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Stromelysin-2 (Matrix Metalloproteinase 10) Is Inducible in Lymphoma Cells and Accelerates the Growth of Lymphoid Tumors In Vivo[1]

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Matrix metalloproteinase (MMP) 10 (stromelysin-2) is known to degrade various components of the extracellular matrix; however, the signals that regulate its expression and its role in lymphoma growth remain unknown. In the present work, we report the up-regulated expression of MMP10 in T lymphoma cells following contact with endothelial cells. The induction of MMP10 was found to be dependent on the specific interaction between LFA-1 and ICAM-1, which play a central role in regulating the expression of genes involved in the rate-limiting steps of lymphoma development. MMP10, but not MMP3 (stromelysin-1), was also up-regulated in human B lymphoma cells following exposure to IL-4, IL-6, and IL-13, but not to IL-1. To gain further insight into the role of MMP10 in lymphoma development, we generated lymphoma cell lines constitutively expressing high levels of MMP10 and studied these cells for their ability to form thymic lymphoma in vivo. Mice injected with lymphoma cells constitutively expressing MMP10 developed thymic lymphoma more rapidly than those injected with control lymphoma cells. These results provide the first in vivo evidence that overexpression of MMP10 promotes tumor development, and indicate that MMP10 induction is an important pathway activated not only upon ICAM-1/LFA-1-mediated intercellular contact, but also following activation of tumor cells with inflammatory cytokines. ***The Journal of Immunology*****, 2004, 173: 3605–3611.**

Non-Hodgkin's lymphomas, and several other cancers, are associated with severe metastatic disease that results in high mortality rates. The metastatic properties of tumor cells are acquired through the regulation of the expression of various molecules, including some involved in angiogenesis as well as in cellular migration, cell growth, apoptosis, differentiation, and invasion. Matrix metalloproteinases (MMPs)[3] play a central role in all of these events (1–4). In this regard, a direct link between the level of MMP9 (gelatinase B) produced by lymphoma cells and their metastatic potential has been established (5–7). MMPs constitute a family of enzymes that exhibit a high affinity and lytic activity for a variety of extracellular matrix components, including collagens, proteoglycans, elastin, laminin, and fibronectin (8). Recent evidence has also established that MMPs have an important role in processing enzymes that regulate cellular behavior and function by selective proteolysis of cell surface receptors and adhesion molecules, cytokines, and growth factors (9, 10).

Stromelysins are recognized as collagenase-related connective tissue-degrading metalloproteinases capable of degrading multiple components of the extracellular matrix, or stroma (8). Stromelysin-1 (MMP3) and stromelysin-2 (MMP10) isoforms are similar in their amino acid sequence and substrate specificity. The most significant differences between MMP3 and MMP10 reside in their differential patterns of expression on normal and transformed cells (11–14). In both cases, however, their expression has been associated mostly with parenchymal cells found in various tissues, and with increased metastatic behavior, most notably in the case of embryonic fibroblasts and other stromal cells (15–18).

We previously showed that MMP9 expression is regulated by bidirectional signaling during contact between T lymphoma cells and endothelial cells (EC) (19). Because MMP10 expression in normal human tissues has been reported in T cells (20), we investigated in this study its expression and its role in lymphomagenesis. We found that MMP10 gene expression is inducible in T lymphoma cells upon specific contact with EC, an effect that is partially mediated by ICAM-1 and LFA-1 adhesion molecules. Expression of MMP10, but not that of MMP3, was also induced in human B cell lymphoma upon exposure to IL-4, IL-6, and IL-13, but not to IL-1. Finally, using an in vivo experimental model, we found that overexpression of MMP10 by lymphoma cells accelerated the growth of thymic lymphoma. Our results indicate that both intercellular contact and cytokine secretion control the expression of MMP10 in lymphoma cells, which, in turn, plays an important role in the growth of thymic lymphoma.

Materials and Methods

Mice

Male and female C57BL/6 mice were bred in our animal facility and maintained under specific pathogen-free conditions and in accordance with institutional guidelines. Breeder pairs for the C57BL/6 mouse colony were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell lines and reagents

The origin of the 267 and 164T2 T lymphoma cell lines has been described (21). As a model to study lymphoma-EC interaction, we used the endothelioma cell line bEnd.3. In addition to expressing the von Willebrand

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[3] Abbreviations used in this paper: MMP, matrix metalloproteinase; EC, endothelial cell; MT4-MMP, membrane-type 4 MMP; PHEN, phenanthroline; TIMP, tissue inhibitor of metalloproteinase.

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factor, these cells express the same repertoire of cell adhesion molecules than that found on normal EC, including ICAM-1, VCAM-1, E- and P-selectins, CD31, and ICAM-2. The bEnd.3 cells up-regulate the expression of cell adhesion molecules after stimulation with inflammatory cytokines such as IL-1 and TNF-α, with kinetics similar to those reported for primary EC. Endothelioma cell lines have been used by many investigators as an in vitro model to study the ability of leukocytes to interact with vascular endothelium (19, 22–26). The Hi-7 fibroblastic cells were kindly provided by C. Daniel (Institut National de la Recherche Scientifique-Institut Armand-Frappier, Laval, Quebec, Canada) (27). The human Burkitt lymphoma Raji cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The diffuse large B cell lymphomas (OCY lines) have been described previously (28). HUVEC cell lines were obtained from GlycoTech (Rockville, MD) and were maintained in EC complete medium containing EC basal medium supplemented with 2% FBS, 0.01 μg/ml human epidermal growth factor, 0.001 mg/ml hydrocortisone, 0.012 mg/ml bovine brain extract, 2 mM L-glutamine, 10 mM HEPES buffer, and 0.001% gentamicin/amphotericin (Cambrex, Walkersville, MD). Except for the human B lymphoma cell lines, which were maintained in B cell medium (RPMI 1640 supplemented with 10% (v/v) serum, 2 mM L-glutamine, 10 mM HEPES buffer, 0.1 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.075% (w/v) sodium bicarbonate), all other cell lines were maintained in RPMI 1640 complete medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 10 mM HEPES buffer, 0.1 U/ml penicillin, 50 μg/ml streptomycin, and 55 μM 2-ME. The hybridomas R1/2 (anti-VLA-4 used as an isotype-matched control) and I27.7/7 (anti-LFA-1) were purchased from ATCC. The anti-VCAM-1 (429 Ab) has been described (25). These Abs were purified using the standard protein G affinity chromatography. The 3E2 mAb (anti-ICAM-1) was purchased from BD Pharmingen (San Diego, CA). The polyclonal Ab directed against murine MMP10 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated rabbit anti-goat IgG secondary Ab was purchased from Valeant Pharmaceuticals (Irvine, CA). Recombinant human cytokines (IL-4, IL-6, IL-13, and IL-1) were purchased from R&D Systems (Minneapolis, MN).

In vitro lymphoma-EC interactions

EC lines (HUVECs or bEnd.3) were seeded at the indicated density in 6- or 12-well plates (Corning-Costar, Acton, MA) and incubated overnight at 37°C in a 5% CO_2 atmosphere until they reached ~80% confluence. Aliquots of 10^6 lymphoma cells resuspended in complete RPMI 1640 medium were then added to the layer of EC (or control fibroblastic Hi-7 cells), and the cocultures were incubated for the indicated time at 37°C. In some cases, lymphoma or adherent cells were preincubated with blocking mAb (or isotypic controls) for 30 min at 37°C and then washed to remove excess mAb before they were used in cocultures. Lymphoma cells were separated from adherent cells using a warm solution of 0.004% trypsin-0.002% EDTA for 3 min. Resulting cell suspensions were immediately centrifuged and processed for RNA analysis or, in the case of protein analysis, were grown for 3 h in complete medium before centrifugation.

RNA isolation and analysis

Total RNA was isolated using RNeasy kit (Qiagen, Burlington, Canada), according to the manufacturer's instructions. First-strand cDNA was synthesized from 2–3 μg of total cellular RNA using the Superscript II (Invitrogen Life Technologies, Carlsbad, CA). For RNA analysis by PCR amplification, oligonucleotide primers were designed according to DNA sequences for mouse stromelysin-2 (MMP10) and membrane-type 4 MMP (MT4-MMP; MMP17) obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD) and chosen to have ~50% GC content. In the case of mouse MMP10, the sense (5′-AGG GAC CAA CTT ATT CCT GG-3′) and antisense (5′-CAG TAT GTG TGT CAC CGT CC-3′) primers were located in exons V and X, respectively. The murine MT4-MMP-specific primers were 5′-CTG TCC AAA GCG ATT ACT GC-3′ (sense) and 5′-AGT GCT AGA CCG ATT GTT GG-3′ (antisense), and the murine E-selectin-specific primers were 5′-GGA CTG TGT AGA GAT TTA CAT CC-3′ (sense) and 5′-GCA GGT GTA ACT ATT GAT GGT-3′ (antisense). Primers for human MMP10 were 5′-GTC ACT TCA GCT CCT TTC CT-3′ (sense) and 5′-ATC TTG CGA AAG GCG GAA CT-3′ (antisense); primers for human MMP3 were 5′-AGA GGT GAC TCC ACT CAC AT-3′ (sense) and 5′-GGT CTG TGA GTG AGT GAT AG-3′ (antisense); primers for human tissue inhibitor of metalloproteinase-1 (TIMP-1) were 5′-AGC GCC CAG AGA GAC ACC-3′ (sense) and 5′-CCA CTC CGG GCA GGA TT-3′ (antisense), respectively. Primers for GAPDH were 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ (sense) and 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ (antisense), while the β-actin-specific primers were 5′-CAT GGA TGA CGA TAT CGC TGC GC-3′ (sense) and 5′-GCT GTC GCC ACG CTC GGT CAG GAT C-3′ (antisense). Amplification of GAPDH or β-actin cDNA was used as RNA loading controls. Amplification of specific genes was performed in a MJ Research thermal cycler (model PTC-100TM; Cambridge, MA) using the following parameters: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles, except for β-actin, GAPDH, and E-selectin, which only required 25 cycles. The amplification for each gene was in the linear part of the curve. The reaction mixture was size separated on an agarose gel, and specifically amplified products were detected by ethidium bromide staining and UV transillumination. Quantitative analysis was conducted using a computerized densitometric imager (model GS-670; Bio-Rad, Mississauga, Canada). DNA sequencing of the amplicons confirmed the specificity of PCR.

Western blot analysis

Cells were washed with PBS and homogenized over ice in lysis buffer (10 mM Tris, pH 7.5, 2.5% Triton X-100) supplemented with a mixture of protease inhibitors (Complete; Roche, Laval, Canada). Equal amounts of cell lysates (as determined by Bio-Rad protein assay) were separated onto a 7.5% SDS-PAGE gel and then transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 10% milk in PBS/0.05% Tween 20 overnight at 4°C and then probed for 2 h at room temperature with primary Ab (polyclonal goat anti-murine MMP10 diluted 1/100 in PBS/0.05% Tween 20/5% BSA). After several washes in PBS/0.05% Tween 20, membranes were probed with secondary Ab (HRP-coupled rabbit anti-goat IgG diluted 1/10,000 in PBS/0.05% Tween 20/10% milk) for 45 min at room temperature, followed by several washes in PBS/0.05% Tween 20. Detection was performed using the ECL method (Amersham Biosciences, Baie d'Urfé, Canada).

Stimulation of lymphoma cells with cytokines

Human B lymphoma cells (10^6) were cultured in 1 ml of fresh B cell medium for 14 h in the presence or absence of recombinant human IL-4 (1 U/ml), IL-6 (1.5 ng/ml), IL-13 (6 ng/ml), or IL-1 (100 ng/ml). Cells were then centrifuged, RNA extracted, and processed for analysis of gene expression by RT-PCR.

Generation of stable transfectants expressing MMP10

The 1744-bp cDNA encoding the murine MMP10 gene (kindly provided by M. Madlener and S. Werner, Max-Planck-Institut für Biochemie, Martinsried, Germany) was inserted into the SRα eukaryotic expression vector containing the puromycin selection marker (kindly provided by F. Denis, Institut National de la Recherche Scientifique-Institut Armand-Frappier). To generate stable transfectants of lymphoma cells expressing MMP10 constitutively, 267 T lymphoma cells were transfected by electroporation (Gene Pulser; Bio-Rad) using the following parameters: 40 μg of DNA per 10^7 cells in PBS on ice; 960 μF; 250 mV. Control cells consisted of 267 lymphoma cells transfected with SRα empty vector alone. After 24 h of culture in complete medium, transfected cells were allowed to grow in complete medium containing 5 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO) before individual colonies were selected and expanded. MMP10 expression was assessed by RT-PCR and Western blot analyses.

Invasion assay

The invasive properties of MMP10-expressing lymphoma cell clones were measured by the invasion of cells through Matrigel-coated Transwell inserts (Corning-Costar). Briefly, Transwell inserts with an 8-μm pore size were coated with a 2 mg/ml Matrigel and lymphoma cells (2×10^6 cells) added to the upper chamber inserts in Transwell chamber plates filled with prewarmed complete medium containing supernatant (1:1) from mitogen-activated (Con A) T cell cultures as a source of T cell chemoattractant (29). Plates were incubated for 24 or 36 h in a CO_2 incubator. Percentage of cell invasion was calculated from the ratio of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. In some experiments, lymphoma cells were resuspended in RPMI 1640 containing 5 mM 1,10-phenanthroline (PHEN) and incubated at 37°C for 30 min before their addition to Transwell chamber plates. At least two independent experiments were performed for each lymphoma cell clone, in which each clone was analyzed in duplicate.

Induction of primary thymic lymphoma

Five- to 6-wk-old C57BL/6 mice (5–10 mice per group) were injected intrathymically in each of the two thymic lobes with 5×10^3 MMP10-expressing or control lymphoma cells. Mice were then regularly observed for clinical signs of thymic lymphoma (runting, swelling of the thorax, and dyspnea), which only appear at the end stage of the disease and reveal

imminent death owing to pulmonary compression by oversized thymic tumor. In this thymic lymphoma growth model, injected lymphoma cells generate tumors that grow with various speeds, depending notably on the phenotype of the injected lymphoma cells (in this case, overexpression or not of MMP10). Thus, survival of the animals depends on the growth rate of their thymic lymphoma, which dictates at what moment the animals will display dyspnea and will have to be sacrificed. The presence of thymic tumors is then confirmed at necropsy. All of the animal research was reviewed and approved by the Institutional Animal Care and Use Committee at the Institut National de la Recherche Scientifique-Institut Armand-Frappier in accordance with institutional and Canadian guidelines for animal experiments.

Statistical analysis

Statistical significance was measured using a log rank test, and the level of significance was established at $p < 0.05$.

Results

Basal MMP10 gene expression in lymphoma and endothelioma cells

We began our investigation on the role of MMP10 in lymphoma by measuring constitutive levels of its expression in our lymphoma and EC lines. We found that, in contrast to the endothelioma cells, murine T lymphoma cell lines did not express MMP10 constitutively (Fig. 1*A*). Both cell types expressed, however, MMP17 (MT4-MMP), a GPI-anchored protease that has the capacity to degrade gelatin and activate pro-MMP2 (30).

To study MMP10 gene expression in lymphoma cells upon contact with EC, we cocultured lymphoma cells with bEnd.3 and examined the levels of MMP10 transcripts in lymphoma cells after contact. Lymphoma cells, which firmly adhered to bEnd.3, were detached at the indicated time after initiation of the culture using a low concentration of trypsin-EDTA, as previously described (19). This procedure allowed removal of >95% of lymphoma cells, while <1% of bEnd.3 was detached by this procedure, leaving intact the bEnd.3 monolayer, as assessed by flow cytometric analysis (data not shown). Our results showed that contact with bEnd.3 induced time-dependent de novo expression of MMP10 mRNA in both 164T2 and 267 T lymphoma cells (Fig. 1*B*). The induction was transient, as it reached maximal expression at 12 h postcontact. No such induction was observed upon contact of lymphoma cells with fibroblasts. To ensure that no EC were present among lymphoma cells that were removed after coculture, we routinely measured by RT-PCR the levels of EC-specific E-selectin transcripts in isolated T lymphoma cells; no detectable levels were present (Fig. 1*C*). These results indicated that no significant numbers of EC were present among lymphoma cells after contact and argued against the possibility that MMP10 expression in T lymphoma cells following contact with EC was attributable in part to constitutive expression of MMP10 by contaminating EC. Western blot analysis was performed to confirm the induction of MMP10 at the protein level (Fig. 1*D*). To rule out contamination of lymphoma cell suspension by EC that express endogenous levels of MMP10, Western blot analysis was performed on lysates obtained from lymphoma cells that had been removed from 18-h contact with EC and subsequently cultured alone for 3 h. In these conditions, rare EC that could have contaminated the lymphoma suspension would adhere to the bottom of the plates, while lymphoma cells would remain nonadherent, which facilitated their purification from EC. These procedures were conducted to ensure that the MMP10 protein detected by Western blot in Fig. 1*C* did not originate from contaminating EC. Activation of MMP10, but not of MMP3, gene expression was also observed following contact of human B and T lymphoma cells with HUVECs (data not shown).

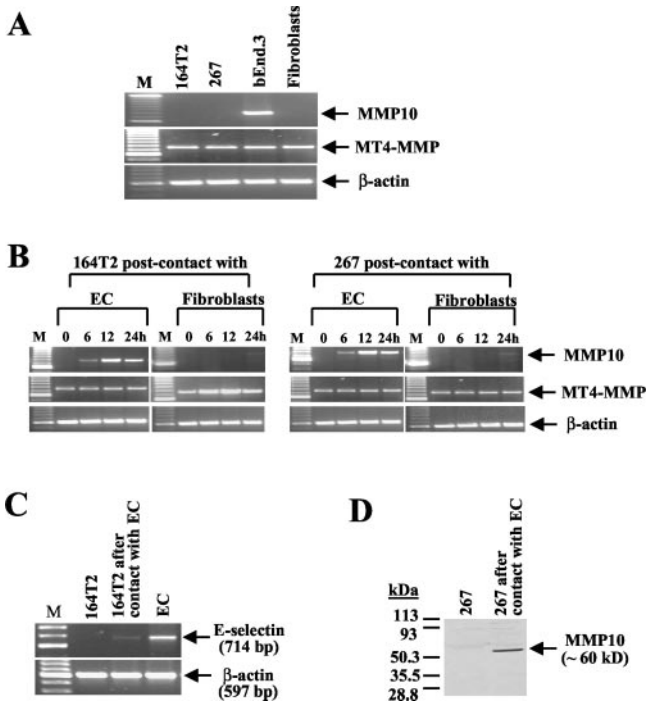

FIGURE 1. MMP10 gene expression is induced in lymphoma cells on contact with EC. *A*, MMP10 gene expression in murine T lymphoma and stromal cells. Levels of MMP10 transcripts were determined by RT-PCR analysis in 164T2 and 267 T lymphoma cells and in bEnd.3 EC and Hi-7 fibroblastic cells. Levels of MT4-MMP transcripts are shown as a specificity control. M, m.w. standards (100-bp DNA ladder). The *lower thick band* represents the 600-bp marker. Results are representative of at least three independent experiments. *B*, Induction of MMP10 in lymphoma cells after contact with endothelioma cells, but not fibroblasts. The bEnd.3 endothelioma or Hi-7 fibroblastic cells were seeded at a density of 0.8×10^5 and 10^6 cells/well (Corning 12-well plate), respectively, and were cultured for 12 h to reach ~80% confluency. The 164T2 or 267 T lymphoma cells (10^6 cells/well) were then added to the endothelial or fibroblastic layers. After indicated periods of intercellular contact, lymphoma cells were collected, total RNA extracted, and RT-PCR analysis using MMP10 and β-actin-specific primers was performed, as described in *Materials and Methods*. M, m.w. standards (100-bp DNA ladder). The *lower thick band* represents the 600-bp marker. Levels of MT4-MMP transcripts are shown as a specificity control. Results are representative of three independent experiments. *C*, Control showing absence of EC in lymphoma cell preparations collected after contact with EC. Transcripts of E-selectin, a specific marker of EC, were found in RNA extracted from endothelioma cells, but were undetectable in RNA samples of lymphoma cells collected after coculture with EC. M, m.w. standards (100-bp DNA ladder). The *lower thick band* represents the 600-bp marker. Results are representative of at least three independent experiments with 164T2 as well as 267 T lymphoma cells. *D*, Induction of MMP10 detected at the protein level. Western blot analysis of MMP10 in 267 T lymphoma cells that had been cultured alone during 3 h after removal from an 18-h interaction with bEnd.3 cells. Proteins (100 μg) from cell lysates were immunodetected with MMP10-specific polyclonal Abs, as described. Results are representative of two independent experiments.

The essential role of LFA-1 and ICAM-1 in the induction of MMP10 expression

Integrins play a key role in intercellular adhesion events, notably in the delivery of signals regulating MMP gene expression (19, 31). Because lymphoma cells express high levels of LFA-1, an integrin involved in the activation of *mmp9* gene in lymphoma cells upon contact with EC (19), we next tested the effect of blocking LFA-1 on the induction of MMP10 in lymphoma cells following contact with bEnd.3. Our results showed that preincubation of

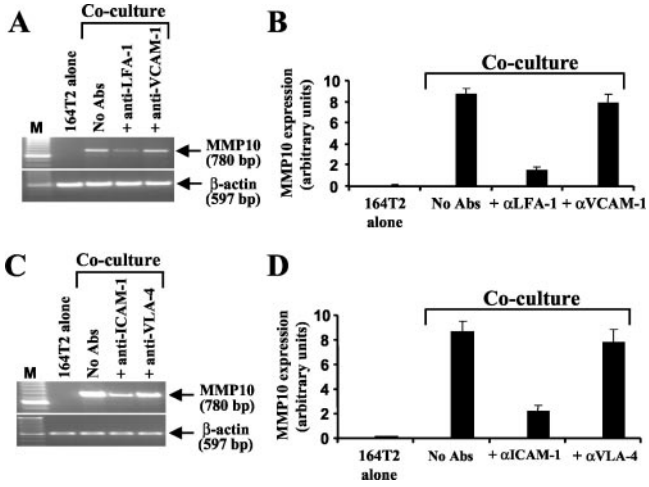

FIGURE 2. The induction of MMP10 expression in lymphoma cells upon contact with EC is LFA-1 and ICAM-1 dependent. Involvement of LFA-1 and ICAM-1 in the induction of MMP10 expression in 164T2 T lymphoma cells after contact with EC was determined by preincubating: *A*, 164T2 T lymphoma cells for 30 min with blocking mAbs specific for LFA-1 (or VCAM-1 as an isotypic control), or *C*, EC with mAbs specific for ICAM-1 (or VLA-4 as an isotypic control), and then washing pretreated cells to remove excess Ab, before lymphoma and EC were cocultured for 12 h. MMP10 gene expression was then analyzed at the mRNA level by RT-PCR, as previously described, and quantitative analysis of Ab inhibition was performed by imaging densitometry (*B* and *D*). M, m.w. standards (100-bp DNA ladder). The *lower thick band* represents the 600-bp marker. Results are representative of three independent experiments.

T lymphoma cells with anti-LFA-1 mAb reduced the de novo induction of MMP10 in pretreated lymphoma cells after contact with EC by ~80% (Fig. 2, *A* and *B*). This effect specifically blocked LFA-1 signaling, as no such reduced induction was observed after preincubation of lymphoma cells with an anti-VCAM-1 control mAb, VCAM-1 being constitutively expressed on bEnd.3 cells, but not on T lymphoma cells (data not shown). Alternatively, to determine whether ICAM-1, a major ligand for LFA-1 (32), played a role in the interaction between tumor cells and EC, we pretreated bEnd.3 EC with anti-ICAM-1-blocking mAb and observed a decrease of ~75% in the induction of MMP10 expression in 164T2 T lymphoma cells after contact with pretreated EC (Fig. 2, *C* and *D*). Again, this effect specifically blocked ICAM-1 signaling, as no such decrease was observed when EC were pretreated with an anti-VLA-4 control mAb (VLA-4 is not expressed by bEnd.3 EC).

Induction of MMP10 in human B lymphoma cells following stimulation with cytokines

MMP gene expression, in addition to being regulated by intercellular contact, most notably in B lymphoma cells, has been shown to be under the control of cytokines (33–36). Because previous studies have shown that cytokines such as IL-4, IL-6, and IL-13 play a significant role in lymphomagenesis (37–39), we investigated whether these cytokines could modulate MMP10 expression and found that exposure of Raji lymphoma cells to IL-4, IL-6, and IL-13 induced the expression of MMP10 (Fig. 3*A*). This induction was specific, as no such induction was observed with MMP3, another member of the stromelysin subfamily of MMP, although MMP3 transcripts were detected in HT1080-positive control cell line; moreover, no modulation of TIMP-1 expression was observed. The ability of IL-13 to up-regulate MMP10 was not specific to the Raji B cell line, as other, albeit not all, B lymphoma cell lines could express MMP10 in response to this cytokine (Fig. 3*B*).

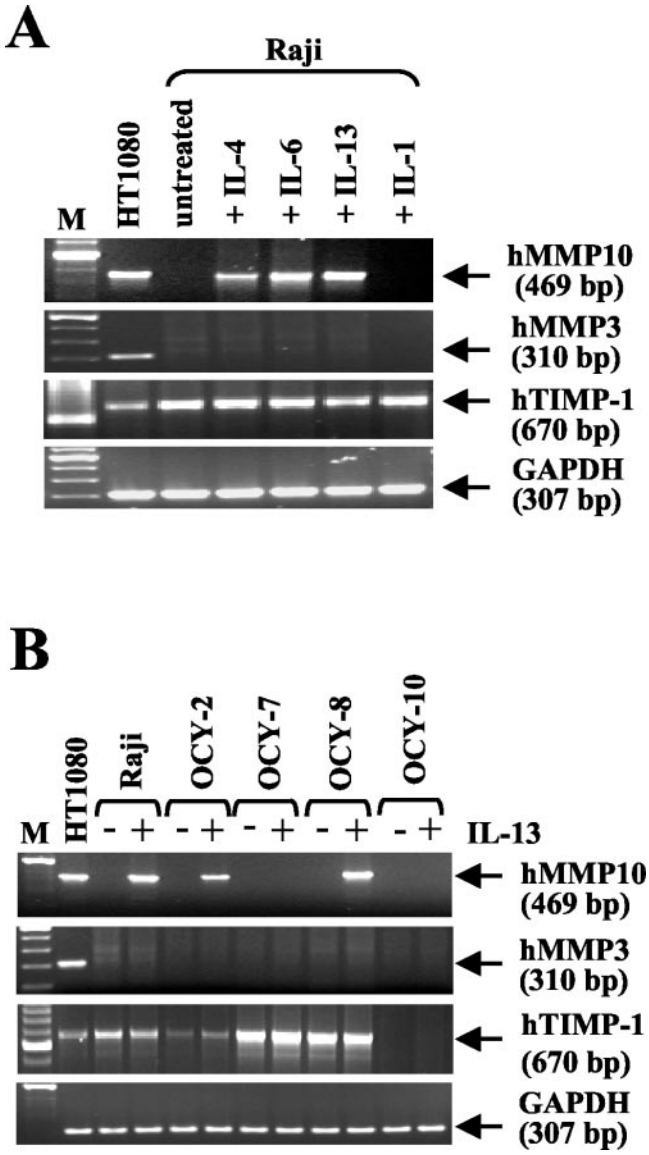

FIGURE 3. Up-regulation of MMP10 in lymphoma cells upon exposure to cytokines. Raji cells (*A*) and several B lymphoma cell lines (*B*) were stimulated with various cytokines, including IL-13, for 14 h. MMP10, MMP3, and TIMP-1 mRNA expression was then measured by RT-PCR, using HT1080 fibrosarcoma cell line as a positive control. Results are representative of at least three independent experiments. The expression of GAPDH was included as an RNA loading control. Results are representative of at least two independent experiments.

Invasive in vitro behavior of lymphoma cells overexpressing MMP10

Extracellular proteases have been shown to cooperatively influence tumorigenicity through proteolytic cascades, with individual proteases having distinct roles in matrix degradation, invasion, and migration. To determine whether increased expression of MMP10 conferred to lymphoma cells an increased proteolytic activity, we first generated T lymphoma cell lines overexpressing MMP10. Murine T lymphoma cells (267 cell line) were transfected with the pSRα expression vector encoding the murine MMP10 cDNA, and puromycin-selected cell clones were isolated. These clones were characterized for MMP10 expression at the mRNA and protein levels by RT-PCR and Western blot analyses (Fig. 4, *A* and *B*). MMP10 mRNA and protein were detected in cells transfected with the MMP10 cDNA (clones C7, A2, and C4), but not in lymphoma

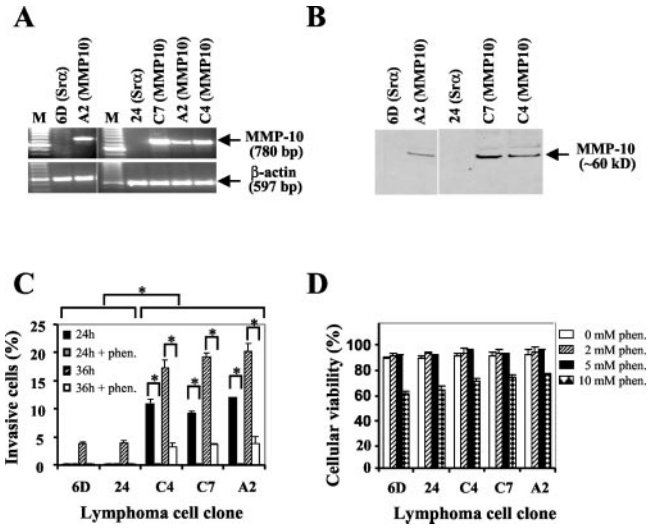

FIGURE 4. Generation of transfectant lymphoma cell clones overexpressing MMP10 and measure of their invasive behavior. mRNA (*A*) and Western blot (*B*) (100 μg/lane) analyses of MMP10 expression in 267 lymphoma cells transfected with the empty vector (SRα), or with the MMP10 cDNA. Clones were selected using puromycin-containing medium. Both control (empty SRα vector) and all MMP10-expressing clones shown were used for independent in vivo studies. M, m.w. standards (100-bp DNA ladder). Results are representative of at least two independent experiments. *C*, The effect of MMP10 on the invasive behavior of lymphoma cells overexpressing MMP10 was determined using Matrigel-coated Transwell inserts. Invasion assays were conducted for 24 or 36 h in serum-free medium after pretreatment or not of transfectant lymphoma cells with 5 mM 1,10-PHEN for 30 min. Results are means $\pm$ SE for two independent experiments, each performed in duplicates. $*$, $p \leq 0.05$. *D*, Lack of cytotoxicity of PHEN treatment (5 mM for 30 min). The different lymphoma cell transfectants were incubated with various concentrations of PHEN for 30 min before cell count with trypan blue. Results are means $\pm$ SE for two independent experiments, each performed in duplicates.

cells transfected with the control vector (clones 6D and 24). We then measured the effect of MMP10 expression on the ability of lymphoma cells to migrate through a matrigel-based matrix. We found that overexpression of MMP10 by lymphoma transfectants significantly improved their ability to migrate through the matrix compared with cells transfected with the control vector (Fig. 4*C*). The ability of the metalloproteinase-specific inhibitor PHEN to inhibit the invasive behavior of lymphoma transfectants shows that this effect is indeed MMP dependent. It is worth noting that the observed decrease in invasive behavior associated with pretreatment of lymphoma transfectants with PHEN was not caused by cytotoxicity (Fig. 4*D*).

MMP10 accelerates lymphoma growth in vivo

To measure the effect of MMP10 overexpression in thymic lymphoma growth, we compared the capacity of control (Srα clones) and MMP10 transfectants to modulate tumor growth in vivo following intrathymic injection in young C57BL/6 syngeneic mice. In this orthotopic thymic lymphoma growth model, injected lymphoma cells generated tumors that grew with various speeds, depending notably on the phenotype of the injected lymphoma cells (in this case, overexpression or not of MMP10). Clinical signs of thymic lymphoma (runting, swelling of the thorax, and dyspnea) only appeared at the end stage of the disease and were a sign of imminent death owing to pulmonary compression by oversized thymic tumor. Thus, survival of the animals depended on the growth rate of their thymic lymphoma, which dictated at what moment the animals would display dyspnea and would have to be sacrificed. Two clones of control (Srα clones) and MMP10-overexpressing lymphoma transfectants were thus injected intrathymically in mice, and the frequency of tumor development as well as the mean survival time were determined. Our results showed that expression of MMP10 by lymphoma cells significantly ($p = 0.0001$) accelerated the growth of thymic lymphoma in vivo, because mice injected with MMP10 transfectants presented dyspnea earlier and had to be sacrificed more rapidly than those who received control lymphoma cells (Fig. 5). Indeed, mean survival time values were significantly reduced when MMP10-expressing cells were injected as compared with control cells (22 $\pm$ 3 days vs 41 $\pm$ 4 days, respectively). Similar results were obtained with the clone A2 (data not shown).

Discussion

We have shown in this study that: 1) MMP10 expression can be induced in murine T lymphoma cells after contact with EC, an effect that is partially mediated by ICAM-1 and LFA-1 adhesion molecules; 2) MMP10 expression can also be induced in human B lymphoma cells after exposure to cytokines such as IL-4, IL-6, and IL-13, but not IL-1; and 3) MMP10 expression by lymphoma cells

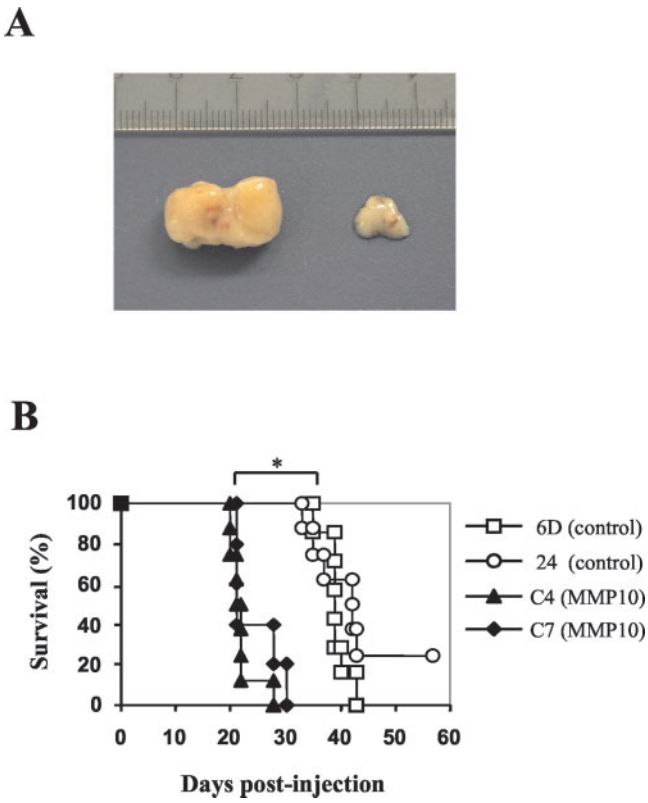

FIGURE 5. Effect of MMP10 on lymphoma growth. Lymphoma cell transfectants (5×10^3) were injected in each of the two thymic lobes in young C57BL/6 syngeneic mice. Mice exhibiting signs of distress and becoming moribund were killed by carbon monoxide anesthesia and autopsied. Thymic lymphomas were confirmed at necropsy and fixed for histological examination. *A*, Representative thymic lymphoma harvested at necropsy following intrathymic injection of lymphoma transfectant compared with a normal thymus on the right. *B*, Survival analysis of tumor-free mice over a time period of 57 days after tumor injection. Thymic lymphoma induced by intrathymic inoculation of T lymphoma cells is restricted to the thymus, and does not metastasize to peripheral organs (40). The results are representative of two independent experiments with three different clones expressing MMP10. $*$, $p \leq 0.05$.

accelerates the growth of thymic lymphoma. To our knowledge, the present study is the first to directly address the expression of MMP10 in lymphoma and the signals that regulate its expression in lymphoma cells; moreover, it provides the first indication for a role of MMP10 in tumor growth.

We found that both T and B lymphoma cells of human and mouse origin secrete MMP10 on contact with EC, but not with fibroblasts. It must be noted that the inability of fibroblasts to induce MMP10 gene expression is not due to their lack of ICAM-1 expression, as Hi-7 cells transfected with ICAM-1 were unable to induce MMP10 gene expression in lymphoma cells (data not shown). This suggests that binding to ICAM-1 is insufficient to induce MMP10 expression in lymphoma cells, and that a costimulatory signal generated from molecules other than the LFA-1/ICAM-1 complex is necessary for the induction of MMP10. A similar situation was observed in the case of MMP9 induction upon contact with EC (19). In fact, these data are consistent with those obtained from coculture systems using Transwells, in which lymphoma cells were physically separated from EC by a porous filter that only allowed selective exchange of small molecules. In these experiments, we did not observe induction of MMP10 gene expression in lymphoma cells (data not shown), indicating that cell-cell contact was necessary. Although membrane-bound chemokines/growth factors are prime candidates for signals inducing MMP10 in lymphoma cells, further studies using gene-profiling experiments on EC and blocking Abs will help to determine the repertoire of factors expressed by these cells and the signal(s) involved.

Stromelysins are considered collagenase-related connective tissue-degrading metalloproteinases. The ability of MMP10 to promote neoplasia has therefore been inferred to its secretion by peritumoral cells in response to the presence of signals provided by the tumor cells (16). Our study, however, clearly shows that tumor cells themselves can secrete MMP10, notably following exposure to cytokines such as IL-4, IL-6, and IL-13. It is likely that these cytokines can be provided by a variety of peritumoral cells, including EC, macrophages, or even infiltrating activated lymphocytes. IL-13, as well as IL-6, has been shown to be involved in the pathophysiology of human lymphomas (37–39). These results are in fact consistent with the observation that Hodgkin/Reed-Sternberg cells, which are responsive to IL-13 (41), express MMP10 in vivo (42). This suggests that the contribution of these cytokines to the pathophysiology of lymphoma can be attributed, at least in part, to their ability to up-regulate MMP genes, such as MMP10. We have observed that IL-13, however, selectively induces expression of MMP10 in some B lymphoma cells such as Raji cell line, but not in others. This distinct ability to express MMP10 following exposure to IL-13 may vary according to: 1) the levels of expression of functional IL-13Rs, 2) the integrity of the IL-13 signaling cascade involved in the activation of the MMP10 promoter, or 3) the clinical status of the patient, and/or the type of lymphoma. Further studies with more patients will thus be required to determine whether the expression of MMP10 in non-Hodgkin's lymphoma is associated with a particular growth phenotype or with a particular clinical outcome. Moreover, our results showing that IL-4 stimulates MMP10 secretion in both human T and B lymphoma cells have important implications for the success of IL-4 as a treatment for low and high grade lymphomas. They may explain, at least in part, the limited success of clinical trials of using human rIL-4 in patients with non-Hodgkin's lymphoma (43).

Although MMP3 and MMP10 are encoded by different genes, both enzymes are considered as isozymes, as they share significant structural characteristics and substrate specificity. The most significant differences between these enzymes are within their differential pattern of expression (8), despite the fact that both enzymes are encoded by genes flanked by common consensus sequences specific for transcription factors such as AP-1 and PEA3. MMP10, for example, has been shown to be much less responsive than MMP3 to growth factors such as IL-1 in human fibroblasts (11, 44). We found a similar pattern in human lymphoma cells, namely that MMP10, but not MMP3, was induced by IL-4, IL-6, and IL-13. However, lymphoma cells and fibroblasts have distinct abilities to express MMP10 and MMP3 following exposure to cytokine, because IL-1 is ineffective in inducing MMP3 and MMP10 expression in lymphoma cells. This concept is also supported by our data showing that in lymphoma cells, MMP10 can be induced by IL-4, a cytokine previously associated with down-regulation of IL-1-induced stromelysin expression in stromal cells (45–47). It is also important to note that while MMP10 mRNA levels were increased in lymphoma cells upon treatment with IL-4, IL-6, or IL-13, the mRNA level of TIMP-1, a natural inhibitor of MMPs, including MMP10, was not modulated by these cytokines, suggesting that stimulation of lymphoma cells by these cytokines shifted the equilibrium between MMP and their inhibitors. The limited inductibility of MMP3 in lymphoma cells suggests that its expression is under tight regulatory control, possibly involving epigenetic mechanisms such as DNA methylation. We and others have indeed shown that the expression of some members of the MMP family is subjected to changes in the methylation status at the level of their promoter in lymphoma cells (48–50). Future investigations on the signaling cascade leading to activation of both genes in lymphoma cells should clarify this issue.

In light of the side effects that have been observed in clinical trials, recent studies have focused on the importance of using specific rather than broad-spectrum MMP inhibitors for the treatment of cancer (7). For this reason, it is of prime importance to understand MMP pathophysiology in cancer. The combined use of in vitro and in vivo experimental models has allowed us to identify MMP10 has a new target for lymphoma therapy. Although the underlying mechanisms by which MMP10 accelerates lymphoma growth in our model remain to be elucidated, our results showing that expression of MMP10 increases the ability of lymphoma cells to break down the extracellular matrix are likely to play an important role in promoting lymphoma growth. This would lead, for instance, to the release of growth factors and cytokines, and/or directly regulate the bioactivity of soluble mediators. Our model may thus be suitable to assess the effects of specific inhibitors of lymphoma growth.

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