Role of Interleukin 10 and Transforming Growth Factor β1 in the Angiogenesis and Metastasis of Human Prostate Primary Tumor Lines from Orthotopic Implants in Severe Combined Immunodeficiency Mice

Mark E. Stearns,[1] Fernando U. Garcia, Kim Fudge, Johng Rhim, and Min Wang

Medical College of Pennsylvania and Hahnemann University, Department of Pathology and Laboratory Medicine, Philadelphia, Pennsylvania 19102-1192 [M. E. S., F. U. G., K. F., M. W.], and NIH-National Cancer Institute, Frederick, Maryland 21702 [J. R.]

ABSTRACT

Transfection of primary human prostate tumor cells (*i.e.*, HPCA-10a, 10b, 10c, and 10d lines) with the *transforming growth factor* (*TGF*)-β*1* gene stimulated anchorage-independent growth and promoted tumor growth, angiogenesis, and metastasis after orthotopic implantation in severe combined immunodeficiency mice. In contrast, *interleukin (IL)-10* transfected cells or cells cotransfected with these two genes exhibited reduced growth rates and significantly reduced angiogenesis and metastasis after 8, 12, and 16 weeks. Enzyme-linked immunosandwich assays confirmed that the respective tumors expressed elevated levels of TGF-β1 and IL-10 *in vivo*. ELISAs further showed that TGF-β1 expression induced matrix metalloproteinases-2 (MMP-2) expression, whereas IL-10 down-regulated MMP-2 expression while up regulating TIMP-1 in the transfected cells. Also, tumor factor VIII levels correlated with TGF-β1 and MMP-2 expression and inversely with IL-10 and TIMP-1 levels. More importantly, mouse survival was zero after 4–6 months in mice bearing TGF-β1- and MMP-2-expressing tumors and increased significantly in mice implanted with IL-10- and TIMP-1-expressing tumors (*i.e.*, to >80% survival). Analysis of the metastatic lesions showed that they expressed TGF-β1 and MMP-2 but barely detectable levels of IL-10 or TIMP-1, suggesting that IL-10 and TIMP-1 might normally block tumor growth, angiogenesis, and metastasis.

INTRODUCTION

IL-10[2] may play an important role in controlling tumor growth and metastasis. The biological effects of IL-10 on tumor growth have ranged from modulating tumor growth (via indirect effects on the immune system) to inhibiting tumor angiogenesis and metastasis. Huang *et al.* (1) found that human melanoma A375P cells transfected with a murine IL-10 cDNA exhibited reduced growth and metastatic abilities that correlated with a significant decrease in neovascularity of the tumors. IL-10 produced by the A375P-IL-10 cells was found to down-regulate expression of vascular endothelial growth factor, IL-1β, tumor necrosis factor-α, IL-6, and matrix metalloproteinase 9 in activated macrophages that normally infiltrated the tumor tissues. The authors suggested that the production of IL-10 by tumor cells might inhibit macrophage-derived angiogenic factors to block tumor growth and metastasis indirectly (1). Alternatively, IL-10 might sensitize tumor cells to natural killer cells, which blocked metastasis as shown in a murine model of breast cancer (2). In similar studies, Richter *et al.* (3) reported that IL-10 blocked tumor growth apparently by blocking angiogenesis and macrophage penetration of the tumor tissue. Kundu *et al.* (2) also found with studies of IL-10-transfected murine mammary tumor cell lines that tumor growth was completely inhibited and metastasis was reduced by ~90% in syngeneic BALB/ccByJ mice. The effect appeared to be independent of T-cell activity but was dependent on natural killer cell function. These observations suggest that the production of IL-10 by tumor cells might inhibit macrophage-derived angiogenic factors to block tumor growth, angiogenesis, and metastasis indirectly (2). In apparent contrast to these findings, Sato *et al.* (4) found that IL-10 expression was characteristic of human metastatic melanomas, implying that IL-10 might down-regulate the antitumor activities of monocytes and macrophages, blocking production of antitumor effector molecules (5), to thereby enable metastasis (4). Additional studies are required to resolve the role of IL-10 in tumor growth and metastasis as a function of the tissue of origin, the type of cancer, or whether differences exist between tumors in rodents and humans.

The role of other cytokines in the process will also be critical. Recently, several studies have reported that EL4 tumor cells secreted TGF-β and IL-10 *in vitro* (6, 7) and *in vivo*. Studies in C57BL/6 mice indicated that EL4 tumor growth

Received 8/10/98; revised 11/19/98; accepted 12/8/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Department of Pathology and Laboratory Medicine, MCP-HUH University, MS 435, Broad and Vine Street, Philadelphia, PA 19102-1192.

[2] The abbreviations used are: IL-10, interleukin 10; TGF-β1, transforming growth factor β1; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinase-2; HPCA, human prostate cancer adenocarcinoma; SCID, severe combined immunodeficiency; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

avoided macrophage-immune surveillance by secreting TGF-β to regulate macrophage secretion of antitumor components (8). The amounts of tumor-secreted TGF-β increased with tumor growth and appeared to induce IL-10 production by the macrophages, which in turn suppressed the antitumor activities of the macrophages (8). The studies failed to assess whether IL-10 also influenced TGF-β production by the tumor cells or whether IL-10 expression modulated tumor growth and metastasis.

Differences among organ sites of the tumor origin might be a factor influencing cytokine activity. For example, Greene *et al.* (9) have elegantly shown that the elevated expression of epidermal growth factor receptor, basic fibroblast growth factor, IL-8, MMP-2, MMP-9, and multidrug resistance-1 correlated with the increased metastatic potential of human prostate PC-3M cancer cells implanted into the prostate of nude mice (9). Their data demonstrated that the organ site of tumor cell implantation strongly modulated gene expression and metastasis of the tumor cells.

Utilizing a similar approach, in this report we have examined whether permanent IL-10 and *TGF-β1* gene transfection of four different HPCA-10a, 10b, 10c, and 10d-HPV-18 lines [*i.e.*, derived from microdissections of Gleason score 10 glands and immortalized with HPV-18 infection (10)] influenced growth *in vitro* plus tumor cell growth, metastasis, and mouse survival rates after orthotopic implantation of the cells in SCID mice. In addition, the influence of these genes on the expression of TIMP-1, MMP-2, and factor VIII levels (*i.e.*, the degree of tumor angiogenesis) has been measured in relation to tumor metastasis and mouse survival. The results showed that: (*a*) IL-10 up-regulated TIMP-1 and down-regulated MMP-2 to block angiogenesis, growth, and metastasis of tumors; and (*b*) TGF-β1 up-regulated MMP-2 to stimulate tumor growth, angiogenesis, and metastasis. Increased mouse survival was correlated with IL-10 activity and inversely correlated with TGF-β1 expression, indicating that IL-10 might be of therapeutic value in treating patients with cancer who have a high probability of metastasis.

MATERIALS AND METHODS

Cell Cultures. HPCA-10a–d cell lines were generated by HPV-18 immortalization of primary epithelial cultures derived from human prostate Gleason score 10 cancer glands microdissected from sagittal sections of human prostate. The epithelial nature of the cultures was confirmed by cytokeratin 8/18 antibody labeling (10). Cells were maintained at low passage (<10 passages) and cultured as described previously by our laboratory using a keratinocyte medium supplemented with pituitary extract and insulin-transferrin-selenium (Clonetics, San Diego, CA; Ref. 10).

***IL-10* and *TGF-β1* Gene Transfections.** Total cytoplasmic RNA was isolated from 10^7 human prostate PC-3 cells treated with 10 μg/ml ConA. RNA was reverse transcribed into cDNA using oligo dT primer as described by others (11), then amplified 40 cycles using two oligonucleotide primers derived from a published IL-10 sequence (12), including 5′-AATGGAGCTCGCTCTAGAATGCACAGCTCAGCACTG-3′ and 5′-AATGGATATCGCGAATTCTTTCTCAAGGGGCTGGGT-3′, incorporating an *Sst*I and an *Eco*RV site, respectively (underlined), and from a TGF-β1 sequence (13), including: TGF-β8, 5′-GAGGTCACCCGCGTGCTAATG-3′; and TGF-β9, 5′-GGCCAGGACCTTGCTGTACTG-3′, incorporating an *Sst*I and an *Eco*RV site, respectively (underlined). PCR was carried out for 20 s at 94°C, 20 s at 60°C, and 20 s at 72°C for 40 cycles, followed by a 10-min extension at 72°C. The PCR product was then cloned into a Bluescript vector (Stratagene, La Jolla, CA), and several independent clones were sequenced to confirm that the *IL-10* and the *TGF-β1* gene had been cloned. One of each of the cDNAs was subcloned into a expression vector pCEP4 (Invitrogen, San Diego, CA). HPCA-10a, 10b, 10c, and 10d cells were transfected with the pCEP4 vector alone (HPCA-10a, 10b, 10c, and 10d-HPV-18 Mock) or with either the pCEP4-TGF-β1, the pCEP4-IL-10 vector independently, or with both simultaneously (*i.e.*, HPCA-10a-IL-10 and TGF-β1 Mock) using the Lipofectamine method (Oncogene Science, Bedford, MA). After transfection, hygromycin (400 μg/ml)-resistant cells were selected over a 2-month period, the cells were pooled, and single cell clones were generated by limited dilution in 96-well dishes. At least two different clones were generated and characterized for each vector. The clones were amplified in five passages, and cells were frozen in liquid nitrogen for future studies. Cells were revived and used at a final passage <5 in all experiments. ELISAs (see methods below) with IL-10 and TGF-β1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) confirmed that each of the clones still produced IL-10 and TGF-β1 (>10 pg/ml).

Anchorage-dependent Cell Proliferation Assay. The biological activity of IL-10 and TGF-β1 was determined using the colorimetric MTT (Sigma Chemical Co., St. Louis, MO) dye-reduction assay (14, 15). In brief, the HPCA-10-HPV-18 cells (1×10^4 cells/well in 200 μl of medium containing 2% FCS) were exposed to 30 ng/ml IL-10 or 10 ng/ml TGF-β1 for 72 h in a 96-microtiter well. Ten μl of 5 mg/ml MTT was added and incubated for 4 h, the cells were then detergent-lysed in absolute ethanol and the plates were read for absorbance at 570 nm. Cell viability in the experiments was estimated by trypan blue exclusion assays as >95%.

Immunohistochemistry. Tissues were processed for antibody labeling according to methods described previously (16) using primary IL-10 (Schering-Plough, Kennilworth, NJ) and TGF-β1 (Santa Cruz Biotechnology) antibodies at a dilution of 1:500 and secondary peroxidase-anti-peroxidase antibodies (Sigma) at a dilution of 1:400. At least three sections were labeled from each tissue, and one section was labeled with secondary antibody only as a control. One additional section was stained with H&E for identification of the cells.

ELISAs. ELISAs were carried out as described previously (16) using well-characterized IL-10 (courtesy of Dr. N. Sawant, Schering-Plough, Kennilworth, NJ); TGF-β1 (Santa Cruz Biotechnology), and TIMP-1 and MMP-2 monoclonal antibodies (16). Standard curves were developed previously for each of these antibodies (16) to enable measurements of the antigen levels in crude protein extracts. Factor VIII antibodies were from Sigma. ELISAs (*A*, 490 nm) were performed using aliquots of crude protein extracts (16) to coat the EIA-plates (Dynatech, Chantilly, VA). After blocking with 5% bovine serum albumin, excess primary antibody (100 μl/well at 1 μg/ml) was added and incubated at 37°C for 2 h. A Vector Elite

Table 1 Colony-forming ability in soft agar of HPCA-10a clones

Clone	Vehicle	IL-10		TGF-β1	
		10 ng/ml	30 ng/ml	1 ng/ml	10 ng/ml
HPCA-10a	244 ± 20	255 ± 17	380 ± 33 $P < 0.001$	291 ± 18 $P < 0.05$	256 ± 20
HPCA-10a-TGF-β1	339 ± 26	384 ± 29	244 ± 26	389 ± 20	444 ± 30 $P < 0.001$
HPCA-10a-IL-10	222 ± 16	284 ± 19 $P < 0.05$	214 ± 11	282 ± 23 $P < 0.05$	334 ± 20 $P < 0.001$
HPCA-10a-TGF-β1-IL-10[a]	230 ± 22	252 ± 30	280 ± 19	301 ± 21 $P < 0.05$	350 ± 40 $P < 0.001$

[a] HPCA-10a-TGF-β1 cells were cotransfected with the *IL-10* gene. All experiments were carried out in medium containing 2% serum. Each data point represents the total number of colonies counted per 12-mm dish after plating with 1×10^4 cells for 72 h. Each data point represents the mean ± 1 SD from three dishes/experiment and three experiments.

kit (Vector, Burlingame, CA) was used to detect the bound antibody with goat or rabbit anti-mouse secondary antibody and *o*-phenylenediamine as peroxidase substrate. Amounts of antigen in crude tumor extracts were determined from the standard curves.

The absorbance readings (*A*, 490 nm) were obtained for three protein concentrations of the crude protein extract from cells or tumor tissue (0.25, 0.50, and 1.0 mg of protein), and the actual amounts of IL-10, TGF-β1, TIMP-1, and MMP-2 antigen produced by each cell line were determined from a standard curve comparing absorbance readings to known amounts of purified IL-10 (Schering-Plough) or TGF-β1 (Sigma), TIMP-1 and MMP-2 as described previously (16). The readings were then normalized for 1.0 mg of protein. Protein levels were measured according to the method of Bradford *et al.* (17).

Mice. Pathogen-free male SCID/SCID mice were purchased from Taconic Laboratories (Albany, NY) and housed in barrier cages in a barrier facility. Mice were fed a standard laboratory chow *ad libitum* and were used at 8–10 weeks of age. Orthotopic injection was carried out according to methods described by Greene *et al.* (9). After experimental treatment, the mice were sutured with staples and watched for 3 days to ensure recovery.

Statistical Analysis. Differences in the number of colonies of the lung, liver, and peritoneum were analyzed using the Mann-Whitney *U* test. Statistical significance was determined by a two-way analysis of variance or by the two-tailed Student's *t* test (Minitab, Statistical Software 8.2), and results are expressed as the mean ± 1 SD of replicate determinations.

RESULTS

Anchorage-dependent Growth Assays

The HPCA-10 cells (seeded at 1×10^4 cells/well in 200 μl of medium containing 2% FCS) were exposed to 30 ng/ml IL-10 or 10 ng/ml TGF-β1 or both for 72 h. MTT assays (*A*, 570 nm) revealed that in comparison with the untreated cells, the cell number increased ~180, ~250, and ~300% in response to either IL-10, TGF-β1, and IL-10 plus TGF-β1, respectively. Moreover, trypan blue exclusion assays showed that cell viability was >95% in the absence or presence of increased amounts of either cytokine.

Colony-forming Assays in Soft Agar

Colony-forming assays in soft agar further revealed that the different HPCA-10a–d lines grew under anchorage-independent conditions in the presence of 2% FCS (and vehicle) for 72 h (Table 1). The degree of colony formation by HPCA-10a cells were supported IL-10 and TGF-β1 (*i.e.*, by 30 ng/ml IL-10 and by 1 ng/ml TGF-β1) in the presence of 2% FCS (Table 1). HPCA-10a clones independently transfected with *TGF-β1* and *IL-10* genes (or cotransfected with both genes) tended to exhibit a colony-forming ability comparable with the nontransfected HPCA-10a cells. Exogenously supplied IL-10 (1 and 10 ng/ml) had little noticeable effect, but 10 ng/ml TGF-β1 tended to stimulate colony formation by a significant degree (Table 1). Similar results were observed with HPCA-10 c–d clones (data not shown).

In Vivo Growth Studies

Tumor Growth in Orthotopic Implants. The growth of: (*a*) HPCA-10a, 10b, 10c, and 10d lines; (*b*) IL-10-transfected HPCA-10a, 10b lines; (*c*) TGF-β1-transfected HPCA-10a, 10b lines; and (*d*) IL-10 and TGF-β1 cotransfected HPCA-10a, 10b lines was examined after orthotopic injection in the prostate gland of SCID mice ($n = 10$ mice/cell line) according to methods described by Greene *et al.* (9). In Fig. 1, studies with nontransfected (*panel 1*) or mock-transfected HPCA-10a clones (*panels 2* and *6*) revealed that the average size of the tumors increased from <1 mm^3 at 8 weeks (*column a, panels 1, 2,* and *6*) to ~1.5 mm^3 at 12 weeks (*column b, panels 1, 2,* and *6*) and ~2.7 mm^3 at 16 weeks (*column c, panels 1, 2,* and *6*). Similarly, the tumor volume of the HPCA-10a-TGF-β1 (*panel 4*)-transfected cells increased from ~0.5, 1.8, and 3 mm^3 by 8, 12, and 16 weeks, respectively. Although the data were not statistically different from nontransfected cells ($P > 0.1$), TGF-β1 appeared to promote growth. In contrast, the tumor volume of HPCA-10a-IL-10 (*panel 3*) and HPCA-IL-10 and TGF-β1 (*panel 5*)-transfected cells was significantly reduced and ranged from 0.2 to 0.5 mm^3 after 8, 12, and 16 weeks (*columns a, b,* and *c; panels 3* and *5*), indicating IL-10 expression retarded tumor growth *in vivo* ($P < 0.001$). Other experiments showed that after mixing of equal numbers of cells from the HPCA-10a-IL-10 and HPCA-10a-TGF-β1-transfected clones (and injected at a final concentration of 1×10^7 cells in 0.05 ml), the tumor volume

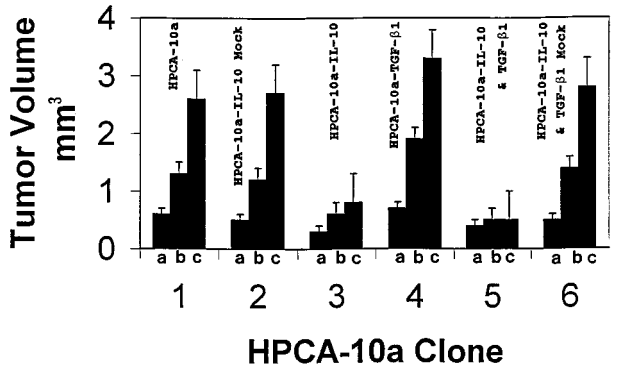

Fig. 1 Correlation of the tumor volume (*Y* axis, mm^3) after 8 (*columns a*), 12 (*columns b*), and 16 (*columns c*) weeks. *Panel 1*, HPCA-10a; *panel 2*, HPCA-10a-IL-10 Mock cells; and HPCA-10a cells transfected with: *panel 3*, IL-10; *panel 4*, TGF-β1; *panel 5*, IL-10 and TGF-β1; and *panel 6*, HPCA-10a-IL-10 and TGF-β1 Mock cells. Values averaged from $n = 10$ mice/data point and represent the means; *bars*, 1 SE.

was also reduced to <0.5, 1, and 1.5 mm^3 by 8, 12, and 16 weeks, respectively ($P < 0.05$; data not shown). Presumably, IL-10 produced by the HPCA-10a-IL-10 cells retarded growth of the HPCA-10a-TGF-β1 cells. Similar results were observed in comparable studies with the HPCA-10b, 10c, and 10d clones (data not shown).

Metastasis Studies

The frequency of HPCA-10a tumor cell metastasis from orthotopic implants was examined in SCID mice ($n = 20$ mice/experiment, Table 2). The data showed that HPCA-10a parent; HPCA-10a-IL-10 Mock; HPCA-10a-TGF-β1 Mock, HPCA-10a-IL-10 and TGF-β1; and HPCA-10a-IL-10 and TGF-β1 Mock cells metastasized at a very low frequency after 16 weeks. The majority of the metastases were observed in the peritoneum (in 5 of 20 to 6 of 20 mice) as compared with the lung (1 of 20 mice), liver (2 of 20 to 3 of 20 mice), brain (0 of 20 mice), or bone marrow (1 of 20 mice). The number of tumor nodules/organ site was also small (*i.e.*, <5/organ site). Likewise, the HPCA-10a-IL-10-transfected clones produced metastases to different organ sites in a small number of mice (ranging from 1 of 20 to 3 of 20 mice) with <2 nodules/organ site. In contrast, the HPCA-10a-TGF-β1 clones exhibited a significantly elevated frequency of metastasis to different organ sites including the lung (5 of 20 mice; $P < 0.001$), liver (7/20 mice; $P < 0.001$), and peritoneum (14 of 20 mice, $P < 0.001$) compared with controls. The numbers of tumor foci per organ site of metastasis varied depending on the organ size but were significantly elevated in the TGF-β1-transfected clones (ranging from 15 to 30 nodules/organ site in the lung, liver, and peritoneum; $P < 0.001$). Interestingly, the degree of metastasis to the brain and bone marrow (*i.e.*, 1 of 20 to 2 of 20 mice) was comparable with controls. Similar results were observed with the three other HPCA-10b, 10c, and 10d clones transfected with HPCA-10a-IL-10 and TGF-β1.

Immunolabeling Studies

Immunoperoxidase labeling was initially used to assess the relative levels of IL-10, TGF-β1, TIMP-1, and MMP-2 expressed by the epithelial cells in tumors from HPCA-10a, HPCA-10a-IL-10, HCA-10a-TGF-β1, and the HPCA-10a-IL-10 and TGF-β1 cotransfected cultures. Comparisons of IL-10 levels in nontransfected (Fig. 2*A*) or TGF-β1-transfected cells (Fig. 2*B*) with that observed in the IL-10 transfected cells (Fig. 2, *C* and *D*) clearly indicated that IL-10 expression was elevated in the latter tumors. Likewise, TGF-β1 levels were moderate in nontransfected HPCA-10a (Fig. 3*A*) and in HPCA-10a-IL-10 (Fig. 3*C*) and HPCA-10a-IL-10 and TGF-β1 cotransfected cells (Fig. 3*D*) but elevated in the HPCA-10a-TGF-β1-transfected tumor cells (Fig. 2*B*). The TIMP-1 levels correlated with IL-10 expression in the HPCA-10a-IL-10-transfected cells (compare Fig. 4, *A* and 4*B*, with *C* and *D*). In contrast, MMP-2 was highly expressed in nontransfected HPCA-10a and HPCA-10a-TGF-β1-transfected cells (Fig. 5, *A* and *B*), only faintly expressed in the HPCA-10a-IL-10-transfected cells (Fig. 5*C*), and moderately expressed in the HPCA-10a-IL-10 and TGF-β1 cotransfected cells (Fig. 5*D*). Similar results were observed with the different HPCA-10b, 10c, and 10d clones described in Tables 1 and 2 (data not shown).

ELISAs

IL-10 and TGF-β1. ELISA standard curves were developed previously for measurements of IL-10 in crude cell and tissue extracts (16). Similarly, a standard curve for ELISA measurements of nanogram amounts of TGF-β1 or factor VIII in crude cell extracts of tumor cells was established. In the presence of excess antibody (200 ng/ml), the ELISA reading (*A*, 490 nm) increased in a linear manner as a function of the amount of pure antigen (*i.e.*, 1–160 ng). Likewise, preabsorption assays showed that with TGF-β1 and factor VIII antibodies (100 ng/ml) preabsorbed with increased amounts of antigen (*i.e.*, 1–110 ng), the absorbance decreased in a linear manner from 1.0 to 0.

ELISA measurements confirmed that IL-10-transfected and IL-10 plus TGF-β1 cotransfected HPCA-10a tumor cells expressed increased levels of IL-10 after implantation orthotopically in SCID mice for 8, 12, or 16 weeks (*i.e.*, 40–90 ng/mg protein, Fig. 6, *panels 1–3, columns c* and *e*, respectively). Controls showed that relatively low levels of IL-10 were expressed by the HPCA-10a parent cells (Fig. 6, *panels 1–3, column a*), HPCA-10 IL-10 Mock (Fig. 6, *panels 1–3, column b*), and HPCA-10a-IL-10 and TGF-β1 Mock (Fig. 6, *panels 1–3, column f*). Likewise, TGF-β1 and IL-10 plus TGF-β1 cotransfected tumor cells expressed significantly elevated levels of TGF-β1 (*i.e.*, 35–90 ng/mg protein, Fig. 7, *panels 1–3, columns d* and *e*) after orthotopic implantation for 8, 12, and 16 weeks. Controls showed that relatively low levels of TGF-β1 were expressed by the HPCA-10a parent cells (Fig. 7, *panels 1–3, column a*), HPCA-10a-IL-10 Mock (Fig. 7, *panels 1–3, column b*), HPCA-10a-IL-10 (Fig. 7, *panels 1–3, column c*), and HPCA-10a-IL-10 and TGF-β1 Mock (Fig. 7, *panels 1–3, column f*). Measurements of mouse prostate tissue showed that neither cytokine was expressed to a significant degree by nontumor tissue (*i.e.*, the levels were <2 ng/mg protein).

Additional ELISA studies showed that the average amount of IL-10 varied from <0.1 ng/mg to ~10 ng/mg in crude protein extracts from the tumor tissue derived from the three different HPCA-10a, 10b, 10c, and 10d tumor clones at 16 weeks. The

Table 2 Frequency of metastasis—16 weeks

Clone	Lung	Liver	Peritoneum	Brain	Bone marrow
HPCA-10a parent	1/20[a]	2/20	6/20	0/20	1/20
	2 ± 1[b]	4 ± 1	4 ± 1	ND[c]	1 ± 0
HPCA-10a IL-10-Mock	1/20	3/20	6/20	0/20	1/20
	3 ± 1	5 ± 1	5 ± 1	ND	1 ± 0
HPCA-10a	2/20	2/20	3/20	1/20	1/20
IL-10	3 ± 1	3 ± 1	4 ± 2	ND	2 ± 1
HPCA-10a	1/20	2/20	5/20	0/20	1/20
TGF-β Mock	5 ± 1	4 ± 1	3 ± 1	ND	1 ± 0
HPCA-10a	5/20	7/20	14/20	1/20	2/20
TGF-β1	13 ± 3	23 ± 6	14 ± 3	ND	2 ± 1
HPCA-10a	1/20	5/20	5/20	0/20	1/20
IL-10 and TGF-β1	3 ± 1	5 ± 1	2 ± 1	ND	1 ± 0
HPCA-10a	1/20	5/20	5/20	0/20	1/20
IL-10 and TGF-β1 Mock	4 ± 1	4 ± 1	3 ± 1	ND	1 ± 0

[a] The number of mice with tumor metastases of 20 mice examined.
[b] The average number of tumor foci detected per organ site. Values represent the average ± 1 SD from six measurements/experiment.
[c] ND, not done.

TGF-β1 levels ranged from ~10 to 30 ng/mg protein in these tumors. Likewise, tumor tissue from four different IL-10-transfected HPCA-10a, 10b, 10c, and 10d clones ranged from 70 to 120 ng/mg in crude protein in 16-week tumors. The TGF-β1-transfected HPCA-10a, 10b, 10c, and 10d clones each expressed TGF-β1 at levels ranging from 60–90 ng/mg protein after 16 weeks, confirming that the data presented in Figs. 6 and 7 were not artifactual.

TIMP-1 and MMP-2. The relative levels of TIMP-1 and MMP-2 expressed by different HPCA-10a clones were measured after 8, 12, and 16 weeks (Figs. 8 and 9, *columns a*, *b*, and *c*, respectively). Fig. 8 showed that the TIMP-1 levels ranged from ~10 to 30 ng/mg protein in tumors formed from HPCA-10a (*panel 1*); HPCA-10a-IL-10 Mock (*panel 2*); HPCA-10a-TGF-β1 (*panel 4*); and HPCA-10a-IL-10 and TGF-β1 Mock (*panel 6*) lines after 8, 12, and 16 weeks (*columns a*, *b*, and *c*, respectively). In contrast, the TIMP-1 levels were significantly elevated in tumors from HPCA-10a-IL-10 (*panel 3*); and HPCA-10a-IL-10 and TGF-β1 (*panel 5*)-transfected tumors after 8, 12, and 16 weeks (*columns a*, *b*, and *c*, respectively). Fig. 9 showed that the MMP-2 levels ranged from ~30 to 70 ng/mg protein in tumors formed from HPCA-10a (*panel 1*); HPCA-10a-IL-10 Mock (*panel 2*); and HPCA-10a-IL-10 and TGF-β1 Mock (*panel 6*)-transfected lines after 8, 12, and 16 weeks (*columns a*, *b*, and *c*, respectively). The MMP-2 levels were significantly elevated in tumors from HPCA-10a-TGF-β1-transfected lines (*panel 4*). In contrast, the tumors from HPCA-10a-IL-10 (*panel 3*) and HPCA-10a-IL-10 and TGF-β1 (*panel 5*) transfected tumors failed to express significant amounts of MMP-2 after 8, 12, and 16 weeks (*columns a*, *b*, and *c*, respectively).

ELISAs of factor VIII levels showed that none of the tumors exhibited detectable antigen by 8 weeks (Fig. 10, *panels 1–6*, *column a*). However, in nontransfected HPCA-10a cells (*panel 1*) HPCA-10a-IL-10 Mock (*panel 2*), HPCA-10a-TGF-β1 (*panel 4*), and HPCA-10a-IL-10 and TGF-β1 Mock (*panel 6*)-transfected tumors, the factor VIII levels ranged from ~20 to 30 ng/mg; and from 30 to 48 ng/mg protein in the 12- and 16-week tumors, respectively (Fig. 10, *columns b* and *c*). In comparison, the HPCA-10a-IL-10-transfected (*panel 3*) and HPCA-10a-IL-10 and TGF-β1 cotransfected (*panel 5*) cell tumors exhibited factor VIII levels of ~1 ng/mg at 8, 12, and 16 weeks (Fig. 10, *columns a*, *b*, and *c*), indicating that little or no tumor-associated angiogenesis had occurred.

Finally, the levels of each antigen were analyzed in metastatic nodules from the lung, liver, peritoneum, and brain. Fig. 11 showed that in metastases to the peritoneum, the levels of TGF-β1 ranged from 20 to 25 ng/mg protein in the HPCA-10a-IL-10 and TGF-β1 Mock (*panel 1*); HPCA-10a-IL-10 (*panel 3*); and HPCA-10a-IL-10 and TGF-β1 (*panel 4*)-transfected clones (Fig. 11, *column a*). TGF-β1 levels were ~60 ng/mg protein in metastases to the peritoneum by the HPCA-10a-TGF-β1-transfected clones (*panel 2*, *column a*). However, IL-10 levels were uniformly low (<2 ng/mg protein) in all peritoneum metastases from the different clones (Fig. 11, *columns b*). The TIMP-1 levels were also uniformly low (~10 ng/mg protein, Fig. 11, *columns c*), but the MMP-2 levels were uniformly high (~40 to 60 ng/mg protein, Fig. 11, *columns d*) in peritoneum metastases from all four HPCA-10a clones.

Overall, TGF-β1 levels in lung, liver, and peritoneum metastases from TGF-β1-transfected and IL-10 and TGF-β1 cotransfected cell primary tumors ranged from 23 to 30 ng/mg crude protein extract from the tumor nodules. Little or no TGF-β1 (<2 ng/mg) was normally detected in the host tissues from the same organ sites. IL-10 was also not detected (<1 ng/mg) in any of the tumor metastasis or the host tissues. Likewise, IL-10 was not detected (<1 ng/mg) in any of the metastases from any of the IL-10-transfected or the HPCA-10a-IL-10 and TGF-β1 cotransfected lines.

Attempts to measure the levels of TIMP-2 and MMP-9 by ELISA yielded consistently low levels (<5 to 10 ng/mg protein) in either the primary tumors or the metastases from any of the HPCA-10 clones, and significant changes in the levels of these two proteins were not observed. Finally, positive control measurements with β-actin antibodies showed that the levels ranged from 300 to 350 ng/mg protein in the tumor extracts, and there was no significant change in the levels of these proteins as a function of gene transfection or metastatic behavior of the

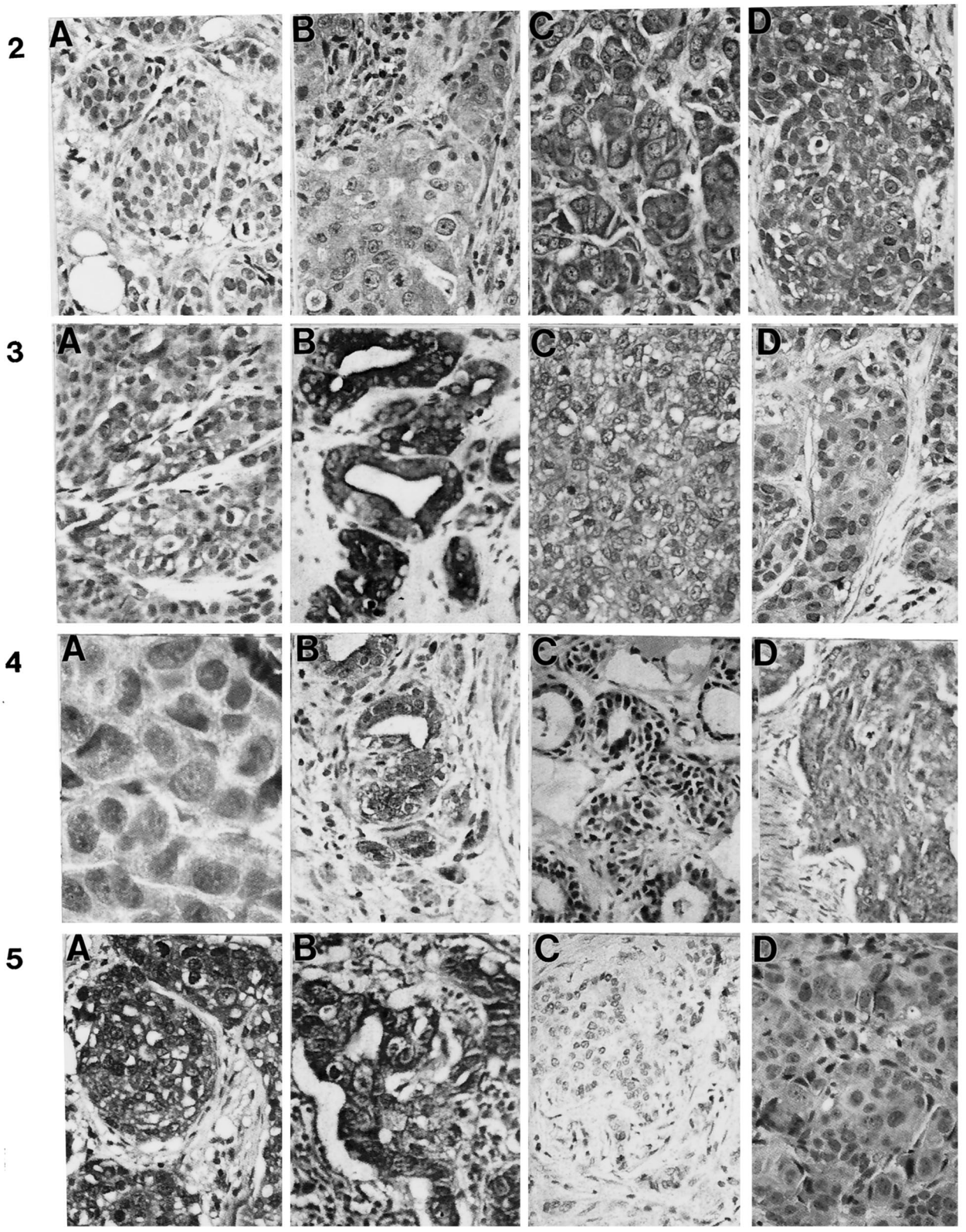

Fig. 2–5 Immunoperoxidase-labeled sections of tumors from: *A*, HPCA-10a; *B*, HPCA-10a-TGF-β1; *C*, HPCA-10a-IL-10; and *D*, HPCA-10a-IL-10 + TGF-β1-transfected cells. Antibodies included: IL-10 (Fig. *2*); TGF-β1 (Fig. *3*); TIMP-1 (Fig. *4*); and MMP-2 (Fig. *5*).

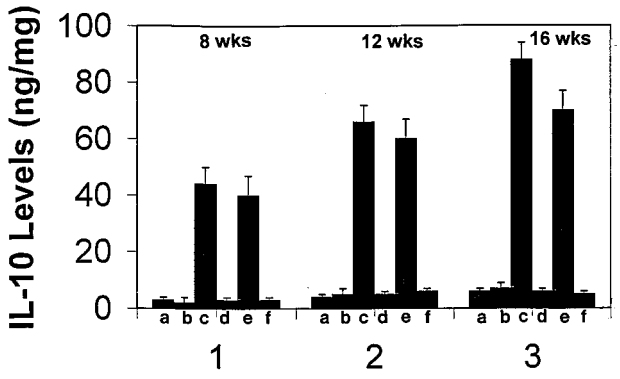

Fig. 6 ELISA measurements of IL-10 levels in orthotopic tumors from 8 (*panel 1*), 12 (*panel 2*), and 16 (*panel 3*) week tumors. The HPCA-10a lines included: *a*, HPCA-10a; *b*, HPCA-10a-IL-10 Mock; *c*, HPCA-10a-IL-10; *d*, HPCA-10a-TGF-β1; *e*, HPCA-10a-IL-10 and TGF-β1; and *f*, HPCA-10a-IL-10 and TGF-β1 Mock.

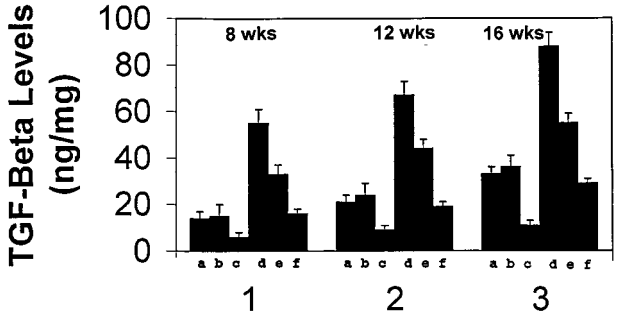

Fig. 7 ELISA measurements of TGF-β1 levels in orthotopic tumors from 8 (*panel 1*), 12 (*panel 2*), and 16 (*panel 3*) week tumors. The HPCA-10a lines included: *a*, HPCA-10a; *b*, HPCA-10a-IL-10 Mock; *c*, HPCA-10a-IL-10; *d*, HPCA-10a-TGF-β1; *e*, HPCA-10a-IL-10 and TGF-β1; *f*, HPCA-10a-IL-10 and TGF-β1 Mock.

tumors. Negative controls with secondary IgG antibodies produced basal levels of antigen binding (*i.e.*, <0.5 ng/mg protein) in all the assays, and the values were corrected for this background level of nonspecific antibody binding.

Finally, duplicate studies with the three different HPCA-10a, 10b, 10c, and 10d tumor clones transfected with IL-10 or TGF-β1 yielded results similar to that described for the HPCA-10a clone (data not shown).

Mouse Survival Studies

Mouse survival studies showed that mice implanted with HPCA-10a-TGF-β1-expressing cells all died by 2–6 months (Fig. 12). The mice implanted with either HPCA-10a-IL-10 Mock or HPCA-10a-TGF-β1 Mock cells all died by 3–6 months. In contrast, the mice implanted with HPCA-10a-IL-10 and HPCA-10a-IL-10 and TGF-β1-transfected HPCA-10a cells had a >80% survival by 6 months (Fig. 12). Again, duplicate studies with the three different HPCA-10a, 10b, 10c, and 10d tumor clones transfected with IL-10, TGF-β1, or both yielded results similar to that described for the HPCA-10a clone (data not shown).

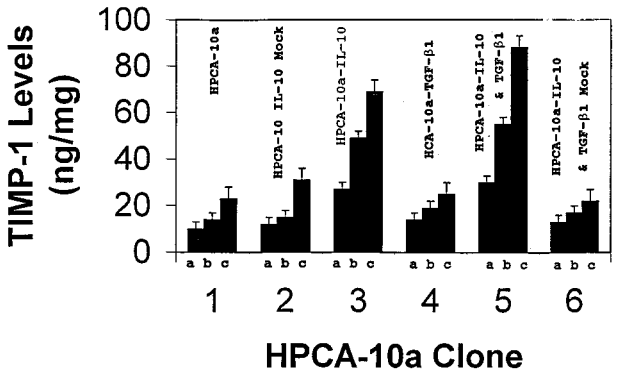

Fig. 8 ELISA measurements of TIMP-1 levels in orthotopic tumors from HPCA-10a (*panel 1*), HPCA-10a-IL-10 Mock (*panel 2*), HPCA-10a-IL-10 (*panel 3*), HPCA-10a-TGF-β1 (*panel 4*), HPCA-10a-IL-10 & TGF-β1 (*panel 5*), and HPCA-10a-IL-10 & TGF-β1 Mock (*panel 6*) lines. Tumors were extracted after 8 (*columns a*), 12 (*columns b*), and 16 (*columns c*) weeks.

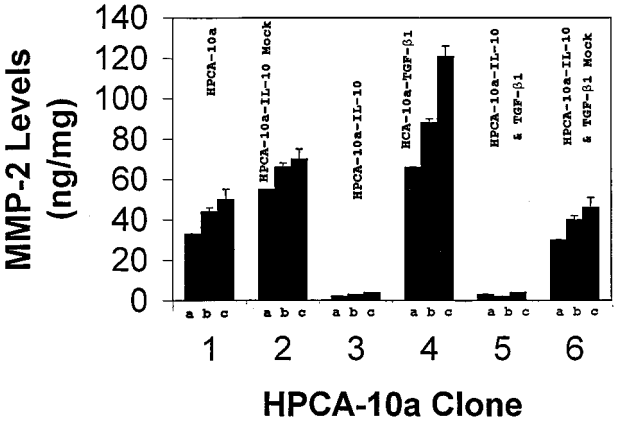

Fig. 9 ELISA measurements of MMP-2 levels in orthotopic tumors from HPCA-10a (*panel 1*), HPCA-10a-IL-10 Mock (*panel 2*), HPCA-10a-IL-10 (*panel 3*), HPCA-10a-TGF-β1 (*panel 4*), HPCA-10a-IL-10 & TGF-β1 (*panel 5*), and HPCA-10a-IL-10 and TGF-β1 Mock (*panel 6*) lines. Tumors were extracted after 8 (*columns a*), 12 (*columns b*), and 16 (*columns c*) weeks.

DISCUSSION

The goal of this study was to assess the influence of IL-10 and TGF-β1 on TIMP-1 and MMP-2 expression plus tumor survival and metastasis by primary human prostate tumor cells. The initial studies demonstrated that anchorage-dependent and -independent growth of the different HPCA-10 clones was supported by either IL-10, TGF-β1, or IL-10 plus TGF-β1. Likewise, clones transfected with *TGF-β1* and *IL-10* genes (or cotransfected with both genes) grew in colony-forming assays. The clones transfected with TGF-β1 tended to exhibit an enhanced colony-forming ability compared with the nontransfected HPCA-10a cells. Immunolabeling and ELISAs confirmed that the transfected clones expressed TGF-β1 and IL-10 *in vitro* and *in vivo* in orthotopic tumors.

Immunolabeling and ELISAs further showed that the IL-10-transfected clones up-regulated TIMP-1 and down-regulated

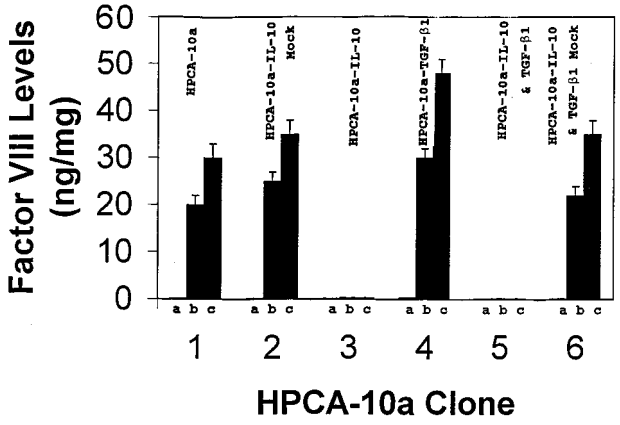

Fig. 10 ELISA measurements of factor VIII levels in orthotopic tumors from HPCA-10a (*panel 1*), HPCA-10a-IL-10 Mock (*panel 2*), HPCA-10a-IL-10 (*panel 3*), HPCA-10a-TGF-β1 (*panel 4*), HPCA-10a-IL-10 & TGF-β1 (*panel 5*), and HPCA-10a-IL-10 and TGF-β1 Mock (*panel 6*) lines. Tumors were extracted after 8 (*column a*), 12 (*columns b*), and 16 (*columns c*) weeks.

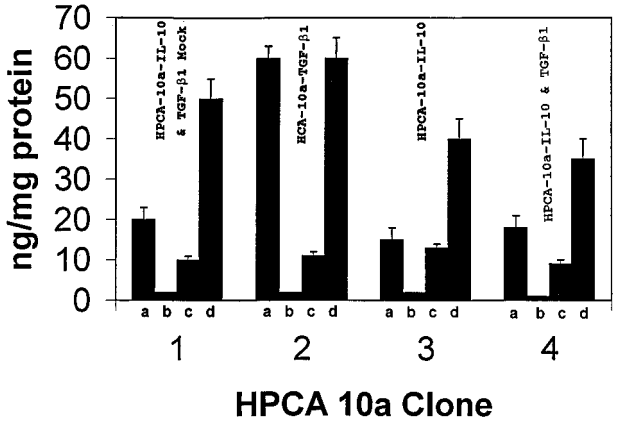

Fig. 11 ELISAs showing the levels of TGF-β1 (*a*), IL-10 (*b*), TIMP-1 (*c*), and MMP-2 (*d*) detected in peritoneum metastases by HPCA-10a-IL-10 and TGF-β1 Mock (*panel 1*), HPCA-10a-TGF-β1 (*panel 2*), HPCA-10a-IL-10 (*panel 3*), and HPCA-10a-IL-10 and TGF-β1 (*panel 4*) transfected cells.

MMP-2 expression. In contrast, TGF-β1 transfection had little effect on TIMP-1 but did up-regulate MMP-2. In the cotransfected cells, the MMP-2 levels were reduced significantly (compared with that observed in nontransfected and TGF-β1-transfected tumor), presumably as a result of IL-10 effects. We have attempted to compare the relative levels of IL-10, TGF-β1, TIMP-1, and MMP-2 expression with tumor growth and metastasis. The data showed that cells overexpressing IL-10 and TIMP-1 (with reduced MMP-2 levels) failed to grow large tumors or metastasize, whereas the converse was observed for tumors overexpressing TGF-β1 and MMP-2. In accordance, the levels of factor VIII were very low in the IL-10-expressing tumors and elevated in the TGF-β1-expressing tumors by 8–12 weeks, indicating that IL-10, TGF-β1, TIMP-1, and MMP-2 expression might somehow indirectly influence angiogenesis.

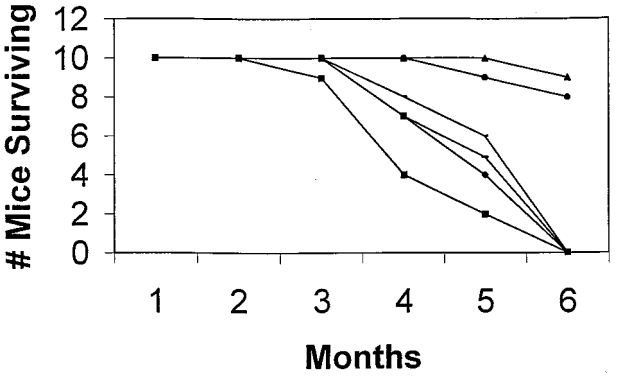

Fig. 12 SCID mice (n = 20/clone tested) survival curves after orthotopic injection of the different clones including: ◆, HPCA-10a Mock-IL-10 cells; —, HPCA-10a; and HPCA-10a cells transfected with IL-10 (▲), TGF-β1 (■), IL-10 plus TGF-β1 (●), and HPCA-10a Mock-TGF-β1 (—) cells.

Studies of the metastatic lesions revealed that only a few tiny lesions developed from the HPCA-10-IL-10 clones or HPCA-10-IL-10 and TGF-β1 clones, suggesting that IL-10 blockage of angiogenesis also blocked metastasis.

ELISAs indicated that the metastatic nodules uniformly expressed little or no IL-10 but did express relatively high levels of TGF-β1 (*i.e.*, in lung, liver, and peritoneum metastases from HPCA-10a-TGF-β1 clones). ELISAs further showed that the metastases expressed little or no TIMP-1 but overexpressed MMP-2, indicating that metastasis was associated with the expression of these genes. Mouse survival studies further showed that mice implanted with HPCA-10a-IL-10 and HPCA-10a-IL-10 and TGF-β1-transfected HPCA-10a cells had a >80% survival rate by 6 months, whereas mice injected orthotopically with HPCA-10a-TGF-β1 clones died by 2–6 months. We conclude that IL-10 induction of TIMP-1 and inhibition of MMP-2 expression are inversely associated with metastatic ability and increased mouse survival. Conversely, TGF-β1 induction of MMP-2 directly correlates with metastatic frequency and reduced mouse survival.

The results from the studies presented here were strongly supported by earlier *in vitro* studies from our laboratory, which showed that IL-10, IL-4, and to a lesser extent IL-6 stimulated the expression of TIMP-1 and decreased the levels of MMP-2 expression in primary prostate epithelial cell cultures (10). Earlier studies by our laboratory also showed that IL-10 transfection also blocked tumor growth and metastasis by human prostate PC-3 ML tumor cells after orthotopic implantation in the prostate gland of SCID mice (18). Measurements of tumor volume after 5, 8, and 12 weeks indicated that tumor volume and metastasis were negatively correlated with the amount of IL-10 production. Likewise, mouse survival rates increased to >80% in mice implanted with the IL-10-transfected PC-3 ML clones (18). We suggest, therefore, that IL-10 expression and therapeutic approaches designed to enhance TIMP expression while reducing MMP-2 levels might be of therapeutic benefit in treating advanced prostate cancer.

A clear association between increased MMP-2 production and malignant aggressiveness has been observed in a number of

different cancer types including prostate cancer (19). Stetler-Stevenson and others (20–25) have shown clearly that an increased expression of MMP-2 is normally observed in human malignant breast cancer (21, 22), colon adenocarcinomas (23, 24), and gastric cancer (24, 25). The investigators observed that the invasive ductal and lobular tumor cells expressed high levels of MMP-2, and increased collagenase expression was associated with the progression of tumors. In human prostate cancer, we have compared benign and malignant prostate tissue from transurethral resection of the prostate ($n = 111$, Ref. 26). *In situ* labeling clearly showed that the amounts of MMP-2 increased and TIMP-1 decreased with tumor progression to higher Gleason grades.

A number of laboratories have also investigated the effects of different cytokines on TIMP and MMP expression in a variety of cell lines. Studies examining the role of IL-6 on collagenase and TIMP-1 production in rat hepatocytes (27) and human fibroblasts, synoviocytes, chondrocytes (28), and macrophages (29) have indicated that IL-6 does not stimulate collagenase production but is a potent inducer of TIMP-1. In comparison, IL-4 and IFN-γ were found to inhibit collagenase expression but not influence TIMP secretion (28). Lacraz *et al.* (28) originally reported that IL-4 and IFN-γ suppressed matalloproteinase synthesis in macrophages without affecting TIMP levels. In a recent paper, Lacraz *et al.* (29) compared the effects of IL-10, IL-4, IL-2, IL-6, and INF-γ on MMP-9, interstitial collagenase, and TIMP-1 synthesis in human macrophages and monocytes. They reported that IL-10 and IL-4 inhibited the production of MMP-9. IL-10 also stimulated TIMP-1 synthesis. Their data indicated that IL-10 controlled MMP-9 and TIMP-1 expression at a pretranslational phase, although steady-state and half-life mRNA studies were not carried out to assess whether IL-10 affected mRNA stability. Interestingly, they further found that IL-10 failed to influence MMP or TIMP production by human fibroblasts, suggesting that cell type-specific responses might be involved. Overall, these data are in strong agreement with the observations reported here. Others have further shown that IL-1, tumor necrosis factor-α, and phorbol esters, which stimulate collagenase and stromelysin gene expression, also up-regulate TIMP expression. On the other hand, TGF-β and retinoic acid, which repress MMP-1 and MMP-3 gene expression, are potent activators of TIMP expression (19). Clearly, these studies and our data strongly suggest that the regulation of MMPs and TIMPs are through independent mechanisms.

We believe that the orthotopic implantation of human tumor cells might be clinically relevant, because the natural regulation of gene expression and metastasis is no doubt strongly influenced by the host tissue stroma and microenvironment (9). In and elegant study, Greene *et al.* (9) have shown that several genes expressed by highly metastatic PC-3M cells were up-regulated by implantation in the prostate gland and down-regulated by implantation s.c. in nude mice. Highly metastatic PC-3M cells expressed high levels of epidermal growth factor receptor, basic fibroblast growth factor, MMP-2, MMP-9, and multidrug resistance-1 mRNA transcripts after orthotopic implantation in the prostate gland compared with the low levels of these genes expressed by PC-3M cells injected ectopically (9). More importantly, they reported a direct correlation between the orthotopic implantation, an elevated expression of these genes, and an increased frequency of metastasis (9), indicating that the regulation of gene expression by the host tissue plays a major role in modulating metastasis. The data presented here strongly support these results and further validate the orthotopic model as ideal for analysis of genes regulating metastasis.

REFERENCES

1. Huang, S., Xie, K., Bucana, C. D., Ullrich, S. E., and Bar-Eli, M. Interleukin 10 suppresses tumor growth and metastasis of human melanoma cells: potential inhibition of angiogenesis. Clin. Cancer Res., *2:* 1969–1979, 1996.

2. Kundu, N., Beaty, T. L., Jackson, M. J., and Fulton, A. M. Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. J. Natl. Cancer Inst., *88:* 536–541, 1996.

3. Richiter, G., Kruger-Krasagakes, S., Hein, G., Huls, C., Schmitt, E., Diamantstain, T., and Blankenstein, T. Interleukin 10 transfected into Chinese hamster ovary cells prevents tumor growth and macrophage infiltration. Cancer Res., *53:* 4134–4137, 1993.

4. Sato, T., McCue, P., Masuoka, K., Salwen, S., Lattime, E. D., Mastrangelo, M. J., and Berd, D. Interleukin 10 production by human melanoma. Clin. Cancer Res., *2:* 1383–1390, 1996.

5. Nabioullin, R., Sone, S., Mizuno, K., Yano, S., Nishioka, Y., Haku, T., and Ogura, T. Interleukin-10 is a potent inhibitor of tumor cytotoxicity by human monocytes and alveolar macrophages. J. Leukocyte Biol., *55:* 437–442, 1994.

6. Weiskirch, L. M., Bar-Dagan, Y., and Mokyr, M. B. Transforming growth factor-β mediated down regulation of antitumor cytotoxicity of spleen cells from MOPC-315 tumor bearing mice engaged in tumor eradication following low dose melphalan therapy. Cancer Immunol. Immunother., *38:* 215–236, 1994.

7. Gorelik, L., Prokhonova, A., and Mokyr, M. B. Low dose melphalan induced shift in the production of a Th-2 type cytokine to the Th1-type cytokine in mice bearing a large MOCP-315 tumor. Cancer Immunol. Immunother., *39:* 117–123, 1994.

8. Maeda, H., Kuwahara, H., Ichimura, Y., Ohtsuki, M., Kurakata, S., and Shiraishi, A. TGF-β enhances macrophage ability to produce IL-10 in normal and tumor bearing mice. J. Immunol., *81:* 4926–4932, 1995.

9. Greene, G. F., Kitadai, Y., Pettaway, C. A., von Eschenbach, A. C., Bucana, C. D., and Fidler, I. J. Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an *in situ* messenger RNA hybridization technique. Am. J. Pathol., *150:* 1571–1582, 1997.

10. Stearns, M., Wang, M., and Stearns, M. E. Cytokine (IL-10, IL-6) induction of tissue inhibitor of metalloproteinase 1 in primary human prostate tumor cell lines. Oncol. Res., *7:* 173–181, 1995.

11. Dellabona, P., Casorati, G., Friedli, B., Angman, L., Sallusto, F., Tunnacliffe, A., Roosneck, E., and Lanzavecchi, A. *In vivo* persistence of expanded clones specific for bacterial antigens within the human T cell receptor α/β $CD4^{-}8^{-}$ subset. J. Exp. Med., *177:* 1763–1771, 1993.

12. Tepper, R. I., and Mule, J. J. Experimental and clinical studies of cytokine gene-modified tumor cells. Hum. Gene Ther., *5:* 153–164, 1994.

13. Marzo, A. L., Fitzpatrick, D. R., Robinson, B. W. S., and Scott, B. Antisense oligonucleotides specific for transforming growth factor β2 inhibit the growth of malignant mesothelioma both *in vitro* and *in vivo*. Cancer Res., *57:* 3200–3207, 1997.

14. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, *65:* 55–63, 1983.

15. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchel, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res., *47:* 936–942, 1987.

16. Stearns, M. E., and Wang, M. Immunoassays of the metalloproteinase (MMP-2,9) and tissue inhibitor of metalloproteinase (TIMP-1,2)

levels in non-invasive and metastatic PC-3 clones. Effects of Taxol. Oncol. Res., *6:* 195–202, 1996.

17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem., *72:* 248–254, 1976.

18. Stearns, M. E., Fudge, K., Garcia, F., and Wang, M. Interleukin 10 inhibition of human prostate PC-3 ML cell metastases in SCID mice. IL-10 stimulation of TIMP-1 and inhibition of MMP-2/MMP-9 expression. Invasion Metastasis, *17:* 62–74, 1997.

19. Denhardt, D. T., Feng, B., Dylan, R. E., Cocuzzi, E. T., and Malyankar, U. M. Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions. Pharmacol. Ther., *59:* 329–341, 1993.

20. Stetler-Stevenson, W. G. Type IV collagenase in tumor invasion and metastasis. Cancer Metastasis Rev., *9:* 289–303, 1990.

21. Monteagudo, C., Merino, M. J., San-Juan, J., Liotta, L. A., and Stetler-Stevenson, W. G. Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. Am. J. Pathol., *136:* 585–592, 1990.

22. Ogilvie, S. J., Hailey, J. A., Juacaba, S. F., Lee, E. C. G., and Tarin, D. Collagenase secretion by human breast neoplasms: a clinicopathologic investigation. J. Natl. Cancer Inst., *74:* 19–28, 1985.

23. Levy, A. T., Cioce, V., Sobel, M. E., Garbisa, S., Grigioni, W. F., Liotta, L. A., and Stetler-Stevenson, W. G. Increased expression of the M_r 72,000 type IV collagenase in human colonic adenocarcinoma. Cancer Res., *51:* 439–444, 1991.

24. D'Errico, A., Garbisa, S., Liotta, L. A., Castronovo, V., Stetler-Stevenson, W. G., and Grigioni, W. F. Augmentation of type IV collagenase, laminin receptor, and Ki67 proliferation antigen associated with human colon, gastric, and beast carcinoma progression. Mod. Pathol., *4:* 239–246, 1991.

25. Hewitt, R. E., Leach, I. H., Powe, D. G., Clark, I. M., Cawston, T. E., and Turner, D. R. Distribution of collagenase and tissue inhibitor of metalloproteinase (TIMP) in colorectal tumours. Int. J. Cancer, *49:* 666–672, 1991.

26. Wood, M., Fudge, K., Mohler, J., Frost, A., Garcia, F., and Stearns, M. E. *In situ* hybridization studies of metalloproteinases 2 and 9 and TIMP-1 and TIMP-2 expression in human prostate cancer. Clin. Exp. Metastasis, *15:* 246–258, 1997.

27. Roeb, E., Graeve, L., Hoffmann, R., Decker, K., Edwards, D. R., and Heinrich, P. C. Regulation of tissue inhibitor of metalloproteinases-1 gene expression by cytokines and dexamethasone in rat hepatocyte primary cultures. Hepatology, *18:* 1437–1442, 1993.

28. Lacraz, S., Nicod, L., Rochemonteix, B. G., Baumberger, C., Dayer, J. M., and Welgus, H. G. J. Suppression of metalloproteinase biosynthesis in human alveolar macrophages by interleukin-4. Clin. Invest., *90:* 382–388, 1993.

29. Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G., and Dayer, J-M. IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J. Clin. Invest., *96:* 2304–2310, 1995.

Clinical Cancer Research

Role of Interleukin 10 and Transforming Growth Factor β1 in the Angiogenesis and Metastasis of Human Prostate Primary Tumor Lines from Orthotopic Implants in Severe Combined Immunodeficiency Mice

Mark E. Stearns, Fernando U. Garcia, Kim Fudge, et al.

Clin Cancer Res 1999;5:711-720.