Abstract Hypoxia contributes to cytotoxic chemotherapy and radiation resistance and may play a role in the efficacy of antiangiogenesis cancer therapy. We have generated a series of cell lines derived from the colon adenocarcinoma models HT29 and HCT116 by exposing cells in vitro to repeated sublethal periods of profound hypoxia. These cell lines have altered sensitivity to hypoxia-induced apoptosis: those derived from HT29 are resistant, whereas those from HCT116 are more susceptible. We used cDNA selected subtractive hybridization to identify novel genes mediating sensitivity to hypoxia-induced apoptosis and isolated macrophage migration inhibitory factor (MIF) from the hypoxia-conditioned cell lines. MIF expression correlates with susceptibility of the cell lines to apoptosis. In hypoxia-resistant cells, the induction of apoptosis by hypoxia can be restored by the addition of exogenous recombinant MIF protein, suggesting that resistance may result in part from down-regulation of MIF production possibly through an autocrine loop. Inhibition of MIF using small interfering RNA in the susceptible lines conferred resistance to hypoxia-induced cell death. The relative expression of MIF in the hypoxia-conditioned cells implanted s.c. in severe combined immunodeficient mice in vivo was similar to that observed in vitro. In an analysis of 12 unrelated colon tumor cell lines, MIF expression and response to hypoxia varied widely. Cell lines in which MIF was inducible by hypoxia were more sensitive to oxaliplatin. In human colon tumor specimens analyzed by immunohistochemistry, MIF expression was similarly variable. There was no detectable expression of MIF in normal colon mucosa or adenoma but positive staining in all carcinomas tested. Taken together, these data indicate that MIF may be a determinant of hypoxia-induced apoptosis in vitro and that its variable expression in human colon cancers may indicate a functional role in vivo. We suggest that MIF expression in colorectal cancer may be a marker of susceptibility to therapies that may depend on induction of hypoxia, possibly including antiangiogenic therapy.
a 24-hour hypoxic challenge, whereas those from HCT116 surprisingly were more sensitive. Sensitivity to cisplatin and oxaliplatin in vitro correlated with that to hypoxia, suggesting a more general change in pathways leading to apoptosis following a potentially lethal event. We have used these cell lines to identify transcripts that may underlie resistance to apoptosis under hypoxia.

Our initial approach to identify candidate genes used PCR-selected cDNA subtractive hybridization, from which we identified several candidate genes. We selected macrophage migration inhibitory factor (MIF) for additional study. Originally described as a T-cell–derived soluble peptide that altered the mobility of macrophages (8), MIF is released from the anterior pituitary in response to physiologic stress, including endotoxic shock, hypersensitivity, and adult respiratory distress syndrome, as well as in immune disorders, such as glomerulonephritis and arthritis (9). Its effect is to oppose the immunosuppressive effects of glucocorticoids, but recent data propose additional roles for MIF in cell proliferation, survival, and angiogenesis (10). These observations have increased interest in MIF in cancer research.

In the current article, we describe the identification of MIF as a determinant of the apoptotic response to hypoxic exposure in colon cancer cells with varying resistance/sensitivity to hypoxia. MIF expression was induced by hypoxia in HCT116 and in the cell line derived from it by repeated exposure to hypoxia, HCP40. Expression was decreased in HT29 and in a similarly derived hypoxia-conditioned cell line HP40. Expression correlated with the apoptotic response to hypoxia in these cell lines. The addition of exogenous recombinant MIF (rMIF) rescued resistant cells from an antiapoptotic effect of its down-regulation. In an unrelated series of colon cancer cell lines, MIF expression and its inducibility by hypoxia varied. The ability to up-regulate MIF was positively correlated with cytotoxicity from oxaliplatin, supporting a more general involvement of MIF in apoptotic responses. MIF expression was also specific for carcinoma in samples derived from patients with colorectal cancer, in which expression varied widely at all stages of the disease.

**Materials and Methods**

**Cells, tumor samples, and treatment.** The cell lines were obtained from American Type Culture Collection (Manassas, VA). The human colon cancer specimens were collected on a University of Pennsylvania clinical protocol from unselected patients with colorectal cancer who provided full informed consent for these analyses. The establishment and characterization of hypoxia-conditioned cell lines from HT29 and HCT116 have been described previously.4 Briefly, the two colon cancer cell lines were exposed to repeated 4-hour periods of sublethal hypoxia thrice weekly, resulting in the generation of a series of cell lines derived from each: from HT29, the HP series up to HP40, reflecting 40 exposures to hypoxia, and from HCT116, the HCP series up to HCP40. These cell lines have now been maintained stably in culture for >2 years. The cells were cultured in MEM supplemented with 10% fetal bovine serum. For the hypoxia treatments to induce apoptosis, the cells were exposed to profound hypoxia (95% N$_2$/5% CO$_2$, with <10 ppb O$_2$) in a Forma anaerobic chamber (Forma Scientific, Inc., San Jose, CA) for 8 hours and harvested for RNA and protein isolation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (11). For cytotoxicity under hypoxia, cells were plated at a density of 3,000 cells per well in 96-well plates. The plated cells were incubated overnight at 37°C at 5% CO$_2$ and 95% air. The cells were transferred to the incubator at 37°C at 5% CO$_2$ and 95% air for additional 48 hours, after which the MTT assay was done: 40 μL of 5 mg/mL MTT were added per well. After 2 hours

![Fig. 1. Sensitivity of HT29 and HCT116 and their hypoxia-conditioned derivatives (HP40 and HCP40, respectively) to profound hypoxia for 8, 24, and 48 hours as measured by the MTT assay.](image)

![Fig. 2. Northern analysis (A) and Western analysis (B) of colon cancer cell lines for MIF under normal oxic conditions or following an 8-hour exposure to profound hypoxia.](image)

---

4 K.S. Yao et al., submitted for publication, 2005.
at 37°C, the cells were lysed by adding 100 μL of 20% (w/v) SDS and 50% (v/v) N,N-dimethylformamide (pH 4.7) and incubated 3 hours at 37°C. The absorbance at 570 nm was determined using a microplate reader (BioTek Instruments, Inc., Winooski, VT). The reported values are the result of duplicate determinations. The data shown in MTT assay are means ± SD of two independent experiments done in triplicate. The statistical significance of the differences between control and hypoxia-treated groups was assessed by the two-sample Student’s t test between two paired samples.

**PCR-selected cDNA subtractive hybridization.** Subtractive hybridization was done using a commercially available kit (Clontech Laboratories, Inc., Palo Alto, CA) following the manufacturer’s instructions. mRNA from all four cell lines (HCT116 and HCP40 and HT29 and HP40) was isolated and converted into double-stranded cDNA. Both parental cell line mRNAs were used as drivers and that of the conditioned cell lines were used as testers. The tester and driver double-stranded cDNAs were separately digested by the RsaI restriction enzyme to obtain shorter, blunt-ended molecules. After ligation of two different adaptors (Adaptor 1 and Adaptor 2R provided by BD Biosciences, Palo Alto, CA) to the tester cDNA but not to the driver cDNA, the tester cDNA with adaptor and driver cDNA without adaptor were subjected to a first hybridization to remove common transcripts as well as to enrich for differentially expressed sequences. The samples were then hybridized a second time to further enrich for double-stranded cDNAs containing both adaptors. The double-stranded cDNA mixture was then subjected to two rounds of PCR amplification using PCR primers [primer 1 and primer 1R in the first PCR reaction and PCR-nested primer 2 with nested primer 2R in the second reaction (all primers provided by BD Biosciences)] to generate differentially expressed sequences. Finally, the PCR products were cloned into TA-vector (Invitrogen, Carlsbad, CA) for sequencing, and transcripts of interest were identified using Genbank.

**Northern blotting.** Total RNA was isolated using the Trizol reagent (Life Technologies, Grand Island, NY). Briefly, 10^7 cells were lysed in 3 μL Trizol reagent and incubated for 5 minutes at room temperature. After adding 0.6 μL chloroform, the tube was vortexed and incubated at room temperature for 5 minutes. The sample was centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase was mixed with 1.5 mL isopropyl alcohol to precipitate the RNA and again centrifuged at 12,000 × g for 15 minutes at 4°C. The RNA pellet was washed once with 70% ethanol and dissolved in diethyl pyrocarbonate–treated water. RNA was electrophoresed in a 1% agarose denaturing gel. The RNA was transferred to nylon membranes and hybridized to a 575-bp cDNA MIF probe (from the MIF coding sequence). A β-actin probe was used as a loading control. Membranes were washed for 1 hour in 2× SSC containing 0.5% SDS and at 60°C followed by 1 hour in 0.1× SSC containing 0.5% SDS. The filters were exposed to X-ray film at 70°C for 1 to 5 days.

**Western blotting.** Proteins from a total cell extract (20 μg/lane) were separated by electrophoresis in a SDS-12% polyacrylamide gel and transferred to a Hybond-P membrane (Amersham, Arlington Heights, IL). Western blotting was carried out using MIF antibody (R&D Systems, Inc., Minneapolis, MN) and horseradish peroxidase–conjugated serum as the second antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The Enhanced Chemiluminescence Plus detection system (Amersham) was used to develop the signal.

**Immunohistochemistry.** The formalin-fixed, paraffin-embedded mouse tumors were sectioned to 5 μm thickness and fixed onto slides. After dewaxing and rehydrating, the slides were subjected for antigen retrieval by boiling in the microwave for 10 minutes with 0.01 mol/L citrate buffer (pH 6.0). After antigen retrieval, nonspecific binding of antibody was blocked with 1% bovine serum albumin/TBS for 15 minutes. After PBS wash, slides were incubated with polyclonal rabbit anti-human MIF antibody (1:50 dilution; Santa Cruz Biotechnology) at 4°C overnight followed by incubation with biotinylated anti-rabbit antibody (DAKO Corp., Carpinteria, CA) and horseradish peroxidase–conjugated streptavidin (DAKO) with 3,3′-diaminobenzidine as the chromogen. The slides were counterstained with hematoxylin. Normal rabbit antibody was used as a negative control. A similar procedure was used for the human tumor specimens. Positive control slides were provided by the Cooperative Human Tissue Network.

Slides were evaluated by estimating the percentage of cells exhibiting MIF staining that was clearly distinct from any background staining. Faint staining that could not be convincingly positive was considered negative. Tumor cells and, when present, adenomatous and normal enterocytes were all evaluated for staining. Lymphocytes present in the colon served as an internal positive control.

**Macrophage migration inhibitory factor stimulation and neutralization studies.** We incubated colon cancer cells with both full-length rMIF and the inactivating antibody (both a gift from Dr. Gao; R&D Systems, Minneapolis, MN) using concentrations and exposure times as described previously (12). In brief, the cells were cultured overnight in DMEM containing 10% fetal bovine serum. The medium was replaced with fresh medium supplemented with purified rMIF at a final

---

**Fig. 2. continued.** C. Immunohistochemistry of paraffin-embedded tumor sections from HT29, HP40, HCT116, and HCP40 grown s.c. in severe combined immunodeficient mice by the injection of 1 × 10^7 cells. After growing for 14 days, tumors were excised and fixed for immunohistochemical analysis as described in Materials and Methods. D. Immunohistochemistry of frozen sections of tumors derived from HT29 and HP40. Pimonidazole staining to detect hypoxic cells in the tumor in HT29 and HP40 cells (hypoxic regions) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay by 3,3′-diaminobenzidine staining (apoptosis).
concentration of 100 ng/mL with or without a neutralizing anti-human MIF monoclonal antibody at 100 μg/mL. The cells were treated overnight and harvested for further analysis.

**Macrophage migration inhibitory factor small interfering RNA treatment.** The nucleotide sequences of dsRNA and complementary dsRNA for the construction of antisense to human MIF mRNA were based on MIF sequences (Genbank) as follows: [sense small interfering RNA (siRNA)] 5′-ACACCAACGUCGCCCGCGCdTdT-3′ and (antisense siRNA) 5′-GCCGCCGCCCGCGCGCdTdT-3′. As a control, we used a RNA duplex constructed from a scrambled sequence as follows: 5′-CGAUGACUCGCGCGCdTdT-3′. The MIF siRNA was effective in RNA interference–mediated suppression of MIF in our cell lines. The oligonucleotides were synthesized and purified by Integrated DNA Technologies (Coralville, IA). HCT116 and HCP40 cells (1 × 10⁶) were used for transfection experiments. The cells were transfected with either the MIF siRNA or the control scrambled RNA duplex using the Genesilencer siRNA Transfection Reagent (Genlantis, San Diego, CA) according to the manufacturer’s procedure. After 24 hours, the cells were exposed to hypoxia and evaluated by MTT assay as described above.

**Statistics.** Comparisons of quantitative measures between the cell lines were done using t tests, and P ≤ 0.05 was accepted as significant.

**Results**

**Differentially expressed transcripts in hypoxia-conditioned cell lines showing altered sensitivity to hypoxia-induced cell death.** We have described elsewhere the derivation and characterization of a series of hypoxia-conditioned colon cancer cell lines.⁴ Through repeated exposure to sublethal periods of profound hypoxia, the colon cancer cell lines HCT116 and HT29 exhibited altered morphology, growth characteristics, and responses to hypoxic challenge.⁴ We and others showed previously that apoptosis is a physiologic response to hypoxia in colon cancer cell lines (13–15). Both HCT116 and HT29 have reduced survival in a MTT assay at 8, 24, and 48 hours after incubation in a hypoxic environment (Fig. 1). Under hypoxic conditions as shown previously,⁴ the cell lines derived from these parental lines differ in susceptibility to hypoxia-induced cell death: the cell line derived from HCT116 (after 40 thrice-weekly hypoxic exposures), HCP40, is more susceptible to hypoxia and shows reduced survival (P < 0.005 for all time points). A similarly derived line from HT29 (HP40) is more resistant to hypoxia (Fig. 1; P < 0.02 at 24 and 48 hours). These cell lines therefore provide a model for the investigation of mechanisms of sensitivity and resistance to hypoxia-induced apoptosis in colorectal cancer.

Our initial approach to the isolation of candidate genes that might confer altered sensitivity to hypoxia was to use subtractive hybridization. This analysis revealed 31 genes, the expression of which varied proportionally with susceptibility to hypoxia-induced apoptosis, among them MIF (Appendix 1). We focused on MIF based on the recent association of the expression of this cytokine with features of the neoplastic process and confirmed the identification of this gene transcript using Northern and Western analyses. As shown in Fig. 2A and B, MIF expression at both RNA and protein levels is markedly increased in response to hypoxia in both HCT116 and HCP40. By contrast, hypoxia induces a striking decrease in steady-state MIF mRNA in HT29 and a decrease in both mRNA and protein for MIF in the hypoxia-resistant HP40. These results localize the hypoxia-induced changes to the level of transcriptional control and suggest that loss of MIF expression correlates with resistance to hypoxia-induced apoptosis.

To determine if these findings with a cytokine would also be observed in the *in vivo* setting, we implanted the conditioned cell lines s.c. in severe combined immunodeficient mice and examined growth characteristics and microscopic changes. We found that the conditioned cell lines (from both HT29 and HCT116) grew more rapidly *in vivo* than the parental lines and that this behavior was associated with increased angiogenesis in both tumors. This model therefore dissociates angiogenesis from apoptosis *in vivo*. Examination of MIF expression by immunohistochemistry showed that the tumors *in vivo* replicated the expression patterns *in vitro* (Fig. 2C). Thus, HT29 expressed significant amounts of MIF, which were lost in the HP40 tumors, whereas, in HCT116, a modest level of expression in the parental line was greatly increased in the HCP40 tumors. These data validate *in vivo* the model derived *in vitro*.

We were also interested to know if, in the hypoxic cores of tumors grown *in vivo*, differences in the proportion of apoptotic cells might be appreciated. In Fig. 2D, we show regions of high pimonidazole staining reflecting hypoxia in both HT29 and HP40. In HT29 tumors, a substantial proportion of apoptotic cells is observed, but many fewer are present in HP40. This suggests that, *in vivo* also, the HP40 tumors are resistant to hypoxia-induced apoptosis.

**Macrophage migration inhibitory factor content has a direct effect on survival in response to hypoxia.** To determine if MIF production has a functional role in the survival of cells under hypoxic conditions, we added rMIF to the culture medium of HT29 and HP40 cells and examined their responses to hypoxia. We hypothesized that the addition of MIF would render the cells more susceptible to apoptosis as observed in the HCT116 and HCP40 lines. Exposure to hypoxia for 48 hours resulted in a 30% decrease in the survival of HT29 cells, but only a 10% decrease in the resistant HP40 cells (Fig. 3A). The addition of a neutralizing MIF antibody to HT29 cells rendered their profile more resistant but had no effect on that of HP40, which was already resistant (Fig. 3A). Conversely, the addition of rMIF to HT29 had a minimal effect on survival, whereas, in HP40, a marked increase in cell kill was observed, restoring the sensitivity of this line almost to that of the parental HT29. Specificity of this response for MIF was shown by coincubation with both MIF and the MIF antibody, which abolished the effect of MIF on survival in the resistant line. These experiments related the observed changes in gene expression to a functional effect on susceptibility of the cells to hypoxic cell death. The results of a colony-forming assay are shown (Fig. 3B): a similar pattern of cell kill as in the MTT assay is evident.

To analyze further the contribution of MIF, we examined the effects of down-regulating MIF expression in the HCT116/HCP40 pair of cell lines. We used a 21-bp siRNA oligonucleotide and showed that steady-state MIF mRNA content was decreased in a concentration-dependent fashion (Fig. 4A). Almost complete ablation of MIF mRNA content was achievable in both HCT116 and HCP40 cells treated underoxic and hypoxic conditions (Fig. 4B). The effect of this down-regulation on cell survival is shown in Fig. 4C: the increased sensitivity to hypoxia on the part of the high MIF-expressing HCP40 line was abolished by siRNA treatment.
An effect on hypoxia-induced cell kill is also evident in the parental HCT116 line. These findings are consistent with the involvement of MIF in hypoxia-induced apoptosis in these cell lines.

Macrophage migration inhibitory factor expression varies in human colon cancer cell lines and tumor specimens. To examine the broader relevance of these observations to colon cancer, we extended the analysis of MIF expression to a series of 10 additional human colon cancer cell lines and to a series of human colon cancer specimens for which there was matched normal colon. In 12 colon cancer cell lines, we observed a wide variation in MIF expression from 0.1 to 2.2 (arbitrary units, ratio to expression of β-actin; Fig. 5A). Also variable was the response of the cell lines to hypoxic exposure. In seven cell lines, induction or stable expression was found. In five of the lines (HCT15, SW620, BE, HT29, and Colo205), downregulation of MIF occurred on hypoxic exposure. To investigate a relationship between this event and susceptibility to apoptosis induction, we analyzed the relationship between MIF induction and sensitivity to a DNA-damaging agent (oxaliplatin) expressed as IC50 in a colorimetric cell growth assay (Fig. 5B). This analysis revealed that the relative change in MIF expression on hypoxic exposure was predictive of sensitivity to oxaliplatin treatment in vitro ($r \pm SD = 0.378 \pm 0.369$).
As part of study of gene expression in human colon cancer, we had 11 cases for which we had available tumor as well as adenoma and normal mucosa samples and 27 colon cancers in total. We examined these specimens for MIF expression by immunohistochemistry. Among the cases with benign and malignant tissues, we found no detectable staining in any of the normal colon and rare staining in the adenoma samples, whereas all of their malignant tissue counterparts had positive staining that ranged from 5% to 90% of the cells (median, 40%). A comparison of MIF expression in tumor versus normal colon epithelium in the same patient is shown in the Northern analysis of samples from five of these patients (Fig. 5C). A clear relationship with stage could be discerned in this small sample.

Discussion

For antiangiogenic therapy of cancer to be effective, tumor cells must have the capacity to enter apoptosis in response to oxygen and nutrient deprivation. Tumor cells have long been known to be resistant to chemotherapy- and radiation-induced apoptosis in a hypoxic environment. To investigate the basis of this resistance, we used a series of cell lines conditioned by repeated exposure to hypoxia and characterized by varying susceptibility to hypoxia-induced apoptosis. The cell lines are collaterally sensitive and resistant to DNA-damaging platinum compounds.

We analyzed the differences in gene expression among these cell lines to isolate transcripts associated with hypoxia sensitivity and resistance. From a subtractive hybridization screen emerged the cytokine MIF, which was found to be associated with the ability of colon cancer cells to enter apoptosis in response to hypoxia. MIF expression was induced by hypoxia in some but not all colon cancer cell lines, whereas induction has also been reported in glioma and squamous cell cancers (16, 17). In our experiments, the expression of MIF protein was closely correlated with that of the RNA, suggesting regulation at the level of transcription. Additional experiments using a reporter construct containing the MIF promoter confirmed that, in the cells with high MIF expression, transcriptional activity was increased. In this study, we validated MIF expression from the subtractive screen and showed that MIF and its induction by hypoxia were positively correlated with susceptibility to hypoxia- or DNA damage–induced apoptosis in cell lines, with concordant results. We found further that, in both cell lines and human tumors, expression of MIF is variable and, in clinical specimens, independent of stage of disease.

The functional relevance of MIF to hypoxia-induced apoptosis was established by the restoration of hypoxic sensitivity of HP40 by exogenous rMIF and siRNA down-regulation of MIF in the resistant line. However, a similar increase in sensitivity to apoptosis (i.e., to the level of HCT116, for example) was not observed when HT29 cells were similarly treated (data not shown). This finding suggests that increasing MIF alone will not assure a high level of sensitivity to hypoxia-induced apoptosis but that its suppression or absence is sufficient to result in resistance. A potential role for MIF supplementation in resistant tumors merits exploration.

A positive or facilitatory role of MIF in apoptosis may be inferred from several lines of evidence. MIF+/– mice have normal development but are resistant to lethality from bacterial infection and endotoxin (18). Macrophages from such mice are resistant to tumor necrosis factor-α–induced cytotoxicity

For antiangiogenic therapy of cancer to be effective, tumor cells must have the capacity to enter apoptosis in response to oxygen and nutrient deprivation. Tumor cells have long been known to be resistant to chemotherapy- and radiation-induced apoptosis in a hypoxic environment. To investigate the basis of this resistance, we used a series of cell lines conditioned by repeated exposure to hypoxia and characterized by varying susceptibility to hypoxia-induced apoptosis. The cell lines are collaterally sensitive and resistant to DNA-damaging platinum compounds.

We analyzed the differences in gene expression among these cell lines to isolate transcripts associated with hypoxia sensitivity and resistance. From a subtractive hybridization screen emerged the cytokine MIF, which was found to be associated with the ability of colon cancer cells to enter apoptosis in response to hypoxia. MIF expression was induced by hypoxia in some but not all colon cancer cell lines, whereas induction has also been reported in glioma and squamous cell cancers (16, 17). In our experiments, the expression of MIF protein was closely correlated with that of the RNA, suggesting regulation at the level of transcription. Additional experiments using a reporter construct containing the MIF promoter confirmed that, in the cells with high MIF expression, transcriptional activity was increased. In this study, we validated MIF expression from the subtractive screen and showed that MIF and its induction by hypoxia were positively correlated with susceptibility to hypoxia- or DNA damage–induced apoptosis in cell lines, with concordant results. We found further that, in both cell lines and human tumors, expression of MIF is variable and, in clinical specimens, independent of stage of disease.

The functional relevance of MIF to hypoxia-induced apoptosis was established by the restoration of hypoxic sensitivity of HP40 by exogenous rMIF and siRNA down-regulation of MIF in the resistant line. However, a similar increase in sensitivity to apoptosis (i.e., to the level of HCT116, for example) was not observed when HT29 cells were similarly treated (data not shown). This finding suggests that increasing MIF alone will not assure a high level of sensitivity to hypoxia-induced apoptosis but that its suppression or absence is sufficient to result in resistance. A potential role for MIF supplementation in resistant tumors merits exploration.

A positive or facilitatory role of MIF in apoptosis may be inferred from several lines of evidence. MIF+/– mice have normal development but are resistant to lethality from bacterial infection and endotoxin (18). Macrophages from such mice are resistant to tumor necrosis factor-α–induced cytotoxicity

6 K.S. Yao and P.J. O’Dwyer, data not shown.
Similarly, MIF is described as an antagonist of glucocorticoid responses, although it is not reported if this extends to inhibition of glucocorticoid-induced lymphocyte apoptosis (9, 19). Our data suggest that the absence of MIF precludes the induction of hypoxia-induced apoptosis, and the mechanism of this effect will be important to elucidate. These data might seem to be inconsistent with a recent publication from Bernhagen’s group in which MIF was shown to be antiapoptotic in tumor cells treated with camptothecin or exposed to oxidative stress through thiol starvation or diamide treatment (20). In the setting of thiol starvation, the antiapoptotic role of MIF was possibly associated with an apparent inhibition of c-Jun NH2-terminal kinase phosphorylation of c-Jun at a late time point, but these effects are often variable with respect to the models and their influence on apoptosis (21). Interestingly, the effects of MIF in these models seemed to be exerted by different mechanisms. The apoptosis suppression by MIF in thiol-starved cells was dependent on the oxidoreductase Cys-Ala-Leu-Cys motif, because overexpression of the mutant C60SMIF construct failed to influence apoptosis. With camptothecin-induced apoptosis on the other hand, the serine-substituted protein was equally effective in suppressing apoptosis. Other groups have reported the suppression of p53-mediated apoptosis by MIF (22, 23) and suggest a link between the enzymatic redox function of MIF and its role in the regulation of cell survival (24). Nguyen et al. (20) have shown that MIF suppresses pro-oxidative stress-induced apoptosis. It is possible that the effects observed with hypoxia-induced apoptosis may be specific to

![Diagram](https://example.com/diagram.png)

**Fig. 5.** A, Northern analysis of 12 colon cancer cell lines for MIF under normal oxic conditions or following an 8-hour exposure to profound hypoxia. The hypoxic/oxic ratio indicates the ratio of RNA expression in oxic versus hypoxic cells, both expressed relative to &b-actin. B, relationship of inducibility of MIF expression (hypoxic/oxic ratio as in A above) to susceptibility to cell kill by oxaliplatin or cisplatin, defined by IC50. C, Northern blot of five paired samples of normal colon epithelium and colon adenocarcinomas for MIF and &b-actin. D, staining of adenoma-epithelial boundary with anti-MIF in a patient with colon adenocarcinoma.
that stimulus; if supported in further studies in vivo, a clear opportunity for selective therapeutic intervention may be provided.

An extensive prior literature supports a growth-promoting role for MIF, however, and these effects on apoptosis may seem to be in conflict. It has been reported that MIF is expressed in several types of tumors, including myelomonocytic leukemia (25), prostate cancer (26), breast cancer (27), colon cancer (28, 29), and lung cancer (30). However, the precise role of MIF in carcinogenesis and tumor progression remains unclear. In colon cancer cells, Takahashi et al. found that MIF was expressed and that its inhibition by an antisense construct resulted in a 40% decrease in growth (28). In a lymphoma model, similar inhibition of tumor cell growth was observed (31). Interestingly, in this model, there seemed to be minimal effects on the tumor cells, whereas profound inhibition of endothelial cell proliferation occurred. This antiangiogenic effect was confirmed in a melanoma cell model by Shimizu et al. (32). Munaut et al. found that, in human glioblastomas, MIF expression correlated with vascular endothelial growth factor expression (33). Because in the colon cancer cell lines described here there is increased angiogenesis and elevated vascular endothelial growth factor expression in vivo with both conditioned cell lines, MIF expression is dissociated from angiogenesis in this model.

The link with tumor cell proliferation is, however, well preserved in this model: the doubling time of the HCT116 derivative HCP40 is decreased, whereas that of HP40 is increased relative to the parental lines. A contribution of MIF to proliferation is suggested also by the work of Hudson et al. (22), who showed that p53-mediated activation of the cell cycle inhibitor p21 could be abrogated by MIF. Additional support for the importance of MIF in progression of cancer comes from the studies of Bucala’s group, who showed that proliferation and carcinogenesis in MIF knockouts are p53 dependent (34). Because in the colon cancer cell lines described here there is increased angiogenesis and elevated vascular endothelial growth factor expression in vivo with both conditioned cell lines, MIF expression is dissociated from angiogenesis in this model.

The biology of MIF and its effects on proliferation and survival are complex and sometimes contradictory (35). Like other redox proteins, MIF seems to be multifunctional, with both a redox and possibly an isomerase role, the biological substrate for which has not yet been defined (36, 37). Despite extensive efforts, a receptor for MIF has not yet been identified, and its access to cells seems to occur through endocytosis (38). The protein is predominantly localized in the cytoplasm, although a minority of tumors shows nuclear staining on immunohistochemical analysis. Protein partners include CD74, Jab-1 (a c-Jun NH2-terminal kinase – interactive protein), and an antioxidant protein PAG, but the functional implications of these interactions remain to be elucidated (39–41). The activation of mitogen-activated protein kinase signaling pathways by MIF in this model is consistent with previous work (12), and additional participants in signaling pathways, including protein kinase A and phospholipase A2, are subject to MIF regulation (42). Our work supports an autocrine mechanism of MIF regulation in these cells, and elucidation of this pathway may identify additional targets for modifying the expression of MIF in hypoxic cells.

Taken together, these data indicate that MIF may be required for hypoxia-induced apoptosis in vitro and that its variable expression in human colon cancers may indicate a functional role in vivo. We suggest that MIF expression in colorectal cancer may be a marker of susceptibility to therapies that may depend on induction of hypoxia, possibly including antiangiogenic therapy.

### Table 1. Expression of MIF in human colorectal cancers

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>% Cells staining positive for MIF, median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>50 (20-80)</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>70 (20-80)</td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>60 (50-90)</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>80 (5-90)</td>
</tr>
</tbody>
</table>

### Appendix 1. Genes isolated through subtractive hybridization from hypoxia-conditioned cell lines

- Increased in HP40 vs HT29
  - CGI-31 protein (LOC51075)
  - Guanine nucleotide binding protein (G protein), β-polypeptide 2 – like 1
  - Tumor-associated calcium signal transducer 2 (TACSTD2)
  - Lectin, galactoside binding, soluble, 4 (galectin 4)
  - Integrin β1 (fibronectin receptor, β-polypeptide, antigen CD29 includes MDF2 and MSK12, ITGB1)
  - Eukaryotic translation elongation factor 1 α (EEF1A1)
  - Annexin A2 (ANXA2)
  - Voltage-dependent anion channel 1
  - Protein phosphatase 1, catalytic subunit, γ isoform (PPP1CC)

- Decreased in HP40 vs HT29
  - MIF
  - Eukaryotic translation elongation factor 1 γ
  - Small nuclear ribonucleoprotein polypeptide A
  - NAC mRNA
  - Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
  - Protein tyrosine phosphatase, receptor type, F (PTPRF)

- Increased in HCP40 vs HCT116
  - Diazepam binding inhibitor (γ-aminobutyric acid receptor modulator, acyl-CoA binding protein; DBI)
  - Similar to N-myc downstream regulated
  - FAT tumor suppressor homologue 1 (FAT)
  - X-box binding protein 1
  - CD59, a LY-6-like protein regulating complement membrane attack
  - Neural precursor cell expressed, developmentally down-regulated 5 (NEDD5)

- Decreased in HCP40 vs HCT116
  - SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARCD2)
  - Eukaryotic translation elongation factor 1 α (EEF1A1)
  - Glyceraldehyde-3-phosphate dehydrogenase
  - Stem-loop (histone) binding protein (SLBP)
  - Methionine adenosyltransferase II, β (MAT2B)
  - Fatty acid-CoA ligase, long-chain 5 (FACL5)
  - S100 calcium-binding protein A6 (calcyclin)
  - RNA helicase p68 (HUMP68) gene
References


15. Shimizu S, Eguchi Y, Kamike W, et al. Induction of apoptosis as well as necrosis by hypoxia and predomi-


31. Shimizu S, Eguchi Y, Kamiike W, et al. Induction of apoptosis and necrosis by hypoxia and predomi-


Macrophage Migration Inhibitory Factor Is a Determinant of Hypoxia-Induced Apoptosis in Colon Cancer Cell Lines

Kangshen Yao, Seiichiro Shida, Muthu Selvakumaran, et al.


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/11/20/7264

Cited articles This article cites 41 articles, 14 of which you can access for free at: http://clincancerres.aacrjournals.org/content/11/20/7264.full#ref-list-1

Citing articles This article has been cited by 11 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/11/20/7264.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/11/20/7264. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.