PRODUCTION OF TGF-β1 SPECIFIC CHICKEN POLYCLONAL ANTIBODIES AND THEIR USE IN ANALYSIS OF CYTOKINE LEVEL IN HOOKWORM INFECTED HAMSTERS

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Abstract

In vitro production of a potent immunoregulatory cytokine – TGF-β1 in mesenteric lymph node (MLN) cell cultures derived from three age groups of Syrian golden hamsters infected with Ancylostoma ceylanicum was investigated. Expression of TGF-β1 mRNA in the cells was measured by real-time quantitative PCR. Protein concentration in cell culture supernatants was determined by ELISA with the use of generated chicken polyclonal antibodies recognising hamster TGF-β1. The highest concentration of this cytokine was released by cells from the youngest hamsters, which were the most susceptible to hookworm infection. Real-time PCR analysis of TGF-β1 mRNA expression in non-stimulated MLN cells showed that fold changes in mRNA expression in the age groups corresponded to protein concentration in culture supernatants. To our knowledge this is the first report of TGF-β1 production during hookworm infection in hamsters. We hope that our studies provide a basic start for further investigation of TGF-β1 role in immunosuppression during hookworm infection.

Key words: hamster, Ancylostoma ceylanicum, immunosuppression, lymph nodes, cytokines.

TGF-β is a cytokine with a very broad spectrum of induced biological effects, which depend on time, physiological status and cooperation with other interleukins and biomolecules. Most of these interactions are yet to be determined. TGF-β shows a significant role in inducing the inflammatory process by recruitment of monocytes from venous blood and their infiltration to the inflammation site. TGF-β also stimulates phagocytosis, production of chemoattractants for monocytes and expression of integrins, which allow their adhesion to the endothelium (32, 40-42). On the other hand, TGF-β acts as a very powerful silencer of inflammatory reaction (28). It also influences T and B lymphocytes at each stage of their development (5, 25, 27). All these facts show how complicated and undefined interactions are between TGF-β and other cytokines and how distinct effects it has on immunological response and its efficiency.

In many cases helminth infection triggers the Th2 immune responses, which are protective for the host. These have been well documented in murine laboratory model of infection with intestinal nematode parasites (reviewed in 15). However, some helminth infections such as schistosomiasis and filariasis elicit in some cases a ‘modified Th2’ immune response, which is characterised by low levels of Th1 cells and high expression of IL-10, which might indicate a strong regulatory T (Treg) cell activity (33). Individuals with such type of immune response are susceptible to these parasites, but the infection is clinically silent and chronic. Treg activity is associated with high expression of IL-10 and TGF-β. These cytokines are responsible for active downmodulation of T-cell proliferation to filarial antigens, which can be restored in vitro by antibodies specific to IL-10 and/or TGF-β (13, 23). IL-10 and TGF-β are also responsible for enhanced parasite survival during schistosomiasis (37). TGF-β neutralisation in mice infected with Heligmosomoides polygyrus resulted in elevation of cytokines in serum, which coincided with the reduction of worm burdens and faecal egg counts and restored the infiltration of eosinophils into the lamina propria of the small intestine (14). High levels of TGF-β are also coincident with susceptibility to infection with intracellular parasites such as Trypanosoma cruzi (39) and Leishmania species (1).

The idea that TGF-β is responsible for suppression of host immune response is supported by the fact that helminths produce protein homologues of TGF-β that functionally interact with host cytokine receptors. TGF-β like proteins were first identified in a filarial worm Brugia malayi, BmTGH-1 (22) and...
BnTGH-2 (21). BnTGH-2 shares a 32% homology in an active C-domain to human TGF-β, binds to the TGF-β receptor, and shows similar biological effects in vitro (21).

The mechanisms of immunosuppression induced by hookworms, and immune responses of the host have been the subject of many studies (reviewed in 12, 16, 31). Hookworms release many immunosuppressive agents such as neutrophil inhibitory factor NIF (36), metalloproteases which can cleave eotaxin (8), or factors, which are capable of inducing apoptosis in activated T cells (6). The role of cytokines in immunosuppression during hookworm infection remains unknown, although high levels of IL-10 in infected patients suggest the role of Treg cells (19, 38). Recently, it has been shown that hookworm antigens alter dendritic cell differentiation and modify their function, as well as induce generation of CD4 and CD8 suppressor T cells (7, 17).

Levels of TGF-β and its possible role in hamster immune response against hookworms have not been established so far. Therefore, we have decided to investigate the changes of TGF-β1 production during A. ceylanicum infection in Syrian golden hamsters of different age. Because of lack of appropriate immunological reagents we have produced chicken polyclonal antisera recognising hamster TGF-β1. Using those we determined the TGF-β1 concentration in supernatants from cultures of MLN cells. In order to confirm our results, TGF-β1 mRNA expression was also investigated using real-time PCR.

Material and Methods

Expression and purification of hamster recombinant TGF-β1. Total RNA was extracted from hamster spleen with “Total RNA” kit (A&A Biotechnology, Poland) and used as a template for a reverse transcription reaction with RevertAid H Minus M-MulV Reverse Transcriptase (MBI Fermentas). cDNA encoding a fragment of the mature protein was amplified using PCR with the following primers: TGFBL (5’ TTCACTCCACGGAGAGAAGCT 3’) and TGFBR (5’ GGGCTTCGACCCCAGTACTCA A 3’). The primers were designed according to the nucleotide sequence available in GenBank under accession number: X60296. PCR was performed using the following reagents: 25 ng of template cDNA, 1 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each specific primer, and 1U Taq polymerase (MBI Fermentas) for 35 cycles in MJ Research thermocycler under conditions at 94°C for 30 s, 56°C for 30 s, and 72°C for 20 s. The amplified product was then cloned into pET 28a (+) expression vector (Novagen). The obtained recombinant plasmid was sequenced to confirm the proper ORF of the insert and competent E. coli BL21 (DE3) cells were electrotransformed. Expression of recombinant protein was induced in cells in the late log phase by the addition of 1 mM isopropyl thiogalactoside (IPTG) (MBI Fermentas). Recombinant TGF-β1 was purified by the method of affinity chromatography on HiTrap Chelating HP column (Sigma) under denaturing conditions, according to manufacturer’s instructions. The recombinant protein was further electroeluted from the polyacrylamide gel in order to eliminate possible contamination with bacterial proteins. Gel slices containing recombinant TGF-β1 were electroeluted in Bio-Rad Electro-Eluter. The purity and specificity of purified recombinant cytokine was determined by SDS-PAGE and Western blotting with histidine antibodies (Sigma). The concentration was defined using BCA Protein Assay Kit (Pierce).

Production of chicken polyclonal antibodies to hamster TGF-β1. Six-week-old Leghorn white chicken was immunised intramuscularly with 0.5 mg of rTGF-β1 with Freund’s incomplete adjuvant. Booster immunisations were administered three times with alum adjuvant in 2 week intervals. Two weeks after the last immunization, the chicken was sacrificed and serum was collected. The specificity of antibodies to recombinant and native TGF-β1 was determined by Western blotting.

Hamsters. Three age groups of Syrian Golden hamsters: 4-week-old, 8-week-old, and 6-month-old were infected orally with 60 infective larvae (L3) of A. ceylanicum. All groups consisted of 10 animals. 21 d after the infection, the animals were euthanised. Adult worms were removed from the intestine and counted. All animals were maintained under standard conditions according to local regulations. All experiments were approved by the Third Local Ethical Committee on Warsaw University of Life Sciences - SGGW. The study was conducted according to the institution’s guidelines for animal husbandry.

Cell cultures. Mesenteric lymph nodes (MLN) were removed from euthanised hamsters and single-cell suspensions were prepared using a cell dissociation sieve. Erythrocytes were lysed in 170 mM Tris buffer containing 155 mM NH₄Cl for 5 min. The cells were washed and cultured in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, and antibiotic-antimycotic solution (all from Sigma). Then the cells were plated at 2 x 10⁶ cells/well in 12-well plates and cultured with medium alone, ConA (Sigma) at a final concentration of 5 µg/mL, or A. ceylanicum somatic antigen (30 µg/mL). The plates were incubated at 37°C in an atmosphere of 5% CO₂. After 72 h of incubation, cell-free supernatants were collected.

Real-time PCR. After 72 h culture, unstimulated MLN cells (1 x 10⁷ cells) derived from all animals were separated from the culture medium and total RNA was isolated with “Total RNA” kit (A&A Biotechnology) according to manufacturer’s instructions. Prior to CDNA synthesis, equal amounts of RNA were pooled from two individuals into one sample. Five micrograms of total RNA was then used as a template for CDNA synthesis using RevertAid H Minus M-MulV Reverse Transcripase (MBI Fermentas). Real-time PCR analysis was performed in the Light Cycler 2.0 System with the Light Cycler Fast Start DNA Master SYBR Green I Kit (Roche Applied Science).
Amplification was performed under the following conditions: 94°C for 5 s, 58°C for 10 s and 72°C for 5 s. Primers for TGF-β1 were the same as those used for cloning and primers for HPRT were: HPRTF: 5’ TGGCATGTCATGTAGAGATGG 3’ and HPRTR: 5’CATTTTGGGTTGTACTGTTTG 3’. Each reaction was performed in triplicates. Quantification of mRNA expression was analysed with the 2^−ΔΔC_T method (30) and presented as the fold change in gene expression normalised to an endogenous reference gene (HPRT) and relative to the calibrator (4-week-old group).

**ELISA.** TGF-β1 concentrations were determined using direct ELISA. 96-well plates were coated with 100 µl of culture supernatants for 12 h at 4°C. The plates were then washed 3 times with PBS/0.05% Tween (PBS-T) and blocked with 5% non fat milk in PBS-T for 1 h at 37°C. After washing, the plates were incubated for 1.5 h at 37°C with chicken serum recognising hamster TGF-β1 diluted 1:100 in PBS-T. Again the plates were washed and incubated with a 1:30,000 dilution of rabbit anti-chicken IgY HRP-conjugated antibodies (Sigma) for 1 h at 37°C. Following washing, the plates were developed with TMB (Sigma) according to manufacturer’s instructions and read at 450 nm in MRX microplate reader (Dynatech Laboratories). TGF-β1 concentration was determined using standard curves, which were prepared simultaneously with the recombinant hamster TGF-β1.

**Statistics.** Statistical significance of differences between groups was determined with nonparametric Mann-Whitney test. A value of P<0.05 was considered to be significant. Analysis was done using Statgraphics Plus 4.1 software.

**Results**

The amplified cDNA fragment encoding hamster TGF-β1 protein had a molecular mass of 266 bp. The recombinant fusion protein containing the His-tag was produced in E. coli BL21 (DE3) cells and its molecular weight was approximately 11 kDa. The recombinant protein – rTGF-β1 was expressed in an insoluble form, therefore its purification using affinity chromatography was performed under denaturing conditions with 4 M urea. The presence of purified protein was confirmed by the Western blot analysis. However, the SDS analysis showed poor purity of rTGF-β1, therefore, electro elution form the gel slice was applied.

In order to obtain antibodies recognising hamster TGF-β1, the recombinant protein was used to immunize a chicken. The specificity of antibodies to the native form of the cytokine was confirmed by Western blotting. Chicken polyclonal antibodies specifically recognised the native active TGF-β1 homodimer of a molecular weight of 25 kDa in MLNs homogenate, as well as corresponding recombinant protein of molecular weight of about 11 kDa (Fig. 1). Antibodies present in chicken serum additionally recognised two proteins of approximate molecular weight of 60 and 80 kDa in a sample of homogenised MLNs, which correspond to latent forms of TGF-β1.

Out of three A. ceylanicum infected age groups of hamsters, the youngest were the most susceptible to the hookworm infection and the oldest ones were the most resistant. Differences in worm counts between groups were statistically significant (Fig. 2).

The highest expression of TGF-β1 mRNA was noted in MLN cells of the youngest hamsters. In MLN cells derived from 8-week-old hamsters, TGF-β1 expression was lowered by 86% and from 6-month-old lowered by 58% (Fig. 3).
Fig. 3. TGF-β1 mRNA expression in hamster MLN cells. Real-time PCR analysis was performed on cDNA reverse transcribed on total RNA template, isolated from unstimulated MLN cells after 72 h of culture. Samples of RNA derived from all animals were pooled from each two individuals. Data are presented as fold-change in cytokine expression normalised to HPRT expression and relative to cytokine expression in 4-week-old group calculated with the 2ΔΔCt method.

In vitro production of TGF-β1 by unstimulated MLN cells was the highest in cultures from the youngest hamsters (Fig. 4). A significant difference in cytokine concentration was noted between 4- and 8-week-old groups. Mitogen stimulated cells from the oldest animals released a significantly lower amount of TGF-β1 than cells from younger hamsters. The highest concentration of TGF-β1 was noted in cultures of MLN cells from the youngest animals.

Again the difference between 4- and 8-week-old groups was statistically significant.

Fig. 4. In vitro production of TGF-β1 by hamster MLN cells from 4-, 8-week- and 6-month-old animals. Cells were cultured for 72 h in RPMI 1640 medium and stimulated with ConA (5 μg/mL), A. ceylanicum antigens (30 μg/ml) or unstimulated. The concentration of TGF-β1 was determined by direct ELISA using polyclonal chicken antibodies recognising hamster TGF-β1. Conditions of cell culture and ELISA are described in the text. Results are presented as mean ± SD. Statistically significant differences between age groups are indicated with asterisks (* P<0.05, ** P<0.01).

Discussion

We have previously reported a direct ELISA method for measuring hamster IL-4, IL-12, and IFN-γ concentrations (11). The same method was applied for generation of polyclonal antibodies recognising hamster TGF-β1 in order to investigate TGF-β1 production in hookworm infected hamsters. These antibodies effectively recognised hamster recombinant TGF-β1 as well as native, active 25 kDa homodimers of this cytokine. Western blot analysis showed that these antibodies additionally recognise two other 60 and 80 kDa proteins, which are probably latent forms of TGF-β (2, 3, 24). Polyclonal TGF-β1 antibodies were used in a direct ELISA in order to determine the concentration of TGF-β1 in supernatants from hamster cell cultures. Additionally, in order to confirm our results, we performed a real-time quantitative PCR analysis of TGF-β1 mRNA expression in unstimulated MLN cells. Fold changes in mRNA expression in three investigated groups corresponded to protein concentration in culture supernatants.

The intensity of A. ceylanicum infection was the highest in young 4-week-old hamsters, whereas adult 6-month-old animals were infected by the least number of worms. We have examined TGF-β1 concentrations in culture supernatants of MLN cells derived from these infected hamsters. Antigen stimulated and unstimulated cells from the youngest, most susceptible hamsters produced higher amounts of TGF-β1 than cells from older animals, which were more resistant to hookworm infection. In case of mitogen stimulation, cells from the oldest animals released significantly smallest amount of investigated cytokine. It seems that TGF-β1 production in infected hamsters depends on worm burdens and age of the host animal. The influence of age on the intensity of hookworm infection was already observed in dogs. Adult animals acquire natural resistance in the absence of previous exposure to A. caninum, whereas pups are susceptible to infection (35). In neonates and infants, the immunological system is not fully developed and lymphocytes are functionally immature in comparison with adults (10). Production of IL-4 and IFN-γ by neonatal T cells is markedly lower compared to adult T cells (29). Enhanced resistance to hookworms in adult hamsters may also be related to the presence of steroid sex hormones in mature animals and their effect on specific immune response. Sex hormones affect the immunological system by regulation of proinflammatory mediator synthesis by macrophages (9). They might also influence the function of B and T lymphocytes by the induction of apoptosis (4). It is also tempting to claim that young animals are not able to maintain appropriate balance between Th1, Th2, and Treg cells (since immature immune system), which is crucial for effective response and evasion of immunopathologies.

It has been reported that individuals chronically infected with helminths (Necator americanus, Ascaris lumbricoides, Schistosoma mansoni, and Trichuris trichura) have increased populations of Th3/Treg cells and significantly higher levels of TGF-β (26). Production of another immunoregulatory cytokine IL-10 has been determined in hookworm mono-infected patients. Geiger et al. (19) showed that high levels of IL-10 were associated with reduced levels of IFN-γ, IL-5, and IL-13 in N. americanus infected patients. It has been
proven that hookworm antigens, especially ES antigens, efficiently inhibit proliferative responses and cytokine secretion by peripheral blood mononuclear cells (PBMCs) isolated from these patients (18). It is possible that adult hookworms might directly stimulate host IL-10 production, as enhanced IL-10 mRNA expression was also observed in hamsters infected with *A. ceylanicum* (20, 34). Recent experiments prove that hookworm antigens modify functions of dendritic cells, which decrease their ability to present antigens and increase their ability to produce IL-10 and TGF-β, and these cytokines mediate the mechanism of immune suppression by CD4 suppressor T cells (7). TGF-β is therefore an important factor involved in the complicated mechanism of down-modulation of immune responses during hookworm infection.

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**References**