MMP-9 Expression in COH Cells by RNAi Strategy—We used an RNAi method to target MMP-9 in the ES COH cell line, which constitutively expresses high levels of MMP-9. The constructs we designed encoded an RNA that targets the MMP-9 mRNA (Fig. 1A). The 21-nucleotide-long target sequence had no homology with other members of the MMP family. The ds-RNA and Silencer<sup>TM</sup> negative control si-RNA (snc) were each tested for their ability to suppress MMP-9 specifically. We first assessed whether RNAi was dose- and time-dependent. COH cells were transfected with 1–20 nM of the ds-RNA for up 3 days. By RT-PCR analysis, a MMP-9-dependent ds-RNA-mediated inhibition was observed in a dose- and time-dependent manner (Fig. 1B). The snc-RNA (20 nM) was incapable of inhibiting MMP-9 gene expression (Fig. 1B) even when transfected with a 10-fold-excess of the saturating ds-RNA concentration (20 nM) (data not shown). The time-course assay performed with 20 nM ds-RNA-transfected COH cells showed that induced MMP-9 silencing could be maintained for at least 3 days (corresponding to seven generation times) (Fig. 1C). Importantly, snc-RNA and ds-RNA transfection had no effect on the mRNA levels of two unrelated genes i.e. VEGF and integrin β (Fig. 1B).

The RT-PCR results were confirmed by enzyme-linked immunoadsorbent assay. COH snc-RNA-transfected cells cultured up to 3 days spontaneously released high amounts of MMP-9 into the culture conditioned medium whereas ds-RNA-transfected cells showed a marked time- and dose-dependent inhibition in MMP-9 protein levels (Fig. 1D, left panel). In accordance with RT-PCR data, levels of released VEGF by COH cells were not affected by ds-RNA transfection (Fig. 1D, right panel). Zymography analysis of the conditioned media from COH cells before and after snc-RNA treatment indicated the presence of a gelatinase activity at 92 kDa (Fig. 1E, compare lanes 4 and 6) consistent with the pattern of recombinant proMMP-9 (Fig. 1E, lane 1). In contrast, MMP-9 was barely evident in conditioned medium from ds-RNA cells (Fig. 1E, compare lane 8 with lanes 4 and 6) corroborating the undetect-
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We further examined the impact of MMP-9 silencing on adhesion of COH cells to extracellular matrix components. Parental COH cells were shown to express intermediate to high levels of (α1–α5) β1 integrins as determined by FACS analysis (Table I), and adhere to Fn, Lm, and Col but not to BSA (Fig. 4D). No detectable level of β3 and β5 subunits was observed (data not shown). We employed function-blocking Abs to identify the cell adhesion receptors involved in cell attachment. These experiments indicated that cell adhesion was mediated via α1β1 for fibronectin, αβ1 and CD44 for laminin and α1β1 and α5β1 for Col, respectively (Fig. 4D). Day 2 transfection with snc-RNA or ds-RNA did not modify the rates of binding of COH cells to Fn, Lm, and Col (Fig. 4E). However, a significant difference in morphology of attached cells was observed. Adherence of snc-RNA cells, like parental cells (data not shown), was accompanied by an apparent spreading of many cells on each substrate-coated well (Fig. 4F, a, c, and e) whereas adherent ds-RNA transfected COH cells retained a rounded morphology (Fig. 4F, b, d, and f). These morphological changes were, however, independent of the levels of cell surface expression of snc-RNA and ds-RNA cell adhesion molecules involved in COH cell adhesion (Table I).

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Migration toward Fn was significantly reduced for day 2 ds-RNA COH cells (50% inhibition) compared with day 2 snc-RNA cells or parental cells (Fig. 4A). Visualization of migrated cells indicated cell integrity for both snc-RNA and ds-RNA cells (Fig. 4C). A kinetic course similar to that of cell–cell contact formation was observed for inhibition of migration (maximal inhibition at day 2–3; data not shown for day 3). Altogether, these experiments support a positive role for proMMP-9 in reduced COH cell migration secondary to increased cell–cell adhesion.

MMP-9 Silencing Inhibits COH Cell Spreading to Fibronectin-, Laminin-, and Collagen IV-coated Surfaces—We further examined the impact of MMP-9 silencing on adhesion of COH cells to extracellular matrix components. Parental COH cells were shown to express intermediate to high levels of (α1–α5) β1 integrins as determined by FACS analysis (Table I), and adhere to Fn, Lm, and Col but not to BSA (Fig. 4D). No detectable level of β3 and β5 subunits was observed (data not shown). We employed function-blocking Abs to identify the cell adhesion receptors involved in cell attachment. These experiments indicated that cell adhesion was mediated via α1β1 for fibronectin, αβ1 and CD44 for laminin and α1β1 and α5β1 for Col, respectively (Fig. 4D). Day 2 transfection with snc-RNA or ds-RNA did not modify the rates of binding of COH cells to Fn, Lm, and Col (Fig. 4E). However, a significant difference in morphology of attached cells was observed. Adherence of snc-RNA cells, like parental cells (data not shown), was accompanied by an apparent spreading of many cells on each substrate-coated well (Fig. 4F, a, c, and e) whereas adherent ds-RNA transfected COH cells retained a rounded morphology (Fig. 4F, b, d, and f). These morphological changes were, however, independent of the levels of cell surface expression of snc-RNA and ds-RNA cell adhesion molecules involved in COH cell adhesion (Table I).

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MMP-9 Silencing Impairs COH Cell Migration—In parallel, to analyze the impact of MMP-9 silencing on migration of COH cells, we used transmigration assays with Transwells (Costar) where the lower chamber was uncoated or coated with ECM proteins Fn, Lm, and Col. As shown in Fig. 4A, a significant percentage of parental COH cells spontaneously transmigrated, and Fn significantly increased their migration. The two other ECM proteins Col and Lm did not promote as efficient migration as Fn (data not shown). Importantly, COH cell migration was not inhibited by the MMP-9 inhibitor 2(R)-1-(4-biphenylsulfonyl)laminol-3-phenylpropionic acid (Fig. 4A). As expected, gelatinolytic activity of recombinant proMMP-9, as well as that present in the conditioned medium of COH cells, was down-regulated by this inhibitor (Fig. 4B) ascertaining its potency.

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**Expression of cell-cell and cell-ECM adhesion molecules on COH cells before and after MMP-9 silencing**

Parental COH cells (control) and snc-RNA and ds-RNA COH cells cultured under similar conditions for 2 days were then assayed for expression of I-CAM-1, V-CAM, E-cadherin, N-CAM, CD44, α1-α5, and β1 integrins by immunofluorescence in a BD Biosciences flow cytometer, as described under “Materials and Methods.” Percentages of positive cells are means ± S.D. of three to five separate experiments. The relative intensity of fluorescence (number in parentheses) is an indication of the level of expression.

|                  | Parental | snc-RNA | ds-RNA |
|------------------|----------|---------|--------|
| I-CAM-1          | <5       | <5      | <5     |
| V-CAM            | 22 ± 14 (4) | 15 ± 8 (4) | 13 ± 6 (4) |
| E-cadherin       | 23 ± 10 (135) | 31 ± 19 (138) | 59 ± 26 (135) |
| N-CAM            | 37 ± 10 (18) | 40 ± 7 (18) | 44 ± 12 (18) |
| α1               | 59 (16) | 64 ± 24 (19) | 69 ± 28 (17) |
| α2               | 77 (35) | 61 ± 17 (13) | 58 ± 18 (11) |
| α3               | 64 (22) | 79 ± 3 (10) | 60 ± 3 (10) |
| α4               | 33 (6) | 27 ± 12 (5) | 36 ± 15 (5) |
| α5               | 91 ± 3 (49) | 91 ± 6 (47) | 93 ± 5 (47) |
| α6               | 20 (5) | 18 ± 15 (5) | 16 ± 5 (5) |
| β1               | >95 (116) | >95 (107) | >95 (110) |
| CD44             | 66 ± 12 (24) | 54 ± 27 (19) | 60 ± 23 (21) |

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**Effect of an MMP-9 inhibitor on cell-cell adhesion molecules on COH cells in absence or presence of an MMP-9 inhibitor**

COH parental cells were cultured in absence or in presence of the MMP-9 inhibitor 2(R)-2-[[4-biphenylsulfonyl]amino]-3-phenyl propionic acid (25 μM) or its Me2SO control for 1 day. Then, cells were assayed for expression of I-CAM-1, V-CAM, E-cadherin, and N-CAM by immunofluorescence.

**Table II**

| Phenotype | Percentages of positive cells |
|-----------|------------------------------|
|           | Relative intensity of fluorescence |
|-----------|--------------------------------|
|           | Parental | + MeSO | + Inhibitor |
| E-cadherin| 36 (138) | 20 (138) | 30 (135) |
| N-CAM     | 50 (20)  | 54 (20)  | 52 (20)   |
| I-CAM-1   | <5       | <5      | <5        |
| V-CAM     | <10      | <10     | <10       |

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**Effects of MMP-9 Silencing on Paxillin Phosphorylation and RhoA Activity**—Because adhesive interactions (between adjacent cells or cells and ECM) are critically influenced by the organization of the cytoskeleton, we further explored the effects of MMP-9 silencing on various cytoskeletal related proteins. COH snc-RNA cells, like parental cells, express different proteins of the cytoskeleton including actin, talin, vinculin, and paxillin (Fig. 5A). The total steady state protein levels of actin, vinculin, and talin were not modified upon 3 days of ds-RNA treatment (Fig. 5A). With regard to α-actinin, a time-dependent increased signal in the Western blot was unexpectedly observed under both snc- and ds-RNA conditions (Fig. 5A).

Paxillin migrates in SDS-PAGE as two close bands between 66 and 70 kDa (49) and in lower molecular mass forms (44–46 kDa) (50, 51). COH snc-RNA cells exhibited high molecular mass forms of paxillin around 66 kDa (Fig. 5A). Total steady state protein levels of 66-kDa paxillin increased with time in COH cells transfected with ds-RNA compared with the snc-RNA cells, and this was associated with the clear appearance of 44–46-kDa paxillin forms on day 2 (Fig. 5A). The 66-kDa form of paxillin contains two critical tyrosine phosphorylation sites at Tyr31 and Tyr118 (52). Immunoblot analysis with an Ab against phosphotyrosine residue 118 (pTyr118-paxillin) showed a significant decrease (3-fold) in the phosphorylation of paxillin. Activation of the kinase activities of FAK and PYK2, implicated previously (52) in the phosphorylation of paxillin. Activation of the kinase activities of FAK and PYK2 leads first to autophospho-
FIG. 4. Impact of MMP-9 silencing on COH cell adhesion to Fn-, Lm-, and Col-coated surfaces and COH migration toward Fn. A, COH parental cells were cultured in the absence or presence of the MMP-9 inhibitor 2(R)-2-[4-biphenylsulfonyl)amino]-3-phenylpropionic acid (25 μM) or its Me₂SO (DMSO) control for 1 day prior to being plated (1 × 10⁵/chamber) in the Transwells in which the lower side was uncoated (−) or coated with Fn (+) overnight at 37 °C in serum-free RPMI medium containing 0.1% BSA. snc-RNA (20 nM) and ds-RNA (20 nM) COH cells were cultured for 2 days prior to being plated (1 × 10⁵/chamber) in the Transwells. The data represent the mean number of cells that migrated to the lower chamber in triplicate samples for one experiment, representative of three separate experiments for parental, snc-RNA, and ds-RNA cells. B, gelatinolytic activities of recombinant pro-MMP-9 (0.5, 1, and 10 ng) and in the culture media of COH parental cells were analyzed using zymography performed in the absence (minus) or in the presence of MMP-9 inhibitor (10⁻⁴ M). C, light microscopy of adherent snc-RNA and ds-RNA cells that migrated in the lower chamber. Magnification, ×160. D, COH parental cells were added to Fn-, Lm-, Col-, and BSA-coated wells, and the plates were incubated at 37 °C for 60 min. Attached living cells were quantitated as described under “Materials and Methods.” 100% relative adherence represented adherence of untreated COH cells. Data represent the mean ± S.D. of four separate experiments performed in
FIG. 5. Expression of cytoskeletal-related proteins in COH cells before and after MMP-9 silencing. A, equivalent amounts of cell extracts (40 μg) obtained from COH cells treated with snc-RNA or ds-RNA (20 nM) for 1–3 days were loaded on 8% ProSieve® gels in reducing conditions. Immunoblotting and protein detection with appropriate Abs to paxillin (PXC-10), pTyr118-paxillin and RhoA (mIgG1, cytoskeleton), vinculin (hVIN-1), talin (8D4), α-actinin (EA53), and actin (AC-15) were performed as described under “Materials and Methods.” The level of GTP-bound RhoA was measured in the total extracts (500 μg of protein) by immunoprecipitation with beads coupled to the Rho-binding domain of the Rho effector Rhotekin. Exposure times were <15 s for total RhoA and actin, 5 min for talin, vinculin, and α-actinin, and >30 min for GTP-RhoA. B, equivalent amounts of total extracts (40 μg) obtained from COH parental cells or treated with snc-RNA or ds-RNA (20 nM) for 2 days were loaded on 8% ProSieve® and immuno blotted using E-cadherin (clone 34) and NCAM Abs. C, total extracts were prepared to subject to immunoprecipitation (IP) with anti-E-cadherin (clone 34) or β-catenin (clone 14) Abs, followed by immunoblotting with anti-E-cadherin (H-108) and β-catenin (clone 14) Abs. Nuclear (EN) and cytoplasmic (EC) extracts (D) or cytosolic and membranous pools obtained from COH parental cells or treated with snc-RNA or ds-RNA (20 nM) for 2 days (E) were fractioned as described under “Materials and Methods.” After solubilization, equivalent amounts of proteins were loaded on 8% ProSieve® gels and immunoblotted using β-catenin (clone 14) and integrin β1 (clone DE9) Abs.
Together, these findings indicate that MMP-9 silencing favored motile properties of COH cells. ProMMP-9 silencing by RNAi significantly decreases spreading of COH cells on Fn, Lm, and Col and strongly reduces their migration toward Fn. Although other authors showed recently (65) that proMMP-9 binding to gelatin or collagen type IV resulted in an enzymatic activation of proMMP-9 without loss of its NH2-terminal propeptide, the scenario is unlikely in our system, because cell migration is not inhibited by an MMP-9 inhibitor. Therefore, our results strongly suggest that MMP-9-silenced transfectants are less motile than their parental cells because of the loss of proMMP-9 protein devoid of enzymatic activity.

COH cells cultured as monolayers express a fibroblastic morphology characteristic of metastatic cells. Our data demonstrate that, concomitantly to the loss of migration, proMMP-9 silencing in COH cells favored a morphology different from that of the parental cells and more specifically cell aggregates. This was because of up-regulation of E-cadherin levels at the cell surface, because an Ab against E-cadherin efficiently blocked cell-cell adhesion. Malignant transformation is often characterized by disruption of cell-cell adhesion, which can be achieved by down-regulating the expression of cadherin and/or β-catenin (22). Moreover, clustering of cell surface cadherins is associated with the recruitment of β-catenin to the cadherin-associated complex (17). Although the levels of total E-cadherin and β-catenin remained unchanged upon ds-RNA treatment, we demonstrated an enhanced physical association between E-cadherin/β-catenin proteins by coimmunoprecipitation. As expected, β-catenin was detected at the plasma membrane at cell-cell junctions of MMP-9-silenced cells. Together, our data indicate that surface stabilization of E-cadherin/β-catenin complex in MMP-9-silenced COH cells leads to the induction of E-cadherin-mediated cell-cell adhesion, which, in turn, diminishes the motility. Our data are in agreement with the literature documenting the involvement of the cadherin/β-catenin pathway in tumorigenesis (23, 66). Disruption of adhesion systems can contribute to tumor development (22, 23) whereas increased levels of cadherins reduce tumorigenic properties (67).

In addition, biological and synthetic inhibitors of MMP activities have been suggested to promote cell-cell adhesion by at least preventing the cadherin ectodomain cleavage, thus stabilizing cadherin-mediated cell-cell contacts (68, 69). MMP-3 can cleave E-cadherin (67) whereas MMP-9 cleaves ICAM-1 (70). Similarly, an MMP activity seems to be responsible for the shedding of VCAM-1 (71). Here, we demonstrate that an inhibitor of MMP-9 affected neither COH cell adhesion and migration nor the profiles of surface expression of ICAM-1, VCAM, NCAM, and E-cadherin. These results therefore provide evidence of the following. (i) The integrities of CAMs and E-cadherin on COH cells are independent of proMMP-9. (ii) The impact of proMMP-9 silencing on E-cadherin-mediated cell-cell adhesion is not related to decreased proteolysis of surface E-cadherin.

In MMP-9-silenced COH cells, alterations of spreading, migration, and E-cadherin-mediated cell-cell adhesion were indicative of changes in the functional association of cell adhesion molecules (integrins and E-cadherin) with the cytoskeletal network. The proteins of the Rho subfamily Cdc42, Rac, and Rho are signaling molecules primarily involved in remodeling the actin cytoskeleton (17, 20). In particular, Rho proteins are required for cadherin-mediated cell-cell adhesion and consequently influence many aspects of cell shape and movement (17, 19, 20). More specifically, β-catenin, depending on its relative amounts in the cytoplasm/nucleus or associated with cadherins at the plasma membrane, can influence cells toward migration or cell-cell adhesion by regulating the function of Rho proteins (20). Previous studies (20, 72) reported that RhoA activation increases the accumulation of cadherin/β-catenin complexes at cell-cell contact in keratinocytes and epithelial cells. We similarly found a correlation among RhoA activation, preferential accumulation of cadherin/β-catenin complexes at cell-cell junctions, and E-cadherin-mediated adhesion of MMP-9-silenced COH cells.

Among cytoskeletal proteins that play a critical role in cell spreading and migration, paxillin localizes primarily to sites of cell adhesion to the ECM called focal adhesions (51, 52). Paxillin is a 66-kDa cytoplasmic protein that binds to several...
proMMP-9 silencing in the switch between the stationary and migratory state in COH ES cells. The model highlights a role for proMMP-9 in the switch between the stationary and migratory phases of COH cells (for details see “Discussion”).

Cell shape changes result in modulation of various phenotypic changes and alteration of MMP-9 expression (55, 74). Inversely, our data indicate that proMMP-9 extinction induces cell shape changes (particularly those resulting from paxillin/RhoA/β-catenin pathways) that alter the adhesive and migratory properties of COH cells. Therefore, an unanswered question regarding the critical role of proMMP-9 for E-cadherin-mediated cell-cell adhesion and migration is how can proMMP-9 influence motile properties of COH cells? A number of recent papers have demonstrated that the hyaluronan receptor CD44 can serve as a docking molecule to retain secreted MMP-9 (under both pro and active forms) at the cell surface (75, 76). CD44 is involved in cell adhesion and trafficking, as well as in tumor growth, invasion, and metastasis (76–78). The cytoplasmic domain of CD44 interacts with several cytoskeletal proteins including several guanine nucleotide exchange factors leading to activation of Rac1 and, under certain conditions, results in increased Rho activation (76–78). The interaction of CD44 with these guanine nucleotide exchange factors leads to activation of Rac1, which, under certain conditions, results in increased Rho activa-
tion (76). CD44 is involved in cell adhesion and trafficking, as well as in tumor growth, invasion, and metastasis (76, 77). CD44 is involved in cell adhesion and trafficking, as well as in tumor growth, invasion, and metastasis (76, 77). CD44 is involved in cell adhesion and trafficking, as well as in tumor growth, invasion, and metastasis (76, 77).

whether MMP-9 silencing results in the withdraw of nuclear β-catenin via the coordinating signals transduced through β-catenin/TGF-4 pathways has to be considered.

FIG. 7. A model proposed to explain the impact of MMP-9 silencing on induced E-cadherin-mediated adhesion and concomitant loss of migration of COH ES cells. The model highlights a role for proMMP-9 in the switch between the stationary and migratory phases of COH cells (for details see “Discussion”).
Matrix Metalloproteinase-9 Silencing by RNA Interference Triggers the Migratory-adhesive Switch in Ewing’s Sarcoma Cells
Josiane Sancéau, Sandrine Truchet and Brigitte Bauvois

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