



the expression of a number of genes that regulate angiogenesis and invasion (7, 8). The process of invasion is regulated within a complex homeostasis by the expression of enzymes such as the MMPs, which are responsible for the degradation of the extracellular matrix and basal membranes (9, 10) and the adhesion molecule E-cadherin, which promotes homotypic cellular cohesion. The overexpression of the MMPs, especially MMP-9, in tissue, serum, and urine of patients with TCC correlates with disease progression and metastasis (11–21). Likewise, down-regulation of E-cadherin is associated with rapid progression (22–26). We reported previously (27) that the expression of the pro-angiogenic factors bFGF and VEGF predicted for disease recurrence of muscle-invasive TCC treated with systemic M-VAC chemotherapy. In the current study, we demonstrate that the ratio of MMP-9:E-cadherin is a stronger independent predictor for recurrence than either VEGF or bFGF. This suggests the hypothesis that therapy that either down-regulates MMP-9 or up-regulates E-cadherin expression may improve the prognosis for these patients.

IFNs are cytokines that exert pleiotropic antitumoral effects; they regulate cell growth and differentiation, inhibit the expression of oncogenes, up-regulate apoptosis, and activate T lymphocytes, natural killer cells, and macrophages (28–31). IFN- $\alpha$  and - $\beta$  directly inhibit the proliferation of tumor cells of different histological origin (32–39) and are endogenous inhibitors of angiogenesis. Recent studies report that IFN- $\alpha$  down-regulates the *in vitro* expression of the angiogenesis factors bFGF (34), IL-8 (35, 36), and MMP-2/9 (37–39) and up-regulates E-cadherin (40–42). Systemic therapy with IFN- $\alpha$  inhibited the growth of the human TCC cell line 253J B-V<sup>R</sup> growing within the bladder of athymic nude mice by down-regulating the expression of bFGF, resulting in the inhibition of tumor-induced angiogenesis as measured by the reduction in MVD. This antitumor effect was independent of any antiproliferative effects of IFN (43). In this study, we report that systemic therapy of human TCC growing within the bladders of athymic nude mice with IFN- $\alpha$  down-regulated the expression of MMP-9, up-regulating E-cadherin expression, and inhibited tumor growth and spontaneous metastasis. IFN therapy also potentiated the efficacy of paclitaxel against established bladder cancers growing within the bladders of athymic nude mice.

Thus, virulent TCC is characterized by a high MMP-9:E-cadherin ratio. Systemic low-dose IFN decreases this ratio by down-regulating MMP-9 and up-regulating E-cadherin and, in combination with systemic cytoreductive chemotherapy, provides a novel therapeutic strategy for the treatment of advanced TCC.

## MATERIALS AND METHODS

**Surgical Specimens.** Formalin-fixed, paraffin-embedded pretherapy biopsy specimens were available from 55 patients with muscle-invasive TCC who underwent neoadjuvant M-VAC chemotherapy (median of three courses) followed by radical cystectomy and pelvic lymph node dissection. All of the patients were treated at the Kochi Medical School (Kochi, Japan) between 1984 and 1998. Initial clinical stage was determined by pathological examination of the transurethral resection in conjunction with the findings of the examination under an-

esthesia. All of the patients underwent chest X-ray and computed tomographic scans of the abdomen and pelvis, and any patient with lymph node or distant metastasis was excluded from this analysis. The expression of MMP-9 and E-cadherin within viable tumor of each biopsy specimen was determined by ISH. After cystectomy, patients were monitored carefully with periodic measurement of hepatic transaminases and alkaline phosphatase, chest radiography, and abdominal and pelvic computed tomographic scans. Time to recurrence and overall survival were recorded.

**Cell Lines and Culture Conditions.** 253J B-V<sup>R</sup>, a human TCC cell line resistant to the antiproliferative effects of IFN- $\alpha$ , was grown as a monolayer culture in modified Eagle's MEM supplemented with IFN- $\alpha$ -2a (10,000 units/ml), 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin (44).

**In Situ mRNA Hybridization Analysis.** For ISH analysis, specific antisense oligonucleotide DNA probes for MMP-9 (45), E-cadherin (46), VEGF, bFGF, and IL-8 (27) were prepared as described previously. These probes were designed to be complementary to the mRNA transcripts based on published reports of the cDNA sequence, as described previously. The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (Genetics Computer Group, Madison, WI) based on the FastA algorithm. These sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each sequence was also confirmed by Northern blot analysis (47). A poly(dT)<sub>20</sub> oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All of the DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3' end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted using a stock solution to 1  $\mu$ g/ $\mu$ l in 10 mmol/liter Tris (pH 7.6) and 1 mmol/liter EDTA. Immediately before use, the stock solution was diluted with probe diluent (Research Genetics).

*In situ* mRNA hybridization was performed as described previously (47, 48) with minor modifications. ISH was performed using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, PA; Ref. 49). Tissue sections (4  $\mu$ m) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific; Refs. 47, 48). Alternatively, for evaluation of *in vitro* mRNA expression, cells were grown on dry-sterilized ProbeOn slides fixed in 4% paraformaldehyde in diethyl pyrocarbonate-PBS for 20 min at room temperature. The slides were placed in the Microprobe slide holder and then dewaxed and rehydrated with Autodewaxer and Autoalcohol (Research Genetics) followed by enzymatic digestion with pepsin. Hybridization of the probe was performed for 45 min at 45°C, and the samples were then washed three times with 2  $\times$  SSC for 2 min at 45°C. The samples were incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer for 1 min, and incubated with fresh chromogen substrate for 15 min at 45°C. If

necessary, samples were incubated a second time with fresh chromogen to enhance a weak reaction. Complementary mRNA in the sample leads to a red stain in this assay. Control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone. Four samples of normal urothelium were used as reference material to which all of the tumor samples were referenced (27).

**Quantification of Color Reaction.** Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a three-chip charged-coupled device color camera (model DXC-960 MD; Sony Corporation, Tokyo, Japan). The images were analyzed using the Optimas image analysis software (version 6.2; Media Cybernetics, Silver Spring, MD). The slides were prescreened by one of us to determine the range in staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green, and blue modes of the color camera. All of the subsequent images were quantified based on this threshold. The integrated absorbance of the selected fields was determined by the mean log inverse gray scale values multiplied by the area of the field. The samples were not counterstained, so the absorbance was attributable solely to the product of the ISH reaction. For each section, we determined the absorbance in several  $2 \times 2$ -mm zones located at the periphery of the tumor. Five different fields within each  $2 \times 2$ -mm zone were quantified, and an average value was determined (50). The intensity of staining was recorded as a ratio of the observed intensity:the intensity of the integrated absorbance of poly(dT)<sub>20</sub> in the same sections, and this ratio was then normalized by comparison with the integrated absorbance of a reference set of normal urothelium stained simultaneously with the tumor section according to the following equation:  $100 \times (A/B)/(C/D)$ , where *A* is the absorbance of the gene expression in the tumor specimen, *B* is the absorbance of poly(dT)<sub>20</sub> expression in the tumor specimen, *C* is the absorbance of the gene expression in the normal urothelium, and *D* is the absorbance of poly(dT)<sub>20</sub> expression in normal urothelium. An example of ISH and relative gene expression of MMP-9 and E-cadherin is shown in Fig. 1.

**In Vitro mRNA Expression of MMP-9 and E-cadherin after in Vitro Exposure to IFN- $\alpha$ .** We plated  $1 \times 10^6$  253J B-V<sup>R</sup> cells onto ProbeOn slides in 150-cm<sup>2</sup> dishes and incubated the cells continuously with increasing doses of IFN- $\alpha$  for 96 h. ISH with probes for MMP-9 and E-cadherin was performed as described above, and the relative expression of mRNA of each was quantified.

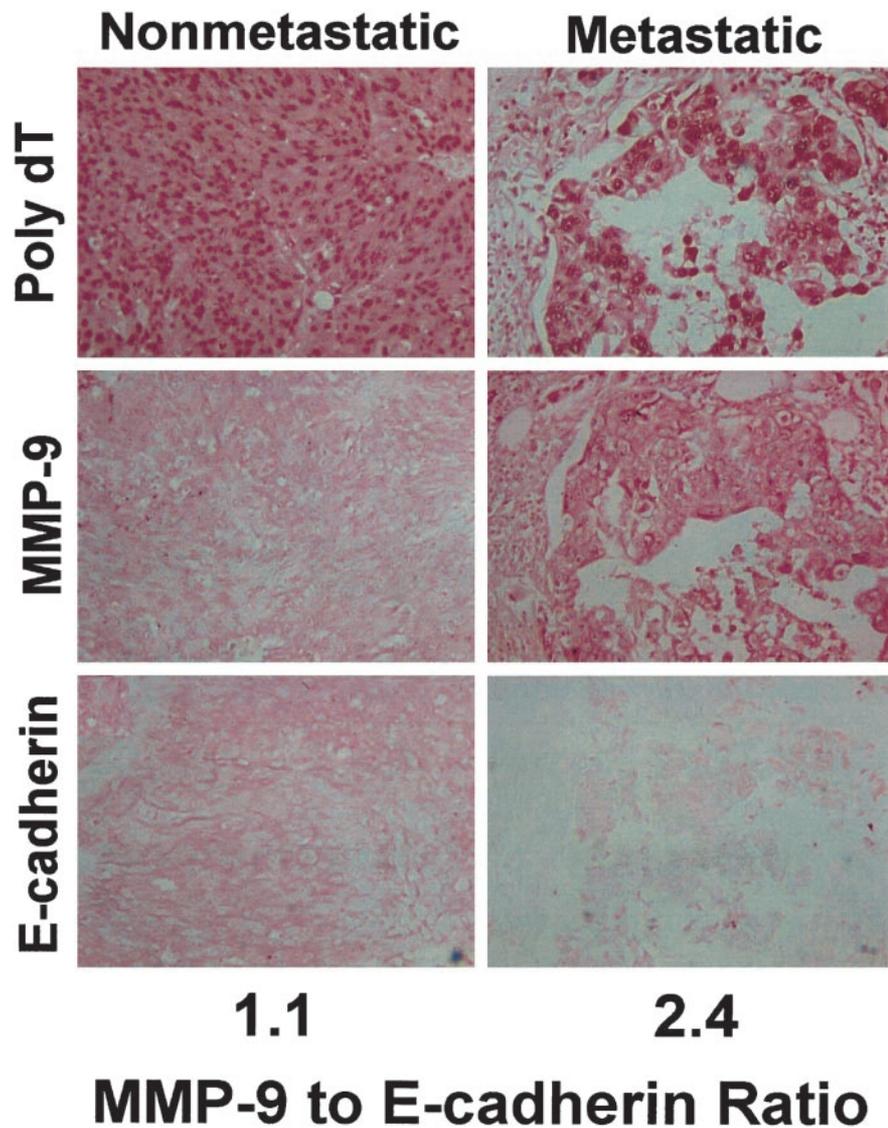
**Collagenase Activity.** To determine collagenase activity, electrophoresis of serum-free conditioned medium was performed as described previously (51). Cells ( $5 \times 10^3$ ) were seeded in six-well plates and grown to 60–70% confluence. The cells were washed with HBSS and grown for 24 h in serum-free medium. The supernatant fluid was collected to determine collagenase activity, and the remaining cells were counted to confirm the cell number. Collected samples were concentrated by centrifugation with MICROCON microconcentrators (Amicon, Inc., Beverly, MA). Thirty  $\mu$ l of each sample were electrophoresed with 10  $\mu$ l of loading buffer (10% SDS) on 20% SDS-polyacrylamide gels containing 1 mg/ml gelatin. After electro-

phoresis, gels were washed in 2.5% Triton X-100 to remove SDS and to allow proteins to renature. Then the gels were immersed in incubation buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 5 mM CaCl<sub>2</sub>, and 1  $\mu$ M ZnCl<sub>2</sub> for 24 h at 37°C. The zymograms were stained with 0.1% (w/v) Coomassie Blue R-250 (Sigma Chemical Co.) and destained in 40% methanol-10% acetic acid. Identification of a transparent band at *M<sub>r</sub>* 72,000 or 92,000 on the Coomassie Blue background of the slab gel indicated enzymatic activity. The collagenase activity was quantified by densitometry (ImageQuant software program; Molecular Dynamics, Sunnyvale, CA) after normalizing for cell number. To determine whether IFN- $\alpha$  mediates a decrease in MMP-9 activity, we incubated 253J B-V<sup>R</sup> cells in the presence of different doses (0–100 units/ml) of IFN- $\alpha$ , and the activity of MMP-9 was determined.

**CAT Assay.** Using the FuGENE 6 protocol (Boehringer Mannheim Corp.), 253J B-V<sup>R</sup> cells were transfected with the basic CAT expression vector with no promoter/enhancer sequences (pCAT-basic) or a control plasmid with SV40 promoter and enhancer (pCAT-control; Promega, Madison, WI). One copy of the full-sequence 570-bp human MMP-9 promoter (a gift of Dr. Seiki Motoharu, Department of Virology and Oncology, Cancer Research Institute, Kanazawa University Kanazawa, Japan) was ligated upstream of the basic CAT expression vector. Cells ( $5 \times 10^3$ /well in a six-well tissue culture dish) were transfected with 2.5  $\mu$ g of the reporter CAT constructs and 2.5  $\mu$ g of a  $\beta$ -actin expression plasmid. After 96 h, extracts were prepared from all of the plates, normalized for  $\beta$ -actin activity, and assayed for CAT activity (52) as described previously by Hudson *et al.* (53). The CAT assay was quantified by densitometry of autoradiographs with the use of the ImageQuant software program (Molecular Dynamics) and was evaluated as the ratio of acetylated species:all species.

**MMP-9 mRNA Half-Life Studies.** To determine whether IFN- $\alpha$  therapy altered the stability of MMP-9 mRNA, the 253J B-V<sup>R</sup> cells were incubated in IFN- $\alpha$  for 96 h, and further transcription in the cells was then blocked by the addition of ActD (Calbiochem-Novabiotechnology, Inc., Lake Placid, NY; final concentration of 5  $\mu$ g/ml). Total RNA was extracted from the cells 0, 1, 2, and 4 h after the addition of ActD, and MMP-9 mRNA expression was determined by Northern blot analysis and compared with the control value at each time point (total RNA extracted from cells before ActD treatment was arbitrarily defined as 100%). The half-life of MMP-9 mRNA was determined by plotting relative MMP-9 mRNA expression levels on a semilogarithmic axis *versus* time (Cricket Software, Malvern, PA). MMP-9 mRNA half-life was not affected by treatment with IFN- $\alpha$  (data not shown).

**Invasion Assay through Matrigel.** Polyvinylpyrrolidone-free polycarbonate filters (8- $\mu$ m pore size; Nuclepore; Becton Dickinson Labware, Franklin Lakes, NJ) were coated with a mixture of basement membrane components (Matrigel; 25  $\mu$ g/filter) and placed in modified Boyden chambers (54). The cells ( $5 \times 10^4$ ) were released from their culture dishes by short exposure to EDTA (1 mmol/liter), centrifuged, resuspended in 0.1% BSA-DMEM, and placed in the upper compartment of the Boyden chamber. Fibroblast-conditioned medium in the lower compartment served as a chemoattractant. After incubation for 6 h at 37°C, the cells on the lower surface of the filter were



*Fig. 1* Examples of ISH. Note the higher expression of MMP-9 (187% compared with 117%) and lower expression of E-cadherin (68% compared with 92%) in the metastatic tumor compared with the nonmetastatic tumor. The ratio of MMP-9:E-cadherin was 2.7 in the metastatic tumor and 1.3 in the nonmetastatic tumor.

stained with Diff-Quick (American Scientific Products, McGraw Park, IL) and quantified with a cooled charge coupled device (CCD) Optotronics Tec 470 camera (Optotronics Engineering, Goletha, CA) linked to a computer and digital printer (Sony Corporation). The results were expressed as the average number within a single 200 $\times$  field in five separate areas on the lower surface of the filter.

**Animals.** Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and used at 8 to 12 weeks of age. All of the facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health.

**In Vivo Therapy with IFN- $\alpha$ .** Experiments were designed to evaluate the effect of therapy with systemic IFN- $\alpha$  on the tumorigenic and metastatic potential of 253J B-V<sup>R</sup> cells growing within the bladder of athymic nude mice and the effect of therapy on the relative expression of MMP-9 and E-cadherin. A lower midline incision was made, and viable tumor cells ( $1 \times 10^6/0.05$  ml) in HBSS were implanted into the bladder wall. The formation of a bulla indicated a satisfactory injection. The bladder was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips (44). Three days after tumor implantation, the mice were treated with either saline daily or IFN- $\alpha$  according to the following schedule: 70,000 units weekly, 35,000 units twice/week, or 10,000 units daily, administered s.c. for 4 weeks. On or around day 35, mice were anesthetized with sodium pentobarbital and killed by cervical dislocation, and the presence of tumor within the bladder and at metastatic sites was evaluated. The bladders were then

**Table 1** Univariate analysis of MMP-9, E-cadherin, and the MMP-9:E-cadherin ratio as predictors for disease-free survival<sup>a</sup>

Parameter	P
E-cadherin	0.023
MMP-9	0.053
MMP-9:E-cadherin ratio	0.002

<sup>a</sup> Cox proportional hazards model.

either quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or frozen in OCT compound (Miles Laboratories, Elkhart, IN).

We also designed a series of experiments to evaluate combination therapy of established 253J B-V<sup>R</sup> tumors growing within the bladder of athymic nude mice with IFN- $\alpha$  and paclitaxel. The 253J B-V<sup>R</sup> cells were implanted into the bladder wall as described previously, and therapy commenced 14 days later when the tumors were palpable. Mice were treated with daily saline, daily IFN- $\alpha$  (10,000 units s.c.), paclitaxel 1 mg/kg weekly, or a combination of IFN- $\alpha$  and paclitaxel. On or around day 45, when control mice were moribund, mice were anesthetized with sodium pentobarbital and killed by cervical dislocation, and the presence of tumor within the bladder and at metastatic sites was again evaluated. The bladders were then either fixed in 10% buffered formalin or frozen in OCT compound.

**Immunohistochemistry.** For immunohistochemical analysis, frozen tissue sections (8- $\mu$ m thick) were fixed with cold acetone. Tissue sections (5- $\mu$ m thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, and transferred to PBS. The slides were rinsed twice with PBS, antigen retrieval was performed with pepsin for 12 min, and endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with the appropriate dilution (1:100) of rat monoclonal anti-CD31 antibody (PharMingen, San Diego, CA; Ref. 55), a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA) or a 1:200 dilution of anti-E-cadherin antibody (Zymed, San Francisco, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody: peroxidase-conjugated goat antimouse IgG1 (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) or antimouse IgG1 (PharMingen). The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill's hematoxylin (Biogenex Laboratories, San Ramon, CA), and again washed three times with PBS. The slides were mounted with a mounting medium (Universal Mount; Research Genetics; Ref. 55).

**Quantification of MVD.** MVD was determined by a light microscope after immunostaining of sections with anti-CD31 antibodies (56) according to the procedure of Weidner *et al.* (57). Clusters of stained endothelial cells distinct from ad-

**Table 2** Multivariate analysis of stage, MVD, and metastasis-related genes as predictors for disease-free survival<sup>a</sup>

Parameter	P
Clinical stage	0.138
MVD	0.146
MMP-9	0.045
E-cadherin	0.356
VEGF	0.181
bFGF	0.250
IL-8	0.766
MMP-9:E-cadherin ratio	0.016

<sup>a</sup> Cox proportional hazards model.

jacent microvessels, tumor cells, or other stromal cells were counted as one microvessel. Microvessels were counted by using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering), linked to a computer and digital printer (Sony Corporation). The density of microvessels was expressed as the average of the five highest areas identified within a single 200 $\times$  field.

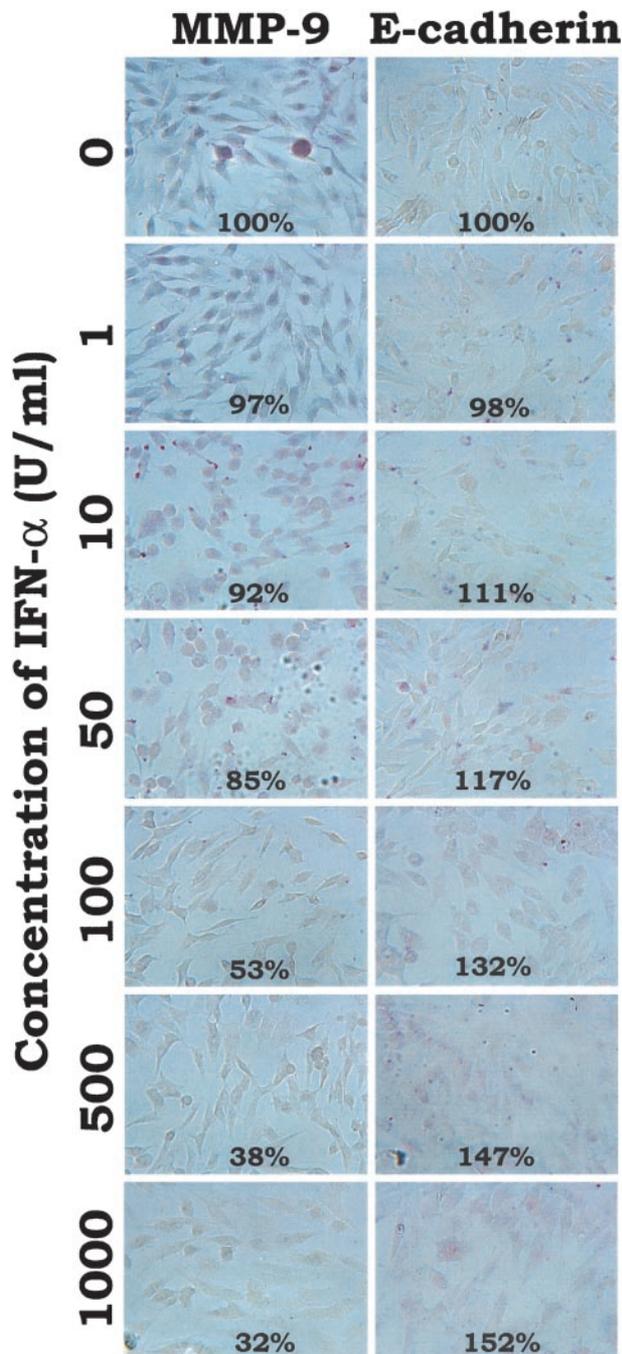
**Quantification of Intensity of Immunostaining.** The intensity of immunostaining of MMP-9, E-cadherin, bFGF, VEGF, and IL-8 was quantified in three different areas of each sample by an image analyzer using the Optimas software program (Bioscan, Edmonds, WA). Three different areas in each sample were quantified to yield an average measurement of intensity of immunostaining. The results were presented as a number of each cell line compared with the control, which was set to 100.

**Statistical Analysis.** The statistical differences in MVD, the expression of bFGF, VEGF, IL-8, MMP-9, and E-cadherin, and the MMP:E-cadherin ratio were analyzed by the Mann-Whitney test (58). The median value of each factor was selected as the cutoff point for designating high or low expression. Multivariate analyses were conducted using the Cox proportional hazards model (59). The incidences of tumor and metastasis were statistically analyzed by the  $\chi^2$  test. Statistical significance was defined as  $P < 0.05$ .

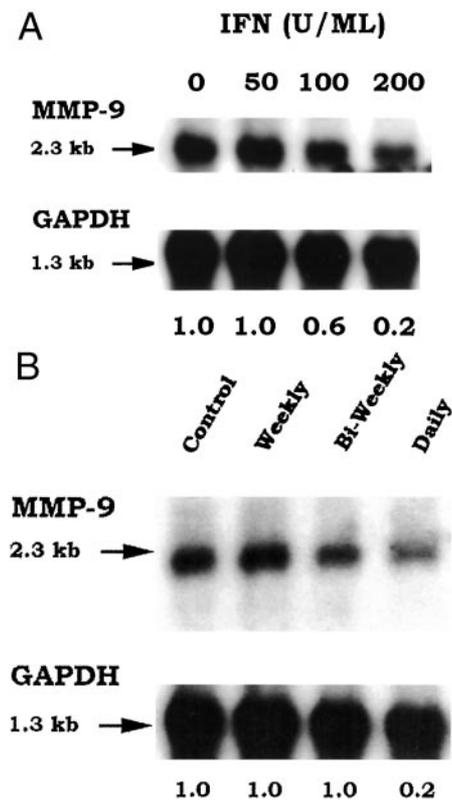
## RESULTS

**Patient Demographics.** The cohort we studied consisted of 46 men and nine women. The median age was 69 years (range, 44–75 years). The median follow-up was 38 months (range, 6–132 months). All of the tumors were grade 2 or 3 by WHO criteria.

**Prognostic Factors in Pretreatment Biopsy Specimens.** The prognostic significance of clinical stage, MVD, and the expression of VEGF, bFGF, IL-8, MMP-9, and E-cadherin as determined by ISH, and the ratio of MMP-9:E-cadherin in the biopsy specimens collected before therapy was evaluated by using the Cox proportional hazards model. As reported previously (27), clinical stage (organ confined *versus* extravesical; two-sided;  $P = 0.062$ ) approached significance as a prognostic factor for disease-free survival, whereas disease-free survival was independent of grade (data not shown). Both VEGF expression and MVD were significant predictors of disease recurrence (27). Univariate analysis indicated that pretherapy values for E-cadherin, MMP-9 expression, and the MMP-9:E-cadherin



**Fig. 2** Down-regulation of MMP-9 mRNA and up-regulation of E-cadherin mRNA expression by IFN- $\alpha$ . *In vitro*, 253J B-V<sup>R</sup> cells were cultured in the presence or absence of IFN- $\alpha$  for 96 h, and the steady-state mRNA expression of each was determined by ISH. The expression of each gene after therapy with 0 units/ml of IFN- $\alpha$  was arbitrarily set at 100%. A dose-dependent decrease in the relative expression of MMP-9 to 53% and 32% of that for untreated controls was observed at 100 units/ml and 1000 units/ml, respectively. E-cadherin expression was 132% and 152% of that for untreated controls at 100 and 1000 units/ml of IFN- $\alpha$ , respectively. This is one representative experiment of two.

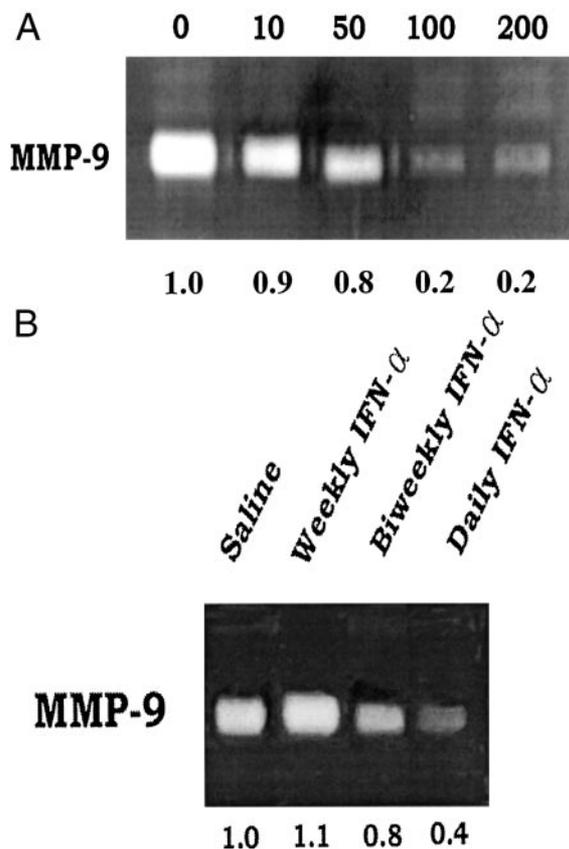


**Fig. 3** Northern blot analysis. *A*, *in vitro* MMP-9 mRNA expression of 253J B-V<sup>R</sup> treated with 0, 50, 100, or 200 units/ml of IFN- $\alpha$  for 96 h. Differences in activity are shown by the ratio of MMP-9 expression of IFN- $\alpha$ -treated cells to that of control cells ratio defined as 1.0. MMP-9 mRNA expression after IFN- $\alpha$  treatment was inhibited in a dose-dependent manner. This is one representative experiment of two. *B*, *in vivo* MMP-9 mRNA expression of extracts from tumors treated with either saline (control) or daily (10,000 units), biweekly (35,000 units), or weekly (70,000 units) IFN- $\alpha$ . MMP-9 expression was inhibited 80% after daily IFN- $\alpha$  therapy. This is one representative experiment of two.

ratio were also significant predictors for disease progression ( $P = 0.023$ ,  $P = 0.053$ , and  $P = 0.002$ , respectively; Table 1). By multivariate analysis using a threshold value of 1.8, the ratio of MMP-9:E-cadherin was the strongest independent predictor for disease-free survival ( $P = 0.016$ ; Table 2), even when compared against VEGF and MVD.

***In Vitro* Expression of MMP-9 and E-cadherin by 253J B-V<sup>R</sup> after IFN- $\alpha$  Therapy.** *In vitro* MMP-9 and E-cadherin mRNA expression after IFN- $\alpha$  treatment was assessed by ISH (Fig. 2). In response to increasing doses of IFN- $\alpha$ , the steady-state gene expression of MMP-9 decreased in a dose-dependent manner from 100% expression at 0 units/ml to 32% at 1000 units/ml (Fig. 2). These results were confirmed by Northern blot analysis (Fig. 3A). By ISH, E-cadherin expression increased in a dose-dependent manner from 100% expression at 0 units/ml to 152% at 1000 units/ml.

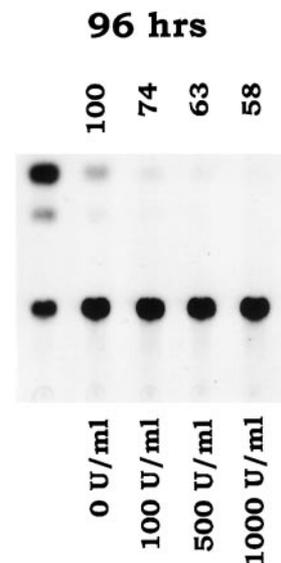
***In Vitro* and *In Vivo* Collagenase Activity.** To demonstrate that IFN inhibited the *in vitro* activity of MMP-9, collagenase activity was determined by zymography. By densitometry after normalizing for cell number, *in vitro* col-



**Fig. 4** Collagenase assay. *A*, gelatinolytic activity of the conditioned medium of 253J B-V<sup>R</sup> cells treated with 0, 50, 100, or 200 units/ml of IFN- $\alpha$  for 96 h after normalizing for cell number. Differences in activity are shown by the ratio of gelatinolytic activity of IFN- $\alpha$ -treated cells to that of control cells (ratio defined as 1.0). Collagenase activity after IFN- $\alpha$  treatment was inhibited in a dose-dependent manner. This is one representative experiment of two. *B*, *in vivo* gelatinolytic activity of extracts from tumors treated with either saline (control) or daily (10,000 units), biweekly (35,000 units), or weekly (70,000 units) IFN- $\alpha$ . Collagenase activity was inhibited 60% after daily IFN- $\alpha$  therapy. This is one representative experiment of two.

lagenase activity was significantly inhibited (up to 3-fold) by continuous exposure to IFN up to a dose of 100 units/ml (Fig. 4A). Increasing the dose above 100 units/ml did not enhance the down-regulation of collagenase activity. *In vivo* gelatinolytic activity of extracts from tumors treated either with saline or with daily (10,000 units), biweekly (35,000 units), or weekly (70,000 units) IFN- $\alpha$  was similarly measured by zymography (Fig. 4B). Collagenase activity was inhibited 2-fold by daily IFN- $\alpha$ , compared with saline or weekly or biweekly IFN therapy ( $P < 0.05$ ).

**CAT Activity.** The full-sequence MMP-9 promoter (570 bp) was linked upstream of the CAT reporter gene and transfected into the 253J B-V<sup>R</sup> cells. The cells were incubated in IFN- $\alpha$ . At 96 h after transfection, cell extracts were prepared, normalized for  $\beta$ -actin activity, and assayed for CAT activity. CAT activity driven by MMP-9 promoter decreased in a dose-dependent manner (Fig. 5). CAT activity driven by the SV40 promoter was the same in both cell populations and served as an



**Fig. 5** Effect of IFN- $\alpha$  on CAT activity driven by the MMP-9 promoter in 253J B-V<sup>R</sup> cells. The CAT activity was evaluated as the ratio of acetylated species:all species. Differences in expression at 96 h are shown as the ratio of CAT activity of treated cells to that of untreated cells (defined as 100). CAT activity levels driven by the MMP-9 promoter were decreased by IFN- $\alpha$  96 h in a dose-dependent fashion. This is one representative experiment of two.

additional internal control for transfection efficiency. Next, the stability of MMP-9 mRNA was investigated by examining its half-life. The half-life of MMP-9 mRNA of 253J B-V<sup>R</sup> treated with IFN- $\alpha$  was similar to untreated controls (data not shown).

**Invasion Assay through Matrigel.** We next evaluated whether the down-regulation of mRNA MMP-9 expression and collagenase activity by IFN- $\alpha$  treatment resulted in decreased invasion through Matrigel. Continuous 96-h exposure of the 253J B-V<sup>R</sup> cells to 100 or 1000 units/ml of IFN- $\alpha$  significantly decreased invasion through Matrigel ( $6 \pm 5$  and  $2 \pm 2$  cells/200 $\times$  field) compared with that observed after therapy with saline or IFN- $\alpha$  concentrations of 1 or 10 units/ml ( $43 \pm 7$ ,  $39 \pm 8$ , and  $21 \pm 3$ , respectively;  $P < 0.05$ ; Fig. 6).

**Tumorigenicity and Metastasis.** We next evaluated the effect of systemic IFN- $\alpha$  therapy on the tumorigenicity and metastasis of human TCC growing within the bladder wall of nude mice. 253J B-V<sup>R</sup> cells were implanted into the bladder wall, and therapy with IFN- $\alpha$  began 3 days later. After 4 weeks of treatment, the mice were killed and necropsied, and tumor growth and metastasis were evaluated. Treatment with daily IFN- $\alpha$  significantly inhibited tumorigenicity compared with the other schedules of IFN- $\alpha$  administration ( $P < 0.001$ ), and metastasis was completely inhibited by daily IFN- $\alpha$  therapy (Table 3).

**Effect of Systemic IFN- $\alpha$  on Tumor-induced Angiogenesis and MMP-9 and E-cadherin Expression Levels.** The *in vivo* expression of MMP-9 mRNA was evaluated by ISH and Northern blot analysis (Table 4; Fig. 3B; and Fig. 7). By ISH, daily IFN- $\alpha$  reduced MMP-9 mRNA 2-fold ( $P < 0.01$ ) compared with saline treatment (Table 4; Fig. 7). This was confirmed by Northern blot analysis (Fig. 3B). *In vivo* collagenase

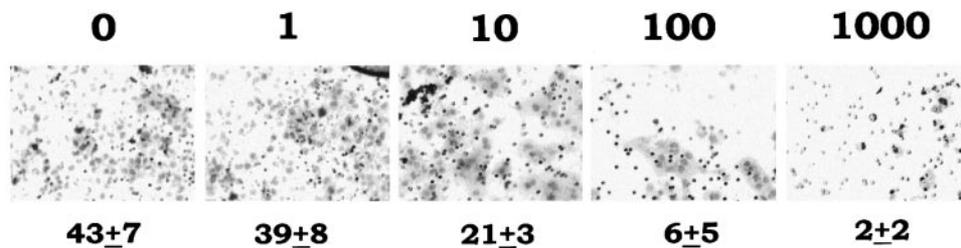


Fig. 6 Invasion through Matrigel-coated plates. Migration of 253J B-V<sup>R</sup> cells ( $2 \times 10^5$ ) after IFN- $\alpha$  treatment through Matrigel-coated filters into fibroblast-conditioned medium as a source of chemoattractants, expressed as the average number of cells in the five highest spots identified within a single  $200\times$  field on the lower surface of the filter. The number of migrated cells that penetrated through the Matrigel-coated filters decreased in a dose-dependent manner.

Table 3 Systemic therapy of 253J B-V<sup>R</sup> cells with IFN- $\alpha^a$

IFN- $\alpha$ treatment		Tumor incidence	Tumor weight (mg)		Spontaneous metastasis	
Dose (units)	Schedule		Median	Range	Lung	Lymph node
Saline	Daily	9/9	357	(185–522)	4/8	8/8
70,000	Once weekly	8/8	279	(162–392)	3/8	8/8
35,000	Twice weekly	8/8	220	(133–456)	4/8	7/8
10,000	Daily	10/10	101	(24–175) <sup>b</sup>	0/8	0/8

<sup>a</sup> 253J B-V<sup>R</sup> cells ( $1 \times 10^6$ ) were implanted into the bladder of nude mice, therapy began on day 3, and mice were treated with s.c. IFN- $\alpha$  for 4 weeks at the indicated doses and schedules. One representative of two experiments.

<sup>b</sup>  $P < 0.001$ .

activity was similarly reduced in tumors treated with daily IFN- $\alpha$  compared with the other treatments (Fig. 4B;  $P < 0.05$ ). Conversely, E-cadherin mRNA increased 2-fold after daily IFN therapy (Table 4; Fig. 7). The MMP-9:E-cadherin mRNA expression ratio was  $4.5 \pm 0.6$  in untreated controls compared with  $1.1 \pm 0.1$  for mice treated with daily IFN- $\alpha$  (Table 4). The MMP-9:E-cadherin protein ratio was similarly decreased from  $2.6 \pm 0.4$  in untreated controls to  $1.3 \pm 0.1$  for mice treated with daily IFN- $\alpha$  ( $P < 0.01$ ; data not shown). Tumor-induced neovascularization (MVD) was determined by immunohistochemical staining using anti-CD31 antibodies (Table 4). Daily IFN- $\alpha$  treatment resulted in a significantly lower value for MVD, with a mean of  $75 \pm 25$  vessels/high-power field in untreated controls compared with  $39 \pm 12$  vessels/high-power field in mice that received daily IFN- $\alpha$  ( $P < 0.01$ ).

**Therapy of Established Bladder Tumors with Paclitaxel and IFN- $\alpha$ .** Because the intent was to develop novel chemotherapeutic strategies for advanced TCC, we next evaluated whether daily IFN- $\alpha$  would potentiate the efficacy of paclitaxel against established bladder tumors. Paclitaxel was chosen for these experiments because it has activity against metastatic TCC when administered as a single agent (5). In these experiments, treatment commenced 14 days after tumor implantation, at which time the tumors were palpable and well established. Therapy continued until all of the control mice became moribund and had to be killed. On or around day 45, mice were anesthetized with sodium pentobarbital and killed by cervical dislocation, and the presence of tumor within the bladder and at metastatic sites was evaluated. Mice treated with IFN- $\alpha$  and paclitaxel together had significantly smaller tumors (median weight, 158 mg; range, 47–241 mg) than did mice treated with saline (median weight, 385 mg; range, 195–510 mg), paclitaxel

alone (median weight, 225 mg; range, 75–326 mg) or IFN- $\alpha$  alone (median weight, 304 mg; range, 222–375 mg). Because the tumors were larger at the initiation of therapy (day 14; mean weight, 233 mg) than at the completion of therapy in mice that received IFN- $\alpha$  plus paclitaxel, this combination induced the regression of established bladder tumors and was not merely cytostatic (Table 5). Therapy with IFN- $\alpha$  and paclitaxel also resulted in a lower expression of MMP-9 and a 2-fold increase in the expression of E-cadherin mRNA (Table 6). Therefore, although IFN- $\alpha$  did not inhibit the growth of established tumors, it potentiated the cytoreductive effects of paclitaxel, and the combination decreased the MMP-9:E-cadherin ratio to 1.0.

## DISCUSSION

Although TCC of the bladder is a chemosensitive tumor, most deaths from bladder cancer are caused by metastases that resist conventional chemotherapy (27). Although up to 70% of patients with advanced TCC have an initial response to chemotherapy, chemoresistant disease will rapidly develop in most of these patients (2–5). New chemotherapeutic strategies are therefore necessary to improve the outcome for patients with advanced bladder cancer. One approach to the development of new treatment strategies is to identify genes that are expressed by tumors that progress despite conventional cytoreductive chemotherapy and then target their expression or activity.

Metastasis of human TCC is mediated by the expression of genes such as E-cadherin or the MMPs (MMP-9) that regulate cellular adhesion and invasion. The ability of a tumor cell to invade the extracellular membrane is a prerequisite for metastasis. To invade, the tumor cell must first overcome homotypic forces that maintain cell-to-cell adhe-

Table 4 mRNA and protein expression of MMP-9 and E-cadherin, the MMP-9:E-cadherin ratio, and MVD after therapy with IFN- $\alpha$ 

IFN- $\alpha$ treatment <sup>a</sup>		mRNA expression <sup>b</sup>			Protein expression <sup>c</sup>			Mean vascular density <sup>d</sup>
Dose (units)	Schedule	MMP-9	E-cadherin	MMP-9:E-cadherin	MMP-9	E-cadherin	MMP-9:E-cadherin	
Saline	Daily	221 $\pm$ 27	48 $\pm$ 6	4.5 $\pm$ 0.6	180 $\pm$ 17	68 $\pm$ 9	2.6 $\pm$ 0.4	75 $\pm$ 25
70,000	Once weekly	203 $\pm$ 21	55 $\pm$ 15	3.7 $\pm$ 0.5	195 $\pm$ 9	61 $\pm$ 15	3.2 $\pm$ 0.6	69 $\pm$ 20
35,000	Twice weekly	195 $\pm$ 14	75 $\pm$ 10	2.6 $\pm$ 0.3	158 $\pm$ 21	72 $\pm$ 7	2.2 $\pm$ 0.3	62 $\pm$ 22
10,000	Daily	113 $\pm$ 13 <sup>e</sup>	106 $\pm$ 9 <sup>e</sup>	1.1 $\pm$ 0.2 <sup>e</sup>	122 $\pm$ 13 <sup>e</sup>	95 $\pm$ 1 <sup>f</sup>	1.3 $\pm$ 0.1 <sup>e</sup>	39 $\pm$ 12 <sup>e</sup>

<sup>a</sup> 253J B-V<sup>R</sup> cells ( $1 \times 10^6$ ) were implanted into the bladder of nude mice, therapy began on day 3, and mice were treated with IFN- $\alpha$  for 4 weeks at the indicated doses and schedules.

<sup>b</sup> ISH was performed on three bladders from each group. Image analysis was performed on five areas in each bladder. Expression of each factor in both the tumor and normal tissue was normalized by dividing by the expression of poly(dT) in the same area. The normalized value in tumor was divided by that of normal bladder mucosa to give the final expression of each factor.

<sup>c</sup> Immunohistochemical analysis was performed on three tumors from each group. Image analysis was performed out on five areas in each tumor and expressed as the mean  $\pm$  SD of the absorbance.

<sup>d</sup> Mean vascular density was determined by identifying the tumor area with the most intense CD31 staining. Mean number of vessels  $\pm$  SD/ $10 \times 100$  fields.

<sup>e</sup>  $P < 0.01$  versus control.

<sup>f</sup>  $P < 0.05$  versus control.

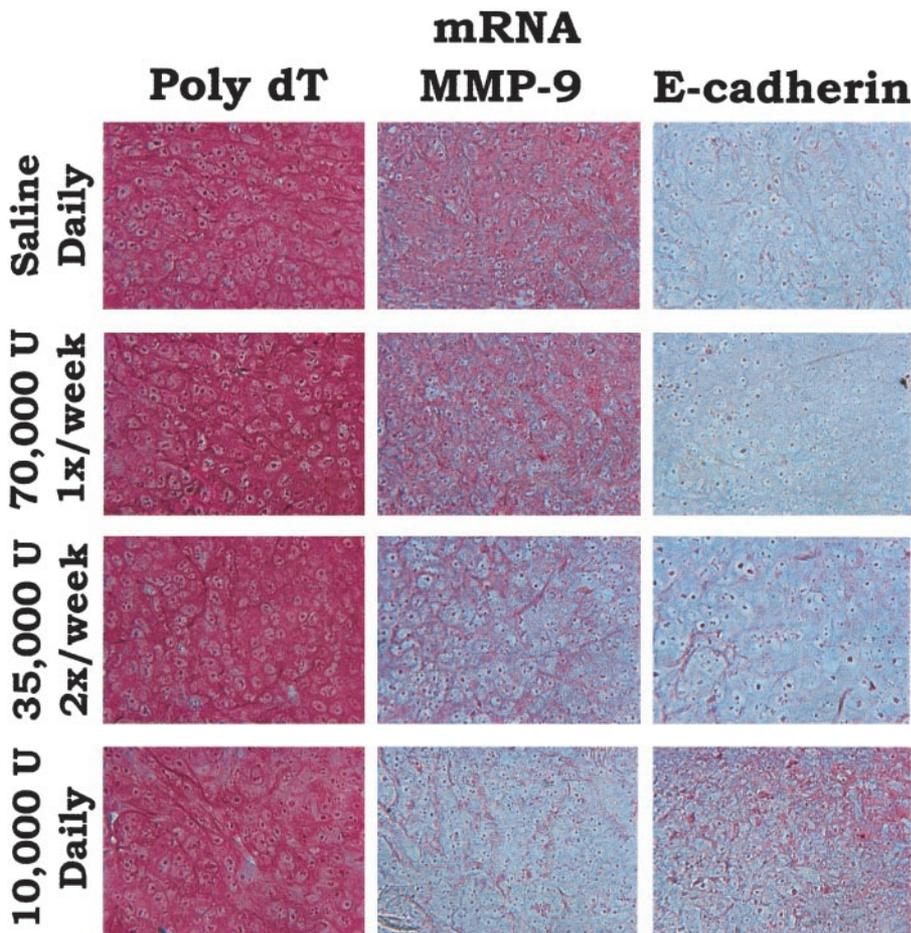


Fig. 7 MMP-9 mRNA expression after systemic IFN- $\alpha$  therapy. MMP-9 mRNA expression was determined by ISH after 4 weeks of therapy with saline (control) or daily (10,000 units), biweekly (35,000 units), or weekly (70,000 units) IFN- $\alpha$ . The intensity of mRNA expression was determined by optical densitometry after comparison with the integrated absorbance of poly(dT)<sub>20</sub>, which was set to 100, and that of the normal urothelium. The mRNA expression of MMP-9 was decreased 2-fold in tumors treated with daily IFN, whereas E-cadherin increased 2-fold. The MMP-9:E-cadherin ratio decreased in a schedule-dependent manner from 4.5 in the untreated controls to 1.1 in the tumors treated with daily IFN- $\alpha$  therapy.

sion within the primary tumor and then traverse through an altered extracellular membrane to enter the systemic circulation. E-cadherin is a cell-surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion (60). It is localized at the epithelial junction complex and is responsible

for the organization, maintenance, and morphogenesis of epithelial tissues (61–64). Reduced levels of E-cadherin are associated with a decrease in cellular and tissue differentiation and increased histological grade in different epithelial neoplasms (61, 62). The loss of E-cadherin expression or

Table 5 Systemic therapy of 253J B-V<sup>R</sup> cells with IFN- $\alpha$  and paclitaxel<sup>a</sup>

Therapy	Tumor weight (mg)	
	Median	Range
Pretreatment control	233	(129–322)
Saline daily	385	(192–510)
IFN- $\alpha$ (10,000 units daily)	304	(222–375)
Paclitaxel	225	(75–326)
Paclitaxel and IFN- $\alpha$	158 <sup>b</sup>	(47–241)

<sup>a</sup> 253J B-V<sup>R</sup> cells ( $1 \times 10^6$ ) were implanted into the bladder of nude mice, therapy began on day 14, and mice were treated with IFN- $\alpha$  or paclitaxel for 3 weeks at the indicated doses and schedules. One representative of two experiments.

<sup>b</sup>  $P < 0.001$  vs. saline;  $P < 0.05$  vs. low-dose paclitaxel, IFN- $\alpha$  only, and pretreatment controls.

activity favors tumor cell invasion, and transfection of E-cadherin-encoding cDNA into invasive cancer cells inhibits their invasiveness (64). A number of investigators (22–26) have reported that decreases in E-cadherin expression correlate with early recurrence and poor survival in patients with invasive TCC.

The MMPs are degradative enzymes that alter the surrounding extracellular membrane and allow tumor cells to migrate into the surrounding matrix (65–68). Their protease function is regulated by activity of membrane-type MMP-1 (69, 70). Elevated expression levels of MMP-2 and MMP-9 are found in the tissue, serum, and urine of patients with invasive bladder tumors (11–21). Tumor growth and metastasis also depend upon the ability of the tumor to induce its own blood supply. Angiogenesis depends on the outcome between stimulatory and inhibitory regulation by the tumor and its microenvironment (7, 8). Overexpression of proangiogenic factors by tumor cells is one mechanism by which tumors can increase neovascularity. Overexpressed levels of angiogenic factors bFGF (71, 72) and VEGF (73–75) have been identified in tissue, serum, and urine of patients with bladder cancer and have also been associated with disease progression. Increased MVD, a histological surrogate for angiogenesis, correlates with the progression of advanced TCC (27).

In our current study, we evaluated the prognostic significance of MVD and of the expression of several metastasis-related genes that regulate cellular adhesion (E-cadherin), invasion (MMP-9), and angiogenesis (VEGF, bFGF, and IL-8) in patients with muscle-invasive TCC treated with neoadjuvant M-VAC and radical cystectomy. Previous reports have shown that the ratio of the expression levels of MMP-9:E-cadherin (determined by colorimetric ISH and image analysis) predicted the metastatic potential of colorectal (76, 77), gastric (78), pancreatic (79), lung (80), and prostate carcinomas (81). Consistent with those findings, we demonstrate here that a high expression level of MMP-9 coupled with a low expression level of E-cadherin was associated with early recurrence and death of patients with TCC. It is unlikely that the expression level of either MMP-9 or E-cadherin directly influences chemosensitivity; rather, these levels identify TCC with a propensity to invade and metastasize. This index of the invasive potential of bladder cancer (MMP-9:E-cadherin ratio,  $>1.8$ ) was a stronger predic-

tor of tumor progression than clinical stage, MVD, or VEGF expression.

Having determined that the relative expression of MMP-9 to E-cadherin correlates with a poor prognosis for patients with muscle-invasive TCC, we evaluated whether therapy with IFN- $\alpha$ , which down-regulated MMP-9 and up-regulated E-cadherin, might inhibit the growth and metastasis of human TCC growing within the bladders of athymic nude mice. For these experiments, we implanted the highly metastatic 253J B-V<sup>R</sup> cell line (which expresses high levels of MMP-9 and low levels of E-cadherin) into the bladders of athymic nude mice and evaluated the effects of treatment with systemic IFN- $\alpha$ .

IFN- $\alpha$  and - $\beta$  have been used to treat a myriad of human solid malignancies with only marginal therapeutic benefit (82–88). In most of these studies, the IFNs were administered at maximal tolerated doses for short durations (3–4 months). In contrast, the long-term administration ( $>8$  months) of low-dose IFN- $\alpha$ -2a to patients with malignant hemangiopericytoma, Kaposi's sarcoma, or life-threatening hemangiomas of infancy has led to remarkable clinical regression of these tumors (89–91). Previous studies with IFN have attributed its antitumor effects to the induction of cell differentiation, inhibition of cell proliferation, modulation of host immunity (92, 93), and alteration of the level of gene expression in target cells (94–97).

We reported recently (43, 97) that the daily systemic administration of low-dose IFN- $\alpha$  to nude mice bearing human TCC in the bladder wall decreased expression levels of bFGF, VEGF, and MMP-9, decreased MVD, and inhibited tumor growth. These effects were independent of the antiproliferative effects of IFN- $\alpha$ . This antiangiogenic activity of IFN- $\alpha$  seems to depend upon both the schedule and dose of IFN- $\alpha$ , because frequent administration of an optimal biological dose as opposed to the administration of IFN at its maximally tolerated dose is necessary to demonstrate this effect (87).

In the present study, we demonstrate that IFN- $\alpha$  down-regulates both the *in vitro* and *in vivo* production of MMP-9, with a concomitant decrease in enzymatic activity (collagenase activity) and invasion through Matrigel. This decrease in MMP-9 was accompanied by an increase in E-cadherin expression. The MMP-9:E-cadherin ratio was reduced from 4.2 to 1.1 in the 253J B-V<sup>R</sup> tumors after therapy with systemic IFN- $\alpha$ , which is well below the index value of 1.8, that discriminated between metastatic and nonmetastatic TCC in the patient population studied. IFN- $\alpha$  reduced the invasive potential of the TCC cells by simultaneously decreasing MMP-9 and increasing E-cadherin expression. These changes in MMP-9 and E-cadherin levels were accompanied by a decrease in tumorigenicity and spontaneous metastasis to the lymph nodes and lungs of the mice. Furthermore, IFN- $\alpha$  potentiated the efficacy of paclitaxel, again with a corresponding reduction in the ratio of MMP-9:E-cadherin to 1.0. This indicates that systemic IFN therapy can be combined effectively with conventional chemotherapeutic agents as a treatment for advanced TCC.

The regulation of MMP-9 by IFN is complex. bFGF is known to up-regulate the production of MMP-9 by human cancer cells. Therefore, IFN- $\alpha$  could reduce MMP-9 indirectly through the inhibition of bFGF (98). However, we suspect that IFN regulates MMP-9 more directly. Results from promoter-reporter (CAT) assays showed that IFN rapidly decreases

Table 6 mRNA and protein expression of MMP-9, E-cadherin, the MMP-9:E-cadherin ratio and MVD after therapy with IFN- $\alpha$  and paclitaxel<sup>a</sup>

Treatment	mRNA expression <sup>b</sup>			Protein expression <sup>c</sup>			Mean vascular density <sup>d</sup>
	MMP-9	E-cadherin	MMP:E-cadherin	MMP-9	E-cadherin	MMP-9:E-cadherin	
Pretreatment control	222 $\pm$ 18	51 $\pm$ 18	4.0 $\pm$ 0.6	165 $\pm$ 15	48 $\pm$ 9	3.4 $\pm$ 0.4	65 $\pm$ 19
Saline daily	201 $\pm$ 18	55 $\pm$ 6	3.7 $\pm$ 0.5	180 $\pm$ 9	61 $\pm$ 11	2.9 $\pm$ 0.3	75 $\pm$ 25
IFN- $\alpha$ daily	146 $\pm$ 17	68 $\pm$ 11	2.3 $\pm$ 0.2	151 $\pm$ 11	89 $\pm$ 16	1.7 $\pm$ 0.2	69 $\pm$ 20
Paclitaxel weekly	162 $\pm$ 14	79 $\pm$ 9	2.0 $\pm$ 0.3	188 $\pm$ 15	69 $\pm$ 9	2.7 $\pm$ 0.4	62 $\pm$ 22
IFN- $\alpha$ + paclitaxel	130 $\pm$ 13 <sup>e</sup>	111 $\pm$ 9	1.0 $\pm$ 0.2	122 $\pm$ 13 <sup>e</sup>	86 $\pm$ 21	1.4 $\pm$ 0.2	39 $\pm$ 12 <sup>e</sup>

<sup>a</sup> 253J B-V<sup>R</sup> cells ( $1 \times 10^6$ ) were implanted into the bladder of nude mice, therapy began on day 14, and mice were treated with IFN- $\alpha$  or paclitaxel for 3 weeks at the indicated doses and schedules.

<sup>b</sup> ISH was performed on two bladders from each group. Image analysis was performed out on five areas in each bladder. Expression of each factor in both the tumor abnormal tissue was normalized by dividing by the expression of poly(dT) in the same area. The normalized value in tumor was divided by that of normal bladder mucosa to give the final expression of each factor.

<sup>c</sup> Immunohistochemical analysis was performed on two tumors from each group. Image analysis was performed out on five areas in each tumor and expressed as the mean  $\pm$  SD of the absorbance.

<sup>d</sup> Mean vascular density was determined by identifying the tumor area with the most intense CD31 staining. Mean number of vessels  $\pm$  SD/10  $\times$  100 fields.

<sup>e</sup>  $P < 0.01$  versus control.

MMP-9 promoter activity, and nuclear run-on assays indicated that IFN-mediated decreases in MMP-9 mRNA levels are not associated with changes in message half-life (99). Computer-assisted analysis of the MMP-9 promoter revealed two consensus (7 of 7) IFN-stimulated response elements within the 700-bp 5' flanking sequence of the *MMP-9* gene.<sup>5</sup> The down-regulation of MMP-9 correlates with decreased MVD, reduced tumor growth, and inhibition of metastasis. The IFN-induced increase in E-cadherin levels may also be mediated via direct signal transduction and activators of transcription (STAT)-dependent regulation of transcription because there is also a perfect IFN-stimulated response element within the E-cadherin promoter as well.<sup>5</sup> In addition, others (100, 101) have shown that transfection of tumor cells with the cDNA of E-cadherin and other cadherins is accompanied by a decrease in collagenase IV levels. This close relationship of MMP-9 and E-cadherin is further supported by a recent study (102) in which treatment of Madin-Darby canine kidney tumor cells with anti-E-cadherin antibodies resulted in up-regulated type IV collagenase. Furthermore, metalloproteinases may enhance tumor cell invasion by cleaving E-cadherin from the cell surface and releasing a soluble E-cadherin fragment that inhibits E-cadherin function in a paracrine fashion (103).

In summary, we found that the ratio of MMP-9:E-cadherin expression levels identified patients with muscle-invasive TCC who had disease progression despite aggressive therapy with neoadjuvant M-VAC and radical cystectomy. Systemic treatment of human TCC growing within the bladders of athymic nude mice down-regulated MMP-9 while up-regulating E-cadherin expression and inhibited tumor growth and metastasis. Moreover, IFN- $\alpha$  potentiated the therapeutic efficacy of paclitaxel. These data support the hypothesis that the optimal integration of systemic IFN- $\alpha$  with cytoreductive chemotherapy may improve the survival of patients with muscle-invasive bladder cancer.

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