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Matrix Metalloproteinase Activity Modulates Tumor Size, Cell Motility, and Cell Invasiveness in Murine Aggressive Fibromatosis

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ABSTRACT

Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) regulate the degradation of extracellular matrix components and play important roles in the progression of select neoplastic processes. The locally invasive soft tissue tumor, aggressive fibromatosis (also called desmoid tumor), is caused by mutations resulting in β-catenin-mediated T-cell factor (tcf)-dependent transcriptional activity. Because β-catenin can regulate MMP expression, we investigated the expression of several MMPs and TIMPs in aggressive fibromatosis tumors that develop in *Apc+/Apc1638N* mice. *Mmp-3* and *Timp-1* were differentially regulated (5-fold and 0.5-fold, respectively) in tumors compared with normal fibrous tissue. Conditioned media from tumor cells showed an increased ability to degrade collagen, and inhibition of MMPs using GM6001 decreased the ability of the tumor cells to invade through Matrigel. Both the treatment of *Apc/Apc1638N* mice with GM6001 or crossing with a transgenic mouse that overexpresses *Timp-1* resulted in a significant reduction in tumor volume. Surprisingly, overexpression of *Timp-1* also resulted in a 50% increase in tumor number. Although TIMP-1 can induce growth stimulatory effects in some cell types, we found no difference in proliferation or apoptosis rate in cells from tumors that developed in the *Timp-1*–transgenic mice compared with mice that did not express the *Timp-1* transgene, suggesting that TIMP-1 promotes aggressive fibromatosis tumor formation through an alternate mechanism. These data suggest that MMPs play a crucial role in regulating the invasiveness of mesenchymal cells and in modulating aggressive fibromatosis tumor progression. Because this is a locally invasive tumor, MMP inhibition could slow tumor growth and may prove to be an effective adjuvant therapy.

INTRODUCTION

Aggressive fibromatosis, also called desmoid tumor, is a locally invasive soft tissue tumor that is difficult to manage using conventional therapies (1). It is composed of a clonal population of mesenchymal, spindle-shaped cells (2, 3) harboring mutations resulting in β-catenin protein stabilization (4–6). β-Catenin stabilization is sufficient for the formation of aggressive fibromatosis tumors, as demonstrated using a transgenic mouse that overexpresses a stabilized form of β-catenin (7). Germline mutations in the *adenomatous polyposis coli* (*APC*) gene, resulting in an elevated level of β-catenin protein, predisposes the gene to the formation of aggressive fibromatosis tumors, especially in cases in which the mutation clusters toward the 3′ end of the gene (8, 9). A mouse with a targeted mutation in *Apc* near the 3′ end, the *Apc/Apc1638N* mouse, develops many aggressive fibromatosis tumors. Male mice develop a larger number of tumors than female mice, averaging 45 aggressive fibromatosis tumors by 6 months of age. These mice also develop gastrointestinal polyps, although at an incidence substantially less than that of the Min mouse, which harbors an *Apc* mutation at a different location (10).

β-Catenin is a central mediator in the canonical Wnt signaling cascade. In the absence of an appropriate ligand, a multiprotein complex including adenomatous polyposis coli phosphorylates NH_2 terminus residues of β-catenin, targeting the protein for degradation. When an appropriate Wnt ligand is present, these sites are not phosphorylated, β-catenin protein becomes stabilized and binds to transcription factors in the tcf-lymphoid enhancer factor family, resulting in transcriptional activation. β-Catenin can also be stabilized by mutations removing the phosphorylation sites or by mutations in members of the multiprotein complex regulating its phosphorylation. In tumors, mutations stabilizing β-catenin protein result in the expression of target genes, altering cell behavior in a way to produce a neoplastic phenotype (reviewed in refs. 11–13). Two such target genes are matrix metalloproteinase seven, which is regulated by β-catenin-mediated tcf-dependent transcription in colonic neoplasia in concert with Ets and c-Jun (14, 15), and membrane-type matrix metalloproteinase-1, which is regulated by β-catenin-mediated tcf-4-dependent transcription in colonic neoplasia (16). However, genes are regulated by β-catenin-mediated tcf-dependent transcription in a cell-context-dependent manner, and β-catenin target genes in one cell type are not necessarily target genes in other cell types (17).

Matrix metalloproteinases (MMPs) are a family of proteinases that are capable of degrading nearly all components of the extracellular matrix, including collagen, fibronectin, gelatin, and elastin. More than 20 different MMPs have been identified, each with a distinct and overlapping expression pattern and substrate specificity. Most MMPs are secreted as zymogens and require the cleavage of the pro-domain to become an active enzyme. Activated MMPs modulate cell behavior by altering cell-extracellular matrix, cell-cell interactions, and can directly affect signaling through cleavage of signaling ligands and receptors (18, 19). Their activity is critical for physiological and pathological processes associated with extensive tissue remodeling, including angiogenesis, bone remodeling, wound healing, inflammatory diseases, and cancer. MMP activities can be inhibited through a family of MMP inhibitors, the tissue inhibitors of MMPs (TIMPs), which reversibly inhibit MMP activity by binding to the substrate binding site (20).

MMPs are expressed at high levels in numerous cancers of epithelial cell origin, and their level of expression correlates with increased aggressiveness of tumors (18). Although it is thought that increased expression of MMPs mainly contributes to the invasion and metastasis of tumor cells, mounting evidence is also placing a prominent role for MMPs in cancer initiation and progression (21). Besides their expression by tumor cells of epithelial origin, MMPs are also produced by the mesenchymal stromal cells sounding these tumors (22). Genetic analysis in mice demonstrated that MMPs play a crucial role in tumor initiation and progression. Overexpression of *Mmp*s causes increased susceptibility to neoplastic formation with or without external stimuli. For example, overexpression of *Mmp-3* induced the spontaneous

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formation of mammary adenoma (23). Tumor development in these mice was attenuated by overexpressing the endogenous inhibitor *Timp-1*. Conversely, deficiency in *Mmp*s decreases tumor formation, as in targeted deletion of *Mmp-7*, which decreases gastrointestinal tumor formation in *Apc^{min}* mice (24). Despite the potential for MMP inhibition as a treatment for cancer based on these, and other, animal investigations, the results of clinical trials of MMP inhibitors have been disappointing. A possible explanation is that although MMP inhibitory drugs slow tumor growth, by the time tumors are well advanced such drugs probably do not produce clinically significant effects (25, 26).

Because MMPs can be regulated by β-catenin-mediated tcf-dependent transcription, we investigated MMP and TIMP expression and function in murine aggressive fibromatosis tumors formed in the *Ap/Apc1638N* mouse. Unlike neoplasms that metastasize, the locally invasive nature of aggressive fibromatosis may make it a lesion that is more amenable to treatment with MMP inhibitors. Furthermore, this model gives an opportunity to investigate the function of MMPs in mesenchymal cells.

MATERIALS AND METHODS

Genetically Engineered Mice. The generation and phenotype of *Apc/Apc1638N* mice has been reported previously. These mice develop about six gastrointestinal lesions by 6 months of age, and male mice develop an average of 45 fibromatoses by the age of 6 months. Female mice develop substantially fewer numbers of fibromatosis tumors then males (10). *Timp-1* transgenics, as described previously (27), express the full-length murine *Timp-1* cDNA under the regulation of the mouse MHC class I (H2) promoter. This mouse expresses the *Timp-1* transgene in almost all tissues, including mesenchymal tissues, skin, and muscle. These mice were crossed, as in our previous investigations (28), to produce *Apc/Apc1638N-Timp-1*–transgenic mice and *Apc/Apc1638N* littermates that do not express the *Timp-1* transgene. In this way, mice overexpressing *Timp-1* are compared with littermate controls. Ten male and ten female mice of each genotype were investigated. Mice were sacrificed at 6 months of age, and size and number of tumors formed were scored as reported previously (10).

Treatment of Mice with GM6001. Three-month old male *Apc/Apc1638N* mice were treated with daily s.c. injections of GM6001 (100 mg/kg; Chemicon, Temecula, CA), as reported previously (29). Control mice received daily injections of carrier (DMSO). Mice were treated for 4 weeks, and after termination of treatment, they were sacrificed and examined for tumor formation, as reported previously (10, 28). Male mice were used for this experiment because they develop significantly more tumors than female mice. The ability of GM6001 to inhibit MMPs in the tumors from these mice was verified using casein zymography. Five-treatment and five-control mice were evaluated.

Cell Culture Studies. Primary cell cultures were established from aggressive fibromatosis tumors and normal fibrous tissue (fascia) samples from nine *Apc/Apc1638N* mice and normal fascial tissue from nine wild-type mice. Monolayer cultures were initially grown in high-glucose-DMEM supplemented with 50% fetal bovine serum then in high glucose-DMEM supplemented with 10% fetal bovine serum after 1 week in culture as reported previously (30). Cell cultures were maintained at 37°C in 5% CO_2 and divided when confluent. Experiments were performed with cells between the first and fifth passage.

Proliferation assays, apoptosis assays, motility studies, and Matrigel invasion studies were performed as reported previously (30–32). Proliferation was measured using BrdUrd incorporation over an 8-hour period. Cells taking up BrdUrd were identified using immunohistochemistry, and the positively stained nuclei were counted over 10 high-powered fields running from the periphery to the center of the cell culture plate. Apoptosis was measured using a flow-cytometry-based technique (31) according to the manufacturer's directions (Molecular Probes, Eugene, OR), as well as by a terminal deoxynucleotidyl transferase-mediated nick end labeling assay, also performed according to the manufacturer's instructions (Roche, Laval, Quebec, Canada). Motility was measured using a Boyden's chamber, in which 50,000 cells from different cultures were seeded into Boyden's motility chambers (10-mm tissue culture inserts with a 8-μm polycarbonate membrane; Becton-Dickinson Labware, Bedford MA) in a 500-μl volume of high glucose-DMEM supplemented with 0.5% fetal bovine serum treated with DMSO or 50 μM GM6001. Cells were incubated in 37°C for 22 h and allowed to migrate. After incubations, cells were fixed in 4% paraformaldehyde/PBS for 20 minutes, and cells inside the chambers were scraped using a cotton swab. Cells that crossed the membrane were stained with 4′,6-diamidino-2-phenylindole fluorescent-mounting media and counted under a fluorescent microscope. The number of cells that crossed the membrane was determined by cell counts in six high-power random-selected fields in each of the triplicates. Invasion chambers were prepared by coating 10-mm tissue culture inserts with 8-μm polycarbonate membranes with Matrigel (85 μg/cm^2, Becton-Dickinson Labware) as per manufacturer's protocol. The cell density, group size, and assay methods used were similar to that of the motility study using the modified Boyden's chamber. A fluorescent fibronectin substrate adhesion assay, as reported previously (32), was performed according to the manufacturer's instructions (Molecular Probes, Eugene OR). All assays using cell cultures were performed 9 times.

RNA Preparation and Semiquantitative Reverse Transcription-PCR. RNA was isolated from aggressive fibromatosis tumor and normal fibrous tissue (fascial tissue) of *Apc/Apc1638N* mice and fascial tissue from wild-type mice by the TRIzol method (Invitrogen, Carlsbad, CA). Nine independent tumors from nine different mice were analyzed as well as normal tissues from the same nine mice. PCR primers were chosen to amplify a section of cDNA that crossed an intron or were selected from previous publications (28). Semiquantitative and real-time PCR was performed as reported previously (28).

Casein and Gelatin Zymogram. Cells (5×10^5) were plated in 150-mm plates and allowed to attach overnight. Cells were then washed with PBS 3 times and incubated with 15 ml of serum-free high glucose-DMEM for 72 h. The medium was recovered and centrifuged at $1,000 \times g$ for 5 minutes. The supernatant of conditioned media was concentrated 10 times using spin centration columns (Millipore, Billerica, MA). Protein concentrations were determined using the Bradford-Lowry method. Fifty micrograms of total protein were loaded for zymography analysis. Samples were run in gelatin and casein zymogram gels (Invitrogen) for 5 hours at 90V and 4°C. The gels were then washed 3 times in 2.5% v/v Triton-X. Gelatin and casein zymograms were incubated in zymography incubation buffer at 37°C for overnight and 48 hours, respectively. After the incubation period, the gel was stained with 0.25% Coomassie blue staining solution (40% methanol and 10% acetic acid) for 1 hour. Gels were destained with water until white clearing could be observed on the Coomassie stained gel. Media was treated with GM6001 to verify that the resultant bands were likely attributable to MMP activity. Media from an aggressive fibromatosis cell culture was diluted 1:1, 1:2, 1:5, and 1:10, and analyzed by zymography to verify that this technique would indeed be able to distinguish between various concentrations.

Western Analysis. Media and cell lysates, under the same conditions used for zymography, were analyzed using Western analysis for expression of MMP-9 and MMP-3. Equal amounts of total protein from normal fibroblasts (NFs) and from aggressive fibromatosis cultures were electrophoresed on an SDS polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Hybridization was carried out overnight using the RM105MMP9 or the RP5MMP3 antibody (Triple Point Biologics, Forest Grove, OR) at a concentration of 1:500 and 1:250, respectively. The antibody was detected using IgG horseradish peroxidase secondary antibody, which was detected using chemiluminescence. The membrane was stripped and reprobed using an antibody to actin (Sigma) as an additional control for protein loading.

Collagen Degradation Assay. Conditioned media from different cell culture samples were prepared as described above. Fifty micrograms of total protein were incubated with 2 μg of DQ-collagen type I and type IV (Molecular Probes). Fluorescent collagen fragments were measured at 490-nm excitation wavelength. Readings were collected at 24 hours after incubation. Collagenase activity was obtained using a standard curve of collagenase activity derived from recombinant collagenases.

Statistical Analysis. Means, SDs, and 95% confidence intervals were calculated for each experiment. Assays using cell cultures were all performed 9 times. Results in this text are given as ± the SD, whereas error bars in figures are given as 95% confidence intervals. The 95% intervals take into account the number of observations for each experimental condition, and as such, significant differences ($P < 0.05$) can be easily observed in the graphical form, as

instances in which the error bars do not overlap the mean of another experimental condition.

RESULTS

Dysregulation of MMPs and TIMP-1 in Murine Aggressive Fibromatosis. *Mmp-3, Mmp-2, Mmp-7, Mmp-9, Mmp-11, Mmp-14,* and *Timp-1* expression were examined using semiquantitative reverse transcription (RT)-PCR comparing expressions among aggressive fibromatosis tumors collected from *Apc*/*Apc1638N* mice and normal fibrous tissues from fascia from the mice, as well as from wild-type littermates. Nine-independent samples of aggressive fibromatosis tumors, fibrous tissue from *Apc*/*Apc1638N* mice, and fibrous tissue from wild-type mice were examined. *Mmp-3* mRNA expression was increased significantly in aggressive fibromatosis tumors compared with normal fibrous tissues (relative densitometry standardized to *Gapdh* is 1.27 $\pm$ 0.02 in aggressive fibromatosis (AF) compared with 0.13 $\pm$ 0.04 in the fascia ($n = 9$ independent tumors and normal fascia, $P < 0.005$). No significant differences were observed in the expression of the other *Mmp*s. *Timp-1* expression was approximately 50% lower in aggressive fibromatosis tumors compared with normal fascia ($n = 9$, $P < 0.05$). The differences in expression of *Mmp-3, Mmp-9,* and *Timp-1* were confirmed using real-time PCR (Fig. 1). There was little sample-to-sample variability in the results, as illustrated by the relatively small 95% confidence intervals. Representative semiquantitative RT-PCR results are shown (Fig. 2, *A* and *B*).

Conditioned media collected from primary cultures of nine independent aggressive fibromatosis tumors, normal fascia from the same mice, and fascia from wild-type mice were analyzed for the presence of caseinolytic proteins using a casein SDS-PAGE gel. A marked increase in the amount of a protein of a size consistent with MMP-3 was observed in the aggressive fibromatosis samples compared with NFs from wild-type and *Apc*/*Apc1638N* mice (Fig. 2*C*). There was a 10-fold increase in the tumors over levels in normal fascia from wild-type mice and a 7-fold increase in the tumors over normal fascia from the *Apc*/*Apc1638N* mice ($n = 9$, $P < 0.005$ and $P < 0.01$, respectively). Western analysis using an antibody to MMP-3 confirmed the increase in protein level between tumors and normal fascia from the *Apc*/*Apc1638N* mice (Fig. 2*D*). Although no differences were observed in *Mmp-9* mRNA expression, casein zymography showed an increase in a band at a size that could be MMP-9. Western analysis, using an antibody to mouse MMP-9, was performed to determine whether MMP-9 protein was present at higher levels in the tumor cells. A slight increase in MMP-9 protein level was found (Fig. 2*D*). This suggests a post-transcriptional mechanism up-regulating MMP-9. Treatment with 10 μmol/L of the broad spectrum MMP inhibitor, GM6001 (33), inhibited the caseinolytic activity, supporting the notion that the bands observed are attributable to MMPs.

Increased Collagen Degradation in Aggressive Fibromatosis Cells. To examine the endogenous activity of MMPs in aggressive fibromatosis, conditioned media collected from primary cultures of AF tumor and skin were used in a collagen degradation assay in which cleavage of type I and type IV collagen released fluorogenic fragments. Conditioned media from aggressive fibromatosis cell cultures showed a significant increase in their ability to degrade type IV collagen, which is a MMP-3 substrate (Fig. 3*A*). The increase in collagen type I processing was more moderate in aggressive fibromatosis-conditioned media in comparison with normal fascial cultures (Fig. 3*B*). These data correspond with the preferential ability of MMP-3 to cleave type IV collagen (34).

MMP Inhibition Decreases Aggressive Fibromatosis Tumor Invasion and Motility. To investigate the effect of MMP inhibition on tumor cell motility and invasion, Boyden's chamber and Matrigel

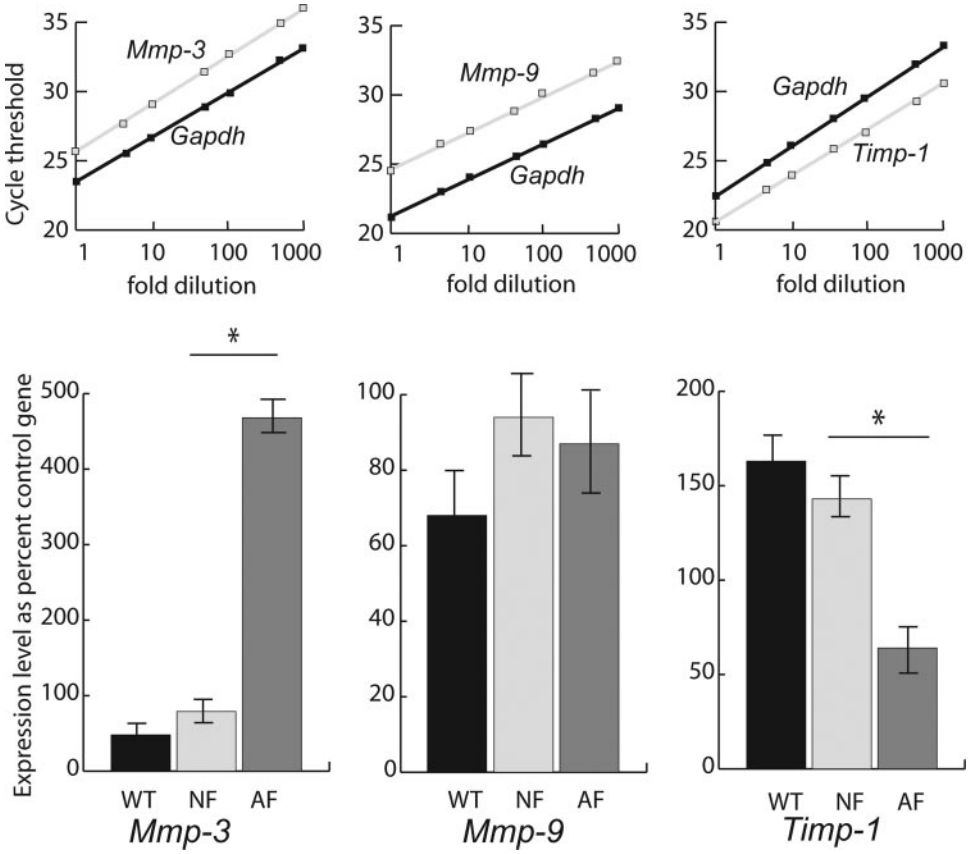

Fig. 1. Real-time RT-PCR data for the expression of *Timp-1, Mmp-3,* and *Mmp-9* in AF and normal fibrous (NF) tissues from *Apc1638N* mice and NF tissues from wild-type (WT) mice. The *top row* shows standard plots of the threshold cycle *versus* fold dilution for various genes of interest and the control gene, *Gapdh*. The *bottom row* shows means and 95% confidence intervals for the level of expression of the genes in comparison with the control gene for analysis from nine independent samples of different mice, *Gapdh*. Significant differences ($P < 0.05$) between expression in the aggressive fibromatosis tumors and in NF tissues are indicated with an *asterisk above a line over the two comparisons*.

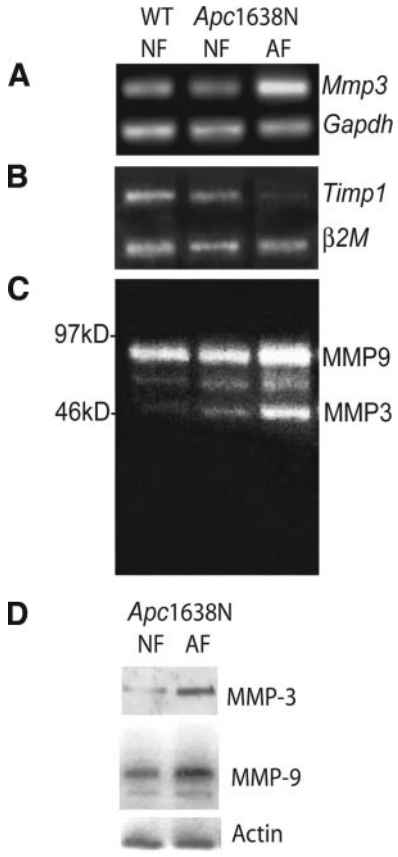

Fig. 2. Murine aggressive fibromatosis tumors exhibit elevated levels of MMPs and decreased levels of Timp-1 compared with normal fibrous tissues. *A*, semiquantitative RT-PCR shows increased expression of *Mmp-3* in AF tumors compared with normal fibroblasts (NFs) from *Apc1638N* mice and wild-type mice (WT). There is a 4-fold increase in *Mmp-3* expression compared with the NFs (1.27 ± 0.02 compared with 0.27 ± 0.08, $n = 9$, in the wild-type NFs and 0.13 ± 0.04, $n = 9$, in fibroblast from *Apc/Apc1638N* mice). *B*, semiquantitative RT-PCR for *Timp-1* from the same tissue types showing a significant decrease in *Timp-1* expression in the tumors. *C*, casein zymogram shows an increase in bands of a size consistent with MMP-3 and MMP-9 in conditioned media from tumors compared with NFs. Treatment with GM6001 completely abolished these bands. *D*, Western analysis for MMP-3 and MMP-9 showing an increase in MMP-3 and MMP-9 protein level in the aggressive fibromatosis over NFs.

assays were used, respectively. Aggressive fibromatosis cells showed a significant increase in their ability to migrate through the Boyden's chamber in comparison with normal fibroblast cells (21.9 ± 3.2 *versus* 12.5 ± 4.3, $n = 9$; Fig. 4*A*). With the addition of the broad spectrum MMP inhibitor GM6001, there was a decline in the number of cells migrating across the membrane in aggressive fibromatosis and normal fibroblast cultures, and there was no difference in the number of cells migrating across the membrane between aggressive fibromatosis and normal fibroblast cultures treated with GM6001 (Fig. 4*A*). Using a Matrigel invasion assay, aggressive fibromatosis cells showed an increased ability to degrade through the matrix in comparison to normal fibroblast cells (Fig. 4*B*). After addition of GM6001, invasion of AF cells was significantly decreased (19.5 ± 4.86 *versus* 8.89 ± 4.42, $n = 9$, for vehicle and GM6001-treated, respectively; Fig. 4*B*). Thus, MMPs play an important role in promoting cell invasion and motility in both neoplastic and normal mesenchymal cells.

In contrast to changes in cell invasion and motility, we found no differences in the proliferation or apoptosis rate after GM6001 treatment in aggressive fibromatosis cultures, as measured using BrdUrd incorporation (21 ± 8 *versus* 19 ± 7, $n = 9$, percentage of cells incorporating BrdUrd), a terminal deoxynucleotidyl transferase-mediated nick end labeling assay (1.8 ± 2.4 *versus* 1.9 ± 2.1, $n = 9$, percentage of positively stained cells), and a flow cytometry technique (30; 2.7 ± 3.4 *versus* 3.0 ± 2.9, $n = 9$, percentage of apoptotic cells).

GM6001 Treatment Decreases the Average Aggressive Fibromatosis Tumor Volume in *Apc/Apc1638N* Mice. Three-month-old male *Apc/Apc1638N* mice were treated with daily s.c. injections of GM6001 (100 mg/kg), as reported previously (29). Control mice received the carrier (DMSO), which was also injected daily. Mice were treated for 4 weeks duration. After termination of treatment, mice were sacrificed and examined for tumor formation as reported previously (10, 28). Male mice were used because they develop significantly more tumors than female mice. The ability of GM6001 to inhibit MMP in the tumors from these mice was verified using casein zymography. The average tumor volume in each mice was significantly reduced in GM6001-treated mice (from 59 ± 28 mm^3 to 22 ± 4 mm^3 per animal, $n = 5$, $P < 0.05$) when compared with animals treated with vehicle alone (Fig. 5). This difference in tumor volume is largely attributed to a decreased number of larger-sized tumors in the GM6001-treated mice. There was no significant difference in the number of aggressive fibromatosis tumors formed by these mice nor was there any difference in the gastrointestinal tumor number or size.

***Timp-1* Overexpression Decreases the Average Volume of Aggressive Fibromatosis Tumors and Increases the Number of Aggressive Fibromatosis Tumors that Form.** *Timp-1*–transgenic mice (27) were crossed with *Apc/Apc1638N* mice. A higher level of *Timp-1* expression in the tumors was confirmed using RT-PCR, where *Timp-1* was increased 50% in tumors from *Timp-1*–transgenic in comparison

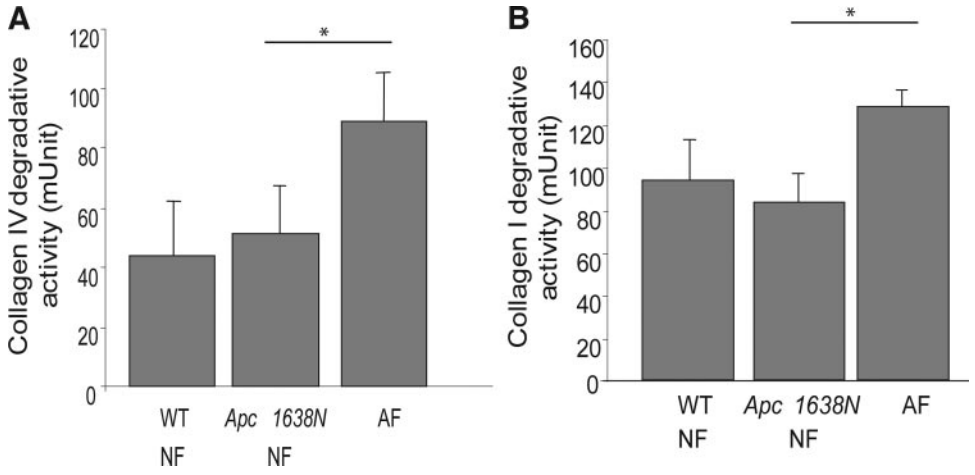

Fig. 3. Cells from murine AF tumors show an increased ability to degrade collagen over NFs. *A*, conditioned media from aggressive fibromatosis showed a significant increase in its type IV collagen degradation ability compared with NFs. *B*, aggressive fibromatosis also showed increased ability to degrade collagen type I compared with NFs. Data are given as means and 95% confidence intervals for data from nine independent tumors. Statistically significant differences ($P < 0.05$) are indicated by an *asterisk over a line above the comparisons*. Data from cells from aggressive fibromatosis tumors are labeled *AF*, normal fibroblasts are labeled *NF*, and cells from wild-type mice are labeled *WT*.

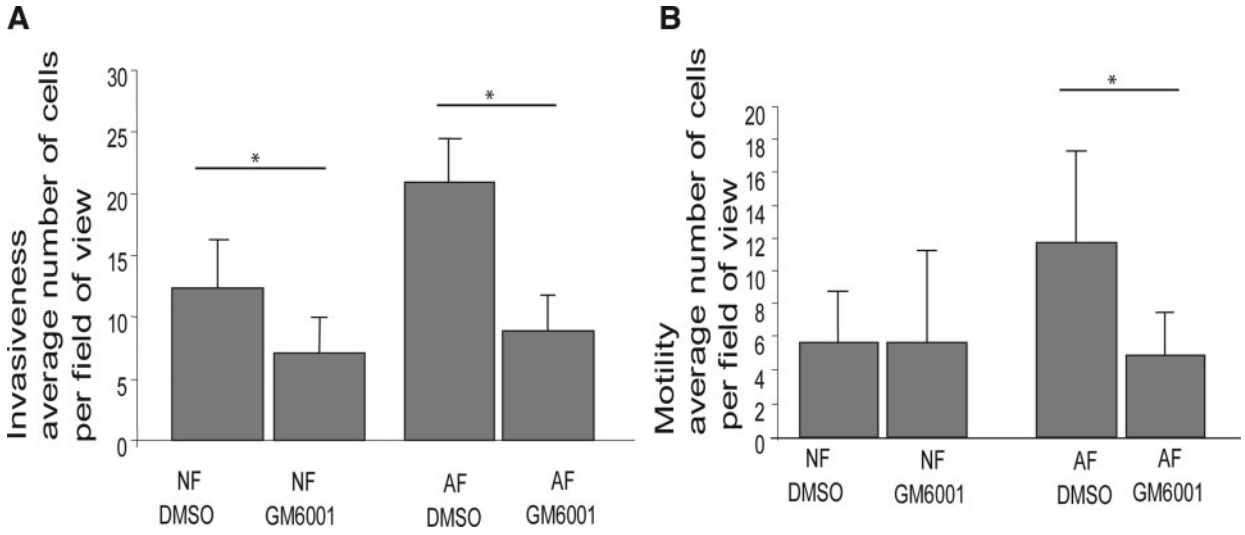

Fig. 4. *A*, inhibition of MMP activity decreased the invasiveness of AF cells as well as NFs as measured by the Matrigel invasion assay (19.5 ± 4.86 cells/field of view *versus* 8.89 ± 4.42 cells/field of view for vehicle, DMSO, and GM6001-treated, respectively, in the case of aggressive fibromatosis, $n = 9$, $P < 0.05$). The number of cells that crossed the membrane was counted by direct visualization of 4′,6-diamidino-2-phenylindole-stained nuclei in six high-power fields. *B*, inhibition of MMP activity in aggressive fibromatosis cells showed a decrease in motility (11.86 ± 5.44 *versus* 4.98 ± 2.55, $n = 9$, $P < 0.05$), as assessed by the number of cells passing through 8-μm polycarbonate filters. Normal fibroblasts did not show a significant difference in cell motility. Data are given as means and 95% confidence intervals (as *error bars*). Statistically significant differences ($P < 0.05$) are indicated by an *asterisk over a line above the comparisons*.

to the non-transgenic littermate (Fig. 6*A*). An inhibition of MMPs was confirmed using casein zymography. Conditioned media collected from *Timp-1* overexpressing aggressive fibromatosis cells showed an almost 3-fold decrease in its ability to degrade fibrillar collagen when compared with conditioned media collected from tumor cells in littermates not overexpressing *Timp-1* (Fig. 6*B*).

Tumor number and size were analyzed and scored as reported previously (10) when the mice reached 6 months of age. These were compared between *Apc/Apc1638N* mice expressing *Timp-1* and littermate *Apc/Apc1638N* mice that did not express *Timp-1*. Surprisingly, male mice developed an increased number of aggressive fibromatosis tumors when compared with the littermate controls (31 ± 13 *versus* 19 ± 7, $n = 10$). However, the tumors in mice overexpressing *Timp-1* were smaller in size than littermate control mice (2.7 ± 1.3 mm^3 *versus* 7.4 ± 4.6 mm^3 volume/tumor, $n = 10$), resulting in an overall

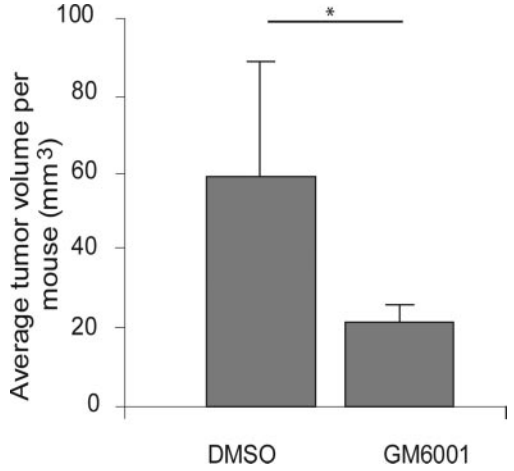

Fig. 5. Treatment of *Apc/Apc1638N* mice with GM6001 decreased the volume of aggressive fibromatosis tumors that formed. Three-month-old male *Apc/Apc 1638N* mice were treated with GM6001at a dosage of 100 mg/kg/day for 3 weeks. A significant difference was observed in the average tumor volume per mouse (58.77 ± 28.4 mm^3/mouse in DMSO-treated *versus* 21.68 ± 4.3 mm^3/mouse in GM6001-treated, $n = 5$). *Error bars* represent 95% confidence intervals. A statistically significant difference ($P < 0.05$) is indicated by an *asterisk above the line over the comparison.*

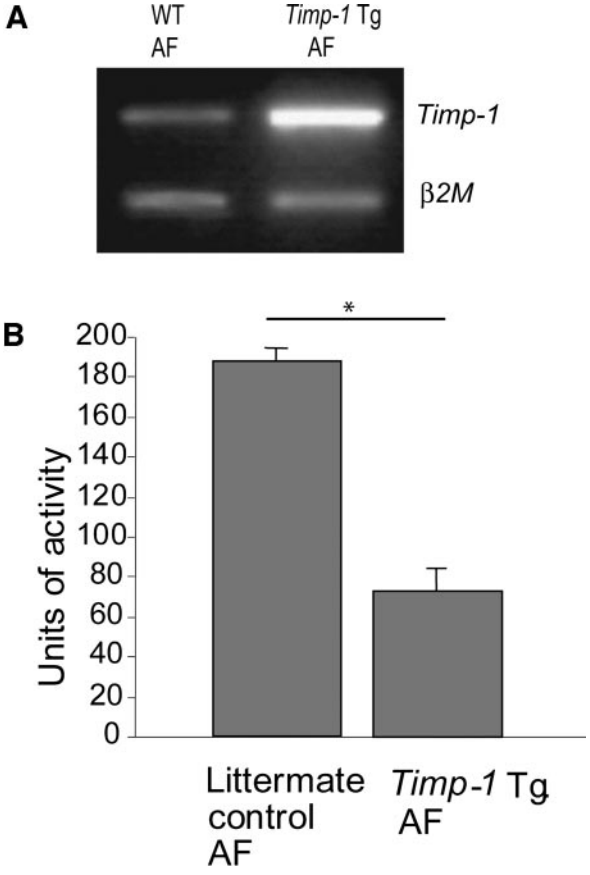

Fig. 6. AF tumors from mice expressing the *Timp-1* transgene have elevated *Timp-1* mRNA levels and show evidence of decreased MMP activity. *A*, semiquantitative RT-PCR showed increased mRNA expression of *Timp-1* in aggressive fibromatosis tumors from *Timp-1*–transgenic *Apc/Apc1638N* mice (*Timp-1* Tg) compared with tumors from their littermate controls that did not express the *Timp-1* transgene (WT). *B*, conditioned media collected from *Timp-1* overexpressing aggressive fibromatosis tumor cells showed an almost 3-fold decrease in its ability to degrade fibrillar collagen type I when compared with conditioned media collected from the tumor cells of mice not expressing the transgene. A statistically significant difference ($P < 0.05$) is indicated by an *asterisk above a line over the comparison*. The data points in *B* are the result of studies from nine independent tumors of different mice ($n = 9$).

decrease in the volume of each tumor and the total tumor volume of each mouse (Fig. 7). Female mice showed a similar decline in tumor volume but did not show a significant difference in tumor number. There were no differences in the size or number of gastrointestinal lesions formed by these mice.

***Timp-1* Overexpression Does Not Affect Aggressive Fibromatosis Cell Proliferation or Apoptosis Rate but Reduces Cell Invasiveness and Motility.** Primary cultures of aggressive fibromatosis cells from both *Timp-1*–transgenic mice and their littermate control were used. Differences in *Timp-1* expression between the cell cultures were confirmed using semiquantitative RT-PCR (Fig. 7*A*). There was no difference in the proliferation rate or apoptosis rate of these cultured cells as measured by BrdUrd incorporation, a terminal deoxynucleotidyl transferase-mediated nick end labeling assay, and a flow cytometry technique (Fig. 8, *B* and *C*). Cell invasiveness and motility, however, were lower in cultures from tumors from the *Timp-1*–transgenic mice (Fig. 8, *D* and *E*).

DISCUSSION

We demonstrated that MMP expression is increased in murine aggressive fibromatosis tumors. The increased MMP activity makes the cells more efficient at processing fibrillar collagens, increases their motility, and increases their ability to invade through Matrigel. Blocking MMP activity, either pharmacologically or by overexpressing *Timp-1*, resulted in *Apc/Apc1638N* mice developing aggressive fibromatosis tumors with a smaller volume. MMP activity did not regulate cell proliferation or apoptosis. These results suggest an important role for MMPs in regulating the ability of tumor cells of mesenchymal origin to locally invade through the extracellular matrix.

Although all cells in *Apc/Apc1638N* mice harbor a mutant *Apc* allele, not all mesenchymal cells become aggressive fibromatosis tumors. One possibility is that secondary genetic events occur in some cells, causing them to go on to become neoplasms. Indeed, previous studies show that the loss of the wild-type *Apc* allele is a frequent event in the aggressive fibromatosis tumors, as well as in other tumors that develop in *Apc* mutant mice (35, 36). β-Catenin protein stabilization seems to be a crucial event in the development of aggressive fibromatosis tumors. Transgenic mice expressing a stabilized form of β-catenin driven by a cytomegalovirus tetracycline-regulated promoter develop aggressive fibromatosis tumors (7), suggesting that β-catenin stabilization is sufficient to cause this tumor type. Aggressive fibromatosis tumors in *Apc* mutant mice have an elevated β-catenin protein level compared with normal fibrous tissues (28), suggesting that the secondary event leading to the development of tumors in *Apc* mutant mice causes β-catenin protein stabilization.

There is a striking difference in the number of aggressive fibromatosis tumors that formed between male and female *Apc/Apc1638N* mice, a finding that is also demonstrated in several previous studies investigating the *Apc/Apc1638N* mice. One possible explanation for this is related to the presence of higher levels of androgens in the male mice. Androgen-receptor activation in several cell types has been shown to activate β-catenin-mediated transcriptional activity (37, 38). This mechanism could play a role in aggressive fibromatosis by elevating β-catenin protein level and thus promoting tumor formation. Aggressive fibromatosis tumor cells may also be responsive to estrogen or androgen, independent of an effect on β-catenin protein level, and these hormones may regulate the expression of other genes important in tumor initiation or progression. Indeed, human aggressive fibromatosis tumor cells express estrogen receptors, which could activate cell signaling pathways regulating tumor cell growth (39). We did not observe the same increase in tumor number associated with expression of the *Timp-1* transgene in the female mice as in male mice. The difference we observed between female and male mice in the response of the neoplastic phenotype to overexpression of *Timp-1* is not a phenomenon limited to *Timp-1*. Differences in the change in neoplastic phenotype between male and female mice have also been

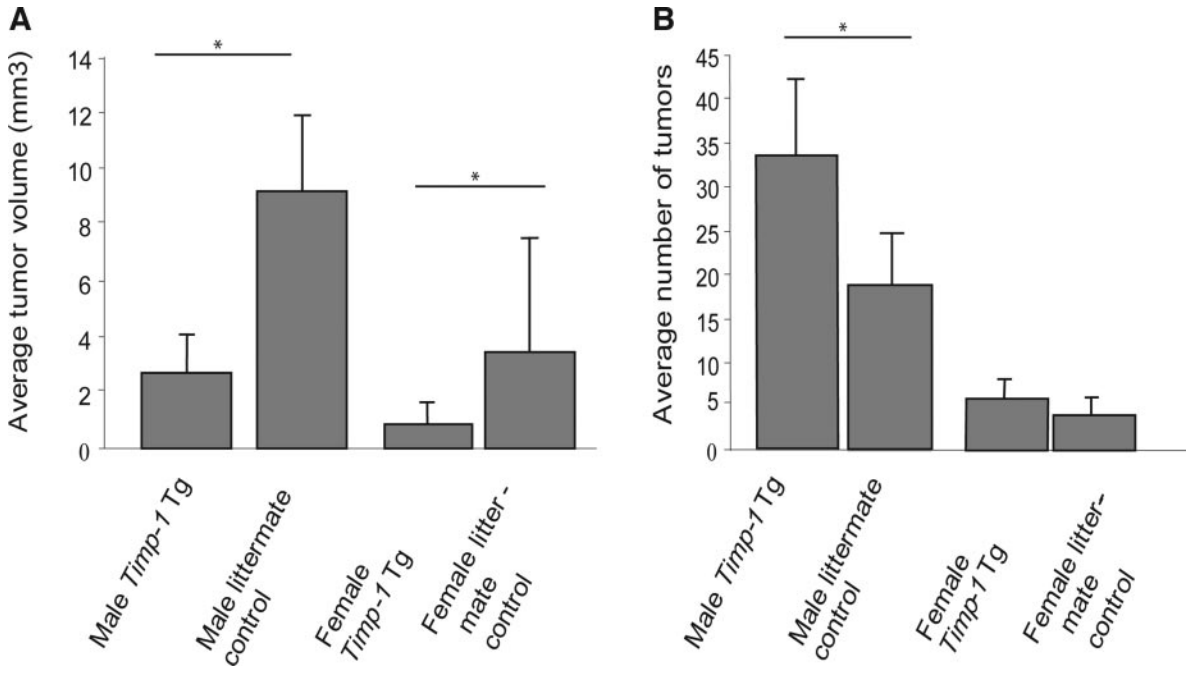

Fig. 7. Size and number of aggressive fibromatosis tumors formed in mice expressing the *Timp*-1 transgene compared with controls. *A*, 6-month-old male and female *Timp-1*–transgenic mice have a decreased volume per tumor compared with littemate controls that did not express the *Timp-1* transgene ($n = 10$; $P < 0.02$ for both male and female mice). *B*, the average number of aggressive fibromatosis tumors in male *Timp-1*–transgenic mice was higher than their littermate controls. Data are given as means and 95% confidence intervals (shown as *error bars*). Statistically significant differences ($P < 0.05$) are indicated by an *asterisk over a line above the comparisons*. Female mice did not show a significant difference in tumor numbers.

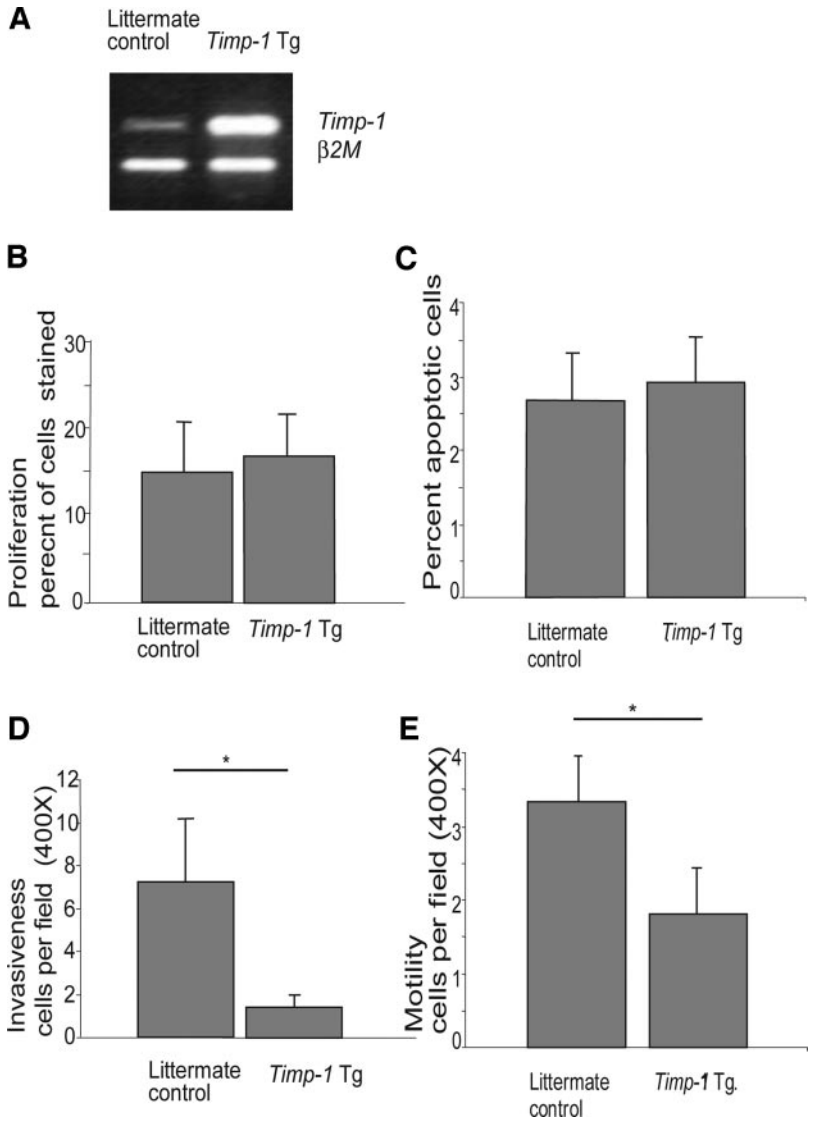

Fig. 8. Expression of the *Timp-1* transgene decreases cell motility and invasiveness in murine aggressive fibromatosis. *A*, cells in culture derived from mice expressing the *Timp-1* transgene (*Timp-1* Tg) expressed *Timp-1* at higher levels than cells from mice not expressing the transgene, as determined using semiquantitative RT-PCR. *B* and *C*, no significant difference in cell proliferation (percentage of cells exhibiting BrdUrd incorporation as shown in *B*) or apoptosis (percentage of apoptotic cells detected using flow cytometry as shown in *C*) between tumor cells expressing the *Timp-1* transgene and tumor cells not expressing the transgene. *D*, *Timp-1* overexpressing aggressive fibromatosis cells showed a decrease in motility, as assessed by the number of cells passing through 8-μm polycarbonate filters compared with littermates that did not express the transgene. *E*, *Timp-1* overexpressing aggressive fibromatosis cells showed decreased invasiveness as measured by Matrigel invasion assays, compared with tumor cells from mice not expressing the transgene. Data are given as means and 95% confidence intervals (as shown in the *error bars*). Statistically significant differences ($P < 0.05$) are indicated by an *asterisk above a line over the comparisons*. Each data point is the result of studies on cell cultures from nine independent tumors of different mice ($n = 9$).

observed in crossing the *Apc/Apc1638N* mice with mice deficient in either cycloxygenase-2 or the receptor for hyaluronan-mediated motility (28, 40). This may relate in part to the smaller number and size of the tumors that form in female mice, but a relatively high degree of variability in tumor number, which requires larger numbers of animals to show statistically significant differences. Another possibility is that the factors responsible for the difference in tumor number and size between male and female mice (*e.g.*, androgen levels) also modulate the ability of TIMP-1 to alter the tumor phenotype.

The level of expression of *Mmp*s is higher in murine aggressive fibromatosis tumors than in normal fibrous tissue from the same mice. In addition, the level of expression of some of the *Mmp*s is also higher in the normal fibrous tissues from the *Apc/Apc1638N* mice than from wild-type mice. This finding shows that a heterozygous *Apc* mutation on its own is insufficient to cause the level of *Mmp* up-regulation seen in the tumors. It is likely that the genetic event(s) in addition to the heterozygous *Apc* mutation that are necessary for the formation of aggressive fibromatosis tumors is also responsible for the elevated level of *Mmp*s. Although it is possible that the secondary event could vary from tumor to tumor, the low variability in the level of expression of the various *Mmp* family members evaluated in 10 independent tumors suggests that whatever these events are, they alter the level of expression of these family members in a relatively consistent manner.

The elevated protein level of β-catenin in aggressive fibromatosis tumors that form in *Apc/Apc1638N* mice (28) and the associated activation of tcf-dependent transcription are likely explanations for the regulation of the various *Mmp*s. Indeed, *Mmp-3*, the predominant *Mmp* expressed in murine aggressive fibromatosis tumors, is a direct target of β-catenin-mediated tcf-dependent transcription, and there are several putative T-cell factor-binding sites in the promoter of *Mmp-3* gene (41).

MMP-3 has a broad spectrum of potential activity in neoplasia. Its substrates include fibronectin, laminin, elastin, collagen IV, and proteoglycans (42, 43). It also directly processes growth factors such as insulin-like growth factor binding proteins, thus having the potential to act as an autocrine or paracrine factor affecting different cellular processes (44). MMP-3 also activates other MMPs such as MMP-9, as shown in a study in which overexpressing cell cultures needed to be supplemented with active MMP-3 to initiate the activation of pro-MMP-9 (45). This is perhaps part of the explanation for increased MMP-9 protein level by zymography, despite finding no difference in expression at the mRNA level in aggressive fibromatosis. MMP-3 can act as a tumor promoter, as demonstrated in the breast, where overexpression of MMP-3 in breast epithelial cells led to the development of premalignant and malignant lesions in the mammary glands of transgenic animals without external stimuli (23). Thus, there are many

ways in which MMP-3 activity could promote a more invasive phenotype in cells from aggressive fibromatosis.

The broad spectrum MMP inhibitor, GM6001, and expression of *Timp-1* both decreased tumor volume in the *Apc/Apc*1638N mice, suggesting an important role for MMP activity in tumor growth in aggressive fibromatosis. Surprisingly, however, mice overexpressing *Timp-1* also developed a significantly greater number of tumors. There are several potential explanations for the different effects observed between chemical and endogenous MMP inhibition. Our data are reminiscent of data in the Min mouse, in which treatment with an MMP inhibitor decreased tumor multiplicity, although overexpression of *Timp-1* increased tumor multiplicity (46). This was thought perhaps attributable to the concentration of TIMP-1, because there is a growth-promoting activity of TIMP-1 observed in the picomolar range, but not in the nanomolar range, which is required for MMP inhibition. However, our data showing no difference in cell proliferation or apoptosis in aggressive fibromatosis tumor cells overexpressing *Timp-1* seems to indicate that this explanation does not hold true for this tumor type. The temporal difference in MMP inhibition may account for the increased number of tumors in *Timp-1*–transgenic mice, because GM6001 was administered to 3-month-old mice, by which time the tumor initiating process has already begun. *Timp-1*, on the other hand, is expressed throughout the development of the mice and during the tumor-initiation phase. MMPs may thus play different roles at different stages of AF tumor development and progression. Another possibility is that TIMP-1 may promote tumor formation independently of its MMP-inhibitory ability. TIMP-1 was first identified as a growth factor with erythroid-potentiating activity, and *in vitro* evidence shows that TIMP-1 is able to induce growth of a variety of normal and malignant cells, including erythroid progenitor cells (47, 48). TIMP-1 can also suppress programmed cell death *in vitro* and promote tumor growth *in vivo* in malignant Burkitt lymphoma cells. In addition, TIMP-1 has been shown to be an immunosuppressive factor, inhibiting T-cell cytotoxicity against autologous cells presenting tumor-associated antigens in Hodgkin lymphomas (49). These activities of TIMP-1 may be independent of its function in MMP inhibition. Our data show that TIMP-1 does not affect aggressive fibromatosis cell proliferation or apoptosis, but it is possible that the effects of TIMP-1 as an immunosuppressive factor may play a role in AF tumorigenesis, potentially by bypassing immune surveillance. Another possibility is that TIMP-1 also induces the growth of mesenchymal progenitor cells in addition to erythroid progenitor cells, which may increase the number of cells that have the potential to become aggressive fibromatosis tumors.

We found that MMP activity is important for aggressive fibromatosis tumors to grow in size. It is likely that degradation of the extracellular matrix is required for these tumor cells to migrate and invade into surrounding areas. The results of MMP inhibition in clinical trials for cancer have been disappointing. In many cases, patients received MMP inhibitors when the cancer is advanced, despite animal studies showing that MMP inhibitors may reduce tumor burden best if administered early in tumor progression. Because aggressive fibromatosis is a locally invasive and not metastatic tumor, it may represent a situation analogous to the early stages of malignancy. In addition, because the tumor does not metastasize, limiting its growth and preventing symptoms can be an effective therapy. As such, MMP inhibition is likely to be an effective therapy in aggressive fibromatosis by slowing tumor progression, as shown in this work. Perhaps the use of an MMP inhibitor along with other drugs known to attenuate the tumor phenotype in aggressive fibromatosis, such as cyclooxygenase inhibitors, would be an effective multi-drug regimen.

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