Involvement of specific matrix metalloproteinases during tumor necrosis factor/IFNγ–based cancer therapy in mice

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Abstract

The potent antitumor activity of tumor necrosis factor (TNF) in combination with IFN-γ can only be applied in local regimens due to their strong proinflammatory properties. It has been shown that the broad-spectrum matrix metalloproteinase (MMP) inhibitor BB-94 protects against TNF/IFNγ-induced toxicity without blocking the antitumor effect. Here, we tried to explain this protective role of BB-94 and sought to assign roles to specific MMPs in TNF/IFNγ-induced toxicity. By studying the expression of MMP genes in different organs and in the tumor, we observed that the expression levels of MMP-7, MMP-8, MMP-9, and MMP-12 and tissue inhibitor of metalloproteinase-4 are clearly up-regulated in the liver during therapy. MMP-8 and MMP-9 are also up-regulated in the lung and kidney, respectively. In the tumor, most MMP genes are expressed, but only MMP-3 is up-regulated during TNF/IFNγ treatment. Using MMP-deficient or double-deficient mice, we have shown a mediating role for MMP-3 during TNF/IFNγ treatment in tumor-free and B16BL6 melanoma-bearing mice. By contrast, MMP-12 seemed to have some protective role in both models. However, because most phenotypes were not extremely outspoken, we have to conclude, based on the set of MMP-deficient mice we have studied, that inhibition of a single MMP will probably not increase the therapeutic value of TNF/IFNγ, but that rather, broad-spectrum MMP inhibitors will be required. [Mol Cancer Ther 2007;6(9):2563–71]

Introduction

About 30 years ago, a soluble cytokine termed tumor necrosis factor (TNF) was identified as a factor that is produced upon activation of the immune system. Soon, it was discovered that this cytokine can exert considerable cytotoxicity on many tumor cell lines (1) and can cause tumor necrosis in animal model systems (2). Moreover, TNF was the first cytokine to be used in cancer biotherapy. Unfortunately, systemic application of high-dose TNF is excluded due to severe cardiovascular side effects. Furthermore, systemic administration of the maximum tolerated dose to humans is not associated with significant antitumor activity, as shown by phases I-II trials (3). However, pioneering work by Lejeune et al. on isolated limb perfusion led to the establishment of a protocol for local high-dose TNF applications that are associated with manageable systemic side effects (4–7). Use of isolated limb perfusion with a combination of TNF and chemotherapy was approved in 1998 for limb salvage of unresectable soft tissue sarcoma. However, the impracticality of administering TNF systemically prevents assessment of this cytokine's effectiveness in terms of overall patient survival, which depends mainly on metastatic spread throughout the body and, thus, is not affected by locoregional treatments. In this respect, the development of novel and safe systemic antitumor therapies based on TNF are still needed; our research is focused on this need.

During the last decade, it has been shown that matrix metalloproteinases (MMP) are associated with all stages of tumor development and metastasis. MMPs are zinc- and calcium-dependent proteolytic enzymes, which regulate various cell behaviors relevant to cancer biology, including cancer cell growth, differentiation, apoptosis, migration and invasion, and the regulation of tumor angiogenesis and immune surveillance (8, 9). Tumor-promoting effects have also been reported for the tissue inhibitors of MMPs (TIMP), although extensive research on the role of TIMPs in cancer have shown that TIMPs also have a pronounced antitumor potential (10, 11).

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Thus, TIMPs can be double-edged swords displaying both antitumor efficacy and tumor-promoting effects.

Using a broad-spectrum metalloproteinase inhibitor (BB-94), which inhibits MMPs and also other metalloproteinases, and using MMP-deficient mice, we have shown that MMPs also play a mediating role in a model of TNF-induced hepatitis. Furthermore, BB-94 confers protection against the lethal effects of TNF/IFNγ in tumor-bearing mice without interfering with the antitumor activity (12). This indicates that MMP inhibitors might be effective for increasing the therapeutic potential of systemic TNF/IFNγ antitumor therapy. Unfortunately, late after the end of the therapy, some mortality occurred, and some tumors relapsed. In addition, BB-94 is insoluble and a nonselective, broad-spectrum inhibitor that would target potentially protective MMPs as well. Therefore, the primary aim of this research was to identify the relevant lethality-mediating MMP(s) with the idea of developing specific inhibitors in the future. To determine the specific MMPs involved in the TNF/IFNγ therapy, we used two strategies. First, we used reverse transcription-PCR (RT-PCR) to assess the expression of MMPs and TIMPs during treatment with TNF/IFNγ. Second, we studied the response to TNF/IFNγ in several tumor-free and B16BL6 melanoma-bearing MMP-deficient mice.

Our studies show that in the TNF/IFNγ antitumor-model, several MMP genes are up-regulated in the liver. In the tumor, most MMP genes are expressed, but only MMP-3 is up-regulated during TNF/IFNγ therapy. MMP-3 seems to be an important mediator, and MMP-12 seems protective in the TNF/IFNγ-based models.

Materials and Methods

Animals

Mice deficient in individual MMP genes were generated by gene targeting as previously described (13–16) or provided by Dr. S. Itohara (MMP-2) and Dr. J. Mudgett (MMP-3). All mutants were backcrossed for at least six generations into a C57BL/6J@ICO background using mice purchased from Charles River France (ICO), after which heterozygotes were intercrossed. Homozygous knock-out and wild-type mice were identified and used for further breeding. All strains were then moved by embryo transfer to a specific pathogen-free animal facility.

MMP-2/9 and MMP-8/9 double-deficient mice with C57BL/6J@ICO background were generated by interbreeding the MMP-2 or MMP-8 knock-out strain with the MMP-9 knock-out strain.

Mice were maintained in a temperature-controlled, air-conditioned animal house with 14- to 10-h-light/dark cycles, and they received food and water ad libitum. They were used between the age of 8 and 15 weeks. All experiments were approved by the local ethics committee.

Cytokines

Recombinant murine TNF and IFNγ were expressed in *Escherichia coli* and purified in our laboratory. TNF had a specific activity of 1.0×10^9 IU/mg. IFNγ had a specific activity of 1.1×10^9 IU/mg. Endotoxin presence in the cytokine preparations could not be detected by a *Limulus amoebocyte* lysate assay.

Tumor Cell Culture

The murine B16BL6 melanoma cell line was a gift from M. Mareel (Ghent, Belgium) courtesy of I. Fidler (Houston, TX). B16BL6 cells were grown at 37°C in DMEM containing 10% FCS, 1% Na-pyruvate, 1% D-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Cells were harvested by treatment for 15 min with enzyme-free cell dissociation buffer (Life Technologies, Invitrogen). After three washes in lipopolysaccharide-free PBS (Life Technologies, Invitrogen), cells were counted, and their concentration was adjusted to 6×10^6 cells/mL. About 100 μL of this solution was injected s.c. in the shaved right thigh.

Disease Models

Because the B16BL6 melanoma expresses most MMP genes, we first studied the effect of daily TNF/IFNγ treatment on a background of complete MMP deficiency. Second, we inoculated the mice with B16BL6 melanoma tumor cells 10 days before starting the treatment. This is a b-restricted tumor that grows in all of our MMP-deficient mice, which were backcrossed into the C57BL/6 b-restricted background. In both models, a mixture of TNF and IFNγ (0.1 mL) is injected s.c. into the shaven, right hind leg for 10 consecutive days. Mortality and tumor size index (TSI) were scored daily. TSI was defined in square millimeters as $a \times b$, with a being the longest diameter of the tumor and b being the longest diameter perpendicular to a.

Semiquantitative RT-PCR

Each of the 36 mice was inoculated with a B16BL6 melanoma tumor. After 10 days, tumor-bearing mice were divided randomly in two groups. One group was treated with 100 μL PBS, and the other group was treated with 6 μg TNF + 5,000 units of IFNγ. After 2, 4, and 6 days of treatment, liver, small intestine, colon, kidneys, lungs, heart, and spleen were isolated, each time of six PBS- and six TNF/IFNγ-treated mice. Total RNA was extracted from all organs using the RNeasy kit from Qiagen. RNA concentrations were determined by measuring absorbance at 260 nm. RNA was stored at −80°C until reverse transcription was done. First-strand cDNA was prepared from 1 μg total RNA using an oligo-dT primer and reverse transcriptase (Superscript; Invitrogen). For semiquantitative RT-PCR, cDNA was diluted 10-fold, and 10 μL of cDNA was amplified using oligonucleotide primers specific for the relevant MMP or actin.

The primer sequences, annealing times and temperatures, and the number of cycles and product sizes are shown in Table 1. We used a 30-s melting step at 94°C and a 30-s extension step at 72°C.

The housekeeping gene actin was also amplified as an internal control for the comparison of the target genes.

After completion of the PCR, 10 μL of each reaction was analyzed by agarose gel electrophoresis (1.5% gel) followed by ethidium bromide staining. The pictures

Table 1. Sequence of primers used for the RT-PCR assays

Gene		Sequence (5′-3′)	Annealing (s, °C)	Number of cycles	Product size (bp)
TIMP-1	S	GGCATCCTCTTGTTGCTATCACTG	20, 68	35	174
	AS	GTCATCTTGATCTCATAACGCTGG			
TIMP-2	S	CTCGCTGGACGTTGGAGGAAAGAA	20, 68	43	161
	AS	AGCCCATCTGGTACCTGTGGTTCA			
TIMP-3	S	CTTCTGCAACTCCGACATCGTGAT	20, 68	43	216
	AS	CAGCAGGTACTGGTACTTGTTGAC			
TIMP-4	S	ACTTGCTATGCAGTGCCATG	30, 60	35	144
	AS	TCGGTACCAGCTGCAGATG			
MMP-2	S	AGATCTTCTTCTTCAAGGACCGGTT	20, 68	43	225
	AS	GGCTGGTCAGTGGCTTGGGGTA			
MMP-3	S	ATTGCATGACAGTGCAAGGG	40, 57	40	673
	AS	TGGAGGACTTGTAGACTGGG			
MMP-7	S	TGGAGTGCCAGATGTTGCAG	30, 55	44	519
	AS	TTTCCATATAACTTCTGAATGCCT			
MMP-8	S	ACATCAACATTGCTTTCGTCT	40, 57	36	476
	AS	TGAGGTCAACTGTTCTCAGC			
MMP-9	S	CCCACATTTGACGTCCAGAGAAGAA	30, 63	43	208
	AS	GTTTTTGATGCTATTGCTGAGATCCA			
MMP-10	S	TCCCGAGCCTGAATTTCAT	30, 60	47	76
	AS	AGCCTCATAGGCAGCATCTAA			
MMP-11	S	ATTTGGTTCTTCCAAGGTGCTCAGT	30, 63	38	155
	AS	CCTCGGAAGAAGTAGATCTTGTTCT			
MMP-12	S	TTGATGGCAAAGGTGGTACA	45, 55	40	236
	AS	AGCAGAGAGGCGAAATGTGC			
MMP-13	S	ATGATCTTTAAAGACAGATTCTTCTGG	30, 63	52	203
	AS	TGGGATAACCTTCCAGAATGTCATAA			
MMP-14	S	GGCCTGGAACATTCTAACGA	30, 55	40	331
	AS	GCATTGGGTATCCATCCATC			
MMP-15	S	GGTACATGTGAAAGCCAACCT	30, 60	47	121
	AS	GTACCAGCCCAGCTTCTCAG			
MMP-16	S	ATCATGGCCCCATTTTATCA	45, 51	40	352
	AS	GCATTGGGTATCCATCCATC			
MMP-17	S	GCCGGGATACTGTGCGT	30, 55	44	519
	AS	CTACCTCGTGGAAGTTCAAGG			
MMP-19	S	TGGGCCACTGGAGAAAGAAG	30, 60	35	157
	AS	TCAGCCCAACCAGCTTTCAC			
MMP-23	S	TTCCCCATTCAGTTTCCGTG	30, 60	35	147
	AS	AAGAAAGCGTGGGCCAGTT			
MMP-24	S	TGAAGAGGTGCCATACCATGA	45, 55	35	124
	AS	ATGGGCTAGGAATCCCCCTT			
Actin	S	CAGGAGGAGCAATGATCTTG	30, 55	25	220
	AS	TTCCGATGCCCTGAGGCTCT			

were scanned (ImageMaster VDS; LISCAP Capture Application, v1.0), and intensities of bands were divided by intensities of the corresponding actin signals (measured with ImageMaster TotalLab, v1.0). Mean and SD of control and treated samples were calculated and compared. RT-PCRs for actin and for each MMP were repeated at least twice.

Zymography

Each of the 12 mice was inoculated with a B16BL6 melanoma tumor. After 10 days, tumor-bearing mice were divided randomly in two groups. One group was treated with PBS and the other group with 6 μg TNF + 5,000 units of IFNγ. After 4 days of treatment, livers were isolated and homogenized. Protein was extracted from the livers, and after normalization of protein concentrations, zymography was done. The samples were separated by electrophoresis in a 10% SDS-PAGE gel containing 1 mg/mL gelatin as a substrate. Gels were then washed for 30 min with 2.5% Triton X-100 and incubated at 37°C for 48 h in 50 mmol/L Tris-HCl (pH, 7.5) containing 10 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, and 0.1% Triton X-100 while gently shaking. Gels were stained with Coomassie brilliant blue and destained.

Statistical Analysis

Survival curves (Kaplan-Meyer plots) were compared using a log-rank test. Final mortality rates were compared

with a χ^2 test. Means ± SD were compared with a Student's t test. *, **, and *** represent $0.01 < P < 0.05$, $0.001 < P < 0.01$ and $P < 0.001$, respectively.

Results

Expression of MMPs in Tumor and Organs during TNF/IFNγ Treatment

TNF is a very strong proinflammatory trigger that leads to activation of the transcription factor NF-κB, binding sites for which have been identified in the promoters of several MMP-encoding genes, e.g., those encoding MMP-1, MMP-3, MMP-9, MMP-14, and MMP-19 (8). Furthermore, MMPs are induced by TNF during liver toxicity (17). Therefore, we investigated whether we could identify significant differences in the expression level of MMPs in liver and other organs during TNF/IFNγ treatment. Expression of the four known TIMPs was also studied. Semiquantitative RT-PCR was done to determine whether normal mouse organs contain mRNAs of several MMPs and TIMPs, and whether this expression is modulated during treatment of tumor-bearing animals with PBS or TNF/IFNγ. The organs and tumors were harvested on days 2, 4, and 6 after the start of the TNF/IFNγ therapy, and total RNA was prepared. We isolated liver, small intestine, colon, kidneys, lungs, heart, and spleen. In addition, the organs of three tumor-free mice were isolated as controls.

As shown in Fig. 1A (which shows the situation on the fourth day of treatment), MMP-9 mRNA was found in livers of tumor-free mice, whereas MMP-7, MMP-8, MMP-12, and TIMP-4 were not detected. Furthermore, when mice were inoculated with a B16BL6 tumor and 10 days later treated daily with PBS, gene expression of these MMPs did not change. However, during treatment of tumor-bearing mice with TNF/IFNγ, expression of MMP-7, MMP-8, MMP-12, and TIMP-4 was induced, and MMP-9 was clearly up-regulated after 4 days. This expression pattern was also observed after either 2 or 6 days of treatment (data not shown). We also observed expression of MMP-14, MMP-16, MMP-19, TIMP-1, and TIMP-2 in the liver, but their expression did not change during treatment. Other MMPs and TIMP-3 were not expressed in this organ.

The expression profiles of MMPs and TIMPs in the liver and in the other organs are summarized in Fig. 1B. MMP-2, MMP-12, MMP-16, and TIMP-2 are constitutively expressed, and with the exception of MMP-12, their expression profiles were changed by neither tumor inoculation nor treatment with TNF/IFNγ. All other MMPs and TIMPs were expressed in at least one of the organs, with the exception of MMP-15. Whenever or wherever MMPs and TIMPs were expressed, most of them did not change in expression during the treatment. However, MMP-9 was up-regulated in the kidney and MMP-8 in the lung (Fig. 1A).

Although most MMPs and TIMPs were expressed in the tumor, with the exception of MMP-7, MMP-8, MMP-11, MMP-15, MMP-24, and TIMP-3 (Fig. 1B), only MMP-3 was significantly up-regulated during therapy (Fig. 1A).

We also examined MMP-2 and MMP-9 protein levels in the liver using gelatin zymography. An overall increase in the levels of MMP-2 and MMP-9 was observed in livers of mice treated for 4 days with TNF/IFNγ when compared with control and PBS-treated livers. However, this was not always the case; levels of MMP-2 and MMP-9 were also increased in one (out of five) PBS-treated sample, whereas levels of both MMPs were not increased in two (out of five) TNF/IFNγ-treated mice. In addition, bands corresponding to their activated forms were also evident (Fig. 1C).

Response of Tumor-Free MMP-Deficient Mice to TNF/IFNγ

Because MMP-deficient mice inoculated with an MMP-expressing tumor (Fig. 1B) cannot be considered as completely deficient for that particular proteinase, we first injected tumor-free knock-out mice with TNF/IFNγ to assess the response of each mouse strain to daily treatments with TNF/IFNγ.

Most tumor-free MMP knock-out mice did not differ from their wild-type counterparts in their response to TNF/IFNγ. However, we observed that mice knocked out for MMP-7, MMP-9, and MMP-12 were slightly sensitized to treatment with TNF/IFNγ (Fig. 2, ns). In contrast, tumor-free MMP-3 knock-out mice were significantly and reproducibly protected against the daily treatment with TNF/IFNγ (Fig. 3; ***, $P = 0.0003$).

Response of Tumor-Bearing MMP-Deficient Mice to TNF/IFNγ

Because the establishment of tumors requires efficient metalloproteinase-mediated invasion and angiogenesis, we evaluated the effect of MMP deficiency on tumor growth. B16BL6 tumors were inoculated into the different MMP-deficient mice, and their growth was monitored for 16 days. The tumor growth rates were not significantly different in most of the different MMP knock-out mice compared with those of the respective wild-type animals (data not shown). However, the tumor growth rate of MMP-2 knock-out mice was significantly slower than that of their wild-type counterparts (data not shown). This had also been observed by the group of Dr. Itoh (18). In addition, during tumor growth and in the absence of TNF/IFNγ, there was no difference in the survival rate of the different MMP knock-out and wild-type mice tested.

The primary aim of this experiment was to examine the effect of MMP deficiency on the lethal outcome of antitumor therapy with TNF/IFNγ. Tumors were grown by s.c. inoculation of B16BL6 melanoma cells, and 10 days later, the mice were treated daily for 10 consecutive days with 10 or 12.5 μg TNF, combined with 5,000 units of IFNγ. MMP deficiency had no effect on TNF/IFNγ-induced tumor regression (data not shown), and again, no real difference was seen in the survival of most MMP-deficient and double-deficient mice compared with their wild-type counterparts (Fig. 3). However, MMP-2 knock-out mice showed an increased susceptibility to the side effects of the therapy. Moreover, the MMP-12 knock-out mice were again more susceptible than their wild-type counterparts.

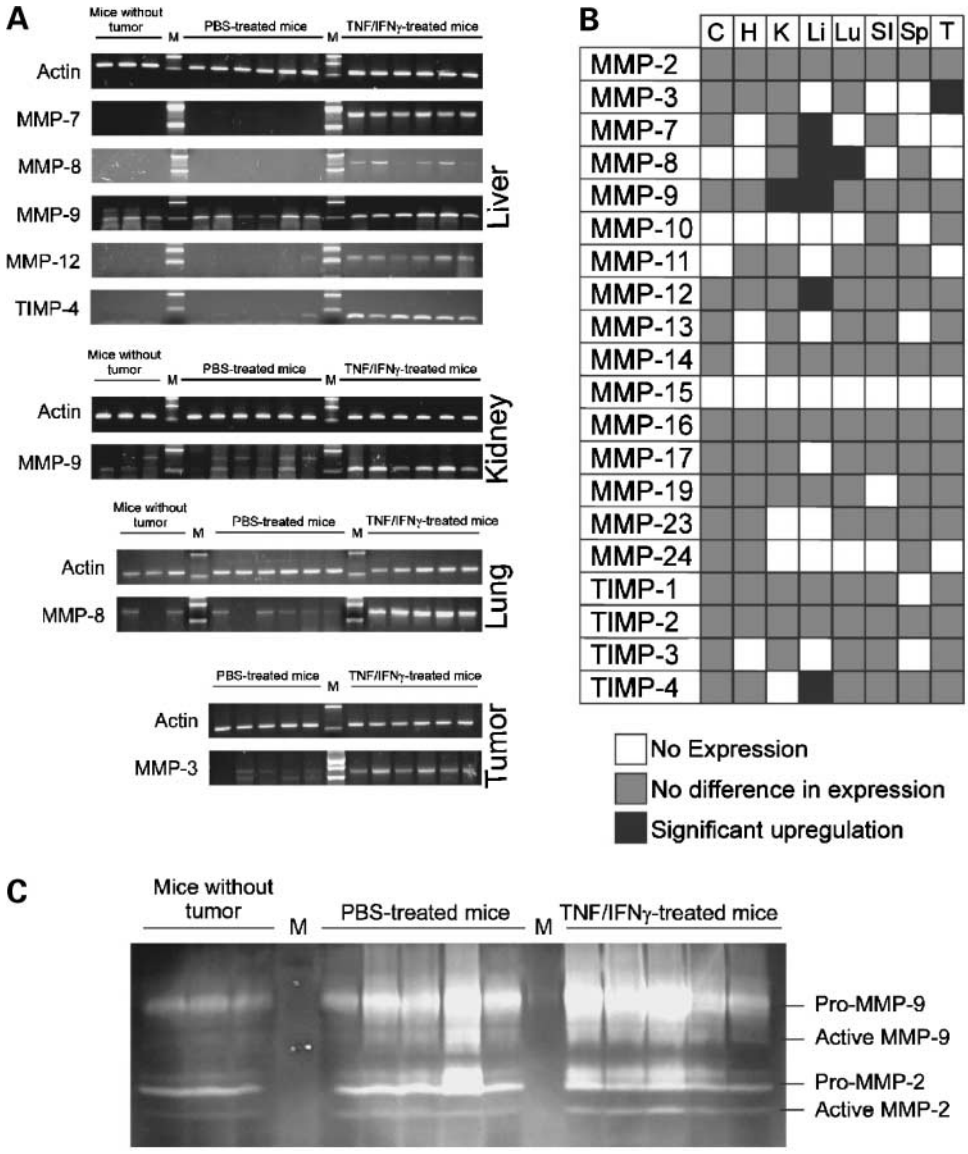

Figure 1. MMP expression during TNF/IFNγ treatment. Tumor-bearing mice were treated with PBS ($n = 6$) or with 6 μg TNF/5,000 units of IFNγ ($n = 6$). After 4 d of treatment liver (*Li*), small intestine (*SI*), colon (*C*), kidneys (*K*), lungs (*Lu*), heart (*H*), spleen (*Sp*), and tumor (*T*) were isolated. The organs of three tumor-free mice were also isolated as a control. Total RNA was extracted from all organs and semiquantitative RT-PCR was done for 23 MMPs and the 4 known TIMPs. **A,** RT-PCR reactions were analyzed by agarose gel electrophoresis and revealed by ethidium bromide staining. **B,** expression profiles of MMPs and TIMPs in different organs. Intensities of bands were measured and divided by intensities of the corresponding actin signals. Mean and SD of PBS- and TNF/IFNγ-treated samples were calculated and compared. □, no expression; ■, no significant difference in mean expression compared with PBS-treated samples; ■, significant up-regulation of expression compared with PBS-treated samples. **C,** protein was extracted from homogenized livers, and gelatin zymography was done on livers of tumor-free control mice ($n = 3$), livers of tumor-bearing mice treated for 4 d with PBS ($n = 6$), and livers of tumor-bearing mice treated for 4 d with 6 μg TNF/5,000 units of IFNγ ($n = 6$).

In addition, MMP-3 knock-out mice were slightly protected against the 10-day treatment with TNF/IFNγ, although not significantly (Fig. 3).

Discussion

There is still a need for development of novel and safer systemic antitumor therapies based on TNF. One possible strategy includes the elucidation and inhibition of critical mediators responsible for the TNF-induced toxicity. We have previously shown that the broad-spectrum MMP inhibitor BB-94 may be useful for this purpose. BB-94 has been reported to inhibit almost all MMPs (19, 20) and some members of the ''a disintegrin and metalloprotease domain'' (ADAM) family (21, 22). We have shown that, when using BB-94 during systemic TNF/IFNγ therapy, it prevents mortality in tumor-bearing mice without interfering with the antitumor activity of TNF/IFNγ (12). However, the protection conferred by BB-94 is not absolute because late deaths occurred in some experiments. This may have been due to some toxicity of BB-94 itself, its poor solubility and pharmacokinetics, or its inhibition of protective molecules. In addition, some tumors relapsed after treatment was stopped. Therefore, we sought to identify the individual MMPs that are expressed in our system and to determine the outcome of TNF/IFNγ treatment in mice lacking a specific MMP. Because tumors are supposed to produce MMPs, we used TNF/IFNγ in tumor-free as well as tumor-bearing MMP-deficient and double-deficient mice.

Because MMPs are induced by TNF during liver toxicity (17) and because several MMP-encoding genes contain NF-κB–responsive elements (8), we looked for changes in expression levels of MMPs and TIMPs in liver and other

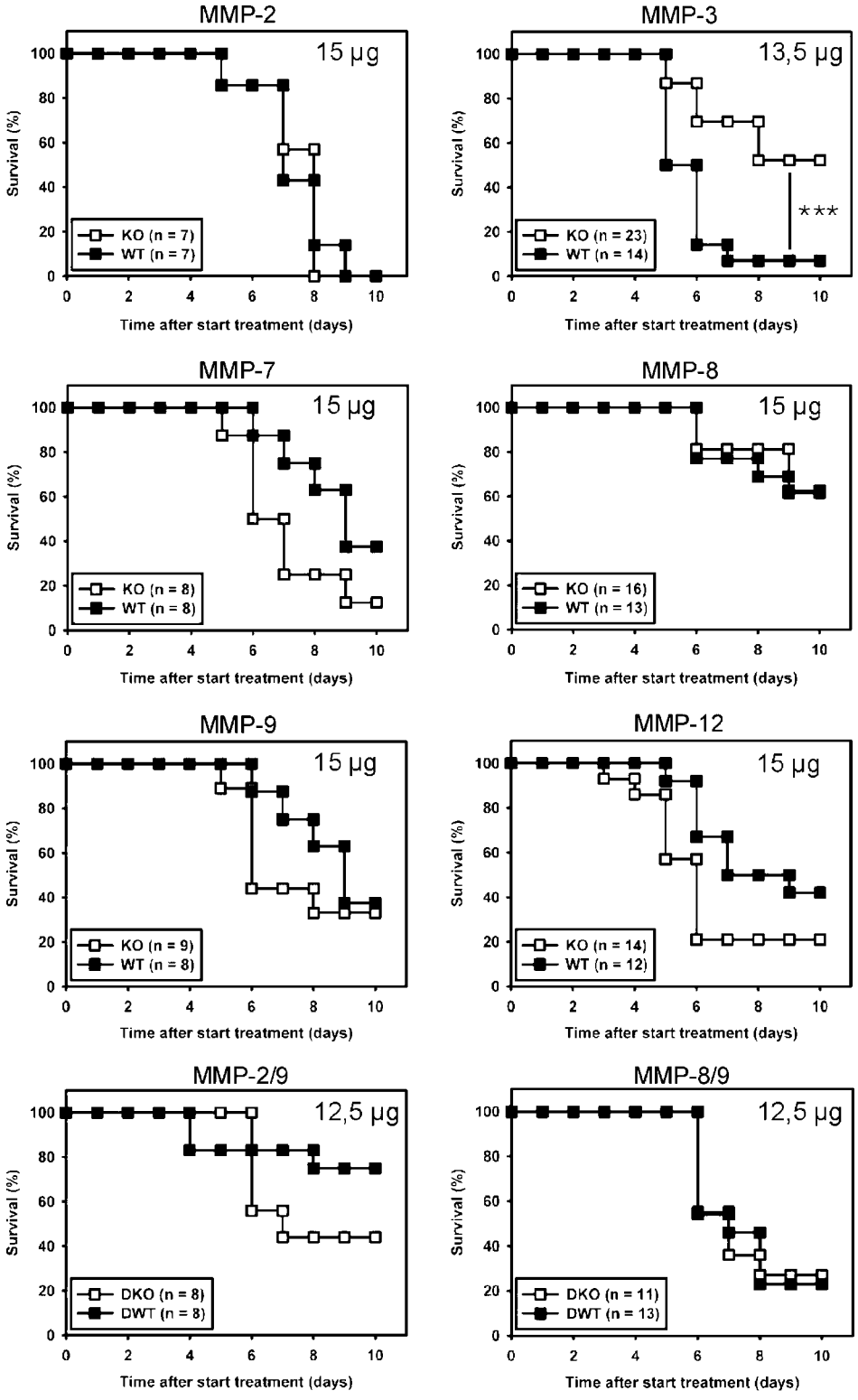

Figure 2. Response of tumor-free MMP knock-out mice in the TNF/IFNγ-model. Survival is plotted as a function of time (days after tumor inoculation). MMP wild-type mice (■; *n*, indicated inside each graph) and MMP knock-out mice (□; *n*, indicated inside each graph) were shaved and treated daily for 10 consecutive days with TNF/IFNγ (amount of TNF combined with 5,000 units of IFNγ is indicated inside each graph). Survival is shown and was recorded until the end of the treatment (no further deaths occurred).

organs during TNF/IFNγ treatment. This study also served to determine which MMP-deficient mice should be tested for their response to TNF/IFNγ. Organs of control mice were compared with organs of tumor-bearing mice treated with PBS or with TNF/IFNγ. We found that several MMPs and TIMP-4 are expressed in the liver during treatment with TNF/IFNγ, MMP-9 is up-regulated in the liver and kidney, and MMP-8 is up-regulated in the liver and lung,

the latter being not surprising because MMP-8 plays a mediating role in liver- and lung-specific inflammation (23, 24). The role of MMP-7 and MMP-12 during acute inflammation in the lung has been described (25–27), but we show for the first time that these two MMPs can also be expressed during inflammatory disorders in the liver. Furthermore, a role for TIMP-4 in inflammatory disorders in the liver has not been previously described. Although

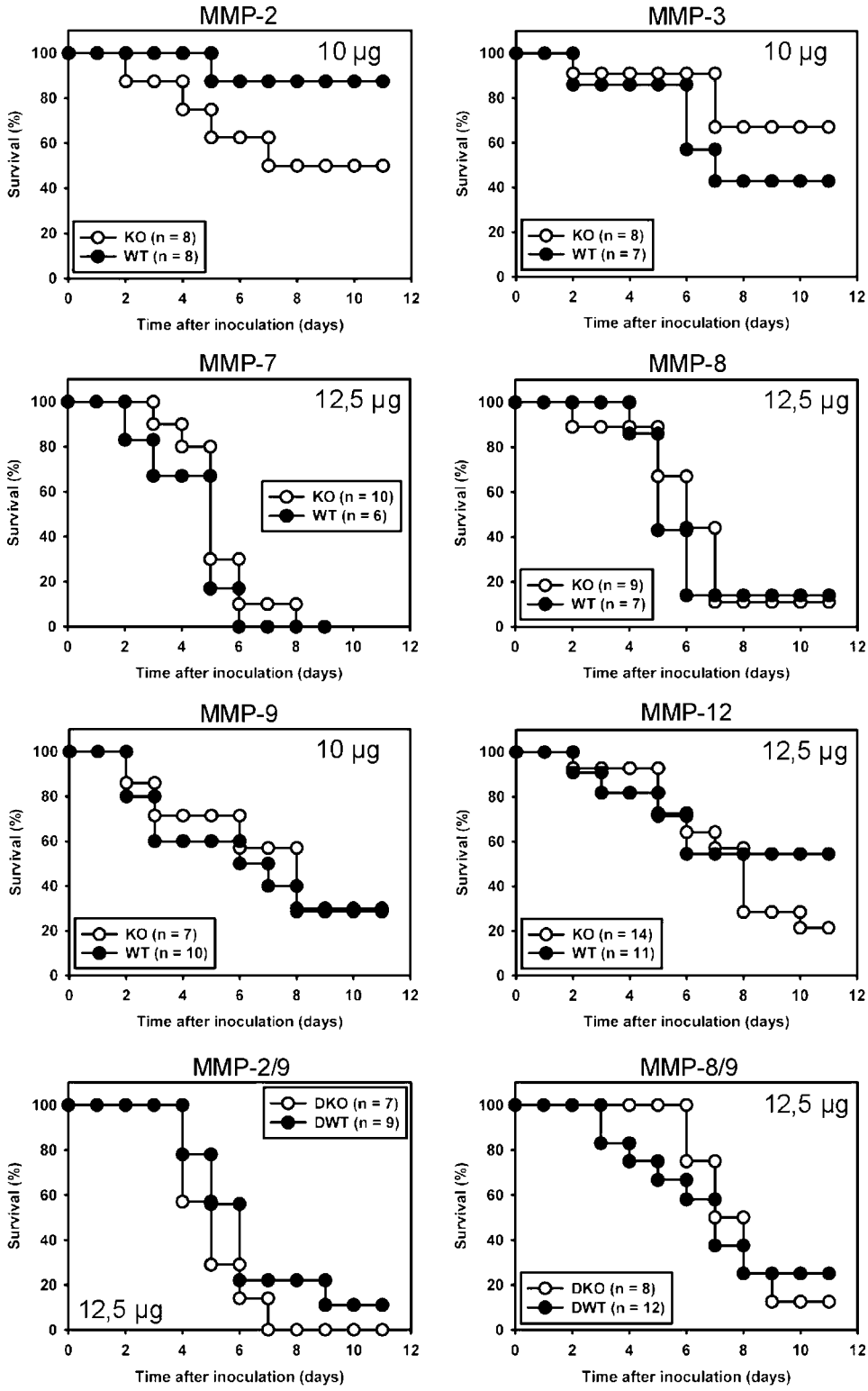

Figure 3. Response of tumor-bearing MMP knock-out mice in the TNF/IFNγ model. Survival is plotted as a function of time (days after tumor inoculation). MMP wild-type mice (●; *n*, indicated inside each graph) and MMP knock-out mice (○; *n*, indicated inside each graph) were inoculated with a B16BL6 tumor on day 0. Ten days later, the mice were treated daily for 10 consecutive days with TNF/IFNγ (amount of TNF combined with 5,000 units IFNγ is indicated inside each graph). Survival is shown and was recorded until the end of the treatment (no further deaths occurred).

the B16BL6 tumor constitutively expresses most MMPs and TIMPs, only the expression of MMP-3 seems to be up-regulated during treatment with TNF/IFNγ. This raises the possibility that MMP-3 mediates some aspect of the antitumor activities of TNF/IFNγ. Indeed, MMP-3 can induce apoptosis when overexpressed in mammary epithelial cells (9). In conclusion, the expression of five different MMP genes is modulated by TNF/IFNγ, namely, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-12, and hence, we studied mice deficient in these MMP genes for their response to treatment with TNF/IFNγ.

Both tumor-free as well as tumor-bearing MMP-3 knock-out mice showed resistance to TNF/IFNγ injections. However, tumor-bearing MMP-3 knock-out mice were less resistant than tumor-free MMP-3 knock-out mice, possibly due to the strong expression of MMP-3 by the tumor. Still, it is surprising that a difference can be found between tumor-free MMP-3 knock-out and wild-type mice because MMP-3 is not up-regulated in any of the organs during TNF/IFNγ treatment, but is constitutively expressed in colon, heart, kidney, and lung. However, apart from gene regulation, TNF/IFNγ treatment could also lead to the activation of MMP-3 (28), and recent studies have shown important proinflammatory roles for MMP-3 (29–31). Another intriguing result is that tumor-free as well as tumor-bearing MMP-12 knock-out mice were slightly, but consistently, more sensitive to TNF/IFNγ than their wild-type counterparts. These data indicate that MMP-12 has a protective effect in TNF/IFNγ-induced inflammation. MMP-12 is expressed by macrophages (32), the influx of which and expression of MMP-12 may be required for clearance of debris in the liver. Indeed, lack of MMP-12 expression by macrophages inhibits their ability to invade Matrigel-impregnated sponges *in vivo* (16). There was no difference in the response between the other tested MMP knock-out and wild-type mice. However, we could observe some disparity in survival between tumor-bearing and tumor-free mice of some groups of animals (both wild-type and knock-out). It could be anticipated that tumor-bearing mice would be generally sicker and, thus, more susceptible to the treatment than tumor-free mice.

Because MMPs as a group have dual roles in TNF-induced inflammation models, some being protective and others sensitizing, MMP inhibitors do not seem to hold much promise to confer protection as adjuvants in TNF/IFNγ therapy. Nevertheless, we repeatedly found that a broad-spectrum inhibitor, BB-94, could protect for a short period against TNF/IFNγ-induced toxicity. Using MMP-deficient mice, MMP-3 in particular seemed to be a mediator during TNF/IFNγ therapy, whereas MMP-12 seemed to have some protective role. None of the MMP-deficient mice exhibited the level of protection conferred by BB-94. This finding might be explained by compensatory mechanisms in which other proteases substitute for the function of the deleted gene. In the future, other MMP-deficient mice will be tested, and the effect of a MMP-3–specific inhibitor, if such inhibitor becomes available, will be evaluated in these experimental models. Expanding the study to ADAM family members is another option: ADAM-10, ADAM-12, and ADAM-17 have been shown to be expressed in particular forms of inflammation (33, 34), and ADAM-17 is involved in the maturation of TNF from the membrane pro-form (35).

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