A Study of CD9 Antigen and GP IIb/IIIa—Like Protein on Activated Human Vascular Endothelial Cells

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ABSTRACT

We analyzed the expression of CD9 antigen and GPIIb/IIIa—like protein by activated human umbilical vein endothelial cells (HUVECs) incubated with tumor necrosis factor—alpha or lipopolysaccharide using flow cytometry and two anti—CD9 monoclonal antibodies (NNKY1—19 and MALL—13; raised using platelets and ALL cells, respectively) as well as an anti—GPIIb/IIIa monoclonal antibody (NNKY2—6). Using the anti—CD9 antibodies, it was found that the fluorescence intensity of activated HUVECs was higher than that of untreated control cells. However, fluorescence for NNKY2—6 showed no significant difference between activated HUVECs and untreated controls.

These findings suggested that CD9 antigen expression by HUVECs increases in response to activation. On the other hand, there was no significant difference of GPIIb/IIIa—like protein expression between activated HUVECs and control cells. It is well known that anti—CD9 antibodies can induce platelet activation, but the actual function of CD9 antigen is still unknown. The results of this study suggest that it plays a role in the adhesion of hematopoietic cells to ECs or in the procoagulant activity of ECs.

I. INTRODUCTION

CD9 antigen was first defined as a cell surface membrane protein with a molecular weight of about 24,000 daltons using a monoclonal antibody (BA—29), produced against an acute lymphoblastic leukemia (ALL) cell line. Extensive studies have shown that this antigen is expressed by a variety of cells, including common ALL cells, platelets, and endothelial cells. Although the widespread distribution of CD9 antigen suggests that it may play an important role in various biological responses, the nature of this role remains obscure. In the case of platelets, CD9 antigen appears to be involved in cell activation. Several authors have studied the effect of anti—CD9 antibodies on platelet function and have found that such antibodies variously induce platelet aggregation, the release of intracellular granule contents, the production of arachidonic acid metabolites, and elevation of the intracellular Ca²⁺ concentration. In the present study, we examined the expression of this antigen during the activation of human vascular endothelial cells by lipopolysaccharide (LPS) and tumor necrosis factor (TNF) using flow cytometry and two anti—CD9 monoclonal antibodies (NNKY1—19 and MALL—13; raised using platelets and ALL cells, respectively) as well as an anti—GPIIb/IIIa monoclonal antibody (NNKY2—6). Using the anti—CD9 antibodies, it was found that the fluorescence intensity of activated HUVECs was higher than that of untreated control cells. However, fluorescence for NNKY2—6 showed no significant difference between activated HUVECs and untreated controls.

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cytometry and monoclonal antibodies (MoAb). Endothelial cells (ECs) also synthesize a membrane complex similar to the platelet glycoprotein (GP) IIb/IIIa complex. Therefore, we also examined the acquisition of this antigen during the activation of human vascular endothelial cells by LPS and TNF using flow cytometry and MoAbs.

II. MATERIALS AND METHODS

1. Endothelial cell culture: Human vascular ECs were isolated from umbilical veins and cultured according to the method of Jaffe et al. with some minor modifications. Human umbilical vein endothelial cells (HUVECs) were cultured as flat monolayers in 25–cm² plastic flasks (Corning Incorporated, Corning, NY, U.S.A.). Cultures were performed in medium 199 (M199; Hazelton, PA) with 10µg/ml endothelial cell growth supplement (ECGS; Biomedical Product Division, Benford, MA), 25U/ml penicillin (Sigma, St. Louis, MO), 25 µg/ml streptomycin (Sigma), 1µg/ml amphotericin B (Flow Laboratories, Irvine, Scotland), and 100µg/ml heparin (Novo Industry A/S, Copenhagen, Denmark), and 15% fetal bovine serum (FBS; Bocknek, Tronto, Canada). HUVECs were identified by their typical characteristics. Cells from the second passage were used in these studies. After reaching confluence, cells were incubated for 48h at 37°C in medium containing E. coli LPS (20µg/ml, Difco Laboratories, Detroit, Michigan, U.S.A.) or medium containing recombinant TNF–alpha (500U/ml, Genzyme Corporation, Cambridge, U.S.A.).

2. Monoclonal Antibody Preparation: The anti–CD9 MoAbs used (NNKY1–19 and MALL–13) have been described previously. NNKY1–
19 was developed by immunizing mice with human platelets and MALL-13 by immunization with common ALL cells. The respective subclass of NNKY1-19 and MALL-13 are IgG1 and IgG2a. The molecular weight of the platelet-membrane antigens recognized by NNKY1-19 and MALL-13 was 24,000 daltons under nonreducing conditions. Another MoAb, NNKY2-6, was used for anti-GPIIb/IIIa antibody.11,12)

3. Immunofluorescence Flow Cytometry: For immunofluorescence flow cytometry, HUVECs monolayers washed twice with phosphate-buffered saline were detached from culture flasks by brief exposure to 0.05% trypsin (Gibco, Grand Island, NY) and 0.02% ethylene diamine tetraacetic acid disodium salt (EDTA), followed by neutralization with soybean trypsin inhibitor (200 μg/ml, Sigma). The HUVECs were then centrifuged, washed twice with phosphate-buffered saline at room temperature, and fixed in 1% paraformaldehyde. Then, MoAbs (100 μl) at an optimal concentration were added and the cells were incubated for 1 hr at room temperature. After being washed three times with phosphate-buffered saline, the cells were incubated with the fluorescein isothiocyanate (FITC)-labeled secondary antibody (goat anti-mouse IgG) for an additional 1 hr at room temperature. After two washes with phosphate-buffered saline, the fluorescent-stained cells were analyzed using a Becton-Dickinson FACStar. Table 1 displays these methods.

III. RESULTS

1. Induction of HUVEC gap formation by LPS and TNF: When LPS (20 μg/ml) and TNF (500 U/ml) were added to monolayer cultures of HUVECs, the cells changed shape and gaps developed in the monolayers after 48h. Figure 1 displays the typical morphological changes with gap formation in the monolayers. Monolayers of HUVECs were incubated with 500 U/ml TNF-alpha for 0h (A) or 48h (B), and were incubated without TNF-alpha for 0h (C) or 48h (D) as the control (Fig. 1).

Fig. 1 TNF-alpha-induced gap formation in cultured HUVEC monolayers.
2. Flow cytometry: After incubation with TNF or LPS for 48h, HUVEC CD9 antigen expression was increased compared with the untreated controls. The cells were stained by the indirect immunofluorescent method using anti-CD9 MoAbs (MALL-13 and NNKY1-19) and an anti-GPIIb/IIIa MoAb (NNKY2-6) followed by fluorescein conjugated goat anti-mouse IgG. The results are displayed as histograms (y axis: relative number of cells, x axis: fluorescence intensity). The fluorescence profiles of cells incubated with LPS and TNF are shown as solid lines, and those of cells incubated without LPS and TNF are shown as broken lines (Fig. 2). An increase of cells with a higher fluorescence intensity was recognized in flow cytometric histograms of TNF- or LPS-treated

![Flow cytometric histograms](image)

**Fig. 2** Flow cytometric histograms of TNF- or LPS-treated HUVECs compared with untreated control cells.

![Histograms and RFI](image)

**Fig. 3** Histograms and RFI that calculated from each histogram according to the definition.

\[
\text{RFI} = \left[ \frac{\text{mean channel of the positive region}}{\text{total cell count in the positive region}} \right] \times 10^{-3}
\]
HUVECs compared with untreated control cells using MALL-13 and NNKY1-19.

In addition, the relative fluorescence intensity (RFI) was higher in treated HUVECs than untreated controls. We defined RFI as follows:

$$RFI = \frac{\text{mean channel of the positive region} \times \text{total cell count in the positive region}}{10^{-3}}$$

**Fig. 3** displays histograms and RFI to give examples.

Left histogram shows the result that recorded from ECs stained with only fluorescein conjugated goat anti-mouse antibody, as negative control. Mean channel of the positive region is 37.79, and total cell count in the positive region is 105. Right histogram shows the result that recorded from ECs stained with an anti-GPIIb/IIIa MoAb (NNKY2-6) and fluorescein conjugated anti-mouse antibody. Mean channel of the positive region is 74.59, and total cell count in the positive region is 9848 (Fig. 3).

However, when NNKY2-6 was used there was no significant difference between the RFI of activated HUVECs and that of untreated control cells. Figure 4 shows the mean (± SD) RFI values as a percentage versus the RFI of the untreated control culture. Following LPS treatment (n = 4), the values were: MALL-13, 128.83 ± 11.24; NNKY1-19, 130.29 ± 5.29; and NNKY2-6, 101.96 ± 15.34. Following TNF treatment (n = 3), they were: MALL-13, 119.51 ± 3.51; NNKY1-19, 123.36 ± 1.87; and NNKY2-6, 104.31 ± 8.29. Following LPS treatment, the RFI values obtained with anti-CD9 MoAbs was higher than that obtained with the anti-GPIIb/IIIa MoAb (p < 0.05): #1. Likewise, the RFI values obtained using anti-CD9 MoAbs were higher than those obtained with the anti-GPIIb/IIIa MoAb after TNF treatment (p < 0.05): #2 (Fig. 4).

**IV. DISCUSSION**

Extensive studies have shown that CD9 antigen is expressed by a variety of cells, including B cells, common ALL cells, platelets, ECs, tonsillar follicular cells, hepatocytes, epithelia cells, and cells of the renal glomeruli and proximal tubules. Although the widespread distribution of this molecule suggests that CD9 antigen may play an important role in basic cell physiology, the nature of this role has remained obscure. In platelets, CD9 anti-
gen appears to be involved in cell activation. Several groups have studied the effect of anti-CD9 antibodies on platelet function and have found that such antibodies induce platelet aggregation, granule secretion, release of intracellular granule contents, production of arachidonic acid metabolites, and elevation of the intracellular Ca\(^{2+}\) concentration. Platelet aggregation is considered to result from association of CD9 antigen with GPIIb/IIIa complex on the platelet membrane. In addition, anti-CD9 antibody has recently been demonstrated to induce homotypic adhesion (aggregation) of pre-B cell lines by some undefined mechanism. One group has reported that anti-CD9 antibodies incubated with ECs rapidly stimulate a large increase in neutrophill adherence to the ECs. 

In this study, monolayers of HUVECs showed the morphological changes with gap formation. It is known that TNF and LPS induce HUVECs activation, and HUVECs change shape with activation. Therefore, we thought that HUVECs were activated with TNF and LPS treatment. And the RFI was increased in HUVECs activated by TNF and LPS according to flow cytometric analysis using anti-CD9 MoAbs. Thus, CD9 antigen expression on HUVECs appears to be increased by activation. In a recent study, the expression of CD9 antigen was induced in various hematopoietic cell lines by treatment with 12-o-tetradecanoyl-phorbol 13-acetate (TPA). Therefore, CD9 antigen expression is induced following the activation of various hematopoietic cell lines and ECs. Activation of cultured HUVECs by inflammatory stimuli such as TNF and LPS increases their surface adhesiveness for leukocytes and related cell lines. In addition, exposure to TNF and LPS induces procoagulant activity in cultured HUVECs. The function of CD9 antigen is still a subject of debate. Our finding that CD9 antigen expression is increased in ECs following activation with TNF and LPS, we suggest the probability that this antigen plays a role in adhesion between hematopoietic cells and ECs or in the procoagulant activity of ECs. GPIIb/IIIa is a member of the integrin superfamily of heterodimeric adhesion receptors, which binds fibronectin, fibrinogen, vWF, and vitronectin. Inter-cellular adhesion molecule (ICAM)-1, one of these adhesion molecules, is known to be increased on HUVECs by TNF and LPS treatment. In this study, however, GPIIb/IIIa-like protein did not increase the RFI of TNF- and LPS-treated HUVECs compared with untreated controls. Therefore, GPIIb/IIIa-like protein expression was not increased by the activation of HUVECs. The concentration of GPIIb/IIIa-like protein occurs at sites of cell-to-cell contact in ECs grown as confluent monolayers. This GPIIb/IIIa-like protein may thus play a role in cell-to-cell adhesion under conditions of confluency, or conversely may be associated with ventral adhesion sites in ECs that are not in permanent reciprocal contact. Platelet GPIIIa and EC GPIIIa are apparently identical, while the GPIIb/IIIa-like protein of ECs appears to be equivalent to the vitronectin receptor and is not platelet GPIIb/IIIa. In this study, RFI using NNKY2-6 was not increased by HUVECs activation with LPS and TNF. NNKY2-6 is one of MoAbs that recognize GPIIb/IIIa complex of platelets. And analysis using MoAs that recognize other epitopes of GPIIb/IIIa is necessary to discuss the increasing of GPIIb/IIIa-like protein expression by the activation of HUVECs. Further studies are required in order to clarify the biological functions of CD9 antigen and the significance of its induction by cell activation.

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