Regulation of the Mr 72,000 Type IV Collagenase by the Type I Insulin-like Growth Factor Receptor

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Abstract

The Mr 72,000 type IV collagenase (matrix metalloproteinase 2 (MMP-2)) is known to play a central role in the process of invasion and metastasis, but its regulation is not clearly understood. We investigated the role of the type I insulin-like growth factor (IGF-I) in the regulation of tumor cell invasion and the synthesis of MMP-2. Highly invasive murine Lewis lung carcinoma subline H-59 cells, in which expression of the IGF-I receptor (IGF-IR) was blocked by antisense mRNA, had a significantly reduced invasion in reconstituted basement membrane (Matrigel) as compared with that of controls. These cells had a decrease of up to 6-fold in the level of MMP-2 mRNA transcripts, as assessed by reverse transcription-PCR, and a corresponding reduction in protein synthesis, as assessed by the Western blot assay and gelatin zymography. Conversely, overexpression of IGF-IR in a second, poorly invasive carcinoma subline (M-27) with low endogenous levels of the receptor increased MMP-2 mRNA and protein expression by up to 7.5- and 4-fold, respectively. Ligand-mediated activation of the IGF-IR induced MMP-2 synthesis in both cell types. The results identify IGF-I as a regulator of MMP-2 expression and cellular invasion.

Introduction

The IGF-IR and its ligand play a critical role in the regulation of cellular proliferation, apoptosis, and transformation (1). We are investigating the role of the receptor in the regulation and maintenance of the metastatic phenotype using a murine carcinoma model of two sublines of Lewis lung carcinoma, H-59 and M-27, with divergent potentials to metastasize to the liver that correlate with IGF-IR expression levels. We reported previously that H-59 cells expressed higher levels of the Mr 72,000 type IV collagenase (MMP-2) than did M-27 cells, and this correlated with invasiveness as measured in the reconstituted basement membrane (Matrigel) assay (2). Suppression of IGF-IR expression in H-59 cells by stable transfection with a plasmid vector expressing IGF-IR antisense cDNA abrogated their metastatic potential (3), whereas overexpression of IGF-IR in M-27 cells resulted in enhanced invasiveness and an increased potential to colonize the liver (4).

The dissolution of ECMs by proteinases is an essential step in the process of metastasis. The MMPs are a family of zinc-binding proteinases that play a role in processes such as inflammation and wound healing. Several members of this family, including type I collagenase (MMP-1), the gelatinases (MMP-2 and MMP-9), and stromelysins (MMP-3 and MMP-10), have been implicated in cancer cell invasion (5). The evidence is particularly compelling for the involvement of MMP-2 in the dissolution of basement membrane barriers, a process thought to be required for tumor cell invasion into blood vessels and for tumor extravasation (5). MMP-2 and other metalloproteinases may also facilitate the expansion of a growing tumor mass in the primary site either directly (by proteolytic cleavage of ECM proteins) or indirectly (by activation of other proenzymes; Refs. 5 and 6). These enzymes can also activate latent ECM-bound growth factors and/or inactivate growth-inhibitory molecules (5, 6), thereby indirectly affecting cellular proliferation.

The finding that, in our murine carcinoma model, the invasive and metastatic potentials of the cells correlated with IGF-I expression and MMP-2 levels prompted us to investigate whether IGF-I plays a role in the regulation of MMP-2 synthesis. To this end, we used clonal populations of the tumors in which IGF-IR levels were either suppressed (H-59) or enhanced (M-27) by gene transfer. Our evidence suggests that IGF-IR can regulate the expression of MMP-2. This implies that in addition to its growth-modulating effects, IGF-I can also impact the metastatic potential by increasing the collagenolytic activity of the cells.

Materials and Methods

Cell Lines. The origin, metastatic phenotypes, and methods of transplantation for H-59 and M-27 tumors were described in detail previously (7). Transfection was performed using Lipofectin (8). The culture medium was supplemented with 200 μg/ml G418 2 days after the transfection and thereafter. All tissue culture reagents were obtained from Life Technologies, Inc. (Burlington, Ontario, Canada). SA-1, SA-8, SA-9, and SA-10 were clonal lines derived from H-59 cells transfected with the CVN plasmid expressing IGF-IR antisense cDNA; clone SS-2 was derived from H-59 cells transfected with the same plasmid expressing the IGF-IR cDNA fragment in the sense orientation. The origin and phenotypes of these clones were described in detail previously (3). Clones R3, R4, and R9 were derived from M-27 cells transfected with the CVN vector expressing the full-length human IGF-IR cDNA; CVN1 and CVN3 are control clones derived from M-27 cells transfected with the vector alone. The NIH 3T3 mouse fibroblast cell line was obtained from Dr. Clifford Stanners (McGill Cancer Center). McGill University, Montreal, Quebec, Canada). It was maintained in RPMI 1640 supplemented with 10% FCS.

Cell Invasion Assay. Tumor cell invasion was assessed in vitro using the reconstituted basement membrane (Matrigel) invasion assay, as described in detail elsewhere (4). Matrigel (Collaborative Research, Bedford, MA) was used at a concentration of 0.23 mg/ml, and rat fibronectin (5 μg/ml; Life Technologies, Inc.) was used as a chemoattractant in the lower chamber.

Reverse Transcription-PCR. Total cellular RNA was extracted, and the reverse transcription reaction was performed as described previously (4). One-tenth of the cDNA product was used in the PCR reaction (4). Primers specific for mouse MMP-2 were designed from the known cDNA sequence (9). The sequence for the upstream primer corresponded to nucleotides 1381–1401, and the sequence for the downstream antisense primer corresponded to nucleotides 2099–2119. Using these primers, a 738-bp product was amplified that hybridized to a [γ-32P]ATP end-labeled MMP-2 oligonucleotide (bp 1742–1769) in a Southern blot assay. Two primers for the murine β-actin gene (corresponding to bp 222–240 and 1052–1070) were used as internal controls (10). The amplified DNA fragments were analyzed without further purification by electrophoresis on 1.2% agarose gels. Total RNA from mouse fibroblast
NIH 3T3 cells were used as a positive control for MMP-2 transcripts (11). The optimal number of PCR cycles for which exponential production of the PCR products of interest (MMP-2 and β-actin) could still be observed was predetermined using NIH 3T3 RNA (Fig. 2a). Tumor-derived cDNA was then amplified using 35 cycles to ensure a linear range. Because the quantity of amplified β-actin cDNA is assumed to be proportional to the initial amount of mRNA, the relative level of expression of MMP-2 could be determined by normalizing to the β-actin level, using densitometry.

Northern Blot Assay. The Northern blot assay was performed essentially as described previously (12). A 32P-labeled 1.1-kb human MMP-2 cDNA fragment (a kind gift from Dr. W. Stetler-Stevenson, NIH, Bethesda, MD) and an 800-bp fragment of rat cyclophilin cDNA (13) were used as hybridization probes. The relative amounts of mRNA transcripts were analyzed by laser densitometry using an LKB Bromma Ultrascan XL enhanced laser densitometer and normalized relative to the internal cyclophilin controls.

Western Blot Assay. To prepare tumor-conditioned media, confluent monolayers (8 × 10^6 cells) were washed extensively to remove the serum, cells were cultured for 72 h at 37°C in serum-free medium with or without 6.67 nM recombinant human IGF-I (Upstate Biotechnology, Lake Placid, NY), and the supernatants were lyophilized and reconstituted (50-fold concentration) before use. Western blot analysis was carried out as described previously (12). The blots were probed with polyclonal antibodies to MMP-2 and TIMP-2 (both of which were kind gifts from Dr. W. Stetler-Stevenson; Ref. 14), and the relative amounts of MMP-2 and TIMP-2 were assessed using a LKB Bromma Ultrascan XL enhanced laser densitometer.

Gelatin Zymography. The gelatinolytic activity of MMP-2 was analyzed by zymography as described previously (2). The concentrated conditioned media from H-59, M-27, and the transfectants were electrophoresed on a SDS-polyacrylamide gel containing 1 mg/ml gelatin. The gels were stained with Coomassie Blue and destained with 10% acetic acid-50% methanol until the desired color intensity was obtained. The gelatinolytic activity seen as a clear zone on the blue background was quantitated by densitometry using photographic negatives of the gels.

Results

To study the effect of altered IGF-IR expression on tumor cell invasion, the Matrigel assay was used. The results shown in Fig. 1 demonstrate that in H-59 cells, the suppression of IGF-IR expression caused a reduction of up to 50% in tumor cell invasion. This was in accord with earlier findings that overexpression of IGF-IR in the poorly invasive M-27 cells significantly augmented (8–10-fold) their invasive capability (4). A major basement membrane-degrading proteinase implicated in Matrigel invasion is MMP-2 (5). We therefore investigated whether its expression in these cells was altered. Quantitative reverse transcription-PCR analysis was used to analyze changes in MMP-2 mRNA levels. The results shown in Fig. 2b indicate that in M-27 cells overexpressing IGF-IR, the expression of MMP-2 mRNA increased 2.5–7.7-fold as compared with that of nontreated cells, which were assigned an arbitrary value of 1.0.

Fig. 1. Suppression of IGF-IR expression by antisense RNA results in decreased tumor cell invasiveness. Tumor cells (5 × 10^4 in 100 µl of medium) were added to the Matrigel-coated filters and incubated for 48 h at 37°C. Results are expressed as the percentage of cells that invaded Matrigel relative to the number of cells that migrated through control filters precoated with 7.3 µg/filter human placental type IV collagen (4). Bars, SD. SA-1 and SA-9, IGF-IR antisense transfectants; SS-2, a sense transfected control.

Fig. 2. Analysis of MMP-2 expression in tumor cells. a, optimal conditions for quantitative analysis of PCR-based amplification of MMP-2 cDNA were determined using cDNA derived from NIH 3T3 fibroblasts. Two sets of primers for MMP-2 and β-actin and 20–41 amplification cycles were used. PCR products were separated by electrophoresis on a 1% agarose gel. The yields were analyzed by densitometry, and the yield of MMP-2 relative to that of β-actin was calculated for each PCR cycle. b, total mRNA was reverse-transcribed, and cDNA was amplified using the same MMP-2 and β-actin primers and 35 cycles. NIH 3T3 fibroblasts were used as controls to quantitate the relative levels of MMP-2. The positions of the size markers are shown on the left. Results of the densitometry were normalized relative to β-actin and are shown in the graph (bottom). c, induction of MMP-2 mRNA synthesis by IGF-1. Total RNA was extracted from H-59 cells that were serum-starved for 24 h and then incubated with 10 ng/ml IGF-1 for the indicated time intervals. The membranes were probed sequentially with 32P-labeled MMP-2 and cyclophilin cDNA probes. Laser densitometry was used to quantitate the levels of MMP-2 mRNA relative to those of control cyclophilin mRNA. Results of this analysis are shown in the bar graph (bottom) and are expressed as the ratios of MMP-2: cyclophilin mRNA in the IGF-1-stimulated H-59 cells relative to those of the control, nontreated cells, which were assigned an arbitrary value of 1.0.
expressing IGF-IR antisense mRNA, MMP-2 mRNA levels were reduced by 2.5–6-fold relative to that of wild-type or control transfectants. In these cells, the expression of TIMP-2 mRNA as measured by the Northern blot assay was unaltered (data not shown). Northern blot analysis performed on RNA extracted from serum-starved H-59 cells at various time intervals after incubation with IGF-I (10 ng/ml) revealed a time-dependent increase in MMP-2 mRNA transcripts that was maximal after 2 h (Fig. 2c).

To assess MMP-2 expression at the protein level, tumor cell-conditioned media were analyzed by the Western blot assay. The results shown in Fig. 3a confirmed that in M-27 cells overexpressing IGF-IR, the increase in MMP-2 mRNA was paralleled by an increase of 2–4-fold in MMP-2 production. Furthermore, when the cells were serum-starved and then cultured in medium supplemented with IGF-I (50 ng/ml), MMP-2 levels in the conditioned media of all cells increased by 2–4-fold relative to that of unstimulated cells. In the same cells, TIMP-2 levels remained unchanged (Fig. 3b).

In cells overexpressing IGF-IR, the increased production of MMP-2 was reflected in increased gelatinolytic activity as assessed by zymography. The results shown in Fig. 4 demonstrate that in tumor-conditioned media, two zones of lysis corresponding to the latent (M, 72,000) and activated (M, 66,000–68,000) forms of MMP-2 (15) were present. These zones of lysis could not be seen in the presence of EDTA (data not shown), indicating that they were produced by a metalloproteinase. These gelatinolytic activities were more prominent in medium conditioned by M-27 cells overexpressing IGF-IR than when they were in media conditioned by wild-type or mock-transfected cells (10–11-fold increase). They could be further enhanced in all of the cells in the presence of IGF-I but not in the presence of epidermal growth factor, which was used as a control (data not shown). Conversely, a 2.5–5-fold reduction in gelatinolytic activity was seen in H-59 cells expressing IGF-IR antisense mRNA as compared with that of wild-type or sense-transfected cells. Moreover, whereas in the latter cells, this activity was enhanced 2-fold after the addition of IGF-I to the culture medium, no increase in gelatinolytic activity was seen when the antisense-expressing cells were treated in a similar manner.

Discussion

Proteolytic degradation of the ECM is an essential and recurring process in the course of cancer metastasis. Cleavage of type IV collagen, the major constituent of basement membranes, permits local invasion at the primary site, is required during tumor cell intravasation and extravasation, and is a major trigger for angiogenesis (6, 16). Prominent among the proteinases involved in basement membrane degradation are the type IV collagenases or gelatinases, also known as MMP-2 and MMP-9 (17). MMP-2 is secreted as a Mr 72,000 zymogen and is activated extracellularly to produce partially and fully activated gelatinases of Mr 62,000–68,000 (15). This enzyme has been implicated in the invasion and metastasis of diverse tumors (5, 6). Suppression of MMP-2 activity by synthetic or natural inhibitors has been shown to block tumor invasion in vitro and metastasis in vivo (16).

The regulation of MMP-2 synthesis in mammalian cells is still not well understood. Unlike other metalloproteinases such as MMP-1, MMP-3, and MMP-9, MMP-2 synthesis is not inducible by cytokines such as tumor necrosis factor α and interleukin 1 (18) and is refractory to the protein kinase C activator phorbol 12-myristate 13-acetate (19). Among the factors reported to induce MMP-2 synthesis are the cytokine transforming growth factor β (19, 20), increased intracellular calcium (19), and two ECM proteins, namely, laminin (which may function through a phospholipase D-activating pathway: Ref. 21) and vitronectin (which activates signal transduction through the integrin vitronectin receptor αvβ3; Ref. 22). Although suggested by some reports, the role of oncogenes such as Ha-ras and c-erb in the regulation of MMP-2 synthesis is still unclear (11). The identification of factors regulating MMP-2 synthesis may be complicated by the apparent involvement of tissue-specific enhancer/promoter elements, as has recently been suggested (23).

Our results provide the first line of evidence that IGF-I is involved in the regulation of MMP-2 synthesis. They therefore add new insight into the role that the IGF-IR plays in tumor progression, implicating it in the regulation of late events in this process, namely, the acquisition by transformed cells of an invasive/metastatic phenotype.
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Fig. 4. Changes in the gelatinolytic activity of tumor cells expressing altered IGF-IR levels. Gelatin zymography was performed on concentrated (×50) serum-free conditioned media harvested after a 72-h incubation of the tumor cells in the presence or absence of IGF-I. Densitometry results are shown in the bottom panel. The values were normalized relative to those of the respective unstimulated, wild-type cells. The estimated molecular weights are indicated to the left.

In cells overexpressing IGF-IR, such as clones R3 and R4, we observed increased MMP-2 levels even without prior stimulation of the cells with IGF-I (Figs. 3 and 4). M-27 and H-59 cells do not produce detectable levels of IGF-I mRNA (12) but express IGF-II transcripts. The increase in MMP-2 levels may have been due, at least in part, to the autocrine effects of IGF-II or to the ligand-independent receptor activation associated with receptor overexpression (24). Alternatively, MMP-2 production could have been triggered by the IGF-I present in the culture medium before serum depletion for these analyses.

IGF-I has been implicated in transcriptional regulation of various genes including early response genes fos (25) and jun (26), cell cycle intermediates such as cyclin D1 (27) and cdc2 (28), and the urokinase plasminogen activator inhibitor PAI-1 (29). IGF-I has also been implicated in the synthesis of ECM proteins such as collagen (30) and proteoglycans (31). The finding that it also regulates MMP-2 synthesis suggests that IGF-I could play a dual role in the coordination of ECM turnover and maintenance of homeostasis. Because IGF-I has been shown to enhance steady-state mRNA levels through a direct effect on gene transcription or by posttranscriptional stabilization of mRNA (29), the precise molecular mechanism involved in the regulation of MMP-2 synthesis remains to be elucidated.

Several signal transduction pathways are known to be turned on in response to ligand binding by IGF-IR. They include a tyrosine phosphorylation cascade involving the major IGF-IR substrate insulin receptor substrate 1 (IRS-1), which links the receptor to the ras signaling pathway, activation of phospholipase C (PLC-γ), and the accumulation of IP3 (reviewed in Ref. 1). Whereas the link to MMP-2 synthesis remains to be elucidated, it is of interest to note that IRS-1 was also found to be associated with the integrin vitronectin receptor αvβ3 (32), which is itself a regulator of MMP-2 synthesis and activity (19, 33). In addition, intracellular calcium mobilization was recently shown to be a critical component of the transforming growth factor β1-mediated activation of MMP-2 synthesis (19). The IGF-I-induced accumulation of IP3, and the resulting increase in cytoplasmic calcium levels may therefore be one mechanism for IGF-I-mediated regulation of MMP-2.

The link between IGF-IR and MMP-2 may be reciprocal. Thus, recent studies have implicated MMP-2 in proteolytic processing of IGF-I-binding proteins 3 and 5 that can result in increased bioavailability of IGF-I (34). This in turn may lead to further amplification of MMP-2 synthesis. Because IGF-binding protein 5 is reportedly incorporated into the ECM, it may also provide the cells with an ECM-bound reservoir of IGF-I that can become accessible during ECM degradation. Enhanced production of MMP-2 may therefore result not only in increased invasion but also in augmented cellular proliferation.

Taken together with our findings, the data suggest that the activities of IGF-I and MMP-2 are coordinated at both the transcriptional and functional levels, and that together, they play a central role in regulating the metastatic phenotype. This and recent findings on the significance of IGF-IR in human cancer (36) provide a compelling rationale for targeting the IGF-IR and IGF-IR-activated signaling pathways in the design of antimitastatic therapy.

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References


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