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Platelets, after Exposure to a High Shear Stress, Induce IL-10-Producing, Mature Dendritic Cells In Vitro

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There is evidence for immune system involvement in atherogenesis. In the present study the effect of platelets on dendritic cells (DC), an important immunologic regulator, was examined in vitro. Platelet-rich plasma, after exposure to shear stress, was added to human monocyte-derived immature DC, which were then examined for surface Ag expression, allogeneic T lymphocyte stimulatory activity, and cytokine production. After exposure, the number of anti-CD40 ligand (anti-CD40L) and anti-P-selectin IgG molecules bound per platelet was increased. These activated platelets induced DC maturation, as revealed by significant up-regulation of CD83, CD80, and CD86 Ags. The addition of platelets in the presence of IFN-γ plus LPS significantly enhanced IL-10 production from immature DC. After platelet addition, mature DC provoked a significant proliferation of allogeneic naive T lymphocytes. These activated T cells showed lower IFN-γ production than those stimulated by LPS- and IFN-γ-treated DC. CD40L on the platelet surface was not involved in maturation of DC, as mAb to CD40L failed to block maturation. The effect of platelets was observed even if platelets and DC were separated using large-pore-sized membranes or when platelets were depleted from plasma by centrifugation. Furthermore, it was abrogated after the depletion of protein fraction. Thus, soluble protein factors excreted from activated platelets contribute to IL-10-producing DC maturation.


The role of cross-talk between platelets and leukocytes in the local inflammatory response, particularly with regard to involvement in the chronic inflammatory aspect of atherosclerosis, has long been discussed (1). Several murine studies revealed that immune systems were tightly involved in the course of atherogenesis (2). Among them, recruitment of monocytes was shown to be important for the formation of atherosclerosis (3). Experiments using IFN-γ receptor-deficient. Apoe−/− mice proved that Th1-skewed immune reactions participate in the formation of atherosclerosis (4). Th1 cells were dominant in early phases of atherosclerosis (5). Dendritic cells (DC), the most potent APC, initiate innate and acquired immune responses (6). After treatment with LPS plus cytokines, DC secrete large amounts of IL-12 and IL-18, both of which are expressed in atherosclerosis lesions, which augment IFN-γ production (7, 8). Although their developmental pathways are quite complex, one DC population differentiates directly from monocytes (monocyte-derived DC) and migrates into endothelial cells using specific chemokine receptors (9), which suppose its tight involvement in the plaque formation similar to monocytes or macrophages. Recent investigations have revealed the presence of DCs at atherosclerosis-related lesions, especially at sites prone to rupture (10, 11).

Whether activated platelets attached to an atherosclerotic lesion influence plaque formation has not yet been elucidated (12). By releasing adhesive ligands, platelet-derived growth factor, or β-amyloid precursor proteins, platelets can provoke activation of APC (1, 13). Platelets express CD40 ligand (CD40L) after stimulation by thrombin (14). The role of CD40L in the progression of atheromatous plaque is of particular interest, as many recent reports indicate the presence of DC40 on the surface of atheroma-related cells, such as endothelial cells, macrophages, and smooth muscle cells (15). As the CD40/CD40L interaction is important in the progression of atherosclerosis (16) as well as plaque rupture mediated by increased matrix metalloprotease production from macrophages and T cells (17), interruption of CD40L binding to CD40 receptors or inhibition of CD40L expression on platelets may be a new therapeutic strategy to prevent progression of atherosclerosis and atheroma rupture. The function of DC is mediated by CD40 ligation by CD40L (18). Recently, Hilf et al. (19) reported that human platelets inhibited DC activation in the presence of heat shock protein and suggested that a low concentration of platelets was a reason for failed DC activation.

We have focused on the direct interaction between platelets and DC. Several groups have proposed that the mechanism of vivo platelet activation might not be the same as that of platelet activation induced by chemical agonists such as thrombin (20). Indeed, multiple receptor-ligand interactions, including many plasma

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and matrix proteins with their corresponding platelet surface receptors, are involved in the process of platelet activation after interaction with subendothelial matrix exposed at sites of endothelial damages (21, 22). Von Willebrand factor (VWF) and its interaction with platelet receptors, gpIb/IX and gpIIb/IIIa, are particularly important, especially at sites exposed to high shear stress (12, 20, 21, 23). Previous reports clearly demonstrated the appearance of immune-related proteins, such as CD40L and P-selectin, on the surface of platelets after exposure to a high shear stress (23, 24). In the present study we clarified the role of activated platelets after exposure to high shear stress on maturation and cytokine production of monocyte-derived DC. DC are currently recognized not only as immunogenic but also as tolerogenic when they are in immature or semimature stages (25). These opposite paths are determined by the release of proinflammatory cytokines, such as IL-12 or IL-10. Mature DC in general, through the production of IL-12, are inducers of Th1 immune effectors, whereas immature or semimature DC induce Th2 immune effectors (26). Thus, key cytokines (IFN-γ for Th1, IL-5 for Th2) which are produced from naive T lymphocytes after stimulation with platelet-activated DC were examined.

Materials and Methods

Blood sample preparation and sheared-induced platelet activation

Blood was collected from 10 healthy adult donors who abstained from the use of drugs known to interfere with platelet function, such as NSAIDs. Blood was immediately treated with trisodium citrate or the specific thrombin inhibitor PPACK to keep physiologic concentrations of divalent cations. Platelet-rich plasma was separated by centrifugation at 100 × g for 15 min. Platelets in 400 μl of platelet-rich plasma were exposed to a high shear rate of 10,800 s⁻¹ for 6 min in an optically modified cone-plate viscometer (20), the shear rate previously shown to induce VWF-gpIIb/IX-mediated platelet activation (20, 23, 24). Shear-induced platelet aggregation was blocked by the specific anti-gpIIb/IIIa agent tirofiban, enabling detection of CD40L and P-selectin molecules expressed on single platelets as we previously reported (24). Umbilical cord blood (UCB) was obtained with written informed consent after approval by the human subjects committee of Tokai University Hospital.

Effects on DC maturation

Mononuclear cells (MNCs) were separated from blood samples by Ficoll-Hypaque density (1.077 g/dl) gradient. CD14⁺ cells were isolated from MNCs using MACS CD14 immunomagnetic beads (Miltenyi Biotec, Gladbach, Germany), followed by a MACS-positive selection column (Miltenyi Biotec). DC were induced from CD14⁺ cells by culture for 7 days in RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS (Life Technologies, Gaithersburg, MD) and supplemented with GM-CSF (100 ng/ml; a gift from Kirin Brewery, Maebashi, Japan), and IL-4 (10 ng/ml; a gift from Ono Pharmaceutical, Osaka, Japan). DC were then incubated with platelet samples. In all experiments platelets were prepared from 10 ml of heparinized human blood and adjusted to a 200-μl final volume. Addition of 100 and 10 μl corresponded to 1000 and 100 for the platelet to DC ratio, respectively. Positive and negative controls for DC maturation were obtained by culture in the presence or the absence of LPS (100 ng/ml; Sigma-Aldrich) and IFN-γ (1000 U/ml; Shionogi, Osaka, Japan). The level of DC maturation was checked by double-immunofluorescence staining using FITC-anti-CD1a mAb (DAKO, Glostrup, Denmark)/PE-anti-CD80 mAb (Immunotech, Marseilles, France), and FITC-anti-CD80 mAb (Immunotech)/PE-anti-CD86 mAb (Immunotech). In additional experiments, anti-CD40L mAb (Ancell, Bayport, MN) was added together with activated platelets to test the role of CD40L on DC maturation. The efficacy of mAb was confirmed by inhibition of the maturation effect by CD40L transfectant (CD40Lþ; provided from Dr. Schultz, Dana-Farber Cancer Institute, Boston, MA). In the next experiment, platelets were placed in the chamber of a Transwell insert with high density-sized pores (BD Biosciences, San Jose, CA), which prevented direct contact between platelets and DC. Plasma, after depletion of shear-stressed platelets by centrifugation (10,800 s⁻¹; 6 min), or plasma without any treatment was added. In the next experiment, protein components among these plasma samples, after depletion of shear-stressed platelets, were depleted by ethanol precipitation, then added to immature DC.

Effects of platelets after shearing on production of cytokines from DC

Immature DC were washed extensively and replated at a density of 10⁵/96-well, flat-bottom plates with 10% FCS containing RPMI 1640. These DC were cultured for 48 h with or without the addition of platelets exposed to a high shear rate in the presence or the absence of LPS (10 μg/ml) plus IFN-γ (1000 U/ml). The culture supernatant was harvested and examined for the quantity of IL-10 and IL-12p70 by ELISA (Immunotech).

Effects of sheared and nonsheared platelets on the proliferation of allogeneic naive T lymphocytes in response to DC and on cytokine production

UCB-MNCs, depleted of CD14⁺ cells by MACS column separation, were added at 6 × 10⁵/well to 96-well, round-bottom plates as responders. Immature DC, DC matured by LPS and IFN-γ, and DC incubated with platelets exposed or not exposed to a high shear stress were serially diluted after radiation (15 Gy) and added as stimulators. The cells were cultured for 6 days in RPMI 1640 with 10% pooled human sera, then counted for their proliferative activity by the [3H]thymidine uptake test. Simultaneously, the culture supernatant was harvested from the cultured wells of immature, LPS- and IFN-γ-treated DC, or DC incubated with 100 μl of platelets as stimulators and examined for the content of cytokines (IFN-γ and IL-5) using a cytometric bead array (CBA) kit (BD Biosciences).

Naive T cells after incubation with DC were cultured for another 4 days in the presence of IL-2 (20 U/ml; Shionogi), incubated with 20 μg/ml PMA (Sigma-Aldrich) and 10 μg/ml ionomycin (Sigma-Aldrich) for 5 h in the presence of brefeldin A (Sigma-Aldrich) and permeabilized, and the intracellular expression of IFN-γ and IL-4 was examined using FITC-IFN-γ and PE-IL-4 (BD Biosciences) by flow cytometry. Also, the cultured cells were extensively washed and replated at a density of 10⁵/200 μl in the presence of PMA/ionomycin, and their supernatant was harvested 16 h later to examine the cytokine (IFN-γ, IL-4, IL-5, and IL-10) concentrations using a CBA kit.

Statistical analysis

All numerical data are expressed as the mean ± SD unless otherwise specified. The difference between two groups of data was tested by Student’s paired t test or unpaired t test. A value of p < 0.05 was considered statistically significant.

Results

Platelet surface expression of CD40L and P-selectin after exposure to a high shear rate

The numbers of platelet-expressing CD40L and P-selectin as well as the mean number of CD40L and P-selectin molecules expressed on the platelet surface were calculated according to a previously described procedure (20, 24). Quantitative analysis revealed that the mean number of anti-CD40L and anti-P-selectin IgG molecules bound per platelet increased from 17 ± 156 and 34 ± 148/platelet before to 388 ± 331 and 1122 ± 554/platelet after shearing (both p < 0.01). These results represent the appearance of CD40L and P-selectin, not representing the nonspecific increase in nonspecific IgG binding to activated platelets, because the number of isotype-matched negative control Ab against thyroglobulin (T-γ) did not increase even after exposure to the same shear stress (24). Shear-induced CD40L and P-selectin expression was mediated by VWF-gpIIb/IX interaction because mAb blocking VWF-gpIIb/IX interaction, both anti-gpIIb/IX and VWF, completely inhibited shear-induced platelet surface translocation of those molecules (24).

Effects of platelets on DC maturation

As shown in Fig. 1, immature DC started to express cell surface markers specific for matured DC, CD80, CD83, and CD86 after being cultured with platelets exposed to a high shear rate. Furthermore, the enhancing effect was dose dependent. However, no maturation was observed when DCs were incubated with platelets not exposed to shear. Stimulation with LPS and IFN-γ significantly enhanced surface expression of CD83, CD80, and CD86 compared...
with no stimulation (Figs. 1 and 2; \( p < 0.01 \)). Also, a statistically significant difference (\( p < 0.05 \)) in Ag (CD83, CD80, and CD86) expression was found between DC without and with platelet addition (platelet/DC ratio, 1000). Unexpectedly, mAb to CD40L did not significantly neutralize the enhancing effect of platelet-induced Ags (Fig. 1B), whereas it prevented an up-regulation of CD83, CD80, and CD86 Ags by CD40L-expressed feeder cells. As shown in Fig. 3A, platelets could still up-regulate the expression of CD86 Ag, even if cell-to-cell contact was avoided using the Transwell insert. Furthermore, plasma, after depletion of shear-stressed platelets, could still show maturation-enhancing effects, as shown in Fig. 3B. Plasma without shearing did not have such a maturation effect. As shown in Fig. 4, these DC maturation effects were completely abrogated after depletion of the protein fraction.

**Effects of platelets on IL-10 and IL-12 production**

Untreated DC did not significantly produce either IL-10 or IL-12, as revealed by ODs showing the lowest level of detection. As shown in Fig. 5A, addition of platelets slightly induced IL-10 production, whereas no IL-12 production was observed. In the presence of LPS and IFN-\( \gamma \), platelet addition did not influence IL-12 production, but significantly enhanced IL-10 production (Fig. 5B). As a result, DC exposed with LPS and IFN-\( \gamma \) plus platelets showed comparable levels of IL-12 and IL-10 production. CD40L mAb did not affect the enhancement of IL-10 production by activated platelets (data not shown).

**Effects of platelets on proliferation of allo-T lymphocytes and cytokine production from T lymphocytes**

As shown in Fig. 6, the magnitude of allogeneic naive T cell proliferation was highest when they were stimulated with DC matured by LPS and IFN-\( \gamma \). Although the magnitude was not as prominent, DC after platelet addition (platelet/DC ratio, 1000) significantly promoted allo-naive T cell proliferation. No significant proliferation was observed when T cells were stimulated with DC cocultured with nonsheared platelets or immature DC. Control platelets did not show any effect of proliferation (data not shown). As shown in Fig. 6, none of the DC samples provoked syngeneic T lymphocyte proliferation.

As shown in Fig. 7, T lymphocytes after incubation with LPS- and IFN-\( \gamma \)-matured DC produced IFN-\( \gamma \), whereas no significant production was obtained when they were cocultured with platelet-matured DC or immature DC. IL-5, one of the main Th2 cytokines, was produced in low concentrations from mixed cultures with immature DC, LPS and IFN-\( \gamma \), or platelet-matured DC. As shown in Fig. 8, CD4\(^{+}\) naive T lymphocytes after incubation with platelet-matured DC exhibited much lower intracellular expression of IFN-\( \gamma \) than those stimulated with LPS and IFN-\( \gamma \)/DC. However, the levels of the other cytokines (IL-4, IL-5, and IL-10) were equivalent in T cells preactivated with LPS and IFN-\( \gamma \)/DC and those with shear-stressed platelets.

**Discussion**

The roles of platelet-derived molecules, including platelet-derived growth factor (1), integrins (1), P-selectin (27), and platelet-derived microparticles (28), in regulating local inflammatory response have been investigated. We have demonstrated that the proteins known to interact with leukocytes, P-selectin and CD40L, were surface translocated by high shear rate conditions, even in the
interaction between thrombin. P-selectin, but not CD40L, plays a role in the physical absence of any known platelet-activating agent, such as ADP or thrombin. P-selectin, but not CD40L, plays a role in the physical interaction between flowing leukocytes and collagen-adherent platelets (29). By contrast, CD40L plays functionally important roles in the maturation of DC, resulting in specific Ag expression, allo-T cell proliferation, and cytokine production (30). These results suggested that platelets play important roles in the regulation of a local inflammatory response at sites exposed to high shear stress by trapping monocytes that can be transformed to DC and by inducing their maturation through stimulation of the CD40 receptor by surface-translocated CD40L. Although CD40L surface translocation can be induced by platelet activation by soluble agonists such as thrombin (14, 31), these are not likely to play important roles in vivo, because high concentrations of soluble agonist are not likely to exist in vivo, especially in the presence of blood flow. VWF-gpIb/IX-mediated platelet CD40L expression is more likely to occur at sites exposed to arterial blood flow conditions generating high shear rates, as animal experiments clearly demonstrate the crucial role of this interaction in arterial thrombosis (32). Similar VWF-mediated CD40L expression is likely to occur on platelets interacting with exposed subendothelial matrix, such as collagen, because VWF has specific collagen binding sites, and the VWF-gpIb/IX interaction plays a crucial role in platelet thrombus formation on collagen (21, 33).

We clearly demonstrate that immature DC become mature when they are incubated with platelets after exposure to high shear stress. DC matured with platelets expressed all CD83, CD80, and CD86 Ags at a significantly high level compared with those without maturation. IL-12 production from immature DC was not enhanced by the addition of shear stress-activated platelets. IL-10 production was promoted by platelet addition in the absence and the presence of LPS and IFN-γ. As a result, naive T cells after stimulation with platelet-matured DC did not acquire the ability to produce the Th1 cytokine, IFN-γ, whereas they maintained IL-5 production. Gatti et al. (34) applied fixed activated platelets to Langerhans cells, which were activated by CD40L on platelets. In contrast, our results have shown that CD40-CD40L interaction did not play an important role in maturation of DC, as revealed by mAb blocking tests. Henn et al. (31) reported that CD40L expressed on the platelet surface is transient and is cleaved to a soluble form after stimulation by soluble agonists, although no soluble CD40L Ag was detected in culture supernatant of DC after exposure to high shear stress by ELISA (data not shown). Hilf et al. (19) recently reported that thrombin-activated platelets expressed heat shock protein (gp96) receptors and down-regulated Gp96-mediated DC maturation. They also observed an effect of platelets on DC without Gp96 maturation, but in contrast to our results, neither a change in surface Ags nor enhanced IL-10 was observed. The ratio of platelets to DC (20:1) was far less than that in our experiment (100:1 to 1000:1), which might be a reason why

![FIGURE 3. Indirect contact between DC and platelets after high shear stress. A, Platelets after high shear stress were placed in the chamber of a Transwell insert with high density-sized pores, which prevented direct contact between platelets (plt) and DC. Under the chamber, DC were cultured for 48 h, and their surface marker expression was compared before and after the addition of platelets. B, Plasma before and after depletion of shear-stressed platelets or plasma without any treatment was added to immature DC, and their surface marker expression was analyzed.](Image)

![FIGURE 4. Effect of protein depletion. Immature DC were left untreated or were incubated with LPS and IFN-γ, with plasma after depletion of shear-stressed platelets (plasma without plt), or with protein-deprived solution from these treated plasma (protein dep.) Maturation of DC was detected by CD1a, CD83, CD80, and CD86 expression by double-immunofluorescence staining.](Image)

![FIGURE 5. Production of cytokines from DC. Immature DC were washed extensively and replated at a density of 10^5/ml, flat-bottom plate with 10% FCS containing RPMI 1640. These DC were cultured for 48 h with or without the addition of platelets exposed to high shear in the absence (A) or the presence (B) of LPS (10 μg/ml) plus IFN-γ (1000 U/ml). The culture supernatant was harvested and examined for the quantities of IL-10 and IL-12p70 by ELISA. The mean ± SD of 10 experiments are shown, and statistically significant differences are indicated (*, p < 0.05). Although not shown in this figure, DC with no treatment were not capable of cytokine production.](Image)
thymidine uptake test. Representative data are shown as the mean ± SD of triplicate wells. **, p < 0.01 compared with immature DC.

discrepant results were obtained. The functionally important roles of platelet-expressed CD40L in local inflammation and progression of atherosclerosis suggest that drugs inhibiting platelet CD40L translocation might prevent atherosclerosis. However, contrary to our initial hypothesis, our results clearly prove that soluble factors, other than CD40L, excreted from platelets after shear stress, including many of the candidate materials released from dense granule, such as soluble P-selectin (27), and those released from dense granule, such as ADP (35), might contribute to maturation of DC. To differentiate whether the soluble factor made by platelets is lipid or protein, protein fraction was depleted by simple methods of ethanol precipitation. This resulted in the total disappearance of maturation effects or promotion of IL-10 production in the presence of LPS and IFN-γ (data not shown). Activated platelets make abundant lysophosphatidic acid (36–38), a bioactive lipid mediator that can enhance the secretion of IL-10 from DC (39). The addition of diacylglycerol pyrophosphate (8:0), which is a selective antagonist of lysophosphatidic acid (37), did not produce any neutralization effect.

FIGURE 7. Effects of sheared platelets on the cytokine production from naive T lymphocytes. UCB-naive T cells were stimulated with immature DC, LPS- and IFN-γ-treated DC, or DC incubated with sheared platelets for 6 days, and the supernatant of mixed culture wells was examined for cytokine concentrations using a CBA kit. The mean ± SD of 10 experiments are shown, and statistically significant differences are indicated (**, p < 0.01).

The roles of macrophages and T cells in the progression of atherosclerosis and the onset of atheroma rupture have previously been established (1). Many immune-related cells, such as chronically activated CD4⁺ T cells, exist in atheroma (40). There are several candidate Ags that mediate specific expansion of T lymphocytes, such as viruses or bacteria and endogenous altered Ags, heat shock proteins, or oxidized low density lipoproteins (41). Those Ags, if presented to professional APC such as DC, can guide naive T cells to Th1-type immune effectors. Actually, CD4⁺ T cell clones in human atherosclerotic lesions specifically respond to oxidized low density lipoproteins in an HLA class II-restricted manner (41). In the present study we focused on DC, which are demonstrated to be in atheroma (10, 11); their role in atherosclerosis was clarified in chronic inflammation related to infection by Chlamydia pneumoniae (42), whereas their important roles in immune regulation need to be studied (6). In the case of Chlamydia infection, host APCs, especially DC, accumulate at infected endothelial cells, take up bacterial Ags, and produce inflammatory cytokines such as IL-6, TNF-α, or IL-12/18, which accelerate atherosclerosis (42). IL-12 and IL-18 cause Ag-specific CD4⁺ T cells to secrete IFN-γ, which is proinflammatory and proatherogenic (7, 8). Platelets accumulate at damaged endothelial cells and are then activated through a gpIIb/IX-VWF-mediated interaction, which can result in IL-10-producing DC maturation, as shown in the present study. Those DC may antagonize IFN-γ production and thereby confer atheroprotection. In vitro and in vivo studies have shown that rIL-10 suppresses progressive generation of atherosclerosis (43).
Our study has methodology limitations, particularly in applying the experimental results to understand the role of platelets in regulating local inflammatory responses in vivo. First, we demonstrated DC maturation in the presence of platelets after exposure to high shear only in a pure culture system, excluding the effects of various cellular and protein components in vivo, such as erythrocytes. Other important contributors, such as endothelial cells, extracellular matrix, and complex blood flow conditions, were not considered in our ex vivo system. Second, we demonstrated platelet surface translocation of CD40L and P-selectin only after exposure to high shear stress and then could not reproduce the complex stress which to platelets are exposed in vivo. Thus, the amounts of CD40L and P-selectin expression may not be physiologic. Although no clinical data regarding platelet surface CD40L are available to date, previous studies have shown P-selectin expression to be induced in a similar manner when platelets pass over the damaged surfaces, i.e., coronary intervention (44). Thus, we believe that platelet activation, with P-selectin and CD40L surface translocation, can be induced by interaction with damaged vascular surfaces under high shear stress conditions in vivo.

In conclusion, it was demonstrated that platelets, after exposure to high shear stress, play an important role in DC maturation and subsequent reactions, including allo-T cell proliferation and cytokine (IL-10) production. Also, the soluble protein factors secreted from these activated platelets contributed to such DC maturation or IL-10 production. Recent in vivo works have shown that platelets were involved in increased risk of atherosclerosis (12, 45). To the contrary, our experimental results suggest that platelets suppress the progression of atherosclerosis through regulating the immunological function of DC. These results show unique, dual functions of platelets and their important roles to maintain equilibrium in the context of local inflammatory responses at sites of vascular injury.

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References


