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A Proinflammatory Cytokine Inhibits p53 Tumor Suppressor Activity

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Summary

p53 has a key role in the negative regulation of cell proliferation, in the maintenance of genomic stability, and in the suppression of transformation and tumorigenesis. To identify novel regulators of p53, we undertook two functional screens to isolate genes which bypassed either p53-mediated growth arrest or apoptosis. In both screens, we isolated cDNAs encoding macrophage migration inhibitory factor (MIF), a cytokine that was shown previously to exert both local and systemic proinflammatory activities. Treatment with MIF overcame p53 activity in three different biological assays, and suppressed its activity as a transcriptional activator. The observation that a proinflammatory cytokine, MIF, is capable of functionally inactivating a tumor suppressor, p53, may provide a link between inflammation and tumorigenesis.

Key words: macrophage migration inhibitory factor • p53 • inflammation and cancer • growth arrest • apoptosis

Elucidating the molecular mechanisms of tumorigenesis is essential for future progress in the diagnosis and treatment of human cancer. Inactivation of tumor suppressor genes is an essential step in the etiology of tumor initiation and growth. A great deal of effort has focused on the role of the p53 tumor suppressor in cancer (1, 2). Its pivotal position is underscored by the observation that mutations in p53 are the most common genetic alteration in human tumors.

p53 has a key role in inducing growth arrest or apoptosis after genotoxic stress (3–8). Cells lacking p53 are capable of proliferation with damaged DNA, and thus are capable of accumulating multiple, potentially oncogenic mutations (9, 10). In addition, p53 controls the onset of cellular senescence, a process which limits the number of times a cell can potentially divide and which may act as an antitumor mechanism (11). Overcoming p53 function extends potential life span and directly contributes to cellular immortalization (12–14).

In a variety of tumors, p53 is functionally inactivated, but the gene remains intact (15–17). In these tumors, the activity of p53 regulators may be altered. Thus, the identification and characterization of novel regulators of p53 activity may have direct consequences for understanding the etiology of multiple tumor types.

Eventual tumor formation has been associated with several chronic inflammatory conditions, although the relationship between inflammation and tumor development remains largely obscure at a molecular level (18, 19). Tumor initiation is precipitated by a combination of oncogenic mutational events and loss of the cellular controls that prevent cell division in the presence of DNA damage, leading to fixation and propagation of these mutations (9). At sites of inflammation, the release of reactive oxygen species from activated phagocytes has been associated with genotoxic damage in adjacent cells (20, 21). However, it has been unclear how these cells could bypass the normal controls to prevent proliferation with damaged DNA.

Here, we have undertaken two functional screens to identify negative regulators of p53 tumor suppressor activity. From each screen we isolated macrophage migration inhibitory factor (MIF).[1] Our observation that MIF, a proinflammatory cytokine released at the sites of inflammation, is capable of functionally inactivating p53, a tumor suppressor that normally functions to prevent proliferation of cells carrying genotoxic damage, may provide a mechanistic link between inflammation and cancer.

Materials and Methods

Construction of tet-GFP-p53 p53$^{-/-}$ Mouse Embryonic Fibroblast Cell Line. p53$^{-/-}$ mouse embryonic fibroblasts (MEFs; from T.

[1]*Abbreviations used in this paper:* FBS, fetal bovine serum; GFP, green fluorescent protein; GSNO, *S*-nitrosoglutathione; MBP, maltose binding protein; MEF, mouse embryonic fibroblast; MIF, macrophage migration inhibitory factor; NO, nitric oxide; SNP, sodium nitroprusside.

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Jacks, Massachusetts Institute of Technology, Cambridge, MA) were sequentially infected with pWZL-Blast-rtta, a blasticidin selectable retroviral vector expressing the reverse transactivator of the tetracycline inducible system (22), and pBabe-puro-tet-GFP-p53-sin, a self-inactivating retrovirus expressing a GFP-p53 fusion protein under the control of the tetracycline inducible promoter. Cells were drug selected, and a clone (TGP53-4) was isolated that showed observable GFP-p53 expression, and growth arrest of the cells after addition of 1 μg/ml doxycycline to the media.

Recombinant MIF. EcoRI and SalI sites were introduced immediately 5′ and 3′ to the open reading frame of human MIF by PCR, and this EcoRI-SalI fragment was cloned into EcoRI-XhoI sites of pMal-C2 (New England Biolabs). A maltose binding protein (MBP)–MIF fusion was expressed in BL21 *Escherichia coli* cells, affinity purified by amylose chromatography, and cleaved using factor Xa. MBP was removed after cleavage by amylose chromatography. Since MBP had no effect in any of the assays used, some experiments were performed using rMIF immediately after cleavage.

Bypass of p53-induced Growth Arrest. TGP53-4 cells were infected with a pHygroMarx I–derived provirus containing MIF cDNA or empty vector control. After hygromycin selection, cells were plated at ~5,000 cells/plate. 1 μg/ml doxycycline was added to induce p53 expression in appropriate plates. Media were replaced every 3 d, containing fresh doxycycline where necessary. After 10 d, cells were fixed in 1% glutaraldehyde and stained with 0.25% crystal violet. For experiments using soluble rMIF, TGP53-4 cells were plated at ~10,000 cells/plate in the presence or absence of 150 ng/ml of rMIF added to the growth media. 24 h later, doxycycline was added to induce p53 expression. Media were replaced every 3 d containing fresh doxycycline and/or rMIF. After 9 d, cells were fixed and stained as above.

Elongation of Life Span of Primary Mouse Fibroblasts. MEFs were prepared from 14-d CD1 mouse embryos, and were repeatedly passaged. Where necessary, cells were infected in passage 2 with pMARXIV-p53αs, pWZLneo-MIF, or control viruses, and selected by drug resistance for the selectable marker. One passage before the onset of senescence (usually around passage 4–5), cells were split and plated at ~300,000 cells/plate in the presence or absence of rMIF. Fresh tissue culture media (containing rMIF where appropriate) were replaced every 3 d. After 15–17 d, cells were fixed in 1% glutaraldehyde and stained with crystal violet. To determine cell concentration, crystal violet was resolubilized in 10% acetic acid and absorbance at 595 nm was analyzed using a Bio-Rad 550 microplate reader.

Apoptosis of Rat-1/mycER Cells. Rat-1/mycER cells were infected with retroviruses expressing LacZ, MIF, or Bcl2 cDNAs. After drug selection, cells were plated onto acid-washed coverslips at low density and shifted to media containing 0.1% fetal bovine serum (FBS) plus 0.1 μM estradiol to induce apoptosis. After 24 h, cells were stained with 4 mg/ml Hoechst 33342 for 10 min, then washed and scored by fluorescent microscopy. Cells containing condensed or fragmented DNA cells were scored as apoptotic cells. At least 100 fields/slide were analyzed by two independent observers.

Apoptosis of RAW264.7 Macrophages. RAW264.7 macrophages were pretreated with varying concentrations of MIF for 24 h, and then treated with 0.25–1.0 mM sodium nitroprusside (SNP) or 0.5–1 mM *S*-nitrosoglutathione (GSNO) for 8 h to 2 d. Cells containing condensed or fragmented DNA after very brief fixing with paraformaldehyde and staining with Hoechst 33258 were scored as apoptotic cells.

Fluorescence Microscopy. TGP53-4 cells were split onto coverslips in the presence or absence of 150 ng/ml MIF. 24 h later, 1 μg/ml doxycycline was added to the media. 16 h after doxycycline addition, cells were washed in PBS and fixed in 2% paraformaldehyde, and GFP-p53 was visualized with a Zeiss Axiophot fluorescent microscope using a standard FITC filter set.

Western Blots. Cells were washed in PBS, harvested in PBS, centrifuged, and lysed. Equal amounts of total protein (30–300 μg) were heat-denatured, separated on a 10% SDS-polyacrylamide gel, and blotted to nitrocellulose. Blots were probed with antibodies that recognize p53 (DO-1, FL-393; Santa Cruz Biotechnology), MDM2 (SMP-14; Santa Cruz Biotechnology), Bax (BAX Δp21; Santa Cruz Biotechnology), or p21 (23) followed by a horseradish peroxidase–conjugated anti–mouse antibody, and detected using enhanced chemiluminescence.

Northern Blots. Total RNA was prepared from TGP53-4 cells after induction of GFP-p53. 10 μg was separated in a 1% formaldehyde gel and blotted to Hybond-N^+ membranes. Blots were probed with random primed radiolabeled probes corresponding to the full-length coding sequence of mouse p21 and cyclin G. Radioactive signals were quantified using a Fuji FLA-200 phospho/fluorescent imager, and normalized to loaded RNA by quantification of fluorescence of ethidium bromide–stained ribosomal RNA bands in the RNA gel, or after blotting to the membrane.

Luciferase Assays. TGP53-4 cells were cotransfected with PG13, a plasmid which carries firefly luciferase under the control of three tandem copies of a p53-responsive consensus sequence, and pCDNA3-β-gal, a plasmid which carries β-galactosidase under the control of the CMV promoter. 1 d after transfection, cells were split, pooled, and replated at ~500,000 cells/plate. 150 ng/ml rMIF was added to half of the plates. The next day, 1 μg/ml doxycycline was added to the media to induce GFP-p53. At 0 and 10 h after induction, extracts were prepared, and luciferase and β-galactosidase activities were assayed using Promega kits. Luciferase reporter activities were normalized to β-galactosidase expression levels.

Results

MIF Isolated in Screens for Negative Regulators of p53 Activity. To identify novel regulators of p53 activity, we undertook a screen to identify genes that, when expressed at high level, were capable of bypassing p53-mediated growth arrest. A $p53^{-/-}$ MEF cell line was engineered to express a GFP-p53 fusion protein under the control of a tetracycline (doxycycline)-inducible promoter (22; TGP53-4 cell line). GFP-p53 fusion proteins are localized normally and can transactivate target genes (24; and data not shown). After addition of doxycycline to the media, the p53 fusion protein was induced, and cells became growth arrested and failed to form colonies.

We used the TGP53-4 cell line in a phenotype-based screen to identify negative regulators of p53 activity. These cells were infected with an A431 epidermoid carcinoma–derived cDNA library in a Moloney murine leukemia virus (MMLV)–based retroviral vector, pHygroMarx I (25). pHygroMarx I contains a bacterial origin of replication, zeocin resistance marker between the LTRs, and a loxP site in the 3′ LTR, which is duplicated upon integration, to facilitate provirus recovery by Cre-mediated excision after in-

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tegration into the genome. LinX (25) ecotropic retrovirus producer cells were transiently transfected with this library, and after 3 d, supernatant was used to infect TGP53-4 cells. Approximately 4×10^6 cells were infected. After drug selection for the library vector, cells were split at varying dilutions, and 1 μg/ml doxycycline was added to the media to induce expression of the GFP-p53 fusion protein. When necessary, cells were split again to improve colony discrimination. Cells that were no longer inhibited by p53 induction gave rise to colonies in the presence of doxycycline. These clones were infected with pBabe-puro-Cre, a Moloney murine leukemia virus–based virus that strongly expresses Cre recombinase to excise the provirus. Proviruses containing cDNAs from positive clones were recovered by Hirt extraction.

Proviruses were recovered from a total of 50 positive colonies. Nucleotide sequencing and database analysis revealed that cDNAs recovered from five different colonies encoded the same protein, human MIF, a cytokine that was shown previously to exert both local and systemic proinflammatory activities (26). All cDNAs encoding MIF were full length and in the sense orientation. The complete upstream regions were sequenced from three of these recovered cDNAs. Two differed in the precise 5′ terminus, indicating that they were derived from independent clones.

A cDNA-encoding MIF was also independently isolated in a similar phenotype-based screen to identify negative regulators of myc-dependent apoptosis in rat fibroblasts. Rat-1 fibroblasts expressing a c-myc–estrogen receptor fusion protein (Rat-1/mycER) were infected with pools of a cDNA library prepared from Rat-1/mycER cells committed to apoptosis in pHygroMarx II. After drug selection, cells were induced to undergo apoptosis by shifting to low serum media (DMEM + 0.1% FBS) plus 0.1 μM estradiol (to induce c-myc activity) for 3 d, followed by 2 d of serum starvation without estradiol. Cells that were protected from apoptosis were recovered in media containing 10% FBS. Rescued cells were subjected to three additional cycles of apoptotic induction. Proviruses were recovered from apoptosis-resistant cells by Cre-mediated excision of genomic DNA line (27). Since this screen was carried out in a cell line expressing wild-type p53, and myc-driven apoptosis is largely p53 dependent (28), inhibitors of p53 function were expected to be recovered from this screen.

MIF Treatment Bypasses p53-mediated Growth Arrest. To confirm that MIF was capable of bypassing p53-mediated growth arrest, a provirus containing MIF or a control provirus was transduced into TGP53-4 cells. Doxycycline was added to induce p53 expression. Numerous colonies formed on plates containing MIF-expressing cells, but few or no colonies formed on plates containing control cells (Fig. 1 A).

Since MIF was originally identified as an extracellular cytokine, we tested whether MIF protein could overcome p53-mediated growth arrest upon addition as a recombinant protein to the culture medium. MIF protein was produced as an MBP fusion protein, and cleaved to separate MIF from MBP (Fig. 1 B). TGP53-4 cells were grown in the presence or absence of recombinantly produced MIF (rMIF) and doxycycline. Colony formation was observed

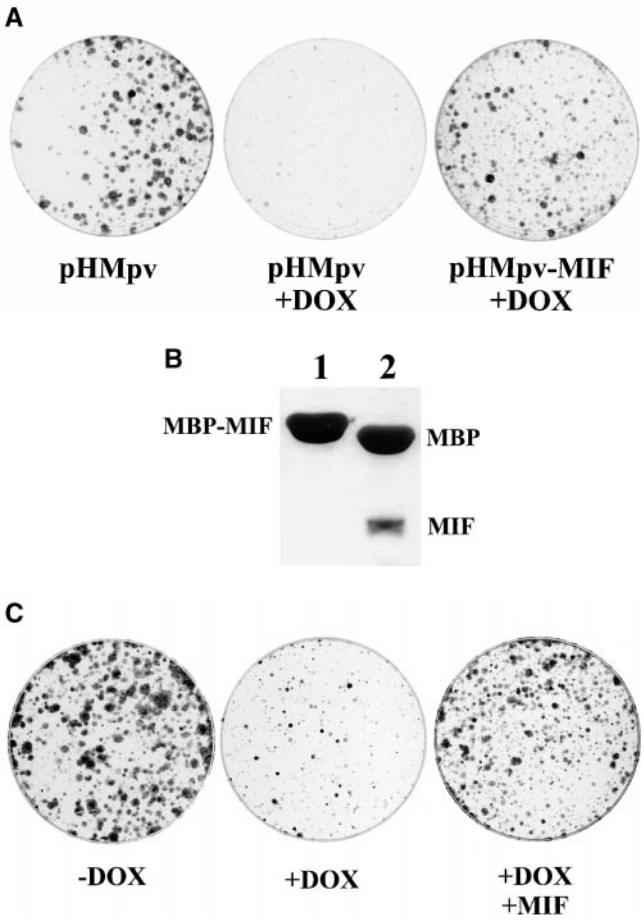

Figure 1. MIF treatment overcomes p53-induced growth arrest. (A) Expression of MIF bypasses p53-induced growth arrest and allows colony formation in a tetracycline-inducible GFP-p53 cell line. pH Mpv, HygroMarx I-based provirus; HMpv-MIF, HygroMarx I-based provirus expressing human MIF; DOX, 1 μg/ml doxycycline. (B) Recombinantly produced MBP-MIF before (lane 1) and after cleavage (lane 2). No contaminating bands were observed in Coomassie blue or Sypro orange–stained gels. (C) Addition of 150 ng/ml soluble rMIF bypasses p53-induced growth arrest of a tetracycline-inducible GFP-p53 cell line.

in the absence of doxycycline, or in the presence of doxycycline, and rMIF, but not in the presence of doxycycline alone. Therefore, MIF was capable of bypassing p53-mediated growth arrest when added as a soluble factor (Fig. 1 C).

MIF Treatment Suppresses p53-dependent Transcriptional Activation. p53 might be inactivated by altering its subcellular localization, by decreasing protein levels, or by suppressing its ability to function as a transcriptional activator. Since GFP-p53 can be visualized directly in cells and shows normal subcellular localization, we analyzed whether p53 showed altered subcellular localization in the presence of MIF. No obvious difference in the subcellular localization of GFP-p53 was observed; p53 showed nuclear localization irrespective of MIF treatment (Fig. 2 A). p53 can also be regulated by altering protein abundance; however, p53 protein levels were not reduced after MIF treatment (Fig. 2 B).

p53 primarily functions via its ability to transactivate gene expression. Therefore, we tested whether MIF treat-

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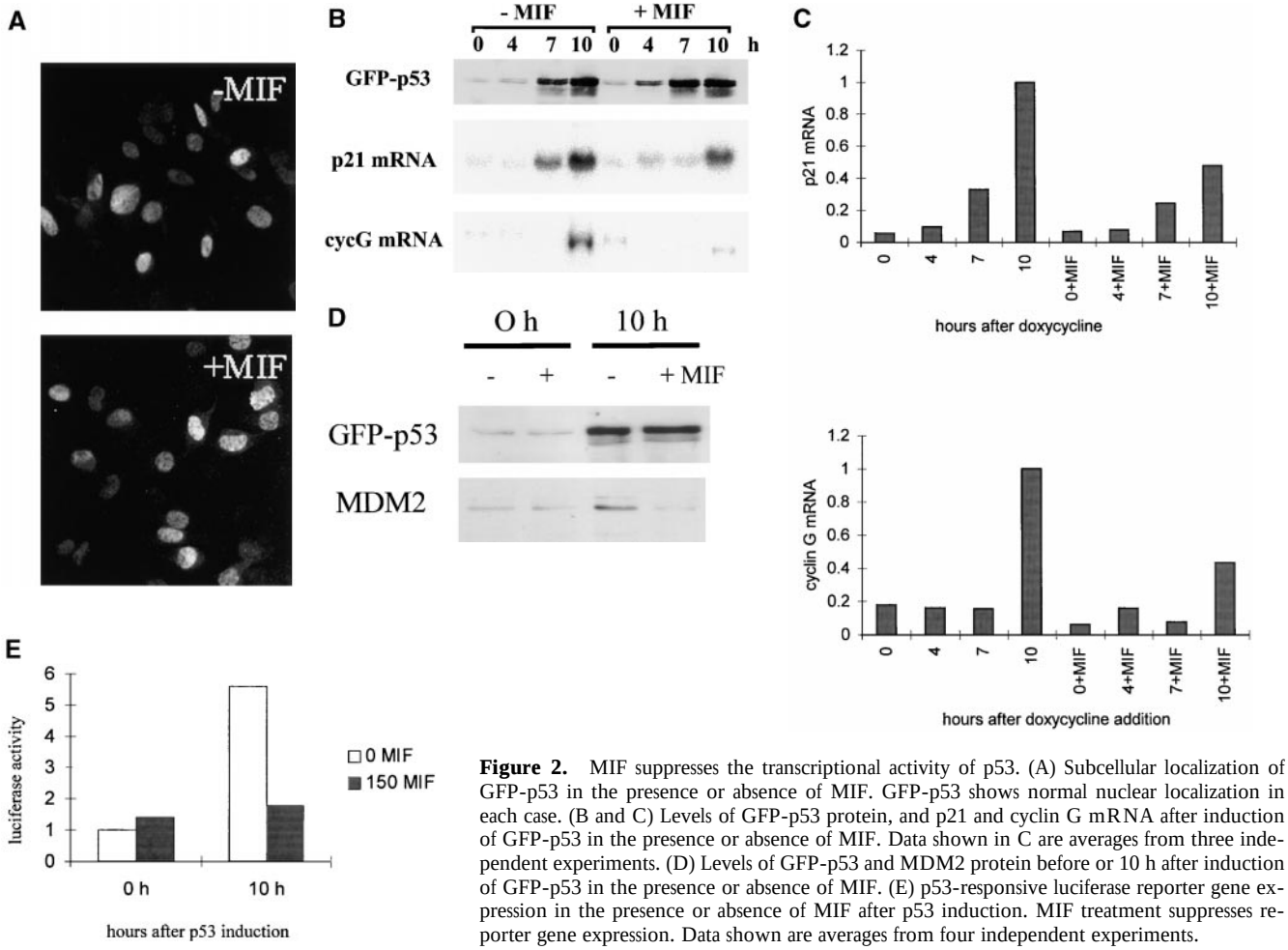

Figure 2. MIF suppresses the transcriptional activity of p53. (A) Subcellular localization of GFP-p53 in the presence or absence of MIF. GFP-p53 shows normal nuclear localization in each case. (B and C) Levels of GFP-p53 protein, and p21 and cyclin G mRNA after induction of GFP-p53 in the presence or absence of MIF. Data shown in C are averages from three independent experiments. (D) Levels of GFP-p53 and MDM2 protein before or 10 h after induction of GFP-p53 in the presence or absence of MIF. (E) p53-responsive luciferase reporter gene expression in the presence or absence of MIF after p53 induction. MIF treatment suppresses reporter gene expression. Data shown are averages from four independent experiments.

ment interfered with this activity. After induction of p53, RNA was prepared from TGP53-4 cells grown in the presence or absence of MIF. The abundance of two p53 transcriptional targets, p21 (29–31) and cyclin G (32), was assessed by Northern blot (Fig. 2 B). Levels of p21 and cyclin G in MIF-treated cells were decreased to ~50 and 40% of control levels (Fig. 2 C). In addition, p53-dependent induction of MDM2, another p53 target which acts in a feedback loop to negatively regulate levels of p53 (33, 34) was decreased in MIF-treated cells (Fig. 2 D).

The effect of MIF treatment on the activity of a p53-dependent reporter was also assayed. TGP53-4 cells were transfected with PG13-luc, a plasmid which carries firefly luciferase under the control of tandem copies of a p53-responsive consensus sequence (35), in the presence and absence of MIF, and luciferase activity was assayed after induction of GFP-p53. Treatment with rMIF suppressed p53-dependent luciferase expression (Fig. 2 E). Considered together, these data suggest that MIF treatment bypassed p53-mediated growth arrest by suppressing p53-dependent transcriptional activation.

MIF Treatment Suppresses p53-dependent Apoptosis. In addition to its ability to induce growth arrest, p53 functions to induce apoptosis in response to cellular stress in susceptible cells (5, 7, 8). As described above, we isolated a cDNA-encoding MIF in a screen designed to identify inhibitors of myc-dependent apoptosis, a process which is largely p53-dependent. To formally confirm that MIF expression could suppress this phenotype, Rat-1/mycER cells were infected with an MIF-expressing virus and control viruses, and apoptosis was induced by serum starvation and estradiol treatment. Cells that expressed MIF were partially protected from apoptosis under these conditions, though not as efficiently as cells that expressed Bcl2 (Fig. 3 A).

Since MIF regulates numerous functions of macrophages in in vitro assays and in vivo, we also tested whether MIF treatment was capable of inhibiting apoptosis in macrophages. After activation, macrophages release nitric oxide (NO) as part of their antimicrobial repertoire. However, high levels of NO can, in turn, cause macrophage apoptosis. For example, apoptosis is induced by treatment of RAW264.7 macrophages with cytokines that induce endogenous production of NO, or with chemical releasers of NO. Apoptosis is associated with induction of p53 and is inhibited by expression of antisense p53 constructs, indicating that NO-induced macrophage apoptosis is p53 dependent (36, 37). To test whether MIF treatment was capable of suppressing NO-induced apoptosis, we treated RAW264.7 macrophages with NO-releas-

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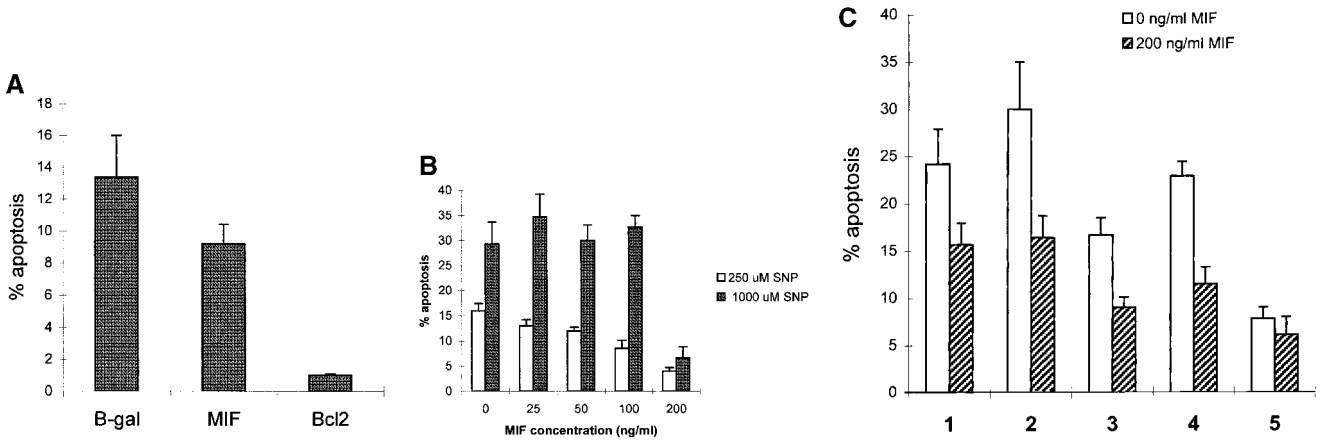

Figure 3. MIF treatment overcomes p53-dependent apoptosis in fibroblasts and macrophages. (A) Apoptosis in Rat-1/mycER cells. Rat-1/mycER cells expressing LacZ, MIF, or Bcl2 cDNAs were shifted to media containing 0.1% FBS plus 0.1 μM estradiol to induce apoptosis. After 24 h, cells were stained with Hoechst 33342 and scored. Cells containing condensed or fragmented DNA cells were scored as apoptotic cells. (B) RAW264.7 macrophages were pretreated with varying concentrations of MIF for 24 h, and then treated with 250 μM or 1 mM SNP. Apoptotic nuclei were scored after 2 d. (C) RAW264.7 macrophages were pretreated with MIF for 16 h, treated with SNP or GSNO for 8 h, and apoptotic cells were scored. 1, 0.5 mM SNP; 2, 1.0 mM SNP; 3, 0.5 mM GSNO; 4, 1.0 mM GSNO; 5, no treatment.

ers, SNP, or GSNO, in the presence of various concentrations of rMIF. MIF treatment suppressed NO-induced apoptosis in a dose-dependent manner (Fig. 3, B and C).

MIF Treatment Extends the Life Span of Primary Murine Fibroblasts. p53 also plays a role in controlling the onset of cellular senescence (12–14). Normal primary mouse fibroblasts are capable of a finite number of divisions in culture, and ultimately arrest with a senescent morphology (11). Loss of p53 allows primary mouse cells to extend their division potential. Thus, in a colony formation assay, cells lacking p53 are capable of forming colonies at passages at which wild-type cells are not. Therefore, we tested whether MIF was capable of elongating the potential life span of primary MEFs. At one passage before the onset of senescence (passage 4–5), primary MEFs were plated in the presence or absence of rMIF. After 15 d, numerous colonies had formed on plates treated with MIF, whereas none were observed in the absence of MIF. This indicated that MIF treatment, like loss of p53, was capable of inducing elongated life span (Fig. 4 A). Colony formation occurred at a frequency of $\sim 10^{-4}$ colonies/cell (the frequency of colony formation observed with cells expressing an antisense or dominant negative p53 under identical conditions is $2–3 \times 10^{-4}$ and $1–3 \times 10^{-3}$ with fibroblasts prepared from a $p53^{-/-}$ mouse; Carnero, A., and D. Beach, unpublished). To determine the concentration of MIF that was optimal for colony-forming activity, we repeated the experiment in the presence of 0–600 ng/ml rMIF. Elongation of life span was dose dependent, with 150 ng/ml giving the most pronounced effect (Fig. 4 B).

Biological Activity of MIF Correlates with Its Ability to Suppress p53-responsive Gene Expression in Extending Life Span of Primary MEFs. Since MIF treatment does not completely negate p53-mediated gene expression, we sought to test whether the ability of MIF to induce a p53-related biological activity correlated with the relative suppression of p53-mediated gene expression. Primary MEFs were infected

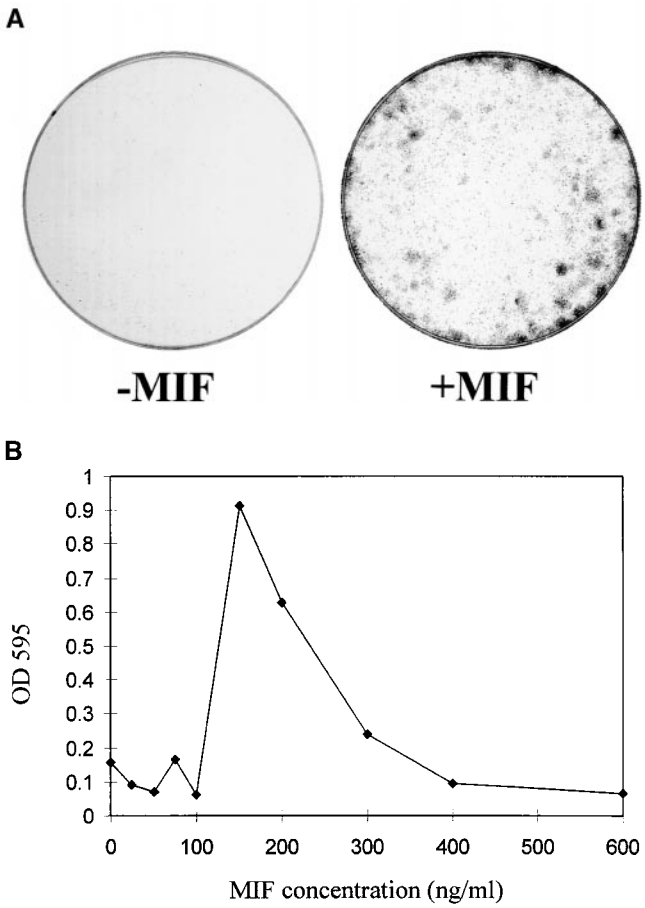

Figure 4. (A) Primary MEFs show extended life span in the presence of 200 ng/ml rMIF. Cells one passage before senescence were plated in the presence and absence of MIF, and stained after 15 d. Numerous colonies were formed only in the presence of MIF. (B) Dose dependency of MIF treatment in inducing extended life span. Primary cells, as in A, were grown in the presence of varying concentrations of MIF. After 17 d, cells were crystal violet stained, and washed. Resolubilized crystal violet was assayed as a measure of cell density.

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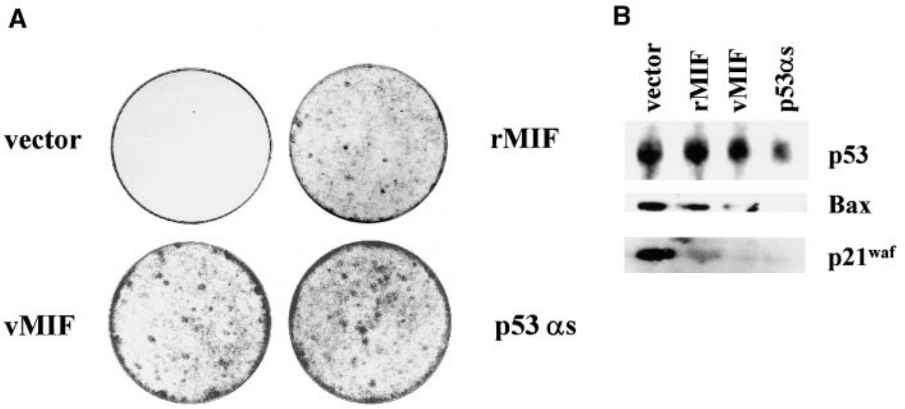

Figure 5. MIF biological activity correlates with suppression of p53-mediated target gene expression. Primary MEFs expressing MIF (vMIF), an antisense directed against p53 (p53αs), control vector (vector), or treated with rMIF were plated in passage 5. 15 d after plating cells were (A) fixed and stained with crystal violet or (B) lysed, and extracts were probed for p53, Bax, or p21 expression by Western blot.

with a virus expressing MIF, an antisense construct directed against p53 or control virus in passage 2. Infected cells were selected for drug resistance, cultured, and plated on duplicate plates in passage 5. At the same time, MEFs in passage 5 were plated in the presence or absence of rMIF. 15 d after plating, one of each duplicate plate was fixed and stained with crystal violet (Fig. 5 A). Protein extracts were prepared from the other duplicate plate, and the levels of p53 and two p53 targets, p21 and Bax (38), were assayed by Western blot (Fig. 5 B). In each case, the number of colonies observed roughly correlated with the relative suppression of p53 target gene expression, consistent with the hypothesis that suppression of p53 activity is largely responsible for this MIF-induced biological activity.

Discussion

We have demonstrated that MIF treatment was capable of overcoming p53 activity in three distinct biological assays. The ability of a secreted factor to overcome a growth-inhibitory pathway that has been associated with cellular mortality and with the response of cells to genotoxic stress may have an important physiological role. At sites of inflammation, MIF is released from T cells and from macrophages (26). High local concentrations of MIF contribute to T cell activation and enhance the antimicrobial activity of macrophages (39, 40). When activated, macrophages release NO and other oxide radicals (41). However, NO can also induce macrophage apoptosis. Since MIF can partially negate the p53 response and can protect macrophages from NO-induced apoptosis, this factor may normally act to protect macrophages from the destructive machinery they use to kill invading organisms.

Inflammatory loci are characterized by high rates of cell death and compensatory proliferation in adjacent cells (42). At the same time, upregulation of p53 is often observed (43, 44). Overcoming p53 activity through MIF action may help to limit the damage response, and therefore to limit the loss of host cells and to permit local cell proliferation for tissue repair. After cessation of the inflammatory state, local levels of MIF decrease, allowing restoration of the normal damage response.

However, chronic bypass of p53 function by MIF could contribute to the development of tumors. Loss of p53 function is one of the most common events in human cancer. Cells that lack p53 function have enhanced proliferative potential and display extended life span. In addition, cells lacking functional p53 are deficient in responding to chromosome damage (9, 10). During inflammation, release of highly reactive oxidants by activated phagocytes has been implicated in the induction of DNA damage in neighboring cells (20, 21). In the chronic presence of MIF, cells with attenuated p53 function might continue to proliferate in the presence of DNA damage, and eventually accumulate multiple oncogenic mutations.

Several chronic inflammatory conditions are strongly associated with eventual tumor formation (18, 19). For example, ulcerative colitis or Crohn's disease is associated with the eventual development of bowel cancer, whereas reflux esophagitis or Barrett's syndrome has been linked to the development of esophageal cancer. Schistosomiasis infection predisposes to the development of urinary bladder cancer, and long term *Helicobacter pylori* infection has been implicated in the development of gastric cancer. In some cases of *H. pylori* infection, ablation of the infectious agent is correlated with reversal of the inflammatory state and with regression of the associated tumor. This suggests that, in this model, at least one tumorigenic event requires continued presence of the inflammatory state, and is reversible (45). The observation that MIF can interfere with p53 function may provide insight into the mechanisms by which certain chronic inflammatory conditions predispose individuals to tumor formation.

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