

Mast Cell-Derived VEGF Enhances the Passage of IgE FE-3 through the Rat Aortic Endothelial Cell Monolayer

Toshiyuki Nakasone^{a,b} Kazuhiko Hanashiro^a Mariko Nakamura^a
Hajime Sunakawa^b Tadayoshi Kosugi^a

^aFirst Department of Physiology and ^bDepartment of Oral and Maxillofacial Surgery, School of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan

Key Words

IgE · Permeability · Endothelial cell · Mast cell · Mast cell-derived VEGF · IgE-mediated inflammation

Abstract

Background: It is well known that the IgE-mediated allergic reaction in various extravascular tissues is induced by the mutual interaction of IgE, target cells (mast cells, MCs) and allergens. However, so far little has been known about the detailed mechanism whereby IgE in the circulating blood is transferred to the extravascular tissue. To examine whether or not MCs are involved in the permeability of IgE across rat aortic endothelial cells (RAECs) in vitro, we determined the permeability constant (PC) of dinitrophenyl-specific rat IgE (IgE FE-3) using a dual chamber system. **Methods:** Isolated RAECs obtained by a primary explant technique were seeded on a collagen-coated membrane in the upper chamber. MCs were collected from the peritoneal cavity of Wistar rats and suspended in the lower chamber. The time-dependent changes in concentration of IgE FE-3 (IgE) in the upper and lower chambers were measured by IgE capture ELISA after addition of IgE (10 µg/ml) to the upper chamber. **Results:** The cultured medium of IgE-stimulat-

ed MCs significantly increased the PC of IgE ($9.86 \pm 0.46 \times 10^{-6}$ cm/s), as compared to the value to which calcium ionophore A 23187-stimulated MCs increased the PC of IgE ($7.97 \pm 0.21 \times 10^{-6}$ cm/s). The increase of PC by IgE-stimulated MCs was most strongly inhibited by suramin ($92.3\% \pm 1.89$), and was weakly inhibited by tranexamic acid, cimetidine and diphenhydramine. In addition, the PC of IgE was increased with the increase in the MC-derived vascular endothelial growth factor/permeability factor (VEGF). **Conclusions:** After IgE is transferred from the circulating blood to the extravascular tissue, it may bind to FcεRI of the MC and the other FcεRI-bearing cells. The MC is then activated through the interaction of IgE and FcεRI. Release of VEGF from the activated MC increases, and the VEGF enhances the permeability of IgE.

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Introduction

It is well known that the IgE-mediated allergic reaction in various extravascular tissues is induced by the mutual interaction of IgE, target cells (mast cells, MCs) and allergens. IgE is a key substance in the allergic reaction occur-

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Correspondence to: Dr. Tadayoshi Kosugi
First Department of Physiology, School of Medicine
University of the Ryukyus, 207 Uehara
Nishihara, Okinawa 903-0215 (Japan)
Tel. +81 98 895 1108, Fax +81 98 895 1402, E-Mail tkosugi@med.u-ryukyu.ac.jp

ring in extravascular tissues. It is produced in the tissue and/or distributed from the circulating blood to the extravascular tissue. The magnitude of the allergic reaction in the extravascular tissue has been inferred, therefore, possibly to be proportional to the degree of IgE passage through the vascular wall. However, on the basis of clinical data, the concentration of IgE in the circulating blood of patients with allergic diseases does not regularly reflect the pathophysiological signs of allergy [1], and it remains unclear whether the IgE level in the circulating blood reflects the amount of IgE in the extravascular tissue or not. Concerning the regulatory mechanisms of IgE passage from the circulating blood to extravascular tissue, it has been suggested that this passage might be related to interactions of IgE with IgE-receptor-bearing cells such as MCs [2] and/or to their regulatory effects on the barrier function of endothelial cells (ECs). However, so far little has been known about the detailed mechanism whereby IgE in the circulating blood is transferred to the extravascular tissue. We need to determine the regulatory mechanism, which governs IgE passage through the vascular wall to the extravascular tissue. It has been reported that histamine can enhance the permeability of IgE across rat aortic endothelial cells [3]. However, vascular endothelial growth factor/permeability factor (VEGF) is also a powerful enhancer of vascular permeability, being 50,000 times more potent than histamine [4]. In addition, there is a report that mouse or human MCs can secrete VEGF, and such secretion is enhanced via upregulation of the IgE receptor of the MCs [5]. The detailed relationship between IgE passage through ECs and MCs has not yet been examined. It remains to be demonstrated whether or not MCs can affect the permeability of IgE using cultured ECs. To resolve these issues in the present study, we investigated whether or not chemical mediators from IgE-stimulated MCs modulated the passage of IgE through an EC monolayer using a dual chamber system.

Materials and Methods

Animals

Wistar rats weighing 250–300 g were employed for the experiments. The rats were purchased from Kyudo Co. (Kumamoto, Japan). We followed the Standards Relating to the Care and Management of Experimental Animals (Notification No. 6, March 27, 1980, from the Prime Minister's Office, Tokyo, Japan) for the care and use of animals, together with the guide for animal experiments issued by the University of the Ryukyus. All animal studies were reviewed and approved by the Animal Care Committee at the University of the Ryukyus.

Reagents

Nutrient mixture F-12 HAM, Kaighn's modification (HAM's F-12K), collagenase type I (cell culture grade), 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), recombinant human VEGF₁₆₅, calcium ionophore A23187, suramin, cimetidine, and diphenhydramine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Tranexamic acid (t-AMCHA) was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Sanko Junyaku (Fukuoka, Japan). Goat anti-human VEGF₁₆₅ polyclonal antibody was obtained from R&D System (Minneapolis, Minn., USA). Falcon Cell Culture Insert® (diameter 6 mm; pore size 3 µm; pore density $8.0 \times 10^5/\text{cm}^2$) and its companion plate were purchased from Becton Dickinson (Franklin Lakes, N.J., USA). Type I collagen from the tail of rats was obtained from Collaborative Biomedical Products (Bedford, Mass., USA). Rat basophilic leukemia cells (RBL-1) and mastocytoma cells (P 815) were obtained from American Type Culture Collection (ATCC) (Rockville, Md., USA). Novo heparin was purchased from Aventis Pharma (Tokyo, Japan). Monoclonal DNP-specific rat IgE (IgE FE-3) was purified by the modified method described previously [6]. Monoclonal antibody against IgE FE-3 (BB-9) was purified by the same method as described previously [7, 8]. DNP-As was purified employing the method described by Strejan and Campbell [9] and Eisen et al. [10]. Biotinylated DNP-As was prepared according to the method of Nerurkar et al. [11]. 2,4-Dinitrobenzene sulfonic acid sodium salt was purchased from Tokyo Kasei (Tokyo, Japan). Diethylether, Tween 20, 3,3'-diaminobenzidine and gelatin fine powder were obtained from Nacalai Tesque (Kyoto, Japan). Amino hexanoyl-biotin-N-hydroxysuccinimide, peroxidase (POD)-conjugated rabbit anti-goat IgG (H+L), and HRP-conjugated streptavidin were obtained from Zymed Laboratories (San Francisco, Calif., USA). CNBr-activated Sepharose 4B, and Protein A-Sepharose CL-4B, FicolTM 400 were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). DiI-Ac-LDL was obtained from Harbor Bio-Products (Norwood, Mass., USA). Unless otherwise stated, only reagent-grade chemicals were used as other reagents.

Cell Culture

The isolation of rat aortic ECs was performed according to the method of MacGuire and Orkin [12]. The full length of the thoracic aorta was aseptically removed from the thoracic cavity of a Wistar rat and rinsed in HAM's F-12K. The vessel was gently cleaned to exclude periaortic fat and connective tissue, and was then cut into flat segments measuring approximately 5 mm × 5 mm. These flat aortic segments were placed endothelial side down on a dish coated with type I collagen from the rat's tail, and 1 ml of culture medium (HAM's F-12K containing 10% FBS, 100 µg/ml of penicillin and 100 µg/ml of streptomycin) was added. The cells were cultured in a humidified incubator (Astec, Fukuoka, Japan) at 37 °C in a 95% air/5% CO₂ atmosphere (CO₂ incubator), according to the method of Hanashiro et al. [3]. To identify the cultured cells as ECs from their uptake of DiI-Ac-LDL, they were subsequently incubated in the presence of 10 µg/ml of DiI-Ac-LDL for 4 h in a CO₂ incubator. Finally, the medium was removed and the cells were washed 3 times with Dulbecco's PBS. The LDL particles were visualized by fluorescence microscopy (Nikon, Tokyo, Japan) employing filters adjusted for rhodamine fluorescence according to the procedure of Pitas et al. [13]. MCs were isolated from the peritoneal cavity fluid of specific pathogen-free Wistar rats according to the procedures of Hayashi et al. [14] and Kuriu et al. [15] with slight modifications. A

nonsensitized Wistar rat was anesthetized with diethyl ether and bled from the cervical vein. 6 ml (v) per 100 g (body weight of rat) of Dulbecco's PBS buffer containing 0.1% gelatin, 1 ml of Novo heparin and 5.6 mM D-glucose was injected into the peritoneal cavity of the rat. The peritoneal cavity cells suspended in this solution were collected using a syringe with a 18-gauge probe and centrifuged at 1,250 rpm for 1 min at 4°C. The pellet was suspended in 20% Ficoll. A discontinuous Ficoll density gradient was set up in a 12-ml tube (bottom layer: 1 ml of 40% Ficoll; middle layer: 3 ml of 30% Ficoll; upper layer: 1 ml of suspended peritoneal cavity cells), which was then centrifuged at 1,400 rpm for 20 min at 4°C. The cells between the middle layer and bottom layer were collected. After the collected cells had been washed twice with serum-free HAM's F-12K (SFM), centrifugation was performed at 800 rpm for 5 min at 4°C. The peritoneal cavity cells were resuspended in 10 ml of SFM. 90% or more viable MCs were contained in the suspension of these cells based on the results of the toluidine blue staining test.

Experimental Protocol for Passage of IgE through the EC Monolayer

The passage of IgE through EC monolayer was monitored as described previously [3]. Polyethylene terephthalate track-etched membranes of the Falcon Cell Culture Insert (upper chamber) were coated with type I collagen from rat's tail. Such upper chambers were placed in each well of a 24-well plate (lower chamber). The cells were removed from the tissue flask by treatment with 0.25% collagenase and centrifuged at 1,500 rpm for 5 min. The cell pellet was resuspended in complete culture medium and 0.45 ml containing 1×10^5 cells was seeded onto each collagen-coated membrane. Experiments using these cells were performed at 48–72 h after seeding. Confluence of monolayer cells on the membranes was evaluated by Millicell-RES (Millipore, Bedford, Mass., USA) and light microscopy. The EC monolayer was judged to be confluent when the endothelial electrical resistance was 8–10 $\Omega \cdot \text{cm}^2$. In addition, cell-to-cell junctions were tightly retained at this electrical resistance based on the findings of light microscopy (data not shown). 0.43 ml of SFM containing IgE (10 $\mu\text{g}/\text{ml}$) was added to the upper chamber and 1 ml of SFM to the lower chamber, and the dual chamber was placed in a CO₂ incubator. IgE was purified from cultured medium of FE-3 cells using BB-9 (monoclonal anti-rat IgE FE-3)-Sepharose 4B as described previously [8]. After 2 μl had been withdrawn from the upper chamber, an equal volume of SFM containing IgE (10 $\mu\text{g}/\text{ml}$) was added to the chamber at different times (1, 2, or 3 h). After 50 μl had been withdrawn from the lower chamber, an equal volume of SFM was added to the chamber at different times (1, 2 or 3 h). The concentration of IgE in the media of the upper and lower chambers was determined by IgE capture ELISA as described previously [8].

To test the effect of activation of MCs by IgE and A23187 on the PC of IgE, MCs had been exposed to IgE at different concentrations (3, 5 and 10 $\mu\text{g}/\text{ml}$) or to A23187 (1, 5, 10 $\times 10^{-6}$ M) for 24 h in a CO₂ incubator, the cultured media of MCs were collected and added to the upper chamber. In the case of IgE stimulation, IgE in the cultured medium was removed using a BB-9-Sepharose 4B column.

Western Blot Analysis

After non-, A23187 (1×10^{-6} M)- or IgE (10 $\mu\text{g}/\text{ml}$)-stimulated MCs had been cultured for 24 h in a CO₂ incubator, the respective cultured media were concentrated by 5-fold using Vivapore (Sartorius, Göttingen, Germany) and were then electrophoresed. SDS-PAGE was performed using Phast gel (gradient 8–25). After comple-

tion of the electrophoresis, the gel was treated by silver staining according to the method of Heukeshoven and Derinck [16]. Separated proteins were blotted onto a nitrocellulose membrane (0.45- μm pore size in roll form; Toyo Roshi Kaisha, Tokyo, Japan). The transfer of protein from the polyacrylamide gel onto this paper was carried out according to the method of Towbin et al. [17]. The membrane was blocked with Dulbecco's PBS containing 1% skimmed milk overnight at 4°C. 2 $\mu\text{g}/\text{ml}$ of goat anti-human VEGF₁₆₅ polyclonal antibody was incubated with the membrane for 2 h at room temperature (RT). After intense washing, the membrane was incubated with HRP rabbit anti-goat IgG ($\times 1/3,000$) as the secondary antibody for 2 h at RT. The membrane was subsequently washed and incubated with a solution consisting of 250 $\mu\text{g}/\text{ml}$ of 3,3'-diaminobenzidine tetrahydrochloride, 0.01% H₂O₂, 50 mM Tris-HCl (pH 7.2) for 3 min at RT. Although we should theoretically use anti-rat VEGF instead of goat anti-human VEGF₁₆₅ antibody to detect rat MC-derived VEGF for Western blot and ELISA, we empirically used the antibody for the following reasons: (1) anti-rat VEGF was not commercially available, (2) the amino acid sequence of rat VEGF (GenBank accession No. AF222779) has 76% identity with that of human (AL136131) and (3) polyclonal goat anti-VEGF₁₆₅ antibody have a very high chance of containing antibodies against common epitopes of rat VEGF.

Estimation of MC-Derived VEGF by ELISA

ELISA was performed according to the modified method of Engvall and Perlmann [18]. The ELISA plate was coated with the sample or human recombinant VEGF₁₆₅ at different concentrations. After blocking with 1% skimmed milk in Dulbecco's PBS for 2 h at RT, the wells were incubated with goat anti-human VEGF₁₆₅ polyclonal antibody (2 $\mu\text{g}/\text{ml}$) in Dulbecco's PBS for 2 h at RT. Following washing, the wells were incubated with POD rabbit anti-goat IgG ($\times 1/3,000$) in Dulbecco's PBS containing 0.05% Tween 20 for 1 h at RT. At 30 min after substrate solution (ABTS) had been added to each well, spectrophotometric readings were made using the λ_1 : 415 nm and λ_2 : 492 nm wavelength filters of a dual wavelength microplate photometer (MTP-22, Corona Electric Co, Ibaragi, Japan).

Purification of MC-Derived VEGF

After cultured medium of IgE-stimulated MCs was applied to a column of HiTrap Heparin HP (Amersham Pharmacia Biotech), nonadsorbed proteins were eluted with 10 mM sodium phosphate buffer (pH 7.2). The adsorbed protein (MC-derived VEGF) was eluted with 10 mM sodium phosphate buffer containing 1.5 M NaCl (pH 7.2). The eluate was dialyzed against distilled water and lyophilized. When used, the lyophilized MC-derived VEGF was dissolved in SFM.

Data Analysis

The PC of IgE was calculated as described previously [3]. The degree of passage of IgE through the ECs was expressed as the upper chamber volume that was cleared of IgE through the EC monolayer according to the method of Cooper et al. [19]. The clearance volume of IgE at each different time point ($V_{\text{IgE } t}$) was calculated by summing up the incremental clearance volumes up to that time point: $V_{\text{IgE } t} = \Sigma(\text{VL} \cdot \Delta[\text{L}]_{\text{IgE}} / [\text{U}]_{\text{IgE}})$, where VL is the volume of the lower chamber at different time points, $\Delta[\text{L}]_{\text{IgE}}$ is the increase in the concentration of IgE in the lower chamber between time points, and $[\text{U}]_{\text{IgE}}$ is the concentration of IgE in the upper chamber at each time point. The change in V_{IgE} over time (dV_{IgE}/dt), which equals the clearance rate

in microliters per minute, was determined by weighted least-square nonlinear regression for the experimental period. Furthermore, V was $(VL \cdot VU)/(VL + VU)$. If $(PC \cdot S/V) \cdot t$ was < 0.1 , then with an error of $< 5\%$, $dV_{IgE}/dt = PC \cdot S$, where PC is the permeability constant, S is the surface area, and VU is the volume of the upper chamber. The total PC of the system with the endothelial monolayer (P_{total}) can be related to the permeability of the EC monolayer alone (P_{ec}) and the permeability of the system with the collagen-coated membrane devoid of EC (P_{filt}) by $1/P_{total} = 1/P_{ec} + 1/P_{filt}$.

The permeability of the EC to protein independent of the collagen-coated membrane and unstirred layers was thus calculated.

The inhibitory ratio induced by the different inhibitors was calculated from the following equation: inhibition (%) = $(A - B)/(A - C) \times 100$, where A is the PC obtained from the cultured medium of IgE- or A23187-stimulated MCs without inhibitors; B is the PC obtained from the cultured medium of IgE- or A23187-stimulated MCs with various concentrations of inhibitors, and C is the PC obtained from the EC monolayer which was exposed to SFM in both the upper chamber and lower chamber.

Statistical Analysis

Statistical analysis was carried out by the unpaired Student t test for between-group comparisons and two-way ANOVA followed by Dunnett's multiple comparison test using commercially available StatView v4.5 and Super ANOVA (Abacus Concepts, Berkeley, Calif., USA). Data are expressed as the means $\pm$ standard deviation (SD). When a p value of less than 0.05 was obtained, the means were considered to be significantly different.

Results

Relationship between Concentration of IgE in the Upper Chamber and PC

To determine an optimal concentration of IgE to estimate the PC of IgE, various concentrations (1, 3, 5, 10, 20 or 40 $\mu\text{g/ml}$) of IgE in 0.43 ml of SFM had been added to the upper chamber and incubated for 3 h. The PC of IgE reached a maximal level ($3.12 \pm 0.52 \times 10^{-6} \text{ cm/s}$) when 10 $\mu\text{g/ml}$ of IgE was added to the upper chamber. Therefore, 10 $\mu\text{g/ml}$ of IgE was selected for use in subsequent experiments (fig. 1).

Effect of MCs on the Passage of IgE through an EC Monolayer

To test whether or not MCs can affect the permeability of IgE through ECs, we compared the PC induced by MCs, RBL-1 and mastocytoma (P 815) cells. 1 ml of SFM containing MCs (1×10^4 or 1×10^5 cells) from the rat peritoneal cavity, RBL-1 (1×10^4 or 1×10^5 cells) or P 815 (1×10^4 or 1×10^5 cells) was added to the lower chamber. The PC of IgE was not significantly increased by the mastocytoma cells as compared with that under cell-free conditions (fig. 2). On the other hand, the PC induced by the MCs or RBL-1 increased significantly, as com-

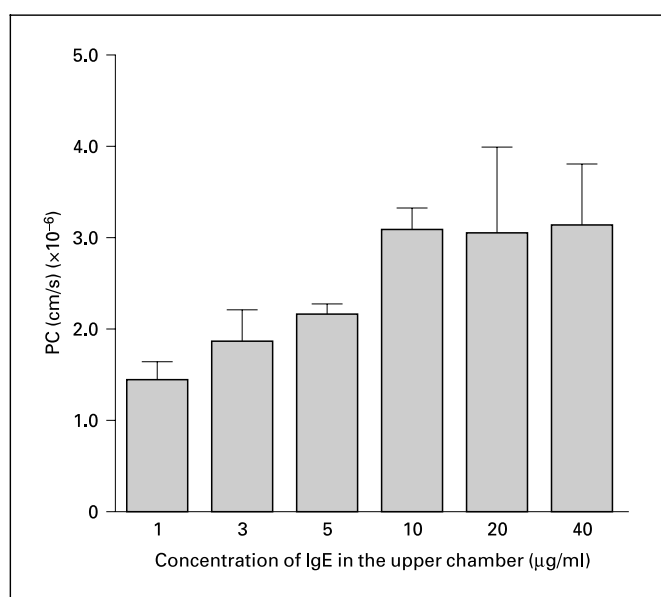


Fig. 1. Concentrations of IgE added to the upper chamber. The columns show the PC obtained with SFM containing different concentrations of IgE. After 0.43 ml of SFM containing the different concentrations of IgE (1, 3, 5, 10, 20 or 40 $\mu\text{g/ml}$) had been added to the upper chamber and incubated for 3 h in a CO_2 incubator, the PC was calculated as described in Materials and Methods. Data are expressed as means $\pm$ SD ($n = 3$).

pared to that induced by the mastocytoma cells at the same cell number. In addition, the PC induced by the MCs and RBL-1 increased with the increase in their cell count.

Time-Dependent Changes in PC following Exposure to Cultured Medium of Nonstimulated MCs

Since it was found that MCs enhanced the PC of IgE, there may be permeability substances that could enhance the PC in the cultured medium of MCs. To test the effect of cultured medium of MCs, 0.43 ml of SFM was added to the upper chamber. 1 ml of 24-hour cultured medium of nonstimulated MCs (1×10^5 cells/ml) was added to the lower chamber, and the EC monolayer was exposed to the cultured medium for different times. The PC of IgE was significantly increased at 12 and 24 h exposure, as compared to that after exposure to SFM containing no MCs, and its value ($5.25 \pm 0.17 \times 10^{-6} \text{ cm/s}$) reached a maximal level in the detectable range at 24 h exposure (fig. 3).

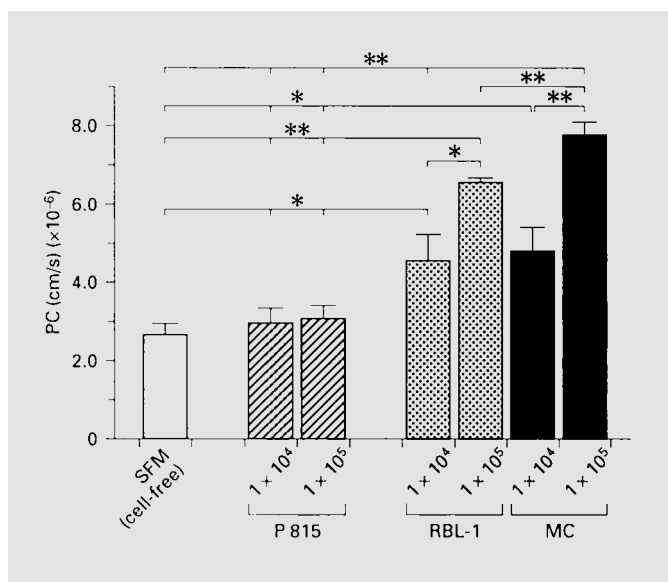


Fig. 2. Effect of MCs, RBL-1 and mastocytoma cells (P 815) on the passage of IgE through an EC monolayer. □ = PC with SFM (cell-free conditions); ▨ = PC with mastocytoma (P 815; 1×10^4 or 1×10^5 cells); ▩ = PC with RBL-1 (1×10^4 or 1×10^5 cells); ■ = PC with MCs (1×10^4 or 1×10^5 cells) from the rat peritoneal cavity. 1 ml of SFM containing MCs (1×10^4 or 1×10^5 cells), RBL-1 (1×10^4 or 1×10^5 cells) or P 815 (1×10^4 or 1×10^5 cells) was added to the lower chamber. Other details of the procedure were as described in Materials and Methods. Data are expressed as means $\pm$ SD (n = 3). * p < 0.05; ** p < 0.01.

Comparison of PCs Induced by Cultured Media of IgE- and A23187-Stimulated MCs

Since the PC induced by MCs (1.0×10^5 cells, fig. 2) was much larger than that by the cultured medium of MCs (at 3 h, fig. 3), the permeated IgE from upper chamber seemed to stimulate MCs to enhance the PC of IgE. Thus, experiments were carried out to evaluate the effect of cultured medium of IgE-stimulated MCs (CM-MC/IgE) or that of calcium ionophore A23187-stimulated MCs (CM-MC/A23187) on the PC of IgE. The PC of IgE was increased by CM-MC/IgE (3, 5 or 10 μ g/ml) in a dose-dependent manner (fig. 4). On the other hand, when the EC monolayer was exposed to CM-MC/A23187 (1 – 10×10^{-7} M), the PC of IgE constantly reached a maximal level and did not change in a dose-dependent manner. The PC value ($9.86 \pm 0.46 \times 10^{-6}$ cm/s) induced by the CM-MC/IgE (10 μ g/ml) was significantly increased as compared to that ($7.97 \pm 0.21 \times 10^{-6}$ cm/s) induced by the CM-MC/A23187 (1 – 10×10^{-7} M). However, the PC induced by the CM-MC/IgE (5 μ g/ml) was almost equal to that induced by the CM-MC/A23187 (1 – 10×10^{-7} M).

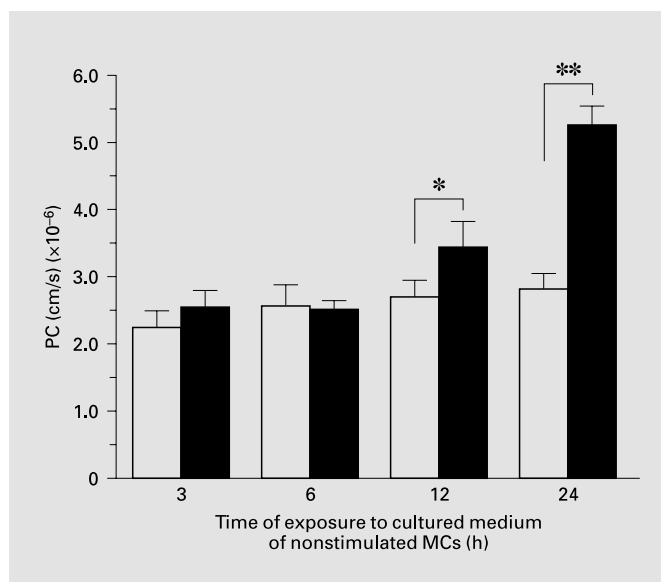


Fig. 3. Time-dependent changes in PC induced by cultured medium of nonstimulated MCs. □ = PC obtained with SFM at the given times; ■ = PC obtained with cultured medium of nonstimulated MCs at the given times. Other details of the procedures were as described in Materials and Methods. Data are expressed as means $\pm$ SD (n = 3). * p < 0.05; ** p < 0.01.

Effect of Various Inhibitors on the Increase of PC by Cultured Media of IgE- and Calcium Ionophore A23187-Stimulated MCs

In order to determine which MC-derived mediator elicited the greatest enhancement of the PC, experiments were carried out using various inhibitors to inhibit the increase of PC by CM-MC/IgE or CM-MC/A23187. Suramin (1×10^{-5} M), t-AMCHA (5×10^{-2} M), cimetidine (1×10^{-6} M) or diphenhydramine (1×10^{-6} M) were tested. The ECs were exposed for 12 h to inhibitors in SFM of upper chamber in the presence of CM-MC/IgE or CM-MC/A23187 in the lower chamber. The PC value for CM-MC/IgE (5 μ g/ml) was $8.04 \pm 0.28 \times 10^{-6}$ cm/s, while CM-MC/A23187 (1×10^{-6} M) was $7.98 \pm 0.47 \times 10^{-6}$ cm/s (fig. 4). The PC values induced by these two cultured media were thus almost equal. Based on the above results, CM-MC/IgE (5 μ g/ml) and CM-MC/A23187 (1×10^{-6} M) were used in the present experiments. The PC induced by the CM-MC/IgE was decreased by suramin (VEGF receptor blocker, 1×10^{-5} M), t-AMCHA (plasminogen activator inhibitor,

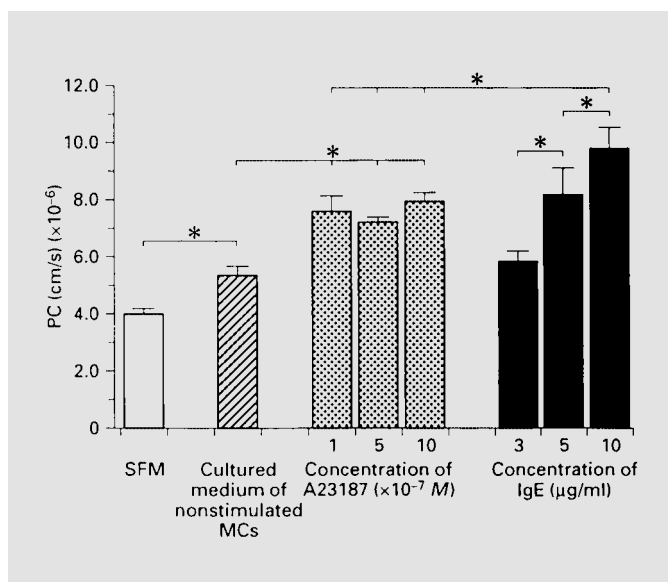


Fig. 4. Comparison of PC induced by cultured medium of IgE- and A23187-stimulated MCs. □ = PC with SFM; ▨ = PC with cultured medium of nonstimulated MCs; ▤ = PC with cultured medium of A23187-stimulated MCs; ■ = PC with cultured medium of IgE-stimulated MCs. The nonstimulated MCs (1×10^5 cells/1 ml) were cultured for 24 h, and the PC with the cultured medium was then calculated. After MCs had been stimulated by A23187 at different concentrations (1 – 10×10^{-7} M) and IgE at different concentrations (3, 5 or 10 μ g/ml), they were cultured for 24 h. The PC was then calculated for the cultured media. Other details of the procedures were as described in Materials and Methods. Data are expressed as means $\pm$ SD ($n = 3$). * $p < 0.05$.

5×10^{-2} M) diphenhydramine (H_1 blocker; 1×10^{-6} M) and cimetidine (H_2 blocker; 1×10^{-6} M). Among these inhibitors, suramin demonstrated intense inhibition ($92.3 \pm 1.89\%$) of the increase of PC induced by the CM-MC/IgE (fig. 5). In addition, the PC induced by the CM-MC/A23187 was weakly inhibited by the above inhibitors. Suramin significantly inhibited the increase of PC induced by the CM-MC/IgE, as compared to the other inhibitors. However, suramin did not significantly inhibit the PC induced by the CM-MC/A23187, as compared to the inhibition by cimetidine.

Western Blot Analysis of Cultured Media of Non-, A23187- and IgE-Stimulated MCs

In lanes A and B, no bands of protein were observed (fig. 6). On the other hand, five bands were noted at 12 kD, 17 kD, 28 kD, 67 kD and 98 kD in lane C. On Western blot analysis, no band was observed in lanes A and B, but one band was noted at 28 kD in lane C.

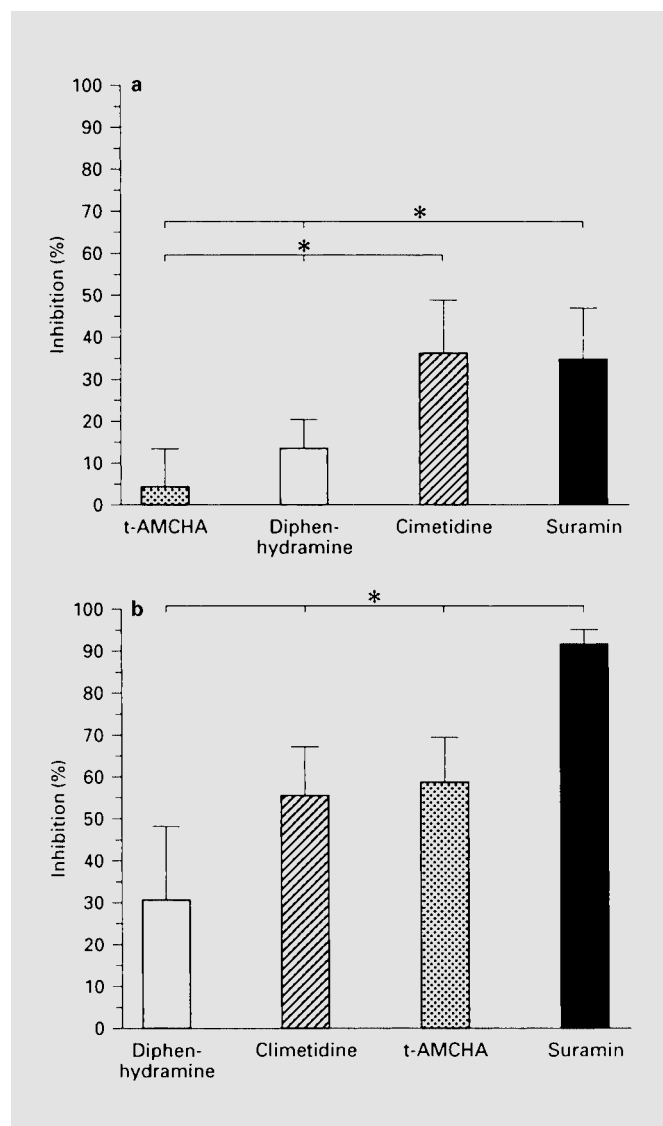


Fig. 5. Effect of various inhibitors on the increase of PC by cultured medium of IgE- (b) or A23187-stimulated (a) MCs. □ = Inhibition by diphenhydramine; ▨ = inhibition by cimetidine; ▤ = inhibition by t-AMCHA; ■ = inhibition by suramin. After MCs had been cultured with IgE (5 μ g/ml) or A23187 (1×10^{-6} M) for 24 h, the cultured media were collected. The IgE contained in the cultured media was removed using a BB-9-Sepharose 4B column. The EC monolayer was exposed to suramin (1×10^{-5} M), t-AMCHA (5×10^{-2} M), cimetidine (1×10^{-6} M) or diphenhydramine (1×10^{-6} M) in the upper chamber, and cultured medium of IgE- or A23187-stimulated MCs was put in the lower chamber. Data are expressed as means $\pm$ SD ($n = 4$). * $p < 0.05$.

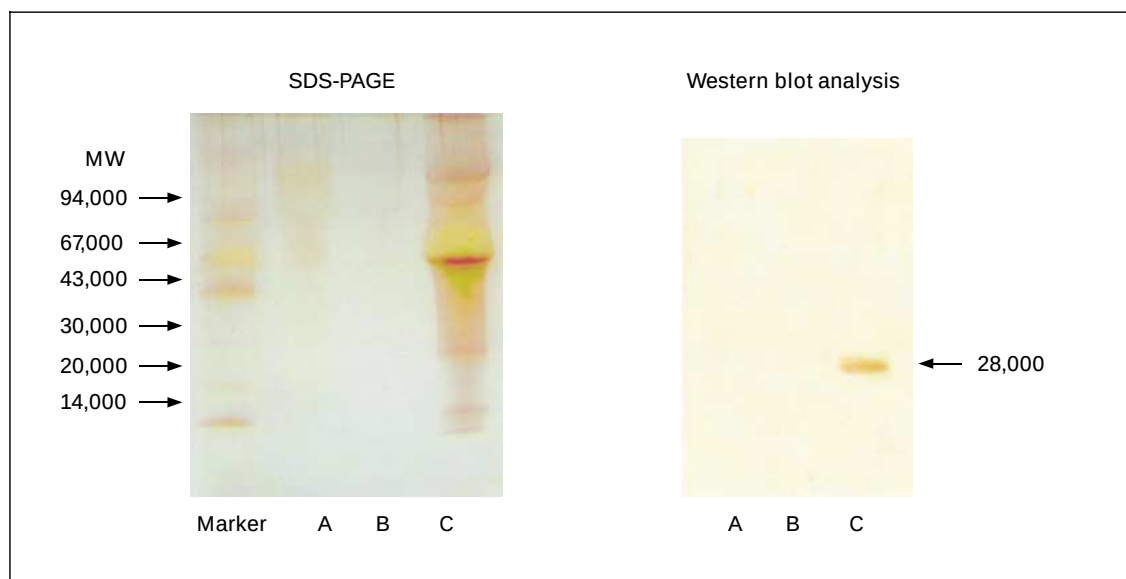


Fig. 6. SDS-PAGE with silver staining and Western blot analysis of cultured medium of non-, A23187 ($1 \times 10^{-6} M$)- and IgE ($10 \mu\text{g/ml}$)-stimulated MCs. Lane A = Cultured medium of nonstimulated MCs was concentrated 5-fold; lane B = cultured medium of A23187-stimulated MCs was concentrated 5-fold; lane C = cultured medium of IgE-stimulated MCs was concentrated 5-fold. Western blot analysis was carried out using goat anti-human VEGF₁₆₅ polyclonal antibody.

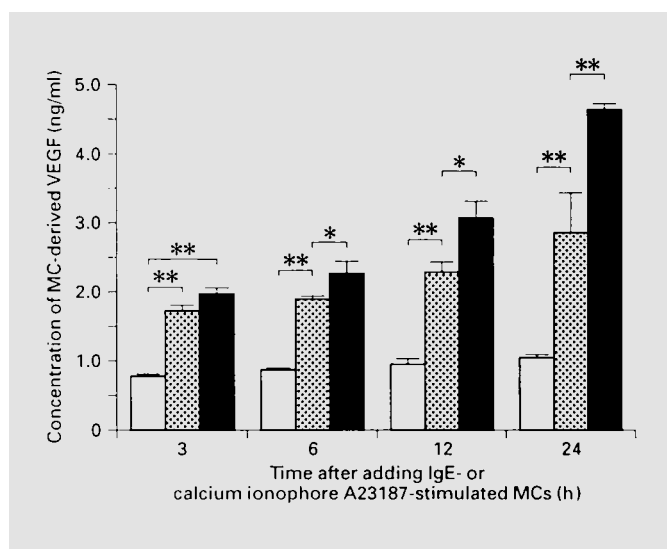


Fig. 7. Time-dependent changes in concentration of MC-derived VEGF induced by non-, IgE- and A23187-stimulated MCs. $\square$ = Non-stimulated MCs; $\square$ = A23187-stimulated MCs; $\blacksquare$ = IgE-stimulated MCs. Data are expressed as means $\pm$ SD ($n = 4$). * $p < 0.05$; ** $p < 0.01$.

Time-Dependent Changes in Concentration of MC-Derived VEGF Derived from Non-, IgE- and A23187-Stimulated MCs

Standard curve for estimation of VEGF on ELISA was made using human recombinant VEGF₁₆₅ (hrVEGF₁₆₅) as the reference protein. The absorbance on ELISA increased linearly with the increase in recombinant VEGF₁₆₅ concentration (data not shown). The lower detection limit for the estimation of VEGF was 2 ng/ml. After MCs (1×10^{-5} cells/ml) in the lower chamber had been exposed to 1 ml of SFM, SFM containing IgE ($10 \mu\text{g/ml}$) and A23187 ($1 \times 10^{-6} M$) for different times (3, 6, 12 and 24 h) in a CO₂ incubator, the cultured media of MCs were collected at each time from the lower chamber. The concentrations of MC-derived VEGF following exposure of MCs to 1 ml of SFM containing IgE and A23187 were then determined by ELISA and PC of IgE was also calculated in the case of IgE stimulation. The concentration of VEGF derived from both IgE- and A23187-stimulated MCs increased from 3 until 24 h (fig. 7). The concentration of VEGF derived from the IgE-stimulated MCs was significantly increased at 6, 12 and 24 h in a time-dependent fashion, but that derived from the A23187-stimulated MCs did not increase significantly in a time-dependent fashion. The VEGF derived from the IgE-stimulated MCs was sig-

Table 1. Time-dependent changes in PC and concentration of VEGF following suspension of MCs in the lower chamber

Time after MCs were suspended in lower chamber, h	Concentration of MC-derived VEGF in lower chamber, ng/ml	PC (cm/s) $\times 10^{-6}$	
		without MCs in lower chamber	with MCs in lower chamber
3	1.93 $\pm$ 0.02	0.22 $\pm$ 0.02	5.24 $\pm$ 0.55 ⁺
6	2.28 $\pm$ 0.26	0.25 $\pm$ 0.03	4.69 $\pm$ 1.28 ⁺
12	3.07 $\pm$ 0.32*	0.27 $\pm$ 0.02	11.33 $\pm$ 1.14 ^{+,o}
24	4.66 $\pm$ 0.15**	0.32 $\pm$ 0.17	9.91 $\pm$ 0.79 ^{+,o}

The time-dependent changes in the PC of IgE and concentration of MC-derived VEGF in the lower chamber were measured concomitantly at different times (3, 6, 12 or 24 h) after incubation of MCs. Following sampling from the upper chamber and lower chamber, the PC was calculated and the concentration of MC-derived VEGF in the lower chambers was also determined. * $p < 0.05$, ** $p < 0.01$ vs. MC-derived VEGF (3 and 6 h); ⁺ $p < 0.05$ vs. PC without MCs in the lower chamber; ^o $p < 0.01$ vs. PC with MCs in the lower chamber (3 and 6 h). Data are expressed as the means $\pm$ SD (n = 3).

nificantly higher than that derived from the A23187-stimulated MCs at 6, 12 and 24 h.

The PC of IgE and concentration of MC-derived VEGF in the lower chamber at 12 and 24 h after MCs had been introduced into this chamber were significantly increased, as compared to those at 3 and 6 h after the MCs were placed in the chamber. The PC of IgE increased with the increase in the concentration of MC-derived VEGF in the lower chamber (table 1). The PC of IgE induced by the suspension of MCs increased significantly until 24 h, in comparison to that induced without the suspension of cells. On the other hand, the concentration of VEGF in the lower chamber with MCs increased in a time-dependent fashion.

Effect of Purified Rat VEGF on PC of IgE

To test the direct effect of exogenous VEGF (purified rat VEGF and hrVEGF₁₆₅) on the PC of IgE, the ECs were exposed to the exogenous VEGF at different concentrations (1, 5 and 10 ng/ml) for 12 h. The PC of IgE was significantly increased by both purified rat VEGF and hrVEGF₁₆₅ in a dose-dependent fashion (fig. 8a). The ECs were then exposed to 1 ng/ml of VEGF for different time periods (3, 6, 12 and 24 h) to test the time-dependent effect of VEGF on the PC of IgE. As shown in figure 8b, the exogenous VEGF, especially purified rat VEGF, increased the PC of IgE in a time-dependent manner.

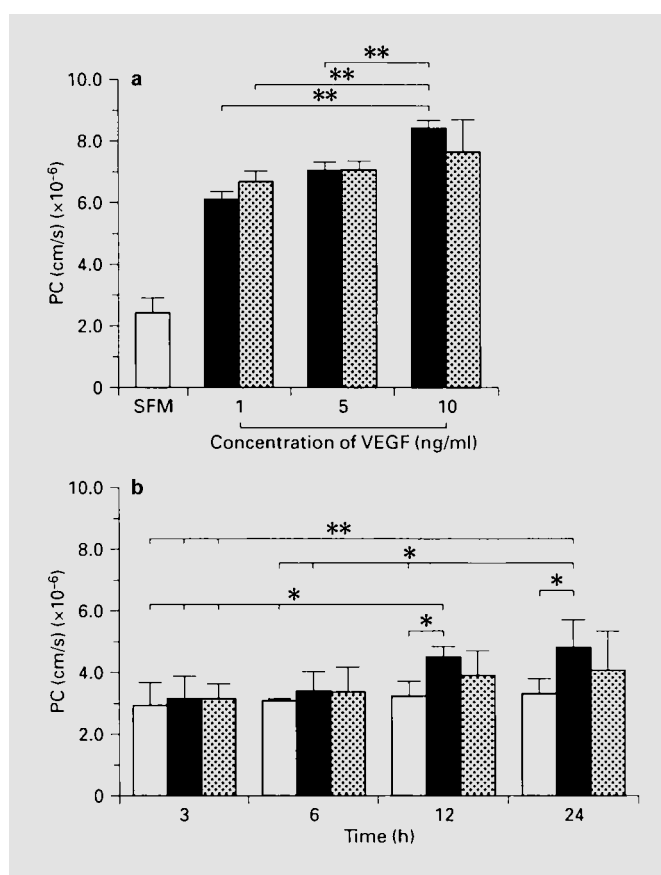


Fig. 8. Direct effect of exogenous rat VEGF and hrVEGF on the PC of IgE. a Dose-response relationship of rat VEGF (■) and hrVEGF₁₆₅ (▨). ECs were exposed to various concentrations of VEGF for 12 h. b Time-dependent changes in the PC of IgE after exposure of ECs to VEGF (1 ng/ml). □ = SFM; ■ = purified MC-derived VEGF (1 ng/ml); ▨ = rhVEGF₁₆₅ (1 ng/ml). Data are expressed as means $\pm$ SD (n = 4). * $p < 0.05$; ** $p < 0.01$.

Discussion

In the present study, we demonstrated that the IgE permeation through ECs involves the secretion of VEGF from MCs and that the secretion of VEGF was enhanced by IgE stimulation of MCs, which was likely mediated by the interaction with high affinity IgE receptor (FcεRI). This was supported by the data that FcεRI-bearing cells (MCs and RBL-1 cells) increased the PC, whereas P 815, which lacks FcεRI, did not (fig. 2). It was also demonstrated that the PC of IgE induced by MCs was significantly larger than that induced by RBL-1. Boesiger et al. [5] demonstrated that the upregulation of FcεRI by preincubation of cultured MCs with IgE stimulated the secre-

tion of VEGF upon stimulation by IgE and antigen. However, Sterk and Ishizaka [20] reported that the numbers of FcεRI on rat MCs are as many as those of RBL-1. Therefore, the difference of PC induced by these cells was probably based not on the number of FcεRI on the cells but on a larger production and secretion of VEGF from the MCs than from the RBL-1.

The CM-MC/IgE significantly increased the PC of IgE, as compared to the case where CM-MC/A23187 was used (fig. 3). Thus, it was inferred that the kind and quantity of permeability substances from the IgE-stimulated MCs differed from those from the A23187-stimulated MCs. Since suramin most potently inhibited the PC induced by either CM-MC/IgE or CM-MC/A23187 as compared to other inhibitors, suggesting that both IgE and A23187 promoted IgE permeation by stimulating VEGF secretion from MCs. Therefore, the difference between A23187 and IgE stimulation of PC might be caused in part by the difference in amount of VEGF secretion by these stimulations (fig. 4). This was supported by the data that the concentration of VEGF derived from the IgE-stimulated MCs was greater than that of the VEGF derived from the A23187-stimulated MCs (fig. 7). We also demonstrated that the PC of IgE was significantly increased with the increase of VEGF secretion from the MCs, and VEGF secretion from the MCs on the basis of the interaction of IgE and FcεRI was enhanced by the increase of IgE passed from the upper chamber to the lower chamber (table 1).

Boesiger et al. [5] demonstrated that VEGF secretion, in bone marrow-derived cultured mast cells, was stimulated by IgE and antigen, phorbol myristate acetate (PMA) and A23187, suggesting that PKC and $[Ca^{2+}]_i$ are involved in VEGF secretion. They also discussed that since PMA induced an even more substantial secretion of VEGF than did A23187 and such PMA-induced VEGF production was associated with minimal release of histamines, there may be endogenous activators of MCs PKC that can induce the release of VEGF preferentially over histamines [5]. Furthermore, Ozawa et al. [21] demonstrated that antigen-induced secretion of hexaminidase is dependent on the Ca^{2+} -independent PKC (δ and ϵ). Although Ca^{2+} -dependent PKC (α and β) also exists in RBL cells, they were not translocated to membrane and were degraded within 6 h by PMA stimulation, indicating that novel PKC rather than conventional PKC is involved in secretion in MCs [21]. Taken together, Ca^{2+} -independent PKC is a possible answer for the question about the difference between A23187 and IgE stimulation of PC.

The increased PC of IgE induced by the cultured medium of the IgE- or A23187-stimulated MCs were

inhibited not only by suramin but also by t-AMCHA, cimetidine and diphenhydramine. This suggests that the strongest effective substance for the PC was MC-derived VEGF, while plasminogen activator and histamine were also involved. Although Sillaber et al. [22, 23] reported that human tissue MCs constitutively produce, express and release tissue-type plasminogen activator (t-PA) and human MCs express the receptor for urokinase (u-PAR), it remains unclear whether or not rat MCs produce, express and release t-PA or u-PA. We previously demonstrated that histamine dose-dependently (from 10^{-13} to 10^{-9} M) increased the PC of IgE, which was completely abolished by cimetidine (10^{-10} M) [3]. Therefore, the concentration of cimetidine (10^{-6} M) we used was high enough to estimate the contribution to the increase in PC by IgE-stimulated MCs. As shown in figure 5, cimetidine partially inhibited the increase of PC by IgE-stimulated MCs, while suramin almost completely inhibited it, indicating that VEGF is a major factor contributing to enhancing the PC of IgE. However, it is possible that there may be a signal link between histamine- and VEGF-induced stimulation of IgE permeation through ECs or the inhibitors tested may have more than one site to act on, because the total inhibition of suramin and cimetidine is more than 100%.

VEGF is angiogenic for ECs and is a potent stimulant for increased vascular permeability [24–27]. Senger et al. [4] reported that VEGF increased the microvascular permeability in vivo with a potency 50,000 times greater than that of histamine on an equimolar basis. VEGF has been shown to be released by monocytes and, more recently, by eosinophils and neutrophils. These observations suggest that VEGF may participate in the acute and chronic phases of inflammation-associated permeability mediated by these cell types. However, it remains unknown whether or not the active transport of IgE on ECs is influenced by the cultured medium of IgE-stimulated MCs.

Transfer of IgE from the circulating blood to extravascular tissue via ECs may be dependent on the concentration of VEGF secreted from MCs in the extravascular tissue, the concentration of transferred IgE to the extravascular tissue and the number of high-affinity IgE receptors of MCs. Galli et al. [5, 28, 29] found that mouse or human MCs could produce and secrete VEGF, and could secrete VEGF upon activation via FcεRI, while the IgE-dependent secretion of VEGF could be significantly increased in MCs that had undergone IgE-dependent upregulation of their surface expression of FcεRI. Furthermore, the transport of macromolecules across these cells is known to

be dependent on both the paracellular and intracellular pathways [30], and the transport of IgE may be dependent on such a mechanism. It seems reasonable to infer that, after IgE has been transferred from the circulating blood to extravascular tissue, it binds to the FcεRI of MCs and upregulates the FcεRI.

In conclusion, we consider that the MC was activated by the interaction of IgE and FcεRI; the release of VEGF from the activated MC was increased, and, thereafter, this VEGF enhanced the permeability of IgE. Thus, IgE-mediated inflammation could be exacerbated by such an increased permeability of IgE.

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