Adenovirus-mediated Transfer of a Truncated Transforming Growth Factor-β (TGF-β) Type II Receptor Completely and Specifically Abolishes Diverse Signaling by TGF-β in Vascular Wall Cells in Primary Culture*

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We constructed an adenoviral vector expressing a mutated human type II transforming growth factor-β (TGF-β) receptor that was truncated of its kinase domain (AdexCAT/βTR) and examined whether this truncated receptor could abolish signaling by TGF-β using arterial endothelial cells and smooth muscle cells, as well as a lung epithelial cell line (Mv1Lu). Infection of cells with AdexCAT/βTR induced expression of the truncated receptor, the amount of which would be excessive compared with those of both full-length type I and type II receptors, as assessed by levels of their mRNAs. The antiproliferative effect of TGF-β was completely eliminated in both endothelial cells and Mv1Lu that were infected with AdexCAT/βTR. The transcriptional activation by TGF-β of plasminogen activator inhibitor-1 and fibronectin was entirely suppressed. Abrogation of the TGF-β-enhanced production of type I collagen in infected smooth muscle cells was confirmed by immuno-cyto-staining and by [3H]proline incorporation in a quantitative manner. Mitogenic response to other growth factors remained unaffected in infected cells. Our data demonstrated that the adenovirus-mediated transfer of a truncated type II TGF-β receptor completely and specifically abolishes the diverse effects of TGF-β as a dominant-negative mutation, supporting the hypothesis that both the type I and type II receptors are required for all signaling by TGF-β. This method may facilitate the clarification of the role of TGF-β both in vitro and in vivo.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates cell proliferation and differentiation and extracellular matrix production (1-3). TGF-β seems to play pivotal roles in embryogenesis (4, 5) and in chronic fibroproliferative pathophysiological conditions in adults such as liver cirrhosis, chronic glomerulonephritis, and atherosclerosis (3). However, partly due to its multiple functions, the exact roles of TGF-β remain controversial. To clarify the actual roles of TGF-β in physiological conditions, an effective and feasible method that inhibits the signaling by TGF-β in a specific manner is required.

TGF-β exerts its effects by binding to and activating specific receptors located on the cell membrane. Two signaling receptors, termed type I and type II receptors, have been cloned recently (6-11). Each type possesses an extracellular region, a single transmembrane portion, and a serine/threonine kinase domain in its cytoplasmic region. Recent molecular and cytogenetic analyses have shown that the type II receptor can itself bind free TGF-β (8), whereas the type I receptor can only recognize TGF-β that is already bound with the type II receptor (7, 9-13), and suggested that formation of a ligand-induced heterodimer (or a tetramer) involving both the type I and type II receptors is required for signaling (11, 13-16). In this model, transphosphorylation of the type I receptor by the type II receptor, which is constitutively phosphorylated, seems to be essential for signal propagation (14-16). Evidence supporting this idea is that a mutated type II receptor that was either truncated of its kinase domain (15) or substituted one critical amino acid resulting in the loss of its transphosphorylation activity (16) inhibited, albeit partially, many of the signaling responses induced by TGF-β. However, other investigators have found that a similar truncated type II receptor inhibited only the antiproliferative effect of TGF-β and not the transcriptional activation of extracellular matrix proteins, suggesting that each receptor may have its own distinct signaling pathways (17). Further, in most studies reported so far, the signaling pathways were investigated either in an established epithelial cell line derived from the mink lung, Mv1Lu cells, which were either intact or chemically mutated, or in COS cells that were transiently transfected with the receptors. The use of cells in primary culture may be necessary to determine whether the truncated type II receptor could abolish all the diverse signaling pathways of TGF-β as a dominant-negative mutation, thus serving as a useful tool for the elucidation of the roles of TGF-β.

It has been shown that an adenoviral vector is remarkably efficient for both in vitro and in vivo gene transfer in a wide...
Adenoviral Transfer of a Dominant-negative TGF-β Receptor

variety of cells and species. In this study, we constructed a replication-defective adenovirus expressing the truncated human type II TGF-β receptor under a powerful constitutive promoter (AdexCATbTR) and investigated whether the adenovirus-mediated expression of the truncated receptor could eliminate the diverse effects of TGF-β as a dominant-negative mutation in cells derived from the arterial wall as well as in Mv1Lu cells. All signals tested elicited by three isoforms of TGF-β were completely suppressed in a TGF-β-specific manner. Our data support the notion that the formation of ligand-induced heteromeric complexes involving both the type I and type II receptors is required for all diverse effects of TGF-β. Furthermore, the results demonstrate that the adenovirus-mediated transfer of the truncated type II TGF-β receptor should prove a useful tool for the clarification of the roles of TGF-β both in vitro and in vivo.

MATERIALS AND METHODS

Cell Culture—Mink lung epithelial cell line, Mv1Lu (CCL-64, American Type Culture Collection), was grown in minimum essential medium (Life Technologies, Inc.) with 10% fetal bovine serum (BioWhit- taker, Walkersville, MD). Arterial endothelial cells (EC) and smooth muscle cells (SMC) were primarily prepared from the bovine thoracic aorta as described previously (18, 19) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) with 10% fetal calf serum (Hyclone Lab., Logan, UT). Each medium contained 100 IU/ml of penicillin and 50 μg/ml of streptomycin. The EC expressed TGF-β and were challenged with 200 pM of TGF-β1 (provided by Kirin Brewery Co. Tokyo, Japan) from either Mv1Lu cells or EC 24 h after infection with AdexCATjTR at various m.o.i. Either total RNA (25 μg) or polyadenyl- lated RNA (5 μg) was separated by electrophoresis on a 1% agarose, 1 × MOPS, 5 mM sodium acetate, 1 mM EDTA, 1.2 M formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham Corp.). The membrane was probed for 16 h at 42 °C with [3P]-labeled CDNA corresponding to either bovine plasminogen activator inhibitor-1 (PAl) (provided by M. Pessin, Weizmann Institute of Science, Rehovot, Israel) or bovine endostatin (provided by P. Farmer, University of California, San Francisco) that was tagged to the N terminus (15) (provided by J. Massague, Memorial Sloan-Kettering Cancer Center) most of the cytoplasmic kinase region deleted (only 7 amino acids remained in the intracellular region), and it was placed into a cassette cosmid vector, pAdexCA2w (provided by I. Saito, University of Tokyo) under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter (23) (pAdexCATjTR). A recombinant adenovirus was constructed by in vitro homologous recombination in 293 cells (24) using pAdexCATjTR and the adenovirus DNA-terminal protein complex by a method previously described (25). The desired recombinant adenovirus, designated as AdexCATjTR, was purified by cesium chloride gradient centrifugation through an extensive dialysis. The titer of the virus stock was assessed by a plaque formation assay using 293 cells and expressed as plaque formation unit. Two control adenoviruses were used: AdexCALacZ expressing bacterial β-galactosidase and Adex1w containing no exogenous gene (20–22). Values were expressed as counts per minute (cpm)/g of DNA. Statistical analysis of values was performed by the Student’s t test with a 0.05 value of significance being considered significant.

RESULTS

Adenovirus-mediated Expression of Truncated Human Type II TGF-β Receptor in Mv1Lu Cells—We first examined the expression levels of the truncated human TGF-β type II receptor in Mv1Lu cells exposed to AdexCATjTR at various m.o.i. As shown in Fig. 1A, mRNA corresponding to the truncated type II TGF-β receptor was detected in Mv1Lu cells infected with AdexCATjTR at m.o.i. 20 or left uninfected and incubated for 48 h in serum-free DMEM. Then cells were treated with or without 100 μg of TGF-β1 for 6 h before cellular RNA was isolated. For the analysis of mRNA levels of wild-type TGF-β receptors, polyadenylated RNA was extracted using Oligotex-dT30 (Takara Shuzo Co. Otsu, Japan) from either Mv1Lu cells or EC 24 h after infection with AdexCATjTR at various m.o.i. Either total RNA (25 μg) or polyadeny- lated RNA (5 μg) was separated by electrophoresis on a 1% agarose, 1 × MOPS, 5 mM sodium acetate, 1 mM EDTA, 1.2 M formaldehyde gel and transferred to a nylon membrane (Hybond-N, Amersham Corp.). The membrane was probed for 16 h at 42 °C with [3P]-labeled CDNA corresponding to either bovine plasminogen activator inhibitor-1 (PAl) (provided by M. Pessin, Weizmann Institute of Science, Rehovot, Israel) or bovine endostatin (provided by P. Farmer, University of California, San Francisco) that was tagged to the N terminus (15) (provided by J. Massague, Memorial Sloan-Kettering Cancer Center) most of the cytoplasmic kinase region deleted (only 7 amino acids remained in the intracellular region), and it was placed into a cassette cosmid vector, pAdexCA2w (provided by I. Saito, University of Tokyo) under a CA promoter comprising a cytomegalovirus enhancer and a chicken β-actin promoter (23) (pAdexCATjTR). A recombinant adenovirus was constructed by in vitro homologous recombination in 293 cells (24) using pAdexCATjTR and the adenovirus DNA-terminal protein complex by a method previously described (25). The desired recombinant adenovirus, designated as AdexCATjTR, was purified by cesium chloride gradient centrifugation through an extensive dialysis. The titer of the virus stock was assessed by a plaque formation assay using 293 cells and expressed as plaque formation unit. Two control adenoviruses were used: AdexCALacZ expressing bacterial β-galactosidase and Adex1w containing no exogenous gene (20–22). Values were expressed as counts per minute (cpm)/g of DNA. Statistical analysis of values was performed by the Student’s t test with a 0.05 value of significance being considered significant.
TGF-β receptor was detected by Northern blotting probed with a 5’ end of the human TGF-β type I receptor cDNA. In Fig 1B, a glycosylated protein of approximately 45 kDa was detected by immunoblotting analysis using an antibody against the HA epitope. The amount detected in both mRNA and protein levels seemed to be m.o.i.-dependent. The result suggested that transfection at m.o.i. 20 would achieve submaximal expression of the truncated TGF-β type II receptor.

We next wanted to know a relative expression level of truncated and full-length type II receptors and whether expression of the full-length receptor was affected by infection with AdexCATβTR. Because the TGF-β receptor is a low abundant protein (8) and not easily detected by Western blotting, we compared the levels of mRNA for the two receptors in EC that had been exposed to AdexCATβTR at various m.o.i. Two mRNAs were detected in Northern blotting using a 5’ end of the human type II receptor cDNA as a probe (Fig 2A). These mRNAs, of 5.5 and 0.9 kb, would correspond to the full-length bovine type II and the truncated human type II receptors, respectively. The mRNA of the truncated receptor in cells infected with AdexCATβTR at m.o.i. 3 was already extremely abundant compared with that of the bovine full-length receptor. Although we did not quantify the receptor proteins, and we might underestimate the mRNA level of the bovine full-length type II receptor due to using a human probe, these levels of mRNA may suggest that a large excess of the truncated receptor over the full-length receptor would be expressed in cells exposed to AdexCATβTR. Fig 2B indicates that transcription of the full-length type II receptor was not significantly altered by the AdexCATβTR-mediated co-expression of the truncated type II receptor; the intensities of mRNA for full-length type II receptor normalized to signals of GAPDH or β-actin were virtually consistent. The mRNA level of the full-length type I receptor was also not changed by infection with AdexCATβTR (Fig 2C).

Suppression of the TGF-β-induced Antiproliferative Effect by Adenovirus-mediated Transfer of the Truncated Type II TGF-β Receptor—TGF-β has been shown to arrest the cells in the late G1 phase of the cell cycle (1, 29, 30). We examined whether adenovirus-mediated transfer of the truncated type II TGF-β receptor could release the antiproliferative effect of TGF-β in either Mv1Lu cells (Fig 3A) or EC (Fig 3B). Cells infected with a control adenovirus, either Adex1w (3A) or AdexCALacZ (3B), as well as intact cells, exhibited an antiproliferative response to TGF-β1 in a dose-dependent manner over the range of concentrations 0–200 pM (Fig 3). However, cells treated with AdexCATβTR at m.o.i. 20 completely lost the TGF-β-induced antiproliferative effect even in the presence of a high concentration of TGF-β1 (200 pM) (Fig 3). This antiproliferative effect of TGF-β1 and its abrogation by infection with AdexCATβTR were also observed with both TGF-β2 and -β3 (data not shown).

Inhibition of TGF-β-induced Transcriptional Activation by Adenovirus-mediated Transfer of the Truncated Type II Receptor—It is well known that TGF-β stimulates the production of extracellular matrix proteins, which may lead to fibroproliferative disorders (3). To examine whether infection with AdexCATβTR could abolish the TGF-β-stimulated increase in transcription, we quantified the static levels of mRNA of PAI-I (Fig 4A) and fibronectin (Fig 4B) in EC. The levels of mRNA for both PAI-I and fibronectin were substantially increased in response to TGF-β1 (100 pM) in EC either uninfected or infected with Adex1w. However, cells treated with AdexCATβTR became completely unresponsive to TGF-β1 even at a higher concentration of TGF-β1 (300 pM) (Fig 4).

Inhibition by AdexCATβTR of the TGF-β-induced Accumulation of Extracellular Matrix Protein in SMC—To confirm the inhibitory effects of AdexCATβTR on the production of extracellular matrix at the protein level, we examined by immunocytochemistry the level of type I collagen in SMC that had been

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**Fig. 1.** Adenovirus-mediated expression of the truncated human type II TGF-β receptor in Mv1Lu cells. A, total RNA was extracted from cells 24 h after infection with AdexCATβTR at various m.o.i. as indicated and subjected to Northern analysis using a 32P-labeled 5’ end of the type II receptor cDNA as a probe. The corresponding RNAs (0.9 kb) were detected by autoradiography. The same membrane was also probed with a cDNA coding GAPDH (1.3 kb) as an internal control to show the references of loaded RNA. B, cell lysates were harvested 72 h after infection with AdexCATβTR at various m.o.i. as indicated, subjected to SDS-polyacrylamide gel electrophoresis and transferred to a membrane. The membrane was probed with a monoclonal antibody against the HA epitope, and the signal was visualized using an alkaline phosphatase-conjugated anti-mouse IgG and chromogenic reagents. Molecular markers are in kilodaltons.

**Fig. 2.** Full-length bovine type I and type II and truncated human type II TGF-β receptor mRNA in bovine EC infected with AdexCATβTR. A, polyadenylated RNA isolated from bovine EC infected with AdexCATβTR at various m.o.i. as indicated was probed with a 5’ end of the human TGF-β type II receptor cDNA. Exposure time for autoradiography was 1 h. B, the membrane corresponding to the full-length bovine TGF-β type II receptor mRNA in A was exposed to a film for a prolonged period of time (16 h). C, the same polyadenylated RNA as in A were hybridized with the human TGF-β type I receptor cDNA, and a film was exposed for 16 h. Signals of both the full-length receptor (B and C) were normalized to their mRNA levels of both GAPDH and β-actin (B) or of GAPDH (C), and the values relative to the normalized value obtained from uninfected cells were shown in the bottom column (B and C). Two other independent experiments gave similar results.
stimulated with TGF-β1 (Fig. 5, B–E). In SMC infected with Adex1w as well as in intact SMC, the immunoreactivity for type I collagen observed mainly in the cytoplasm as fine granules, was significantly enhanced by treatment with TGF-β1 (Fig. 5, B and C). However, in SMC exposed to AdexCATβTR, the immunoreactivity (Fig. 5D) was the same as that in quiescent cells (Fig. 5A), even in the presence of additional TGF-β1. Cells stimulated with TGF-β1 but not treated with a primary antibody showed no staining (Fig. 5E).

To confirm this further, we quantified the production of extracellular proteins by measuring incorporation of [14C]proline into SMC. TGF-β-stimulated incorporation of [14C]proline into both collagenase-digestible protein (Fig. 6A), which represents collagen synthesis, and total protein (Fig. 6B) was completely abolished in SMC that had been infected with AdexCATβTR at m.o.i. 20.

Unaffected Signaling by Other Growth Factors in Cells Infected with AdexCATβTR—Finally, we examined whether infection with AdexCATβTR might affect signaling mediated by other growth factors. DNA synthesis in response to either PDGF-BB or bFGF was measured in Balb/3T3 cells that had been infected with AdexCATβTR. As shown in Fig. 7, neither PDGF-BB- nor bFGF-stimulated DNA synthesis was affected in cells infected with AdexCATβTR, indicating that AdexCATβTR specifically inhibits TGF-β-mediated signal transduction but does not affect signaling by other growth factors.

DISCUSSION

For elucidating the roles of growth factors and cytokines in pathophysiological processes that develop in mature adults, the usefulness of targeted gene-disruption through a homologous recombination would be limited, because such mutations either have lethal effects at early embryonic stages (more often the more critical the molecules are) or show no effects if the target molecule belongs to a multi-gene family such as TGF-β, which comprises at least three isoforms encoded by different genes. It has been demonstrated that a kinase-defective mutated form of receptor specifically abolished the receptor-mediated signaling by PDGF (31, 32), FGF (33–37), epidermal growth factor (38), insulin (39, 40), and vascular endothelial growth factor (41), for example. Based on these findings, in this study we constructed a recombinant adenovirus expressing a human truncated, kinase-defective type II TGF-β receptor (AdexCATβTR) and examined whether AdexCATβTR could serve as a dominant-negative mutation using primary cultures derived from arterial wall, rather than donor cells that had been transplanted and selected or chemically mutated cell lines.

Ligand-induced receptor dimerization and the subsequent autophosphorylation that recruits substrates for the receptor kinases are essential steps in the signal propagation by growth factor receptors with intrinsic tyrosine kinases such as the PDGF, FGF, epidermal growth factor, insulin, and vascular endothelial growth factor receptors. Homodimerization of a single kind of receptor is usual for those receptors, although it has been shown that heterodimers between related receptors can also be formed for signaling (32, 34). In contrast, the molecular mechanism underlying the activation of the serine/threonine kinase receptors for the TGF-β superfamily is still not fully established. It has been shown that the type I receptor binds to the free ligand, then forms a heteromeric complex with the type I receptor, phosphorylates, and activates the type I receptor kinase to initiate intracellular signaling (9, 10, 12–14, 16, 42). As a fact, a truncated type II receptor expressed in Mv1Lu cells formed a heteromeric complex with the type I and type II receptors and partially inhibited both the antiproliferative effect of TGF-β and transcriptional activation by TGF-β.
A similar form of truncated type II receptor transfected in rat cardiomyocytes attenuated the transcriptional response of TGF-β, although the antiproliferative effect was not examined (43). However, in another study a similar truncated type II receptor abolished only the TGF-β-induced antiproliferative effect, without affecting the TGF-β-stimulated production of type I collagen.

**FIG. 5.** **Suppression of TGF-β-induced accumulation of collagen type I in SMC infected with AdexCATβTR.** Confluent SMC were infected with either AdexCATβTR (D) or Adex1w (C) at m.o.i. 20 or left uninfected (A, B, and E). After incubation for 2 days, cells were stimulated with 200 pm of TGF-β1 for 60 h (B–E). Type I collagen protein was detected by immunocyto staining using a monoclonal antibody against human type I collagen. Specificity of type I collagen immunostaining was shown by lack of immunoreactivity when primary antibody was absent (E). Cells were lightly counterstained with hematoxylin. The original magnification was ×100.

**FIG. 6.** **Quantitative analysis of TGF-β-induced collagen synthesis in SMC.** SMC infected with either AdexCATβTR or AdexCALacZ at m.o.i. 20 or left uninfected were stimulated with 160 pm of TGF-β1 for 24 h and then incubated with [14C]proline and TGF-β1 for additional 24 h. The [14C]proline incorporation into collagenase-digestible protein (A) and into total protein (B) fractions was measured. The data are expressed as means ± S.D. (n = 4).
either PAI-I or fibronectin (17). This result would seem to suggest that each type of TGF-β receptor may have its own distinct signaling pathway, each mediating a separate set of TGF-β actions and that the type I receptor-mediated signaling does not require the involvement of type II receptors (17). Similar observations have been made in other settings: 293 cells were stimulated with either PDGF-BB (5 ng/ml) or bFGF (5 ng/ml), and DNA synthesis was measured using [3H]thymidine as described in the legend of Fig. 3. The data are shown as means ± S.D. (n = 5).

In summary, it seems likely that both type I and type II receptors should form a heteromeric complex for generating signaling and that a heteromeric complex, at least in the intracellular region, is essential (48, 49).

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Adenoviral Transfer of a Dominant-negative TGF-β Receptor

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