Overexpression of Transforming Growth Factor β Type I Receptor Abolishes Malignant Phenotype of a Rat Bladder Carcinoma Cell Line

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Abstract
Transforming growth factor β, (TGF-β1), a potent growth inhibitor of bladder carcinoma cells, elicits its effects by binding to cell surface receptors. LMC19, a highly invasive and metastatic rat bladder carcinoma cell line, was insensitive to the growth-suppressive effect of TGF-β1, and it expressed undetectable levels of TGF-β type I receptor (TβRI) mRNA by reverse transcription-PCR and its protein by Western blot analysis. To evaluate the effect of TβRI in reducing the malignant phenotype, we transfected LMC19 with an expression vector containing human TβRI cDNA. Stable transfection with the expression vector yielded five transfectants that expressed the introduced TβRI mRNA. The binding activity of TGF-β1 to TβRI was restored in all of the transfectants. The growth of the transfectants on a plastic surface was markedly inhibited in the presence of TGF-β1 in the culture medium (P < 0.001), whereas the control cells (parental and transfectant with only neo gene) remained TGF-β1 insensitive. The colony-forming efficiency of the transfectants was strongly reduced in soft agar medium containing 5% FCS (P < 0.001) and was restored by the addition of a neutralizing anti-TGF-β antibody. Furthermore, none of the transfectants tested formed tumors in athymic nude mice, whereas the control cells did so in all mice tested. These findings indicate that introduction of TβRI can revert a malignant phenotype to a less aggressive (even benign) phenotype in a rat bladder carcinoma cell line that lacks TβRI, and that reduced expression of TβRI may be associated with the development and progression of bladder carcinomas.

Introduction
TGF-β1, is a potent growth inhibitor in many normal and neoplastic cells in vivo and in vitro (1–3). However, there are several types of malignant cells that are resistant to the growth-inhibitory effect of TGF-β1, and insensitivity to the TGF-β1 effect is closely associated with tumor development and progression (4–6). Such an insensitivity in malignant cells could be due to either loss or reduced expression of specific receptors for TGF-β or to alterations in the postreceptor signaling pathway(s) of TGF-β (5, 7). Moreover, it has been reported that some growth factors, including epidermal growth factor and basic fibroblast growth factor, and extracellular matrix could modulate the growth-inhibitory effect of TGF-β1 (8). TGF-β1 elicits its effects by binding to its cell surface receptors. Three major types of TGF-β binding proteins, TβRI, TβRII, and TβRIII, are known to be widely distributed in most TGF-β-responsive cells. TβRI and TβRII are glycoproteins of M, 55,000 and M, 70,000, respectively, and both are serine/threonine kinase receptors that are necessary for TGF-β signal transduction (9, 10). TβRIII has a large extracellular domain with a relatively small cytoplasmic domain that contains no obvious signaling motif (11).

We reported previously that TGF-β1 inhibited the growth of several types of rat bladder carcinomas and nonmotumogenic urothelial cells, but that one tumor cell line, LMC19, which is highly invasive and metastatic in athymic nude mice, was insensitive to the growth-inhibitory effect of TGF-β1 (12, 13). TβRI mRNA expression is undetectable in LMC19 cells. Therefore, we hypothesized that acquisition of the ability to express TβRII should restore sensitivity to the growth-inhibitory effect of TGF-β1 and should reduce malignancy. To test this hypothesis, we transfected LMC19 cells with an expression vector containing human TβRI cDNA, and we assessed the biological potential of the transfectants in vivo and in vitro.

Results
Effect of TGF-β1 on the Growth of MYP3, MYU3L, and LMC19 Cells in Monolayer Culture. TGF-β1 was added to individual wells at a final concentration of 1, 5, or 10 ng/ml. Seventy-two h later, the number of cells was estimated by the MTT assay. TGF-β1 treatment significantly inhibited the growth of MYP3 and MYU3L (10.1 and 18.5% of the respective untreated controls; P < 0.001), but not that of LMC19 (99.0% of untreated control), when 10 ng/ml of TGFβ1 were

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3 The abbreviations used are: TGF, transforming growth factor; TβRI, TGF-β receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
added (Fig. 1). These findings were similar to those we observed in a previous experiment by using a \[^{3}H\]thymidine incorporation assay (12).

Expression of TβRI and TβRII in MYP3, MYU3L, and LMC19 Cells. Expression of TβRI and TβRII mRNA was examined by the RT-PCR method. Products specific for TβRI (987 bp) were observed only with MYP3 and MYU3L and not with LMC19 (Fig. 1, top panel). In all of the cell lines tested, a band specific for TβRII (700 bp) was observed (Fig. 2, middle panel). The expression of G3PDH mRNA, used as an internal control, was approximately the same in all clones (Fig. 2, bottom panel). Western blot analysis demonstrated immunoreactive TβRII (M, 70,000) in all cell lines (Fig. 3, bottom panel), whereas TβRI expression (M, 55,000) was limited to MYP3 and MYU3L (Fig. 3, top panel).

Isolation of LMC19 Clones That Express TβRI mRNA. After cotransfection with pSV7d-TβRI and pSV2neo, many G418-resistant clones were obtained, and approximately 40% of them expressed TβRI-specific mRNA; five clones that expressed TβRI-specific mRNA (Fig. 4, top panel, Lanes b–f) were selected and designated as LTβRI-5, LTβRI-6, LTβRI-16, LTβRI-20, and LTβRI-21. No specific signals were detected in the control clone transfected with only pSV2neo (LMC19neo; Fig. 4, top panel, Lane a). Expression of β-actin mRNA used as an internal control was approximately the same among the cells tested (Fig. 4, bottom panel). Unique integration of the human TβRI cDNA was confirmed by PCR and Southern blot analysis (data not shown).

Receptor Cross-Linking of \(^{125}\text{I}\)-labeled TGF-β, TβRI Transfectants. The binding of \(^{125}\text{I}\)-labeled TGF-β, to cell surface receptors was investigated on LMC19 transfecteds by receptor cross-linking analysis. The cross-linking analysis clearly demonstrated that the specific binding of \(^{125}\text{I}\)-labeled TGF-β, was only to TβRII and TβRIII in LMC19neo cells (Fig. 5, Lane a). In contrast, the binding activity of \(^{125}\text{I}\)-labeled TGF-β, to TβRI was restored in all of the TβRII transfectants tested (Fig. 5, Lanes c, e, and g). These binding activities were completely inhibited by the addition of 100-fold excess of a competitor, 500 ng/ml unlabeled TGFβ, (Fig. 5, Lanes b, d, f, and h).

Effect of TGF-β, on Growth of the Transfectants. The effect of TGF-β, (5 ng/ml) on the growth of the TβRII transfecants was examined. The number of cells was assessed after 72 h of culture. TGF-β, strongly inhibited the growth of all of the TβRII transfecants (17.2–25.7% of respective untreated controls; \( P < 0.001 \)). In contrast, no significant growth inhibition was observed in parental LMC19 and LMC19neo (98.2 and 109.2% of respective untreated controls; Fig. 6A).
**Fig. 4.** Northern blot analysis for the expression of transfected TβRI mRNA. Cytoplasmic RNA was extracted from early confluent cells. RNA (30 μg/lane) was fractionated on 1.0% denaturing agarose gel, transferred to nylon filter, and hybridized to 32P-labeled probes for human TβRI (top panel) or β-actin (bottom panel). Large arrowhead, 2.5-kb mRNA of transfected human TβRI. Small arrowhead, 2.5-kb mRNA of β-actin. Lanes a, LMC19neo; Lanes b, LTβRI-5; Lanes c, LTβRI-6; Lanes d, LTβRI-16; Lanes e, LTβRI-20; Lanes f, LTβRI-21.

**Fig. 5.** Receptor cross-linking of 125I-labeled TGF-β1. Cells (2.5 × 10^5 cells/100-mm dish) were incubated with 5 ng/ml 125I-labeled TGF-β1 in binding buffer and were cross-linked with disuccinimidyl suberate before analysis by 5-10% SDS-PAGE. Lanes a and b, LMC19neo; Lanes c and d, LTβRI-5; Lanes e and f, LTβRI-16; Lanes g and h, LTβRI-20. In Lanes b, d, f, and h, cells were cocultivated with 125I-labeled TGFβ1, and 500 ng/ml (100-fold excess) unlabeled TGF-β1.

**Anchorage-independent Growth Potential of the Transfectants and Effect of Anti-TGFβ Neutralizing Antibody.** The anchorage-independent growth potential of the transfectants was examined in the soft agar medium containing 5% FCS. All of the transfectants tested demonstrated a significant reduction in colony-forming efficiency (3.3 ± 3.1 to 35.7 ± 8.3 per 1000 cells) as compared to the parental and neo-control cells (210.7 ± 23.5 and 198.3 ± 26.7 per 1000 cells, respectively; Fig. 6B). We concluded that this reduction of the colony-forming efficiency of the transfectants was due to the TGF-β present in 5% FCS; when anti-TGFβ neutralizing antibody was added, the colony-forming efficiency of the transfectants was restored almost to the level of parental LMC19 cells (174.0 ± 29.2 to 214 ± 17.0 per 1000 cells; Fig. 6B).

**Tumorigenicity in Athymic Nude Mice.** The five TβRI transfectants (2 × 10^6 cells) were injected s.c. into the dorsal flanks of nude mice. All mice that received LMC19neo cells developed tumors rapidly, and they were killed at 65 days because of an increasing tumor burden. In contrast, all mice receiving transfectants had failed to develop tumors at 120 days of the experiment (Table 1).

**Discussion**

The results of the present study have demonstrated clearly that introduction of TβRI to bladder carcinoma cells that lack TβRI results in a striking reduction in their malignant phenotype; the growth of the transfectants on a plastic surface was markedly inhibited in the presence of TGF-β1 (P < 0.001); the colony-forming efficiency in soft agar medium was reduced remarkably in the presence of 5% FCS (P < 0.001) and was restored by the addition of anti-TGFβ neutralizing antibody; TβRI transfectants failed to form tumors in athymic nude mice.
Although TGF-β, is a potent growth inhibitor in many normal and malignant cells in vivo and in vitro (1–3), some cancer cells are resistant to this growth-inhibitory effect, and the insensitivity is closely associated with tumor development and progression (4–6). Our previous studies also showed that the growth of nonmalignant rat urothelial cells and many bladder carcinoma cells was inhibited by TGF-β₁, but a few rat bladder carcinomas, including LMC19, were insensitive to its suppressive effect and had a high invasive and metastatic potential (12, 13).

Both TpRI and TpRII are required for TGFβ signaling. TGF-β₁ binds to TpRII, which acts as a primary receptor, and it forms a heterotetramer with TpRI, which acts as a transducer. Then, serine/threonine residues on TpRI are phosphorylated by TpRII kinase. Phosphorylated TpRII induces expression of target genes, including p21 and p15, and release of p27 from Cdk4 and Cdk6 (14). These signaling pathways were mediated by Smad2 and Smad4 proteins (15–17). Mutations of Smad2 and Smad4 as well as TpRI and TpRII are reported to occur in several types of carcinomas that are insensitive to TGF-β action (18–20). Our cross-linking analysis has clearly shown that in the cells that lack TpRI, TGF-β₁ can bind to TpRII but cannot induce a growth-inhibitory effect.

There have been reports showing that reduced expression of TpRII correlates with insensitivity to TGF-β₁ inhibition in many types of tumors, including bladder cancer (21–23). However, there are only two reports correlating a reduced expression of TpRI with TGF-β₁ insensitivity (24, 25); Kim et al. (24) and Carcamo et al. (25) examined the effect of transfected TpRI, but it is only Carcamo et al. (25) who examined its effect on cell growth in vitro, and neither group examined its effect on growth in vivo. We believe that such study would be incomplete until the effect of transfected genes is evaluated in vivo by tumorigenicity assay. We believe that this is the first report clearly demonstrating that the absence of TpRI is associated with aggressive behavior in vivo and in vitro in a rat bladder carcinoma. This finding strongly suggests the possibility that TpRI is a potent tumor suppressor in bladder cancer. In humans, TpRI is mapped to chromosome segment 9q33–q34 (26). Loss of heterozygosity of chromosome 9q has been shown in most carcinomas of the bladder, and it is suggested that this region of the chromosome contains one or more tumor suppressor genes (27). In light of the present study, TpRI is a strong candidate for being a tumor suppressor in bladder cancer.

### Materials and Methods

**Cells and Cell Culture.** LMC19, a rat urinary bladder carcinoma cell line, was established in our laboratory (12). The cells were isolated from a pulmonary metastasis in a nude mouse inoculated s.c. with rat bladder carcinoma cells of cloned origin. LMC19 cells form an invasive and differentiated cell carcinoma at a s.c. site in nude mice, and they metastasize to the lungs (12). LMC19 is resistant to the growth-suppressive effect of TGF-β₁ (12). MYUL3 is another rat bladder carcinoma cell line also established in our laboratory. It is tumorigenic and highly invasive but not metastatic (13). These cell lines were grown in Ham’s F-12 medium supplemented with 5% FCS, 10 μM non-essential amino acids, 100 μg/ml streptomycin, and 100 units/ml penicillin (all from Life Technologies, Inc., Gaithersburg, MD) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. MYP3 is a non-tumorigenic, anchorage-dependent rat uterine cell line (13). Cells were grown in Ham's F-12 medium supplemented with 10% FCS, 10 μM non-essential amino acids, 2.7 mg/ml dextrose (Sigma Chemical Co., St. Louis, MO), 1 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 100 μg/ml streptomycin, and 100 units/ml penicillin (all from Life Technologies, Inc.) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

**Plasmid and Transfection.** An expression vector, pSV7td, containing full-length human TpRI cDNA (pSV7td-TpRII) was kindly provided by Dr. K. Miyazono (Cancer Institute, Tokyo, Japan). Cells (5 × 10⁶ cells/dish) were seeded in 100-mm culture dishes (Falcon, Lincoln Park, NJ) in Ham's F-12 medium with 5% FCS. Twenty-four hours later, cells were transfected with 20 μg of pSV7td-TpRI and 1 μg of pSV2neo (provided by Dr. Y. Ebina) by the Lipofectamine method. Forty-eight hours later, the culture medium was switched to a selection medium containing G418 (800 μg/ml G418; Life Technologies, Inc.). After continuous culture in the same medium for 14 days, G418-resistant clones were isolated and expanded in a 24-well culture dish.

**Isolation of Cytoplasmic RNA, RT-PCR, and Northern Blot Analysis.** Cells grown in monolayers were harvested at an early phase of confluency. RNA was prepared by lysing of cells in a hypotonic buffer containing NP40 (Sigma), followed by removal of nuclei. Expression of rat TpRI, TpRII, and G3PDH mRNA (used as an internal control) was assessed by the RT-PCR method. The nucleotide bases used were 5'-TTGTTGGAGCGAGTTGGTCG-3' as an up-stream primer and 5'-CCATCGTGTGTTGGGATTTAGCT-3' as a down-stream primer for rat TpRI (28), 5'-AGCAAGAGAGCGCCGTCCTTG-3' as an up-stream primer and 5'-GGGCCATGGTATGCTGTGTGT-3' as a down-stream primer for rat TpRII (29), and 5'-TGAAGTGCCTTGTCACAGGATT-3' as an up-stream primer and 5'-CATGTTAGGGCATGTTCACCC-3' as a down-stream primer for G3PDH. One μg of cytoplasmic RNA in a 20 μl mixture with a hexamer random primer (Life Technologies, Inc.) was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) at 42°C for 60 min. Two μl of reverse-transcribed mixture was subjected to PCR as described in our previous report (30). Amplified cDNA was subjected to electrophoresis in 1.5% agarose gels containing 100 ng/ml ethidium bromide. The authenticity of the PCR products was verified by diagnostic restriction digestion with the following enzymes: BamH1 for TpRI and Xhol for TpRII (Life Technologies, Inc.).

For Northern blot analysis, 30 μg of cytoplasmic RNA were electrophoresed onto a formaldehyde/10% agarose gel and blotted onto a nylon filter. The filter was hybridized in Church buffer at 68°C and washed with 0.1 N NaOH and 0.1 N NaOH on ice for 30 min in 50 ml Tris-SC (pH 7.5) containing 150 μg/ml NaCl, 1% Triton X-100, 10 μg/ml aprotinin, 0.1 μM methylosulfate, and 0.1% sodium deoxycholate. The samples were centrifuged at 4°C for 15–20 min, and supernatants were collected. The samples (100 μg protein/ane) were electrophoresed on SDS-polyacrylamide gels. Proteins from gels were transferred to nitrocellulose, and TpRI and TpRII were detected, respectively, with rabbit anti-TpRI and rabbit anti-TpRII (both from Santa Cruz Biotechnology Inc. Santa Cruz, CA), and an ECL kit (Amer sham Corp., Arlington Heights, IL).
Receptor Cross-Linking. Cells were plated at a density of 2.5 × 10^6 cells per 100-mm dish in Ham’s F12 medium containing 5% FCS 24 h prior to cross-linking. The monolayers of cells were washed three times with binding buffer (phosphate-buffered 0.9% saline containing 0.9 mM CaCl₂, 0.49 mM MgCl₂, and 1 mg/ml BSA) and then incubated for 3 h on ice with 5 ng/ml 125I-labeled TGFβ1 (Amersham) or 5 ng/ml 125I-TGFβ, plus 500 ng/ml unlabeled TGFβ1, in binding buffer. Cells were then washed three times with cold BSA-free binding buffer and cross-linked for 15 min on ice in BSA-free binding buffer containing 0.28 mM disuccinimidyl suberate (Pierce, Rockford, IL). The cells were washed once with BSA-free binding buffer and then were lysed on ice for 30 min in 20 ml Tris-HCl (pH 7.5) containing 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 10 mM EDTA, 1.5% Trasylol, and 0.3% phenylmethylsulfonyl fluoride. The samples were centrifuged at 4°C for 15 min, and supernatants were transferred into new tubes. The samples (30 μg protein/well) were electrophoresed on SDS-polyacrylamide gel. The gel was then dried and exposed to an X-ray film (31).

Anchorage-dependent Growth. Cells (1 × 10^5/well) in a 96-well plate containing Ham’s F-12 medium were allowed to grow for 24 h, supplemented with 5% FCS. Then, after 72 h culture in a serum-free medium containing recombinant TGF-β (Life Technologies Inc.; 0–10 ng/ml), cell proliferation was assessed by the addition of 20 μg of the vital dye MTT (1 mg/ml; Sigma) for 4 h. The blue dye taken up by the cells was dissolved in DMSO (100 μl/well), and its absorbance at 495 nm was measured on an automated microplate reader (Bio-Tec, Winoski, VT; Ref. 32). A preliminary study with the MTT assay showed that absorbance was directly proportional to the number of cells.

Anchorage-independent Growth. Cells were suspended at a density of 1 × 10^5 cells in 2 ml of 0.3% Noble agar (Difco, Detroit, MI) in Ham’s F-12 medium containing 5% FCS with or without 50 μg/ml of anti-TGF-β polyclonal neutralizing antibody (R&D Systems, Minneapolis, MN) and layered over 2 ml of 0.6% agar in Ham’s F-12 medium with 5% FCS in 35-mm dishes. The antibody was intended to neutralize any TGF-β that might have been present in FCS. The specificity of the anti-TGF-β antibody was confirmed by its complete inhibition of the growth-suppressive effect of recombinant TGF-β1 (data not shown). The cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 21 days, colonies of more than 20 cells were counted.

Tumorigenicity in Athymic Nude Mice. After trypsinization, cells were washed twice with Ham’s F-12 medium without serum and resuspended in 0.2 ml of the medium without serum. Groups of eight athymic BALB/c nude mice (Harlan Sprague Dawley Inc., Indianapolis, IN) received 2 × 10^5 cells injected s.c. at each dorsal flank. The mice were monitored twice a week for the development of tumors.

References


