Biology of monocyte interactions with the endothelium: the platelet factor

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Chapter 7

“Discussion, concluding remarks and future perspectives”
Cellular components play a major role in atherogenesis and the related, final acute cardiovascular symptoms. While influx of inflammatory cells is seen in early atherosclerotic plaque formation, plaque disruption and endothelial erosion initiate platelet-mediated thrombus formation as a cause for final vascular obstruction in acute coronary syndromes or stroke. The critical role of platelets in atherosclerosis is an old concept, originally proposed by Ross in 1986\(^1\). Since then platelets are considered as crucial mediators of coronary thrombosis in cardiovascular disease and have been identified as the major target in both therapeutic and preventive intervention. The inflammatory nature of atherosclerosis involves chronic stimulation of the endothelial cells that line the intima, the innermost layer of arteries, and an active inflammatory response\(^2,3\) initiating the atherogenic mechanism. While under normal circumstances the endothelial monolayer in contact with flowing blood cells resists firm adhesion of leukocytes, a strong adhesive capacity of platelets and/or monocytes/lymphocytes to the endothelium is essential in the development of atherosclerosis.

In this thesis, important interactions between platelets and leukocytes are highlighted. Platelet binding to monocytes enhances the adhesive capacity of monocytes to the endothelium and results in a general activation of the interacting cells. We thus propose this adhesive synergy as a new proatherogenic mechanism that may become a valuable target of new therapeutic strategies.

**Monocytes and T lymphocytes in atherogenesis**

Adhesion of leukocytes is essential for their role in atherogenesis; however, the normal arterial endothelium resists prolonged contact with leukocytes. Chronic activation of endothelial cells results in increased expression and increased binding affinity of various adhesion molecules on the cell surface that will mediate leukocyte adhesion.

The first steps in the mechanism of leukocyte recruitment to inflammatory sites are tethering and rolling. Two types of leukocyte tethering can be distinguished: primary tethering of leukocytes directly to the endothelial surface and secondary tethering to already adhered leukocytes\(^4,5\). Both mechanisms lead to leukocyte firm adhesion; however, only secondary tethering is associated with cluster formation\(^6\). Secondary tethering facilitates monocyte adhesion downstream to other, already adhered, monocytes. As a result, strings of cells in the direction of the flow are formed\(^5\).

In atherosclerotic inflammation, mostly monocytes and T lymphocytes penetrate the vascular wall along a chemoattractant gradient. Various and specific chemokines can mediate this so-called diapedesis though the endothelium, e.g. monocyte chemoattractant protein-1 (MCP-1) interacts with its receptor CCR2 on monocytes.
Under the influence of oxidized lipoproteins, monocytes can acquire the morphological characteristics of macrophages and later of lipid-loaded foam cells, the latter being characteristic of early atherosclerotic lesions. As monocytes produce and express coagulation-activating tissue factor, monocyte-derived macrophages, in their turn, also play a role in the thrombotic complications of atherosclerosis by producing matrix metalloproteinases (MMPs). MMPs degrade the extracellular matrix leading to increased plaque instability, easy plaque rupture and subsequent thrombus formation (see reference 3 for review).

Besides MCP-1, human atheromas overexpress other chemokines that may contribute to lymphocyte recruitment, including a trio of CXC chemokines induced by interferon-γ (IFN-γ) that bind to the CXCR3 receptor on T lymphocytes. Once resident in the intima, T lymphocytes may become activated by endogenous or microbial antigens and therefore, amplify the inflammatory response e.g. by cytokine release (see reference 3 for review).

Platelets: only important players in the late atherosclerotic process?

Mechanical disruption of the endothelial monolayer and subendothelial matrix exposure e.g. after plaque rupture, lead to immediate strong platelet activation, adhesion and aggregation. Additionally to this primary platelet-mediated hemostasis, tissue factor and platelet-derived phospholipids initiate coagulation and fibrin formation, resulting in a so-called "mixed thrombus" and, sometimes, in fatal occlusion of the blood vessel. Once adhered, platelets participate in plaque progression and complications encountered after plaque rupture whether via surface expression of P-selectin or via release of proinflammatory chemokines. P-selectin is an important adhesion ligand in further leukocyte recruitment to these sites. Indeed, infusion of activated platelets into Apo-E-deficient mice potentiates atherosclerosis by increasing localization of monocytes at the vessel wall 7. Furthermore, a relatively recent study not only shows a proatherogenic role for endothelial-derived P-selectin but also for platelet-derived P-selectin 8.

When platelets are circulating through vessels into an intact, healthy endothelium, the platelets remain in their original, unactivated state. The absence of activating factors and the release of prostacyclin by the healthy endothelium support this state. However, when a platelet encounters a break in the endothelium, it encounters molecules that trigger its activation such as collagen, ADP and thrombin. Platelets will then change in shape and expose certain molecules (e.g. P-selectin) that, altogether, will allow platelets to slow down in their velocity and interact with the injured vessel wall via selectin- and/or vWF-dependent mechanisms 9-11. However, these interactions are often transient, suggesting that platelets are less involved in
early atherogenesis when the endothelial monolayer is not yet disrupted. In this thesis we suggest that activated platelets in the circulation are able to also accelerate the early inflammatory atherogenic process.

**Platelet-derived P-selectin and proinflammatory activity.**

Once activated, platelets can interact with leukocytes via their surface-expressed P-selectin, leading to the formation of leukocyte-platelet aggregates (LPAs). P-selectin on platelets mainly binds to P-selectin glycoprotein ligand-1 (PSGL-1) on resting monocytes, neutrophils or other leukocytes. Normally located randomly on the cell at the tips of the microvilli, upon cell activation PSGL-1 molecules are rapidly redistributed over the cell surface, changing the avidity and affinity for its ligands. In neutrophils, clustering of PSGL-1 leads to weaker interactions with P-selectin; however, this seems to be different for other leukocytes as suggested by the fact most LPAs in the circulation consist of platelets complexed with monocytes or eosinophils. Increased levels of LPA, termed platelet-monocyte complexes (PMC), have been found in stable and acute coronary vascular diseases (CVD) and are currently considered as sensitive markers of vascular disease and its prognosis.

However, besides being markers of CVD, we found that PMC formation also influences the first steps in the process of monocyte recruitment to the endothelium. The suggested role for these complexes in promoting plaque progression is compatible with an earlier report in which it was shown that platelets enhance primary tethering and adhesion of monocytes or THP-1 (monocytoid) cells to endothelial cells.

**Role of platelet-monocyte complexes in promoting plaque progression**

As mentioned before, there are two types of cell tethering: primary and secondary tethering, the latter being associated with downstream formation of cell clusters. PMC appear to be better primary tethers than bare monocytes (Chapter 2). These complexes roll with a lower velocity over the endothelial cell ligands, leading not only to more efficient interactions between adhesion receptors but also to longer interaction times between endothelium-released cytokines and leukocytes and thus to more efficient cytokine activation of additional adhesion molecules. The subsequent secondary tethering of circulating monocytes over previously adhered monocytes is normally mediated by L-selectin interaction with PSGL-1. In addition and as described in Chapter 2, platelet binding to monocytes increases their
clustering capacity. In the presence of PMC, the additional P-selectin-mediated primary and secondary tethering seems to overshadow the typical L-selectin-mediated clustering. Only at higher shear stress, when all adhesive interactions become critical and adhesive redundancy is increased, L-selectin contributes to clustering. This suggests that mainly monocytes with platelets bound to their surface (PMC) are interacting with the already adhered monocytes via P-selectin on their platelets.

PMC were shown to adhere two times better to the endothelium as compared to "bare monocytes" (<5% PMC). This increase in adhesion correlates to an increase in both primary and secondary tether formation, indicating that platelets on the monocyte surface are able to enhance monocyte adhesion and clustering by "bridging" interactions with other monocytes via P-selectin – PSGL-1 interactions.

Altogether, the platelet-mediated adhesion-enhancing mechanism described in Chapter 2 suggests that PMC play a role in promoting plaque progression. The presence of PMC in vivo as a marker of cardiovascular disease may, by this adhesion-enhancing mechanism, accelerate the development of disease and explain its association with disease outcome.

**PSGL-1: crucial molecule that mediates platelet, monocyte and/or platelet-monocyte complex tethering**

PSGL-1 is a general selectin ligand present on most leukocytes and platelets. With the highest affinity for P-selectin, PSGL-1 also binds L- and E-selectin. Leukocyte PSGL-1 mediates primary tethering and subsequently total adhesion of multiple leukocyte subtypes to endothelial P- and E-selectin under physiologically relevant shear stress. By binding to L-selectin on other leukocytes, PSGL-1 is known to be involved in homotypic interactions that result in secondary tethering or cluster formation of neutrophils or monocytes at the endothelium. Although the role of L-selectin, in the presence of PMC, is overshadowed by the PSGL-1 – P-selectin interactions, its general clustering potential becomes clear at higher shear stress (Chapter 2). Additional to its role in secondary tethering, our experiments also showed a role of L-selectin in primary tethering, suggesting the presence of strong endothelial ligands for this selectin. Although several ligands on endothelial cells, e.g CD34 and MadCAM-1, have been associated with L-selectin, so far no specific ligands for selectins on endothelial cells have been described.

Against this background, incubation of endothelial cells with a specific antibody against PSGL-1 provided strong evidence that functional PSGL-1 is present on stimulated endothelial cells. By means of Western blot analysis, flowcytometry and
immunostaining we have additionally shown in Chapter 3 that PSGL-1 is expressed and constitutively present on endothelial cells. Although activation of endothelial cells, with TNF-α or IL-1β, did not affect PSGL-1 expression, activation of cells was necessary for optimal functionality of PSGL-1 as a selectin ligand on endothelium. This may be explained by the fact that PSGL-1 binding to selectins and thus functionality depends on its proper sulfation, fucosylation and sialylation. To be able to bind P-selectin, leukocyte PSGL-1 needs to be tyrosine sulfated and properly decorated with core 2-based O-glycans expressing sialyl Lewis x. In a similar way, functionality of endothelial PSGL-1 seems to depend on sulfotransferases GST-1 and, partially, GST-2, whose expression is indeed dependent on activation of the endothelial cells with TNF-α. The functional presence of this powerful ligand on activated endothelial cells in vitro is responsible for platelet and monocyte primary tethering. PSGL-1 is thus presented as a new ligand for L- and/or P-selectin on endothelium that may be able to modulate platelet and monocyte/PMC primary tethering to the endothelium in vivo.

**PSGL-1 as a signaling molecule**

Previous studies showed platelet involvement in direct interactions between leukocytes and endothelium. However, intravital microscopy and our own observations characterized platelet interactions with the endothelium, at relatively low shear rates, as transient and rapidly reversible. This leads to some speculation on how platelets can mediate monocyte/PMC primary tethering to endothelium, under these conditions.

The expression of P-selectin on the platelet surface following platelet stimulation and granule secretion allows interaction of P-selectin with specific ligands, e.g. PSGL-1, on other cells. In this manner, and as described above, P-selectin mediates cell-cell interactions. Besides this docking function, these adhesion molecules are also involved in signal transduction. Indeed, engagement of the extracellular domain of PSGL-1 by P-selectin, initiates an array of intracellular events within the leukocyte. It has been shown that monocytes, which do not demonstrate constitutive expression of tissue factor under normal conditions, show activation of the tissue factor gene and, in several hours, expression of functional tissue factor after P-selectin ligation to PSGL-1. Some earlier studies also suggested that P-selectin promotes β2 integrin-dependent homotypic neutrophil aggregation and neutrophil-platelet conjugation. Furthermore, platelet-derived P-selectin has also been shown to increase monocyte adhesivity and monocyte adhesive strength to immobilized VCAM-1, indicating that P-selectin binding enhances α4 integrin activity on monocytes. We extended these findings by showing that platelet binding to monocytes not only
provides P-selectin as an extra rolling receptor but also changes the monocyte repertoire of expressed cell adhesion molecules (Chapter 4), both quantitatively and qualitatively. Platelet binding results in decreased expression of L-selectin on the monocyte surface and increased expression and activity of both $\beta_1$ and $\beta_2$ integrins. Altogether, these findings indicate a general activation of the monocyte upon platelet binding. Furthermore, expression and activity of $\alpha_4\beta_1$ and $\alpha_m\beta_2$ integrins were increased upon platelet/P-selectin Fc chimera binding to monocytes. This increased integrin expression and activity correlated with an increase in monocyte/PMC adhesivity to several immobilized $\beta_1$ and $\beta_2$ integrin ligands (fibronectin, VCAM-1, fibrinogen and ICAM-1) and to stimulated endothelial cells (also expressing ICAM-1 and VCAM-1).

Although a general increase in monocyte adhesivity was observed upon either platelet or P-selectin-Fc chimera binding, the strongest effect was obtained after formation of PMC by adding platelets to the monocytes. In agreement, ligation of PSGL-1 by P-selectin was recently shown to induce an intermediate state of neutrophil activation characterized by increased affinity/avidity and subsequent functionality of integrin $\alpha_m\beta_2$ but not by changes in its expression levels. Additional exposure to biologically relevant stimuli such as PAF and IL-8 seems to change the intermediate state of activation to a high affinity one. It is known that platelets, upon binding, are able to release similar stimuli and chemokines. Moreover, monocytes exposed to platelets and the platelet-derived chemokine, RANTES, secrete cytokines such as IL-8 and MCP-1 that are also likely to act in a paracrine stimulating and synergistic fashion. These cytokines or chemoattractants, by enhancing integrin activation, might act in concert with PSGL-1 engagement to induce optimal activation of integrins. Therefore, platelet- and, subsequently, monocyte-released chemokines will optimize monocyte activation, and this may explain the stronger activation of monocytes by platelet binding as compared to P-selectin-Fc chimera that we observed (Chapter 4).

**Other mechanisms of leukocyte recruitment to endothelium**

Other important inflammatory and thus proatherogenic mechanisms and molecules, involved in leukocyte recruitment to endothelium are additionally described in this thesis. Low-density lipoprotein (LDL) receptor-related protein (LRP) is a member of the LDL-receptor family and is known to be expressed in leukocytes and on brain vascular endothelial cells. So far, LRP was shown to be involved in the regulation of the vascular permeability of the blood-brain barrier and to mediate calcium-signaling in primary neurons. In Chapter 5 we describe an additional role of LRP regulating $\beta_2$ integrin-dependent leukocyte adhesion to
endothelium. LRP seems, in this respect, to be required for proper positioning of $\beta_2$ integrins and thus facilitate the interaction with counterreceptors such as fibrinogen, ICAM-1 or ICAM-2. Additionally to mediating these "cis" interactions, LRP seems able to bind $\beta_2$ integrins on other leukocytes. These "trans" interactions may lead to cluster formation and therefore, be strongly involved in leukocyte secondary tethering. Altogether, these evidences link LRP to the inflammatory system.

As we showed in Chapter 3, the functionality of endothelial PSGL-1 is strongly carbohydrate-dependent. This seems to be common to other molecules involved in leukocyte recruitment to the endothelium. Chapter 6 describes a similar mechanism that mediates the interaction between immature dendritic cells (DCs) and the endothelium. DCs are recruited from blood into tissues to patrol for foreign pathogens after antigen intake and processing DCs mature, migrate to secondary lymphoid organs and initiate a specific immune response. Specific homing and trafficking of DCs therefore should be critically organized for the proper function of these later antigen-presenting cells. DCs express a specific C-type lectin, known as DC-SIGN, which not only acts as a recognition receptor but also as an adhesion molecule. DC-SIGN, by interacting with ICAM-2, mediates rolling and adhesion of DCs over endothelial cells. In Chapter 6 we show evidence that expression of the Lewis Y epitope on ICAM-2 is crucial for DC-SIGN-mediated cell rolling interactions. Again, there seems to be a strong carbohydrate-dependency for optimal receptor-ligand interaction and subsequent functionality because ICAM-2 expressed on endothelial cells can only be recognized by DC-SIGN when it is properly glycosylated. Indeed, fucosylation is essential for the rolling and adhesion of DCs to endothelial cells, and Lewis$^Y$ was found to be the major carbohydrate ligand for DC-SIGN. Altogether, the findings concerning both PSGL-1 and DC-SIGN open new possibilities in the modulation of leukocyte migration by post-transcriptional glycosylation of these receptors.

Concluding remarks and future perspectives

Activated platelets can bind monocytes in the circulation, resulting in the formation of PMC. Low numbers of PMC are sufficient to increase both primary and secondary tethering and thus overall monocyte recruitment to the endothelium. This PMC-mediated effect on monocyte adhesion is dependent on the presence of platelet-derived P-selectin as a powerful selectin that is able to overcome monocyte activation-mediated shedding of L-selectin from the surface of monocytes within the PMC. Additionally, platelet binding to monocytes increases expression and functionality of both $\beta_1$ and $\beta_2$ integrins, which also results in stronger firm adhesion capacity. The change in integrin functionality seems to be caused by a combination
of P-selectin-mediated signaling, via PSGL-1, and platelet-released products. Besides its role in firm adhesion, this platelet-mediated increase in integrin functionality also leads to increased migratory capacity of the monocytes. Platelet-induced signaling on monocytes not only increases the monocyte adhesivity and migratory capacity but, in general, it seems to induce a higher activation state and subsequently a stronger proinflammatory and proatherogenic phenotype of the monocyte.

Our studies, by identifying molecules that are crucial in platelet-monocyte contact, and thus platelet-monocyte cross talk, as well as other molecules that mediate monocyte recruitment to the endothelium, are highly relevant to the understanding of potent atherogenic mechanisms. By investigating the consequences of PMC as strong thrombo- and atherogenic entities, our studies have crucial relevance in the development of new therapeutics tools to prevent or improve vascular disease, namely atherosclerosis. In this respect, inhibition of PMC formation by modulation of P-selectin-PSGL-1 interactions and subsequent inhibition of monocyte adhesivity seems to be an interesting and potential approach.

**Remaining questions and remarks:**

1. Platelets, by binding to monocytes and forming PMC, induce monocyte adhesion and clustering to the endothelium by providing P-selectin and thus enhance the monocyte adhesive capacity. Moreover, PMC formation results in upregulation of β₁ and β₂ integrin expression and functionality on monocytes. As a consequence, not only an increase in integrin-dependent firm adhesion of monocytes occurs but also an increase in monocyte migration is observed. It is, so far, unclear how P-selectin binding to monocytes and the triggered signaling pathways further determine the fate of PMC in the vessel wall. This mechanism is however unclear. Preliminary experiments suggest that platelets might act as a touring car, dropping off its passengers (monocytes/eosinophils), at the respective stops (activated endothelial cells), and afterwards detach to further continue their journey, possibly to further pick up and drop off other passengers. In this perspective, engagement of monocyte integrins (β₁ or β₂ integrins such as VLA-4 or Mac-1, respectively) by the respective ligand on the endothelium might act as the detaching (stop) signal for the platelets (touring car). This mechanism, as well as the use of these signaling pathways as targets of therapeutic intervention, needs further investigation. Similarly interesting is the role of additional mechanisms of PMC formation and PMC behavior in vivo.
2. The precise role of activated platelets and PMC in the acceleration of early atherogenesis and cardiovascular disease in vivo still needs confirmation. In this respect, the therapeutic efficacy of inhibitors of PMC formation and function, such as the well-known platelet activation inhibitors or the recently available P-selectin antagonists, should be reviewed against this background. However, such studies should take into account the difficulties related to PMC measurements, because platelets easily become activated in vitro. Preliminary studies in baboons, using orally administered P-selectin antagonists, showed decreased formation of LPA and subsequently of atherosclerosis. In a near future and in the light of our own studies, such an approach may become efficient in preventing cardiovascular disease in humans.

3. By acting in a similar integrin-activating way as platelets, also LRP may play a role in monocyte recruitment to the endothelium. Some preliminary data suggest that LRP may be involved in monocyte secondary tethering. Whether LRP on the surface of one monocyte can interact in “trans” with β2 integrins or even other LRP molecules on the surface of other monocytes and, in such way, lead to the formation of cell strings or clusters is currently under investigation.

4. PSGL-1 was shown to be strongly implicated in monocyte recruitment to the endothelium not only by binding L-selectin and mediating monocyte-monocyte interactions but, more importantly, by binding P-selectin on platelets and thus facilitating PMC formation. Besides being expressed on activated endothelial cells, PSGL-1 is also able to directly interact with ligands on the monocyte and also, in theory, on the platelet surface, and directly recruit these cells to the endothelium. Endothelial PSGL-1 – mediated monocyte recruitment to the endothelium is modulated by activation of the endothelium, which provides crucial changes on the PSGL-1 molecule, thus increasing the affinity for its ligands. This is true for endothelial cell activation with TNF-α. However, whether PSGL-1 functionality is dependent on the type of cytokine or stimulus still has to be investigated. Notwithstanding the evident power of P-selectin/PSGL-1 – blocking antibodies, modulation of endothelial PSGL-1 glycosylation may also become an efficient tool in preventing or diminishing cardiovascular disease. In a similar way, modulation of PSGL-1 glycosylation on monocytes may inhibit PMC formation.

5. Platelet binding to certain leukocytes (monocytes and eosinophils) has been shown to increase the leukocyte recruitment to the endothelium and enhance the development of cardiovascular disease. However, this platelet-mediated enhancement of cell binding capacity is not necessarily always bad. Platelet binding
to other cells, e.g. dendritic cells, may improve the homing of such cells and thus enhance the patrol of foreign pathogens. Possible interactions of platelets with cells other than monocytes or eosinophils and their (patho)physiological role would also be an interesting topic of investigation.
Chapter 7. Discussion and concluding remarks

References


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