Involvement of Adhesion Molecules in Glomerular Endothelial Cell Injury Induced by PMA-Stimulated Lymphocytes

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Abstract. Adhesion of lymphocytes to glomerular endothelial cells might be a critical step in the development of acute and chronic glomerulonephritis. The protective effect of anti-CD11a and anti-intercellular adhesion molecule (ICAM-1) antibodies on the cytotoxicity elicited by phorbol 12-myristate 13-acetate (PMA)-stimulated lymphocytes was investigated by using cultured bovine glomerular endothelial cells (GEN). Both anti-CD11a and CD18 antibodies inhibited GEN injury induced by the activated lymphocytes in a dose-dependent manner. In addition, both antibodies also inhibited the adhesion of the activated lymphocytes to the GEN monolayers. These results suggest that it is important for activated lymphocytes to bind to GEN via adhesion molecules for cytotoxicity to be produced by the activated lymphocytes.

Key words: Lymphocytes, PMA, Glomerular endothelial cells, ICAM-1, CD11a, CD18

INTERACTIONS between leukocytes and endothelial cells have been recognized as critical to the development of acute and chronic inflammatory lesions [1]. Infiltration of tissues by leukocytes, monocytes and lymphocytes depends upon their adhesion to endothelial cells and transmigration through the vessel wall [2-4]. Such interactions occur through specific ligand-receptor interactions. On the membrane of leukocytes, lectin-like molecules (L-selectin), integrins (CD11/CD18 complex, VLA-4) and immunoglobulin superfamily members (CD2, CD31) bind to corresponding endothelial cell membrane ligands, including phospholipids, carbohydrates, lectin-like molecules (P-selectin, L-selectin), and immunoglobulin superfamily members (intercellular adhesion molecule [ICAM]-1 and -2, vascular cell adhesion molecule [VCAM]-1, LFA-1, CD31). Some of these components are rapidly synthesized or are translocated to the endothelial cell surface after stimulation by a variety of substances. Other receptors, including E-selectin, ICAM-1, and VCAM-1, appear later, require new protein synthesis, and are induced mainly by cytokines [5].

CD11/CD18 consists of a common CD18 glycoprotein and three different kinds of CD11 glycoprotein (CD11a, CD11b and CD11c). Although CD11a/CD18 is known to mediate the adherence of the leukocytes to endothelium via ICAM-1 and ICAM-2, the receptors to CD11b/CD18 and CD11c/CD18 on the endothelium are still unknown. It has been reported that endothelial cell injury induced by activated monocytes is regulated by adhesion via integrin family [6]. In addition, the monoclonal antibody (MAB) to CD11b inhibits the degranulation of fMLP- and tumor necrosis factor-stimulated neutrophils [7]. Moreover, CD11b mediates neutrophil aggregation via pro-
tein kinase C-dependent phosphorylation [8] and monocyte adhesion to endothelium by means of protein kinase C [9]. Thus, CD11b as well as CD18, but not CD11a, may regulate some inflammatory changes in leukocytes, monocytes and lymphocytes.

The accumulation of lymphocytes in the extracapillary space of glomeruli may lead to a crescent formation and deterioration of renal function. Even though the role of leukocytes and monocytes in the progression of glomerulonephritis had been reported, the effects of activated-lymphocytes on glomerular endothelial cells were not clearly assessed. In the present study, we studied the effects of MAbs on CD11a and CD18 on glomerular endothelial cell injury induced by phorbol 12-myristate 13-acetate (PMA)-stimulated lymphocytes.

Materials and Methods

Culture of glomerular endothelial cells

Glomerular endothelial cells (GEN) were cultured from bovine adult kidney as described previously [10–12]. GEN were cultivated in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS), 5 U/ml heparin, 2 ng/ml acidic fibroblast growth factor, ITS Premix (5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium) and antibiotics. To characterize the cloned endothelial cells, the uptake of acetylated low-density lipoprotein and the expression of factor VIII-related antigen were tested as previously described [10].

Immunocytochemical detection of ICAM-1 molecule

GEN grown on gelatin-coated plastic coverslips were rinsed with carrier buffer containing phosphate-buffered saline (PBS) with 1% bovine serum albumin (Sigma Co., MO, USA), and fixed with 2% paraformaldehyde (Kanto Kagaku, Tokyo). The sequential series were performed at room temperature. Anti-human ICAM-1 monoclonal antibody (Genzyme Co., CA, USA) or non-specific mouse IgG (Cappel, Denmark) was subsequently added at a concentration of 5 μg/ml in PBS for 1 h. After being washed with carrier buffer, and then incubated with the secondary antibody (Autoprobe™ LM GAM IgG, Janssen Cedara lone, Tokyo) at 1:40 dilution in carrier buffer for 1 h. Subsequently, monolayers were washed with carrier buffer, fixed in 9.25% formaldehyde and 45% acetic acid in PBS for 30s, washed with distilled deionized water and incubated in silver enhancing solution (IntenSE MTM, Janssen Sedarlane) for 25 min. Monolayers were washed again with distilled deionized water and counterstained with Giemsa. After being washed with carrier buffer, the cover slips were mounted in glycerine-PBS and observed under an epifluorescence microscope (Nikon, Tokyo).

Lymphocyte isolation

Human lymphocytes were obtained from heparinized venous blood provided by healthy adult volunteers and Ficoll-Hypaque (Sigma Co., St Louis, USA) was underlaid on blood samples as previously described by Cavender et al. [13]. After centrifugation for 30 min at 400 x g at room temperature, lymphocytes were collected from the interface and washed three times in serum-free RPMI 1640 medium. Lymphocytes were cultured in RPMI medium containing 10% FCS and antibiotics until use. This procedure yielded a 95% effective separation as determined by transmission electron microscope, and >95% cell viability as determined by trypan blue exclusion.

Lymphocyte-GEN adhesion assay

GEN were plated at a density of 1 x 10^5/well on 24-well plates coated with 0.5% gelatin and allowed to form confluent monolayers overnight. GEN were treated for 30 min with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical) in the presence or absence of 10^-7 M H-7 (Sigma Chemical). Lymphocytes were added to GEN monolayers at a lymphocyte to GEN ratio of 10:1 and incubated for 60 min with RPMI medium containing ITS Premix (incubation medium). Lymphocytes were treated with monoclonal antibodies (MAbs) to CD11a and CD18 (Immunotech Co., France) or control mouse IgG for 30 min on ice and the lymphocytes were placed into GEN cultures without washing. After the incubation, the cell layers were washed twice with serum-free RPMI medium by shaking the plates several times by hand until all the non-adhered lymphocytes came off. After being washed, adhered lymphocytes were counted blindly by three observers.
under a phase-contrast microscope. Lymphocytes adhering to GEN were expressed as the percentage of added lymphocytes in 20 light microscope fields (x 200).

Cytotoxicity assay

Cytotoxicity was assessed by lactate dehydrogenase (LDH) release from GEN as described previously [14]. GEN were washed twice with serum-free RPMI medium and treated for 5 h with lymphocytes (lymphocyte-GEN ratio: 1:5 to 1:20) preincubated for 30 min with 10 ng/ml of PMA in the presence or absence of MAbs to adhesion molecules. LDH released from the injured cells were measured with an autoanalyzer (Toshiba, Japan). Results were expressed as a percentage of LDH released. In addition, some experiments were conducted to confirm whether the LDH release is derived from GEN. GEN were preincubated for 30 min with 200 μCi of Na51CrO4 (Amersham Japan, Tokyo) and incubated with PMA-stimulated lymphocytes as described above. The released 51Cr was counted in a gamma counter (LKB, Tokyo).

Statistical analysis

All values are expressed as the mean ± SEM. Data were analyzed by analysis of variance (ANOVA), and a P value of <0.05 was taken as representing a significant difference between group means.

Results

When lymphocytes were added to GEN, visual inspection under a phase-contrast microscope confirmed that the integrity of the GEN monolayers was maintained, and that there were no obvious aggregates of lymphocytes. Two to five percent of the lymphocytes added adhere to resting GEN (Fig. 1A). This adherence was increased when 10 ng/ml PMA-stimulated lymphocytes were added to GEN monolayers (Fig. 1B). Cytotoxicity was not detected at less than 10 ng/ml of PMA and GEN detachment appeared at more than 50 ng/ml of PMA. We therefore used 10 ng/ml of PMA to stimulate the lymphocytes. The addition of lym-
phocytes activated with PMA to GEN monolayers caused a time-dependent increase in lymphocyte adhesion to GEN. The number of lymphocytes which adhered to GEN reached a plateau at 60 min (235 ± 28 cells/× 200 fields of light microscope; GEN: lymphocyte ratio=1:10). Substantial adhesion was detected at lymphocyte-GEN ratios>1:10 and the median percent specific adhesion appeared at a lymphocyte-GEN ratio of 1:10. After a 5 h incubation of GEN with lymphocytes activated with PMA, the lymphocyte binding increased by approximately 100% (Fig. 2). H-7 (10^{-7} M), a protein kinase C inhibitor, suppressed the increase in the adhesion of PMA-stimulated lymphocytes to GEN about 80% (data not shown).

Adhesion molecules have been reported to be involved in protein kinase C-dependent lymphocyte adherence to microvascular endothelial cells [15]. We therefore first assessed whether the ICAM-1 molecule was expressed on GEN. ICAM-1 molecules were slightly but positively expressed on the surface of resting GEN monolayers (Fig. 3A), and negative staining was observed when the primary antibody was changed to non-specific anti-mouse IgG (Fig. 3B). To study the role of LFA-1/ICAM-1 in PMA-stimulated lymphocyte binding to GEN, we added various concentrations of MAbs to LFA-1 α chain (CD11a), β chain (CD18). As shown in Table 1, anti-CD11a antibody inhibited the PMA-induced increase in lymphocyte binding.

![Fig. 2. Effect of PMA on lymphocyte binding to GEN as described in Fig. 1. The adherence of lymphocytes to the GEN was increased after preincubation with 10 ng/ml of PMA approximately two fold and inhibited by 80% with 10^{-7} M of H-7. n=4, *P<0.05.](image)

![Fig. 3. Expression of ICAM-1 molecules on resting bovine GEN. Cells were cultured on plastic coverslips for 24 h and stained with anti-human ICAM-1 antibody (A) and non-specific anti-mouse IgG (B) as described in Materials and Methods. × 200.](image)
INTERACTIONS BETWEEN GLOMERULAR ENDOTHELIAL CELLS AND LYMPHOCYTES

Table 1. Effect of anti-CD11a and CD18 antibodies on the adhesion of PMA-stimulated lymphocytes to GEN

<table>
<thead>
<tr>
<th>dose (μg/ml)</th>
<th>Anti-CD11a antibody ( % of control)</th>
<th>Anti-CD18 antibody ( % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.6 ± 11.6</td>
<td>102.4 ± 12.4</td>
</tr>
<tr>
<td>5</td>
<td>98.2 ± 14.2</td>
<td>96.1 ± 11.8</td>
</tr>
<tr>
<td>10</td>
<td>76.1 ± 10.8*</td>
<td>58.0 ± 13.1**</td>
</tr>
</tbody>
</table>

GEN were incubated for 60 min with lymphocytes stimulated for 30 min by 10 ng/ml of PMA in the presence of various concentrations of anti-CD11a and anti-CD18 antibodies. Data represent the mean ± SEM for 3 experiments and are expressed as percentages of the control data. n=4, * P<0.05, **P<0.01.

by 76% and anti-CD18 antibody inhibited the binding by 58% at a concentration of 10 μg/ml.

On the other hand, our preliminary experiments showed that 10 ng/ml PMA-stimulated lymphocytes induced GEN injury estimated by LDH release. We therefore assessed whether anti-CD11a and anti-CD18 antibodies had any protective effect on the GEN injury induced by PMA-stimulated lymphocytes. As shown in Fig. 4, both anti-CD11a and anti-CD18 antibodies inhibited the GEN injury induced by PMA-stimulated lymphocytes in a dose-dependent manner, but non-specific IgG itself at 10 μg/ml had no inhibitory effect. To confirm whether the LDH release was from GEN, 51Cr-labeled GEN were incubated with PMA-stimulated lymphocytes. Ten-microgram of anti-CD11a and anti-CD18 antibodies inhibited %51Cr release by 68%, 52%, respectively. These results were consistent with those of %LDH release. The LDH measured were therefore released from GEN damaged by PMA-stimulated lymphocytes.

In summary, the present study found that it is important for activated lymphocytes to bind to GEN via adhesion molecules for cytotoxicity to be produced by activated lymphocytes. The damaged GEN may secrete some modulators such as endothelin-1 and prostaglandins to stimulate neighboring mesangial cell proliferation. Taken together, the adhesion of lymphocytes to GEN might be a critical step in the development of glomerulonephritis.
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References