Demonstration That Mutation of the Type II Transforming Growth Factor β Receptor Inactivates Its Tumor Suppressor Activity in Replication-Error-positive Colon Carcinoma Cells*

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Escape from negative growth regulation by transforming growth factor β (TGF-β) as a result of the loss of TGF-β type II receptor (RII) expression has been found to be associated with the replication error (RER) colorectal cancer genotype, which is characteristic of hereditary nonpolyposis colorectal cancers. The RER-positive HCT 116 colon carcinoma cell line was examined for RII mutations. A 1-base deletion was found within a sequence of 10 repeating adenines (nucleotides 709–718), which resulted in a frameshift mutation. Although it is reasonable to predict that the loss of RII function would be an important determinant of malignancy, the large number of potential mutations in cells of this phenotype raises the possibility that an RII mutation may not be a key event in the tumorigenic phenotype of these cells. One way to test directly the importance of RII mutations in determining the malignant phenotype would be to restore its expression. If restoration of expression leads to diminished tumorigenicity, it would indicate that RII mutation is an important determinant of malignancy in the RER phenotype. To determine whether restoration of RII would lead to reversal of malignancy in RER colon cancers, an RII expression vector was transfected into the HCT 116 cell line. RII stable clones showed mRNA and protein expression of transfected RII. The fibronectin mRNA level was increased by exogenous TGF-β1 treatment in a dose-dependent manner in RII-positive clones, whereas the control cells remained insensitive. The RII transfectants showed reduced clonogenicity in both monolayer culture and soft agarose. They were growth arrested at a lower saturation density than control cells. TGF-β1-neutralizing antibody stimulated the proliferation of RII-transfected but not control cells, indicating that the alterations in the growth parameters of the transfected cells were due to the acquisition of autocrine-negative activity. Tumorigenicity in athymic mice was reduced and delayed in RII transfectants. These results indicate that reconstitution of TGF-β autocrine activity by reexpression of RII can reverse malignancy in RER colon cancers, thus verifying that the malignancy of hereditary nonpolyposis colorectal cancer can be directly associated with the loss of RII expression.

Transforming growth factor β (TGF-β) is a multifunctional polypeptide that regulates a number of cellular processes including growth, differentiation, deposition of the extracellular matrix, and immunosuppression (Roberts and Sporn, 1991; Massagué, 1990; Moses et al., 1990). TGF-β exerts its effects through binding to specific cell surface proteins. Three major types of TGF-β receptors have been identified in most cells by receptor affinity labeling assays (Roberts and Sporn, 1991; Massagué, 1990). These receptors have been termed type I (RI), type II (RII), and type III (RIII). RI and RII are glycoproteins of 53 and 75 kDa, respectively, whereas RIII is a proteoglycan of 280–330 kDa. Both RI and RII are transmembrane serine/threonine kinase receptors indispensable for TGF-β signaling (Lin et al., 1992; Wrana et al., 1992; Franzén et al., 1993; Bassing et al., 1994). RIII is a membrane protein lacking a cytoplasmic protein kinase domain (Wang et al., 1991; Morén et al., 1992). The direct involvement of RI and RII in TGF-β signal transduction would suggest that loss of functional RI and/or RII expression could contribute to the loss of TGF-β responsiveness.

An important feature of normal growth regulation is the balance of autocrine-negative and -positive signals regulating the cell cycle. TGF-β has been shown to act as an autocrine-negative growth regulator as evidenced by TGF-β-neutralizing antibody stimulation of several cell lines (Arteaga et al., 1990; Hafex et al., 1990; Singh et al., 1990). Accordingly, cells that lose the ability to express or respond to TGF-β are more likely to exhibit uncontrolled growth and to become tumorigenic. Previous work in our laboratory showed that repression of endogenous TGF-β expression by antisense TGF-β RNA led to malignant progression of colon cancer cells (Wu et al., 1992, 1993). TGF-β antisense transfectcd cells retained sensitivity to exogenous TGF-β, thus suggesting that the loss of autocrine TGF-β function was a key feature in the development of these
transfectants to a more progressed phenotype. The loss of TGF-β receptors in association with the inability to respond to TGF-β has been reported for some tumor cell lines (Arteaga et al., 1988; Kimchi et al., 1988). In particular, some strains of MCP-7 cells appear to be resistant to TGF-β because of the loss of RII expression (Arteaga et al., 1993; Sun et al., 1994). Reconstitution of the autocrine TGF-β loop by reexpression of TGF-β receptors in this breast cancer cell line restored TGF-β sensitivity and reversed malignancy (Sun et al., 1994). Reexpression of RII by complementation of a bladder cancer and a colon carcinoma cell line also led to reversal of tumorigenicity (Geiser et al., 1992). Transfection of an RII expression vector into a human hepatoma cell line with a receptor defect restored TGF-β sensitivity, but in vivo tumorigenicity was not addressed in this study (Inagaki et al., 1993). Taken together, these studies indicated that TGF-β has a significant suppressive role in malignancy.

Hereditary nonpolyposis colorectal cancer has been found to have a high incidence of microsatellite instability (termed RER), which is characterized by genetic alteration of simple repeated sequences (Aaltonen et al., 1993, 1994; Lindholm et al., 1993). Recently, RER was found to be associated with mismatch repair defects, which are responsible for markedly elevated gene mutation rates (Aaltonen et al., 1994; Fishel et al., 1993; Leach et al., 1993). We have shown that TGF-β RII is a downstream mutation target resulting in the disruption of growth regulation of this hereditary form of colon cancer in both cell lines and primary tissues as 9 of 10 RER tumors showed loss of RII transcript, whereas 48 of 53 non-RER tumors expressed the receptor mRNA (Markowitz et al., 1995). Disruption of the RII gene has been noted in gastric cancer, which also has a high incidence of RER (Park et al., 1993, 1994; Thibodeau et al., 1993, 1994, 1995). These studies indicate that RII is a tumor repressor gene in gastrointestinal cancers.

RII is of particular interest as a suppressor gene because of the negative growth regulatory activity of TGF-β. In view of the association of the RER colorectal cancer phenotype with the loss of RII, it is important to determine whether reconstitution of RII would lead to reversal of tumorigenicity. This would provide direct evidence that RII mutation and/or loss of transcript contributes to the malignancy of this hereditary form of cancer. In this report we describe mutational analysis of RII in the RER-positive HCT 116 colon cancer cell line. Stable transfection of an RII expression vector into HCT 116 cells reversed both in vitro and in vivo malignant properties, thus indicating that the malignancy of hereditary nonpolyposis colorectal cancer is directly associated with loss of RII expression.
of 161 amino acids, with the terminal 34 amino acids different from the wild type sequence. Similar RII mutations resulting from 1- and 2-bp deletions within this poly(A) tract have been detected previously in both RER-positive colon cancer cells and primary RER-positive colon cancer tissues (Markowitz et al., 1995). It has been shown that frameshift mutations located in the 5' half of mRNA transcripts can accelerate the decay of mRNAs (Hagan et al., 1995). This likely accounts for the reduced RII transcript levels in HCT116 (Fig. 2) and in previously characterized RER-positive colon cancer cell lines with similar RII mutations.

Expression of RII—Several positive clones (designated as RII clones 17, 21, 26 and 37) with varying levels of transfected RII mRNA expression were obtained (Fig. 2). RII clones 17 and 37 expressed the highest levels of transfected RII. A pool of control plasmid-transfected clones (the NEO pool) expressed little or no endogenous RII mRNA. Consistent with mRNA levels, RII clones 17 and 37 also expressed the highest levels of RII protein as shown by receptor cross-linking assays (Fig. 3, from left, second, fourth, fifth, and sixth lanes), whereas the cell surface receptor expression of RII was undetectable in the NEO pool (Fig. 3, first lane). It appears that restoration of RII expression increased cell surface expression of RI, which is consistent with previous reports that the presence of RII is required for TGF-β binding to RI (Wrana et al., 1992). The specificity of RI and RII was shown by competing 125I-TGF-β₁₁₁ binding to RI and RII with 50-fold unlabeled TGF-β₁ (Fig. 3, third lane). This indicated that transfected RII can be expressed and bind ligand in HCT 116.

Increased Fibronectin mRNA Expression—We determined whether expression of RII, as well as increased TGF-β binding to RI, could restore the TGF-β sensitivity of the RII transfectants. One of the cellular responses to TGF-β is induction of fibronectin expression (Ignatz and Massagué, 1986). Consequently, exponential phase RII clone 17 and the NEO pool were treated with 1.0 and 5.0 ng/ml TGF-β₁ for 24 h. Fibronectin mRNA levels increased in RII-transfected cells in a dose-dependent manner, whereas fibronectin mRNA levels remained the same in the NEO pool cells (Fig. 4). Similar results were also observed in RII clone 37. These observations indicated that RII expression in HCT 116 cells restored TGF-β₁₁₁ sensitivity of extracellular matrix (ECM)-associated molecules (ECM pathway).

Growth Arrest at Low Cell Density—Growth curves for RII clones 17 and 37 and the NEO pool were generated to determine whether RII expression led to alteration of growth parameters in tissue culture (Fig. 5). Growth rates in the exponential growth phase were essentially similar for the NEO pool and RII clones, but some delay in reaching log phase was observed in RII transfectants. The NEO pool had a slightly higher saturation density than the RII-transfected clones.

Plating Efficiency Assay—To assess further the effects of RII expression on cell growth properties, we compared the ability of the control and RII-transfected cells to expand and form colonies at low seeding density. As shown in Fig. 6, RII clones 17 and 37 showed a significant reduction of cloning efficiency (38 and 22% of that of the NEO pool, respectively). HCT 116 cells expressed high levels of TGF-β₁ mRNA (Fig. 2) and secreted TGF-β₁ protein (0.4 ng/ml/10⁶ cells). Taken together with the growth curves, it suggested that endogenous TGF-β₁ was acting in an autocrine-negative manner to inhibit cell proliferation. If this were true, TGF-β₁-neutralizing antibody should be able to uncouple the TGF-β₁ autocrine loop and increase cell proliferation as described previously (Wu et al., 1992, 1993). TGF-β₁-neutralizing antibody increased RII clone 17 cloning efficiency as reflected by increases in the number and size of colonies relative to the control antibody-treated RII clone 17 cells (Fig. 6).
FIG. 4. Fibronectin mRNA induction by TGF-β1 in HCT 116 RII transfectants. HCT 116 NEO pool and RII clone 17 were treated in log phase with 0, 1.0, and 5.0 ng/ml TGF-β1 for 24 h. Total RNA was isolated, and RNase protection assay for fibronectin was performed. Actin mRNA levels were used for normalization.

FIG. 5. Growth curves of HCT 116 NEO pool (□) and RII clones 17 (♦) and 37 (■). Cells were plated at 1,500 cells/well in 96-well plates in 0.1 ml of serum-free medium. The relative cell number was determined using the MTT assay (Carmichael et al., 1987). Values are means ± S.E. of 12 replicates.

FIG. 6. Plating efficiency of HCT 116 NEO control and RII clones 17 and 37. Cells were plated in 24-well plates at 300 cells/well in 1 ml of McCoy's 5A serum-free medium. Cell colonies were observed by MTT staining after 2 weeks of incubation. The relative plating efficiency (%) was determined by the resultant absorbance at 595 nm. Values are the means ± S.E. of four replicates.

FIG. 7A). The proliferation of RII clone 17 was stimulated by 60% after neutralizing antibody treatment, whereas the NEO pool showed no significant response (Fig. 7B). Similar results were seen with RII clone 37 (data not shown).

Anchorage-independent Growth—The ability to form colonies in soft agarose is reflective of malignant transformation. Therefore, to assess the effect of the restoration of autocrine TGF-β activity on the malignant properties of HCT 116 RII transfectants, we compared the ability of the NEO pool and RII-transfected clones to form colonies in soft agarose. As shown in Fig. 8, RII clones 17 and 37 had a significantly lower cloning efficiency in semisolid medium than the NEO pool.

Tumorigenicity—Reduction of the ability for anchorage-independent growth suggested that restoration of autocrine TGF-β activity might also render HCT 116 cells less tumorigenic. To test this hypothesis, we inoculated exponentially growing cells of the NEO pool and RII clones 17 and 37 into nude mice at 5 × 10⁶ cells/site and followed progression of xenograft formation. The NEO pool cells formed xenografts in 10 of 10 inoculation sites, and all grew rapidly. Xenograft formation of RII clones 17 and 37 was delayed compared with the NEO pool (Fig. 9). The time needed to form xenografts of >100 mm³ was 4 days for the NEO pool and 14 days for RII clones 17 and 37; once formed, RII clones 17 and 37 tumors grew at a slower rate than the NEO pool tumors. By day 20, the xenograft size of RII clones 17 and 37 was less than 20% of that of the NEO pool. The experiments assessing the tumorigenicity of RII clones 17 and 37 were performed at different times. Consequently, curves of the NEO controls in these two separate experiments are shown in Fig. 9. This provides a demonstration of the reproducibility of this model system.

DISCUSSION

Many cancers are believed to develop through a series of sequential pathologic steps (Filmus and Kerbel, 1993) that reflect the progressive accumulation of mutations (Fearon and Vogelstein, 1990). Early malignant models of colorectal carcinoma, which are unaggressive and well differentiated, retain some responsiveness to TGF-β growth inhibition, whereas their highly aggressive counterparts do not (Hoosein et al., 1989). Several studies have shown that the progression from adenomas to carcinomas is accompanied by a reduced responsiveness to TGF-β growth inhibition, whereas their highly aggressive counterparts do not (Hoosein et al., 1989; Manning et al., 1991; Markowitz et al., 1994). Since malignant progression in cancer is thought to be related to an accumulation of genetic defects, it was of interest to correlate TGF-β resistance to specific gene mutations or alterations of gene expression. An obvious mechanism that can be proposed when cells develop resistance to TGF-β effects is the loss or significant reduction of TGF-β receptor expression. Our recent studies showed that TGF-β RII, but not RI, can be a down-stream mutation target in hereditary nonpolyposis colon cancers which are characterized by high incidence of RER. Although inactivation of TGF-β RII correlated with DNA repair-defective RER colon tumors (Markowitz et al., 1995), it had not been shown that the loss of the receptor had a direct impact on the malignant phenotype of the cells. To show that the loss of RII expression is directly associated with malignancy of hereditary nonpolyposis colorectal cancer, we restored RII expression in HCT 116 cells. Our results showed that RII expression restored cell growth and anchorage-independent growth in HCT 116 cells.
Autocrine TGF-β activity in HCT 116 RII transfectants. HCT 116 NEO pool and RII clone 17 cells were plated in 24-well plates at 300 cells/well in the presence of 10 µg/ml normal IgG or 10 µg/ml TGF-β1-neutralizing antibody. Cell colonies were stained and photographed, and the relative cell number was determined as described in Fig. 6 after a 2-week incubation. Panel A depicts the effectiveness of the TGF-β1-neutralizing antibody in the clonogenicity of RII clone 17. Panel B shows the quantitation of the colony formation of the NEO pool and RII clone 17 cells. Stimulation by TGF-β1-neutralizing antibody is expressed as the percent increase of absorbance relative to normal IgG-treated cells. The values are the means ± S.E. of four replicates.

Anchorage-independent colony formation in soft agarose of HCT 116 NEO control and RII transfectants. Exponentially growing cells (3 × 10³) were suspended in 1 ml of 0.4% SeaPlaque agarose in McCoy's 5A serum-free medium and plated on top of a 1-ml underlayer of 0.8% agarose in the same medium in a six-well plate. Cell colonies were visualized by staining with 0.5 ml of p-iodonitrotetrazolium violet after 2 weeks of incubation.

In addition to restoration of autocrine-negative activity, the RII-transfected cells displayed sensitivity to exogenous TGF-β for induction of the ECM pathway. However, RII-transfected cells were insensitive to growth inhibition by exogenous TGF-β (data not shown). Segregation of growth inhibition and ECM protein induction in response to exogenous TGF-β has been observed in other colon carcinoma cells (Geiser et al., 1992) as well as other model systems (Ebner et al., 1993). As such, the TGF-β signal transduction pathways for these two types of TGF-β responses may diverge downstream of receptor binding, and the effectors of ECM induction may be more sensitive than growth inhibition effectors. The ECM response is completely absent from untransfected HCT 116 cells, whereas RII clone 26 with a low level of exogenous RII expression showed only 20% as much induction of fibronectin as RII clones 17 and 37 following TGF-β treatment. Thus, the ECM response appears to be a function of available receptors in this model system. Exogenous TGF-β may not be inhibitory because the pathways capable of transducing the inhibitory pathway are saturated by the endogenous TGF-β produced by HCT 116 RII-transfected cells, whereas pathways capable of transducing the ECM pathway are not saturated by these levels of TGF-β and hence are capable of responding to exogenous TGF-β1. For example, HCT 116 cells may have a low potential for induction of cdk inhibitors such as p15 or p27 (Ewen et al., 1993; Hannon and Beach, 1994; Polyak et al., 1994a; Slingerland et al., 1994) which allows for saturation of the pathway by autocrine-negative TGF-β activity. Presumably, the ECM pathway would not be dependent upon the induction of these inhibitors but rather upon specific transactivation factors associated with ECM molecule transcription (Polyak et al., 1994b).

Our finding that the tumorigenicity of HCT 116 is reversible upon restoration of wild type RII expression establishes that RII is a tumor suppressor gene in RER colon cancer. Thus the inactivation of RII in HCT 116 by a 1-base truncation of a polyadenylate sequence is an event that directly promotes tumor progression. Deletions and insertions in repetitive DNA sequences are characteristic of RER tumors (Ionov et al., 1993; Aaltonen et al., 1993; Kim et al., 1994), and the shortening of polyadenylate sequences in RER tumors is particularly common (Chen et al., 1995). The occurrence of a polyadenylate tract within the RII coding region thus renders it particularly vulnerable to mutation in a cell with the RER mutator phenotype.
RII inactivation is thus both a consequence of the RER mutator defect and a mechanism by which the RER defect is able to drive tumor progression forward.

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