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Expression of soluble recombinant TGF-β type II receptor fused with the Fc portion of human IgG1 (sTβRII-Fc) in NS0 cells

Eliza P. Kwiatkowska[1], Urszula Kazimierczak[2], Andrzej Mackiewicz[1,2] and Dariusz W. Kowalczyk[1,2]✉

[1]Department of Cancer Diagnostics and Immunology, GreatPoland Cancer Center, Poznań, Poland; [2]Department of Cancer Immunology, Chair of Medical Biotechnology, Poznań University of Medical Sciences, Poznań, Poland; ✉e-mail: dariusz.w.kowalczyk@wco.pl

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We have constructed and expressed recombinant chimeric soluble TGF-β type II receptor fused with the Fc portion of human IgG1 (sTβRII-Fc) in NS0 mouse myeloma cells and isolated cell lines constitutively secreting very high levels of biologically active protein. The GS-NS0 expression system takes advantage of the strong human cytomegalovirus immediate early promoter expression vector and glutamine synthetase as a selectable marker. The recombinant chimeric receptor could be produced in high amounts and efficiently purified by one step chromatography on a protein A column. Biochemical studies revealed that recombinant sTβRII-Fc binds native TGF-β1 and TGF-β3 isoforms and neutralizes their activity *in vitro*.

Keywords: TGF-β, TGF-β receptor, TGF antagonist, chimeric receptor, GS-NS0 expression system

INTRODUCTION

The transforming growth factor (TGF) family consists of a number of multifunctional growth regulatory factors with important functions in animal cell growth, development, and differentiation (Massague, 1990; Siegel & Massague, 2003). At least three TGF-β isoforms, TGF-β1, -β2, and -β3, which share approximately 70–75% amino-acid sequence homology have been described. Active TGF-β isoforms assemble to make homodimers, which bind to their cognate receptors, but TGF-β1.2 and TGF-β2.3 heterodimers have also been described (Massague, 2000; Shi & Massague, 2003). Most cells express high affinity receptors which can bind TGF-β. Based upon the molecular mass of cross-linked products analyzed by gel electrophoresis three major TGF-β receptors: type I (53 kDa), type II (65 kDa), and type III (100–280 kDa) were identified (Massague, 2000; Shi & Massague 2003). Cross-linking studies of isotope-labelled TGF-β to their receptors indicated that the different types of the receptors displayed different affinities for the TGF-β isoforms. Whereas TGF-β1 and TGF-β3 have very high affinity for type I, II, and III receptors, TGF-β2 binds with high affinity to the type III receptor only (Massague, 2000; Shi & Massague 2003). The TGF-β type III receptor (also referred to as betaglycan) is a membrane-bound proteoglycan. The short intracellular domain of this receptor lacks signal transduction sequences indicating that type III receptor does not play a direct role in TGF-β signalling. Instead, the type III receptor interacts with type II, which results in enhanced TGF-β binding (Massague, 2000; Shi & Massague 2003). In contrast to the type III receptor, TGF-β type I and II receptors possess a cytosolic domain containing a serine/threonine kinase-homologous sequence and directly participate in signal transduction (Massague, 2000; Shi & Massague 2003). Upon TGF-β binding these two receptors form heteromeric complexes which then propagate the signal through activation of Smad2 and Smad4 proteins (Massague, 2000; Shi & Massague 2003).

Undisrupted and properly controlled TGF-β signalling appears to be essential for many physiological functions at virtually all developmental stag-

Abbreviations: BSA, bovine serum albumin; DSS, bis(sulfosuccinimidyl)suberate; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GS, glutamine synthetase; PBS, phosphate-buffered saline; TGF, transforming growth factor.

es (Massague, 1990). Lack of TGF-β expression or altered tissue responsiveness leads to serious pathological conditions such as high embryonic and early postnatal lethality (Shull *et al.*, 1992; Kulkarni *et al.*, 1993), autoimmune disease (Kulkarni *et al.*, 1993; 1995) or cancer (Derynck *et al.*, 2001; Siegel & Massague, 2003; Siegel *et al.*, 2003; Han *et al.*, 2005). Likewise, excessive TGF-β production has been implicated as being responsible for accumulation of detrimental scar tissue (Border & Noble, 1994; Shah *et al.*, 1999; Huang *et al.*, 2002), psoriasis (Li *et al.*, 2004), glomerulonephritis (Clouthier *et al.*, 1997; Reeves & Andreoli, 2000), liver and pulmonary fibrosis (Clouthier *et al.*, 1997; Munger *et al.*, 1999; Kaminski *et al.*, 2000), tumour-induced immunosuppression (de Visser & Kast, 1999) and cancer invasion and metastases (Derynck *et al.*, 2001; Siegel & Massague, 2003; Siegel *et al.*, 2003; Han *et al.*, 2005). Therefore, agents attenuating elevated TGF-β activity may have clinical potential (Sharma *et al.*, 1996; Ziyadeh *et al.*, 2000; Blobe *et al.*, 2000; Huang *et al.*, 2002; Chen *et al.*, 2003; Wojtowicz-Praga, 2003). In this paper we describe designing, construction and production of chimeric soluble TGF-β type II receptor fused to the Fc region of human immunoglobulin G (sTβRII-Fc) with potent anti-TGF-β activity. We show here that NS0 cell-derived recombinant chimeric sTβRII-Fc molecule binds TGF-β and neutralizes its activity in *in vitro* assays.

MATERIALS AND METHODS

Construction of vectors. The cDNA fragment encoding the extracellular domain of human TβRII (Gene Bank: M85079) was amplified by RT-PCR from RNA isolated from human peripheral blood mononuclear cells. The sense primer with a *Hin*dIII restriction site (underlined) preceding the start codon was 5′ CAAAGCTTATGGGTCGGGGGCTGCTC. The antisense primer having a *Xba*I restriction site (underlined) had the sequence 5′ CATCTAGACAAGTCAGGATTGCTGGTGT. The resulting amplified product was cloned into pCR-Blunt II TOPO vector (Invitrogen).

The cDNA fragment encoding the Fc regions of human immunoglobulin G1 (Gene Bank: BC006402) was amplified by RT-PCR from RNA isolated from human peripheral blood lymphocytes. The sense primer containing a *Xba*I restriction site (underlined) had the sequence 5′ CATCTAGACCCAAATCTTGTGACAAAACT. The antisense primer having an *Eco*R I restriction site (underlined) and a stop codon (bolded) was 5′ CAGAATTC**TCA**TTTACCCGGAGACAGGGAGA. The resulting amplified product was cloned into pCR-Blunt II TOPO vector. The cloned cDNAs were sequenced to confirm the fidelity of the PCR reaction, excised from the pCR-Blunt II TOPO vectors and ligated into pEE 12.4 expression vector (Lonza Biologics).

Transfection of NS0 cells. NS0 cells were cultured in RPMI medium (Gibco) supplemented with 10% FCS (Gibco), L-glutamine (2 mM) (Sigma-Aldrich) and gentamycin (80 mg/l) (Roche). Transfection of NS0 cells was performed with DMRIE-C reagent (Gibco BRL). In brief, semi-confluent cells grown on a 78 cm^2 flask were washed with PBS and transfected with 10 μg *Sal*I digested pEE12.4-TβRII-Fc plasmid diluted in 7 ml serum free RPMI medium containing DMRIE-C reagent (84 μl). After a 6-h incubation the culture was supplemented with 5 ml RPMI medium containing FCS and L-glutamine. Fourty-eight hours following transfection the cell culture was diluted ten times with RPMI without glutamine, supplemented with 10% dialyzed FCS (Gibco) and GSM supplement (Sigma-Aldrich) and seeded onto 96-well plates. After thirty days positive clones were isolated and expanded in Hybridoma Serum Free Medium (Sigma-Aldrich) supplemented with 2% dialyzed FCS and GSM supplement.

NS0 transfectant screening with enzyme-linked immunosorbent assay. Plates (Maxisorp, Nunc) were coated with 0.5 μg of polyclonal goat anti-human TβRII antibody (R&D Systems) in 100 μl PBS overnight. They were then blocked overnight with PBS supplemented with 3% bovine serum albumin (BSA) (Sigma-Aldrich). NS0 cell culture supernatants were added at a 1:50 dilution in PBS + BSA. After one hour of incubation at room temperature the plates were washed three times with PBS. They were then incubated with a 1:5000 dilution of a horseradish-conjugated goat anti-human immunoglobulin antibody (Dako Cytomation) for 1 h at room temperature. After extensive washing with PBS, substrate solution (OPD tablets, Sigma-Aldrich) was added for 20 min at room temperature in the dark followed by adding stop solution (3 M HCl) (POCh, Poland). Plates were read in an automated ELISA microplate reader (EL_X808iu, Bio-Tek Instruments) at 492 nm. Background optical density (OD) values were determined on medium samples harvested from untransfected NS0 cells.

Flow cytometry. NS0 clones were tested for sTβRII-Fc production in a flow cytometry assay with anti-TβRII antibody. This method is an adaptation of intracellular staining for cytokine expression in antigen activated T-cells (Kowalczyk *et al.*, 2000). Briefly, NS0 were kept in culture medium containing brefeldin A (1 μl/ml, BD Golgi Plug, Pharmingen) which blocks intracellular protein transport processes. This results in an accumulation of cytokines in the Golgi complex and enhances the detectability of cytokine-producing cells. After four hours of incubation the cells were fixed and permeabilized with BD Cytofix/

Cytoperm reagent (Pharmingen) followed by intracellular staining with FITC-conjugated anti-TβRII antibody (R&D Systems). After extensive washing the cells were analyzed by flow cytometry (FACSCanto, BD Biosciences).

Protein purification and analysis. The human recombinant sTβRII-Fc was purified by one step protein A chromatography using Montage Antibody Purification/Prosep-A Kit (Millipore). Briefly, the tissue culture medium collected from stably transfected NS0 cells was centrifuged, filtered (0.22 μm, Millipore), diluted 1:1 with loading buffer and applied to a protein A column previously equilibrated with PBS. After protein loading, the column was washed with loading buffer to remove non-specifically bound proteins and the soluble TβRII-Fc was eluted with elution buffer. The eluted fraction was immediately neutralized by the addition of a 1/10 vol. of 1 M Tris/HCl, pH 9.0. The recovered protein solution was subjected to buffer exchange and concentration (both procedures on Amicon filters, Amicon Ultra Centrifugal Filter Devices, Millipore), aliquoted and stored at –20°C until further use.

Purified sTβRII-Fc was analysed by 10% SDS/PAGE under reducing (with 0.715 M 2-mercaptoethanol) and non-reducing conditions, followed by Coomassie brilliant blue R staining (EZBlue Gel Staining Reagent, Sigma). The amount of protein was quantified using Bradford Reagent (microtiter plate format) (Sigma).

Western blot analysis. Recombinant sTβRII-Fc was separated by 8% Novex Tris-Glycine Gel (Invitrogen) and transferred to PVDF membrane (Hybond-P, Amersham Biosciences). Following transfer, the membrane was blocked overnight with 2% BSA in PBS supplemented with 0.1% Tween 20 (Sigma-Aldrich). The membrane was incubated with specific polyclonal antibodies: goat anti-human TβRII (R&D Systems) or goat anti-human IgG (Dako Cytomation), then with polyclonal rabbit anti-goat immunoglobulins conjugated to horseradish peroxidase (Dako Cytomation). Recombinant sTβRII-Fc was then visualized with a chemiluminescence immunoassay (ECL Western Blotting Detection Reagent; Amersham Biosciences) following the manufacturer's procedure.

***In vitro* binding to TGF.** TGF-β binding to recombinant sTβRII-Fc was tested in a competition assay with ^{125}I-labeled TGF-β1 as described (Lin *et al.*, 1992). NS0-conditioned medium containing sTβRII-Fc was collected, filtered (0.22 μm, Millipore), chilled on ice and equilibrated with Hepes (20 mM, pH 7.4; Sigma-Aldrich) and phenylmethylsulfonyl fluoride (1 mM, Sigma-Aldrich). ^{125}I-TGF-β1 (70 pM, specific activity 800 Ci/mmol, Amersham) was added in the presence of various concentrations (0–1000 pM) of unlabeled TGF-β1 (R&D Systems) as noted in the figure legends. After incubation overnight at 4°C with constant agitation, soluble receptors and TGF-β were chemically cross-linked with 60 μg/ml DSS (Sigma-Aldrich) for 15 min at 4°C with constant agitation. The reaction was then stopped by addition of 2 mM ethanolamine (Sigma-Aldrich). Samples were then immunoprecipitated with Protein A fast flow beaded agarose (Sigma-Aldrich), resolved by SDS/PAGE and exposed to X-ray film.

Growth inhibition assays. The ability of sTβRII-Fc to act as TGF-β antagonist was tested using HT-2 and TC-1 cells. The HT-2 cell line was maintained in RPMI medium (Gibco) supplemented with 10% FCS (Gibco) and human recombinant IL-2 (100 U/ml) (Pharmingen). For reversal of TGF-β-mediated HT-2 cell growth inhibition, serial dilutions (concentration range: 500–0.12 ng/ml) of purified sTβRII-Fc were preincubated with constant concentration (100 pg/ml) of TGF-β1, TGF-β2, and TGF-β3 isoforms (R&D Systems) for 1 h in assay medium (X-Vivo 15 medium, Cambrex) supplemented with 7.5 ng/ml IL-4 (Pharmingen) in a 96-well microtiter tissue culture plate. Immediately before the assay, HT-2 cells were washed three times with PBS to remove IL-2, resuspended in assay medium, and added to the assay plate at a concentration of 10^4 cells per well. The cells were incubated at 37°C, 5% CO_2, for 48 h and pulsed with ^{3}H-labeled thymidine (1 μCi/well) (Amersham Biosciences) for the last 6 h. Following culture, the cells were collected with an automated cell harvester and tested for radioactivity in a scintillation counter. For reversal of TGF-β growth stimulatory activity 1×10^4 TC-1 cells were seeded on a 96-well plate in DMEM medium (Gibco) supplemented with 2% FBS with or without TGF-β1 (10 ng/ml) and sTβRII-Fc (300 ng/ml). After 24 h the cells were pulsed with [^{3}H]thymidine (1 μCi/well), harvested and tested for radioactivity in the scintillation counter.

Statistics. Where appropriate data are expressed as mean ±standard deviation (S.D.). Statistical analysis was performed using Student's *t*-test, *P* values less than 0.05 were considered significant.

RESULTS

Transfection of NS0 cells with sTβRII-Fc cDNA

The cDNA encoding chimeric sTβRII-Fc molecule was created by in-frame ligation of cDNA encoding truncated TGF-β type II receptor (amino-acid residues 1–159) and the cDNA encoding the Fc portion of the human IgG1 heavy chain. After sequence verification the cDNA was cloned into pEE12.4 expression vector (Fig. 1).

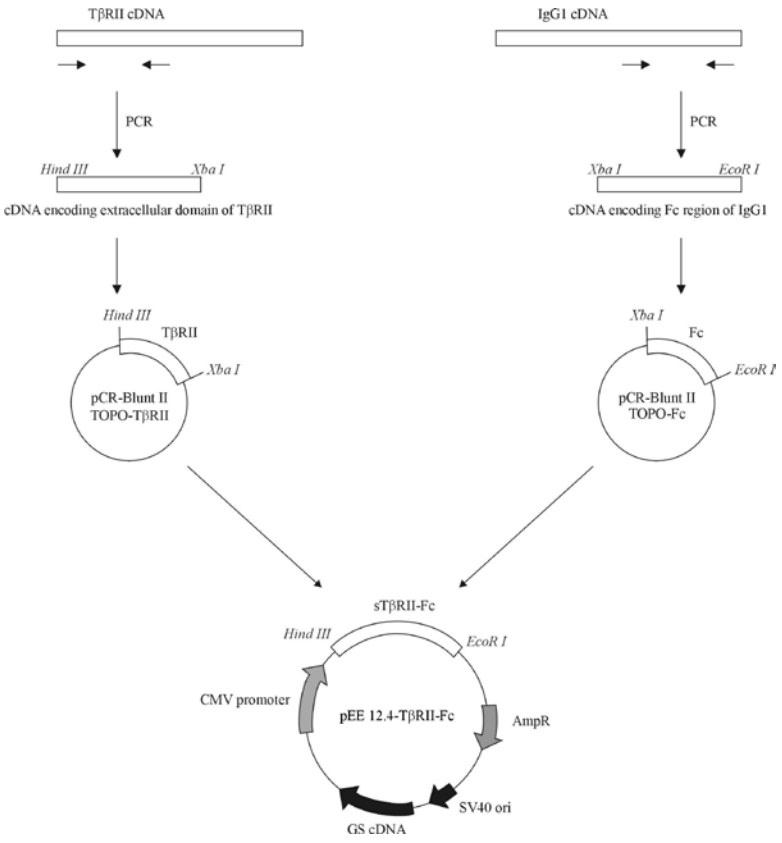

Figure 1. Strategy for sTβRII-Fc cDNA construction.
The cDNA encoding extracellular domain of TβRII and the Fc fragment of human IgG1 were obtained by RT-PCR from mRNA isolated from human peripheral blood lymphocytes. PCR products were directly cloned into pCR-Blunt II TOPO vector. After insert verification with DNA sequencing cDNA was excised with *Hin*dIII, *Xba*I or *Xba*I, *Eco*RI restriction enzymes and cloned into pEE12.4 expression vector.

NS0 cells were then transfected with *Sal*I-linearized pEE12.4:sTβRII-Fc vector and seeded on glutamine-free selection medium. After 30 days, glutamine-independent single clones were harvested and screened by ELISA for transfectants that were positive for sTβRII-Fc expression. For screening the ELISA plates were coated with polyclonal goat anti-human TβRII antibody and detection was performed with goat anti-human immunoglobulin antibody. Thus, the recombinant protein containing both sTβRII and the Fc fragment of human IgG could be captured and detected (not shown). To further confirm the product specificity, the positive clones were tested by flow cytometry with FITC-conjugated anti-TβRII antibody. As shown in Fig. 2 a clone which was determined by ELISA as positive for the sTβRII-Fc, stains positively with the anti-TβRII antibody indicating that the recombinant protein produced in these cells consists of TβRII and the Fc portion of human IgG.

Positive clones were expanded and after cell number normalization further selected according to their growth and productivity characteristics. On average, we harvested over thirty positive clones from 6×10^6 cells used for transfection and placed on a total of 594 wells. From thirty positive clones we chose three high producers which were adapted for growth in low (2%) serum medium. Cell cultures were then maintained as monolayers in Hybridoma Serum Free Medium supplemented with 2% serum.

Production and purification of sTβRII-Fc

An examination of the time course of sTβRII-Fc production by NS0 cells revealed that the levels of secreted recombinant protein peaked at a concentration of about 2 μg/ml between 2 and 3 days af-

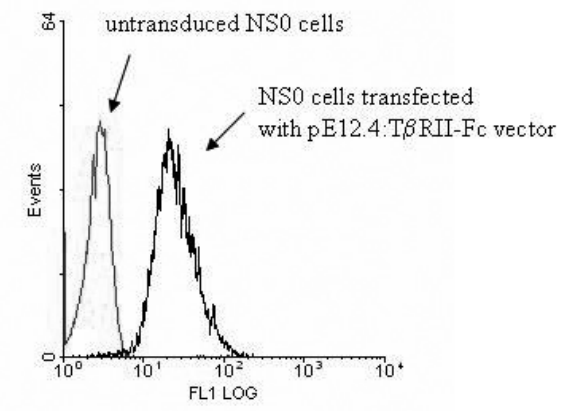

Figure 2. Flow cytometric analysis of a clone expressing sTβRII-Fc recombinant molecule.
NS0 cells were stably transfected with pEE12.4:TβRII-Fc vector and seeded in selective glutamine-free medium. After 30 days single clones were expanded and tested for transgene expression by flow cytometry: cells were incubated for 4 h in the presence of brefeldin A, and after fixation/permeabilization with BD Cytofix/Cytoperm solution incubated with FITC-labeled goat anti-human TβRII antibodies. Untransfected cells were used as a negative control.

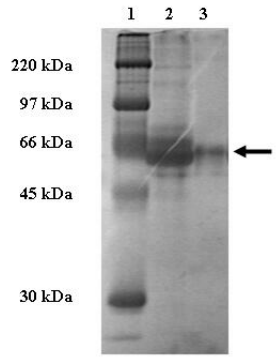

Figure 3. SDS/PAGE analysis of purified chimeric sTβRII-Fc molecule.
sTβRII-Fc was purified from culture medium by one step protein A Sepharose chromatography and subjected to polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the gel was stained with Coomassie brilliant blue. Lane 1, molecular-mass standard (in kDa); lane 2, medium containing sTβRII-Fc; lane 3, purified sTβRII-Fc.

ter medium exchange, as determined by ELISA (not shown).

sTβRII-Fc was purified from culture medium in a single step protein A affinity chromatography, and desalted and concentrated by filtration on Amicon columns. The product analyzed by SDS/PAGE was estimated to be >95% pure by Coomassie brilliant blue R staining (Fig. 3).

Biochemical characteristics

To further confirm product specificity and activity we performed several tests. The sTβRII-Fc fusion molecule consists of the extracellular portion

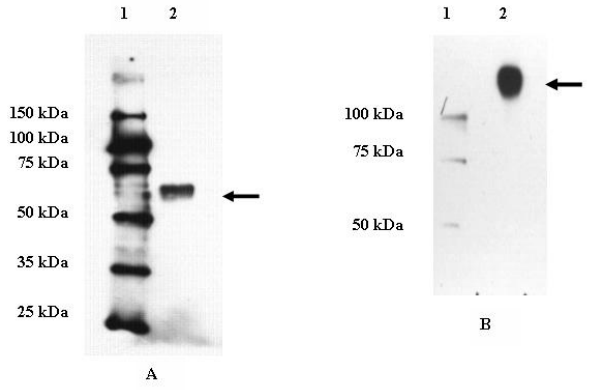

Figure 4. Western blot analysis of the chimeric sTβRII-Fc molecule.
sTβRII-Fc was purified from culture medium by one step protein A Sepharose chromatography and subjected to polyacrylamide gel electrophoresis under reducing (A) or non-reducing (B) conditions. Following electrophoresis the resolved samples were transferred onto PVD membrane and immunoblotted with a goat anti-human TβRII antibody. The position of molecular mass standards is indicated on the left.

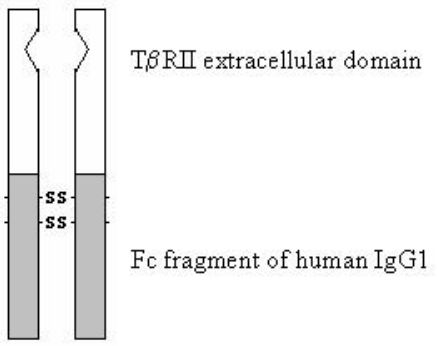

Figure 5. Schematic representation of recombinant chimeric soluble TβRII-Fc molecule.
TβRII-Fc is a homodimer containing two chimeric chains covalently linked by disulfide bonds. The TβRII extracellular domain is responsible for specific TGF-β binding and the Fc fragments of human IgG1 impose dimer formation increasing the receptor affinity and allow for easy one step purification of TβRII-Fc.

of the TGF-β type II receptor lacking the transmembrane domain, the IgG heavy-chain hinge region Cys residues and the CH2 and CH3 domains of the IgG constant region. The preserved structure of the IgG Fc portion imposes homodimer formation by virtue of its hinge region. Analysis of the purified chimeric receptor by 10% SDS/PAGE was performed under reducing (with 0.715 M 2-mercaptoethanol) and non-reducing conditions followed by Coomassie brilliant blue R staining (Fig. 3). Whereas under reducing conditions a product of a molecular mass around 65 kDa was obtained, under non-reducing conditions it migrated at approx. 130 kDa, which confirms the predicted homodimer structure of the native chimeric molecule.

The Western-blot analysis showed that the produced recombinant protein was recognized by a polyclonal antibody raised against TβRII (Fig. 4) and against human IgG (not shown). These results indicate that the recombinant protein consist of the extracellular domain of sTβRII and the Fc fragment of human IgG and forms homodimers by disulfide bonds (Fig. 5).

Next we tested the ability of recombinant soluble type II TGF-β receptor to bind native TGF-β. Conditioned medium from NS0 cells transfected with the pEE12.4:sTβRII-Fc plasmid was incubated with 70 pM ^{125}I-TGF-β1, chemically cross-linked with DSS, and immunoprecipitated with protein A agarose beads, resolved by electrophoresis and visualized by autoradiography. As it is shown in Fig. 6, bands corresponding to ^{125}I-TGF-β1/sTβRII-Fc complexes are clearly visible indicating that the soluble secreted receptor is able to bind and be chemically cross-linked to ^{125}I-TGF-β1. These cross-linked complexes appear to be specific, since the ^{125}I-TGF-β1 binding can be effectively outcompeted by unlabeled TGF-β1 in a dose dependent manner.

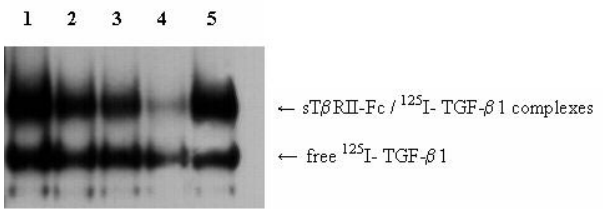

Figure 6. *In vitro* binding of chimeric soluble type II TGFβ-receptor to ^{125}I-TGF-β1 and competition by unlabeled TGF-β1.
Conditioned medium from NS0 cells, containing sTβRII-Fc, was incubated with ^{125}I-TGF-β1 (70 pM) in the absence (lane 5) or presence (lanes 1–4) of increasing amounts of unlabeled TGF-β1. After chemical cross-linking with DSS, the samples were subjected to immunoprecipitation with protein A immobilized on beaded agarose which binds to the Fc fragment of IgG. Following immunoprecipitation, the samples were boiled in Laemmeli buffer, resolved by PAGE and exposed to X-ray film. Lane 1, 10 pM; lane 2, 20 pM; lane 3, 40 pM; lane 4, 400 pM; lane 5, no TGF-β competitor (0 pM).

Since these chemically cross-linked complexes can be easily precipitated by protein A agarose, this indicates that the complexes have protein A binding domains such as IgG Fc fragments.

We therefore concluded that the extracellular domain of the type II TGF-β receptor fused with the Fc portion of human IgG can bind TGF-β1.

sTβRII-Fc biologic activity *in vitro*

The recombinant chimeric sTβRII-Fc molecule was expected to act as a potential TGF-β antagonist. To test this possibility we performed two functional tests where sTβRII-Fc could attenuate the biologic effects of TGF-β. In the first set of experiments we

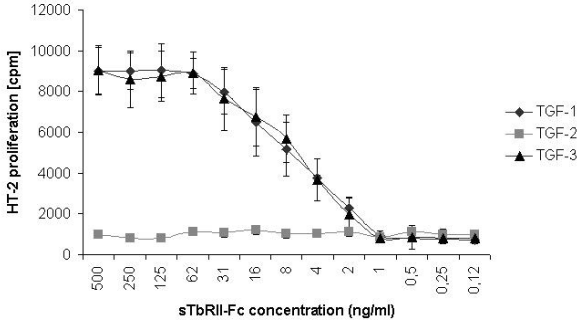

Figure 7. sTβRII-Fc neutralizes TGF-β growth inhibitory activity *in vitro*.
Increasing doses of NS0-derived sTβRII-Fc are able to reverse the proliferation-inhibiting activity of TGF-β1 and TGF-β3 as measured using HT-2 cells. Assay medium containing TGF-β isoforms: TGF-β1, TGF-β2 and TGF-β3 was pre-incubated with serial dilutions of recombinant sTβRII-Fc (0.0012–0.5 μg/ml) and then applied on HT-2 cells to be assayed for growth inhibition. After 12 h the cells were pulsed with [^{3}H]thymidine and assayed for proliferation. The inhibitory activity of TGF-β1 and TGF-β3 but not that of TGF-β2 was reversed by sTβRII-Fc in a dose dependent manner.

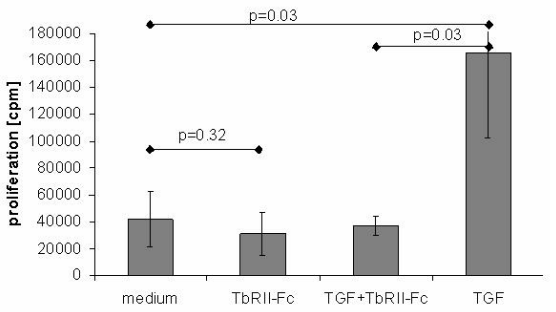

Figure 8. sTβRII-Fc suppresses TGF-β1 growth-promoting activity *in vitro*.
TC-1 tumour cells were cultured in the presence of TGF-β1, sTβRII-Fc or their mixture. After 24 h the cell proliferation was measured by [^{3}H]thymidine incorporation. Addition of TGF-β1 leaded to enhanced tumour cell growth which was inhibited by sTβRII-Fc.

tested the sTβRII-Fc ability to reverse the growth inhibition of HT-2 cells induced by TGF-β. TGF-β inhibits the proliferative response of HT-2 cells to added IL-4 (Ruegemer *et al.*, 1990). The TGF-β isoforms: TGF-β1, TGF-β2 and TGF-β3, were preincubated at 100 pg/ml with increasing amounts of sTβRII-Fc (0.0012–0.5 μg/ml) and then added to the HT-2 cells. As it is shown in Fig. 7 the chimeric sTβRII-Fc was able to reverse the inhibitory effect of TGF-β1 and TGF-β3, however, it was almost ineffective against the TGF-β2 isoform. The relative effective dose for TGF-β1 or TGF-β3 inhibition was determined as 8 ng/ml sTβRII-Fc in this setting. Therefore we concluded, that sTβRII-Fc has high affinity for TGF-β1 and TGF-β3 but not for TGF-β2. The addition of sTβRII-Fc into culture medium without TGF-β did not have any influence on HT-2 cells growth (data not shown) indicating that sTβRII-Fc does not display any stimulatory activity on HT-2 cells which could overcome the TGF-β inhibition.

In contrast to the HT-2 cell line, TC-1 cells growth is stimulated by TGF-β (Kowalczyk, unpublished). To reverse this stimulatory activity of TGF-β, TC-1 cells were incubated with or without sTβRII-Fc and TGF-β1. As it is shown in Fig. 8, sTβRII-Fc treatment attenuated TGF-β growth promoting activity in TC-1 cells. The tumour growth inhibition activity of sTβRII-Fc when added alone is most likely due to neutralization of endogenous TGF-β which may stimulate tumour cell growth in an autocrine manner.

These results demonstrated the potent ability of sTβRII-Fc to act as a TGF-β antagonist.

DISCUSSION

Here we presented the design, generation in the GS-NS0 system and biochemical characterization of recombinant soluble TGF-β type II receptor fused with the Fc portion of human IgG1. The GS-

NS0 expression system is the most industrially important expression systems and as such has proven useful for overexpressing a number of cytokines and other bioactive molecules (Bebbington *et al.*, 1992). The system is the preferred one for so called difficult proteins (Bebbington *et al.*, 1992). Although it has been reported that protein production in insect cells far exceeded that observed for mammalian cell systems, with process optimization concentrating on growth medium and feeding regimens, recombinant protein production can be improved significantly in the GS-NS0 system. For some proteins, accumulated yields of 100–600 mg/l (!) may be routinely achieved, with production rates of 10 to 50 $\mu g/10^6$ cells per day (Bibila & Robinson, 1995; Dempsey *et al.*, 2003). Our attempts to produce significant amounts of sTβRII-Fc in a baculovirus expression system generally failed. At the moment we do not have any clear-cut explanation for these difficulties.

The addition of the IgG Fc portion to the soluble TGF-β type II receptor has generated several advantages. It enabled a relatively easy isolation and purification of the recombinant protein by one step chromatography. The disulfide bonds in the Fc fragment imposed homodimer formation which increased the receptor affinity for its cognate cytokine (Lin *et al.*, 1992). Moreover, the increased molecular mass exceeding 60 kDa may extend *in vivo* half-life by protecting it from renal clearance.

The sTβRII-Fc chimeric molecule produced in the GS-NS0 system was shown to bind native TGF-β1 in standard biochemical assays indicating proper molecular conformation of the recombinant protein. Moreover, in more complex biologic *in vitro* systems chimeric sTβRII-Fc displayed a high neutralizing activity against TGF-β1 and TGF-β3.

To show the potential of recombinant sTβRII-Fc as a TGF-β antagonist we used two different functional assays. In the first assay with the HT-2 cells we blocked the antiproliferative TGF-β activity showing that sTβRII-Fc treatment is able to reverse TGF-β action and restore proliferation. This system may thus mimic attenuation of excessive, inhibitory TGF-β activity on immune cells where TGF-β appears to be a major natural immunosuppressant (de Visser & Kast, 1999). In the second experimental system we showed that sTβRII-Fc was able to neutralize TGF-β in conditions where this cytokine plays a stimulatory role and promotes tumour cell proliferation. These two systems revealed the ability of sTβRII-Fc to efficiently prevent TGF-β activity regardless of the TGF-β receptor level. While HT-2 cells have an extremely low number of TGF-β receptors on the cell surface, TC-1 cells show a much higher number of TGF-β receptor molecules (Massague, 1992).

In the TC-1 cells system TGF-β neutralization with sTβRII-Fc also appeared to significantly inhibit proliferation of tumour cells on its own. This action is most likely mediated by inhibition of endogenously-derived TGF-β which acts in an autocrine manner. Moreover, this *in vitro* anti-tumour activity was also observed against several murine and human cell lines (not shown, manuscript in preparation).

The strategy of using a soluble cytokine receptor fused with the immunoglobulin Fc portion as a cytokine antagonist has already been proved clinically beneficial. Examples include attenuation of TNF-α activity in numerous pathological conditions (WGET Research Group, 2005; Madhusudan, 2005; Nash & Florin, 2005). Thus, a similar approach might be feasible for TGF-β which is involved in pathogenesis of many serious clinical conditions (Flanders & Burmester, 2003).

TGF-β overexpression has been observed in glomerulonephritis, pulmonary fibrosis, liver cirrhosis, and keloids implying that agents with anti-TGF-β activity may have clinical potential in the treatment of these diseases (Border & Noble, 1994). Besides benign conditions, TGF-β is involved in tumour development, invasion and metastasis. Moreover, many tumours secrete increased amounts of TGF-β leading to immunosuppression and in such cases TGF-β neutralization may be a valuable approach in anticancer therapy (Siegel & Massague, 2003; Wojtowicz-Praga, 2003; Flanders & Burmester, 2003).

Strategies for blocking excessive TGF-β activity include gene therapy with soluble TβRII fragments and dominant negative mutants (Fakhrai *et al.*, 1996; Sakamoto *et al.*, 2000; Gorelik & Flavell, 2001), treatment with decorin (Kolb *et al.*, 2001), tranilast (Kosuga *et al.*, 1997), neutralizing antibodies (Hill *et al.*, 2001), threonine kinase inhibitors (Callahan *et al.*, 2002) and RNA expression inhibitors such as anti-sense expression vectors or blocking oligonucleotides (Maggard *et al.*, 2001). Given that their specific application may differ depending on particular clinical situation, diversification in TGF-β neutralization strategies will only expand therapeutic options.

In conclusion, the GS-NS0 expression system was found to be an excellent approach for high-titer production of biologically active and difficult-to-express proteins such as sTβRII-Fc. Our results clearly demonstrated that recombinant sTβRII-Fc had a sufficient ability to bind TGF-β and compete with the natural, membrane-bound form of the receptor. Therefore, recombinant sTβRII-Fc could be employed as a TGF-β1 and TGF-β3 antagonist *in vitro* and *in vivo*.

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U.K. is a student of the Postgraduate School of Molecular Medicine

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