The Propeptide Mediates Formation of Stromal Stores of PROMIC-1: Role in Determining Prostate Cancer Outcome

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Abstract

The extracellular matrix (ECM) is a reservoir of cellular binding proteins and growth factors that are critical for normal cell behavior, and aberrations in the ECM invariably accompany malignancies such as prostate cancer. Carcinomas commonly overexpress macrophage inhibitory cytokine 1 (MIC-1), a proapoptotic and antitumorigenic transforming growth factor–β superfamily cytokine. Here we show that MIC-1 is often secreted in an unprocessed propeptide containing form. It is variably processed intracellularly, with unprocessed forms being secreted from several tumor lines, including prostate carcinoma lines, PC-3 and LNCaP. Once secreted, only unprocessed proMIC-1 binds ECM, demonstrating for the first time the occurrence of extracellular stores of MIC-1. The propeptide mediates this association via its COOH-terminal 89 amino acids. Xenograft models bearing tumors secreting various engineered forms of MIC-1 show that the propeptide regulates the balance between ECM stores and circulating serum levels of mature MIC-1 *in vivo*. The absence of propeptide results in ~20-fold increase in serum MIC-1 levels. The significance of stromal MIC-1 stores was evaluated in prostate cancer tissue cores, which show major variation in stromal levels of MIC-1. Stromal MIC-1 levels are linked to prostate cancer outcome following radical prostatectomy, with decreasing stromal levels providing an important independent predictor of disease relapse. In low-grade localized prostate cancer (Gleason sum score ≤6), the level of MIC-1 stromal stores was the best predictor of future relapse when compared with all other clinicopathologic variables. The secretion and ECM association of unprocessed proMIC-1 is likely to play a central role in modulating local bioavailability of MIC-1 which can affect patient outcome in prostate cancer and other epithelial tumors. (Cancer Res 2005; 65(6): 2330-6)

Introduction

Macrophage inhibitory cytokine 1 (MIC-1), a divergent member of the transforming growth factor–β (TGF-β) superfamily, was first isolated from a U937 cell subtraction cDNA library enriched for genes associated with macrophage activation (1). MIC-1 is also known by other names including PLAB, placental TGF-β, prostate-derived factor, GDF-15, and NAG-1 (reviewed in refs. 2, 3). Like many TGF-β superfamily cytokines, MIC-1 is expressed very widely, but under resting conditions, placenta is the only tissue expressing large amounts of MIC-1 (3). Epithelial cells, in a wide variety of other organs such as prostate and colon, express lower amounts of MIC-1 mRNA. MIC-1 expression is however, dramatically increased in inflammation, injury, and malignancy (3–7).

Increased MIC-1 expression is a feature of many cancers including breast, colon, pancreas, and prostate. Many epithelial tumor cell lines secrete high levels of MIC-1 (8) and several studies (5–7) show major up-regulation of MIC-1 mRNA and protein in cancer biopsies. High tumor expression is also associated with an increase in serum MIC-1 levels suggesting the use of serum MIC-1 measurement for the diagnosis and management of cancer (7, 9, 10).[8] Serum MIC-1 levels can be markedly elevated in metastatic cancer and seem to parallel the stage and extent of disease, particularly in colorectal cancer (9). A number of studies show an antitumorigenic role for MIC-1 where it induces apoptosis and inhibits proliferation of several tumor cell lines (8, 11–14).

MIC-1, like other TGF-β superfamily proteins, is synthesized as a precursor containing an NH_2-terminal propeptide and a COOH-terminal mature MIC-1 domain. This undergoes disulfide-linked dimerization in the endoplasmic reticulum, but unlike other characterized TGF-β superfamily proteins, the MIC-1 propeptide is not required for the correct folding and secretion of disulfide-linked dimers (15, 16). It is however, required for quality control. Only correctly folded and dimerized MIC-1 precursor leaves the endoplasmic reticulum for the Golgi apparatus, where it is thought a furin-like proconvertase proteolytically cleaves it at the conserved RXXR site (amino acid 196). This separates propeptide from the mature COOH-terminal domain and MIC-1 is released as a 24.5-kDa disulfide-linked dimer (1, 15).

In this study, we have continued our investigation of the role of the propeptide in regulating the secretion of MIC-1, in the context of prostate cancer. We show that prostate carcinoma cell lines largely secrete MIC-1 in the unprocessed form, which associates with the extracellular matrix (ECM). We show the propeptide is mediating this ECM association and identify the COOH-terminal 89 amino acids as responsible for the interaction. Using a nude mouse tumor xenograft model, we find the presence of propeptide is an

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[8] D.A. Brown, et al. Serum MIC-1 levels for the diagnosis and tumor grading of prostate cancer, submitted for publication.

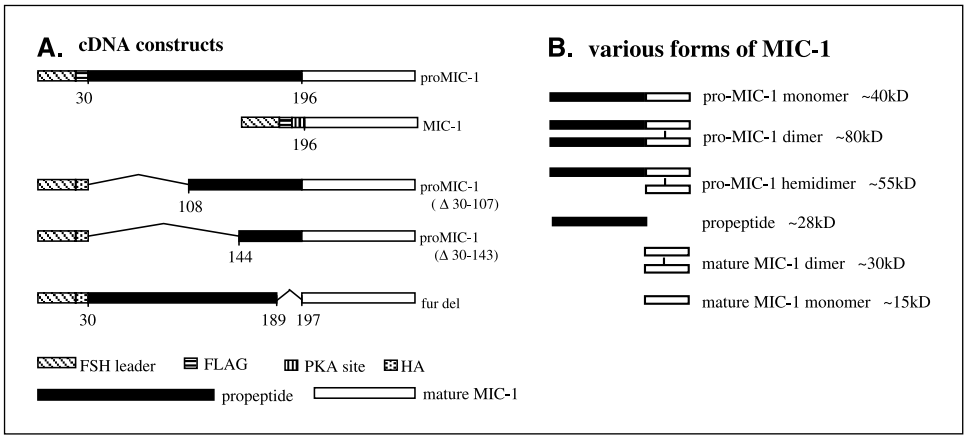

Figure 1. *A,* schematic representation of MIC-1 constructs used in this study. Numbers, amino acid residue positions. *B,* schematic representation of the various forms of MIC-1.

important *in vivo* mechanism for regulating the relative distribution of MIC-1 between the circulation and tissue ECM stores. Finally, we have shown the biological importance of variation in local stores of MIC-1, in prostate cancer, a common tumor overexpressing MIC-1, by linking stromal levels of MIC-1 in prostate cancer tissue cores to patient outcome.

Materials and Methods

Cell Culture. MDCK Type II cells, DU145, Panc-1, LNCaP, and PC-3 cells were maintained in culture as recommended by the American Type Culture Collection (Rockville, MD). MDCK and DU145 cells were stably transfected with the various constructs using LipofectAMINE (Life Technologies, Gaithersburg, MD) as described (1, 15).

Antibodies. Sheep polyclonal antiserum (233) to mature MIC-1 was used in immunoprecipitation and immunoblot analysis as described (15). Control IgG was purified from sheep serum using caprylic acid-ammonium sulfate. Affinity-purified antibodies to mature MIC-1 and MIC-1 propeptide were prepared using Sepharose to which recombinant antigen had been coupled, using the amino-link kit (Pierce, Rockford, IL). This was dialyzed into PBS, concentrated, and IgG concentration determined (bicinchoninic acid assay, Pierce).

Preparation of Macrophage Inhibitory Cytokine 1 cDNA Constructs. Flag-tagged mature MIC-1, proMIC-1, and propeptide deletion constructs were prepared as described (refs. 15, 16; Fig. 1). For mutagenesis, proMIC-1 was cloned into POCUS 2 (Novagen, Madison, WI) and using relevant primers, was subjected to a whole plasmid PCR reaction as described (16). For the fur del mutant, the last seven amino acids (RGRRRAR) of the propeptide encoding two furin-like cleavage sites were deleted using primers 5′-GCGCGCAACGGGGACCAC-3′(forward), 5′-GGCGGCTTGCGGCCGCAA-3′(reverse). All constructs were subcloned into pIRES-2-EGFP (Clontech, Palo Alto, CA) for transfection into MDCK and DU145 cells.

Immunoprecipitation, Immunoblot, and ELISA Analysis. Conditioned media from cell lines (collected over 24 hours) were either analyzed directly by immunoblot with anti-MIC-1 or immunoprecipitation with anti-MIC-1 was carried out at a dilution of 1:10,000 before immunoblot analysis. Samples were analyzed by either reducing or nonreducing SDS-PAGE and immunoblot essentially as described (1, 15). Blots were probed with anti-MIC-1 at a dilution of 1:7,000 followed by biotinylated anti-sheep (1:5,000; Sigma, St. Louis, MO). Blots were developed with streptavidin conjugated horseradish peroxidase (1:5,000) and bands visualized using chemiluminescence reagent (NEN). In house MIC-1 ELISA was done essentially as described (17, 18) and all samples assayed in duplicate.

Immunofluorescence. Immunofluorescence was done essentially as described (19) except cells were not permeabilized. Primary antibodies used were anti-MIC-1 (233) and antipropeptide serum (1:1,000). Secondary antibody was FITC-conjugated anti-sheep antibody (Silenus Laboratories, Hawthorn, Victoria, Australia). Cells were mounted and viewed using UV fluorescence microscopy.

Isolation of Extracellular Matrix. LNCaP cells were grown over a prolonged period of time in the same culture dish, lifted with 6 mmol/L EDTA in PBS, and ECM remaining on the culture dish was extracted with boiling SDS-PAGE sample buffer. Isolation of ECM from MDCK cells was as described (20), then extracted with boiling SDS-PAGE sample buffer.

Xenograft Model. Male BALB/c nude mice (Animal Resources Centre, Murdoch, Western Australia, Australia), at 8 weeks of age, were injected s.c. on their right flank with 5×10^6 transfected DU145 cells in 200 μL of PBS. The DU145 cells had been transfected with either pIRES2-EGFP containing mature MIC-1, a furin deletion MIC-1 mutant (fur del), or empty vector. Once visible, the tumor size was measured twice weekly. After 48 days, the mice were bled by cardiac puncture, sacrificed, and tumors excised, fixed in 10% neutral buffered formalin then wax embedded in paraffin blocks.

Tissue Microarray Construction. Tissue microarrays were constructed from radical prostatectomy specimen blocks of 199 patients treated for clinically localized prostate cancer at St Vincent's Hospital Campus, Sydney, Australia between February 1989 and June 1997 (21). Cores of benign and malignant prostate tissue (1-mm diameter) were incorporated into seven tissue microarrays (22). Specimen and clinical data collection were with the written informed consent of patients. Follow-up data collection was prospective from 1990 with the approval of the St. Vincent's Campus Research Ethics Committee (reference H00/088). The date of disease relapse was defined as the date of the first increase in serum prostate-specific antigen ≥0.4 ng/mL when it was followed by consecutive, further increases.

Immunohistochemistry. Immunohistochemistry of tissue microarrays was done essentially as described.[9] Except affinity-purified anti-MIC-1 IgG was optimized at a concentration of 5 μg/mL which best reflected stromal levels of MIC-1. Control staining was done with purified sheep IgG at the same concentration. Immunostaining of epithelial cells was scored by microscopic assessment of the percentage of the lesional cells with positive cytoplasmic staining. Epithelial and stromal staining intensity were graded between 0 and 3, with 0 being the least staining. There were one to four cores for each patient and the final score was an average. As prostate acinar adenocarcinoma does not have a clearcut morphologically definable tumor stroma and tends to insinuate between nonneoplastic glands, tumor stroma was defined as being <1 mm from malignant epithelial cells. Scoring was done by a single observer (K.K.R.) who was blinded to patient outcome. A final percentage of positively stained cells was calculated by averaging the lesional percent positivity across the cores representing each patient. Final intensities of stromal and epithelial staining were similarly calculated by averaging the scores across the cores representing each patient.

Immunohistochemistry of xenograft tumor sections was done essentially as described[9] using affinity purified anti-MIC-1 IgG, affinity purified antipropeptide IgG and purified normal sheep IgG (4 μg/mL).

[9] K.K. Rasiah, et al. Aberrant expression of neuropeptide Y and macrophage inhibitory cytokine-1 are associated with premalignant prostate disease and prostate cancer progression after radical prostatectomy, submitted for publication.

Statistical Analysis. Variables were compared between patients as well as benign and malignant tissues using unpaired and paired Student's *t* tests, respectively. Immunostaining, clinical, and pathologic data were evaluated for relapse prediction in Cox proportional hazards models and Kaplan-Meier analysis, using relapse as the end point. All data were analyzed using StatView v 5.0 statistical software (SPPS, Inc., Cary, NC). $P < 0.05$ was considered significant.

Results and Discussion

Prostate Cancer Cells Secrete Large Amounts of Unprocessed, Propeptide Containing Macrophage Inhibitory Cytokine 1. An investigation of MIC-1 secretion from several carcinoma lines reveals a large amount of MIC-1 is secreted in the unprocessed form (Fig. 2*A*). Indeed, the major form of MIC-1 from the PC-3 prostate carcinoma line is unprocessed. Proteolytic processing of precursor proteins by proconvertases commonly occurs in the trans-Golgi network (23, 24). However, it is clear many tumor lines secrete MIC-1 in either unprocessed or partially processed forms with at least some processing occurring extracellularly. We have previously shown secretion of unprocessed endogenous MIC-1 from other cell types such as BeWo choriocarcinoma cells (17) and U937 monocytoid cells.[10] Indeed, pulse chase analysis of MIC-1 secretion from U937 cells shows MIC-1 is secreted largely unprocessed, but is processed extracellularly over the 24 hours following secretion. Therefore, Western blot analysis of conditioned medium collected over 24 hours is likely to underestimate the amount of unprocessed MIC-1 secreted. This suggests MIC-1 is secreted unprocessed from a large proportion of tumor cell lines.

Unprocessed proMIC-1 Associates with the Extracellular Matrix. To investigate whether MIC-1 interacts with ECM produced by LNCaP human prostate cancer cells, these cells were grown over a prolonged period of time to allow matrix formation. ECM extract was analyzed by immunoblot analysis with anti-MIC-1 antibody. This shows the presence of both unprocessed proMIC-1 dimer and hemidimer. Mature MIC-1 was mainly present in the conditioned medium (Fig. 2*B*). This is the first demonstration that proMIC-1 associates with the ECM.

The Propeptide Determines Extracellular Matrix Association of proMIC-1. The previous experiment suggested that ECM association was mediated by the propeptide. To confirm the role of the propeptide in mediating this association, Chinese hamster ovary (CHO) cells were transfected with proMIC-1 or mature MIC-1 (lacking the propeptide domain; Fig. 1). Nonpermeabilized CHO cells were analyzed by immunofluorescence with anti-MIC-1 and antipropeptide antibody. Strong immunofluorescence between cells is visible in CHO transfected with proMIC-1 but not CHO transfected with MIC-1, suggesting ECM association of MIC-1 only occurs in the presence of propeptide (Fig. 2*C*).

To identify the domain of the propeptide involved in ECM association, we stably transfected MDCK cells with proMIC-1, mature MIC-1 and various propeptide deletion mutants of proMIC-1, having progressively larger NH_2-terminal deletions (Fig. 1). Immunoblots of ECM extracts from these cells shows in wild-type proMIC-1 transfectants, mainly unprocessed proMIC-1 dimer and hemidimer (only one furin site cleaved) is present. Mature MIC-1 is mainly present in culture medium and was confirmed in mature MIC-1 MDCK transfectants. Deletion of the first 30 and 90 amino acids resulted in similar ECM association of unprocessed proMIC-1 forms to that of wild-type proMIC-1. However, deletion of the first 115 amino acids substantially reduced ECM association (Fig. 2*D*). This shows the COOH-terminal 89 amino acids of the propeptide are necessary for ECM association.

Unprocessed proMIC-1 is secreted and stored locally as a means of regulating MIC-1 activity. This would ensure a storage reservoir of latent MIC-1 in the ECM, where processing to active MIC-1 might occur under specific conditions. This could be accomplished by matrix associated proconvertases such as PACE 4 and PC6 which have been found associated with the ECM (25). Processing of proMIC-1 localized to the ECM, under certain conditions, would allow a rapid increase in local concentration of MIC-1 and a prolonged local response.

The level of processing of proMIC-1 by a furin-like proconvertase, either intracellularly or extracellularly, will modulate the presence and size of these stores. Furthermore, proMIC-1 is secreted basolaterally from epithelial monolayers,[10] a mechanism likely to facilitate matrix association and the formation of ECM stores. It is likely that MIC-1 binds to heparan sulfate in the matrix as the propeptide of MIC-1 binds heparin Sepharose.[11] Heparin interaction often reflects interaction with heparan sulfate proteoglycans, present in the ECM. Thus, proMIC-1 may be released from the matrix by the activity of heparanase or sulfatases or the presence of soluble secreted glycosaminoglycans such as heparin. This may in turn serve to facilitate proconvertase-mediated processing of proMIC-1, which may be sterically hindered by matrix association. The amount of matrix-associated proMIC-1 is also likely to be influenced by the composition of the matrix itself and the fine balance between the activity of extracellular proteases, such as matrix metalloproteases and their inhibitors. Such regulatory influences occur in inflammatory processes and are a feature of many cancers and may represent another mechanism by which a local disease process can modulate tissue MIC-1 levels.

In Animal Models, the Presence or Absence of Propeptide Determines whether Tumor-Derived Macrophage Inhibitory Cytokine 1 Is Deposited Locally in Extracellular Matrix or Secreted into Serum. The association of proMIC-1 with ECM suggests processed mature MIC-1, which has little affinity for ECM, would diffuse and be released into the circulation, while unprocessed proMIC-1 would remain localized until processed. To investigate this *in vivo*, we used a tumor xenograft model in nude mice. DU145 human prostate carcinoma cells, which makes no detectable endogenous MIC-1 (8), were stably transfected with (i) mature MIC-1 (no propeptide), (ii) proMIC-1 with the furin-like proconvertase site deleted (prevents processing and release of mature MIC-1 from propeptide), and (iii) vector only negative control (see Fig. 1). Clones expressing very similar amounts of the two different MIC-1 constructs were selected (Fig. 3*A*). These cells were injected s.c. into the flank of immunodeficient BALB/c *nu/nu* nude mice. After 6 weeks, serum was collected by cardiac puncture, animals sacrificed and their tumors excised. Expression of MIC-1 in tumors was confirmed by immunohistochemistry with both anti-MIC-1 and antipropeptide (Fig. 3*B*). The presence of proMIC-1 was apparent in stroma surrounding the tumor expressing the furin deletion construct. In contrast, little mature MIC-1 was visible in

[10] A.R. Bauskin, et al. The propeptide facilitates secretion and determines sorting of MIC-1, in preparation.

[11] Unpublished data.

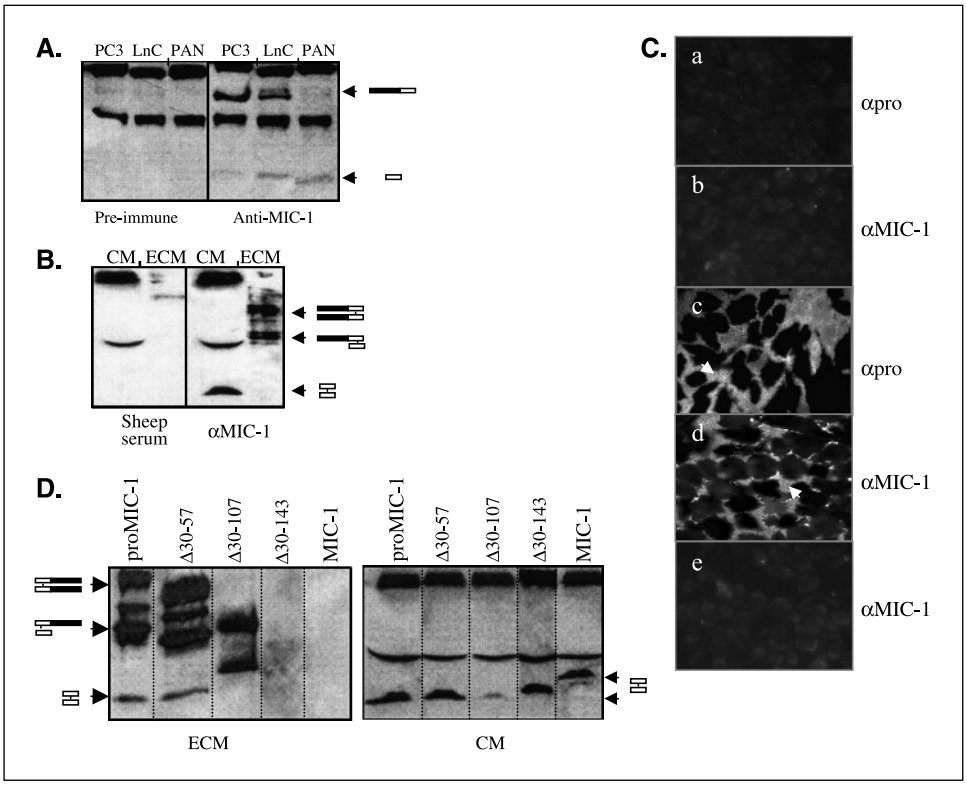

Figure 2. Propeptide facilitates association of unprocessed proMIC-1 with ECM. *A,* endogenous MIC-1 is secreted in both processed and unprocessed forms. Conditioned media from LNCaP, PC-3, and Panc 1 cells was collected over 2 days and analyzed by immunoprecipitation with sheep polyclonal anti-MIC-1 serum, then reducing SDS-PAGE and immunoblot with sheep polyclonal anti-MIC-1. (Major nonspecific bands are IgG heavy and light chains). *B,* endogenous proMIC-1 secreted by LnCAP cells associates with the ECM. Conditioned medium (*CM*) and ECM extracts from LnCAP cells were analyzed by immunoblot with sheep polyclonal anti-MIC-1. *C,* immunofluorescence of fixed nonpermeabilized CHO cells with sheep polyclonal anti-MIC-1 (αMIC-1) or antipropeptide (αpro) antiserum. Untransfected CHO cells (*a* and *b*), CHO cells transfected with proMIC-1 (*c* and *d*), CHO cells transfected with MIC-1 (*e*). *Arrows*, ECM staining between cells. *D,* COOH-terminal amino acids 107 to 195 of the propeptide mediate ECM association. Conditioned medium (*CM*) and ECM extracts from MDCK cells transfected with proMIC-1 (with propeptide), MIC-1 (without propeptide), proMIC-1 Δ30-57, proMIC-1 Δ30-107, and proMIC-1 Δ30-143 were analyzed by immunoblot with sheep polyclonal anti-MIC-1. (Mature MIC-1 from MIC-1 transfectants runs slower than wild-type mature MIC-1, due to the presence of the FLAG epitope, which is known to retard migration on SDS-PAGE; ref. 34).

stroma surrounding tumor expressing only mature MIC-1. Serum levels of circulating human MIC-1 were determined by ELISA specific for human MIC-1, (unable to detect endogenous mouse MIC-1; ref. 17; Fig. 3*C*) and these levels normalized for the tumor volume of each mouse. There was an ~20-fold increase in the level of circulating MIC-1 from tumors expressing only mature MIC-1, compared with tumors expressing only proMIC-1. This shows the propeptide region of proMIC-1 modulates circulating serum levels of MIC-1, by determining the proportion of matrix-bound proMIC-1 to freely diffusible MIC-1.

Macrophage Inhibitory Cytokine 1 Is Present in Both the Epithelium and Interstitium of Tissue Cores from Prostate Cancer Patients. MIC-1 expression is strongly up-regulated in prostate cancer (5, 7), and the two prostate cancer cell lines (PC-3 and LNCaP) studied above secrete MIC-1 which is largely unprocessed and matrix bound. We therefore reasoned that we may see substantial variation in stromal staining of MIC-1 in prostate cancers. To investigate the presence of MIC-1 stromal stores, we evaluated MIC-1 staining of prostate cancer cores.

Prostate cancer develops as a progression from normal epithelium through a series of increasingly dysplastic lesions: low-grade prostatic intraepithelial neoplasia, high-grade prostatic intraepithelial neoplasia, culminating in invasive prostate cancer (26). Representation of several of these grades within the one prostate gland is common. A tissue microarray representation of this progression model was constructed containing cores of benign, premalignant (high-grade prostatic intraepithelial neoplasia) and prostate cancer tissue from 199 patients who underwent radical prostatectomy (22, 27).[9] Immunohistochemistry of MIC-1 on 168 benign and 190 prostate cancer cores showed MIC-1 expressed not only in tumor epithelium, but also revealed stromal staining for MIC-1 which varied substantially from patient to patient (Fig. 4*A*).

To quantify tissue MIC-1, the intensity of staining was graded from 0 to 3, with 0 being the least staining. Additionally, the number of MIC-1-positive epithelial cells in benign or prostate cancer core samples was quantified and expressed as a percentage of the total cells counted. The number of epithelial cells positive for MIC-1 in malignant tissue was significantly higher than benign tissue (mean = 62.9%, SD = 27.6; range, 0-99; mean = 36.2%, SD = 34.4; range, 0-99; $P < 0.0001$; paired t-test). The intensity of epithelial MIC-1 staining was greater in malignant than benign samples (mean = 1.875, SD = 0.772; range, 0-3; mean = 1.45, SD = 0.825; range, 0-3; $P < 0.0001$; paired t-test); which is similar to the findings of Rasiah et al.[9]

Stromal intensity of MIC-1 staining in core samples displaying cancer (malignant stroma) was graded for 188 cases with a mean intensity of 1.075 (range, 0-3; SD = 0.85). Stromal intensity of MIC-1 staining in core samples from areas of the prostate showing no malignancy (benign stroma) was available on 163 subjects with a mean of 0.5 (range, 0-3; SD = 0.85). Using a paired t test, the malignant stromal intensity was significantly higher than benign stromal intensity ($P < 0.0001$). Thus, prostate cancer produces significantly more MIC-1 compared with benign tissue and in addition, there is significantly more MIC-1 associated with tumor stroma.

Macrophage Inhibitory Cytokine 1 Staining of Prostate Epithelium and Interstitium Predicts Disease Outcome. In malignant epithelial cells, Cox univariate analysis (Table 1) showed that decreasing intensity of MIC-1 staining was associated with a greater risk of recurrence of prostate cancer following radical prostatectomy ($P = 0.0360$). Additionally, a trend towards statistical significance was found between increasing numbers of MIC-1-positive malignant epithelial cells and decreased risk of disease recurrence ($P = 0.0680$; Cox univariate analysis; Table 1). To obtain an indication of tumor production of MIC-1, we multiplied the proportion of

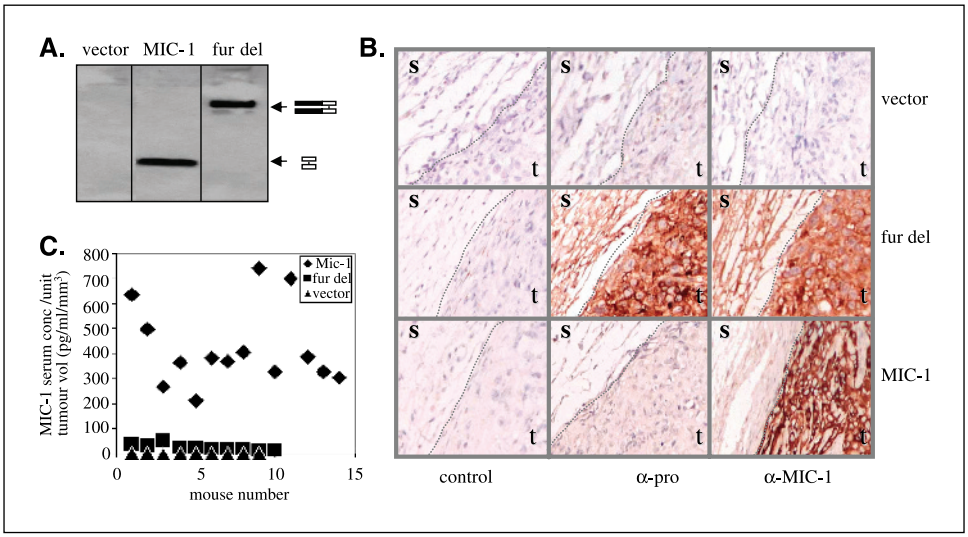

Figure 3. Presence of the propeptide plays a role in determining circulating serum MIC-1 levels. *A,* conditioned medium from subclones of DU145 cells transfected with either mature MIC-1 only or proMIC-1 furin deletion mutant (which does not process into mature MIC-1, fur del) and vector only control were analyzed by SDS-PAGE and Western blot with anti-MIC-1. Conditioned media was collected over 24 hours from freshly plated cells to minimize the amount of ECM and resultant ECM association of proMIC-1. *B,* immunostaining of MIC-1 on tumor epithelial cells in s.c. xenograft tumors derived from proMIC-1 (fur del), MIC-1 and vector transfected DU145 cells. Tumor sections were stained with affinity-purified anti-MIC-1 antibody, affinity-purified antipropeptide antibody, and purified control IgG from normal sheep serum (4 μg/mL). *s,* stroma; *t,* tumor. *Dotted line*, approximates division between stroma and tumor. *C,* human MIC-1 serum levels were determined by ELISA and corrected for tumor volume. MIC-1: n = 14, mean = 422 pg/mL per mm^3 of tumor volume, SD = 161, and range = 213-740. Fur del: n = 10, mean = 24 pg/mL per mm^3 of tumor volume, SD = 11.9, and range = 12-51. Vector: n = 9, mean = 0.5 pg/mL per mm^3 of tumor volume, SD = 0.4, and range = 0-1.

cells with MIC-1 staining by the intensity of that staining within the tumor. Analysis of this surrogate marker of MIC-1 production (mean = 0.785; SD = 0.757; range, 0-2.97) indicated that greater production of MIC-1 in the tumor led to less risk of relapse (P = 0.0213; Cox univariate analysis; Table 1). A similar surrogate marker of benign epithelial production of MIC-1 showed no association with risk of relapse (P = 0.8598; Cox univariate analysis).

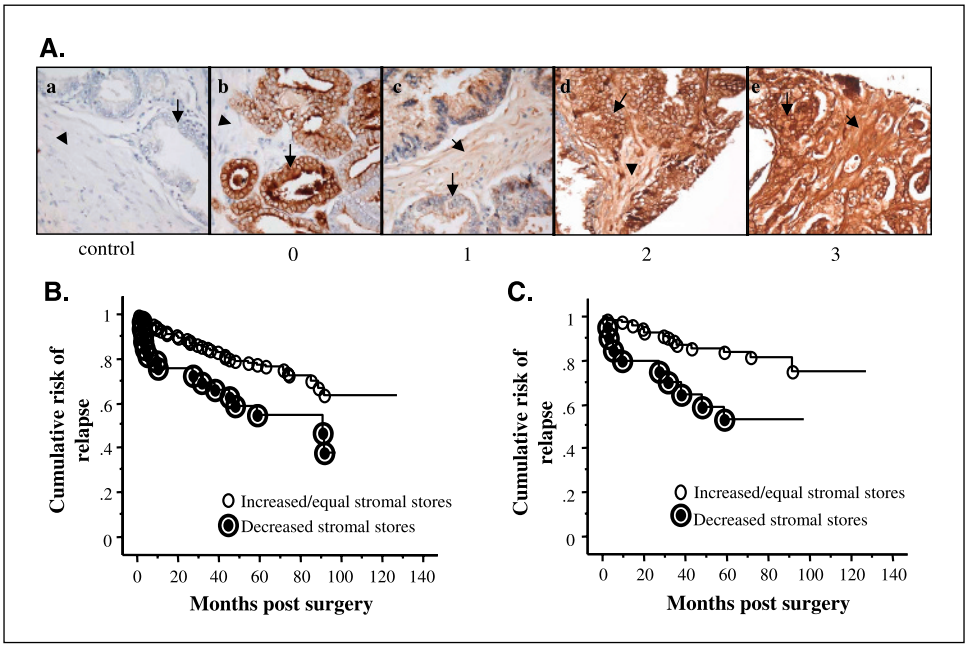

Figure 4. Stromal stores of MIC-1 are a significant predictor of disease relapse following radical prostatectomy. *A,* MIC-1 is present in both the epithelium and stroma of prostate cancer. Photomicrographs of MIC-1 immunoreactivity in prostate cancer, to show the various observed stromal staining patterns in different patients. Prostate cancer patient 1 stained with purified control IgG from normal sheep serum (*a*) and prostate cancer patients 1 to 4, stained with affinity purified anti-MIC-1 antibody and representing stromal staining (*b-e*): 0 (undetectable), 1 (weak), 2 (medium), 3 (strong). Magnification 200×. *Arrows*, epithelial cells; *arrowheads*, stroma. *B,* decreasing stromal stores of MIC-1 are associated with disease relapse in prostate cancer patients following radical prostatectomy. Kaplan-Meier analysis for the effect of stromal MIC-1 storage on relapse of 159 prostate cancer patients. P = 0.0134 [log-rank (Mantel-Cox)]. *C,* decreasing stromal stores of MIC-1 are associated with disease relapse in prostate cancer patients with a Gleason sum score of ≤6 following radical prostatectomy. Kaplan-Meier analysis for the effect of stromal MIC-1 storage on relapse of prostate cancer in 116 patients with a Gleason sum score of ≤6. P = 0.00064 [log-rank (Mantel-Cox)].

Table 1. Cox proportional hazards univariate analysis for relapse of prostate cancer

Risk factor	HR (95% CI)	*P*
Extracapsular spread (>pT2C)	2.960 (1.7496-5.018)	<0.0001
Gleason score*	1.552 (1.289-1.868)	<0.0001
Positive lymph nodes	8.227 (2.545-26.589)	0.0004
Pre-op PSA >10 ng/mL	2.287 (1.356-3.857)	0.0019
Surgical margin involvement	2.088 (1.241-3.513)	0.0056
Surrogate malignant epithelial prod†	0.918 (0.854-0.987)	0.0213
Malignant epithelial intensity†	0.705 (0.508-0.977)	0.0360
Malignant stroma intensity <1†	1.657 (1.003-2.738)	0.0488
Malignant epithelial (% positive)†	0.992 (0.984-1.001)	0.0680
Benign epithelial (% positive)†	1.002 (0.994-1.009)	0.6664
Surrogate benign epithelial prod†	1.000 (0.999-1.001)	0.8598
Benign epithelial intensity†	1.021 (0.725-1.437)	0.9056

Abbreviations: prod, production; pre-op PSA, preoperative serum levels of prostate-specific antigen.
*Gleason score was analyzed as a continuous variable.
†MIC-1 staining variables are described in the text.

To determine if there was any relationship between stromal MIC-1 staining and future recurrence of disease, stromal staining was separated into two groups. It was considered low if it was <1 (n = 116) and high if it was ≥1. MIC-1 staining of >1, in malignant stroma, was associated with decreased risk of relapse of disease (P = 0.0488; Cox proportional hazards univariate analysis; Table 1). When this variable was included in a multivariate Cox proportional hazards analysis with lymph node status, preoperative prostate-specific antigen, the presence of extracapsular spread, involvement of the surgical margin and the Gleason score of the resected tumor, stromal MIC-1 staining remained an independent predictor of future disease relapse [P = 0.0332; hazard ratio (HR), 1.756; 95% confidence interval (95% CI), 1.046-2.949; Table 2].

Decreasing Stromal Stores of Macrophage Inhibitory Cytokine 1 Are Associated with Disease Relapse. To further determine if variation in tumor stromal stores of proMIC-1 had biological significance, we compared benign and malignant stromal staining, obtained from staining of separate core samples from benign and malignant regions of the same prostate gland. To simplify, we decided to define stromal stores as increased/equivalent if the tumor stromal MIC-1 intensity was greater than or equal to the benign stromal intensity. Tumor stromal MIC-1 staining intensity less than benign stroma, indicated decreased stromal stores. Kaplan-Meier analysis revealed that decreased amounts of stromal MIC-1 were associated with significantly earlier disease relapse [P = 0.0134; log-rank (Mantel-Cox); Fig. 4*B*].

Tumors with a Gleason sum of ≤6 have a significantly better prognosis. However, a significant number of these patients will have disease progression. We examined 116 patients with a Gleason sum of ≤6. None had lymph node involvement and 31 patients had a relapse of disease after surgery. Univariate Cox proportional hazard analysis in patients with a Gleason sum of ≤6 revealed that the level of MIC-1 stromal stores, as defined above, was the best predictor of future relapse when compared with all the other variables specified in Table 1, many of which are commonly used outcome variables for prostate cancer (28). Increased/equivalent stromal stores of MIC-1 were associated with a significantly greater chance of remaining disease free (P = 0.0096; HR, 3.1; 95% CI, 1.3-7.2). Tumor stage greater than IIC was the only other variable in these patients that significantly predicted relapse of disease (P = 0.0301; HR, 2.3; 95% CI, 1.1-4.7). Using multivariate Cox proportional hazard analysis with surgical tumor stage and MIC-1 stromal levels, only the latter significantly predicted relapse of disease (P = 0.0326; HR, 2.6; 95% CI, 1.1-6.5). In Kaplan-Meier analysis, patients with decreased MIC-1 stromal levels, as determined by differential stromal staining, relapsed significantly earlier (P = 0.0064; Fig. 4*C*).

MIC-1 clearly has a role in cancer biology. It is one of the major proteins whose expression is induced by p53 (11, 14, 29, 30) although a number of p53 independent mechanisms have also been identified. MIC-1 protein and mRNA expression is up-regulated in a number of carcinomas and its expression is induced in human colorectal cancer cells in a prostaglandin-independent manner, by several nonsteroidal anti-inflammatory drugs (31), as well as by a variety of antitumorigenic compounds such as resveratrol, genistein, and PPAR ligands (32). A number of studies show that MIC-1 has proapoptotic and antitumorigenic activities (16–19). This is supported by epidemiologic studies on the single nucleotide polymorphism in MIC-1, which replaces a His with an Asp residue at position 6 in mature MIC-1, that is linked to cancer. This polymorphism is associated with altered outcome of patients with colon cancer (9) and recent data, from a large population study (1,383 prostate cancer patients and 780 control subjects), shows the H polymorphism is a significant risk factor and the D polymorphism a protective factor in the development of prostate cancer. In this study, 19.2% of familial and 7.2% of sporadic prostate cancer were attributable to the H genotype (33).

These studies have shown for the first time the occurrence of tissue stores of MIC-1 and show that increased stromal stores of MIC-1 may protect against invasive prostate cancer. We have determined the mechanisms in regulating its partitioning to this site and have revealed a novel role for the propeptide in the creation of ECM-bound stores and modulating circulating serum levels of mature MIC-1. Finally, we have been able to show the significance of stromal stores of MIC-1 in prostate cancer by linking their presence with prostate cancer outcome. The occurrence of localized stromal stores of MIC-1 is likely to play a central role in modulating local bioavailability of MIC-1, which can affect patient outcome with prostate as well as other epithelial tumors.

Table 2. Cox proportional hazards multivariate analysis for relapse of prostate cancer

Risk factor	HR (95% CI)	*P*
Extracapsular spread (>pT2C)	2.230 (1.163-3.646)	0.0158
Gleason sum score*	1.464 (1.194-1.795)	0.0002
Positive lymph nodes	6.811 (1.979-23.447)	0.0023
Pre-op PSA >10 ng/mL	2.112 (1.223-3.646)	0.0073
Surgical margin involvement	0.846 (0.432-1.656)	0.6255
Malignant stroma intensity <1†	1.756 (1.046-2.949)	0.0332

Abbreviations: pre-op PSA, preoperative serum levels of prostate-specific antigen.
*Gleason sum score was analyzed as a continuous variable.
†MIC-1 staining variable.

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