Increased production of chemotactic cytokines and elevated proliferation and expression of intercellular adhesion molecule-1 in rat mesangial cells treated with erythrogenic toxin type B and its precursor isolated from nephritogenic streptococci

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Abstract

Background. Previous reports have demonstrated the presence of streptococcal erythrogenic toxin type B (ETB) as well as proliferation and expression of adhesion molecules along with leukocyte infiltrations in biopsies from patients with acute post-streptococcal glomerulonephritis (APSGN). The purpose of the present study was to correlate infiltrative and proliferative events with interactions between ETB or its precursor (ETBP) and intrinsic mesangial cells.

Methods. Rat mesangial cells were cultured with ETB or ETBP (50 μg/ml) while measuring production of monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) and while examining proliferation and expression of intercellular adhesion molecule-1 (ICAM-1). After 24, 48 and 96 h of incubation, MCP-1 and MIP-2 in culture supernatants were assessed by enzyme-linked immunosorbent assay (ELISA). Cells were assessed for proliferation by incorporation of radioactive thymidine and expression of ICAM-1 was measured by indirect immunofluorescence and by cellular ELISA.

Results. Compared with controls, treatment with either ETBP or ETB significantly increased MCP-1 and MIP-2 levels in mesangial cell cultures. Mesangial cells also showed elevated proliferation at 96 h of culture when treated with streptococcal proteins. Although production of MCP-1 and MIP-2 was not correlated with proliferation, treatment with ETBP resulted in a significant correlation between MCP-1 production and proliferation. Immunofluorescence studies revealed an increased expression of ICAM-1 in ETBP/ETB-treated mesangial cells. In addition, cellular ELISA studies showed increased absorbance in cultures treated with ETBP/ETB. Finally, low serum concentrations in the culture medium potentiated the stimulatory effect of ETB on MCP-1 production.

Conclusions. Our findings, by demonstrating a role for cationic streptococcal ETB or ETBP in the induction of chemotactic molecules as well as the proliferation and expression of adhesion molecules, delineate an additional possible pathway for the pathogenesis of APSGN.

Keywords: erythrogenic toxin; glomerulonephritis; intercellular adhesion molecule-1; macrophage inflammatory protein-2; monocyte chemoattractant protein-1; proliferation

Introduction

Acute post-streptococcal glomerulonephritis (APSGN) is a diffuse proliferative endocapillary nephritis that occurs during the convalescent period of group A streptococcal infections [1]. One of the relevant renal histological features of APSGN is an increased glomerular cellularity [2–4]. Previous studies indicate that the streptococcal erythrogenic toxin type B (ETB) and its precursor (ETBP) could be involved in the pathogenesis of APSGN. In this regard, ETB has been found in renal biopsies from patients with APSGN and APSGN sera react preferentially with these streptococcal proteins [5–8]. The presence of streptococcal proteins in the renal microenvironment creates possible interactions between these proteins and intrinsic glomerular cells and this interaction may lead to increased glomerular cellularity. In support of this we previously reported increased glomerular and interstitial leukocyte infiltration after in vivo renal perfusion of ETB or ETBP, an effect that may have been
mediated by chemotactic and macrophage migration inhibition factor (MIF) activities [9]. We additionally reported that ETB and ETBP have capacities to induce proliferation in human mononuclear leukocytes [10]. On the basis of these observations, combined with findings that infiltration, proliferation and expression of adhesion molecules are involved in the hypercellularity observed during the course of glomerulonephritis [1–4,11], we studied the effects of ETB and ETBP on the proliferation and production of monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2) and expression of intercellular adhesion molecule-1 (ICAM-1) in cultured mesangial cells. Our findings demonstrated that ETB and ETBP were capable of inducing increased production of MCP-1 and MIP-2 and of upregulating proliferation and increased expression of ICAM-1 by mesangial cells.

**Subjects and methods**

**Mesangial cell cultures**

Mesangial cells were isolated from the glomeruli of Sprague–Dawley rats using a differential sieving technique. Briefly, cortices were removed and minced before passage through a series of steel sieves with decreasing pore sizes (200, 150 and 75 μm). Isolated glomeruli were then digested with collagenase. Digested glomeruli were cultured in RPMI 1640 combined with antibiotics (100 μg/ml penicillin and 10 μg/ml streptomycin) and 20% fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO) until mesangial cells reached confluence. The cells were passaged using trypsin/EDTA and cells between passages 3 and 6 were used for experiments. Cells were characterized and the homogeneity of mesangial cells was assessed by identifying typical morphology and patterns of staining with antibodies against smooth muscle cell-specific actin, Thy 1.1, factor VIII antigen, leukocyte common antigen and by the presence of hillocks [12].

**Cells**

Cells were characterized and the homogeneity of mesangial cells was assessed by identifying typical morphology and patterns of staining with antibodies against smooth muscle cell-specific actin, Thy 1.1, factor VIII antigen, leukocyte common antigen and by the presence of hillocks [12]. Mesangial cells were cultured until subconfluence (70%) and treated with 50 μg/ml cationic ETB or ETBP (final concentration) for 24, 48 and 96 h at 37 °C with 5% CO2 in the supplemented media as previously described. Blocking experiments were performed by incubation of ETB-treated mesangial cell cultures with a rabbit anti-ETB polyclonal antibody (100 μg/ml). To determine the effect of low level FBS on the production of chemotactic cytokines, mesangial cell cultures were incubated for 96 h with or without ETB in RPMI, penicillin/streptomycin and 0.5% FBS. Control experiments used cellular cultures without streptococcal proteins. The cationic streptococcal protein concentration was chosen from previous investigations [12,14] that reported doses ranging from 20 to 500 μg of cationic antigens to induce in situ immune complex glomerulonephritis in rats and from our demonstration of an optimal proliferative-inducer effect of ETB and ETBP at a dose of 50 μg/ml in human mononuclear leukocytes [10]. After incubation, cells or supernatants were tested for production of MCP-1 and MIP-2 and proliferation and expression of ICAM-1.

**Isolation of ETBP and ETB**

The streptococcal ETB and ETBP were kindly donated by Drs Arnold Vogt and Stephen Batsford. To isolate these, we used two β-haemolytic group A streptococcal strains isolated from patients with APSGN. The bacteria were kept on blood agar at 4 °C and pre-cultured in chemically defined medium (CDM), pH 6.9 at 37 °C for 6 h. Following this, 20 ml of bacterial culture were added to 180 ml of fresh CDM and the mixture was cultured until it was cloudy (~6 h). Two-hundred ml of the pre-culture were added to the main culture medium (800 ml CDM and 1000 ml Todd-Hewitt, 1% glucose, pH 6.9). Bacteria were cultured for several hours and pH was maintained by adding of 0.5 N NaOH with 50% glucose. Then, pH was reduced to 5.9 and was maintained at this level for 12 h with a 0.5 N NaOH solution containing 50% glucose. The culture was centrifuged (10 800 × g × 15 min) and the supernatant was sterile filtered, concentrated to a factor of 1000 in an Amicon system using a YM-5 membrane and finally run on a Mono S cation exchange column (Pharmacia HR 5/5) in a fast-performance liquid chromatography system. The system conditions included: pH 6.0, flow rate 1 ml/min and a buffer gradient of 3.5–250 mM MES. The ETBP eluted before ETB and the fractions were tested in SDS–PAGE under non-reducing conditions. Then, streptococcal antigens were run in isoelectric focusing to determine pI. Under these conditions, ETBP was 44 kDa, pI 8.2 and ETB was 30 kDa, pI 9.0. ETBP was exposed to iodoacetamide to avoid its autocatalytic transformation into ETB. A Limulus amebocyte lysate assay revealed that streptococcal proteins solutions were free of endotoxin.

**Detection of MCP-1 and MIP-2 in culture supernatants**

Supernatants from mesangial cell cultures in the absence or presence of streptococcal proteins (ETB/ETBP) were harvested at 24, 48 and 96 h and were centrifuged and stored at −80 °C until use. MCP-1 and MIP-2 secreted into the supernatant were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., CA, USA) according to the manufacturer’s protocol. Amounts of MCP-1 and MIP-2 were expressed as pg per 10 μg cellular culture protein. For the protein assay, mesangial cell cultures were lysed for 10 min at 4 °C in a lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl and 1% Triton X-100). The lysates were collected and protein content was determined by the Lowry protein assay.

**Determination of ICAM-1 protein in mesangial cells**

To examine the expression of ICAM-1 in mesangial cell cultures in the presence or absence of streptococcal proteins, mesangial cells were plated on 8-well plastic chamber slides (Nunc, Roskilde, Denmark) and incubated until subconfluence. Treated and untreated cultures were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min. After washing, cells were reacted with monoclonal antibody against rat ICAM-1 (Seikagaku Co., Tokyo, Japan) for 30 min and thereafter, a fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used (Accurate Chemical Co., NY, USA) to localize the first antibody. As a negative control mesangial cells were incubated, as previously described, with an irrelevant monoclonal antibody. ICAM-1 expression was also measured by ELISA. In brief, mesangial cells were cultured in flat bottom 96-well plates. Cells were incubated in the absence or presence of ETB or ETBP and incubated for 24, 48 and 96 h. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Cells were then washed with PBS and blocked with 3% ovalbumin in PBS. The cells
were then incubated with an anti-rat ICAM-1 monoclonal antibody (1:100). After incubation for 30 min at room temperature, cells were washed with 3% ovalbumin and incubated with a goat anti-mouse IgG–horseradish peroxidase conjugated antibody (1:200; Pierce, IL, USA) for an additional 30 min. After washing, the ELISA was developed using 3,3′,5′-tetramethylbenzidine (Sigma Chemical Co., St Louis, MO, USA) in citrate buffer containing 0.006% hydrogen peroxide. Absorbance was measured at 450 nm using a Benhmark model microplate reader (Biorad, CA, USA).

**Proliferation assay**

Subconfluent mesangial cell monolayers in 96-well flat-bottom tissue culture plates with RPMI 1640 supplemented with 20% FBS were incubated with 50 μg/ml (final concentration) ETB or ETBP for 24, 48 and 96 h at 37°C in 5% CO2 atmosphere. Control cultures received supplemented medium without streptococcal proteins. Eighteen hours before cell harvest, cells were pulsed with 0.5 μCi/well of 3H-thymidine (NEN Research Products, DuPont, MA, USA). Adherent cells were trypsinized and harvested on individual filter discs, dried and placed in 3 ml scintillation fluid. Radioactivity was counted in a beta scintillation counter. Control and experimental cultures were performed in triplicate and data were expressed as counts per minute (CPM).

**Statistical analysis**

Results in the groups are shown as means ± SEM and represent data from three to four individual experiments. Comparisons between groups were performed by ANOVA and associations between variables were analysed by linear correlation (Pearson). Two-tailed P-values of < 0.05 were considered statistically significant.

**Results**

**Induction of chemokines and proliferation of cultured mesangial cells caused by ETB and ETBP**

The incubation of mesangial cells with ETB or ETBP led to increased amounts of MCP-1 and MIP-2 in the supernatant cultures. The production of both chemokines was already demonstrable at 24 h and increased over time (Figures 1 and 2). Mesangial cells incubated in the absence of streptococcal proteins had basal levels of MCP-1 and MIP-2 at 24 h; there was no significant spontaneous chemokine production at 48 and 96 h of culture (Figures 1 and 2). Treatment of ETB mesangial cell cultures with an anti-ETB polyclonal antibody abolished the stimulatory effect of ETB on MCP-1 production (Figure 3). ETB-treated mesangial cell cultures under low serum conditions (0.5% FBS) showed increased concentrations of MCP-1.
in the supernatants compared with 20% FBS cultures (Figure 4).

Proliferation of mesangial cells was upregulated by ETB and ETBP. In the presence of streptococcal proteins, mesangial cells reached a significant uptake of $^3$H-thymidine by cellular cultures at 96 h of culture. Control experiments used unstimulated cultures. Values are expressed as means±SE from four independent experiments, each conducted in triplicate. *$P<0.01$.

Effect of ETBP and ETB on ICAM-1 expression in cultured mesangial cell

To investigate whether streptococcal proteins could also stimulate ICAM-1 expression in mesangial cells, cellular cultures were incubated with 50 μg/ml of ETBP or ETB for 24, 48 and 96 h. Although basal expression of ICAM-1 was found in untreated cultures, stimulation with ETB or ETBP increased expression of ICAM-1 (Figure 7). These observations were confirmed by cellular ELISA (Figure 8).
Overall renal histologic patterns in APSGN consistently show moderate to marked hypercellularity that is primarily due to proliferation of mesangial and endothelial cells. In the very early stage there is increased non-glomerular cell infiltration, consisting of polymorphonuclear leukocytes and monocyte/macrophage cells [1–4]. The renal tissue inflammation observed in APSGN is generally believed to result from actions of anaphylatoxins generated by the classic complement pathway [11,15,16]. However, *Streptococcus pyogenes* produces several extracellular proteins, including ETB, that may be involved in APSGN-stimulated renal pathology. The role of streptococcal ETB and ETBP in APSGN has been documented by the increased reactivity to ETB and ETBP in APSGN sera and by the presence of ETB in kidney biopsies from APSGN patients [5–8]. In this regard, our data suggesting that ETB/ETBP may be involved in the renal hypercellularity after *in vivo* renal perfusion indicate that ETB and ETBP are potentially nephritogenic by themselves. This effect could be mediated by chemotaxis mechanisms and by MIF activity [9]. ETB/ETBP may also induce proliferation of human mononuclear leukocytes [10], an important cellular component during APSGN [1–4].

Our experiments were designed to elucidate whether the interaction between ETB/ETBP and mesangial cells is related to the renal hypercellularity observed in APSGN. This study provided evidence that ETB/ETBP from nephritogenic streptococci can induce mesangial cells to increase the production of MIP-2 and MCP-1. MIP-2 is an alpha chemokine and a major neutrophil chemoattractant contributing to influx of neutrophils in several forms of glomerulonephritis [17–20]. MCP-1 is a beta chemokine that is very important in recruiting and activating monocyte/macrophages during the course of certain types of glomerulonephritis [19,21–24]. In addition to the chemokine-inducer effects of ETB/ETBP, these streptococcal products were capable of inducing proliferation of cultured mesangial cells. Both proliferative and chemotactic mechanisms are involved in the hypercellularity observed during the course of several experimental and human forms of nephritis [1,3,11,25]. The presence of streptococcal proteins in the mesangial microenvironment [5–7,9] indicates a possible interaction between ETB/ETBP and intrinsic mesangial cells during APSGN, leading to increased expression of MCP-1 and MIP-2 and further influx of neutrophils and monocytes into renal tissues. These events along with the increased proliferation of mesangial cells may play a role in the pathogenesis of APSGN. The increased influx of monocytes and neutrophils induced by MCP-1 and MIP-2 could promote increased production of macrophage and neutrophil inflammatory cytokines and oxygen reactive species to cause further tissue damage [26,17–20]. In turn, certain macrophage products may induce increased expression of MCP-1 by mesangial cells in a paracrine manner [27–29]. In general, the chemokine inducer and the proliferative effects of ETB/ETBP were not correlated, suggesting that the increased production of chemokines was not only due to the proliferation of mesangial cells. In addition, the expression of chemokine concentration per cellular protein content showed increased production of chemokines. However, MCP-1 production and proliferation were correlated when mesangial cells were treated with ETBP. We have no clear explanation for this effect, but activation of a common transcription factor for both processes by streptococcal products could be involved [30–33]. Unexpectedly, stimulation of mesangial cells with ETB during low serum concentrations (0.5% FBS) caused a greater production of MCP-1 than during 20% FBS, suggesting the presence of inhibitory factors in FBS. However, histological...
examination revealed that low serum cultures had morphological cellular alterations and cellular detachment. In addition, low serum cultures had decreased protein content in the cellular lysate (20% serum: 114 ± 27.05 μg/ml; 0.5% serum: 52.21 ± 21.88 μg/ml). We therefore decided to perform the remainder of the experiments under 20% serum conditions.

In the present study, we also documented increased expression in ICAM-1 on ETB/ETBP-treated cultured mesangial cells. This finding suggests that resident mesangial cells may increase ICAM-1 expression in APSGN following interaction with streptococcal proteins and may partly explain the increased expression of glomerular ICAM-1 found in early biopsies from patients with APSGN [34]. Since macrophages accumulate in the mesangium during APSGN, the increased expression of mesangial ICAM-1 may potentiate the proliferative effect of direct macrophage–mesangial cell contact on mesangial cells [35]. Increased ICAM-1 expression on the mesangial cell surface may also promote the recruitment of leukocytes in the mesangium by interaction with its LFA-1 ligand that may be relevant in mesangial hypercellularity. In support of this, increased expression of glomerular LFA-1 positive cells and increased expression of glomerular ICAM-1 have been reported in APSGN [34]. Previous work has shown the importance of ICAM-1 in the antigen presentation function in mesangial cells [36]. Presentation of specific antigens by mesangial cells to T cells that are initially engaged by ICAM-1 may lead to a rapid T-cell activation and production of cytokines that could increase major histocompatibility complex class II antigens on mesangial cells to regulate the specificity of the immune response and amplify its intensity [37–40]. Since both the presence of streptococcal antigens and increased expression of ICAM-1 and T lymphocytes have been demonstrated in the renal mesangium of patients with APSGN [2,3,5–7,9,34], it is possible that ETB/ETBP may induce a local immune response that contributes to the mesangial expansion observed in APSGN.

In conclusion, we found that ETB/ETBP from nephritogenic streptococci may induce increased production of chemokines and cause increased proliferation and expression of ICAM-1 in mesangial cells, which provide mechanisms that may partly explain the inflammatory process observed during the nephritis. These effects of streptococcal proteins on mesangial cells, along with other inflammatory mechanisms, underline their potential pathogenic role in APSGN.

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References


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