Evaluation of Specific Purified TCR Effect on the Immunoregulatory Potential of TGF-β

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ABSTRACT

Transforming growth factor beta (TGF-β) is a mediator released by nearly all cell types. It has suppression activity on the immune system, but exactly how this effect is carried out is not clear. Previous experiments showed that IgG interacts with or carriers active TGF-β, that could suppresses cytotoxic T-cell responses to an immunogenic tumor in mice. Since T cell receptor (TCR) has structural similarities with IgG, we asked the question whether a specific TCR could interfere with and enhance the suppressive effect of TGF-β on T-cell proliferation. T-cell lines were established by limiting dilution and specific TCR were extracted and purified. Mixed lymphocyte reaction (MLR) was carried out using DA ($RT1^a$) vs. LEW ($RT1^l$) lymph node cells and DA vs. PVG ($RT1^u$) lymph node cells. DA cells were used as responder cells and PVG/LEW as stimulator cells. Proliferation of DA cells was examined with different concentration of TGF-β by adding 1μci ^{3}H-thymidine 24 hours prior to harvesting the cells. The results showed that the presence of a specific TCR does not have any effect on the percentage of suppression when already fully suppressed by TGF-β. However, it does have an effect on TGF-β stimulated suppression under certain conditions. When TCR was added at the same concentration as TGF-β (1-2 ng/ml), inhibited TGF-β stimulated suppression of proliferation, but when added at higher concentration than TGF-β, this effect disappeared, and the proliferation was suppressed in the same way, as TCR was absent. Thus, TCR interaction with TGF-β could play an important role in the homeostasis of immunity by augmenting the proliferation of activated dominant lymphocyte clones. This would promote suppression of activation/proliferation of new specific antigen-reactive clones that may arise during ongoing immunity, and also suppressing some autoimmune diseases. *Iran. Biomed. J. 9 (1): 9-14, 2005*

Keywords: Transforming growth factor beta (TGFβ) , Immunoregulation, TCR, T-cell line

INTRODUCTION

The cell growth requires highly complex interactions between ligands (growth factors) and receptors to induce both cellular and molecular events for acceleration or suppression of cell growth [1]. Transforming growth factor beta (TGF-β), is produced by both normal and neoplastic cells, and has been implicated in development of both immune response and cancer [2-4]. TGF-β was first described as an activity produced by a murine sarcoma virus-transformed cell line [5]. Further molecular analyses revealed that this factor contains two distinct classes: TGF-α and TGF-β. TGF-β with molecular weight of 25 kDa has five isoforms and only three of them are present in humans [6]. The mature form of TGF-β homodimer is a

biologically inactive or latent form which can not bind to TGF-β receptors. Further studies revealed that this high molecular weight latent form is due to non-covalent binding with an N-glycosylated precursor, termed TGF-β1 latency-associated protein (TGF-β1-LAP) [7]. The ability of immunocompetent, cells such as T and B cells, to produce TGF-β which in turn regulates the cell response, suggests that this cytokine plays a crucial role in the induction of immune responses. However, the exact immuno-regulatory effect of TGF-β remains to be clarified [8, 9]. Renee and Rowley [10] found that IgG is associated with or carries active TGF-β, which suppresses cytotoxic T-cell responses to an immunogenic tumor in mice. IgG-TGF-β also delivers the mediators upon binding via the F_c receptor to effector cells such as macrophages, since the latent TGF-β contains glycosylation sites that are necessary for this inactive form. They further suggested that IgG and TGF-β may be linked through carbohydrate chains. By cleaving these chains, the active 25 kDa TGF- β will be released. Immunoglobulins are known to be hold together by carbohydrate chains, linking the two constant regions. The structures of these carbohydrates have been found to be mainly of the complex type, ending up in N-acetyl-glycosamine-galactose (sialic acid) in cattle and humans [11, 12].

Experiments in rabbits showed that when galactose and sialic acid subunit are present together in the pocket between the two Ig heavy chains, the pocket would be accessible for small molecules [13]. Kehrl *et al.* [14] showed that latent TGF-β becomes activated upon binding to sialic acid and mannose-6-phosphate. Also, other carbohydrates, such as N-acetyl glucosamine, mannose and mannose-1-phosphate were able to activate latent TGF-β. TCR has structural similarities with IgG and contains carbohydrates which are added during the development in thymus. It is known that a typical α and β chain from mammals contains several N-linked glycosylation sites. Previous study by Cross and Cambier [15] showed that α-chain was relative more acidic than β-chain and this difference disappeared upon Endo-F digestion (by internal carbohydrate bonds breakage). Since sialic acid is charged carbohydrate, it is reasonable to believe that the different appearance in isoelectric focusing is due to the sialic acid of α-chain. Is it possible that TGF-β will bind TCR similar to IgG and promote suppression of proliferation upon activation? The aim of this report was to study whether a specific T-cell-receptor from a T-cell line could interfere with and enhance the suppressive effect of TGF-β on T cell proliferation.

MATERIALS AND METHODS

Animals. In all experiments, LEW (RT^1), DA (RT^a) and PVG ($RT1^u$) male rats (6-8 weeks old) bred in the University of Manchester, Biological Service Unit, were used.

T cell lines. Pooled DA lymph node (LN) cells (5×10^6 cells) were cultured with 5×10^6 irradiated (3,000 rads) LEW LN cells in RPMI-1640 containing 10% FCS, 2 mM L-glutamine, 12.5 mM HEPES, 100 U penicillin, 100 mg streptomycin and 5×10^{-5} M 2-mercaptoethanol for 10-14 days at 37ºC with 5% CO_2 [16]. Blast cells were harvested after ficoll hypaque preparation. The DA cells (25-50 cells) were cultured with 1×10^6 irradiated LEW stimulator cells in the presence of 15-20% concanavalin (con A) supernatant (Sigma-Aldrich, St. Louis, Missouri, USA). Cultures were maintained for approximately 6-8 weeks with fresh concanavalin A supernatant being added every 3-4 days and (with) fresh irradiated stimulator cells every 8-10 days. All lines were allo-stimulated six times before analysis.

TCR extraction. Proliferating DA T-cell lines were centrifuged at 300 ×g for 5 minutes. Pellets were re-suspended in lysis buffer (150 mM NaCl, 1% w/v NP-40, 0.01 M TEA buffer pH 7.8-8.0) and 10% phenylmethylsulfonyl fluoride [m1](10 mg/ml in isopropanol) and left on ice for 30 minutes. The solutions were centrifuged at 300 ×g for 15 minutes and then the supernatant was collected. After another 25 minutes at 0ºC, the supernatant was ultra centrifuged at 39,000 ×g for 45 minutes (Beckman Ultra-centrifuge, UK) and dialyzed against PBS 4 times each for 12 hours at 0ºC and finally stored at -80º C.

Isolation of monoclonal antibody (R73) against TCR. Cell clone R73 (ECACC 90091904) was grown in complete DMEM medium for 3 weeks, expanded every 5-7 days and left to die 1 week before collection. The supernatant was collected and stored at -20ºC. The monoclonal antibody R73 was purified on protein G-Sepharose 4 FF column (MABTrap™ G Kit, Pharmacia, Uppsala, Sweden), and collected fractions were examined for proteins at 280 nm. The supernatant (300 ml) was purified and 1.27 mg of protein was obtained. The final volume was dialyzed against PBS for 24 hours. Monoclonal antibody R73 was concentrated 10 fold on a B15 Minicon® concentrator (Pharmacia, Uppsala, Sweden).

TCR purification. TCR was purified by affinity chromatography. CNBr-activated Sepharose (1 g) was

mixed with 10 ml 1 mM HCl for 15 minutes. The supernatant was removed after centrifugation at 300 ×g for 2 minutes. The Sepharose was washed in 1 mM HCl and in coupling buffer (PBS, 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.5). The final volume was adjusted to 5 ml and purified mAb from R73 cell clone was added and left for 22 hours. Coupled Sepharose was resuspended in inactivating buffer (1 M ethanolamine-HCl, pH 8.0) and left for 3 hours to block Sepharose non-occupied sites. The mixture was finally washed 3 times in washing buffer I (1 M NaCl, 0.1 M sodium acetated, 0.02% sodium azide, pH 4.0), 3 times in washing buffer II (0.025 M Tris-ethanolamine, 1M NaCl, 0.02% sodium azide, pH 8.5s sodium azid, pH 7.3) and then added to a minicolumn. TCR solution was added on the top of the gel and allowed to bind to R73 during half an hour. After binding, the column was washed 3 times with stabilizing buffer using a peristaltic pump. TCR was eluted using elution buffer (0.05 M diethylamide, pH 11.5) and the fractions were neutralized with 40 µl of 0.1M Tris-HC, pH 4.5. TCR was concentrated with Minicon and protein was measured at 280 nm. Finally, TCR was freeze dried and stored at -80ºC. TCR purity was determined by SDS-PAGE.

Mixed lymphocyte reaction (MLR). MLR were carried out in 96−well flat−bottomed tissue culture plates using DA vs. LEW lymph node cells and DA vs. PVG lymph node cells. DA cells were used as responder cells and PVG and LEW as stimulator cells (both irradiated 1 hr, 2000 rad and used at $5 \times 10^6 - 1 \times 10^7$ cells/ml). Cells were suspended in 100 µl of complete medium (RPMI 1640 containing; 25 mM HEPES, 2 mM L−glutamine, 1 mM sodium pyruvate, 0.1 mM non−essential amino acids, 5×10^{-5} M 2−ME, 10% FCS, 0.5 U/ml penicillin and 0.5 mg/ml streptomycin) and 100 µl of RPMI 1640 without FCS. Cultures were incubated at 37ºC, 5% CO_2, and 100% humidity. The proliferation of DA cells was examined with different concentration of TCR and TGF−β1 (R & D systems Europe Ltd., UK)

Fig. 1. TCR affinity chromatography. Profile of TCR purification by affinity chromatography. R73 clone was grown in complete DMEM and the supernatant was purified on a protein G column. Fractions 4 and 5 were collected and pooled.

on a Beckmann LS1801 Beta counter by adding 1 µCi ^{3}H-thymidine 24 hours prior to harvesting of the cells.

Fig. 2. LEW × DA mixed lymphocyte reaction. Proliferation of DA lymph node cells stimulated with LEW irradiated cells (3000 rads) TGF-β was added alone or with equal amounts of TGF-β + TCR (specific).

RESULTS

The supernatant from R73 producing clone was collected when 50% of the cells were dead. After collecting the fractions with the highest optical density (OD), the concentration was 0.094 mg/ml.[m2]

TCR was purified by affinity chromatography using monoclonal antibody R73 The profile of protein after the purification step is seen on Figure 1. Fractions 4 and 5 were pooled, giving a final concentration of 0.38 mg/ml.

To see whether the specific TCR had any influence on the suppressive effect of TGF-β, a panel of mixed lymphocyte reaction was set up. Using DA rat cells as responder to either irradiated PVG or LEW cells as stimulators. The reaction was set up with a broad range from 0.2 ng to 30 ng of TGF-β and TCR in equal amounts. The results (Figs. 2 and 3) indicated the proliferation vs. concentration of TGF-β alone or TGF-β and TCR (equal amount) for both LEW and PVG stimulated growth. The percentage of suppression obtained from MLR experiments is showed in Figures 4 and 5. In both experiments, the TCR does not have any effect on proliferation when added in higher concentration. But at concentrations around 1-2 ng the effect was remarkable, presumably, the soluble TCR was capable of binding to the TGF-β and therefore inhibiting the

normal suppression of proliferation.

Interestingly, this effect did not exist at a lower concentration (0.2 ng). To see whether the concentration of TCR was crucial, experiments with constant TGF-β concentration and two different concentrations of TCR were done. The results are showed in Figures 6 and 7. Both LEW and PVG stimulated proliferation at higher concentration of TCR compared to TGF-β that had no effect on proliferation (it may even enhancing it a little, while lower concentrations prevent suppression).

Fig. 3. PVG × DA Mixed Lymphocyte Reaction. Proliferation of DA lymph node cells stimulated with PVG irradiated cells (3000 rads) TGF−β was added alone or with equal amounts of TGF−β + TCR (specific).

Fig. 4. PVG × DA Mixed Lymphocyte Reaction. Percentage of suppression of proliferation after stimulation with PVG lymph node cells and either with TGF-β alone or TGF-β + TCR in equal concentration.

DISCUSSION

The antiproliferative activity of TGF-β has been described in numerous cell systems *in vitro* [17]. The ability of TGF-β to enhance or inhibit cellular functions often depends on the state of differentiation or activation of the target cells, the concentration of TGF-β, and the presence of other growth factors [18-19]. Furthermore, TGF-β has been shown to block IL-2 production in T cells and cell lines [20], and with the over production of IL-2 in the TGF-β (-/-) mice [21].

The aim of this study was to examine the suppression role of TGF-β with a specific TCR on the T cell proliferation. Previous studies showed that glioblastoma and few other tumors secrete latent TGF-β that are associated with the immune suppression. An immunogenic murine tumor transfected to secrete latent TGF-β failed to stimulate CTL *in vivo* and *in vitro* [22-24].

Fig. 5. LEW × DA mixed lymphocyte reaction. Percentage of suppression of proliferation after stimulation with LEW lymph node cells and either with TGF-β alone or TGF-β + TCR in equal concentration.

Although the data presented here show that the presence of a specific TCR does not have any effect on the percentage of suppression by TGF-β when already fully suppressed. However, the results indicate that the specific TCR does have an effect on TGF-β stimulated suppression under certain conditions.

Fig. 6. Suppression of proliferation of DA cells after stimulation with irradiated PVG lymph node cells (3000 rads) and adding variable amounts of TCR (ng) to same amount of TGF-β (ng).[m3]

Fig. 7. Suppression of proliferation of DA cells after stimulation with irradiated LEW lymph node cells (3000 rads) and adding variable amounts of TCR (ng) to same amount of TGF-β (ng).

When TCR was added in the same concentration as TGF-β (1-2 ng), it does inhibit TGF-β stimulated suppression of proliferation. But, when TCR was added in higher concentrations than TGF-β, this effect

disappears, and proliferation is suppressed in the same way as when TCR is not present. The effect is seen not only when LEW cells are used as stimulators, but also when PVG cells are used. Since the TCR used is from a DA line specific against LEW, we do not expect any effect of adding TCR to PVG stimulated DA cells, since the result is the same. This result suggests the possibilities that first, PVG and LEW must have shared antigenic epitopes so that TCR will be able to bind both lines. Second, TCR uses an antigen independent pathway that will affect both T-cell lines.

When TCR is added in the same concentration as TGF-β, the number of molecules is much less for TCR than for TGF-β, since the molecular molarity is about three times higher. So, it is possible that there is not enough TCR present to bind and mediate the effect of TGF-β, or TCR is fully saturated with TGF-β, which will fully inhibit any suppressive effect by TGF-β. At higher TGF-β concentrations, proliferation is fully inhibited, and the addition of TCR does not have any effect. In these cases, there is enough TGF-β to suppress the normal direct pathway, and the addition of TCR in the same concentrations is not enough to change suppression. At lower concentrations (0.2 ng), there is no effect either. The explanation for this finding is possibly because of the higher affinity of TGF-β for cell receptors than for soluble TCR. Renee and Rowley [10] found that antibodies to TGF-β are effective in bioassays only when they are preincubated with active TGF-β before addition to target cells because of the higher affinity of TGF-β for receptors than for antibody. [m4]

Glick *et al.* [25][m5] demonstrated that fresh sera from mice immunized by bearing an immunogenic tumor or by repeated injections of allogeneic spleen cells or xenogeneic erythrocytes powerfully suppress cytolytic T cell responses in one-way MLR. Suppression is not antigen specific, though is mediated by IgG specific for the immunizing antigen. Suppression caused by IgG mimics the effect of active TGF-β.

On a broader scale, the effects of TGF-β are pleiotropic and depend on the type and state of activation or maturation of target cell in many different tissues, suggesting that the activity of TGF-β must be highly restricted or regulated at different sites [26-27]. Thus, the idea that the activity of TGF-β in immunity is modulated by Ig suggests one kind of strategy for limiting the activity of TGF-β to the relevant system. This activity is compared to the association of TGF-β2 with a-fetal protein which suggests a different strategy for limiting activity of TGF-β to the site of maternal-fetal interaction [28].

Therefore, TCR localizing TGF-β at antigenic sites could play an important role in the homeostasis of immunity. This is done by augmentation proliferation of already activated dominant lymphocyte clones [29]. Clones would promote suppressing activation/proliferation of new specific antigen-reactive clones that may arise during ongoing immunity, and suppressing some autoimmune diseases. On the other hand, TGF-β can promote abnormal scarring as well as wound healing, and stimulate growth of some malignancies, so that TCR carrying TGF-β to antigen target sites in some autoimmune diseases or cancer may have deleterious effects. [m6]

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