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Cell Adhesion Molecule Mediates Endothelial Cell Injury Caused by Activated Neutrophils

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Abstract. Addition of PMA (phorbol myristate acetate)-stimulated neutrophils to an endothelial cell monolayer caused a significant increase in the intracellular peroxide level of the endothelial cells after 15 minutes and endothelial cell injury after 5 hours. Both the early and the late events were abolished in the presence of specific antibodies against CD (cluster of differentiation) 11a, CD11b, CD18 and ICAM (intercellular adhesion molecule) 1, but not CD11c. These antibodies affected neither the production of active oxygen species by the neutrophils nor the rate of adhesion of neutrophils to endothelial cells. Pretreatment of endothelial cells with allopurinol caused significant inhibition of both the early and the late events, suggesting that the binding of adhesion molecules may trigger the activation of XO (xanthine oxidase) of endothelial cells, and have the cells produce more hydrogen peroxide and ferrous ions, followed by producing more hydrogen peroxide. The hydrogen peroxide produced by endothelial cells themselves and by neutrophils may be converted to hydroxyl radicals by ferrous ions, which may cause lethal cell damage. Examination of XO activity in endothelial cells showed that the enzyme activity increased double within 15 minutes after the addition of PMA activated neutrophils. Monoclonal antibodies against CD11a and CD18 significantly inhibited the increased conversion of XD (xanthine dehydrogenase) to XO induced by PMA-activated neutrophils. Moreover, tyrosine kinase inhibitors also inhibited the increased conversion of XD to XO. These results indicate that the adhesion of activated neutrophils to endothelial cells via CD11a/CD18-ICAM-1 is involved in the conversion of XD to XO in endothelial cells, which results in endothelial cell injury. (Keio J Med 45 (3): 207-212, September 1996)

Key words: endothelial cell injury, xanthine oxidase, neutrophil, cell adhesion molecule, active oxygen

Introduction

It is well known that leukocytes play some important roles in many pathophysiological conditions, such as inflammation, atherosclerosis, myocardial infarction, and so on.1 Because of their anatomical location, vascular endothelial cells are the only kind of cells exposed directly to various forms of oxidative stress due to the blood stream other than blood cells. Endothelial cell injury elicited by activated leukocytes is known to be the initial stage of various vascular disorders and is mainly due to active oxygen species released from activated leukocytes such as the superoxide anion, hydrogen peroxide, and hydroxyl radicals.2,3 Recently, the phenomenon of adhesion of monocytes to the endothelium has been recognized as an important process for monocytes to cause endothelial cell injury.4 The adhesion of leukocytes to the endothelium is mediated by various kinds of adhesion molecules.5-13 It has been reported that endothelial cell injury induced by activated monocytes was inhibited by blocking the adhesion of the leukocytes to the endothelium using monoclonal antibodies against CD11a, CD11b, and CD11c as well as CD18.4 Data showing that the binding of adhesion molecules was deeply involved in endothelial cell injury caused by PMA-stimulated neutrophils have also been reported.14 By using similar methods, Shappell et al showed that CD11b, but not CD11a, was involved in hydrogen per-
oxide production at the time when leukocytes adhere to endothelial cells. These papers suggest that the function of adhesion molecules such as CD11/CD18 may be to shorten the distance between leukocytes and endothelial cells, which may facilitate the attack of short-lived active oxygen species to endothelial cells and may have some priming effect on the production of active oxygen species by leukocytes. However, in this paper, we show the increased intensity of intracellular peroxide level in endothelial cells exposed to PMA-stimulated leukocytes. The increase was due to hydrogen peroxide derived from both leukocytes (approximately 40%) and endothelial cells (approximately 60%), and the latter was produced by endothelial cells themselves by xanthine oxidase present in the cells. Examination of the effects of monoclonal antibodies against CD11a, CD11b, CD11c, CD18, and ICAM-1 on the increase in intracellular fluorescence level and cytolyis in endothelial cells exposed to PMA-stimulated leukocytes showed that the binding of all these adhesion molecules except CD11c can trigger the activation of xanthine oxidase in endothelial cells to produce hydroxyl radicals enough to injure themselves.

Materials and Methods

Anti-CD11a (clone 25.3.1), anti-CD11b (clone BEAR 1), anti-CD11c (clone BU15), and anti-CD18 (clone BL5) antibodies were purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Anti-ICAM-1 antibody (clone 84H10) was obtained from Immunotech S.A. (Marseille, France). Catalase, superoxide dismutase, deferoxamine mesylate, and allopurinol were from Sigma Chemical Co. (St. Louis, Mo). 2'-7'-Dichlorofluorescein diacetate (DCFDA), mouse Ig G, and hydrogen peroxide were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan).

Endothelial cells were isolated from freshly excised bovine carotid arteries. Human leukocytes (PMN) were isolated from normal healthy donors. All the procedures such as measurement of intracellular peroxides, cytotoxicity assay, assaying of cell adhesion, measurement of chemiluminescence, hydrogen peroxide assay have been described in our previous papers. Expression of adhesion molecule has been described in a previous paper.

Xanthine oxidase and xanthine dehydrogenase activities were assayed as described. Endothelial cell monolayers were washed twice with a HBSS. The buffer alone or together with neutrophils (4.7·10^6 cells/well) was then added to the monolayers. The wells were kept at 37°C in a CO₂ incubator for 15 min, and then treated with PMA (final concentration, 10 ng/ml) for 30 min, except for in the time-course studies. When indicated, monoclonal antibodies, the control anti-body or elastase inhibitors were added before the addition of neutrophils.

After incubation, the medium was removed, and the wells were washed twice with a KPO₄-buffered saline (10 mM, pH 7.4). After being mechanically harvested with a rubber policeman in 1 ml of ice-cold KPO₄ (50 mM, pH 7.8) containing EDTA (1 mM), DTE (1 mM), and PMSF (1 M), the cells were immediately homogenized with a homogenizer at 100 watts for 1 min on ice. The homogenate was centrifuged at 25000 × g for 15 min at 4°C. The supernatant (400 µl) of each sample was incubated at 37°C for 3 h with xanthine (100 µM) to measure xanthine oxidase, or with xanthine plus NAD⁺ (300 µM), LDH (70 U), and pyruvate (1.75 mM) to measure xanthine oxidase plus xanthine dehydrogenase. The reactions were stopped by the addition of perchloric acid and the precipitate was removed by centrifugation. The samples stored frozen in tightly capped vials at −80°C for storage until analysis, usually within 48 h, by HPLC. This period of storage did not cause any artifactual alteration in the amount of detectable uric acid product. Uric acid was quantified by high-pressure liquid chromatography on a Shimadzu C-18 reverse phase column (Shimadzu, Osaka, Japan) with a mobile phase of 20 mM KH₂PO₄, pH 5.7, plus methanol (3%) at 1 ml/min. The peak of uric acid was detected at 290 nm.

Results and Discussion

Some papers suggested that endothelial cell injury induced by PMA-stimulated leukocytes was due to both hydrogen peroxide and hydroxyl radical releases from the leukocytes. In our assay system, however, the endothelial cell injury induced by PMA-stimulated leukocytes was only partly suppressed (% inhibition = 42.2 ± 3.2) by catalase (700 U/ml), a hydrogen peroxide decomposing enzyme, while it was almost completely inhibited (% inhibition = 95.6 ± 1.2) by deferoxamine mesylate (1 mM), a chelating agent of Fe²⁺, in other words, an inhibitor of hydroxyl radical production. The addition of superoxide dismutase, a superoxide-anion decomposing enzyme, to the assay system had little effect on endothelial cell injury due to PMA-stimulated leukocytes (% inhibition = 0.7 ± 0.2). To elucidate the contribution of hydrogen peroxide released from leukocytes in the assay system of the endothelial cell injury, we first measured the amount of hydrogen peroxide produced by PMA-stimulated leukocytes alone. The concentration of hydrogen peroxide produced by 10⁶ leukocytes activated by PMA (10 ng/ml) — the number of the leukocytes was equivalent to that used in our experiments — was 12.7 ± 1.5 µM, which is almost the same concentration as previously reported. However, when even as much as 50 µM hydrogen peroxide was added to the culture medium, it did not induce endothelial cell injury at all (% specific ²⁵⁴₁Cr release = 1.9 ± 0.2), although 100 µM could cause severe endothelial cell injury (%
DCFDA, which is a fluorescent marker of the production experiments. When endothelial cells prelabeled with phenomena more precisely, we performed the following CD11/CD18 (except CD11c) and ICAM-1 binding. cell injury due to the PMA-stimulated leukocytes. The produced by the leukocytes.

We have found that the endothelial cell injury induced by PMA-stimulated leukocytes was completely inhibited on separation, by 1 mm, of the leukocytes and the endothelial cell monolayer by using a special culture dish named Intercell (% specific $^{51}$Cr release = Intercell(-): 20.3 ± 2.9, Intercell (+): 0.8 ± 0.1). This suggests that the adhesion of leukocytes to endothelial cells may be very important for the leukocytes to induce cytotoxicity. Therefore, we examined this phenomenon from the view point of cell adhesion molecules. First, we examined what kinds of adhesion molecules were expressed on the surface of leukocytes after the PMA treatment. Five hours after the PMA treatment, significant increases in the expression of CD11a, CD11b, CD11c, and CD18 on leukocytes were observed. Then we examined what kinds of adhesion molecules between leukocytes and endothelial cells were involved in the endothelial cell injury due to PMA-stimulated leukocytes. For this purpose, we examined the effect of cleaving the bindings in which these cell adhesion molecules were involved on the endothelial cell injury. Monoclonal antibodies against each subunit of CD11/CD18 had an inhibitory effect on the endothelial cell injury due to the PMA-stimulated leukocytes, although the effect of the anti-CD11c antibody was negligible. Mouse IgG (10 μg/ml) as a control antibody had no inhibitory effect on the injury elicited by PMA-stimulated leukocytes (% of control = 91.0 ± 3.0). At this time, none of the monoclonal antibodies (10 μg/ml) suppressed the production of the luminol-chemiluminescence due to the PMA-stimulated leukocytes significantly. Moreover, none of the antibodies (10 μg/ml) had any significant suppressive effect on adherence of the PMA-stimulated leukocytes to the endothelium (% adherence: control, 100.0 ± 0.9; anti-CD11a, 97.3 ± 0.8; anti-CD11b, 86.5 ± 5.6; anti-CD11c, 101.3 ± 0.8; anti-CD18, 98.5 ± 1.0, anti-ICAM-1, 10.14 ± 2.8; n = 4, not significant). The anti-ICAM-1 antibody also caused significant inhibition of endothelial cell injury due to the PMA-stimulated leukocytes. The inhibitory effects of these antibodies on endothelial cell injury were dose dependent. These results indicate that the endothelial cell injury induced by PMA-stimulated leukocytes is closely related to the adhesion through CD11/CD18 (except CD11c) and ICAM-1 binding.

To elucidate the mechanism of the above-mentioned phenomena more precisely, we performed the following experiments. When endothelial cells prelabeled with DCFDA, which is a fluorescent marker of the production of peroxides, were exposed to PMA-stimulated leukocytes, the fluorescence intensity in the endothelial cells increased significantly after 15 minutes. Nontreated endothelial cells exhibited a low fluorescence intensity (purple color), but the endothelial cells exposed to PMA-stimulated leukocytes exhibited higher intensity (blue-green-red-white color, depending on the conditions). Similar increased fluorescence was also noted in DCFDA-prelabeled human umbilical vein endothelial cells exposed to PMA-stimulated leukocytes as well as endothelial cells isolated from bovine carotid artery, which were used in the series of experiments in the present work (data not shown). It is interesting that the cells exhibited little variety in the response. The intracellular fluorescence significantly increased within 5 minutes after the addition of leukocytes and PMA. The level of the intracellular fluorescence increased at least up to 10 minutes. The addition of PMA alone or unstimulated leukocytes alone to endothelial cell monolayers did not cause any increase in the intracellular fluorescence level at all.

We examined whether or not the intracellular fluorescence level, i.e., intracellular peroxide level, was related to endothelial cell injury. Certain adhesion molecules were found to be involved in the endothelial cell injury induced by PMA-stimulated leukocytes. Therefore, the possibility that the antibodies against these adhesion molecules might affect the intracellular peroxide level in endothelial cells was examined. The simultaneous addition of each antibody against CD11a, CD11b, CD11c, CD18, and ICAM-1 to the assay system abolished significantly the increase in fluorescence in endothelial cells after exposure to PMA-stimulated leukocytes, with the exception of the anti-CD11c antibody. Mouse IgG (10 μg/ml) as a control antibody had no inhibitory effect on the peroxide production elicited by PMA-stimulated leukocytes (% inhibition = 7.5 ± 4.8). This inhibitory pattern was completely the same as those in the case of endothelial cell injury. The time course of the inhibitory effect of the antibodies against CD11a, CD11c, and ICAM-1 on the intracellular increase in fluorescence intensity was examined. Almost similar results to anti-CD11a antibody were obtained in the case of anti-CD11b and anti-CD18 antibodies. The antibodies against CD11a, CD11b, CD18, and ICAM-1 inhibited the time-dependent increase in fluorescence significantly. The anti-CD11c antibody was essentially inactive. These data strongly suggest that the early increase in intracellular peroxide level is closely related to the late endothelial cell injury induced by PMA-stimulated leukocytes.

Next, we investigated the origin of the peroxides, i.e., hydrogen peroxide, the direct precursor of the hydroxyl radical in endothelial cells. To examine how large the influx of hydrogen peroxide from activated leukocytes is, we examined the effect of catalase on the increase in intracellular fluorescence intensity. Since catalase is a
macromolecule enzyme and it cannot penetrate cell membrane, by using catalase we can tell the contribution of hydrogen peroxide from leukocytes. Catalase caused significant, but not complete, inhibition of the intracellular peroxide level. Next, to examine the possibility that endothelial cells generate peroxides by themselves via the xanthine-xanthine oxidase system, allopurinol, an inhibitor of xanthine oxidase, was added to the assay system. Pretreatment of endothelial cells with allopurinol caused significant inhibition of the increase in the fluorescence in the cells exposed to PMA-stimulated leukocytes. Allopurinol was found to be effective enough under the pretreatment conditions, in which allopurinol was washed away and was not present at the time of the assay. No difference was observed between the absence and presence of allopurinol at the time of the assay. Next, the effect of allopurinol pretreatment on endothelial cell injury induced by PMA-stimulated leukocytes was examined. As expected, the allopurinol pretreatment inhibited the endothelial cell injury significantly (% inhibition = 58.5 ± 4.5), but not completely.

Next, we examined the time course of the inhibitory effect of allopurinol on the increase in the intracellular peroxide level after conditioning of PMA-stimulated leukocytes. In allopurinol-pretreated endothelial cells, the production of hydrogen peroxide was almost completely inhibited at least up to 5 minutes; however, after 10 minutes, the intracellular peroxide level increased gradually, suggesting that some hydrogen peroxide produced by leukocytes must penetrate into endothelial cells. Therefore, we repeated the allopurinol experiment in the presence of catalase. The concomitant addition of catalase to the assay system for the allopurinol-pretreated endothelial cells caused complete inhibition of cytolysis in endothelial cells treated with PMA-activated leukocytes.

To confirm further our hypothesis (Fig 1) that the binding of CD11/CD18 and ICAM-1 could activate xanthine oxidase, thereby causing an increase in intracellular fluorescence intensity, we performed another experiment and found that even dead leukocytes are able to increase fluorescence intensity in endothelial cells. For this experiment, leukocytes were activated with PMA for 5 hours so that their adhesion molecules were expressed on the surface of the cell membranes. Then the leukocytes were fixed with ethanol to kill them. The fixed leukocytes were not able to release active oxygen any more, but they exhibited some ability to adhere to endothelial cells through their adhesion molecules. The endothelial cells to which the dead leukocytes adhered showed a significant increase in fluorescence intensity, suggesting that xanthine oxidase was activated by the binding of particular adhesion molecules.

Finally, we examined the change in xanthine oxidase activity of endothelial cells.20 A significant increase in xanthine oxidase activity was observed within 15 minutes after the addition of PMA-stimulated leukocytes. The XO activation in endothelial cells due to the PMA-activated leukocytes was significantly blocked by specific antibodies against CD11a & CD18, suggesting that the LFA-1 and ICAM-1 binding is deeply involved in this process. The XO activation in endothelial cells was also brought about by thrombin or LTB4, activated leukocytes as well as PMA-activated leukocytes. These are the

Fig 1 A possible mechanism by which activated neutrophils injure endothelial cells.

Fig 2 Mechanism of XD-XO conversion induced by stimulated leukocytes.
substances actually produced in vivo situation. The XO activation in endothelial cells due to the PMA-activated leukocytes was significantly inhibited by staurosporine and herbimycin, specific inhibitors of tyrosine kinase, suggesting tyrosine kinase is involved in the XO activation. The XO activation in endothelial cells due to the PMA-activated leukocytes was not affected by various kind of protease inhibitors at all, suggesting that proteases produced by the activated leukocytes have nothing to do with the XO activation.

As mentioned so far, activated leukocytes adhere to endothelial cells with particular adhesion molecules causes activation of XO. Tyrosine kinase is involved in this process (Fig 2).

**References**


**Discussion (Sei-itsu Murota)**

**Deli:** Did you measure the xanthine oxidase and dehydrogenase enzyme activities? What was the ratio of XO and XD under control conditions?

**Murota:** Yes, we did. As you know, the total activity of XO plus XD is constant. The conversion ratio of XO to the total (XO plus XD) in the normal endothelial cells used in our experiments under control conditions was usually 12–13%.

**Hallenbeck:** 1) Can cross-linking with antibodies to CD11a, CD11b, CD18 or ICAM-1 reproduce the results? This would eliminate effects mediated by activated leukocyte products. 2) Have you considered cross-linking the adhesion molecules with antibodies to eliminate the need for leukocytes? This could help to address the Steven Weiss hypothesis that free radicals create an environment in which granule-based enzymes (elastase, gelatenase, collagenase) can attack the endothelium.

**Murota:** 1) That is a good question. I think theoretically you are right; I mean, cross-linking with antibody to ICAM must reproduce the same results. We have examined several kinds of antibodies against ICAM-1, which we managed to obtain, but have failed so far to get good results. As one reason for this, there is the possibility that we still did not have a proper antibody to recognize specific epitopes of ICAM. Another possibility is related to the fact that the endothelial cells we used were isolated
from bovine artery, and it is very hard to get specific antibodies against bovine ICAM-1. I know we must develop some specific antibodies against bovine origin adhesion molecules in the future. Anyway, your question is very good and relevant. 2) The slide showing the data on dead leukocytes which I presented in my talk, may answer your question. In that experiment, leukocytes were activated with PMA for 5h to have them express their adhesion molecules on the surface of the plasma membrane, and then the leukocytes were fixed with ethanol to kill them. Subsequently, the fixed leukocytes were washed twice with a PBS. Therefore, even though granule-based enzymes were released from the leukocytes during the PMA treatment, they must have been washed out. By using these dead leukocytes, we could eliminate the effects of these proteolytic enzymes completely. Our data showed that the dead leukocytes had some activity to adhere to endothelial cells with their expressed adhesion molecules, and the endothelial cells to which the dead leukocytes adhered demonstrated activation of XO. So I can say that the released elastase, collagenase and other such enzymes from the activated leukocytes had nothing to do with the endothelial cell injury in our assay system.

**Kogure:** This is just a brief comment. In the case of an in vivo system, the leukocyte after expression of adhesion molecules both on the surface of the leukocyte and the endothelium penetrates the endothelium and immigrates into the parenchyma. Under such circumstances, the source of active oxygen species can be multiple, and through some reason, the endothelium itself seems to be rather durable against oxygen toxicity.

**Murota:** Thank you for your nice comment, Prof. Kogure. When leukocytes invade into the extravascular tissue, they use LFA-1-ICAM-1 binding to pass through the vascular wall. At this time, even though the adhesion molecule binding is formed, the endothelial cells do not get injured as you just said. This is because in the usual case, endothelial cells have a large capacity to resist oxidative stress, and this capacity may be strengthened by various chemical mediators of inflammation. When endothelial cells are exposed to various inflammatory cytokines and other types of inflammatory mediators, intracellular catalase, SOD, glutathione peroxidase, etc. may be induced. Therefore, in the case of inflammation, endothelial cells are more resistant to leukocytes attack than usual case. However, in the case of ischemia-reperfusion, especially after repeated ischemia-reperfusion, endothelial cells become very weak and they lose their defense system against oxidative stress. On the other hand, their XO is rather activated and they are easily injured.

**Nagy:** In the mechanism characterized by leukocyte-induced endothelial cell damage, the possible role of leukocyte elastase-induced cell injury has not been discussed. What is your position on this question?

**Murota:** As I showed in my talk, that various kinds of protease inhibitors including specific inhibitor of neutrophil elastase did not affect the activated-leukocyte-caused XO activation. I think these data tell us that leukocyte protease has nothing to do with the leukocyte-induced endothelial cell damage, at least in our assay system. Thus, the dead leukocyte data can also exclude the role of elastase in the endothelial cell injury.

**Raivich:** This is a very interesting presentation, linking endothelial xanthine oxidase to endothelial cell damage. My question concerns the specificity of allopurinol. Have you looked at whether just xanthine oxidase is involved, or could some of the damage be mediated by a similar enzyme blocked by allopurinol? Did you address this question using an anti-sense approach against xanthine oxidase or by using xanthine oxidase knockouts?

**Murota:** No, we have not done such experiments using anti-sense against XO or using XO knockouts yet. Regarding allopurinol, I don’t know whether allopurinol blocks any other enzymes than XO. In our experiments, allopurinol was used only under pretreatment conditions, i.e., the allopurinol used was washed out soon after the finish of the treatment, so that allopurinol was absent from the medium during the assay to avoid active oxygens being produced from the allopurinol itself.

**Shin-ichi Yoshida (Saitama Medical Center, Neurorsurgery):** Do you have any evidence that hydroxyl radical production is increased in the endothelial cells which are adhered by stimulated PMNL?

**Murota:** We have only indirect evidence to prove the presence of hydroxyl radicals in endothelial cells after stimulation with activated leukocytes. The indirect evidence is that blockade of the Fenton reaction by chelating ferrous ion caused complete abolition of the endothelial cell damage due to the activated leukocytes, as shown in my talk. I recall that someone may have proved the production of hydroxyl radicals by means of ESR in endothelial cells under ischemia-reperfusion conditions.

**Tomita:** I would like to add some details of our observations on interactions between activated PMNL and HUVEC. When a PMNL adhering to a HUVEC was exposed to water, the PMNL burst leaving intracellular cytosome strings attached to the endothelial cell. This indicates that the adhesion of the two cells is not due simply to membrane-membrane adhesion, but that some intracellular structural connection must be involved. It seems quite likely that the basis of this apparent transcellular continuity of structures could be the microfilaments, which play a role in such intercellular signal transmission as reported by Professor Murota.