Macrophage inhibitory cytokine-1: A novel biomarker for p53 pathway activation

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Abstract
The p53 tumor suppressor protein plays a key function in the cellular response to stress by activating a subset of genes responsible for cell cycle arrest and apoptosis. Activation of the p53 pathway in tumor cells has been proposed as a novel approach to cancer therapy and substantial efforts have been dedicated to the discovery of pharmacological p53 activators. Here, we show that the transforming growth factor-β superfamily cytokine, macrophage inhibitory cytokine-1 (MIC-1), can serve as a secreted biomarker for activation of p53 in both cellular and xenograft models of human cancer. Using doxorubicin treatment in the HCT116 colon cancer cell line, we have shown that MIC-1 secretion into culture media is correlated with p53 pathway activation as measured by the up-regulation of its downstream transcriptional target p21. When transplanted into nude mice, HCT116 cells continued to secrete human MIC-1 and mouse plasma levels correlated well with tumor volume. Treatment of these animals with a single dose of doxorubicin led to elevation of the plasma MIC-1 level, which was paralleled by p21 induction in the tumor xenografts. Estimation of MIC-1 concentration, both in vivo and in vitro, represents a novel tool for the study of p53 pathway and development of p53-activating therapeutics. (Mol Cancer Ther. 2003;2:1023 – 1029)

Introduction
The p53 tumor suppressor pathway plays a pivotal role in prevention of cancer development and is disabled by mutations or deletions in 50% of all human malignancies (1–3). p53 is a potent transcription factor that can activate a subset of genes controlling the progression of the cell cycle and the activation of proapoptotic signals (4, 5). In normal proliferating cells, p53 is kept at a very low level by MDM2, which inhibits p53 transcriptional activity and facilitates its ubiquitin-dependent degradation (6). However, the p53-MDM2 regulatory loop can be disrupted in response to stress signals. This leads to p53 protein stabilization, nuclear accumulation, and activation of the p53 pathway, resulting in growth arrest or apoptosis (7).

The consequences of p53 pathway activation have suggested that it could offer a novel approach to cancer therapy and substantial experimental efforts have been directed toward the discovery of pharmacological activators of p53 (8). In general, the strategies have been aimed at restoring the transcriptional activity of mutant p53 protein (9), reintroducing wild-type p53 by gene therapy (10), or activating the p53 pathway in cancer cells with wild-type p53 by blocking its MDM2-mediated degradation (11–13). All strategies for development of p53-based therapeutics can benefit significantly from a reliable molecular marker of p53 pathway activation. The cyclin-dependent kinase inhibitor p21Waf1/Cip1 has been considered best suited for this purpose, as p21 is positioned immediately downstream of p53 and mediates cell cycle arrest (14, 15). However, p21 is not secreted, thus necessitating mRNA or protein isolation from cell lysates for analysis, techniques unsuitable or very cumbersome for a larger-scale screening. This is especially challenging if p53 activation is to be followed in animal models of human cancer. The recently cloned member of the transforming growth factor-β (TGF-β) superfamily, macrophage inhibitory cytokine-1 (MIC-1), which is secreted and powerfully induced by p53, suggests an alternate strategy.

MIC-1 is a divergent member of the TGF-β superfamily originally identified based on increased mRNA expression associated with macrophage activation (16). It has subsequently been reported under a wide variety of other names, including prostate-derived factor (17), growth/differentiation factor-15 (18–20), and placent TGF-β (21, 22). The major function of this protein is still uncertain, but it has been suggested to have a number of different roles (16, 17, 23–25) including growth inhibition (26) and induction of apoptosis in epithelial and other tumor cell lines (21, 22). A sensitive, species-specific ELISA for human MIC-1 (hMIC-1) has also been developed and has made it possible to measure MIC-1 levels in serum. MIC-1 can be detected in the serum of all individuals and increases dramatically in pregnancy (27), and more modest increases have been linked to an elevated risk for developing stroke and myocardial infarction (28).

The MIC-1 promoter contains two consensus p53-binding sites and can be activated by the tumor suppressor in vitro (21, 22). It is activated by wild-type p53 but not by...
the transcriptionally inactive mutant p53 (21). MIC-1 is expressed and secreted in cells with wild-type p53 but not in cancer cell lines with mutant p53 status (21). Consequently, it has been postulated that MIC-1 acts as a p53 pathway mediator and induces growth arrest and apoptosis by a paracrine mechanism (21, 22).

In this report, we show that MIC-1 induction and secretion in response to p53 activation is comparable with p21 induction and can serve as a secreted biomarker for activation of the p53 pathway in both in vitro and in vivo models of human cancer.

Materials and Methods

Cells and Drug Treatment

HCT116, H460a, PC3, MCF7, and H1299 cells were purchased from American Type Culture Collection (Manassas, VA), MDA-MB-435 cells were a gift from Dr. P. Steeg (National Cancer Institute/NIH), and RKO cells were provided by Dr. B. Vogelstein (Johns Hopkins Oncology Center). Cells were grown in the recommended media supplemented with 10% heat-inactivated fetal bovine serum. Media and serum were purchased from Invitrogen (Carlsbad, CA). For drug treatment, 1.5 × 10^6 cells were seeded in 75-cm² tissue culture flask in 10 ml of growth media 30 h before treatment. They were incubated with doxorubicin (10-mM stock solution in DMSO; Sigma, St. Louis, MO) at various concentrations (0.03–1.0 μM) for 24 h or with a fixed concentration (1 μM) for various periods. Control cells were treated with an equivalent amount of DMSO. For determination of the secreted MIC-1, tissue culture media from the treated cells was collected, clarified by brief centrifugation, and kept frozen at −80°C until analysis. The concentration of MIC-1 was measured by a sandwich ELISA as described (27). As the ELISA is human specific and does not cross-react with mouse MIC-1, we were able to determine total hMIC-1 concentrations in mouse plasma. Western blot analysis was performed as previously described (29) using antibodies specific for human p53 (SC263, Santa Cruz Biotechnology, Santa Cruz, CA), p21 (OP64, Oncogene Research Products, Boston, MA), MIC-1 (anti-PTGF-β, N0017, US Biological, Swampscott, MA), and β-actin (Sigma).

Quantitative PCR

Tumor tissues (0.1–0.5 g) were homogenized in a Polytron (Brinkmann, Westbury, NY) PT3100 high-speed tissue disintegrator and the total RNA was isolated using the Ultraspec RNA kit (Biotecx, Houston, TX) following the manufacturer’s instructions. Aliquots containing 5-μg total RNA were used to generate cDNA using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, Foster City, CA). For determination of p21 and MIC-1 gene expression, cells were seeded in 96-well plates (10^3 cells/well) 24 h before doxorubicin treatment. Cells were lysed and total RNA was isolated using the ABI 6700 robotic workstation (Applied Biosystems) and converted to cDNA as described above. The relative quantity of transcripts was determined by real-time PCR using p21-specific (forward: CTGAGACTCTGACGGTGCGAA, reverse: CCGCGTTTGGAGTGTA-GAA, probe: TTGACTCAGCTGAAGCTGCCCTTT) and MIC-1-specific (forward: CCATGGTCTCTTCAAAGAAC-GAC, reverse: GAAGGCCAGACTGCTCAT, probe: TGAACCTCGCTAGCCAAAGACTCCACTGCA) primers and the TaqMan Gold RT-PCT Kit in the Applied Biosystems 9700 thermocycler. The expression of p21 and MIC-1 genes was normalized to 18S rRNA using probes and primers from Applied Biosystems.

In Vivo Studies

Athymic nude mice (Nu/Nu-nuBR, 6–8-week-old female) were purchased from Charles River Laboratories (Wilmington, MA). HCT116 (3 × 10^6), H460a (5 × 10^6), and MDA-MB-435 (5 × 10^6) cells in 0.2-ml PBS were injected s.c. Tumor volumes were measured and calculated as described previously (29). Mice were bled by cardiac puncture or retro-orbital bleeding. Blood plasma was collected and kept at −80°C. Drug treatment began after establishing a s.c. tumor averaging 100–300 mm³ in size. Mice were randomized in two experimental groups (vehicle and drug treated). Doxorubicin was suspended in 0.9% sterile saline and formulated at appropriate concentrations to allow the required dose (10 mg/kg/mouse) to be achieved with administration of 0.2-ml doxorubicin (10 mg/kg) and the vehicle (0.9% saline) were administered i.v. via the caudal tail vein. In the doxorubicin experiment, the animals were bled (5 mice/time point) via cardiac puncture at 8, 24, 48, and 72 h (HCT116) or 24 h only (H460a and MDA-MB-435) after drug treatment.

Results

MIC-1 Is Secreted from Cancer Cell Lines as a Consequence of p53 Activation

It has been shown that the expression of the MIC-1 gene is under the control of a p53-dependent promoter and can be elevated in response to treatment with genotoxic drugs that activate the p53 pathway (21). This effect is dependent on the presence of transcriptionally active p53, reinforcing the notion that MIC-1 functions as a downstream transcriptional target of p53 (21, 22). To assess the utility of MIC-1 as a p53 biomarker, we chose the human colon cancer cell line HCT116, which expresses wild-type p53 and the genotoxic drug doxorubicin. HCT116 cells have been shown to respond to genotoxic insults, such as doxorubicin treatment, by stabilization and accumulation of p53 protein and by induction of p21 expression, leading to cell cycle arrest (30).

Treatment of exponentially growing HCT116 cells with doxorubicin for 24 h showed a dose-dependent elevation of p53, p21, and MIC-1 proteins (Fig. 1A). The most effective doxorubicin concentration tested (1 μM) suppressed the growth of the cells but did not cause extensive cell death for up to 48 h. Using this concentration, we treated HCT116 cells and followed the accumulation of cellular p53, p21, and MIC-1 by Western blotting (Fig. 1A) and the level of MIC-1 secreted in the tissue culture media by sandwich ELISA (Fig. 1B). Results revealed a significant p53 accumulation 8 h after drug treatment, reaching a maximum at
The levels of p21 and MIC-1 increased with a delay of several hours, consistent with their role as downstream targets of activated p53. As expected, with the elevated expression of MIC-1 in cells, levels of secreted MIC-1 increased significantly after 24 h of drug treatment, reaching nearly 8-fold over the baseline concentration at 32 h (Fig. 1B). The noticeable drop in the relative level of the secreted protein was due to an increase in the release of MIC-1 from untreated cells and not to an absolute decrease of MIC-1 secretion from the treated cells. This most likely reflected stress-related p53 activation and/or increase in the number of dead or dying cells in the control cultures. While doxorubicin-treated cells were growth arrested due to the activation of the p53 pathway and were kept subconfluent, control cells continued to grow, leading to nutrient depletion.

In a separate experiment, we compared the changes in the level of secreted MIC-1 with p21 and MIC-1 gene expression in doxorubicin-treated HCT116 cells determined by quantitative PCR (Fig. 1C). After 20 h of treatment, secreted MIC-1 increased in a dose-dependent manner (~4-fold at 1 μM) in close correlation with the increase in MIC-1 gene expression. As shown earlier, MIC-1 gene expression correlated well with p21 expression in response to drug treatment. These results suggested strongly that MIC-1 expression and secretion can serve as an indicator of p53 activation in HCT116 cells.

To expand our observation with HCT116 cells and further validate the p53-dependent nature of MIC-1 activation, we selected five additional cancer cell lines with different p53 status: two with wild-type p53 (MCF7 and H460a), two with mutant p53 (MDA-MB-435 and PC3), and the p53-null cell line H1299. Exponentially growing cells were treated with a range of doxorubicin concentrations for 20 h and the expression of p21 and MIC-1 genes was followed by real-time PCR. In agreement with our HCT116 results, MIC-1 gene was found activated in a dose-dependent manner only in cells with wild-type p53 but not in cells with mutant or deleted p53 gene (Fig. 1D). In comparison, p21 gene expression was elevated in all
tested cells, thus suggesting that in addition to being a transcriptional target of p53, p21 can be activated by a p53-independent mechanism. Activation of the p53 pathway by doxorubicin, followed by a release of MIC-1 in the culture media as estimated by Western analysis, was observed also in RKO and H460a cells with wild-type p53 but not in the mutant p53 breast cancer cell line MDA-MB-435 (data not shown). These experiments confirmed and expanded previous observations that cancer cells with wild-type p53 respond to genotoxic insult by significant elevation of the cellular MIC-1 protein. Because the induction of MIC-1 is likely to be primarily due to activation of its transcription by p53, the level of secreted MIC-1 can serve as a marker for the intracellular level of active wild-type p53 protein.

**MIC-1 Plasma Levels Correlate with Tumor Volume in Mouse Xenograft Models**

The fact that cancer cells with wild-type p53 release MIC-1 in the culture media and significantly elevate its level in response to p53 activation offers the possibility of using MIC-1 as a biomarker of both tumor mass and p53 pathway activation in human cancer xenograft models. To validate this hypothesis, we measured the plasma levels of MIC-1 in 50 nude mice bearing s.c. HCT116 xenografts that varied in size between 100 and 3000 mm³. The data demonstrated a good correlation between tumor xenograft volume and plasma levels of hMIC-1, with a correlation coefficient of 0.89 (Fig. 2A). The plasma level of hMIC-1 (1.0–1.5 pg/ml/mm³ of tumor) was lower than the MIC-1 found in tissue culture media of doxorubicin-treated cells (200–400 pg/ml/10⁶ cells) but well above the detection limit of the sandwich ELISA (5–10 pg/ml). A smaller group of nine animals with H460a lung tumor xenografts showed similar correlation between MIC-1 and tumor volume \((r = 0.94)\), but the relative hMIC-1 level was 3–4-fold lower (Fig. 2B).

The lower plasma level of MIC-1 in the H460a xenograft model compared with HCT116 model correlated well with the 3-fold lower basal expression of the MIC-1 gene determined in the untreated control cells by PCR (see Fig. 1, B and C; data not shown).

Next, we investigated the variability of hMIC-1 blood level and its dependence on tumor volume. Ten animals were each transplanted with equal number of HCT116 cells, and their tumor volume and blood hMIC-1 level were followed on days 14, 25, and 36 by periodic retro-orbital bleeding of the same animals. hMIC-1 serum levels showed both interanimal and time-related variability (Fig. 3A). With increasing tumor size from day 14 to day 36, relative MIC-1 secretion almost doubled (Fig. 3B). At the same time, the average tumor volume increased nearly 10-fold. These results revealed that hMIC-1 is stable in mouse blood, and the basal level of secreted MIC-1/mm³ of tumor can serve as an indicator of tumor burden in mouse xenograft models using wild-type p53 tumors.

**Measurement of Plasma MIC-1 in Mice with Tumor Xenografts Reflects p53 Pathway Activation in Tumor Cells**

To evaluate the utility of MIC-1 as a biomarker for activation of the p53 pathway, nude mice with HCT116 xenografts ranging in volume between 200 and 350 mm³ were given a single injection of doxorubicin and the plasma levels of hMIC-1 were measured 8, 24, 48, and 72 h later. Doxorubicin-treated non-tumor-bearing mice were used as controls. The results showed a 3.4-fold increase in the relative MIC-1 concentration 24 h post-treatment and the level remained in the 3-fold range for another 48 h (Fig. 4). The blood level of hMIC-1 in the non-tumor-bearing control mice was below the detection limit in both the presence and the absence of doxorubicin, confirming that the ELISA is specific for the human form of the protein (data not shown).

To determine whether the hMIC-1 serum levels were representative of the relationship between induction of MIC-1 and p21 observed in cultured cells, we isolated mRNA from the HCT116 tumor xenografts. Tumors were collected from the animal groups treated with doxorubicin for 8 and 24 h and p21 gene expression level was determined by real-time PCR. Concordant with plasma MIC-1 level, there was elevated p21 expression (2.8-fold) in the xenografts of doxorubicin-treated animals at 24 h but not at 8 h (Fig. 4B). The correlation between level of p21...
transcription and plasma hMIC-1 in this model confirmed that secreted MIC-1 reflects p53 pathway activation in response to doxorubicin treatment. Similar elevation of the plasma MIC-1 concentration was observed in the H460a lung cancer xenograft model. Nude mice with established tumors (100–200 mm³) were given a single doxorubicin injection (10 mg/kg) and plasma MIC-1 was determined 24 h later. This treatment led to a 3.89-fold increase in plasma MIC-1 (1.11 ± 0.23 pg/ml/mm³) compared with the vehicle control (0.285 ± 0.03 pg/ml/mm³).

Although our cell-based data were fully consistent with a p53-dependent mechanism of MIC-1 activation and secretion, one cannot exclude the possibility that an unknown p53-independent mechanism triggered by doxorubicin in vivo could contribute to the elevated MIC-1. To address this possibility, we chose one of the mutant p53 cell lines, MDA-MB-435, which has a transcriptionally inactive mutant p53 and did not respond to doxorubicin by MIC-1 induction (Fig. 1D). Nude mice carrying established MDA-MB-435 xenografts (100–400 mm³) received a single dose of doxorubicin (10 mg/kg) and the level of MIC-1 in the plasma was analyzed 24 h later. The basal level of MIC-1 (vehicle control) was found below the detection limit after doxorubicin treatment. This result indicated that under our experimental conditions, doxorubicin is not able to produce significant p53-independent elevation of hMIC-1 in mouse plasma. Therefore, we concluded that secreted hMIC-1 reflected p53 pathway activation in the xenograft tumors.

**Discussion**

Several studies have suggested that harnessing the growth-suppressive and proapoptotic activity of the p53 pathway may offer a novel approach to the treatment of cancer (8). A reliable secreted biomarker for p53 pathway activation represents a powerful tool in the preclinical development of p53-activating therapies. In this report, we have shown that MIC-1 can serve this role both in vitro and in vivo.

MIC-1 is the only known secreted p53-regulated gene, which is expressed strongly in the presence of active p53 (21, 22). The secretion of MIC-1 by cells with wild-type p53 correlates closely with the expression of p21, a well-recognized marker of p53 pathway activation. The transcription and plasma hMIC-1 in this model confirmed that secreted MIC-1 reflects p53 pathway activation in response to doxorubicin treatment. Similar elevation of the plasma MIC-1 concentration was observed in the H460a lung cancer xenograft model. Nude mice with establish tumors (100–200 mm³) were given a single doxorubicin injection (10 mg/kg) and plasma MIC-1 was determined 24 h later. This treatment led to a 3.89-fold increase in plasma MIC-1 (1.11 ± 0.23 pg/ml/mm³) compared with the vehicle control (0.285 ± 0.03 pg/ml/mm³).

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accumulation of p53 in HCT116 cells in response to doxorubicin-induced DNA damage was followed closely by activation of both p21 and MIC-1 gene expression (Fig. 1, A–C), supporting the notion that, similar to p21, MIC-1 is positioned immediately downstream of p53 in the pathway. The induction of MIC-1 gene expression was followed very closely by increased protein secretion (Fig. 1B), suggesting that secreted MIC-1 can be used as a quantitative indicator of p53 pathway activation. Consequently, the availability of a sensitive assay for MIC-1 quantitation represents an important tool for investigation of this pathway in numerous experimental models.

Several lines of evidence suggest that the activation of MIC-1 in tumor cells is dependent solely on the presence of transcriptionally active p53 protein and is not a p53-independent mechanism. First, MIC-1 promoter contains p53-binding sequences and can be activated by wild-type p53 in vitro and in vivo (21, 22). Second, activation of MIC-1 expression has been observed only in cells with wild-type p53 but not in cells with transcriptionally inactive mutant p53 (21, 22). In this study, we have extended this observation by adding seven more cell lines with either wild-type or mutant p53. In all cell lines examined so far (21, 22; this study), MIC-1 expression has been found activated only in the presence of wild-type p53 but not of mutant p53, with the exception of mutant p53-218G (21). Third, nearly identical up-regulation of MIC-1 and p21, a well-characterized p53 response gene, in doxorubicin-treated HCT116 cells (Fig. 1) indicates a common transcriptional activator. Taken together, the data strongly suggest that MIC-1 is a p53-regulated gene and its expression and secretion can serve as a marker for activation of the p53 pathway. In this role, MIC-1 appears to offer better specificity for the p53 pathway than p21, which was activated by doxorubicin in a p53-independent manner (Fig. 1D).

Measurement of tumor-derived MIC-1 in the blood of mice bearing human xenografts offers a new in vivo tool with wide applicability in the preclinical development of cancer therapeutics, as it is secreted in large amounts by tumors, especially of epithelial origin. MIC-1 can be used as a tumor burden marker, and most importantly, it can also be used to assess therapies directed at p53 pathway activation. It allows for rapid (within 24 h) determination of the ability of novel therapeutic molecules to penetrate tumor xenografts and activate the p53 pathway. Determination of MIC-1 secretion is also likely to be useful in models where cells having mutant p53 have the wild-type protein reintroduced by gene therapy (10) or restored through modulation of the p53 conformation (9). Furthermore, it is not limited to s.c. tumor xenografts but can be used as well in metastatic models of human cancer where tumor sampling represents a significant challenge. Most cytotoxic drugs currently used in the clinic or in development can activate p53 as a result of primary genotoxic activity (e.g., doxorubicin) or by causing cellular stress; therefore, their activity can be assessed using MIC-1 as a pharmacodynamic biomarker both in vitro and in vivo.

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