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). 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 */2* mouse Embryonic Fibroblast Cell Line. p53 */2* mouse embryonic fibroblasts (MEFs; from T. 

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.
Published November 15, 1999

pMIF 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<sup>−/−</sup> MEF cell line was engineered to express a GFP:p53 fusion protein under the control of a tetracycline (doxycline)-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, pHygroMARII (25). pHygroMARII 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-
MIF Treatment Bypasses p53-mediated 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, HygroMarx II-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-
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 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-
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.

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 with p53, p53Δm, or p53ΔmΔN, 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 ~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 × 10⁻⁴ and 1–3 × 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).

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.
MIF Inhibits p53 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 H. 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.

We thank Lin Xie for the use of LinX retrovirus producer cells, D. Conklin for the use of the A431 cDNA library in pHygroMarxI, and Michela Armellin for her assistance in scoring apoptotic cells. Many thanks to P. Otavio de Campos Lima, P. Sun, R. Levinsky, and D. Conklin for helpful discussions and additional reagents.
This work was supported by a grant from the Cancer Research Campaign (to J. Hudson and D. Beach). J. Hudson was supported by a grant from the Leukaemia Research Fund. A. Carnero was supported by an EMBO long-term fellowship. R. Maestro was supported by a grant from the Italian Association for Cancer Research. G. Hannon is a Pew Scholar in the Biomedical Sciences. D. Beach is supported by the Hugh and Catherine Stevenson Fund.

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Submitted: 26 July 1999 Accepted: 5 August 1999

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