Expression of Transforming Growth Factor β Type III Receptor Suppresses Tumorigenicity of Human Breast Cancer MDA-MB-231 Cells*

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Transforming growth factor β (TGF-β) promotes tumor progression in some model systems including human breast cancer cells. In this study, we report that human breast cancer cell lines express reduced amounts of TGF-β type III receptor (RIII) when compared with untransformed human mammary epithelial cells. Consequently, we examined whether expression of RIII in human breast cancer MDA-MB-231 cells could reduce TGF-β’s tumor promoting activity by sequestering active TGF-β isoforms produced by the cells. A tetracycline-repressible human RIII expression vector was stably transfected into the cell line. RIII expression in a pool of transfected clones and a single clone was found to be reversibly repressed by tetracycline treatment. Expression of RIII reduced the amount of active TGF-β₁ and TGF-β₂ in the conditioned medium. The medium conditioned by control cells showed a significantly higher growth inhibitory effect than that conditioned by RIII-transfected cells on the growth of the mink lung epithelial CCL64 cells. A conditioned medium collected from RIII-transfected cells treated with tetracycline significantly increased its growth inhibitory activity to that of control cells. Expression of RIII also reduced tumor incidence and growth rate in two separate experiments when the cells were inoculated in athymic nude mice. Treatment of the mice with tetracycline repressed RIII expression in the tumors generated by RIII-transfected cells and increased tumor incidence and growth rate. These results suggest that TGF-β RIII can reduce tumorigenicity of MDA-MB-231 cells apparently by sequestering TGF-β isoforms produced by these cells.

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1 The abbreviations used are: TGF-β, transforming growth factor β; bFGF, basic fibroblast growth factor; RI, RII, and RIII, TGF-β receptor types I, II, and III, respectively.

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TGF-β RIII Suppresses Tumorigenicity

paracrine TGF-β tumor-promoting activity in highly progressed cancers may offer a novel approach to reduce malignancy.

Since TGF-β RIII binds all three TGF-β isoforms with high affinity with Kr values ranging from 50 to 300 pM depending on cell type (4, 47, 48), we hypothesized that cell surface RIII can cause sequestration of TGF-βs to antagonize their paracrine tumor-promoting activity. To test this hypothesis, we expressed RIII in human breast cancer MDA-MB-231 cells. Our studies show that RIII-transfected cells released less active TGF-βs in the conditioned medium and were less tumorigenic in athymic nude mice than the control cells. These results suggest that RIII could be a novel target for antagonizing paracrine TGF-β tumor-promoting activity.

MATERIALS AND METHODS

Cell Culture—Human breast cancer cell lines MDA-MB-231, ZR75–1, and Hs578T, human untransformed mammary myoepithelial cell line Hs578Bst, and mink lung epithelial cell line CCL64 were originally obtained from the Michigan Cancer Foundation. These cell lines were cultured in McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, antibiotics, and 10% fetal bovine serum (49). Human colon cancer cell lines CBS and FET were generously provided by Dr. M. G. Brattain (50). They were adapted to serum-free McCoy’s 5A medium supplemented with pyruvate, vitamins, amino acids, 10 ng/ml epidermal growth factor, 20 μg/ml insulin, and 4 μg/ml transferrin (49). 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.

RIII Expression Vector Construction and Transfection—The full-length cDNA of human TGF-β R III, generously provided by Dr. C.-H. Heldin, was subcloned into a tetracycline-repressible expression system as described previously (19). The sense orientation of the RIII cDNA was confirmed by restriction digestion and agarose gel electrophoresis. We used the tetracycline-repressible expression system to express RIII because we wished to demonstrate the specificity of the effect of RIII expression on extracellular mature TGF-β levels and on in vivo tumorigenicity within the same transfected clone.

The expression vectors were linearized and transfected into one of MDA-MB-231 limiting dilution clones with a BTX Electro Cell Manipulator at 250 V and 950 microfarads. The control cells were transfected with the empty vectors. The transfected cells were plated in 10-cm culture dishes and maintained in the 10% fetal bovine serum medium for 2 days. Selection of stable transfecants was accomplished by adding 1 mg/ml Geneticin (G418 sulfate; Life Technologies, Inc.) to the culture medium at 600 μg/ml. G418-resistant, RIII-transfected clones were pooled in one dish and ring-cloned in another dish. Control clones were pooled and designated as Neo pool. The cell lines were expanded for screening of RIII expression.

RNA Analysis—Total RNA from various cell lines was isolated by guanidine thiocyanate homogenization and acidic phenol extraction (51). To isolate total RNA from xenografts, they were first pulverized in liquid nitrogen with a mortar and a pestle. The tissue powder was then homogenized in a 4× guanidine thiocyanate solution. Total RNA was isolated by ultracentrifugation through a cesium gradient as described by Chirgwin et al. (52). To measure RIII mRNA levels in various cell lines, we constructed a human RIII riboprobe by inserting the full-length RIII cDNA into pBSK(+) plasmid (Stratagene Cloning Systems). The recombinant plasmid was linearized and transfected into one of MDA-MB-231 cells. The transfected cells were plated in 6-well plates and treated with or without 0.1 μg/ml tetracycline until they reach 100% confluence. The media were replenished and conditioned for 72 h. The media were then collected and cell number in each well was counted with a hemocytometer. The conditioned media (10 μl) from duplicate wells for each cell type were added to CCL64 cells after they were plated in a 96-well plate. After 5 days of incubation, relative cell number in each well was obtained with 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (55) and expressed as optical density.

RESULTS

RIII mRNA Levels in Human Breast Cancer Cells—Human breast cancer cell lines have been shown to express readily detectable amounts of one or more TGF-β isoforms (21, 58). Yet, many of them are resistant to TGF-β in growth inhibition assays (21, 22, 59). As a result, the TGF-β isoforms in these cells could act in a paracrine fashion to promote tumor growth. To assess the potential use of RIII to antagonize the paracrine TGF-β activity, we first compared RIII expression levels in four human breast cancer cell lines (MDA-MB-231, ZR75–1, Hs578T, and MCF-10A) with those in two untransformed human mammary cell lines (Hs578Bst and MCF-10A) and two well differentiated, TGF-β-sensitive human colon cancer cell lines (CBS and FET). RNase protection assays revealed that all four breast cancer cell lines express very low levels of RIII mRNA as shown in Fig. 1. In contrast, the untransformed mammary epithelial MCF-10A cells, myoepithelial Hs578Bst cells, and the two well differentiated colon cancer CBS and FET cells express relatively high levels of RIII mRNA. These results suggest that RIII may be down-regulated during mammary neoplastic transformation and progression.

RIII Expression in MDA-MB-231 Cells—To test our hypothesis that re-expression of RIII in breast cancer cells could lead to sequestration of endogenous TGF-β and consequently suppression of its paracrine tumor-promoting activity, we transfected tetracycline-repressible human RIII expression vectors 25368 methanesulfonyl fluoride. Equal amounts of cell lysate protein were electrophoresed in 4–10% gradient SDS-polyacrylamide gel electrophoresis under reducing conditions and exposed for autoradiography.

Measurement of Growth Inhibitory Activity of Media Conditioned by Control and RIII-transfected Cells—A bioassay was used to measure growth inhibitory activity of the media conditioned by control and RIII-transfected cells on the growth of the mink lung epithelial CCL64 cells as described previously (56). Briefly, control and RIII-transfected cells were plated in 6-well plates and treated with or without 0.1 μg/ml tetracycline until they reach 100% confluence. The media were replenished and conditioned for 72 h. The media were then collected and cell number in each well was counted with a hemocytometer. The conditioned media (10 μl) from duplicate wells for each cell type were added to CCL64 cells after they were plated in a 96-well plate. After 5 days of incubation, relative cell number in each well was obtained with 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (55) and expressed as optical density.
into a typical limiting dilution clone of MDA-MB-231 cells. We chose this cell line for our study because it was shown that endogenous TGF-β isoforms could increase tumorigenicity of MDA-MB-231 cells (60).

After selecting the transfected cells with G418, we used RNase protection assays to screen a pool of G418-resistant clones as well as several individual clones for RIII expression and found that both the pool and one of the clones expressed higher levels of RIII mRNA than the control vector-transfected cells (Neo pool) as shown in Fig. 2. They were designated the RIII pool and RIII clone, respectively. We then used receptor cross-linking assays to examine TGF-β RIII protein levels on the surface of Neo pool, RIII pool, and RIII clone cells. Consistent with the RNA data, RIII pool and RIII clone cells also expressed higher levels of cell surface RIII protein than Neo pool cells (Fig. 3). The specificity of the increased 125I-TGF-β
to RIII was confirmed by competition with a 100-fold excess of unlabeled TGF-β (Fig. 3, third lane). Treatment of RIII pool and RIII clone with 0.1 μg/ml tetracycline for 4 days prior to the receptor cross-linking assay reduced their RIII protein levels to that of the Neo pool, indicating that tetracycline could reversibly regulate the expression of transfected RIII. This was particularly useful in subsequent experiments for us to demonstrate the specificity of the effect of RIII expression on the growth inhibitory activity of the media conditioned by RIII-transfected cells and on the tumorigenicity of MDA-MB-231 cells.

Growth Inhibitory Activity of Conditioned Media—MDA-MB-231 cells are known to express TGF-β1 and TGF-β2 mRNA (58) and to release active TGF-β into culture media (15, 21). Therefore, we hypothesized that RIII expression should result in sequestration of active TGF-β isoforms and reduction of active TGF-β isoforms in conditioned media. To test this hypothesis, we first measured the effect of the media conditioned by Neo pool or RIII clone on the growth of mink lung epithelial CCL64 cells which are highly sensitive to TGF-β’s growth inhibitory activity. While Neo pool-conditioned medium significantly (p < 0.0005 by Student’s t test) inhibited CCL64 cell growth in comparison with no conditioned medium in Fig. 4, RIII clone-conditioned medium had almost no growth inhibitory activity. In contrast, the medium conditioned by RIII clone that was treated with 0.1 μg/ml tetracycline showed growth inhibitory activity similar to that of the medium conditioned by Neo pool treated with tetracycline. This suggests that cell surface RIII can regulate the level of growth inhibitory activity in the conditioned medium.

To confirm that the reduced growth inhibitory activity in RIII clone-conditioned medium was due to a reduced amount of active TGF-β1 and TGF-β2, we used enzyme-linked immunosorbent assays to measure active TGF-β1 and TGF-β2 in the media conditioned by Neo pool and RIII clone. While we could detect active TGF-β1 and TGF-β2 in Neo pool-conditioned medium at 49 and 21 pg/10⁶ cells/72 h, respectively, we could not detect either active isoform in RIII clone-conditioned medium suggesting that RIII significantly reduced the amount of active TGF-β released into the culture medium.

Tumorigenicity—The MDA-MB-231 cell line used in this study was insensitive to the growth inhibitory activity of TGF-β, similar to what was described by Kalkhoven et al. (22). Expression of RIII did not alter its TGF-β sensitivity and in
vitro growth property (data not shown). Since our hypothesis was that RIII could antagonize the tumor-promoting activity of paracrine TGF-β by sequestering active TGF-β isoforms released by tumor cells, we inoculated exponentially growing cells of Neo pool, RIII pool, and RIII clone into athymic nude mice at 3 × 10^6 cells per site and followed progression of xenograft formation. As shown in Table I, Neo pool cells formed xenografts in 6 of 8 inoculation sites, whereas both Neo pool and RIII clone cells formed only one xenograft out of 8 inoculation sites in a period of 10 weeks. The average volume of Neo pool-formed tumors was larger than those formed by RIII pool or RIII clone at the end of the experiment. Thus, it appears that RIII expression reduced both tumor incidence and tumor growth rate.

A second experiment was performed to test whether the reduced tumorigenicity of RIII-transfected MDA-MB-231 cells was specifically due to RIII expression. Ten nude mice were inoculated with each cell type at 4 × 10^6 cells per site. Half of the mice were given 5% sucrose solution as drinking water, while the other half were given 5% sucrose solution containing 0.5 mg/ml tetracycline. Tetracycline treatment had no noticeable effect on the growth and behavior of the mice. Consistent with the results from the first experiment, Neo pool cells generated higher tumor incidence (6 out of 10) than RIII clone cells (3 out of 10) during an 8-week period of study (Table II). The average volume of Neo pool-formed tumors was more than 2-fold larger than that of RIII clone-formed tumors at the end of the experiment. While tetracycline treatment had no effect on the tumorigenicity of Neo cells (Table II and Fig. 5A), it increased tumor incidence of RIII clone cells from 30 to 90% (Table II), and the tumor growth rate as well (Fig. 5B). The terminal average volume of RIII clone-formed tumors was increased by more than 3-fold after tetracycline treatment. These results suggest that the reduction of tumorigenicity of RIII-transfected cells was specifically due to RIII expression.

To confirm that tetracycline treatment repressed the expression of the transfected RIII in the RIII clone-formed tumors, we extracted total RNA from resected tumors. RNase protection assays were performed with a human-specific RIII riboprobe to measure human RIII mRNA levels in the tumor RNA samples. Fig. 6 shows human RIII mRNA levels in two xenografts from each treatment. The endogenous RIII levels in Neo pool-formed tumors were similar between the control (lanes 3 and 4) and tetracycline treatment (lanes 5 and 6). The RNA sample in lane 3 was somewhat less than those in other lanes as indicated by its actin mRNA level. Tetracycline treatment reduced RIII mRNA levels in RIII clone-formed tumors (lanes 7 and 8) to the levels (lanes 9 and 10) similar to those of Neo pool-formed tumors suggesting that RIII expression can also be regulated in vivo by tetracycline.

![FIG. 4. Growth inhibition of CCL64 cells by conditioned media from control and RIII-transfected cells. The medium (10 µl) conditioned by Neo pool (Neo) or RIII clone (RIII) cells that were treated with or without tetracycline (Tet.) was collected from two duplicate wells and added to newly plated CCL64 cells in a 96-well plate. After 5 days of incubation, the relative cell number in each well was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described under “Materials and Methods” and expressed as optical density. Each column represents the mean ± S.E. from 8 wells. The relative cell number in the wells that did not receive any conditioned medium is labeled as “no CM.”](Image 80x528 to 275x729)

![FIG. 5. Xenograft growth curves of MDA-MB-231 transfectants. Exponentially growing cells (4 × 10^6) of Neo pool (panel A) and RIII clone (panel B) were subcutaneously inoculated behind the anterior forelimb of 4-week-old athymic nude mice. Ten mice were inoculated at both sides of the back with each type of cells. Half of the mice were given tetracycline-containing drinking water. Tumors were measured externally in two dimensions using a caliper. Volume was calculated according to the equation: V = (L × W^2) × 0.5, where L is length and W is width of a tumor. Due to different tumor incidence under different treatment, the mean and S.E. of each treatment was calculated from different number of tumors as depicted in the parentheses.)](Image 314x301 to 557x385)

### Table I

| Cell line | Tumors at 6 weeks | Tumors at 8 weeks | Tumors at 10 weeks | Terminal mean tumor volume |
|-----------|------------------|------------------|--------------------|---------------------------|
| Neo pool  | 2/8              | 5/8              | 6/8               | 865 (n = 6)               |
| RIII pool | 0/8              | 1/8              | 1/8               | 518 (n = 1)               |
| RIII clone| 0/8              | 0/8              | 1/8               | 219 (n = 1)               |

One of the mice in this group had to be euthanatized before the termination of the experiment because one of its tumors grew too fast. Therefore, the terminal mean tumor volume was obtained from seven tumors instead of nine.

**FIG. 6** shows human RIII mRNA levels in two xenografts from each treatment. The endogenous RIII levels in Neo pool-formed tumors were similar between the control (lanes 3 and 4) and tetracycline treatment (lanes 5 and 6). The RNA sample in lane 3 was somewhat less than those in other lanes as indicated by its actin mRNA level. Tetracycline treatment reduced RIII mRNA levels in RIII clone-formed tumors (lanes 7 and 8) to the levels (lanes 9 and 10) similar to those of Neo pool-formed tumors suggesting that RIII expression can also be regulated in vivo by tetracycline.

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Enhanced autocrine TGF-β isoforms, TGF-β have been suggested to stimulate tumorigenesis (60). For example, in vivo injection of TGF-β into FET and CBS cell lines led to increased tumorigenicity (67). A more recent study showed that a rat gliosarcoma cell line with reduced TGF-β expression by antisense TGF-β/RNA could be used as a vaccine to immunize gliosarcoma-bearing rats and suppress tumor growth (68). TGF-β has also been shown to be angiogenic (43). Overexpression of TGF-β1 in Chinese hamster ovary cells significantly stimulated tumor growth and angiogenesis when they are inoculated into nude mice and the effects could be attenuated by peritumoral injection of a TGF-β neutralizing antibody (44). Therefore, aberrant expression of TGF-β isoforms could promote tumor progression.

In this study, we used RIII to antagonize the paracrine TGF-β activity in human breast cancer MDA-MB-231 cells. Expression of RIII reduced the levels of active TGF-β1 and TGF-β2 in the conditioned medium to an undetectable level. More importantly, RIII-transfected cells showed reduced tumor incidence and growth rate when inoculated in nude mice. Our results are consistent with an earlier study which showed that intraperitoneal injection of a TGF-β neutralizing antibody, which recognized all three TGF-β isoforms, was able to suppress tumorigenicity of MDA-MB-231 cells when inoculated intra-abdominally in nude mice during a 3-week study (60). Since we could reversibly regulate the expression of transfected RIII, the amount of extracellular free TGF-β isoforms, and the tumorigenicity of this cell line in vivo using tetracycline, our system is uniquely suited for the investigation of the role of endogenous TGF-β in tumorigenesis, angiogenesis, host immune surveillance, and tumor cell invasion and metastasis.

In addition to TGF-β RIII, a number of other proteins have been shown to bind TGF-β isoforms and regulate TGF-β activity, such as decorin, endoglin, and TGF-β1 latency-associated peptide. Human decorin binds TGF-β isoforms with lower affinity than RIII (69). Its effect on TGF-β activity is quite controversial. It has been found to antagonize TGF-β activity (70), enhance it (71), or have no consistent effect on it (69). Human endoglin binds TGF-β1 and TGF-β3 with high affinity, but does not bind TGF-β2 (72). The affinity of TGF-β1 latency-associated peptide for three TGF-β isoforms was shown to be similar to or lower than that of RIII (73). It can inhibit TGF-β activity in vitro and in vivo (74, 75). However, there are important differences between RIII and these TGF-β-binding proteins. In addition to its high affinity for all three TGF-β isoforms, RIII was recently shown to restore autocrine TGF-β activity in human breast cancer MCF-7 cells (10). This suggests that expression of RIII in cancer cells can not only antagonize paracrine tumor-promoting activity of endogenous TGF-β isoforms, but also possibly enhance their autocrine tumor-suppressing activity if the cancer cells are inhibited by TGF-β. As such, RIII may be a better candidate than other TGF-β-binding proteins to be targeted for regulation of TGF-β activity in favor of cancer chemotherapy.

Being a membrane heparan/chondroitin sulfate proteoglycan, RIII has been shown to bind basic fibroblast growth factor (bFGF) in addition to TGF-β isoforms (76). bFGF binds heparan sulfate with low affinity and is known to stimulate angiogenesis by regulating the growth and migration of endothelial cells (77). Thus, even though RIII has high affinity for TGF-β isoforms and was shown to reduce TGF-β levels in the conditioned medium, we cannot rule out the possibility that the reduction of tumorigenicity after RIII expression was partially due to the sequestration of bFGF by RIII. In COS-1 cells, overexpression of RIII did not increase the amount of bFGF binding to the cell surface (76), suggesting that although RIII can bind bFGF, it does not significantly contribute to the overall bFGF binding to the cell surface. Whether heparan sulfate domain of RIII is necessary for RIII-mediated tumor suppression in MDA-MB-231 cells remains to be elucidated.

**Fig. 6. RIII mRNA levels in xenografts.** At the termination of the tumorigenicity experiment, total RNA was extracted from two randomly selected xenografts formed in the mice that were inoculated with Neo pool (Neo) or RIII clone (RIII) and treated with or without tetracycline (Tet). RNase protection assays were performed using human-specific RIII and actin antisense riboprobes and 20 μg of total RNA to determine RIII levels in the xenografts from different treatments. Human actin mRNA levels were used for normalization.

**DISCUSSION**

TGF-β isoforms are potent growth inhibitors in a variety of cell types including some carcinoma cell lines in vitro (61, 62). However, their role of controlling tumor cell growth in vivo is quite controversial. Suppression of TGF-β1 and TGF-β2 expression in two well differentiated, TGF-β-sensitive colon carcinoma FET and CBS cell lines led to increased tumorigenicity in vivo (17, 18), suggesting that autocrine TGF-β activity is tumor-suppressive. In contrast, overexpression of TGF-β in other cell types, including those that are inhibited by TGF-β in vitro, resulted in enhanced tumorigenicity in vivo (45, 46, 63, 64). Enhanced autocrine TGF-β activity by receptor overexpression in cell lines or TGF-β1 overexpression in transgenic mice has been shown to suppress colon and mammary tumorigenesis in vivo (19, 20, 65, 66). Therefore, it is likely that if a cell is inhibited by TGF-β and expresses enough TGF-β receptors to bind active TGF-β isoforms in the extracellular compartment, suppression of endogenous TGF-β expression would attenuate the autocrine inhibitory activity elicited by the endogenous TGF-β and consequently increase tumorigenicity. On the other hand, if a cell expresses a high level of TGF-β and inadequate amount of TGF-β receptors, TGF-β would bind to its receptors in stromal cells to generate a paracrine activity.

As a multifunctional growth factor, some of TGF-β's effects have been suggested to stimulate tumorigenesis (60). For example, TGF-β isoforms are potent immune suppressors in that they can inhibit proliferation, activation, and differentiation of various types of lymphocytes (42). Overexpression of TGF-β1 in highly immunogenic murine tumor cells was shown to stimulate tumor growth by helping tumor cells escape host immune surveillance (67). A more recent study showed that a rat gliosarcoma cell line with reduced TGF-β expression by antisense TGF-β/RNA could be used as a vaccine to immunize gliosarcoma-bearing rats and suppress tumor growth (68). TGF-β has also been shown to be angiogenic (43). Overexpression of TGF-β1 in Chinese hamster ovary cells significantly stimulated tumor growth and angiogenesis when they are inoculated into nude mice and the effects could be attenuated by peritumoral injection of a TGF-β neutralizing antibody (44). Therefore, aberrant expression of TGF-β isoforms could promote tumor progression.

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