Pergamon

Thrombosis Research 97 (2000) 505–511

THROMBOSIS RESEARCH

BRIEF COMMUNICATION

Receptor Expression for IgG Constant Fraction in Human Umbilical Vein Endothelial Cells

María F. Alberto[1], Emilse I. Bermejo[1] and María A. Lazzari[1,2]
[1]Department of Thrombosis and Hemostasis,
Hematological Research Institute, National Academy of Medicine, [2]CONICET, Buenos Aires, Argentina.

(Received 2 February 1999 by Editor J. Aznar; revised/accepted 14 October 1999)

Key Words: Fcγ receptors; Human endothelial cell; Flow cytometry

Endothelial cells (EC) lining the vasculature form a natural barrier between the circulating blood and the underlying tissue. One of the major functions of these cells is to prevent platelet deposition and activation of the coagulation cascade [1].

Clinical and laboratory observations suggest that the endothelium plays an active role in the inflammatory and coagulation systems, and also that their responses are interrelated.

In the resting state, EC exert anticoagulant effects through different molecular systems present at the luminal side of their membrane. When submitted to certain injuries (i.e., inflammatory agents), EC exert procoagulant activities at their surface by expressing tissue factor [2,3] and consequently their anticoagulant properties decrease.

In systemic lupus erythematosus (SLE) the clinical picture encompasses a vasculitic syndrome and multiple coagulation abnormalities in addition to autoimmune phenomena. A close correlation exists between the activity of the disease and the immune complex levels, whereby patients with the highest disease scores tend to have the highest level of immunocomplexes (IC) [4]. Moreover, patients with active SLE contain IC that bind to EC of human umbilical vein endothelial cells (HUVEC), altering prostacyclin secretion [5]. It has also been demonstrated that HUVEC produce procoagulant tissue factor after exposure to increasing quantities of heat-aggregated IgG (HA-IgG) and IC [6]. Receptors for the Fc moiety of IgG (FcγRs) may play a role in the development of these processes, but the FcγR expression on HUVEC is still controversial [7,8].

The present study investigates the binding to HUVEC of HA-IgG and of a panel of monoclonal antibodies (mAb) that recognize the three different types of FcγR expression in leukocytes (FcγR I, II, and III). It was performed on HUVEC, both in the resting state and after stimulation with tumor necrosis factor α (TNFα), interleukin 1β (IL1β), interferon γ (INFγ), lipopolysaccharide (LPS), and human thrombin (thr).

We show here that HUVEC express a binding activity to HA-IgG and that the mAb to FcγR I recognize the type I receptor on the EC surface. The binding activity to HA-IgG and FcγR I expression levels were not modified with the stimulation of the different agents assayed.

Abbreviations: EC, endothelial cells; SLE, systemic lupus erythematosus; IC, immunocomplexes; HUVEC, human umbilical vein endothelial cells; HA-IgG, heat-aggregated IgG; FcγRs, receptors for the Fc moiety of IgG; mAb, monoclonal antibodies; TNFα, tumor necrosis factor α; IL1β, interleukin 1β; INFγ, interferon γ; LPS, lipopolysaccharide; EDTA, ethylenediaminetetraacetic disodium salt; thr, human thrombin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; hvWf, human von Willebrand factor.

Corresponding author: María F. Alberto, Hematological Research Institute, National Academy of Medicine, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. Tel: +54 (11) 48050712; Fax: +54 (11) 48050712; E-mail: <emilse@connmed.com.ar>.

PII S0049-3848(99)00203-0

1. Materials and Methods

1.1 Cell Culture

HUVEC were isolated by collagenase (from *Clostridium histolyticum* type IV, Sigma Chemicals Co., St. Louis, MO, USA) treatment of human umbilical veins according to the method of Jaffe et al. [9]. HUVEC were cultured at 37°C in 5% CO_2 in 25 cm^2 tissue culture flasks, pretreated overnight by incubation in 1% gelatin (Sigma Chemicals Co.). Cultures were established in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 20% fetal bovine serum (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM of L-glutamine (Sigma), and 50 μg/mL endothelial cell growth supplement (Sigma). Culture media were changed twice weekly. Confluent HUVEC were passaged by short exposure of monolayers to 0.05% trypsin-ethylenediaminetetraacetic disodium salt (EDTA) (Gibco). HUVEC from passage 1–2 were used for all studies.

1.2. Cytokines, LPS, and Thrombin Treatment

The following sources of cytokines were used in this study: human r-TNFα (biological activity 5.3×10^6 U/mg), human r-IL1β (biological activity 1×10^8 U/mg), and human r-IFNγ (biological activity 1×10^7 U/mg protein) (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA); LPS from *Escherichia coli* 055:B5 (Sigma); and human thrombin (Ortho Diagnostic Systems, Raritan, NJ, USA).

Doses used for the treatment of HUVEC in the culture were: 2 UI/mL thrombin [10] and IFNγ 100 and 500 U/mL [11]. A dose-response curve was done for TNFα, IL1β, and LPS; no modifications in HA-IgG binding were found (data no shown). Only results for the maximal doses tested are shown (i.e., 250 U/mL, 1000 U/mL, and 100 ng/mL, respectively.

HUVEC grew to confluence in 25 cm^2 cell culture flasks and were rinsed twice with RPMI 1640. Cytokines, LPS, and thrombin were diluted in serum-free RPMI 1640 at the chosen concentrations. After a 6-hour incubation with all agents, cells were detached and immediately fixed in 1% paraformaldehyde (Immunotech, Marseille, France), rinsed twice with phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA) and then stained. Each agent was assayed in parallel with a HUVEC control in the resting state derived from the same primary culture at the same level of passage.

1.3. Antibodies

The following antibodies were used: mAb 197 (anti-CD64), mAb 32.2 (anti-CD64), mAb IV.3 (anti-CD32), mAb 3G8 (anti-CD16) (Medarex Inc., West Lebanon, NH, USA), mAb antihuman von Willebrand factor (anti-hvWf), isotype controls (mouse IgG1, IgG2a, and IgG2b), biotin-SP-conjugated affinity pure $F(ab')_2$ fragment goat anti-mouse IgG, biotin-SP-conjugated affinity pure $F(ab)_2$ fragment goat antihuman IgG, B-phycoerythrin-conjugated streptavidin (Immunotech).

1.4. Cell Staining

Before cell staining, confluent monolayers of HUVEC, were detached by treating them with trypsin-EDTA or PBS 0.05% EDTA (PBS-EDTA). The first method implied a proteolytic treatment of monolayers that could modify the surface phenotype. We compared HA-IgG binding on HUVEC detached by both methods.

Following cell isolation, HUVEC were immediately fixed with 1% paraformaldehyde for indirect immunofluorescence. After 30 minutes, the cells were washed with PBS-BSA, incubated with an unlabeled mAb for 40 minutes (197, 32.2, IV.3, 3G8, anti-hvWf, or isotype controls), and diluted in PBS-BSA according to the instructions provided by the manufacturer. Then the cells were washed with PBS-BSA, incubated for 40 minutes with biotin-conjugated $F(ab')_2$ antimouse IgG (1:200 in PBS-BSA), then washed with PBS-BSA, incubated 40 minutes with B-phycoerythrin-conjugated streptavidin (1:50 in PBS-BSA), and washed again. Finally, the cells were fixed in 1% paraformaldehyde for 30 minutes and analyzed by flow cytometry using a FACScan cytometer (Becton Dickinson).

1.5. HA-IgG Binding Assay

Before each experiment, HA-IgG was prepared by heating monomeric IgG (Calbiochem) at 63°C for 20 minutes and centrifuged at $3000\times g$ for 15 minutes to remove insoluble aggregates [12]. Once detached, cells were immediately fixed in 1% paraformaldehyde for 30 minutes, washed with PBS-BSA,

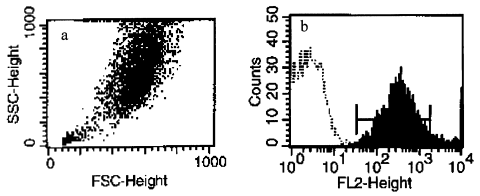

Fig. 1. (a) Dot-plot, side scatter (SSC) vs. forward scatter (FSC) of HUVEC. (b) Histogram of fluorescent signal of hvWf expression on cells cultured from human umbilical vein. In all tested cultures the majority of cells expressed hvWf, indicating its endothelial origin.

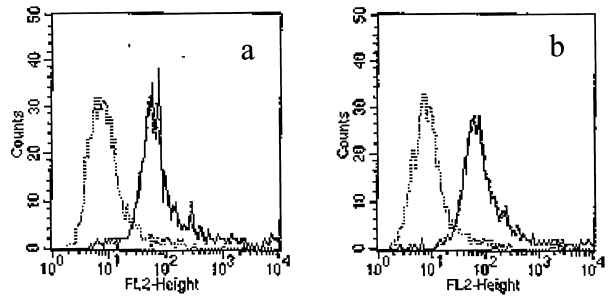

Fig. 2. HA-IgG binding on HUVEC detached by (a) proteolytic trypsin treatment or (b) PBS-EDTA treatment. The percentage of cells that bind to HA-IgG was similar for both methods.

and incubated for 40 minutes with HA-IgG diluted in PBS-BSA to reach a final concentration of 250 μg/mL [5]. Next, cells were washed with PBS-BSA and incubated for 40 minutes with biotin-conjugated $F(ab')_2$ antihuman IgG (1:200 in PBS-BSA). They were then washed twice, incubated for 40 minutes with B-phycoerythrin-conjugated streptavidin (1:50 in PBS-BSA), and washed again. Cells were fixed in 1% paraformaldehyde for 30 minutes and analyzed by flow cytometry. As negative control we used cells incubated directly with biotin-conjugated $F(ab')_2$ antihuman IgG and B-phycoerythrin-conjugated streptavidin.

1.6. FACScan Analysis

HUVEC were identified by their light scattering properties on the cytogram [i.e., right angle scatter (SSC on a linear scale) and forward angle scatter (FSC on a linear scale)] (Figure 1a). For analysis, a gate was set around the HUVEC population and 5000 to 10000 cells were acquire.

For the HA-IgG binding assay, results are expressed as the percentage of cells that produce fluorescent displacement compared to the negative control. Similarly, for each mAb assayed the results are expressed as a percentage of cells that produce fluorescent displacement compared to the isotype-negative control. In both cases, results represent the average of three or four assays performed with HUVEC derived from independent primary cultures.

2. Results

2.1. Characterization of HUVEC in Culture

HUVEC of different preparations were separately seeded in gelatin flasks. Immediately after cell isolation, the majority of cells in the final preparation had a round shape. After a short period of culture, the adherent cells had a fibroblastoid appearance, and cell confluence was reached after 3 to 6 days. Confluent monolayers evaluated by phase contrast microscopy demonstrated the typical cobblestone morphology. To check the purity of isolated cells, the cell preparation was routinely evaluated with mAb to hvWf by flow cytometry. 96±3% of cells expressed hvWf, indicating that the majority of the cultured cells were endothelial cells (Figure 1b).

2.2. HUVEC Binding to HA-IgG and to FcγR Panel

To test whether the detachment method could modify surface phenotype, we assessed the HA-IgG binding to detached HUVEC by proteolytic trypsin treatment or PBS-EDTA treatment. The results represent the average of three assays performed with HUVEC derived from independent primary culture. The viability and the percentage of cells that bind to HA-IgG (Figure 2) were similar for both methods, 65.2±22.3% and 60.9±16.8%, respectively (n=3). Consequently, we chose the trypsin treatment since it was easier and faster.

All experiments were performed using fixed cells immediately after trypsin detachment. There was always HA-IgG binding activity when the cells were in the resting conditions: 59.5±21.4% (n=16) of cells produced fluorescent displacement.

When the reactivity of these HUVEC was evaluated with a panel of monoclonal antibodies that bound to FcγRs present in leukocytes, the HA-IgG binding activity correlated with positive fluo-

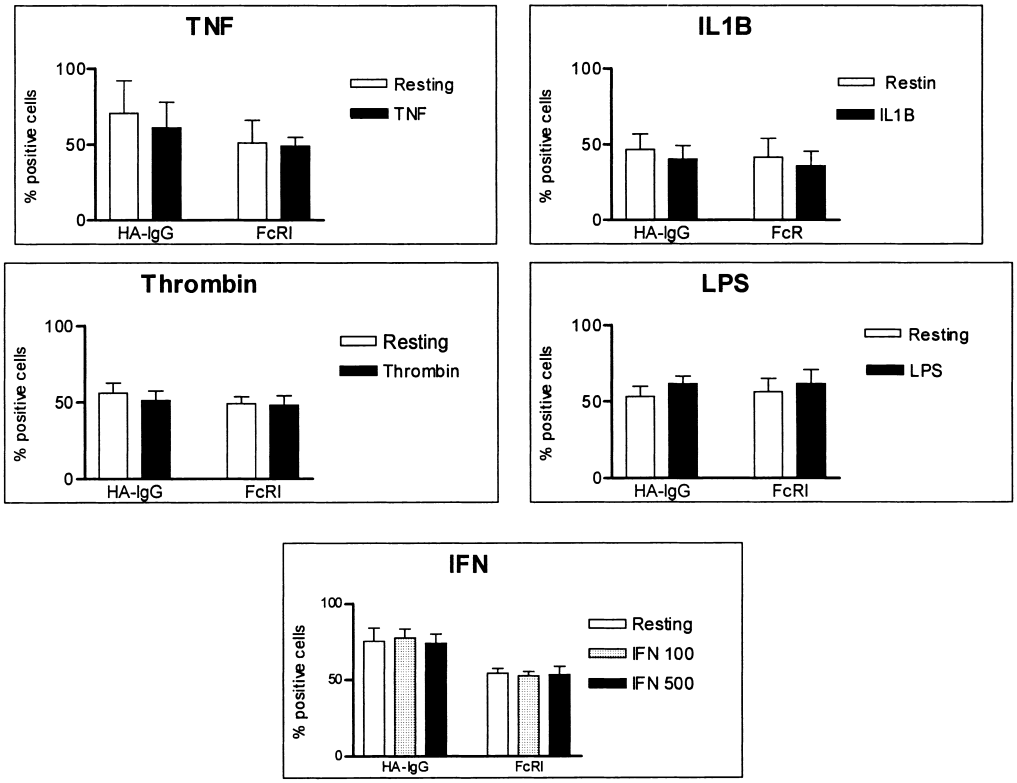

Fig. 3. Expression of the HA-IgG binding and FcγR I on HUVEC after stimulation with TNFα, IL1β, thrombin, LPS, and IFNγ. Each agent was assayed in parallel with a HUVEC control in the resting state derived from the same primary culture.

rescent signal to anti-FcγR I: 49.8±17.0% (n=16) of cells produced fluorescent displacement.

Moreover, HA-IgG binding activity and FcγR I expression were not significantly modified after the stimulation treatments (Figure 3). Table 1 summarizes HA-IgG binding activity and FcγR I expression on HUVEC in resting conditions and after stimulation with the different agents.

The positive expression of FcγR I (anti-FcγR I 197) was confirmed with another antibody (anti-FcγR I 32.2) that recognized a different epitope (Figure 4). The level of fluorescent signals was similar for both antibodies, 46.7±12.3% and 57.2±15.1%, respectively (n=3).

3. Discussion

Endothelium plays an active role in the inflammatory and coagulation systems. Patients with SLE have an increased incidence of arterial and venous thrombosis of uncertain etiology.

Disturbances in endothelial cell function could initiate thrombosis in patients with SLE by altering the local balance between procoagulant and anticoagulant factors within regions of the vessel wall.

In this sense, it has been demonstrated that endothelial cells incubated with HA-IgG or with fractions of SLE sera containing monomeric as well as high molecular weight fractions of IgG produce a significant increase in tissue factor expression compared with equivalent fractions of normal sera [6]. The expression of tissue factor is not an immediate consequence of cell injury per se, but requires several hours of protein synthesis for its expression. In vivo, the production of tissue factor may depend on exposure of the relevant target that permit binding of the antiendothelial antibodies or IC.

It is evident that receptors for the Fc moiety of IgG (FcγR) may play a role in the development of these processes. It was demonstrated that human epidermal microvascular endothelial cells express FcγR II (CD 32) proteins on their cell surface [8]. This fits with the more general concept that

Table 1. Results of HA-IgG binding and FcγR I expression on HUVEC, stimulated vs. resting conditions

Treatments	HA IgG-binding, mean and range (%)	FcγR I expression, mean and range (%)
IL1β (n=4)		
Resting	46.6 (22.4–71.3)	41.2 (11.6–70.4)
Stimulated	40.2 (22.1–64.6)	35.8 (13.8–60.0)
TNFα (n=3)		
Resting	61.3 (27.9–90.3)	51.1 (22.0–70.4)
Stimulated	61.0 (31.0–89.4)	48.9 (37.3–56.4)
Thrombin (n=3)		
Resting	56.0 (45.5–67.0)	49.0 (45.0–58.0)
Stimulated	51.0 (43.0–63.0)	54.0 (43.0–60.0)
LPS (n=3)		
Resting	53.1 (43.1–63.0)	56.2 (40.0–73.4)
Stimulated	60.3 (52.7–70.2)	61.4 (43.2–71.1)
IFNγ (n=3)		
Resting	75.3 (63.7–92.1)	54.3 (49.4–53.4)
Stimulated 100	77.5 (70.3–89.3)	52.5 (49.0–58.4)
Stimulated 500	73.8 (65.4–86.0)	53.3 (45.3–63.3)

microvascular endothelial cells not only modulate inflammatory reactions by leukocyte-endothelial cell adhesion molecules but also participate in Ag-dependent immune responses. However, on HUVEC the FcγR expression is controversial. Cines et al. [5] have demonstrated that HUVEC are capable of binding complex IgG (HA-IgG) but two studies of antigen expression on HUVEC by flow cytometry demonstrated no expression of FcγR I, II, and III (CD 16, CD 32, CD64, respectively) [7,8]. Probably, a different FcγR not recognized by the assayed monoclonal antibodies may be responsible for the HA-IgG binding activity.

To test this hypothesis, we evaluated the binding ability of HUVEC to HA-IgG and the surface fluorescent pattern of a panel of mAb to FcγRs by flow cytometry. HUVEC monolayers were tested in the resting state and after treatment with agents that modify their functional status such as IFNγ, IL1β, TNFα, thrombin, and LPS.

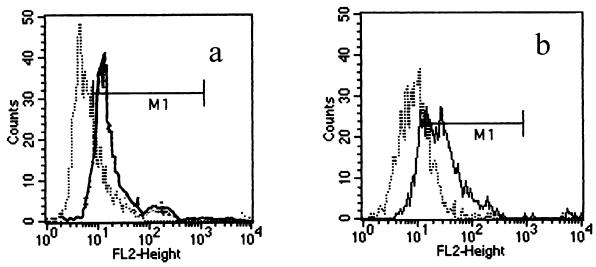

Fig. 4. Expression of FcγR I on HUVEC evaluated with two antibodies that recognize different epitopes: (a) 197/CD64 and (b) 32.2/CD64. The level of fluorescent signal was similar for both antibodies.

We demonstrated that HUVEC bound HA-IgG in the resting state. Surprisingly, the HA-IgG binding activity correlated with positive fluorescent signal to anti-FcγR I.

This HA-IgG binding activity and FcγR I expression level were not modified by the stimulation treatment with the agents mentioned above. We tested the effects of the different stimuli separately, but we cannot exclude synergistic actions of different cytokines as it was demonstrated in aortic endothelial cells between the TNFα and IFNγ, which significantly increase the expression of FcγR II and III [13].

It was used another antibody (mAb 32.2/CD64) to confirm FcγR I expression, which recognizes a different epitope than 197. Both monoclonal antibodies bound to HUVEC evidencing FcγR I expression.

Discrepancies with other authors [7,8], who have not found expression of FcγR I in HUVEC surface, could be attributed to a different methodology employed by cell cytometric analysis. In our case, cells were fixed immediately after the monolayer was treated with trypsin-EDTA. This differs from Ver-

mont-Desroches et al. [7] and Gröger et al. [8], who did not fix the cells. The preparation of endothelial cells for flow cytometry usually requires trypsin treatment to give a single cell suspension, it has been demonstrated that trypsin itself activates surface P-selectin expression and hvWf release [14]. P-selectin expression is transient and disappears from the endothelial cell surface by reinternalization [15]. To prevent reinternalization, endothelial cells have to be fixed with paraformaldehyde and thus preserve the activation phenotype [13]. Although the fixation procedure may alter the reactivity of some antigens (a decreased reactivity to specific antibodies was usually described [16]), it is difficult to consider that the reactivity to two anti-FcγR I is an artifact induced by fixation. A likely explanation for FcγR I surface expression would be that, like P-selectin, it is transient and becomes evident when HUVECs are activated. However, this is only a hypothesis. It has already been demonstrated in platelets that the number of FcγR II on the surface increases when activated by several agonist [17].

In this study we used HUVEC as a model of vascular endothelium. It is widely known that this cell type does not necessarily reflect the in vivo situation [18]. Since it is documented that endothelial cells from diverse tissues are also heterogeneous with respect to their constitutive as well as cytokine-induced phenotype [18], these results cannot be extrapolated to endothelial cells of different origin. In this sense, it has been demonstrated that dermal microvascular endothelial cells express FcγR II [8] and that the aortic endothelial cells express FcγR II and III [13]; both receptors were not found in HUVEC either by us or by others authors [7].

In agreement with FcγR I expression on HUVEC is the stability of this receptor to the trypsin treatment (monolayers detached with PBS-EDTA or triysin are capable to bind HA-IgG) [19,20]. Furthermore, Cines et al. [5] demonstrated that HA-IgG, monomeric IgG, and heat-aggregated Fc fragments bind to HUVEC. In spite of the fact that they did not characterize the receptor type, the binding pattern of aggregated and monomeric IgG suggests the presence of a high affinity receptor such as FcγR I as it was demonstrated in the present paper.

We would like to thank the obstetrical nursing staff of Clinica Mater Dei from Buenos Aires, for their help in the collection of umbilical cords. This work was supported, in part, by grants from CONICET (PICT 0173), Roemmers Foundation and René Barón Foundation.

References

1. Bombeli T, Meuller M, Haeberli A. Anticoagulant properties of the vascular endothelium. Thromb Haemost 1997;77:408–23.
2. Stern DM, Bank I, Nawroth PP, Cassimeris J, Kisiel W, Fenton II JW, Dinarello C, Chess L, Jaffé EA. Self-regulation of procoagulant events on the endothelial cell surface. J Exp Med 1985;162:1223–35.
3. Galdal KS, Lyberg T, Evensen SA, Nilsen E, Prydz H. Thrombin induces thromboplastin synthesis in cultured vascular endothelial cells. Thromb Haemost 1985;52:373–6.
4. Lawley TJ, Hamburger MI, Brown EJ. Immunoglobulin G Fc receptor-mediated clearance in autoimmune diseases. NIH Conference. Ann Inter Med 1983;98:206–18.
5. Cines DB, Lyss AP, Reeber M, Bina M, De Horatius RJ. Presence of complement-fixing anti-endothelial cell antibodies in systemic lupus erythematosus. J Clin Invest 1984;73: 611–25.
6. Tannenbaum SH, Finko R, Cines DB. Antibody and immune complexes induce tissue factor production by human endothelial cells. J Immunol 1986;137:1532–7.
7. Vermont-Desroches C, Marchand D, Roy C, Wijdenes J. Heterogeneity of antigens expression among human umbilical cord vascular endothelial cells: Identification of cells subsets by co-expression of haematopoietic antigens. Immunology Letters 1995;48:1–9.
8. Gröger M, Sarmay G, Fiebiger E, Wolff K, Petzelbauer P. Dermal microvascular endothelial cells express CD32 receptors in vivo and in vitro. J Immunol 1996;156:1549–56.
9. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphological and immunological criteria. J Clin Invest 1973;52:2745–56.
10. Kamed H, Morita I, Handa M, Kaburaki J,

Yoshida T, Mimori T, Murota S, Ikeda Y. Re-expression of functional P-selectin molecules on the endothelial cell surface by repeated stimulation with thrombin. British J Haematol 1997;97:348–55.
11. Guyre PM, Morganelli PM, Miller R. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. J Clin Invest 1983; 72:393–7.
12. Mauer SM, Fish AJ, Blau EB, Michaej AF. The glomerular mesangium. I. Kinetic studies of macromolecular uptake in normal and nephrotic rats. J Clin Invest 1972;51:1092–1101.
13. Pan LF, Kreisle RA, Shi YD. Detection of Fcγ receptors on human endothelial cells stimulated with cytokines tumour necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ). Clin Exp Immunol 1998;112:533–8.
14. Collins PW, Macey MG, Cahill MR, Newland AC. Von Willebrand factor release and P-selectin expression is stimulated by thrombin and trypsin but not IL-1 in culture human endothelial cells. Thromb Haemost 1993;70: 346–50.
15. Sugama Y, Tiruppathi C, Janakidevi K, Andersen TT, Fenton JW II, Malik AB. Thrombin-induced expression of endothelial P-selectin and intracellular adhesion molecule-1. J Cell Biol 1992;119:935–44.
16. Favaloro EJ, Moraitis N, Bradstock K, Koutts J. Co-expression of haemopoietic antigens on vascular endothelial cells: A detailed phenotypic analysis. Br J Haematol 1990;74:385–94.
17. McCrae KR, Shattil SJ, Cines DB. Platelet activation induces increased Fc receptor expression. J Immunol 1990;144:3920–7.
18. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998;91:3527–61.
19. Frey J, Engelhardt W. Characterization and structural analysis of Fc receptors of human monocytes, a monoblast cell line (U937) and a myeloblast cell line (HL-60) by a monoclonal antibody. Eur J Immunol 1987;17:583–91.
20. van de Winkel JGJ, van Ommen R, Huizinga TWJ, de Raad MAHVM, Tuijnman WB, Goenen PJTA, Caapel PJA, Koene RAP, Tax WJM. Proteolysis induces increased binding affinity of the monocyte type II FcR for human IgG. J Immunol 1989;143:571–8.