Interleukin 10 Blocks Matrix Metalloproteinase-2 and Membrane Type 1-Matrix Metalloproteinase Synthesis in Primary Human Prostate Tumor Lines

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INTRODUCTION

The expression of MT1-MMP2 and activation of MMP-2 have been found to be strongly associated with tumor invasion and metastases (1–4), making the MT1-MMP/MMP-2 “hetero-molecular” complex an attractive target for the prognosis and prevention of tumor progression. For example, immunoperoxidase labeling with MMP-2 antibodies and ELISAs has clearly shown that malignant prostate epithelial tumor cells and associated stromal cells strongly overexpress MMP-2 (5, 6). In animal model studies, Greene et al. (6) demonstrated elegantly that an elevated expression of IGF-IR, basic fibroblast growth factor, IL-8, MMP-2, MMP-9, and mdr-1 strongly correlated with the increased metastatic potential of human prostate PC-3-M cancer cells implanted into the prostate of nude mice (6). Similarly, we found that elevated levels of MMP-2 were associated with PC-3 ML cell bone metastasis in SCID mice (7), indicating that the enzyme might play a critical role in cancer progression.

The transcription regulation of MMP-2 synthesis is only partially understood (8), but IGF-I appears to play an important role. For example, in highly invasive murine Lewis lung carcinoma sublines, Long et al. (8) found that IGF-I up-regulated MMP-2 synthesis and related invasive activities (in Boyden Chamber assays) via activation of the IGF-IR signal pathway. Transient transfection of the cells with IGF-IR antisense constructs significantly inhibited the expression of MMP-2 expression and related invasion in the Boyden chamber assays, indicating that the IGF-I:IGF-IR receptor axis might be an important regulator of metastatic behavior in advanced cancer.

The regulation of MMP-2 and its putative receptors (i.e., MT1-MMP) expression may also depend on a variety of cytokines, specific receptor-mediated trans-activating signals, and/or activation of their promoters (9). Preliminary studies in our laboratory showed that IL-10 and IL-4 (but not IL-6) down-regulated MMP-2 mRNA and protein levels after 24–36 h in primary human prostate cell lines (5). Conversely, IL-10, IL-6, and IL-4 up-regulated TIMP-1 protein and mRNA expression in these cells (5). IL-10 had an identical effect on gene expression in human PC-3 cells in vivo and in vitro (7). The data suggest

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2 The abbreviations used are: MT1, membrane type 1; MMP-2, matrix metalloproteinase 2 (latent pro-zymogen); wop53, matrix metalloproteinase 2 promoter without the p53 binding site; wp53, matrix metalloproteinase 2 promoter containing the p53 binding site; EMSA, electrophoretic mobility shift assay; pCAT, plasmid catecholamine acetyltransferase; IGF-I, insulin growth factor-1; IGF-IR, insulin growth factor-I receptor; IL-10, interleukin 10; IL-10R, interleukin 10 receptor; HPCA-10, human prostate cancer-10 primary prostate tumor cell strains; TIMP, tissue inhibitor of metalloproteinase; MGEM, mammary epithelial cell medium.

ABSTRACT

Insulin-like growth factor (IGF) I has been shown previously to up-regulate matrix metalloproteinase-2 (MMP-2) production, whereas the interleukin (IL) 10/IL-10 receptor axis has been found to down-regulate MMP-2 synthesis in tumor cells. In this paper, we showed that IL-10 activation of the IL-10 receptor blocked MMP-2 and membrane type 1 (MT1) -MMP transcription and protein synthesis in nonimmortalized primary human prostate cell strains (i.e., HPCA-10a and HPCA-10c) derived from high-grade cancer. Northern blots, Western blots, and ELISAs showed that IL-10 suppressed IGF-I induction of MMP-2 and MT1-MMP mRNA synthesis in these cell strains (P < 0.001). Inhibition studies with IL-10 and IGF-I receptor antibodies plus transfection experiments with IL-10 sense, and IGF-I receptor antisense constructs confirmed these results. Finally, transient transfection experiments and chloramphenicol acetyltransferase assays with different regions of the 5′ promoter region of the MMP-2 gene (−1659 to −555 bp) additionally showed that IGF-I stimulated p53-dependent plasmid catecholamine acetyltransferase activity and that IL-10 blocked IGF-I-induced plasmid catecholamine acetyltransferase activity. Electrophoretic mobility shift assays revealed that IL-10 induced protein(s) binding to a putative “silencer element” (−1309 to −555 fragment) downstream of the p53 binding site (−1649 to −1640). The data show that IL-10 blocks IGF-I activation of MMP-2 and MT1-MMP mRNA expression and protein synthesis in primary prostate cell strains.
that cytokine up-regulation of TIMP-1 and coordinate down-regulation of MMP-2 expression might control the level of protease activity, matrix turnover, and associated invasive behavior of malignant cells.

In this paper, Northern and Western blots plus ELISAs were used to examine the expression of MMP-2 and MT1-MMP in human prostate primary tumor strains derived from high-grade cancer tissue (i.e., HPCA-10a and HPCA-10c strains). The data showed that IL-10 blocked MMP-2 and MT1-MMP transcription and protein synthesis. Specifically, IL-10-suppressed IGF-I-IGF-I receptor axis induced MMP-2 and MT1-MMP synthesis. Transient transfection experiments and CAT assays with different regions of the 5′ promoter region of the MMP-2 gene (−1529 to −555 bp) additionally showed that IGF-I stimulated p53-dependent pCAT activity and that IL-10 blocked IGF-I-induced pCAT activity. EMSAs indicated that IL-10 induced protein(s) binding to a putative silencer element downstream (−1305 to −555 bp) of the p53 binding site (−1649 to −1630 bp). In sum, the data indicate that the IL-10R axis and IGF-I might function independently to regulate MMP-2 and MT1-MMP transcription in primary prostate tumor strains.

**MATERIALS AND METHODS**

**Cell Cultures.** The HPCA-10a and HPCA-10c cell strains were isolated from Gleason score 10 prostate glands and maintained in culture at low passage (<5 passages; Ref. 5). These epithelial cells and human papillomavirus-18 immortalized strains derived from the HPCA-10a and HPCA-10c strains have been characterized previously, and found to express prostate specific antigen and cytokeratins 8 and 18 (5). Cultures were maintained in MGEM medium supplemented with pituitary extract and 0.1% insulin, transferrin, selenium according to Clonetics (San Diego, CA). Unless otherwise stated, the cells were treated with IL-10 at 15 ng/ml and IGF-I at 10 ng/ml.

**Western Blots.** Western blots were according to the methods of Towbin et al. (10) using well-characterized MMP-2 and MT1-MMP monoclonal antibodies plus peroxidase-antiperoxidase secondary antibodies (Sigma, St. Louis, MO). Whole cell lysates were prepared by solubilization of the cells in buffer containing 0.1 M Tris–HCl, (pH 6.8), 0.1% SDS, 5% glycerol, 0.005% bromphenol blue, 0.005% pyronine Y, and 1% β-mercaptoethanol. About 30 μl of buffer containing a lysate of 5 × 10^6 cells (10 μl protein/lane) was loaded per lane of a polyacrylamide-SDS gel with 10% running gel and a 4.6% stacking gel. β-Actin or GADPH antibodies (Sigma Chemical Co., St. Louis, MO) were used for control blots.

**ELISAs.** ELISAs were performed using standard curves developed for each antibody for converting the ELISA reading (A405 nm) to amounts of protein (5, 11). In experiments, increasing amounts of crude protein extract (5, 10, 15, and 20 μg/ml) were applied to 96-well plates and air dried (3 wells/protein concentration tested). Wells were blotted with an excess of each primary antibodies (i.e., IL-10, IGF-I, MMP-2, and MT1-MMP) and avidin-biotin-labeled peroxidase antiperoxidase secondary antibody (Sigma). ELISA readings for the four different protein concentrations were averaged from triplicate wells per experiment and the data normalized for 10 μg of crude protein extract. The amounts of each antigen (ng/10 μl total protein) in crude protein extract were calculated (mean ±1 SD) from the standard curves developed for each antibody for converting ELISA readings to amounts of protein (5, 11). Protein measurements were according to Bradford (12).

For the experiments described in Table 1, A and B, the cells were seeded at 1 × 10^5/ml for 2 h with 1% serum and 10 mM

### Table 1 Elisas

<table>
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<tr>
<th>Experiment</th>
<th>IGF-IR (ng/mg)</th>
<th>MMP-2 (ng/mg)</th>
<th>MT1-MMP (ng/mg)</th>
<th>IL-10 (ng/mg)</th>
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<td>A. HPCA-10a cells</td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
<td>10 ± 2</td>
<td>54 ± 7</td>
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<td>40 ± 3</td>
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<td>44 ± 3</td>
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<td>55 ± 6</td>
<td>8 ± 1</td>
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dihydrotestosterone, and treated with vehicle, 15 ng/ml IL-10, or 10 ng/ml IGF-I for 48 h. Cells were transiently transfected with either the IL-10 sense or the IGF-IR antisense cDNA overnight before treatment. Alternatively, cells were pre-exposed to IGF-IR antibodies (1:100 dilution) for 1 h and to the antibodies in the presence of IGF-I. Mock-transfection experiments were carried out with the vector alone lacking the IL-10 or IGF-IR gene. The values were normalized for transfection efficiency with ELISA measurements of β-galactosidase. Values represent the average ± 1 SD from three experiments, and P < 0.001.

**Northern Blotting.** The isolation, electrophoresis, and blotting of RNA was performed as described previously (10, 13). The mRNAs encoding MMP-2 (3.1 kb) and MT1-MMP (4.6 kb) were detected by hybridization with cDNAs. MMP-2 and MT1-MMP cDNAs were provided by Dr. William Stetler-Stevenson, (NIH-National Cancer Institute, Division of Pathology, Bethesda, MD). RNA loads on the filters were determined by ethidium bromide staining and by control hybridizations with a full-length β-actin probe or GADPH cDNA (13). Densitometric scans of the gels (A_{600nm}) were carried out to quantify gene expression.

**Sense and Antisense Transient Transfection Studies.** Transient transfections were carried out using a Ad5CMV vector and the LipofectAMINE method (Life Technologies, Inc., Bethesda, MD) according to methods described previously (14, 15). Cells were transfected with the Ad5CMV IL-10 sense constructs (S) or Ad5CMV IGF-IR antisense cDNA constructs (16) overnight before experimental treatment of the cells. Controls included cells transfected with the Ad5CMV-LacZ control virus (i.e., mock transfections). Recombinant protein expression in the transfected cells was normalized for β-galactosidase.

**MMP-2 Promoter.** The MMP-2 promoter was a gift of Junhui Bian and Yi Sun (Parke Davis Inc., Chicago, IL; Ref. 17). A 1746-bp DNA fragment (bp −1659 to +57) upstream from the transcription initiation site of the MMP-2 gene was PCR amplified with Expand High Fidelity DNA polymerase (Boehringer-Mannheim, Chicago, IL). The primers used were T4.01(5’-CACACCCAGACAGAAGGCT) and T4.02 (5’-AAGCCCGATGCGCAGGCT). The p35 binding site was located at nucleotides −1649 to −1630 (with no spacer between the two motifs; Ref. 17). The fragment was gel purified and subcloned into TA vector (Invitrogen, San Diego, CA). Both strands were sequenced with the DNA Sequenase kit (Amerham, Chicago, IL) as well as with an automatic DNA sequencer (Prism 377 DNA sequencer; ABI). There was 100% homology with the published sequence for human MMP-2 promoter (14). After pretreatment of the HPCA-10c cells with IGF-I for 0, 6, 24, and 36 h (Fig. 1), the levels of both mRNAs were diminished to barely detectable levels after 24, 36, and 48 h (Fig. 2). More importantly, after pre-exposure of the cells to IGF-I for 6 h, subsequent exposure to IL-10 (15 ng/ml) down-regulated both MMP-2 and MT1-MMP mRNA to undetectable (or barely detectable) levels after 24, 36, and 48 h (Fig. 1B, Lanes 2–4). Note that in the latter experiments little or no change in mRNA was observed by ~6 h (Fig. 1B, Lane 1) because of the relatively long half-lives of the mRNA (11).

**CAT Assay.** To construct reporter plasmids, the sequences were amplified by the PCR and subcloned into a plasmid carrying the chloramphenicol acetyltransferase gene (18). For transfection, cells were seeded at 2 × 10^5/240-mm dish, and cultured for 16 h in MGEM plus 5% FCS. Reporter CAT plasmids (2–10 μg) were cotransfected with or without 1 μg of the MMP-2 expression plasmid by the LipofectAMINE method according to Life Technologies, Inc. (Kemilworth, NJ). After a 36-h incubation, the cells were harvested, and CAT activity was determined (15, 18). The CAT assays were according to Promega’s protocol (Promega Inc., Madison, WI). The CAT activity was measured in the crude cell extracts using the CAT enzyme liquid scintillation counting assay and TLC methods of Promega. As controls, cells were transfected with the pCAT-promoter-minus an enhancer region (negative control), and pCAT-control plasmids (positive control). The percentage of CAT activity was determined by subtracting measurements from the pCAT-control plasmid values (i.e., the positive control). Measurements were from three experiments (three measurements per experiment) and represent the mean ± 1 SD.

**EMSAs.** EMSAs were carried out according to procedures modified from Finbloom and Weinstein (15).

**Cytokine and Growth Factor Studies.** IL-10 and IL-10 receptor antibodies were kindly provided by Schering-Plough (Kemilworth, NJ) and DNAX (San Diego, CA). IGF-I and IGF-I receptor antibodies were purchased from R&D Systems (San Diego, CA). Stock solutions were made up in 0.9% NaCl solution (pH 7.2) and diluted in serum-free MGEM before the treatment of cells.

**Source of Agents.** The agents used were [3H]chloramphenicol (Amersham Life Science), FCS (Biofluids Inc., Rockville, MD), and DMEM (Mediatech, Herndon, VA). β-Galactosidase assay kit was from Stratagene Inc. (La Jolla, CA), and IgG antibodies were from Sigma. MMP-2 antibodies characterized previously were used (11), and MT1-MMP antibodies were obtained from Sigma.

**RESULTS**

**Northern Blots.** We hypothesized that IGF-I and IL-10 might regulate the transcription of MMP-2 and MT1-MMP mRNA. Northern blots and densitometric scans (A_{600nm}) showed that IGF-I (10 ng/ml) up-regulated MMP-2 (approximately 7–10-fold) and MT1-MMP (~5-fold) mRNA levels after ~6, 14, and 24 h in HPCA-10c cells (Fig. 1A, Lanes 1–4). More importantly, after pre-exposure of the cells to IGF-I for 6 h, subsequent exposure to IL-10 (15 ng/ml) down-regulated both MMP-2 and MT1-MMP mRNA to undetectable (or barely detectable) levels after 24, 36, and 48 h (Fig. 1B, Lanes 2–4). Note that in the latter experiments little or no change in mRNA was observed by ~6 h (Fig. 1B, Lane 1) because of the relatively long half-lives of the mRNA (11).

**Transfection Studies.** After pretreatment of the HPCA-10c cells with IGF-I (10 ng/ml for 24 h) to boost mRNA levels (Fig. 2, Lane 1), the cells were transfected with IGF-IR sense or IGF-IR antisense cDNA (overnight). Subsequent treatment of IL-10 sense-transfected cells with IGF-I for 0, 6, 24, and 36 h had no stimulatory effect on MMP-2 and MT1-MMP mRNA levels (Fig. 2, Lanes 1–4). In fact, the levels of both mRNAs were reduced slightly by 6–24 h (Fig. 2, Lanes 2 and 3) and barely detectable by 36 h (Fig. 2, Lanes 4 and 7). In comparison, IGF-IR mRNA levels were not significantly altered in the transfected cell strains (Fig. 2, Lanes 1–4, and 7). However, in HPCA-10c cells transfected with the IGF-IR antisense cDNA, IGF-IR mRNA levels were diminished to barely detectable levels by ~24 h, but the MT1-MMP and MMP-2 levels re-
IL-10 Blocks IGF-induced MMP-2/MT1-MMP Synthesis

**Fig. 1** Northern blots of total RNA (10 μg/lane) isolated from HPCA-10c cells. A, MMP-2 and MT1-MMP mRNA levels in cells treated with IGF-I (10 ng/ml) for: Lanes 1, 0 h; 2, 6 h; 3, 14 h; and 4, 24 h. B, MMP-2 and MT1-MMP levels in cells pretreated with IGF-I (10 ng/ml for 6 h) and then exposed to either IL-10 (15 ng/ml) or IGF-I (10 ng/ml) + IL-10 (15 ng/ml) for: lanes 1, 0; 2, 6 h; 3, 12 h; and 4, 48 h. Top, ethidium bromide-stained gel showing 28S and 18S RNA. Bottom, β-actin.

**Fig. 2** Northern blots of crude RNA extracts (10 μg/lane) from HPCA-10c cells after pre-exposure to IGF-I (10 ng/ml for 24 h) to boost mRNA levels and then transient transfection overnight with (Lanes 1–4, 7) IL-10 sense; (Lane 5) IGF-IR antisense; (Lane 6) IGF-IIR mock; (Lane 8) IL-10 mock. Cells were subsequently incubated in the presence of IGF-I (10 ng/ml) for: lanes 1, 0 h; 2, 6 h; 3, 24 h; and 4–8, 36 h. Top, ethidium bromide-stained gel showing 28S and 18S RNA. Bottom, glyceraldehyde-3-phosphate dehydrogenase.

mRNA levels of all three of the genes were not reduced after exposure to IGF-I; and 15, IGF-I + IGF-IR antibodies. Cells were also transfected with: lanes 3–6, and 9, IL-10 sense; 10, IL-10 mock; and 11–14, IGF-IR mock cDNAs. Transfected cells were then treated with IGF-I (10 ng/ml for 48 h) in lanes 3–6 and 11–14. Blots were with MMP-2, MT1-MMP, and IGF-IR antibodies. Load equals 10 μg protein/lane.

**Fig. 3** Western blots of crude protein extracts from HPCA-10c cells pretreated for 48 h with: lanes 1, IL-10; 2 and 7, IGF-I; 8, IL-10 + IGF-I; and 15, IGF-I + IGF-IR antibodies. Cells were also transfected with: lanes 3–6, and 9, IL-10 sense; 10, IL-10 mock; and 11–14, IGF-IR mock cDNAs. Transfected cells were then treated with IGF-I (10 ng/ml for 48 h) in lanes 3–6 and 11–14. Blots were with MMP-2, MT1-MMP, and IGF-IR antibodies. Load equals 10 μg protein/lane.

**Western Blots.** Western blots supported the Northern blot studies, and revealed that IL-10 down-regulated MMP-2 and MT1-MMP expression (in HPCA-10c cells) in the absence (Fig. 3, Lane 1) or presence of IGF-I (Fig. 3, Lane 8) by ∼48 h.

IGF-I alone up-regulated gene expression by 48 h (Fig. 3, Lanes 2 and 7). After transient transfection with increased amounts of IL-10 sense cDNA overnight (5–20 μg DNA; i.e., before exposure to IGF-I for 36 h) the expression of MMP-2 and MT1-MMP was blocked in the presence of IGF-I (Fig. 3, Lane 3–6) or absence of IGF-I (Fig. 3, Lane 9). Control experiments revealed that transfection with IL-10 mock (Fig. 3, Lane 10) or IGF-IR mock constructs (Fig. 3, Lanes 11–14) did not substantially reduce MMP-2 or MT1-MMP expression, or block IGF-I-induced expression by 48 h (Fig. 3, Lanes 10–14). Finally, IGF-IR antibodies partially blocked IGF-I-stimulated MMP-2 (but not MT1-MMP expression in this experiment) by ∼5-fold (Fig. 3, Lane 15) indicating that IGF-I ligand effects were mediated through the IGF-IR signal pathway. Note that the levels of IL-10 were not significantly influenced by IGF-IR antibodies (Fig. 3, Lane 15) or IL-10 and IGF-I (Fig. 3, Lanes 1–14).

**ELISAs.** ELISAs were used to quantify the effects of IGF-I and IL-10 on MMP-2 and MT1-MMP expression. The data showed that both 1% serum (Fig. 4, bar 1) had little effect on expression. In contrast, 5% serum (Fig. 4, bar 2) and IGF-I (Fig. 4, bar 4) induced a significant increase in MMP-2 (left side) and MT1-MMP (right side) production in HPCA-10c cells by 48 h (i.e., over that detected in cells treated with 1% serum; Fig. 4, bar 1; P < 0.005). In the presence of IL-10, both 5% serum (Fig. 4, bar 3) and IGF-I for 24 and 48 h (Fig. 4, bars 5 and 6) had limited (at 24 h) or no (at 48 h) stimulatory effect on the levels of either protein. In other experiments, after exposure to IL-10 for 48 h, cells allowed to recover in fresh medium in the absence of IL-10 expressed a significant increase in MMP-2 and MT1-MMP by 48 h in the presence of IGF-I (Fig. 4, bar 7; P < 0.005).

Predictably, IGF-I antibodies blocked the stimulatory effects of IGF-I by 48 h (Fig. 4, bar 8). Also, IL-10R antibodies blocked IL-10 inhibition of IGF-I after 48 h (Fig. 4, bar 9). Measurements of MT1-MMP levels revealed that the relative levels of MT1-MMP (albeit considerably lower than MMP-2) mimicked MMP-2 levels in the above experiments (Fig. 4, bars 1–10).

The ELISAs were used to additionally compare MMP-2 and MT1-MMP levels in protein extracts from two different primary prostate cell strains (i.e., HPCA-10a and HPCA-10c...
strains). The studies revealed that HPCA-10a cells (Table 1A) expressed relatively low levels of IGF-IR, MMP-2, and MT1-MMP compared with HPCA-10c cells (Tables 1B). ELISAs additionally showed that IGF-I (10 ng/ml for 24 h) stimulated increased MMP-2 (approximately 2–3-fold) and MT1-MMP (approximately 4–5-fold) levels in both cell strains compared with vehicle (Table 1, A and B). In contrast, IL-10 (15 ng/ml for 48 h) in the absence or presence of IGF-I significantly reduced the MMP-2 and MT1-MMP levels to basal levels in both cell strains (Table 1, A and B). In the presence of IGF-I (10 ng/ml for 48 h), IL-10 (15 ng/ml for 48 h) also down-regulated MMP-2 and MT1-MMP levels to near zero in both cell strains (Table 1, A and B). IGF-IR antibodies (1:200 dilution) completely blocked IGF-I induction of MMP-2 or MT1-MMP in these cells. Likewise, IL-10R antibodies (1:200 dilution) blocked IL-10 inhibition of IGF-I signaling in HPCA-10c cells.

After transient transfection of HPCA-10c cells with IGF-IR antisense, IGF-I no longer induced MMP-2 or MT1-MMP expression in either HPCA-10a (Table 1A) or HPCA-10c cells (Table 1B). Similarly, transfection with IL-10 sense down-regulated MMP-2 and MT1-MMP expression, and blocked IGF-I induction of expression. However, IGF-IR levels were not influenced by vehicle or IL-10, or transient transfection with IL-10 mock, IL-10 sense, and IGF-IR antisense cDNAs (Table 1, A and B). IGF-IR antibodies also failed to influence IGF-IR expression in these cells.

Controls showed that low levels of each protein were detected in the vehicle (0.9% NaCl)-treated cells or in cells transduced with IL-10 mock constructs. Likewise, cells transduced with the IGF-IR mock construct (i.e., vector alone) did not show any significant change in protein expression (data not shown), and IGF-I induced normal increases in MMP-2 and MT1-MMP in these cells (Table 1, A and B).

Finally, ELISAs showed that although HPCA-10a and HPCA-10c cells normally did not express IL-10, the IL-10 sense-transfected cells (but not the IL-10 mock-transfected cells) expressed abundant IL-10 after 24–48 h (10–20 ng/ml; Table 1, A and B) and after 3–5 passages (data not shown).

Note that the measurements were normalized relative to ELISA measurements of β-galactosidase levels expressed by the transfected cells to account for variations in transfection efficiency and that each data point was significant compared with untreated cells (P < 0.001).

pCAT Studies. pCAT promoter constructs were used to evaluate the direct effects of IGF-10 and IGF-I on MMP-2 expression. Previous studies with the 5’ promoter region of the MMP-2 gene identified a p53 enhancer element (−1649 to −1630 bp), and showed that IGF-I activated p53 signaling and MMP-2 transcription (17). To determine whether IL-10 specifically blocked IGF-I-dependent p53 signaling, the HPCA-10a and HPCA-10c cells were transiently transfected with pCAT constructs of the MMP-2 promoter. Two overlapping regions of the MMP-2 5’ promoter were examined in these studies, including a 1.1-kb fragment containing the p53 binding site (i.e., wp53: −1659 to −555 bp) and a 0.75-kb fragment lacking the p53 site (i.e., wop53: −1309 to −555 bp).

Initially, TLC analysis revealed that the endogenous CAT activity of the wp53 and wop53 pCAT constructs was faint in transiently transfected HPCA-10a cells. IGF-I failed to stimulate detectable CAT activity in cells transfected with the wop53 pCAT construct. However, after exposure of the cells to IGF-I for 2 h the CAT activity was significantly elevated in a dosage-dependent manner. In the presence of IL-10 (30 ng/ml), IGF-I failed to stimulate significant CAT activity (data not shown).

Measurements of the percentage of CAT activity confirmed the qualitative TLC results and showed that IGF-I stimulated the CAT activity in cells transfected with the wp53 construct (Fig. 5A). Control experiments showed that cells transfected with pCAT-control plasmid (not shown), or with the wp53 or wop53 vector exhibited only background levels of CAT activity in response to 1% serum or 1% serum plus IL-10 (i.e., <10% increase in CAT activity; Figs. 5A and 5B, bars 1–3). However, in HPCA-10a (Fig. 5B, bar a) or HPCA-10c (Fig. 5B, bar b) cells transfected with the wp53 construct, IGF-I stimulated a significant increase in the percentage of CAT activity (i.e., approximately 80–100% by 2 h; Fig. 5A, bars 4–5). Similar experiments with the wp53-transfected cells yielded very low CAT activities (i.e., 10–15%; Fig. 5B, bars 4–5). In the presence of IL-10, IGF-I failed to stimulate an increase in the percentage of CAT activity in cells transfected with the wp53 (Fig. 5A, bars 6–7) or wop53 (Fig. 5B, Lane 6) construct. Finally, after treatment with IGF-I plus IL-10 for 2 h (Fig. 5A, bar 7), the wp53-transfected cells recovered after washing, incubation in fresh medium for 8 h, and re-exposure to IGF-I for 8 h (Fig. 5A, bar 8). Here, IGF-I stimulated a significant increase in the percentage of CAT activity (i.e., approximately 70–80%). Compared with untreated cells the experimental data presented in Fig. 6, A and B, were significant (P < 0.001). Note that the data were normalized for β-galactosidase levels expressed in the transfected cells as described in Table, A and B.

EMSA. EMSAs were carried out to confirm whether IL-10 and IGF-I directly regulated protein binding to the MMP-2 promoter to stimulate or inhibit transcription. EMSAs revealed that IGF-I and IL-10 both induced protein(s) binding to
IL-10 Blocks IGF-induced MMP-2/MT1-MMP Synthesis

The mechanisms by which cells regulate MMP-2 and its putative receptor, MT1-MMP, may depend, in part, on the differential effects of growth factors and cytokines (9). For this reason we have examined the differential effects of IL-10 and IGF-I on MMP-2, and MT1-MMP expression in nonimmortalized human prostate tumor epithelial strains derived from high (Gleason score 10) -grade prostate cancer tissues. In this paper, we show that IGF-I up-regulates MMP-2 and MT1-MMP synthesis, whereas the IL-10/IL-10R axis functions to down-regulate MMP-2 and MT1-MMP synthesis in these cells. North...
ern and Western blots plus ELISAs independently verified these results in two different primary cell strains, which differed significantly in their relative degree of expression of these proteins. Moreover, transient transfection with IL-10 sense and IGF-IR antisense cDNAs provided positive confirmation of these results. EMSAs and CAT assays were carried out subsequently to measure the relative influence of IL-10 and IGF-I on both p53 binding to the MMP-2 promoter and activation of CAT activity in the two different primary prostate tumor cells (HPCA-10a and HPCA-10c). IGF-I induced p53 binding and activation of CAT activity, whereas IL-10 stimulated protein binding to the promoter to block IGF-I-stimulated CAT activity by 2 h. Control studies showed that IGF-I and IL-10R antibodies blocked IGF-I and IL-10 effects, indicating that specific signal pathways were involved. EMSA studies of the MMP-2 promoter revealed that IGF-I induction of p53 binding to the enhancer element appeared to occur independent of IL-10-stimulated activation of protein binding to a putative silencer element and vice versa. The implication is that transcriptional regulation of MMP-2 expression (and perhaps MT1-MMP) is controlled by the positive and negative regulatory elements binding protein(s) activated by the IGF-I and IL-10R axis, respectively. Taken together, the data indicate that the IL-10R axis and IGF-I might function independently to suppress and activate MMP-2 transcription, respectively. That is, the experiments with the wop53 fragment (1309 to 1630 bp) indicate that the tentative IL-10 responsive silencer element might be located downstream of the p53 site (1649 to 1630 bp; i.e., located in the wp53 fragment). Future studies with the intact promoter construct will be undertaken to understand the import of the putative silencer/enhancer elements in the context of transcription initiation.

It is important to point out that the CAT assays showed that IGF-I-induced gene transcription was inducible by approximately 2–6 h. Attempts to quantify mRNA levels revealed that there was a significant increase in MMP-2 and MT1-MMP by approximately 6–24 h. However, consistent differences in mRNA and protein expression were more uniform after 24–48 h, and most of the measurements were carried out at this time point. The ELISA measurements in particular were more reproducible in assays of cells treated for 48 h, because the amounts of protein and the differences in MMP-2 and MT1-MMP expression between experiments were more significant (P < 0.001).

Conversely, attempts to down-regulate MMP-2 and MT1-MMP expression required prolonged treatment intervals of...
24–48 h, because the mRNAs were very stable and had relatively long half-lives (i.e., approximately 24–48 h). Consequently, IL-10 or IL-10 sense transfection required ~36 h to reduce mRNA and protein levels to barely detectable levels. In agreement with this observation, “nuclear run-on” assays showed previously that the MMP-2 mRNA half-life was approximately 36–48 h in PC-3 ML cells (11).

Overall, the results presented here strongly supported earlier IL-10 studies with the HPCA-10 strains (5). We examined previously the influence of different cytokines (IL-10, IL-4, IL-6, IL-2, and IFN-γ) on TIMP-1, TIMP-2, MMP-2, and MMP-9 protein, and mRNA expression in human papillomavirus-18 immortalized HPCA-10a, HPCA-10b, HPCA-10c, and HPCA-10d human prostate cell strains derived from the primary cultures. Western blot and Northern blot analysis revealed that IL-10, IL-6, and IL-4 each up-regulated TIMP-1 expression after 16–36 h. In contrast, IL-10 and IL-4 (but not IL-6) down-regulated MMP-2 mRNA and protein levels to different degrees over 24–36 h. The levels of TIMP-2 and MMP-9 protein and mRNA were not influenced substantially by any of the cytokines. Also, IL-2 and IFN-γ had little or no effect on any of these genes (5). The data showed that the IL-10/IL-10R axis can function to up-regulate TIMP-1 expression in primary tumor strains. These data combined with the studies reported here on the nonimmortalized primary cell strains (HPCA-10a and HPCA-10c) have demonstrated that cytokines (particularly the IGF-IR axis) might play a critical role in controlling the molar ratio of TIMP-1 and MMP-2/MT1-MMP to influence the level of protease activity and perhaps the invasive behavior of malignant cells in vivo.

Studies by other laboratories have also implicated other cytokines in the regulation of TIMP and MMP expression in a variety of cell strains (9). Studies examining the role of IL-6 on collagenase and TIMP-1 production in human fibroblasts, synoviocytes, chondrocytes, and macrophages (19) have indicated that IL-6 does not stimulate collagenase production, but is a potent inducer of TIMP-1. In comparison, IL-4 and IFN-γ were found to inhibit collagenase expression but not influence TIMP secretion (19). Lacraz et al. (20) compared the effects of IL-10, IL-4, IL-2, IL-6, and IFN-γ on MMP-9, interstitial collagenase, and TIMP-1 synthesis in human macrophages and monocytes. They reported that IL-10 and IL-4 inhibited the production of MMP-9. Note that we attempted to measure MMP-9 levels in preliminary studies, but very low levels of MMP-9 were detected in HPCA-10a and 10c cells, and additional experiments were not pursued.

The import of these findings in terms of protease activation of substrate solubilization are only partially understood. To exert its enzymatic activity, MMP-2 requires cleavage and activation by MT-MMPs, including MT1-MMP (1). Both MMP-2 activation and MMP-2 activity also appear to be modulated by TIMP-2 (2) and perhaps ανβ3 integrin binding (1, 2). Recently, the proteolytic activity of MT1-MMP and MMP-2 on the cell surface has been localized to invadopodia of human RPMI7951 melanoma cells and was shown to initiate a proteolytic cascade important for cell invasion (21). TIMP-2 was found to play an integral role in MMP-2 activation (22, 23), and insufficiency and excesses of TIMP-2 both inhibit MMP-2 activation by MT1-MMP (2). Although TIMP-1 appears to be incapable of complex formation with the MMP-2 proenzyme (24), TIMP-1 has also been found to target the active site of MMP-2 (1, 2) to block activity. Likewise, TIMP-1/TIMP-2 both inhibit MT1-MMP activity (25). Thus, differential regulation of TIMP-1 and MMP-2 plus MT1-MMP expression by cytokines (IL-10) and growth factors (IGF-1) could directly impact the net proteolytic activity at the invasive forefront of tumor cell lamellipodia.

Unfortunately, the role of growth factors in regulating protease expression and activation in human prostate cancer is a relatively unexplored area. Although a number of growth factors (IGF-I, IGF-I, fibroblast growth factor, and platelet derived growth factor AA) might be involved, recent studies have strongly implicated IGF-1 in both prostate tumor growth and protease production leading to metastasis (26, 27). Studies of rat prostate adenocarcinoma PAIII cells showed that inhibition of IGF-IR by antisense transfection significantly reduced expression of tissue type plasminogen activator, urokinase-type plasminogen activator, and IGF-IR (26). These cells subsequently failed to grow (or produced tiny tumors) and failed to invade the brain parenchyma of nude mice (26), implicating that the IGF-IR axis might play a role in metastasis. Studies in our laboratory supported this suggestion, and indicated MMP-2 levels were significantly elevated in advanced prostate cancer (28) and associated with PC-3 ML tumor metastasis in SCID mice model studies (7). Clearly, additional studies of the role of IGF-1 and inhibitors of IGF-1 signaling (i.e., IL-10) are warranted.

In sum, the studies reported here significantly advance the work on several fronts by showing: (a) IL-10 down-regulates MMP-2 and MT1-MMP in primary prostate tumor cells (HPCA-10a and 10c cells); (b) that the mechanism of action is dependent on a potentially novel signal mechanism that activates a putative silencer element; (c) that IL-10 signaling is IL-10 receptor dependent; and finally (d) that the novel signal element blocks IGF-I-dependent signaling and binding of p53 to the p53 enhancer site. The relevance to prostate cancer is related directly to the possible role of IL-10 in inhibition of tumor growth and metastasis by virtue of the ability of IL-10 to block MMP-2 production and inhibit IGF-I-stimulated signaling. Obviously, the work implicates IL-10 or the IL-10 receptor as tentative therapeutic targets in the treatment of prostate cancer.

REFERENCES


Primary Human Prostate Tumor Lines

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