Reduced Expression of Transforming Growth Factor β Type I Receptor Contributes to the Malignancy of Human Colon Carcinoma Cells*

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Transforming growth factor β (TGFβ) type I (RI) and type II (RII) receptors are essential for TGFβ signal transduction. A human colon carcinoma cell line, designated GEO, is marginally responsive to TGFβ and expresses a low level of RI mRNA relative to colon carcinoma cells, which are highly responsive to TGFβ. Hence, the role of RI as a limiting factor for TGFβ sensitivity and the contribution of low RI levels to the malignant phenotype of GEO cells were examined. Stable transfection of a tetracycline-regulatable rat RI cDNA increased TGFβ1 binding to RI and resulted in increased growth inhibition by exogenous TGFβ1. In contrast, although stable transfection of an RII expression vector into the same GEO cells increased TGFβ2 binding to RII, growth inhibition by exogenous TGFβ2 was not altered. This indicated that the low level of RI is a limiting factor for the growth-inhibitory effects of TGFβ in GEO cells. RII-transfected cells were growth-arrested at a lower saturation density than GEO control cells. They also showed reduced growth and clonogenicity in plating efficiency and soft agarose assays, whereas RII-transfected cells did not show any differences from the NEO control cells in these assays. Tetracycline repressed RI expression in transfected cells and reversed the reduction in plating efficiency of RI-transfected clones, confirming that growth effects were due to increased RI expression in transfected cells. TGFβ1 neutralizing antibody stimulated the proliferation of RI-transfected cells but had little effect on GEO control cells, indicating that increased autocrine-negative TGFβ activity also resulted from increased RI expression. Tumorigenicity in athymic nude mice was significantly delayed in RI-transfected cells. These results indicate that low RI expression is a limiting factor for response to exogenous TGFβ, as well as TGFβ autocrine-negative activity, and that reduction of RI expression can contribute to malignant progression.

Transforming growth factor βs (TGFβs) are a family of multifunctional cytokines that regulate many aspects of cellular function including proliferation, differentiation, adhesion, and migration (1-3). The role of TGFβ in growth regulation is of particular interest in cancer. TGFβ has been shown to be an autocrine-negative growth factor, as evidenced by stimulation of growth of several cell lines treated with TGFβ-neutralizing antibody (4-8). Loss of autocrine TGFβ activity and/or responsiveness to exogenous TGFβ appears to provide cells with a growth advantage leading to malignant progression. For example, loss or reduction of TGFβ response has been shown to be associated with tumor development and progression in a number of cancer cell lines as well as the conversion of colon adenomas to carcinomas (9-12). Previous work in our laboratory showed that suppression of autocrine TGFβ activity by constitutively repressing endogenous TGFβ expression without eliminating the ability to respond to exogenous TGFβ led to a more progressed phenotype in colon cancer cells (6, 7). This suggested that loss of autocrine TGFβ function was a key feature in the development of malignant properties of these colon carcinoma cells. Restoration of TGFβ responsiveness and/or autocrine TGFβ activity by reconstitution of the autocrine TGFβ loop led to reduced malignancy in several carcinoma cell lines (8, 10, 13).

TGFβs exert their effects through binding to specific cell surface proteins. Three major types of TGFβ receptors, type I (RI), type II (RII), and type III (RIII), have been identified in most cells by receptor-affinity labeling assays (1, 2). RI and RII are glycoproteins of M, 53,000 and M, 75,000, respectively, whereas RIII is a proteoglycan of M, 280,000-330,000. TGFβ signals through a heteromeric complex of RI and RII. Both RI and RII belong to the transmembrane serine/threonine kinase receptor family (14-17). It has been shown that RII is required for the binding of TGFβ to RI, whereas RI is required for signal transduction (15, 18). The ability of the two receptors to undergo heteromeric interactions and the necessity of phosphorylation of RI by RII kinase to confer signal transduction further reinforces the evidence that both receptors are required for TGFβ response (18, 19). The direct involvement of RI and RII in TGFβ signal transduction would suggest that loss of functional RI and/or RII expression could contribute to loss of TGFβ responsiveness, resulting in tumor progression. The lack of response to TGFβ in some types of cancer and tumor cell lines

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The abbreviations used are: TGFβ, transforming growth factor β; RI, RII, RIII, receptor types I, II, III, respectively; tTA, tetracycline-controlled transactivator; MTI, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); ECM, extracellular matrix.
has been reported to be associated with the loss of RII. Re-expression of RII has restored TGFβ sensitivity and generated reversal of malignant properties in some of these systems (8, 10, 13, 20–23). Since RII is as necessary as RII for TGFβ signal transduction, it would seem likely that disruption of this TGFβ receptor could also contribute to loss of TGFβ response and tumor-suppressive activity. Resistance to TGFβ resulting from RII loss has been shown in mutagenized mink lung cells, and re-expression of this receptor led to regeneration of TGFβ response (17, 24–26). However, loss or reduced expression of RII has not been directly demonstrated to have a role in determining malignant properties.

We identified a group of colon carcinoma cell lines that showed reduced RI expression (21). One of these cell lines, designated GEO, has been characterized as being relatively insensitive to TGFβ inhibition (9). Here we report that reconstruction of RI expression in GEO cells increased TGFβ growth-inhibitory effects and autocrine TGFβ activity with a concomitant reduction of tumorigenicity in athymic mice. In contrast, increased expression of RII in GEO cells did not affect TGFβ sensitivity or other GEO biological properties, thus indicating that RI is the limiting factor for TGFβ signal transduction in these cells.

MATERIALS AND METHODS

Cell Culture—The GEO human colon carcinoma cell line was established in vitro from a primary tumor, as described previously (27). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2. In McCoy's 5A serum-free medium (Sigma) supplemented with pyruvate, vitamins, amino acids, antibiotics, 10 ng/ml epidermal growth factor, 20 µg/ml insulin, and 4 µg/ml transferrin (28).

Rat RI Expression Vector Construction and Transfection—The rat TGFβ RI cDNA (~3 kilobases; Ref. 29) was subcloned into a tetracycline-regulated expression system kindly provided by Dr. H. Bujard at University of Heidelberg, Heidelberg, Germany (30). As described previously (13), this system includes a tTA, which is generated by fusing the tetr repressor with the activating domain of virion protein 16 of herpes simplex virus, and an expression vector that consists of a tet operator sequence and a minimal promoter sequence derived from the human cytomegalovirus promoter. When the tetracycline repressor of the tTA binds to tet operators, the virion protein 16 domain of the tTA can activate the minimal promoter to start transcription. Tetracycline can block this activation by preventing the tTA from binding to the tet operator sequence. A neomycin-resistant gene under control of the mouse β-globin promoter was subcloned into the tTA-containing plasmid. This plasmid (2 µg) and the rat RI expression vector (20 µg) were linearized and transfected into GEO cells. Electroporation was carried out at 250 V, 960 microfarads with a GenePulser (Bio-Rad). The control cells were similarly transfected with 2 µg of NEO-containing plasmid and 20 µg of the expression vector without rat RI cDNA. The transfected cells were allowed to grow for 2 days before being subjected to selection with 600 µg/ml geneticin (G418 sulfate; Life Technologies, Inc.). Stable cell clones resistant to G418 sulfate were cloned after transfection control pool for transfected RI and RII mRNA levels by NEO clones 32, 46, and 53 and RII clone 37) were compared with the NEO control, TGFβRI and human TGFβRII cDNA plasmid (205-base pair) was iodinated by the chloramine-T method (32) and utilized to visualize TGFβ receptors after cross-linking with disuccinimidyl suberate, as described previously (8, 13). DNA Synthesis Assay—[3H]Thymidine incorporation was used to determine TGFβ sensitivity of the control and transfected cells to exogenous TGFβ treatment (13). The cells were plated in 24-well tissue culture plates at a density of 3.0 × 10^4 cells/well with various concentrations of TGFβRI. Exponential cells were labeled on day 4 with [3H]thymidine (7 µCi; 46 Ci/mmol; Amersham Corp.) for 1 h. DNA was then precipitated with 10% trichloroacetic acid and solubilized in 0.2 M NaOH. The amount of [3H]thymidine incorporated was analyzed by liquid scintillation counting in a Beckman LS5700 scintillation counter.

Plating Efficiency Assay—Plating efficiency assays were performed as described previously (8) to study the effects of increased RI or RII expression on clonogenic potential at a low seeding density. GEO cells and GEO RI and RII transfecants and control cells were plated in 24-well plates at a density of 400 cells/well with McCoy's 5A serum-free medium. TGFβRI neutralizing antibody (R&D Systems) was produced in chickens immunized with purified recombinant human TGFβ1. This antibody neutralizes the biological activity of recombinant human TGFβ1, porcine TGFβ1, and porcine TGFβ12. TGFβ1 is the only isoform expressed by GEO cells. TGFβ1 neutralizing antibody was added to the medium at a final concentration of 10 µg/ml to determine autocrine TGFβ activity.

Cell colonies were visualized by staining with MIT (Sigma) and then solubilized with dimethyl sulfoxide for optical density measurements.

Soft Agarose Assay—Soft agarose assays were performed as described previously (6, 8, 13) to compare the clonogenic potential of control cells and RI- and RII-transfected cells in semisolid medium. Briefly, cells were suspended at 3.0 × 10^3 cells/ml in 1 ml of 0.4% SeaPlaque agarose in McCoy's 5A serum-free medium and plated on top of 1 ml of 0.8% agarose in the same medium in 6-well tissue culture plates. Plates were incubated for 2–3 weeks at 37°C with 5% CO2 in a humidified incubator. Cell colonies were visualized by staining with 0.5 ml of p-iodonitrotetrazolium violet (Sigma).

Tumorigenicity—Tumorigenicity studies were performed as described previously (6, 8, 13). Briefly, exponentially growing GEO transfecants and control cells were inoculated subcutaneously behind the anterior forelimb of 4-week-old athymic mice (athymic nu/nu-CWRU Cancer Center athymic mouse colony). Mice were maintained in a pathogen-free environment. Growth curves for xenografts were determined by externally measuring tumors in two dimensions. The volume was determined by the following equation: \( V = (L \times W)^2 \times 0.5 \), where \( V \) is volume, \( L \) is length, and \( W \) is width.
RESULTS

Expression of Transfected RI and RII—To demonstrate that RI is the limiting receptor in TGF β signal transduction in GEO cells, we stably transfected rat RI and human RII cDNA separately into GEO cells that expressed a low level of endogenous RI and normal levels of RII relative to TGF β-sensitive human colon carcinoma cell lines (data not shown). Several positive clones (designated RI clones 32, 46, and 53 and RII clone 37) with high levels of transfected RI or RII mRNA expression were obtained (Fig. 1). The addition of tetracycline in the culture medium almost completely suppressed the transfected RI mRNA expression (Fig. 1A, from the left, lanes 3, 5, and 7), indicating that the tetracycline-regulatable expression system was functional in GEO cells. Receptor cross-linking assays confirmed that RI clones 32, 46, and 53 expressed higher levels of RI protein, whereas RII clone 37 expressed a higher level of RII protein as compared with the control NEO pool (Fig. 2). The addition of tetracycline in the medium also inhibited RI protein expression (data not shown). The cross-linking assays showed increased TGF β binding to RI but not to RII in RI-transfected cells and increased TGF β binding to RII but not to RI in RII-transfected cells.

TGF β Sensitivity—We next examined whether increased expression of RI or RII alone could affect the sensitivity of the transfectants to TGF β growth inhibitory activity. The NEO pool and RI- and RII-transfected cells were treated with increasing concentrations of TGF β (0.2–25 ng/ml) for 4 days. Relative to the NEO pool, all RI clones showed increasing inhibition of DNA synthesis in a dose-dependent manner, whereas RII clone 37 did not show any increase in inhibition of DNA synthesis (Fig. 3). These results showed that increased RI expression could render the GEO cells more sensitive to growth inhibition by exogenous TGF β, but increased RII expression had no effect. This indicated that RI but not RII is a limiting factor in TGF β signal transduction in GEO cells.

Fibronectin and Integrin α5 mRNA Expression—We then determined whether increased RI expression could induce expression of ECM molecules. Exponentially growing NEO pool and RI clones were treated with increasing concentrations of TGF β (0.2–25 ng/ml) for 4 days. RNase protection assays were performed, and fibronectin, integrin α5, and actin mRNA levels of the NEO pool and RI clone 32 are shown in Fig. 4. Contrary to our expectations, fibronectin mRNA levels remained unchanged in the NEO pool and RI clone 32 after TGF β treatment (Fig. 4A). Similarly, the expression of integrin α5 was also not induced by TGF β treatment, although the expression level of integrin α5...
was low in the GEO cells (Fig. 4B). Similar results were also observed in RI clone 46.

Growth Arrest at Low Cell Density—Growth curves for RI clones and the NEOpool were performed to determine whether increased expression of RI would lead to alterations of growth properties of the cells (Fig. 5). Growth rates in the exponential phase were essentially similar for the NEOpool and RI clones, but RI clones 32 and 46 entered log phase (day 6) later than the NEOpool (day 4). All three RI clones had a slightly lower saturation density than the NEOpool.

Plating Efficiency Assay—Since the growth curve data suggested that RI affected the growth properties of GEO cells in the lag phase when the cell density was low, we compared the ability of the NEOpool and RI and RII clones to expand and form colonies at a low seeding density. The NEOpool and RI and RII clones were plated in 24-well plates in McCoy's 5A serum-free medium at 400 cells/well. After 2 weeks of incubation, RI clones showed a significant reduction of colony formation as compared with the NEOpool. As shown in Fig. 6A, RI clone 46 had fewer and smaller colonies than the NEOpool, whereas RII clone 37 had a similar number and size of colonies as that of the NEOpool. Absorbance measurements showed that the clonogenic efficiency of RI clones 32 and 46 were about 25 and 33%, respectively, of that of the NEOpool, whereas RII clone 37 showed no reduction of clonogenic efficiency relative to the NEOpool (Fig. 6B). The RNase protection assay (Fig. 1) showed that tetracycline could suppress the expression of the transfected RI. To confirm that the reduction of clonogenic efficiency of RI clones was due to expression of RI, tetracycline was added to the culture medium to rescue the cells from RI effects. The clonogenic efficiency of RI clones 32 and 46 was stimulated by 67 and 48%, respectively, after tetracycline treatment, whereas the NEOpool showed no significant response (Fig. 7).

Taken together with the growth curves, these data suggest that transfection of RI but not of RII enhanced autocrine-negative TGFβ activity as well as the inhibitory response to exogenous TGFβ. To test this hypothesis, TGFβ neutralizing antibody was used to suppress autocrine-negative activity of TGFβ, as described previously (8). As expected, TGFβ neutralizing antibody increased colony formation of RI clones, as reflected by an increase in the number and size of colonies. As shown in Fig. 8A, the stimulatory effect of the TGFβ neutralizing antibody on RI clone 32 but not on the NEOpool cells is significant compared to the control antibody-treated cells. Absorbance measurements showed that the cloning efficiency of RI clones 32 and 46 was increased after neutralizing antibody treatment by 60 and 47%, respectively, whereas that of the NEOpool was not significantly increased (Fig. 8B). Addition of TGFβ together with the neutralizing antibody reversed this increase in proliferation (Fig. 8), thus demonstrating that the RI expression increased autocrine-negative TGFβ activity.

Anchorage independent Growth—The ability to form colonies in soft agarose is reflective of malignant transformation. Therefore, to assess the effect of increased RI or RII expression on the malignant properties of GEO transfectants, we compared the colony formation of the NEOpool and RI and RII clones in soft agarose. RI clone 46 showed a striking reduction in cloning efficiency in semisolid medium compared to the NEOpool, whereas RII clone 37 had a similar cloning efficiency as the NEOpool (Fig. 9). These results indicated that increased expression of RI but not of RII reduced anchorage-independent growth of GEO cells, further demonstrating that RI is the limiting receptor in determining the lack of TGFβ responsive-
Tumor suppressor activity (21), we hypothesized that loss or reduction of RI expression could lead to an increase in the malignant properties of carcinoma cells. The results from this study support this hypothesis. Increased RI expression in one of the colon carcinoma cell lines with a low level of RI expression increased TGFβ responsiveness as well as autocrine-negative activity and reduced in vivo malignancy, indicating that low RI expression can be a limiting factor for TGFβ response and autocrine-negative activity. In addition, these studies experimentally demonstrate the importance of the relative stoichiometry of both receptor types for TGFβ signal transduction.

We used rat RI cDNA for transfection to differentiate the transfected RI mRNA from the endogenous RI mRNA. Rat RI protein is predicted to be the same size as human RI (29). Rat and human RI show approximately 77% homology in terms of amino acid identities (33). However, 125I-labeled TGFβ cross-linking assays indicated a lower molecular weight for rat RI. This may be due to differences in the degree of glycosylation between rat and human RI proteins. Similar species differences in glycosylation have been noted for RII (34). It has been shown that TGFβ receptors from different species have varying degrees of affinity to one another (35). However, in our system, rat RI had the ability to complex with human RI1, bind ligands, and propagate signals downstream, as previously shown by Feng et al. (36).

Transfection of RI into GEO cells led to increased autocrine TGFβ activity with alterations of growth parameters of the cells similar to our previous reports for RII transfection (8, 13). Briefly, the transfected cells showed a delay in reaching log phase, a low saturation density, reduced clonogenicity in a plating efficiency assay, and diminished in vivo tumorigenesis relative to the control cells. Despite increased autocrine TGFβ activity and striking effects on plating efficiency and tumorigenicity, RI-transfected cells showed only a modest enhancement in growth-inhibitory response to exogenous TGFβ.

RI-transfected cells displayed increased sensitivity to TGFβ growth-inhibitory effects. However, there was no induction of fibronectin and integrin α5 expression by TGFβ1 in these cells (Fig. 4). Uncoupling of the growth-inhibitory and ECM inductions to TGFβ has been observed in several other studies (8, 10, 35, 37–39). Although it is still possible that there may be activation of some other ECM-related genes after TGFβ treatment of these cells, it may be that TGFβ signal transduc-

**DISCUSSION**

Based on the identification of a subset of colon tumors with reduced RI expression and the demonstration that RII has
tion pathways for these two types of TGFβ responses diverge downstream of receptor binding. Thus, the effectors of ECM induction may be less sensitive than growth inhibition effectors after enhancement of RI expression in contrast to other studies involving RII reconstitution (8, 10), or there may be defects in the specific transactivating factors associated with transcription of ECM molecules in GEO cells.

There are several possible explanations as to why increased RI expression would lead to elevated TGFβ sensitivity. One is that an increased amount of RI expression leads to increased RI activation by RII, and RII complexes. This suggests that excessive RII levels may form inactive homomeric complexes, preventing the formation of active heteromeric complexes between RI and RII. Increased RI expression in GEO cells with low endogenous RI expression would change the ratio of RI and RII and thus prevent the sequestration of homomeric RI/RII complexes. Further biochemical analysis of complex formation by RI and RII will be necessary to determine which of these possibilities occurs. However, since overexpression of RII did not lead to additional loss of TGFβ sensitivity, it would suggest that RI transfection simply leads to increased heteromeric RI/RII complex formation with increased RI activation by RII.

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