p53 Down-regulates Human Matrix Metalloproteinase-1 (Collagenase-1) Gene Expression*

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Recent studies show that the p53 tumor suppressor protein is overexpressed in rheumatoid arthritis (RA) synovium and that somatic mutations previously identified in human tumors are present in RA synovium (Firestein, G. S., Echeverri, F., Yeo, M., Zvaifler, N. J., and Green, D. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10895–10900; Firestein, G. S., Nguyen, K., Aupperle, K. R., Yeo, M., Boyle, D. L., and Zvaifler, N. J. (1996) Am. J. Pathol. 149, 2143–2151; Remé, T., Travaglio, A., Gueydon, E., Adla, L., Jorgensen, C., and Sany, J. (1998) Clin. Exp. Immunol. 111, 353–3581). We hypothesize that the abnormality of p53 seen in RA synovium may contribute to joint degeneration through the regulation of human matrix metalloproteinase-1 (hMMP-1, collagenase-1) gene expression. Transcription assays were performed with luciferase reporters driven by the promoter of the hMMP-1 gene or by a minimal promoter containing tandem repeats of the consensus binding sequence for activator protein-1, cotransfected with p53-expressing plasmids. The results revealed that (i) wild-type (wt) p53 down-regulated the promoter activity of hMMP-1 in a dose-dependent fashion; (ii) four of six p53 mutants (commonly found in human cancers) lost this repression activity; and (iii) this p53 repression activity was mediated at least in part by the activator protein-1 sites found in the hMMP-1 promoter. These findings were further confirmed by Northern analysis. The down-regulation of hMMP-1 gene expression by endogenous wt-p53 was shown by treatment of U2-OS cells, a wt-p53-containing osteogenic sarcoma line, and Saos-2 cells, a p53-negative osteogenic sarcoma line, with etoposide, a potent inducer of p53 expression, p53, activated by etoposide, appears to block hMMP-1 promoter activity induced by etoposide in U2-OS cells. In summary, we have shown for the first time that the hMMP-1 gene is a p53 target gene, subject to p53 repression. Because MMP-1 is principally responsible for the irreversible destruction of collagen in articular tissue in RA, abnormality of p53 may contribute to joint degeneration through the regulation of MMP-1 expression.

Rheumatoid arthritis (RA) is marked by destruction of the extracellular matrix and it is believed that, among other factors, matrix metalloproteinases (MMPs) play an important role in mediating the degradation of connective tissue matrix components such as collagens and proteoglycans (4, 5). Collagenase-1 (MMP-1), stromelysin (MMP-3), gelatinase A and B (MMP-2 and MMP-9), and collagenase-3 (MMP-13) are all present at significantly elevated levels in cartilage, synovial membranes, and synovial fluid of patients with RA (6–8). The synovium produces substantial amounts of MMP-1, the major matrix metalloproteinase involved in the degradation of interstitial collagen, specifically, types I–III. MMP-1 expression has been shown to be stimulated by native collagen type I and collagen fragments, phorbol esters, growth factors, and cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor-α (9–12). The activity of MMP-1 is stringently regulated at three levels: the promoter, the activation of proenzyme, and the inhibition of active enzyme. The activator protein-1 (AP-1) binding sites found in the promoters of human collagenase have been shown to be critical to the expression of human collagenase (13–16).

The protein product of the p53 tumor suppressor gene plays a very important role in cell growth control, DNA repair, and apoptosis (17). It has been proposed that p53 acts as an “emergency brake” inducing G1 arrest and apoptosis after DNA damage, either by halting cell division until the damage is fully repaired or by eliminating the cells with DNA that is irreparably damaged (18–20). Mutational inactivation of p53 is the most frequent genetic alteration in human cancers, indicating that p53 plays an important role in human carcinogenesis. p53 mutants often lose wild-type p53 (wt-p53) activity, and of these, some gain oncogenic activity to promote cellular transformation (21–23).

p53 is a transcription factor that recognizes a specific consensus DNA sequence consisting of two copies of a 10-bp motif, 5′-PuPuPuPuCc(AT)/T/A/GPyPyPy-3′, separated by 0–13 bp. Wild-type p53 (but not mutants) efficiently binds to this sequence and transactivates expression of the target genes (24–28). p53 can also repress a wide variety of cellular and viral promoters that lack p53 binding sites, including c-fos, bcl-2 and insulin-like growth factor I receptor (29–34). Furthermore, p53

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** The abbreviations used are: RA, rheumatoid arthritis; MMPs, matrix metalloproteinases; IL-1α, interleukin 1α; PMA, phorbol 12-myristate 13-acetate; hMMP1luci, human matrix metalloproteinase-1 promoter/luciferase reporter plasmid; hMMP1SEAP2, human MMP1 promoter/secreted form of human plasental alkaline phosphatase reporter plasmid; HFF, human foreskin fibroblast; AP-1, activator protein-1; wt, wild-type; DEME, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CMV, cytomegalovirus.

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can bind to the TATA-binding protein and repress promoter activity (35, 36). p53 has also been demonstrated to interact with other transcription factors: Sp1 (37), CCAAT-binding factor (38), cAMP response element-binding protein (39), and glucocorticoid receptors (40). Taken together these observations strongly imply that p53 interacts directly with the transcription machinery to modulate gene expression. Although wt-p53 can exert its repressive activity on many different genes, most mutants of p53 have lost this repression activity. For example, the mutant p53-143A fails to repress the promoter activities of c-fos (32), Rb (41), and proliferating cell nuclear antigen (33). Mutant p53-175H is unable to repress the promoter activity of hsp 70 (38) and fails to bind to the TATA-binding protein to repress transcription from a minimal promoter (36).

In an earlier study, Firestein et al. (2) reported that the p53 tumor suppressor protein is overexpressed in RA synovium and fibroblast-like synoviocytes. Subsequent studies showed that mutant p53 transcripts previously identified in human tumors are present in RA synovium and fibroblast-like synoviocytes (1, 3). We, therefore, examined whether p53 inactivation could play a role in the overexpression of hMMP-1 seen in RA by testing the hypothesis that the gene transcription of hMMP-1 is subject to wt-p53 regulation. We report here that, indeed, wt-p53 inhibited hMMP-1 gene expression in a dose-dependent manner, and that four of six p53 mutants (commonly found in human cancers) lost most of this repressive activity. These data provide evidence that p53 is involved in regulating hMMP-1 gene transcription and that extracellular matrix degradation attributable to high-level MMP-1 in RA and in certain human tumors may be, at least partially, related to p53 inactivation.

**MATERIALS AND METHODS**

Recombinant human IL-1β was obtained from R & D Systems (Minneapolis, MN). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM), OPTIMEM I medium, McCoy's 5A medium, fetal bovine serum (FBS), stock antibiotic-antimycotic mixture (10,000 units/ml penicillin base, 10,000 µg/ml streptomycin base, and 25 µg/ml fungizone) were products of Life Technologies, Inc. Etoposide was purchased from Sigma. All other chemicals were from the Sigma Chemical Co.

**DNA Plasmids and Probes—** The hMMP1 promoter/luciferase reporter plasmids (−4327hMMP1luci, −3400hMMP1luci, −2900hMMP1luci, −1600hMMP1luci, and −512hMMP1luci) used in this study contain the firefly luciferase gene under the transcriptional control of the hMMP1 promoter and have been described previously (42). The −4327hMMP1SEAP2 reporter plasmid was constructed by subcloning the −4327hMMP1 promoter into the pSEAP2-Basic Vector (CLONTECH). wt-p53 and mutant p53-280T (Arg to Thr at amino acid 280) constructs were generated by reverse transcription polymerase chain reaction (43). Other p53 mutant constructs encoding five p53 mutants commonly seen in human cancers were obtained from Dr. A. J. Levine (osteogenic sarcoma line) cells were propagated in McCoy's medium (1010/200 ml). Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem. Saline supplemented with 0.1 M HEPES, 2 mM MgCl2, 5 mM K3Fe(CN)6, and 5 mM K4Fe(CN)6 in phosphate-buffered saline. All experiments were performed using the LipofectAMINE reagent (Life Technologies), and 50% formamide. The membrane was washed twice in 1× SSC buffer by capillary action using a sponge. The RNA was fixed to the nylon membrane for glyceraldehyde-3-phosphate dehydrogenase to correct for slight differences in the staining results, because the differences in the staining results were at least partially attributable to the detection limit of the 5-bromo-4-chloro-3-indolyl β-D-galactosidase direct staining method. The 5-bromo-4-chloro-3-indolyl β-D-galactosidase staining results may not be a true transfection efficiency indicator in this specific situation although a better one than expression activity. We have seen that the percentage of cells stained blue when cotransfected with wild-type p53 expression plasmid (10–200 ng) was ~10–30% less than that cotransfected with vector parent, whereas under the same conditions the −4327hMMP1 promoter activity was repressed ~70–90%. Data were expressed as the mean ± S.E., and the repression of the promoter activity of hMMP-1 by wt-p53 or mutant p53 was calculated based on the maximum level of promoter activity of hMMP-1 in the presence of pCMV.

**RNA Isolation and Northern Analysis—**Saos-2 cells in 100-mm plates were transiently transfected with 24 µg of wt-p53- or mutant p53-175H-expressing plasmid. After 18 h, fresh McCoy's medium containing 0.5% serum or 8% serum was added. The cells were harvested 24 h later, and total cellular RNA was isolated using RNazol reagent (Cinna-TECH). Total RNA samples (25 µg) were subjected to Northern blot analysis. After electrophoresis in 4% polyacrylamide gel, the RNA was transferred to a nitrocellulose membrane and stained with ethidium bromide for visualization.

**DNA Transient Transfection and Luciferase/SEAP Assay—** Transfections were performed using the LipofectAMINE reagent (Life Technologies) following the manufacturer's instructions. Exponentially growing cells were plated at a density of 5 × 104 cells in six-well cluster plates (Costar, Cambridge, MA) in 2 ml of DMEM and 10% FBS (HHF) or in 2 ml of McCoy's 5A medium and transfected with varying amounts of p53-280T expressing plasmid and 1.5 µg of hMMP1luci reporter plasmid/or 0.5 µg −4327hMMP1SEAP2 reporter plasmid using 5–7 µl of LipofectAMINE reagent/well. The parent vector (pCMV) without the p53 coding sequence was used as control and to maintain the total DNA transfected constant. After 18 h, the cells were gently washed with DMEM, subsequently incubated with fresh DMEM containing 0.5% serum with or without 5 ng/ml IL-1β (HHF), fresh McCoy's medium with or without PMA at a final concentration of 200 nm (Saos-2), or fresh McCoy's medium containing 8% serum with or without 10 µM etoposide (Saos-2 and U2-OS). The cells were harvested 24 h later by lysis with reporter lysis buffer (Promega, Madison, WI), and luciferase activity was determined on an ML 2250 microtiter plate luminometer and reported as relative light units. For some experiments, the medium was withdrawn and assayed for SEAP activity following the manufacturer's instructions (CLONTECH chemiluminescent assay), cells were harvested, and extracts were assayed for luciferase activity. Three transfections, each in triplicate, were performed. The luciferase activity was normalized relative to the amount of protein in the lysates as determined by using a protein assay kit from Pierce. We were unable to normalize to β-galactosidase activities, because p53 represses the traditional viral promoters that drive expression of these reporters. However, in some experiments 0.3–0.5 µg of pCMV-β-galactosidase (Strategene) was also included and stained with 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside, 2 mM MgCl2, 5 mM K3Fe(CN)6, and 5 mM K4Fe(CN)6 in phosphate-buffered saline. Routinely, 5–10% of cells took up enough of the pCMV-β-galactosidase plasmid to stain an intense blue (when the amount of pCMV-β-galactosidase plasmid cotransfected increased, the percentage of cells stained blue increased). We did not normalize reporter activities against the average percentage of cells stained blue (it gives results similar to normalizing to total protein; data not shown). Instead, we presented data as the average of the reporter activities normalized to total protein and the reporter activities corrected with the staining results, because the differences in the staining results were at least partially attributable to the detection limit of the 5-bromo-4-chloro-3-indolyl β-D-galactosidase direct staining method. The 5-bromo-4-chloro-3-indolyl β-D-galactosidase staining results may not be a true transfection efficiency indicator in this specific situation although a better one than expression activity. We have seen that the percentage of cells stained blue when cotransfected with wild-type p53 expression plasmid (10–200 ng) was ~10–30% less than that cotransfected with vector parent, whereas under the same conditions the −4327hMMP1 promoter activity was repressed ~70–90%. Data were expressed as the mean ± S.E., and the repression of the promoter activity of hMMP-1 by wt-p53 or mutant p53 was calculated based on the maximum level of promoter activity of hMMP-1 in the presence of pCMV.
and 1.5
varying amounts of the wild-type p53 expression plasmid pCMVp53
HFF cells (triplicate) were cotransfected with
inhibit the PMA- or IL-1
m
(luci) when cells were cotransfected with 0.2
wt-p53 in Saos-2 cells in the same manner as seen in the HFF
expression plasmid (Fig. 1, lane 4)
and a 3-fold IL-1β-induced promoter activity in HFF cells (Fig. 2, bottom).
p53 Mutants either Lost Most or Only Retained Part of This

RESULTS

Suppression of hMMP-1 Transcription by the wt-p53 Protein—To analyze the effect of wt-p53 protein on the hMMP-1 gene expression, HFF cells harboring endogenous wt-p53 were
transfected with −4327hMMP1luci reporter plasmid (1.5 μg), a luciferase reporter driven by a 4327-bp fragment of the hMMP-1 promoter, together with varying amounts of pCMVp53. The total amount of DNA transfected was kept constant with pCMV. As shown in Fig. 1, expression of wt-p53 downregulated the hMMP-1 promoter activity in a dose-dependent manner (blank bars). To rule out the possibility that this transcriptional regulation was resulting from the interference of the p53-expressing plasmid by endogenous p53, we also used the human osteosarcoma cell line Saos-2 in the transient transfection assays. Saos-2 cells have no endogenous p53 (45). Transcription from the −4327hMMP1 promoter was repressed by wt-p53 in Saos-2 cells in the same manner as seen in the HFF cells (Fig. 1). We observed up to a 20-fold reduction in SEAP activity and up to an 8-fold increase in luciferase activity (p53-luci) when cells were cotransfected with 0.2 μg of wt-p53 expression plasmid (Fig. 1, filled bars).

wt-p53 Reduced the Activities of hMMP-1 Promoter Induced by PMA and IL-1β—PMA and IL-1β are known to induce hMMP-1 gene expression (16, 46). To examine whether p53 can inhibit the PMA- or IL-1β-induced hMMP-1 gene transcription, Saos-2 or HFF cells were cotransfected with −4327pMMP1luci (1.5 μg) together with 100 ng of pCMVp53 or pCMV as control. Half of the cells were subsequently either stimulated with PMA at a final concentration of 200 nm (top) or treated with IL-1β at a concentration of 5 ng/ml (bottom) for 24 h; the other half were incubated in fresh medium without serum as control. Cells were harvested, and protein extracts were assayed for luciferase activity. Three independent transfections, each run in triplicate, were performed, and the results are expressed as the means ± S.E.M. RLU, relative light unit.

Repression Activity on the hMMP-1 Promoter—We next examined whether p53 mutants (commonly found in human cancer) have lost their ability to repress the luciferase reporter activity driven by the hMMP1 promoter. As shown in Fig. 3, wt-p53 (100 ng) induced −20-fold repression of luciferase activity. In contrast, cotransfection of −4327phMMP1luci with the mutant p53 constructs (100 ng) resulted in either minor repression or reduced repression activity. Of the six mutants tested, four mutants, p53-143A, p53-175H, p53-280T, and p53-281G, lost this repression activity almost completely, and the mutant p53-273H lost approximately one-third of this repression activity compared with wt-p53. The remaining mutant, p53-248W, retained most of this repression activity.

Repression of Endogenous hMMP-1 Messenger by wt-p53—We examined the hMMP-1 mRNA levels after transfection of the Saos-2 cells with p53-expressing plasmid. Northern blot (Fig. 4) showed that the hMMP-1 message was detectable at 0.5% serum (lane 1) and induced by 8% serum (lane 2). Significantly, serum-induced hMMP-1 expression was completely suppressed by wt-p53 (Fig. 4, lane 3) but not by p53 mutant pCMV-175H (Fig. 4, lane 4).

Endogenous wt-p53 Inhibited the Promoter Activity of hMMP-1—The repression of hMMP-1 promoter activity by ectopic expression of wt-p53 suggests that one of the functions of endogenous p53 is to inhibit or modulate hMMP-1 promoter activity. To test whether the p53 repression of hMMP-1 is a physiologically relevant response, the ability of induced endogenous p53 to alter the hMMP-1 promoter activity was examined by the treatment of cells with etoposide, a topoisomerase II inhibitor and a known potent p53 inducer (47). We reasoned that if hMMP-1 is a p53 target gene for repression, the hMMP-1 promoter activity should be reduced after p53 induction in cells containing wild-type p53 but not in p53-negative cells. The optimal time of induction of endogenous p53 in U2-OS cells by etoposide has been shown to be at 24 h (47). As shown in Fig. 5, etoposide induced a 5-fold induction of the luciferase activity of −512hMMP1luci reporter construct in Saos-2 cells but not in U2-OS cells. The results suggested that etoposide can induce hMMP-1 expression via signal pathway(s).
FIG. 3. Loss of hMMP1 repression by some p53 mutants commonly found in human cancers. One set of Saos-2 cells was cotransfected with −4372phMMP1luci (1.2 µg) and pCMV-β-galactosidase (0.3 µg) together with 100 ng of wt-p53- or p53 mutant-expressing plasmids. The other set was cotransfected with −372phMMP1luci (1.2 µg) and pCMV-β-galactosidase (0.3 µg) together with 100 ng of pCMV as control. Transfected cells were subsequently incubated with fresh medium containing 8% serum for 24 h. Half of the cells were lysed, and protein extracts were assayed for luciferase activity (triplicate), and the other half were stained for β-galactosidase as detailed under “Materials and Methods.” The average percentage of cells stained blue was within the range of 5–6.1% for all transfections. Relative promoter activity was calculated by arbitrarily setting the activity of the control (cotransfected with the parent vector) as 100.

FIG. 4. Repression of endogenous hMMP-1 messenger by wt-p53. Total RNA was isolated from Saos-2 cells after p53 transfection and subjected to Northern analysis. Northern blot showed that p53.

FIG. 5. Blockage of etoposide-induced activation of the hMMP-1 promoter by endogenous wt-p53. U2-OS cells, a human osteogenic sarcoma line harboring endogenous wt-p53, and Saos-2 cells, a human p53-negative osteogenic sarcoma line, were transiently transfected with −512phMMP1luci. After the transfection, half of the cells were treated with etoposide (10 µM), an anticancer drug and a known p53 inducer, in McCoy’s medium containing 8% serum for 24 h; the untreated other half (in the same medium without etoposide) were used as control. The means ± S.E.M. were derived from three independent transfections and assays, each run in triplicate. Relative promoter activity was calculated by arbitrarily setting the activity of the control as 100.

FIG. 6. Mapping of the minimal promoter sequence required for p53 repression. Saos-2 cells were transiently transfected with a series of 5' deletion hMMP1/luciferase reporter plasmids (−4372phMMP1luci, −3400phMMP1luci, −2900phMMP1luci, −1600phMMP1luci, and −512phMMP1luci) together with 100 ng of pCMV-wt-p53 plasmid or pCMV as control. Transfected cells were subsequently incubated with McCoy’s medium containing 8% serum for 24 h. Cells were harvested, and protein extract was assayed for luciferase activity. The means ± S.E.M. were derived from three independent transfections and assays, each run in triplicate. RLU, relative light unit.

expression plasmid or pCMV as control. As shown in Fig. 7, the luciferase activity of pAP1-luc reporter, but not pSRF-luc reporter, was repressed substantially by wt-p53. This result indicated that the p53 repression activity on hMMP-1 promoter could be mediated through the AP-1 sites found in the hMMP-1 promoter and suggested a potential interaction or cross-talk between AP-1 and p53 on the regulation of hMMP-1 expression.

DISCUSSION

Overexpression of MMPs has been implicated in a number of diseases, including arthritis (11, 46, 48) and tumor invasion and metastasis (49, 50). In RA, a large quantity of MMP-1 produced by the synovium is mainly responsible for the irreversible degradation of collagen in the articular joint tissue (6, 12). Similarly, destruction of the interstitial collagen is also an integral part of tumor invasion and metastasis (49, 50). Despite the extensive studies on the regulation of hMMP-1 gene expression, little is known about negative regulators, which may, when inactivated, contribute to the elevated level of hMMP-1 gene expression in RA or cancer cells. Here we have tested the hypothesis that abnormality of p53 in RA or cancers may contribute to the joint degeneration seen in RA or tumor invasion and metastasis through the modulation of hMMP-1 expression.
Our results strongly suggest that hMMP-1 is a p53 target gene subject to p53 repression. Overexpression of human wt-p53 can exert a strong inhibitory effect on human hMMP-1 gene expression, but mutation of p53 at codons 143, 175, 280, and 281 abrogates this inhibition substantially.

It is interesting to observe that p53 repression of the hMMP-1 promoter was mediated at least in part by AP-1. Because the AP-1 sites at −72 and −181 of the hMMP-1 promoter play a prominent role for the basal and the PMA- and IL-1β-induced promoter activity of hMMP-1 (11, 15, 16, 51), and because wt-p53 can repress c-fos gene expression (32), it is possible that the repression activity of wt-p53 on the hMMP-1 promoter could be mediated partially through the AP-1 sites found in hMMP-1 promoter (the −72 and −181 AP-1 sites). wt-p53 protein could decrease the binding of the Fos and Jun families of transcription factors to the −72 and −181 AP-1 sites by repression of c-fos expression, and in consequence, suppress the promoter activity of hMMP-1 gene. The fact that the hMMP-1 promoter lacks the p53 binding site indicates that p53-induced hMMP-1 repression is mediated by a p53 binding site-independent mechanism, consistent with the findings in other known p53-repressed genes (29–34). It will be of great interest to elucidate the mechanism of potential cross-talk between p53 (negative) and Ap-1 (positive) in the regulation of hMMP-1 expression.

Two lines of evidence suggest that p53 repression of hMMP-1 is a physiologically relevant response. First of all, serum-stimulated expression of endogenous hMMP-1 is completely repressed by wt but not mutant p53. Second, hMMP1luc reporter activities were stimulated by etoposide significantly only in p53-negative Saos-2 cells but not in p53-positive U2-OS cells (Fig. 5). Because etoposide induces both AP-1 and NF-kB (52), two positive regulators of hMMP-1 expression, it is not surprising to see hMMP-1 induction by etoposide in Saos-2 cells. Lack of hMMP-1 induction by etoposide in U2-OS cells, however, implies that etoposide-activated p53 executes a suppressive activity, thus diminishing the Ap-1/NF-kB effect (11, 13, 15, 16).

During the preparation of this manuscript, Aupperle et al. (53) reported that the growth rate, invasiveness, and resistance to apoptosis induced by reactive oxygen species in rheumatoid and normal fibroblast-like synoviocytes increased significantly after endogenous p53 protein was inactivated with virus 18 E6 protein. However, no effect on collagenase mRNA accumulation was observed. One possible explanation for the discrepancy between these findings and our data could be attributable to the fact that the wt-p53 protein level is too low in nontransformed fibroblast-like synoviocytes to induce a detectable change in collagenase mRNA level. In addition, although E6 protein can bind to p53 protein to interfere with the transcrip-
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