Reversion of malignancy in human gastric cancer MKN-45 cells through the transfection of transforming growth factor- β type II receptor gene

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ABSTRACT

Human gastric cancer MKN-45 cells which are resistant to TGF- β growth inhibition and possess TGF- β type I and type III receptors, but not type II receptors, have been used as a model system to reconstitute these cancer cells with TGF- β RII cDNA. The results of these experiments indicated that the reexpression of TGF- β RII gene in MKN-45 cells can restore their sensitivity to TGF- β growth inhibition, decrease their growth rate, reduce their cloning efficiency in soft agar and tumorigenicity in nude mice in stable transfectants, in comparison with their control MKN-45 cells. Among different RII transfectants, their difference in the changes of these parameters, as a result of the regain of autocrine negative growth control by TGF- β , is roughly proportional to their level of expression of transfected RII mRNA. From these data, it is concluded that the inactivation of TGF- β RII gene is related to the escape of growth control by TGF- β in MKN-45 cells. The importance of the study of the interplay of TGF- β and its receptor system in the negative growth control of gastric cancer, and possibly also of other cancers, is discussed.

Key words: *Gastric cancer cell, TGF- β RII gene re-constitution, growth inhibition, reduced tumorigenicity.*

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INTRODUCTION

Normal cell growth regulation is controlled by the balance of both autocrine negative and positive growth factors. Transforming growth factor- β (TGF- β) represents the prototype of a large family of multifunctional peptides, known as TGF- β superfamily, which have been shown to regulate cell proliferation, differentiation and extracellular matrix production[1, 2]. As a preeminent negative growth factor, TGF- β is known to inhibit the growth of many normal as well as transformed cell types, especially epithelial and lymphoid cells. Thus, after many years of researches during late eighties in the growth control by TGF- β and its implications in oncogenesis, it was found that malignant cells which escape from normal growth control are often related to their altered response to TGF- β [3]. With the advent of the recent molecular cloning of TGF- β receptors, it is now possible to study the mechanism of the unresponsiveness of cancer cells to TGF- β on receptor-basis. The TGF- β type I, II and III receptors (referred to as RI, RII, RIII respectively) are commonly present on most cells in tissue culture and are best characterized. RI and RII are glycoproteins of 53 Kd and 75 Kd and belong to transmembrane serine-threonine kinases mediating signaling activities[4, 5]. RIII, also known as betaglycan, is a cell surface proteoglycan of 280-330 Kd, known to act as a reservoir for TGF- β ligand and does not play any role in signaling[6, 7].

With respect to RI and RII implicated in TGF- β signaling, it is now clear that signaling requires a cooperation between the two receptors. TGF- β binds directly to RII, but not to RI. Bound TGF- β is then recognized by RI which is recruited into the receptor complex and becomes phosphorylated by RII. Phosphorylation of RI leads to its propagation of signal to downstream substrates[7-9]. Now, it is interesting to note that the first series of cancers found with the mutation or reduced expression of TGF- β -RI or RII belonged to epithelial and lymphoid cancers, e.g. colon cancer[10, 11], gastric cancer[12, 13], head neck cancer[14], breast cancer[15], and T cell lymphoma[16]. These results later led to the suggestion that the type II TGF- β receptor can be considered as a tumor suppressor gene[17].

In case of gastric cancer, Ito et al. found that the expression of TGF- β RI was reduced in many human gastric carcinoma tissues as compared to the normal tissues[12], while Park et al. reported that genetic changes of TGF- β RII gene itself or altered expression of its mRNA might be linked to the escape of growth control by TGF- β in human gastric cancer cells[13]. However, the reconstitution experiment, through the transfection of RI or RII gene into the highly malignant and receptor defective human gastric cancer cells in order to verify the possible reversion of their negative growth control by TGF- β and their malignant behaviors, has never been reported. Here, we will present the data that a TGF- β resistant human gastric cancer cell line MKN-45, upon transfection of RII gene, could restore TGF- β sensitivity and show growth arrest at low cell density and reduction of cloning efficiency in soft agar and tumorigenicity in athymic nude mice. These results supported the

contention that the inactivation or reduced expression of TGF- β RII is related to the escape of growth control by TGF- β in MKN-45 cells.

MATERIALS AND METHODS

Cell lines and cell culture

The cells of a human gastric cancer cell line MKN-45, derived from a poorly differentiated adenocarcinoma, were maintained in DMEM (GIBCO/BRL) containing 10% heat-inactivated FBS.

Expression plasmid and transfection

The TGF- β RII cDNA (~3.0 kilobase) (Lin et al., 1992)[5] was subcloned into the mammalian expression vector pcDNA1/neo (INVITROGEN). Recombinant plasmid DNAs (20 μ g), purified by Qiagen-pack 500 (QIAGEN), were transfected into MKN-45 cells via standard calcium phosphate precipitate method. Twenty-four hours after transfection, cells were treated with Geneticin (G418)(400 μ g/ml) (GIBCO/BRL). G418-resistant clones were ring-cloned and expanded. These clones were screened for the expression of TGF- β RII mRNA by dot blot analysis.

Southern blot analysis

Genomic DNA was isolated by a standard SDS and proteinase K protocol. Ten micrograms of DNA was digested to completion with EcoR I, electrophoresed in 1% agarose gel, and transferred to nylon membrane (GIBCO/BRL). Prehybridization and hybridization with the TGF- β RII cDNA probe were performed according to standard protocols[18].

Northern blot analysis

Total RNA was isolated with guanidinium isothiocyanate/phenol/chloroform. Poly(A)⁺ RNA was prepared with oligo(dT) columns (PHARMACIA). Two micrograms of poly(A)⁺ RNA was electrophoresed on a 1% agarose-formaldehyde gel and transferred to nylon membrane. Prehybridization and hybridization with TGF- β RII cDNA probe were performed according to standard protocols[18].

DNA synthesis and cell growth

[³H] Thymidine incorporation was used to determine TGF- β sensitivity of MKN-45 control cells and RII-transfected cells to exogenous TGF- β 1 treatment. About 0.5×10^4 cells were plated in 96-well culture plates in the presence of various concentrations of rhTGF- β 1 (0.63-20ng/ml)(GIBCO/BRL) and cultured for 48 h. The cells were then incubated with [³H] thymidine (0.5 μ Ci/well, 87 Ci/mmol, AMERSHAM) for 6 h. DNA was then precipitated with 10% trichloroacetic acid and the amount of [³H] thymidine incorporation was analyzed by liquid scintillation counter. The cell number was determined by the MTT assay[19].

Soft- agar assay

Soft agar assays were performed as described previously (Wu et al., 1992)[20] with slight modification. Briefly, 1×10^3 cells were suspended in 1 ml of 0.37% agar in DMEM medium containing 10% FBS and plated on top of 1 ml of 0.5% agar in the same medium in six-well culture plates. The plates were incubated for 2 w at 37°C with 5% CO₂ in a humidified incubator. Cell colonies were visualized by staining with 1 ml of p-iodonitrotetrazolium violet (SIGMA).

Tumorigenicity

Tumorigenicity studies were performed as described previously (Wu et al., 1993)[21]. Briefly, 3×10^6 cells from exponential cultures of MKN-45 control cells and RII transfectants were inocu-

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lated subcutaneously into 4-week-old athymic nude mice. Mice were maintained in a pathogen-free facility. Growth curves for xenografts were determined by the measurement of tumor size in two dimensions using a caliper. Volume (V) was determined by the following equation, where L is length and W is the width of the xenograft: $V = (L \times W^2) \times 0.5$.

RESULTS

Reexpression of TGF- β RII

In previous studies, we reported that MKN-45 cells, which was resistant to growth inhibition by TGF- β 1, did not express detectable level of TGF- β RII mRNA but did retain the expression of TGF- β RI and RIII (paper in preparation). It is very probable that TGF- β insensitivity of MKN-45 cells may be due to the lack of TGF- β RII expression. To ascertain this possibility, we constructed a recombinant plasmid by inserting the full-length cDNA of TGF- β RII into the mammalian expression vector pcDNAI/neo. MKN-45 gastric cancer cells were transfected with the vector DNA in form of calcium phosphate precipitate, and clones were selected by growing in G418 containing medium. G418-resistant clones were expanded and screened for the presence of RII mRNA by dot blot. Three clones (designated as RII clone 1, 3, and 4) were obtained to express the higher level of TGF- β RII mRNA among RII transfectants (data not shown). Northern blot analysis (Fig 1) showed that three RII transfectants expressed TGF- β RII mRNA with slightly different levels, while MKN-45 control cells did not express it. Among them, RII clone 3 had the highest

Fig 1. Expression of TGF- β RII mRNA in MKN-45 cells and three RII transfectants. Each lane contains two micrograms of poly(A⁺) RNA. β -actin probe was used as internal standard.

Fig 2. DNA analysis by southern hybridization. Ten micrograms of genomic DNA prepared from MKN-45 cells and three RII clones were digested by either EcoRI(E) or HindIII(H).

RII expression level and clone 1 had the lowest one. Genomic DNAs were digested with EcoRI or HindIII, electrophoresed, transferred, and analyzed on a Southern blot with RII cDNA probes (Fig 2). Transfection of TGF- β RII cDNA resulted in the appearance of recombinant TGF- β RII-specific DNA bands (~ 3 kb) with EcoRI restriction enzymes. The different Southern patterns with HindIII showed independent integration events of the TGF- β RII cDNA in the host cell genome, thus proving the clonal origin of these three RII clones.

Restoration of TGF- β sensitivity

To examine whether reexpression of TGF- β RII gene could restore the sensitivity of MKN-45 cells to TGF- β growth inhibitory activity, three RII transfectants were plated in 96-well plates in the presence of TGF- $\beta 1$ at the concentration from 0.63 to 20 ng/ml and incubated for forty-eight hours. As shown in Fig 3, the growth of MKN-45 controls was not inhibited by TGF- $\beta 1$, whereas all three RII transfectants were significantly inhibited in a dose-dependent manner. The IC₅₀ was, approximately, 1.25 ng/ml for clone 3, 2.5 ng/ml for clone 4 and 5 ng/ml for clone 1.

Growth Arrest at low cell density

Growth curves for RII clones 1, 3, 4 and MKN-45 control cells were generated to determine whether TGF- β RII expression led to the alteration of growth parameters in tissue culture (Fig 4). Growth rates of MKN-45 control cells and RII transfectants were similar at the exponential growth phase, but some delay in reaching lag phase were observed in RII transfectants. As a result, RII transfectants had a slightly lower cell density than MKN-45 controls on day 14. RII clone 3 (with the highest RII expression) had the lowest cell density and clone 1 (with the lowest RII expression) had the highest cell density among three RII transfected clones.

Anchorage-independent Growth Assay

The ability to form colonies in soft agar is reflective of malignant transformation. To assess the effect of RII expression on the malignant properties of MKN-45 cells, we compared the ability of MKN-45 cells and RII transfected cells to form colonies in soft agar. The test cells were plated in six-well culture plates at 1×10^3 cells per well. After 2 w of incubation, colonies were stained with p-iodonitrotetrazolium violet. Compared with MKN-45 cells, all three RII transfectants showed a significantly lower cloning efficiency (Fig 5). Among these, RII clone 3 showed the greatest reduction of anchorage independent growth, while clone 1 showed the least.

Tumorigenicity

Reduction of the ability for anchorage independent growth suggested that the restoration of TGF- β sensitivity might also render MKN-45 cells less malignant. To test this hypothesis, we inoculated exponentially growing cells of the control MKN-

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Fig 3. Growth inhibition of RII transfectants by TGF- β 1. MKN-45 cells and three RII clones were plated in 96-well plates at 5×10^4 cells per well in the presence of various concentrations of TGF- β 1. DNA synthesis was assayed 48 h later by measuring [3 H] thymidine incorporation during a 6-h pulse.

Fig 4. Growth curves of MKN-45 cells and RII transfectants. MKN-45 control cells and three RII transfected clones were plated in 96-well culture plates at 5000 cells per well. The relative cell number was determined by the MTT assay.

Fig 5. Anchorage-independent colony formation in soft-agar of MKN-45 control and RII transfectants. Exponentially growing cells (1×10^3) were suspended in 1 ml of 0.37% agar in DMEM medium containing 10% FBS and plated on top of 1 ml of 0.5% agar in the same medium in 6-well culture plates. After two weeks of incubation, cell colonies were visualized by staining with 1 ml of p-iodonitrotetrazolium violet. Two wells showed duplicated assay for the same clone.

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45 cell line and three RII transfected clones into athymic nude mice at 3×10^6 cells per site and followed the progression of xenograft formation. MKN-45 cells formed xenografts in 10 of 10 inoculation sites, and all grew rapidly, while xenograft formation of three RII transfectants was delayed as compared to MKN-45 controls (Fig 6). The time needed to form xenografts of $>100 \text{ mm}^3$ was 12 d for MKN-45 cells and 18 d for RII clone 3 and 4. Once the tumorous nodules were formed, those from RII clones also grew at a slower rate than those from MKN-45 cells.

Fig 6. Xenograft growth curves of MKN-45 cells and RII transfectants. Exponentially growing cells (3×10^6) of MKN-45 cells and three RII transfectants were subcutaneously inoculated in athymic nude mice. Tumors were measured externally on the indicated days in two dimensions using a caliper. Volume was determined from the equation: $V = (L \times W^2) \times 0.5$, where L is length and W is width of the tumor. Each point represents a mean volume $\pm$ S.E. of 6 xenografts.

DISCUSSION

The incidence of gastric cancer is rising sharply in China in these years and becoming one of the most common malignancies with high mortality. Like many other cancers, gastric carcinogenesis is also hypothesized as a multistep and multifactorial process from evidences in pathology and epidemiology[22]. However, this is not yet genetically defined as in the case of colorectal cancer[23]. Recently, Park et al. has mentioned in their paper that there occurred the genetic alteration of a group of oncogene, tumor suppressor gene and other genes in gastric cancer[13]. As mentioned earlier in this paper, the possible role played by the potent negative growth regulator TGF- β in tumorigenesis has long been suspected and now the growing ev-

idences accumulated in recent years inevitably suggested that TGF- β receptor gene (especially RII gene) may be a candidate of tumor suppressor gene[17]. In human gastric cancers, Park et al. have shown that genetic changes of TGF- β RII gene or altered expression of its mRNA occurred in gastric cancer cell lines[13]. Ito et al. have reported the reduced expression of TGF- β RI gene in human gastric cancer tissues, as compared with normal gastric tissues[12]. Recently, we have also examined the expression of TGF- β RII gene in some surgical human gastric cancer specimens, and in 11 out of 18 cases, there was a distant reduction of TGF- β RII gene expression at mRNA levels (unpublished data).

Our previous work has shown that MKN-45 cells did not express detectable amount of TGF- β RII mRNA, but did retain the expression of RI and RIII mRNA. This means that MKN-45 cell will be a very nice model system to study the function of TGF- β RII gene and its cooperation with other receptor genes to execute the normal TGF- β signaling. Now, we have stably transfected MKN-45 cells with a mammalian expression vector pcDNA1/neo inserted with a full length cDNA of TGF- β RII. Three clones (RII clones 1, 3, 4) with relatively higher expression level of RII mRNA were selected, and among them, the level of the expression of RII mRNA was in the order of clone 3 > clone 4 > clone 1. They were then used for the study of the effect of reexpression of RII gene on their sensitivity to growth inhibition by TGF- β 1, growth behavior in tissue culture, cloning efficiency in soft agar and the rate of xenograft formation in athymic nude mice. From the data shown in Fig 3, it is clear that RII transfectants were sensitive to TGF- β 1 growth inhibition, while MKN-45 cells were not inhibited by exogenous TGF- β 1. The inhibition induces IC50 were approximately 1.25 ng/ml for clone 3, 2.5 ng/ml for clone 4 and 5 ng/ml for clone 1 which were roughly proportional to their transfected RII gene expression levels. The growth rates of RII transfectants and control MKN-45 cells were similar during the exponential growth phase, but there was some delay in RII transfectants in the lag phase as shown in Fig 4. Such restoration of TGF- β sensitivity after TGF- β RII transfection in TGF- β insensitive cell line has also been observed in the case of human hepatoma cells[24] and breast cancer cells[15].

Furthermore, whether the reexpression of TGF- β RII gene in MKN-45 cells could reduce their *in vitro* clonogenicity and *in vivo* tumorigenicity was also investigated. From Fig 5, it is evident that all three RII transfectants displayed a marked decrease in cloning efficiency in soft agar, with clone 3 having the greatest reduction of anchorage independent growth. Data shown in Fig 6 gave the results obtained from xenografting experiment in athymic nude mice. All three RII transfectants showed a significant decrease in growth rate. The time needed to form xenograft of (100 mm³) was 12 d for MKN-45 control cells and 18 d for RII clone 3. Similar reduction in tumorigenicity in TGF- β resistant cancer cell lines transfected with TGF- β RII gene has also been reported in colon carcinoma cells[10] and breast cancer cells[15].

From the above data, it is clear that the observed differences between three clones of RII transfectants and their parent MKN-45 cells in (1) growth inhibition by TGF-

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β as represented by IC₅₀, (2) growth behavior in tissue culture as represented by their saturation density and (3) reduction of cloning efficiency in soft agar and growth rate of xenografts in nude mice can be explained by the regain of the autocrine growth control by TGF- β after the transfection of TGF- β RII gene. Comparing the differences among three RII clones in different parameters studied, the differences were more or less inversely related to their expression level of RII gene transfected, and this is especially evident in case of growth inhibition index IC₅₀ and cloning efficiency in soft agar for clone 3 in comparison with other two clones. These results forced us to conclude that the inactivation of TGF- β RII gene is related to the escape of growth control by TGF- β in MKN-45 gastric cancer cells. This is in line with the conclusions reached by most authors in the case of gastric-intestinal cancers and breast cancers.

From our work and informations available to us from other workers, we are of opinion that it is worthwhile to pay more attention to study the changes of TGF- β -receptor system in the evolution of human gastric cancer for several reasons. In the first place, TGF- β RII gene has been shown to be a possible tumor suppressor gene in many epithelial cancers. Its exact role in cancer genesis and cancer progression need to be further investigated. In the second place, it was reported that human gastric cancer cells have a high expression of TGF- β protein[25]. What is the cause of this elevation? Is there any connection to the change of the activity of receptor system? Thirdly, the growth inhibition pathways involving TGF- β is closely connected with the interaction of TGF- β with nuclear phosphoproteins regulating cell cycle. For example, TGF- β can induce the expression of p15 protein which in turn leads to the accumulation of unphosphorylated Rb protein and hence cell cycle arrest[26]. Therefore, the interplay of TGF- β and its receptor system in negative growth control will be an attractive field in cancer researches, not only for gastric cancer, but also for many other types of cancer.

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