Macrophage Inhibitory Cytokine 1 Reduces Cell Adhesion and Induces Apoptosis in Prostate Cancer Cells

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ABSTRACT

Macrophage inhibitory cytokine 1 (MIC-1), a divergent member of the transforming growth factor-β superfamily, is linked to the pathogenesis of cancer. To delineate possible roles for MIC-1 in prostate cancer, a number of prostate epithelial cell lines have been studied, including PZ-HPV-7, DU-145, PC-3, and LNCaP cells. Factors regulating the production of MIC-1 protein by these cells and some of the effects of MIC-1 on them were investigated. Although PZ-HPV-7 and DU-145 produced no MIC-1 protein, PC-3 and LNCaP cells secreted MIC-1 protein at high levels. The secretion of MIC-1 in LNCaP cells was modulated by both androgen and estrogen. Although neither MIC-1 nor anti-MIC-1 antibody had any effect on the proliferation of epithelial cells, MIC-1 induced changes in DU-145 cells. These cells became flattened and more spread out, and this was accompanied by reduced intercellular actin filaments and intercellular junctions. The DU-145 cells then detached from their substrate and underwent caspase-dependent apoptosis. To define some of the genes responsible for these changes, cDNA microarrays, followed by confirmatory reverse transcription-PCR, was used to analyze differential gene expression induced by MIC-1. The antiapoptotic gene metallothionein 1E and cell adhesion genes RhoE and catenin δ1 were down-regulated by more than 2-fold by MIC-1, suggesting that they were, at least in part, responsible for the observed changes in the behavior of DU-145 cells. These findings suggest that although MIC-1 has no effect on cell proliferation, it reduces cell adhesion and consequently induces cell detachment. It is likely that caspase-dependent apoptosis is secondary to loss of cell adhesion and may suggest a role for MIC-1 in tumor dissemination in vivo.

INTRODUCTION

Prostate cancer is the most common noncutaneous male malignancy and the second greatest cause of male cancer death in most Western societies (1). The development of prostate cancer is a multistep process regulated by hormones and growth factors including those of the TGF-α/β and fibroblast growth factor superfamilies. A series of molecular changes take place to promote malignant transition and then progression from being confined in the organ to an invasive and metastatic phenotype. Growth factor cytokines also make a significant contribution to the transition from androgen-dependent to androgen-independent states (2), and one of the most important of these is TGF-β.

TGF-β is a multifunctional cytokine, the biological roles of which include cell cycle control, regulation of early development, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis, immune regulation, and the induction of apoptosis (reviewed in Ref. 3). TGF-β is well known for its inhibition of the proliferation of epithelial cells, such as Mv1Lu mink lung epithelial cells and prostate DU-145 cells. It has also been shown to induce apoptosis in normal prostate epithelial cells and prostatic carcinoma cells both in vivo and in vitro (4–6). This growth inhibition and apoptosis can be correlated with its function as a tumor suppressor.

Another TGF-β superfamily cytokine linked recently to epithelial malignancies is MIC-1, which is also known by other names including prostate-derived factor, growth differentiation factor-15, placental bone morphogenetic protein (PLAB), placental transforming growth factor β, and nonsteroidal anti-inflammatory drug-activated protein 1. It is a divergent member of the TGF-β superfamily (7, 8) and was first isolated from a subtracted cDNA library enriched for genes associated with macrophage activation (8). It is synthesized as a 62-kDa protein, which, after cleavage by a furin-like protease, is secreted as a 25-kDa, disulfide-linked dimer. MIC-1 shares relatively low sequence homology with other family members and does not cluster within existing TGF-β families (8). Under resting conditions, MIC-1 mRNA is expressed highly in placenta, moderately in adult prostate, and at lower levels in brain, liver, kidney, and pancreas (9, 10).

Relatively little is known about the function of MIC-1, but a number of factors link it to epithelial tumors. MIC-1 is powerfully induced by p53 (11), and MIC-1 concentrations in serum and MIC-1 protein production in colon and prostate cancer tissues were up-regulated compared with their normal counterparts (12, 13). Marked up-regulation of MIC-1 mRNA in the transition from normal to prostate cancer cells and from androgen-dependent to androgen-independent prostate cancer cells has been demonstrated with microarray analysis (14, 15). Additionally, nonsteroidal anti-inflammatory drugs that inhibit tumor development also induce HCT-116 colon cancer cells to undergo apoptosis, possibly mediated by autocrine/paracrine induction of MIC-1 (16). MIC-1 has also been reported to inhibit prostate epithelial proliferation (17), induce colon and mammary epithelial cancer cell apoptosis in vitro, and inhibit colon and glioblastoma tumor growth in vivo (11, 16, 18, 19).

To better understand the hormonal factors that may modulate prostate MIC-1 expression and some of the possible consequences of MIC-1 overexpression in prostate cancer, studies of widely available prostate cancer lines were undertaken. These indicate that MIC-1 production by hormone-sensitive prostate cancer cells is modulated by hormones, and that MIC-1 has no effect on cell proliferation but reduces cell adhesion and induces apoptosis. By comparing the transcriptome of MIC-1-treated and -untreated cells, we have been able to determine that these effects are likely to be at least in part mediated by MT1E, catenin δ1, and RhoE.

MATERIALS AND METHODS

Cell Culture. PZ-HPV-7 nonmalignant epithelial prostate cells (ATCC, Rockville, MD) were grown in keratinocyte, serum-free medium plus epidermal growth factor, and supplemented with 2 mM L-glutamine.
MvILu mink lung epithelial cells (ATCC) were grown in MEM plus nonessential amino acids (Life Technologies, Inc.) and 10% fetal bovine serum. PZ-HPV-7, DU-145, PC-3, and LNCaP cells were cultured in the standard 75-cm²-sized tissue culture flasks (Becton Dickinson, Oxford, United Kingdom). When cells reached ~90% confluency, the culture medium was exchanged for fresh medium. After an additional 24 h, conditioned medium was collected and stored at 4°C for the MIC-1 sandwich ELISA assay. To assess whether MIC-1 production was modulated by hormones, hormone-responsive LNCaP cells were plated in 96-well plates at 4500 cells/well in RPMI 1640 plus 10% fetal bovine serum for 24 h. Cells were then washed with serum-free medium and then RPMI plus 10% dextran-coated, charcoal-stripped fetal bovine serum with DHT (Sigma) or β-estradiol (Sigma) at 10⁻¹⁰ to 10⁻¹² M, or control was added in quadruplicate cultures. Cell culture medium was collected 6, 24, 72, or 96 h after treatment for estimation of MIC-1 concentration, and cells were collected for cell number determination.

**Fluorescence Immunocytochemistry.** PZ-HPV-7, DU-145, PC-3, and LNCaP cells were plated on sterile coverslips in 24-well culture plates. Twenty-four h later, the cells were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. After incubation with 2% BSA and 2% fetal bovine serum in PBS, the cells were incubated with 1:400 dilution of sheep anti-hMIC-1 polyclonal antibody 23B3 (20) or control sheep antibody and streptavidin-horseradish peroxidase conjugate (Genzyme) for 30 min, according to the manufacturer’s instructions, with 5 μg/ml FITC-conjugated VAD-FMK, which specifically binds activated caspases (21). Floating and adherent cells were then collected separately and analyzed using a flow cytometer. Total cells and FITC-positive cells were counted using TRUCount absolute count tubes (Becton Dickinson, San Jose, CA) by comparing cellular events with bead events, according to the manufacturer’s directions.

**cDNA Microarray.** DU-145 cells were plated in large flasks at 2 × 10⁶ cells/flask in RPMI 1640 with 10% FCS. Cells were washed with RPMI 1640 SFM and incubated in this medium for 4 h. After treatment with 10 ng/ml hMIC-1 or control for 1, 3, or 9 h, cells were lysed, and RNA was extracted with the standard guanidium/phenol/chloroform method. Direct labeling cDNA microarray experiments were carried out as described previously with some modification (22). Briefly, 10 μg of total RNAs from MIC-1 and control treated samples were used separately to synthesize cDNA with the incorporation of Cy3- or Cy5-dUTP. After cleavage of the PCR products mixed together with blocking agents including poly(dA) and COT-1 DNA, a set of cDNA microarray slides with 19k clones purchased from Ontario Cancer Institute, Toronto, Ontario, Canada were prehybridized with 0.1% BSA. The probe mixture was poured onto the microarray slides. After overnight hybridization, the slides were washed, dried, and scanned with the GenePix Scanner (Axon Instruments, Union City, CA). Data were then processed with GenePix and GeneSpring (Silicon Genetics, Redwood City, CA) software. A postnormalization cutoff of 2-fold up- or down-regulation was used to define differential gene expression. The results were then validated with real-time RT-PCR.

**Validation of Microarray Results with Real-Time RT-PCR.** Confirmation of microarray data was carried out with real-time RT-PCR with RNAs from three independent cell treatment experiments. All results of RT-PCR for each gene were presented as mean ± SE from two PCRs for each RNA sample. Identification of mycogenes was based on microarray data validation with real time RT-PCR reported previously (23). Glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene to control for input RNA because it showed high expression levels, which did not differ between samples tested. Specific primers used for PCR were as follows: 5’-GCTTGTGTGCTACTGTTG-3’ and 5’-CAGGGTGTGAGCTGGTG-3’ for MTIE; 5’-ACCCAAAACATGCTCGGAG-3’ and 5’-TCTT- TTGGCTGGCTTCAA-3’ for complement component 5; 5’-AACGTCGCTGAAAGCCAGACG-3’ and 5’-GGGATGATCACAAATGGC-3’ for integrin α3; 5’-TTGCGTCCTTGGCAAATCC-3’ and 5’-CAGTCTGATCCTTCGCTG-3’ for cation 61; 5’-GTGGGTTGACGGCATGAC-3’ and 5’-CAACCCGGGAGATCTGCC-3’ for RhoE. Oligonucleotide primers were synthesized by GeneWorks (GeneWorks, Adelaide, South Australia, Australia). One μg of total RNA was reverse transcribed using the Superscript II RNase H-Reverse Transcriptase System (Invitrogen, Carlsbad, CA). Diluted cDNA samples (1:5) were subjected to PCR amplification with 30 μm MgCl₂, Taq DNA polymerase (InVitrogen), and SYBR Green I dye (Molecular Probes). The PCR reaction was carried out in a Rotor-Gene real-time PCR machine (Corbett Research, Mortlake, New South Wales, Australia). The reaction mixture was denatured at 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, annealing at 58 to 62°C for 20 s, and extension at 72°C for 30 s. The specificity of the products was verified by melting curves generated by Rotor-Gene software and the sizes on electrophoretic gels. Because the kinetics of fluorescence accumulation during thermocycling are directly related to the starting number of cDNA copies, relative mRNA
expression levels were determined on the basis of the kinetic approach using the Rotor-Gene real-time PCR software and the standard curves constructed with series purified cDNA dilutions (23). The target mRNA concentration of control cells, normalized to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA, was ascribed a fold induction of 1.0.

Statistical Analysis. All data for statistical analysis were presented as mean ± SE. Differences were analyzed for significance using ANOVA among groups. A probability value of 0.05 or less was considered significant.

RESULTS

MIC-1 Is Secreted by Prostate Cancer Cell Lines. To explore the roles of MIC-1 in prostate cancer cells, nonmalignant and malignant prostate epithelial cell lines were first examined for production of significant amounts of MIC-1. Immunofluorescence revealed that the majority of LNCaP cells and PC-3 cells stained positively with sheep anti-hMIC-1 antibody (Fig. 1B). No staining was seen in LNCaP and PC-3 cells incubated with control sheep antibody. Neither DU-145 nor PZ-HPV-7 cells exhibited any intracellular MIC-1 staining (Fig. 1A).

To quantify the production of MIC-1 by these cells, cell line culture supernatants were assayed for MIC-1 content using a sensitive MIC-1 sandwich ELISA. Results were consistent with the immunocytochemical data. Conditioned culture medium from subconfluent PZ-HPV-7 and DU-145 cells showed no detectable MIC-1 secretion over a 24-h period. In contrast, LNCaP cells grown under similar conditions secreted a huge amount of MIC-1 (>1000 ng/ml/24 h). PC-3 cells showed moderate MIC-1 production (110 ng/ml/24 h).

Both Androgens and Estrogens Inhibit MIC-1 Secretion by LNCaP Cells. Because hormones and growth factors are two of the most important factors in prostate cancer progression, the effect of hormones on MIC-1 production was investigated. To be able to correct MIC-1 secretion for cell number, the effects of hormones on this parameter were examined. Consistent with the literature, both DHT and β-estradiol enhanced LNCaP cell proliferation. As shown in Fig. 2A, total cell numbers increased after treatment with DHT for 72 h. The highest increase, of >40%, was seen at a DHT dose of 10⁻¹⁰ m. Cell numbers were also increased by β-estradiol, and 10⁻⁹ m seemed to be the most effective dosage (198,149 ± 5,3511 dpm for β-estradiol versus 96,895 ± 16,721 dpm for control). When cell numbers were not considered, β-estradiol had little effect on MIC-1 secretion into conditioned medium, whereas DHT, at the dose of 10⁻⁹ m, significantly decreased MIC-1 concentration in the culture medium by nearly 20% (Fig. 2B; P < 0.05). The inhibitory effect of DHT became more marked after MIC-1 concentration was adjusted for cell numbers (Fig. 2C). The greatest effect was again seen with 10⁻⁹ m DHT, which decreased MIC-1 production by 55%. Estradiol also decreased MIC-1 production per 10⁶ cells. At 10⁻¹⁰ m, β-estradiol reduced MIC-1 production per 10⁶ cells to the same extent as the same dose of DHT.

The inhibitory effect of DHT and β-estradiol on MIC-1 production by LNCaP cells was time dependent (Fig. 2D). The effect started to be obvious at 6 h of treatment, and by 24 h, MIC-1 per 10⁶ cells was reduced significantly by both DHT and β-estradiol, although β-estradiol was still not as effective as DHT at the same dose. At 96 h after treatment, the inhibitory effects of DHT and β-estradiol were similar.

MIC-1 Has No Effect on Epithelial Cell Proliferation. TGF-β is well known to inhibit epithelial cell proliferation, and whether MIC-1...
MIC-1 REDUCES CELL ADHESION AND INDUCES APOPTOSIS

A

Fig. 3. Effects of TGF-β, MIC-1, and anti-MIC-1 antibody on [3H]thymidine incorporation by epithelial cell lines. A, hTGF-β inhibited [3H]thymidine incorporation in Mv1Lu mink lung epithelial cells, whereas neither hMIC-1 nor murine MIC-1 had any effect. B, hMIC-1 did not have an effect on [3H]thymidine incorporation in DU-145 cells in SFM or in medium with 1 or 10% FCS. C, anti-human MIC-1 antibody had no effect on [3H]thymidine incorporation in LNCaP cells in all of the three medium conditions tested. Results are presented as means of [3H]thymidine incorporation from at least quadruplicates; bars, SE. *, P < 0.05.

had similar effects was investigated. At the dosages of 0.5 to 500 pg/ml, TGF-β decreased [3H]thymidine incorporation by Mv1Lu mink lung epithelial cells in SFM in a dose-dependent fashion (Fig. 3A). However, neither human MIC-1 nor murine MIC-1 had any effect on Mv1Lu epithelial cell proliferation in SFM. Consistent with previous reports, 500 pg/ml TGF-β decreased [3H]thymidine incorporation by DU-145 cells in SFM by ~50% at 48 h (data not shown). At the dosages of 0.1 to 500 ng/ml, however, hMIC-1 had no effect on DNA synthesis in DU-145 cells in SFM or in medium with 1 or 10% FCS (Fig. 3B).

Because LNCaP cells secrete a large amount of MIC-1 into conditioned medium, we determined whether a high-affinity polyclonal anti-MIC-1 antibody would modify the proliferation of these cells. At a dilution of 1:200 to 1:100,000, polyclonal sheep anti-human MIC-1 antibody 233B3 had no effect on [3H]thymidine incorporation of these cells compared with preimmune sheep IgG (Fig. 3C). Similar results were also obtained with PC-3 cells, which also secrete substantial amounts of MIC-1 (data not shown).

Although it is difficult to interpret the results on PC3 and LNCaP cells, because mutations of receptor and/or signaling pathway are common in malignant cells secreting inhibitory molecules, this is less likely to be the case with DU-145, which secretes no MIC-1. The lack of effect of MIC-1 on DU-145 growth suggests a lack of effect of MIC-1 on proliferation of prostate cancer cells.

MIC-1 Induces Morphological Changes and Detachment in DU-145 Cells. Because DU-145 was the only prostatic cancer cell line not secreting MIC-1, it was the most appropriate cell in which to examine, in more depth, the effect of this cytokine. After treatment with MIC-1 for 3 days in SFM, significant morphological changes could be seen. Whereas control cells looked round, cells treated with MIC-1 looked flattened and more spread out (Fig. 4A and 4.1B). This effect was visible only in a proportion of DU-145 cells located at the periphery of large clusters of cells and in very small clusters. After the 4th posttreatment day, significantly more cells treated with MIC-1 than control detached and floated in the medium. Visually, on the 4th day, the majority of the floating cells looked healthy and were viable on trypsin blue staining. However, from the 5th day onward, they became smaller in size and looked unhealthy or nonviable.

To understand the basis of the morphological changes, we stained the cells with fluorescent probes for actin, vimentin, and tubulin. These showed that MIC-1 induced a dramatic reduction in intercellular filamentous actin filaments with the disappearance of intercellular junctions in about 5–10% of the total cell population. There was no effect on vimentin and tubulin distribution and no clear actin cytoskeleton change inside the cells (Fig. 4.1C and 4.1D).

To quantify and evaluate apoptosis in the DU-145 cells detached after incubation with MIC-1, flow cytometry analysis was performed on cells stained with the activated caspase marker CaspACE™ FITC-conjugated VAD-FMK in TRUCOUNT absolute count tubes. This indicated that MIC-1 increased the total number of detached cells and the number of detached cells negative to FITC-conjugated VAD-FMK (nonapoptotic cells). Treatment with 0.1, 1.0, and 10.0 ng/ml MIC-1 for 6 days resulted in increased numbers of detached cells by 213, 354, and 565%, respectively, compared with control, and increased FITC-conjugated, VAD-FMK-negative cells by 39, 94, and 168%, respectively (Fig. 4.2).

MIC-1 Induces Caspase-dependent Apoptosis in DU-145 Cells. The morphological appearance of the MIC-1-treated, nonadherent DU-145 cells suggested that they might have undergone apoptosis. Assessing this with FITC-conjugated VAD-FMK showed that MIC-1 did indeed induce apoptosis in DU-145 cells. This occurred in a dose-dependent manner with maximal effect occurring at 10 ng/ml (Fig. 5), at which concentration ~10% of the cells had undergone apoptosis by 6 days. This corresponded to a more than 8-fold increase in apoptotic cells compared with controls (Fig. 5).

When adherent and detached cells were analyzed separately, it was clear that a lower proportion of the adherent cells was apoptotic compared with their nonadherent counterparts (Fig. 5). This may indicate that reduction in cell adhesion possibly plays a role in the induction of apoptosis.

DNA Microarray Analysis of MIC-1-induced Changes in DU-145 Gene Expression Profile. To understand, on a more global basis, the alterations that MIC-1 was inducing in DU-145 cells, its effects on a large proportion of the transcriptome were assessed using cDNA microarrays. A set of cDNA microarray slides with 19k cDNA clones purchased from Ontario Cancer Institute were hybridized with cDNA synthesized from RNA isolated from cells treated with MIC-1 or control. About 30% of the cDNA clones showed signals at least 2-fold higher than background. At 1 and 3 h after treatment with 10 ng/ml
MIC-1, only MT1E (GenBank accession number H93127) consistently showed a difference of >2-fold in gene expression. MIC-1 decreased MT1E mRNA by 0.41. Several other genes were down-regulated by MIC-1 treatment by just under 2-fold. These included expressed sequence tags, highly similar to SMHU1B, human metallothionein 1B (0.52; GenBank accession number R99207); 60S acidic ribosomal protein P1 (0.54; GenBank accession number R99207); metallothionein 1H (0.55; GenBank accession number R99207); and other expressed sequence tags, similar to RNA helicase-related protein (0.55, GenBank accession number R99207).

Microarray data showed more gene expression changes after 9 h of MIC-1 treatment. At this time point, MIC-1 increased gene expression of complement component 5 (GenBank accession number 114869) by 2.3-fold and decreased gene expression of THO2 (GenBank accession number AA044429) by 0.48, RhoE (GenBank accession number N35505) by 0.47, integrin α3 (GenBank accession number H41926) by 0.46, catenin δ1 (GenBank accession number T95499) by 0.46.

Real time RT-PCR of total RNA from three separate experiments at each time point was used to validate selected microarray data. Specific primers for MT1E, complement component 5, THO2, RhoE, integrin α3, and catenin δ1 were designed and synthesized. Real-time RT-PCR confirmed that MIC-1 treatment for 1 h decreased MT1E gene expression by 0.369 ± 0.050 (P < 0.05) and 3 h MIC-1 treatment by 0.423 ± 0.030 (P < 0.05). At 9 h after treatment, MIC-1 decreased catenin δ1 expression by 0.348 ± 0.071 (P < 0.05) and RhoE expression by 0.463 ± 0.026 (P < 0.05). THO2 mRNA was decreased by MIC-1 to a lesser extent, 0.734 ± 0.08, whereas a decrease of integrin α3 mRNA and an increase of complement component 5 mRNA could not be confirmed in RNA samples not used in microarray experiments. One possible reason for the inconsistency was contamination of cDNA clones on the microarray slides. Inconsistency is known to be an issue with DNA microarrays, and this is largely thought to be attributable to quality control issues associated with their preparation (24). For example, ~38% of cDNA clones from IMAGE Consortium, which was one of the main suppliers of cDNA clones for Ontario Cancer Institute microarray slides, were proved to be contaminated (25).

DISCUSSION

This study shows that MIC-1 secretion is markedly increased in the malignant prostate LNCaP and PC-3 cell lines compared with malignant DU-145 and nonmalignant PZ-HPV-7 prostate cell lines. The reason why the brain-derived metastatic prostate cancer cell line DU-145, unlike lymph node- and bone marrow-derived PC-3 and LNCaP cells, did not produce a detectable amount of MIC-1 is unknown. However, overall, these observations are similar to those for secretion of TGF-β superfamily members such as TGF-β1, TGF-

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β2, and activin, which are produced in both malignant and nonma-
lignant prostate epithelial cell lines as well as in human prostatic
tissues (26). However, malignant cells produce higher levels than their
normal counterparts (26). Our data, as well as already published
reports, suggest that MIC-1 falls into the same pattern. MIC-1 protein
is overexpressed in colon and prostate cancer tissues compared with
normal neighboring areas (12, 13), and MIC-1 is one of the most
significantly overexpressed genes in prostate cancer tissues compared
with normal prostate (14).

MIC-1 secretion in hormone-sensitive LNCaP cells is down-regu-
lated by both androgen and estrogen. Although the presence of estro-
gen receptors in LNCaP cells has been reported, LNCaP cells contain
a mutated androgen receptor, which is capable of responding to
estrogen and adrenal steroids as well as androgen (27, 28). Therefore,
it is likely that in these cells, estrogen is reducing MIC-1 production
through the same receptor and signal pathway as androgen. Because
androgens are an important factor in prostate cancer development and
progression, the interaction between MIC-1 and hormones may well be
important and might even be part of the mechanism by which
androgens exert their effects in this disorder.

MIC-1, unlike TGF-β, did not have an effect on the proliferation of
Mv1Lu epithelial and various prostate epithelial tumor cell lines. In
the case of the former, this is contradictory to a previous report, which
used partially purified MIC-1 protein from conditioned medium from
DLD-1 colon tumor cells transfected with a MIC-1-expressing plas-
mid. In this construct, MIC-1 was Flag tagged at the COOH terminus.
However, in TGF-β superfamily proteins, the COOH terminus is
buried in the dimer interface, and hence addition of a Flag tag in this
location may well result in the expression of misfolded MIC-1. Our
data, however, are consistent with a recent report that LN-Z308
glioblastoma cells and primary neuronal cells do not show growth
inhibition by MIC-1 (19), whereas glioblastoma cells could be growth
inhibited by TGF-β. Because MIC-1 does not inhibit cell prolifera-
tion, it is likely to have a different receptor activation and/or signal
pathway from that of TGF-β.

This study further supports existing data (11, 16, 18) that MIC-1 is
proapoptotic in at least some epithelial cancer cell lines. However, this
proapoptotic effect may be secondary to altered adhesion of DU-145
cells. It was strikingly obvious that prior to undergoing apoptosis,
MIC-1-treated DU-145 cells showed significant morphological
changes, followed by reduced cell-cell adhesion and finally cell de-
tachment. Because cell detachment preceded apoptosis, it is likely that
cell detachment, at the very least, contributed to the development of
apoptosis. If a similar loss of adhesion was stimulated by MIC-1 in
vivo, it would suggest that MIC-1 might facilitate cancer cell detach-
ment, migration, and metastasis.

Although MIC-1 clearly reduced DU-145 cell adhesion and induc-
ed cell apoptosis, these processes seemed to be confined to a small
proportion of the total cell population (<10%). The small number of
these apparently responsive cells may relate to particular cell-cell and
cell-matrix contacts or a particular stage of the cell cycle. This is
supported by the finding that morphological changes, cell detach-
ment, and apoptosis occurred at the periphery of large clusters of cells and
in cells in very small clusters.

Whatever the reason was for the effect being observed only in a
minority of cells would significantly decrease the capacity of any
microarray-based strategy to detect affected gene transcripts. This
may help explain why only a limited number of altered gene tran-
scripts were detected. However, the changes noted in MT1E, catenin
δ1, and RhoE gene expression levels are remarkably consistent with
the functional and morphological data described and suggest likely
mechanisms.

MT1E is the only metallothionein family member that is expressed
in estrogen receptor-negative cells but not in estrogen receptor-posi-
tive cells (29). Metallothionein has been documented extensively to
protect cells from apoptosis and reactive oxidative stress by stabiliz-
ing DNA binding of nuclear factor-κB and inhibiting caspase activa-
tion (30, 31). Catenin δ1 and RhoE are both related to cadherin.
Cadherins are cell-cell adhesion receptors essential for the establish-
ment of the epithelial cell shape, maintenance of the differentiated
phenotype, and formation of adherence junctions between cells (32).
Inhibition of endogenous Rohr in epithelial cells dismantles adherence
junctions by removing cadherin receptors and decreasing cell-cell
adhesion and actin accumulation at the site of cell contact (32). RhoE

Fig. 5. MIC-1 induced DU-145 cell apoptosis in a dose- and
time-dependent fashion. A, MIC-1, at all three concentrations
used, increased the percentage of apoptotic cells compared with
control (P < 0.001). B, MIC-1 decreased the total number of
viable (nonapoptotic) cells at all three tested concentrations
(P < 0.001). C, compared with control (□), MIC-1 (■) signifi-
cantly increased the number of adherent apoptotic cells on the
4th (P < 0.01) and 6th (P < 0.01) posttreatment days. D,
compared with control (□), MIC-1 (■) significantly increased
the number of detached apoptotic cells on the 4th (P < 0.01) and
6th (P < 0.001) posttreatment days. Results are presented as
means of apoptotic/total cells (□) or cell numbers (□-□) from
at least triplicates; bars, SE. *, P < 0.01.
especially has been suggested to have a role in epithelial cell adhesion, because of its cellular localization at the lateral membranes of these cells (33). Catenin δ1 localizes at both cellular junctions and the nucleus. Similar to other catenins, catenin δ1 interacts with cadherin at intercellular junctions. Although most results suggest that it anchors cadherin complex to the actin cytoskeleton and enhances cell-cell adhesion and intercellular junctions, there has also been evidence indicating that it inhibits anchoring under certain experimental conditions (34).

In summary, MIC-1 is overexpressed in malignant PC-3 and LNCaP cells but not in nonmalignant PZ-HPV-7 and malignant DU-145 cells. The production of MIC-1 in hormone-sensitive LNCaP cells is down-regulated by both androgen and estrogen. Although MIC-1 does not inhibit proliferation of prostate epithelial cancer cells, it induces caspase-dependent apoptosis that, at least in part, is likely to be attributable to down-regulation of MT1E gene expression. Furthermore, MIC-1 reduces cell-matrix and cell-cell adhesion, at least partly through decreasing RhoE and catenin δ1 gene expression. Although cell detachment may partly contribute to apoptosis in vitro, it does not always do this in vivo, where detachment is necessary for migration, which in cancer often leads to metastasis in vivo. Additional studies of the interaction of hormones and MIC-1, and the effect of MIC-1 on prostate cancer metastasis in vivo, will provide important information for the management of prostate cancer.

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