Selectin on activated platelets enhances neutrophil endothelial adherence in myocardial reperfusion injury

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Abstract

Objectives: The glycoprotein P-selectin is an adhesion molecule that is rapidly expressed on the surface of platelets and endothelium during the inflammatory process. P-selectin on endothelium has been reported to play an important role in reperfusion injury. However, little is known regarding P-selectin on platelets in contributing to the pathophysiology of myocardial reperfusion injury. In this study, we hypothesized that P-selectin on platelets may enhance neutrophil endothelial adherence and this may play a role in neutrophil-mediated reperfusion injury.

Methods: Endothelial cells, cardiomyocytes, platelets and neutrophils were isolated from adult rats. Endothelial cells and cardiomyocytes were cultivated in a co-culture system. After exposure to hypoxia and reoxygenation, neutrophil adherence and migration were examined.

Results: After exposure to 6 h of hypoxia, endothelial cells co-incubated with platelets showed significantly greater neutrophil adherence (63.1±4.0\%) and migration (78.2±6.7\%) than endothelial cells alone (adhesion: 44.2±2.8\%, migration: 57.9±4.9\%). These increases were significantly inhibited (adhesion: 42.1±3.5\%, migration: 65.5±3.8\%) by an anti-P-selectin monoclonal antibody. Moreover, the superoxide-anion production was significantly elevated when activated platelets were added to neutrophils. This enhanced production was also inhibited by anti-P-selectin antibody.

Conclusion: The presence of activated platelets enhanced neutrophil adhesion and migration process after hypoxia-reoxygenation. This process may occur following platelet-neutrophil interactions via P-selectin and subsequent neutrophil activation.

Keywords: Experimental; Heart; Pathophysiology; Cell communication; Leukocytes; Platelets; Reperfusion; Rat

1. Introduction

Reperfusion injury in myocardium is still an important problem in several clinical situations. Recently, the expression of adhesion molecules has been found to be involved in the pathophysiology of neutrophil-mediated reperfusion injury [1]. The glycoprotein P-selectin also has been reported to play a role in the neutrophil “rolling” on endothelium during reperfusion [2]. The P-selectin ligand on neutrophils is thought to be a sialyl LewisX oligosaccharide presented on PSGL-1 [3]. The P-selectin is expressed on not only endothelial cells (i.e., Weibel-Pla德 bodies) [4] but also platelets (i.e., alpha granules) [5]. It is stimulated by thrombin, histamine, or free radicals, and is functionally expressed by extrusion to the cell surface without de novo protein synthesis within a 5 to 10 min period [6,7]. Although there is much evidence that P-selectin on endothelial cells is involved in the early stage of reperfusion events related to neutrophil-endothelial cell interactions [1,8], the biological relevance of P-selectin on platelets during reperfusion injury is only speculated [9]. More recently, it was reported that platelets and neutro-

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phils act synergistically in provoking cardiac dysfunction after ischemia and reperfusion [10]. However, its precise mechanism especially regarding P-selectin mediated pathogenesis has never been elucidated. Clarifying a role of P-selectin on platelets using an in vitro model would confirm that the interactions of platelets and neutrophils with the vessel wall are key factors in ischemia reperfusion injury.

The aim of this study was to elucidate whether P-selectin on platelets may play a role in myocardial reperfusion injury. For this purpose, we used isolated neutrophils and platelets, and a co-culture system of isolated endothelial cells and cardiomyocytes. We evaluated the effect of a species-specific monoclonal antibody in blocking the biological function of P-selectin on platelets in vitro.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody APR2-4, which was raised against recombinant rat P-selectin, was a gift from the Sumitomo Pharmacy (Osaka, Japan). This antibody specifically bound to rat P-selectin and showed no cross-reactivity to rat E- and L-selectins. In fluorescence activated cell sorter analysis, this antibody detected P-selectin induced on rat platelets after stimulation with thrombin. The complete characterization of this antibody has been previously described [11].

2.2. Rat platelet preparation

Surgical procedures and animal care strictly conformed to the recommendation from the “Guide for the Care and Use of Laboratory Animals” published by the US National Institute of Health. Blood obtained from male Sprague-Dawley rats (weighing 250–300 g) by intracardiac puncture with acid citrate dextrose was centrifuged at 300×g for 20 min at 30°C. The supernatant (platelet-rich plasma) was removed and recentrifuged at 2500×g for 10 min at 30°C to form a platelet pellet. The platelet pellet was washed and the platelets were resuspended in PBS without Ca²⁺ and Mg²⁺ and counted.

2.3. Rat neutrophil isolation and labeling

Rat neutrophils were prepared according to the method of Yuan and Fleming [12]. Briefly, the blood-cell pellet from the same blood from which platelet-rich plasma was removed was resuspended in 2 volumes of 2% gelatin in Hanks’ buffer solution. After sedimentation of erythrocytes at 1 g for 45 min at 37°C in the gelatin solution, the supernatant was removed and centrifuged at 400×g for 10 min at 30°C. Neutrophils were selected from what remained of the pellet with the Percoll (47%) gradient method for 20 min at 30°C. The cells isolated by this method were 95% neutrophils. These neutrophils were washed and suspended in Hanks’ buffer solution and counted. Then the neutrophils were labeled with non-specific fluorescent dye (PKH2, Zynaxis Cell Science) according to the method of Yuan and Fleming [12].

2.4. Isolation of microvascular endothelial cells and cardiomyocytes

Microvascular endothelial cells and cardiomyocytes were prepared by the procedure of Eghbali et al. [13] with minor modifications. In brief, the heart was perfused at 32°C for about 25 min with Ca²⁺ free, oxygenated Krebs-Henseleit solution containing 200 units/ml of collagenase (CLN-III E730, Worthington) and 300 units/ml of dispase. Cell suspensions were removed from the minced tissue and centrifuged for 5 min at 38×g. The pellets were fractionated by centrifugation on gradients of Percoll (52% final concentration) for 30 min at 25°C and rectangular myocytes were obtained after washing with HES and plating on laminin-coated dishes containing M-199 medium. The yields averaged approximately 2 to 4×10⁶ cells/heart. The supernatant was centrifuged for 10 min at 300×g and endothelial cells were obtained from the pellets after washing with HES and plating on collagen-coated dishes or membranes containing DMEM medium. The yields averaged approximately 1 to 2×10⁵ cells/heart. These cultured cells were identified as endothelial cells by their characteristic cellular morphology, growth pattern and the presence of the factor VIII-related antigen.

2.5. Experimental protocol for in vitro study

To investigate neutrophil adherence to endothelial cells, a confluent culture of endothelial cells after 2 or 3 passages on collagen-coated dishes was used. After exposure to 6 h of hypoxia in the anaerobic incubator with a closed gas-pack system (GasPak Plus, Becton Dickinson), fluorescence (PKH2)-labeled neutrophils (5×10⁶/mL) with and without platelets (5×10⁷/mL), and thrombin (at a final concentration of 5 U/mL) were applied to the dishes and subsequently they were incubated for 30 min under normoxia. After reoxygenation these dishes were washed with PBS and the fluorescence intensity of the neutrophils was measured with fluorescent spectroscopy FR 1500 (Shimadzu, Kyoto, Japan). The proportion of the fluorescence of the neutrophils that had adhered to endothelial cells relative to that of all neutrophils applied was considered as the neutrophil adhesion rate. To investigate neutrophil migration across endothelial cells, we used the 3-dimensional double-chamber co-culture system (Transwell-COL, Costar) in which endothelial cells were cultured on collagen membranes in the upper chamber and car-
diomyocytes were cultured in the lower chamber [14]. After exposure to 6 h of hypoxia, labeled neutrophils (5×10^5/ml) with or without platelets (5×10^5/ml), and thrombin (at a final concentration of 5 U/ml) were applied to the upper chamber and subsequently they were incubated for 1 h under normoxia. The proportion of the fluorescence of the neutrophils that had migrated into the lower chamber relative to that of all neutrophils applied to the upper chamber was considered as the neutrophil migration rate. These in vitro models were divided into 4 groups: group 6H (exposed to 6 h of hypoxia and reoxygenation, neutrophil and thrombin), and group 6H+P (exposed to 6 h of hypoxia and reoxygenation, neutrophil and platelets and thrombin), and 6H+P+Ab (6H+P with 4.2 μg/ml of monoclonal antibody APR2-4), and 6H+Ab (6H with 4.2 μg/ml of monoclonal antibody APR2-4). Moreover, superoxide anion generated by neutrophils in the supernatant was determined by the reduction of cytochrome c as previously described [15].

2.6. Statistical analyses

Results were expressed as mean±SEM. ANOVA followed by Bonferroni’s test was used to compare the data for the three or more groups. A value of P<0.05 was considered as statistically significant.

3. Results

3.1. Adherence of neutrophils to endothelial cells

The percentage of neutrophils that adhered to endothelial cells in group 6H+P (63.1±4.0%) was significantly higher than in group 6H (44.2±2.8%) (P<0.01). This increase in the neutrophil attachment rate was significantly inhibited by addition of anti-P-selectin monoclonal antibody APR2-4 (group 6H+P+Ab, 42.1±3.5%) (P<0.05). On the other hand, this antibody did not suppressed the neutrophil adherence in group 6H+Ab (53.7±9.7%) (Fig. 1).

3.2. Neutrophil migration

The neutrophil migration rate in group 6H+P (78.1±6.7%) was significantly greater than that in group 6H (57.9±4.9%) (P<0.01). This increase in this parameter was significantly inhibited by treatment with anti-P-selectin monoclonal antibody APR2-4 (group 6H+P+Ab, 65.5±3.8%) (P<0.05, Fig. 2).

3.3. Superoxide-anion production

The levels of superoxide-anion production were elevated about five times (3.6±0.31 vs. 0.8±0.25 nmol/10^9 cells) when activated platelets were added to the neutrophils. This enhanced production was significantly inhibited by 2.1 μg/ml of anti-P-selectin monoclonal antibody APR2-4 (2.1±0.18 nmol/10^9 cells) (P<0.01, Fig. 3).

3.4. Discussion

We previously reported findings that neutrophils were involved in a mechanism of postischemic reperfusion injury in myocardium [16,17] and CD18 and ICAM-1 were involved in the neutrophil migration into injured myocardium [18]. Our in vitro study, presented in this
Inhibited by monoclonal anti-P-selectin antibody APR2-4. Our in vitro study showed that anti-P-selectin monoclonal antibody in vivo. Weyrich et al. demonstrated the cardioprotective effects of monoclonal antibody (PB1.3) to human P-selectin in a feline in vivo model of myocardial ischemia and reperfusion [8]. They stated that PB 1.3 affected the P-selectin on endothelial cells. On the other hand, our in vitro study showed that anti-P-selectin mono-

paper, demonstrated that the presence of activated platelets significantly enhanced neutrophil adherence and migration to endothelial cells after hypoxia-reoxygenation. The superoxide-anion production of neutrophils was also enhanced by activated platelets. These enhancements were significantly inhibited by a species-specific monoclonal anti-P-selectin antibody. These results suggested that platelets might contribute to neutrophil-mediated reperfusion injury, specifically the involvement of platelet P-selectin.

Although there is much evidence that neutrophils contribute to the injury seen after ischemia and reperfusion [17], Alloatti et al. also reported that reperfusion of isolated rabbit hearts with neutrophils did not affect mechanical recovery, while reperfusion with neutrophils and platelets caused further deterioration [19]. Our in vitro data showed that neutrophil adherence after hypoxia-reoxygenation was significantly increased when platelets were added. Platelets normally circulate without firmly attaching to intact vascular endothelium, but there is some evidence that platelets can adhere to thrombin-treated endothelial cells in vitro [20,21] and ex vivo in a model of thrombin-treated rat lung [22]. Recently, Frenette et al. showed directly that platelets roll on endothelium in vivo and endothelial stimulation increased this platelet rolling [23]. Interestingly, they also reported that activated platelets bound to leukocytes and then rolled together with them. Our present results confirm that the coexistence of platelets and neutrophils may enhance the initial cascade of neutrophil-mediated effects that result in myocardial tissue injury.

In the present study, the enhancement of neutrophil adherence coexistent with platelets was significantly inhibited by monoclonal anti-P-selectin antibody APR2-4. Although P-selectin might be also expressed on the endothelial cells and APR2-4 might act on them, the neutrophil adherence without platelets was not significantly reduced by APR2-4 in our experiment. This fact might have likely been because these assays were performed under static and not flow conditions. Also, the neutrophil adherence to endothelial cells seemed to be multifactorial. In our assay, thrombin in the cell suspensions was not washed out so that thrombin activation of neutrophils and endothelial cells could be other mechanisms that would lead to adhesion via molecules other than P-selectin, for example ICAM-1 and beta2 integrins [24]. However, potential effects of thrombin on neutrophils and endothelial cells were same through the entire study group. Palabrica et al. demonstrated that leukocyte accumulation promoting fibrin deposition was mediated in vivo by P-selectin on adherent platelets [25]. This is consistent with our hypothesis that increased neutrophil adherence after hypoxia-reoxygenation occurred following platelet-neutrophil interactions via P-selectin.

It has been proposed that neutrophil-mediated tissue injury occurs in several discrete steps involving: rolling, adhesion, activation, and migration into the surrounding tissue [1,26]. Our data, obtained with a double-chamber co-culture system, demonstrated that neutrophil migration through the endothelial-cell layer into the cardiomyocyte layer was significantly increased after reoxygenation when platelets were co-included. Moreover, this increase was significantly inhibited by blocking P-selectin. Transmigration of neutrophils has been shown to be mediated by CD18, ICAM-1 [27] and PECAM-1 [28]. In our experiment, the biological blocking of P-selectin on platelets would inhibit the adherence of neutrophils to endothelial cells and the activation of neutrophils that results in an inhibition of neutrophil migration. These considerations for the role of P-selectin in neutrophil activation seem similar to those of the other adhesion molecules.

One of the important functions of activated neutrophils is the production and release of superoxide anions, which play a role in the pathogenesis of reperfusion injury. It has been reported in human samples that activated platelets induced superoxide-anion release by neutrophils through P-selectin [29,30]. In our rat study, the level of superoxide-anion production was significantly elevated when activated platelets were added to neutrophils. This enhanced production was partially inhibited by APR2-4, suggesting that P-selectin on activated platelets may take part in the step involving the activation of neutrophil functions.

Several investigators have shown the reduction of ischemia and reperfusion injury by anti-P-selectin monoclonal antibody in vivo. Weyrich et al. demonstrated the cardioprotective effects of monoclonal antibody (PB1.3) to human P-selectin in a feline in vivo model of myocardial ischemia and reperfusion [8]. They stated that PB 1.3 affected the P-selectin on endothelial cells. On the other hand, our in vitro study showed that anti-P-selectin mono-
clonal antibody also affected the interaction between platelets and neutrophils in reperfusion injury. Recently Lefer et al. clearly demonstrated that platelets and neutrophils act synergistically in provoking postreperfusion cardiac dysfunction in perfused rat heart experiments [10]. These findings are identical to those obtained in the present study. The results of these studies suggest that an anti-P-selectin monoclonal antibody is functionally effective possibly because of the block of P-selectin on platelets as well as on endothelial cells. This also suggests a clinical application of anti-P-selectin monoclonal antibody. For example, in open-heart surgery cardioplegia with blood containing anti-P-selectin antibodies may constitute a new method for inhibiting reperfusion injury and preserving cardiac function, although further investigations are needed to determine the clinical application of P-selectin.

In summary, the presence of activated platelets enhanced neutrophil adhesion and migration process after hypoxia-reoxygenation. This process may occur following P-selectin-mediated adhesive interactions between platelets and neutrophils, and subsequent neutrophil activation. We also demonstrated that a species-specific monoclonal antibody to P-selectin might provide a useful tool for inhibiting adhesive interactions between platelets and neutrophils.

References

[22] Palabrica T, Lobb R, Furie BC et al. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on platelets and neutrophils in reperfusion injury. Recently Lefer et al. clearly demonstrated that platelets and neutrophils in reperfusion injury after hypoxia-reoxygenation. This process may occur following P-selectin-mediated adhesive interactions between platelets and neutrophils, and subsequent neutrophil activation. We also demonstrated that a species-specific monoclonal antibody to P-selectin might provide a useful tool for inhibiting adhesive interactions between platelets and neutrophils.

References

