A Soluble Transforming Growth Factor β Type III Receptor Suppresses Tumorigenicity and Metastasis of Human Breast Cancer

MDA-MB-231 Cells

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ABSTRACT

Transforming growth factor β (TGF-β) can promote late stage tumor progression in a number of model systems. In the present study, we have examined whether expression of a truncated soluble extracellular domain of TGF-β type III receptor (sRIII) in human breast cancer MDA-MB-231 cells can antagonize the tumor-promoting activity of TGF-β by sequestering active TGF-β isoforms that are produced by the cancer cells. The secretion of sRIII reduced the amount of active TGF-β1, and TGF-β2 in the conditioned medium. This led to a significant reduction of the growth-inhibitory activity of the medium conditioned by sRIII-expressing cells on the growth of mink lung epithelial CCL64 cells in comparison with the medium conditioned by the control cells. The tumor incidence and growth rate of all of the three sRIII-expressing clones studied were significantly lower than those of the control cells in athymic nude mice. Four of five control cell-inoculated mice showed spontaneous metastasis in the lung, whereas none of the sRIII-expressing cell-inoculated mice had any lung metastasis. Thus, our results suggest that the sRIII may be used to antagonize the tumor-promoting activity of TGF-β.

INTRODUCTION

Breast cancer incidence and mortality rate are still high in the United States and around the world (1, 2). The increasing number of the new cases of breast cancer every year, and the limited therapeutic avenues provide a strong stimulus for the search of new molecular targets for therapy.

TGF-β is a potent regulator of cell proliferation, differentiation, extracellular matrix formation, and immune response (3). Development and progression of many types of cancers including breast cancer are often associated with the increased expression of TGF-β isoforms (4, 5). On the other hand, tumor progression is often marked with diminished or loss of TGF-β growth-inhibitory response mainly due to the down-regulation or inactivation of TGF-β receptors in many types of cancers including breast, colon, gastric, squamous, and prostate carcinomas, retinoblastoma, and B- and T-cell lymphomas (4). As a result, an excessive amount of free TGF-β can be detected in the stroma of malignant lesions (6), which could act in a paracrine fashion on the stromal components to promote tumor progression. Indeed, the overexpression of TGF-β1 in human breast cancer MCF-7 cells showed increased, estrogen-independent tumor formation in athymic nude mice (7). Thus, the sequestration of overexpressed endogenous TGF-β isoforms to antagonize its paracrine tumor-promoting activity in highly progressed cancers may offer a novel and promising approach to treat some malignant tumors.

TGF-β RIII or betaglycan binds all of the three TGF-β isoforms (TGF-β1, -β2, and -β3) with high affinity (8). It apparently has two TGF-β binding sites (9), yet it does not seem to directly transduce the TGF-β signal (10). Several studies have shown that RIII can enhance TGF-β binding to signaling receptors and restore autocrine TGF-β activity in different model systems (10–12). Our current study is designed to determine whether the extracellular domain of RIII can antagonize TGF-β-mediated tumor progression. Expression of a secreted soluble RIII in the human breast cancer MDA-MB-231 cells led to a large reduction of the level of active TGF-β isoforms in the culture medium. sRIII-expressing cells were much less tumorigenic and metastatic than control cells when inoculated s.c. in athymic nude mice, which suggests that the sRIII may have potential utility in cancer treatment.

MATERIALS AND METHODS

Cell Lines. The human breast cancer cell line MDA-MB-231 and mink lung epithelial cell line CCL64 were originally obtained from the American Type Culture Collection. These cell lines were cultured in McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, antibiotics, and 10% FBS (13). Working cultures were maintained at 37°C in a humidified incubator with 5% CO2. MDA-MB-231-limiting dilution clones were obtained by plating parental cells into 96-well culture plates at 0.5 cell/well.

Vector Construction and Transfection. To construct a soluble RIII-expression plasmid, we deleted the 3’ portion of the human RIII cDNA downstream from the BstBI restriction site, which is located in the extracellular domain of RIII (14). The deleted portion was replaced with a PCR-amplified fragment encompassing the BstBI site and the junction between the extracellular and transmembrane domains of RIII. The antisense primer 5’-GTT-CAGTCCAGACCATGGAAAATTGG-3’ for PCR contained a stop codon as underlined. The PCR-amplified fragment was sequenced to confirm its accuracy. The cDNA encoding the complete extracellular domain of RIII was then subcloned into a tetracycline-repressible mammalian expression system as described previously (11). The sense orientation of the RIII cDNA was confirmed by restriction digestion and agarose gel electrophoresis.

The expression vectors were linearized and transfected into the MDA-MB-231-limiting dilution clone used previously (13) with a BTX Electro Cell Manipulator at 250 V and 950 μF. The control cells were transfected with the empty vectors. The transfected cells were plated in 10-cm culture dishes and maintained in the 10% FBS medium for 2 days. Selection of stable transfectants was accomplished by adding Geneticin (G418 sulfate; Life Technologies, Inc.) to the culture medium at 325 μg active compound per milliliter medium. G418-resistant control clones were pooled and designated as Neo pool. The sRIII-transfected clones were individually ring-cloned and expanded for the screening of sRIII expression.

RNA Analysis. Total RNA from cell lines was isolated by guanidine thiocyanate homogenization and acidic phenol extraction (15). To measure sRIII mRNA levels in transfected cells, we constructed a human sRIII riboprobe by inserting the sRIII cDNA into pBSK (-) plasmid (Stratagene Cloning Systems). The recombinant plasmid was then linearized with BstBI. T7 RNA polymerase was used to synthesize a radioactive antisense human RIII riboprobe of 500 bases. RNase protection assays were performed as described previously (11) to measure sRIII mRNA levels in the transfected clones using this radioactive antisense riboprobe that protected a 416-base fragment of sRIII mRNA.

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**The abbreviations used are:** TGF-β, transforming growth factor(s); RII, type II receptor (TGF-β type II receptor); sRIII, soluble RIII (soluble TGF-β RIII); FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Receptor Cross-Linking. The cell surface level of RII was measured with receptor cross-linking assays using $^{125}$I-labeled TGF-β1, as described previously (11). To measure the sRII levels in the conditioned media, $^{125}$I-labeled TGF-β1 (200 pm) was incubated with 100 µl of the medium conditioned by the transfected cells that were cultured in the presence or absence of 0.1 µg/ml tetracycline. The $^{125}$I-labeled TGF-β1-sRII complex was cross-linked with disuccinimidyl suberate and visualized after SDS-PAGE and autoradiography (11).

Measurement of Secreted Active TGF-β1, TGF-β2, and sRII in Conditioned Media. To measure the amount of active TGF-β1, TGF-β2, and secreted sRII in the media conditioned by control or sRII-transfected clones, monolayer cells in 6-well culture plates were cultured in the 10% FBS medium until confluence. The cells were washed twice with a serum-free McCoy’s 5A medium and incubated in 1.5 ml of this serum-free medium for an additional 48 or 72 h. The conditioned medium was then collected in siliconized micro-centrifuge tubes, and the cell number in each well was counted with a hemocytometer after trypsinization. The amount of mature, active TGF-β1 and TGF-β2 in the conditioned medium was determined, respectively, with a TGF-β1 ELISA kit or a TGF-β2 ELISA kit from R&D Systems according to the manufacturer’s instructions. The sRII in the conditioned medium was determined by an ELISA assay that we developed. Briefly, sRII in 100 µl of conditioned medium was allowed to adhere to the bottom of a 96-well Immuno Plate (Dynatech Laboratories, Inc.) overnight at 4°C. The wells were then washed with PBS and blocked with 1% BSA. The adhered sRII was detected by the following equation: 

\[ V = (L \times W)^2 \times 0.5, \text{where} \ L \text{is the length and} \ W \text{is the width of a xenograft.} \]

Tumor Metastasis Study. Lungs and tumors were harvested from the mice inoculated with both control and sRII-transfected cells after the termination of the tumorigenicity study described above. Tumors and small portions of the lungs were fixed in 10% buffered formalin for immunohistochemical studies. The remaining portions of the lungs were analyzed for the metastatic dissemination of tumor cells according to the method of Aslakson and Miller (16) with several modifications. Lungs were minced into 1–2 mm pieces and digested with an enzyme cocktail containing 0.5 mg/ml Collagenase type IV (Sigma) and 1.0 units/ml Elastase (Sigma) in a shaking water bath at 37°C for 45 min. Cell suspensions were passed through a 18-G needle, filtered through 149 Šm Nitex filter (Small Parts, Inc.), centrifuged at 200 × g for 5 min, and plated in a 6-well plate with the 10% FBS medium containing 325 µg/ml active G418 (Life Technologies, Inc.). Medium was changed after 48 h and incubated for 2 weeks at 37°C. The positive colonies, if any, were stained with MTT.

For the immunohistochemical localization of metastatic cells in lungs, formalin-fixed, paraffin-embedded tissue sections of both the lungs and the tumors were cut into 5-µm sections and stained for p53 with an antihuman p53 antibody (DACO) with the ABC staining kit from Vector Laboratories, Inc.

RESULTS

Endogenous TGF-β isoforms have been shown to increase tumorigenicity and metastatic potential of the MDA-MB-231 cells (17). Hence, to test our hypothesis that the enhanced expression of sRII in breast cancer cells can sequester endogenous TGF-β and consequently suppress its paracrine effects on tumor growth and metastasis, we transfected this human breast cancer cell line with the tetracycline-repressible sRII expression plasmids.

Expression of TGF-β sRII in MDA-MB-231 Transfectants. After selecting the transfected cells with G418, we performed RNase protection assays to screen for sRIII-expressing clones. Three clones (designated 34, 41, and 44) were found to express similar levels of sRIII mRNA when compared with the control cells (Neo Pool) as shown in Fig. 1.

We then performed receptor cross-linking assays to ascertain that the sRIII mRNA-positive clones secreted functional sRIII protein. Cross-linking of $^{125}$I-labeled TGF-β1 to the cell surface receptors revealed that the sRII-transfected clones had the same level of membrane-bound RII as the Neo pool (data not shown). However, when the media that were conditioned by the control and sRII cells were incubated with $^{125}$I-labeled TGF-β1, those condition by sRII clones contained a TGF-β binding protein that is characteristic of sRIII (Fig. 2). The binding of $^{125}$I-labeled TGF-β1 could be partially blocked off by 100-fold cold TGF-β1 as shown in the Lane labeled “cold TGFβ” in Fig. 2. Treatment of sRII-transfected cells with 0.1 µg/ml tetracycline (Tet.) before the collection of the conditioned media reduced the TGF-β-binding protein level in the sRII cell-conditioned media to that of the control cell-conditioned medium (Fig. 2). These results suggested that the sRII-transfected clones expressed and secreted high levels of sRIII protein.

We have also developed an sRIII ELISA to quantitate the amount of secreted sRIII in the conditioned media. As shown in Fig. 3, sRIII clones produced significantly higher levels of sRIII than the Neo pool. This is consistent with the affinity-labeling results presented in Fig. 2.

sRIII Cell-conditioned Media Had Reduced Amount of Active TGF-β Isoforms and Growth-inhibitory Activity. Previous studies have shown that MDA-MB-231 cells secrete active TGF-β1, and TGF-β2 into culture medium (13, 18). Because the sRIII was able to bind TGF-β1 in our cross-linking assays, we examined whether the expression of sRIII reduced active TGF-β isoforms in the media conditioned by sRIII clones. Measurement of active TGF-βs with
ELISA kits showed that TGF-β2 levels were much reduced and TGF-β1 levels were reduced to an undetectable level in the media conditioned by sRIII clones in comparison with the Neo pool controls (Fig. 4), which suggests that the active TGF-β isoforms were sequestered by the secreted sRIII. This was confirmed with a bioassay in which we measured the effect of the conditioned medium by the Neo pool or sRIII clone 34 on the growth of mink lung epithelial CCL64 cells that are highly sensitive to the growth-inhibitory activity of TGF-β. The Neo pool-conditioned medium showed significantly (P < 0.01) higher inhibitory activity than the sRIII clone 34-conditioned medium on CCL64 cell growth (Fig. 5A). This inhibitory activity was due to the presence of active TGFβ1 and TGFβ2 because the addition of TGFβ1- and TGFβ2-neutralizing antibodies significantly (P < 0.01) nullified the inhibitory activity of the Neo cell-conditioned medium (Fig. 5B). On the other hand, treatment of sRIII clone 34 with 0.1 μg/ml tetracycline before the collection of the conditioned medium restored the growth-inhibitory activity of its conditioned medium to the level of the Neo cell-conditioned medium (Fig. 5A). These results demonstrated that the sRIII could sequester extracellular active TGF-β isoforms and antagonize their function.

**sRIII Expression Reduced Tumorigenicity in Vivo.** Because sRIII was able to sequester extracellular active TGF-β isoforms, which have been shown to promote tumorigenesis and metastasis in vivo (17), we next tested whether sRIII expression can suppress the in vivo tumorigenicity of the MDA-MB-231 cells. Exponentially growing cells of the Neo pool and three sRIII-expressing clones were inoculated into the inguinal mammary fat pads of athymic nude mice in two separate sets of experiment. Tumor incidence and growth were monitored. In the first set, Neo pool cells formed tumors in 6 of 10 inoculation sites, whereas sRIII clones 41 and 44 formed tumors in only 1 of 10 and 1 of 8 inoculation sites, respectively, over a period of 8 weeks (Table 1). Similar results were also obtained in the second set of experiments. Neo pool cells showed higher tumor incidence (10 of 10) than sRIII clone 34 (1 of 10) and clone 44 (5 of 10; Table 1). TheNeo cell-formed tumors also grew consistently faster in both sets of the experiments (Fig. 6, A and B). Thus, it is evident that sRIII expression reduced both tumor incidence and tumor growth rate.
sRIII Expression Abrogated Lung Metastasis. Increased production of TGF-β by the tumors has been implicated in the development of a metastatic phenotype (19–21). Because MDA-MB-231 cells were shown to be moderately metastatic (17, 22), we further tested whether sRIII cells showed reduced spontaneous lung metastasis after being inoculated in the mammary fat pad. At the termination of the tumorigenicity study, the lungs from the mice inoculated with Neo pool, sRIII clone 34, or clone 44 cells were aseptically removed, digested enzymatically, and cultured in the presence of G418 to select the metastatic tumor cells. Cells isolated from the lungs of a Neo pool-inoculated mouse formed numerous colonies after G418 selection. In contrast, no colonies were observed in the culture wells plated with cells from the lungs of a sRIII clone 34-inoculated mouse (Fig. 7). Using this method, we found that 4 of 5 mice inoculated with Neo pool cells showed lung metastatic cells, whereas all 10 of the mice inoculated with either sRIII clone 34 or clone 44 cells showed no metastatic cells in their lungs. Using an immunohistochemical method, we also stained for human p53 protein as a marker to detect the presence of metastatic MDA-MB-231 cells in the mouse lung tissue sections. The nuclear staining of p53 with an antihuman p53 antibody was evident in the lung tissue sections from Neo pool-inoculated mice, whereas no staining was observed in the lung tissue sections from sRIII cell-inoculated mice. Fig. 8 shows one such example. Thus, it is apparent that sRIII expression abrogated the metastatic potential of the MDA-MB-231 cells.

**DISCUSSION**

TGF-β has been shown to be both tumor-suppressive and tumor-promoting, largely due to its multifunctional nature. The tumor-suppressing activities of TGF-β are mediated by the induction of growth arrest or apoptosis. TGF-β can also contribute to tumor progression by promoting angiogenesis, invasion, and metastasis. The soluble form of TGF-β type III receptor (sRIII) has been shown to suppress TGF-β signaling by binding to the extracellular domain of the receptor and blocking the interaction with the ligand. This study demonstrates that expression of sRIII in MDA-MB-231 cells abrogated the metastatic potential of these cells, providing evidence for the role of sRIII in modulating TGF-β signaling and tumor progression.
suppressive activity of TGF-β is demonstrated or suggested from the studies on the role of TGF-β and its receptors during neoplastic transformation and tumor progression. Overexpression of TGF-β₁ in transgenic mice was shown to inhibit the formation of carcinogen-induced mammary and skin tumors (23, 24), whereas the blockade of TGF-β signaling in transgenic mice overexpressing a dominant negative mutant TGF-β type II receptor rendered the mice more susceptible to carcinogen-induced mammary gland tumor formation (25). Enhancement of autocrine TGF-β activity by the overexpression of TGF-β receptors was shown to suppress tumorigenicity and revert malignant phenotypes in cancer cells with down-regulated or inactivated TGF-β receptors (4). These and many other published studies indicate that autocrine TGF-β signaling is tumor-suppressive in epithelial cells. Thus, it is not surprising that impaired or lost autocrine TGF-β signaling is often a hallmark during malignant progression in many types of cancer (4).

On the other hand, despite the down-regulation or loss of TGF-β receptors or its intracellular signaling components, neoplastic transformation and malignant progression in various cancers have been shown to be associated with increased expression of TGF-β isoforms. They include breast (19, 26), colon (27, 28), prostate (21), bladder (29), pancreatic (30), and gastric cancers (31), and melanoma (32). More significantly, many of these studies showed that the increased TGF-β production was also associated with poor pathological or clinical outcomes such as higher tumor grade, greater vascular counts, more metastases, and shorter survival time, which suggests that the excessive amount of TGF-β may promote malignant progression. Indeed, ectopic expression of TGF-β₁ in various cell lines has been shown to promote tumor progression (7, 33, 34). On the other hand, the administration of a TGF-β neutralizing antibody inhibited the tumorigenicity of the MDA-MB-231 cells in nude mice (17). Therefore, tumor cells seem to down-regulate their own TGF-β signaling components to evade the autocrine growth suppression but to up-regulate TGF-β production to enhance its paracrine tumor-promoting activity. These findings suggest that a useful strategy for the therapeutic manipulation of the TGF-β system may include the augmentation of the suppressive autocrine TGF-β activity and/or attenuation of the tumor-promoting paracrine TGF-β activity.

In this study, we investigated whether soluble RIII can act as a TGF-β sink to antagonize its paracrine tumor-promoting activity. A soluble RIII had previously been shown to inhibit TGF-β₁ and TGF-β₂-binding to cell surface receptors and to antagonize TGF-β₂ activity in vitro (35). Because the MDA-MB-231 cells produce both TGF-β₁ and TGF-β₂, which were shown to contribute to the malignant properties of this cell line (17), we overexpressed an sRIII in this cell line. Overexpression of the sRIII reduced extracellular levels of active TGF-β₁ and TGF-β₂. Consequently, the medium conditioned by sRIII cells showed significantly lower growth-inhibitory activity in CCL64 cells than the medium that was conditioned by the control cells. When inoculated into nude mice, the sRIII cells were consistently less tumorigenic than the control cells in two separate experiments. The expression of sRIII also suppressed lung metastasis of the cells. Thus, our study demonstrates that the tumor-suppressive activity of RIII that was described previously (13) does not seem to require the membrane anchorage and its intracellular domain.

Although proteoglycans have been implicated in tumorigenesis, their precise role remains to be defined. Studies have shown that the biosynthesis of proteoglycans is generally associated with enhanced tumorigenicity and metastasis (36). Thus, it seems unlikely that the sRIII-mediated tumor suppression was simply due to its being a proteoglycan. On the other hand, previous studies have shown that the antagonization of tumor-cell-produced TGF-β can suppress the malignant phenotype of tumor cells (17, 37), whereas overexpression of, or pretreatment with, TGF-β can stimulate the invasion and metastasis of transformed cells (20, 23). Therefore, the mechanism of the sRIII-mediated suppression of the malignant phenotype seems to be due more consistently to its ability to antagonize the tumor-promoting activity of TGF-β. It is also worth noting that the majority of tumors formed by sRIII cells in our study grew significantly slower than those formed by the control cells (Fig. 6). The restricted tumor cell proliferation in vivo may be due to limited nutrient supply, which is consistent with the role of TGF-β in stimulating angiogenesis in vivo (5). Moreover, the relatively lower tumor incidence in the sRIII cell-inoculated mice may be due to the clearance of the sRIII cells by natural killer cells in nude mice before they can form any visible tumors. This notion is supported by the fact that TGF-β can suppress the proliferation and cytolytic activity of natural killer cells (38) and that neutralization of tumor cell-produced TGF-β can stimulate natural killer cell activity (17). Thus, sRIII may be a potent TGF-β antagonist on many levels.

Several truncated soluble growth-factor receptors have recently been shown to possess potent antagonistic activity against their ligands. For example, a soluble insulin-like growth factor-I receptor was shown to inhibit in vivo metastasis of human breast cancer MDA-MB-435 cells (39). Similarly, expression of a soluble vascular endothelial growth factor receptor in human cancer cells was shown to significantly inhibit their growth and metastasis in vivo and prolong the survival time of xenograft-bearing mice (40). In the case of TGF-β, the utility of its antagonist may not be limited to its antagonization of TGF-β-induced angiogenesis, invasion, metastasis, and suppression of host immunosurveillance. Recent studies have shown that expression of TGF-β renders cancer cells more resistant to cytotoxic drugs, which can be reversed by its neutralizing antibody (41, 42). Being a potent suppressor of many immune system functions (38), TGF-β isoforms produced by cancer cells will undoubtedly negate the effectiveness of a cancer immunotherapy. The development of a TGF-β antagonist such as the sRIII may be potentially useful in cancer treatment not only because it can directly suppress malignancy but also because it can increase the efficacy of cytotoxic chemotherapy or immunotherapy.

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