Thrombosis is a complex and dynamic phenomenon, involving interaction of endothelial and blood cells, plasma clotting, fibrinolysis and hemorrhheologic factors. Platelets play an essential role in the process of thrombosis\cite{1}. Interaction of platelets with collagen and/or thrombin initiates a complex biochemical sequence of signal transmission resulting in a functional cell response.

There are several signal mechanisms involved in platelet activation, including specific receptors, G proteins, phosphatidyl inositol metabolism, synthesis of thromboxane A$_2$ (TXA$_2$) and other eicosanoids, changes in cytosolic calcium, and protein phosphorylation, in serine/threonine and tyrosine residues\cite{2}. These signal mechanisms result in a conformational change in the GP IIb IIIa receptor, platelet secretion, platelet–platelet aggregation, exposure to procoagulant phospholipids in the membrane, clot retraction, and synthesis of different metabolic products.

We have found that the products released by activated platelets have a proaggregating action on the target platelets, favoring platelet recruitment and thrombus growth\cite{3-5}. To study these processes, we have developed an experimental procedure that allows activation and platelet recruitment to be assessed separately\cite{4-6}.

Platelets, leukocytes and erythrocytes appear in the thrombus\cite{7}. This might indicate that the interaction of platelets with other blood cells is important in regulation of the process of thrombosis. Platelet–leukocyte interaction inhibits platelet reactivity, whereas erythrocyte–platelet interaction enhances it\cite{3-6}. It has also been shown that this cell interaction alters the antithrombocytic effect of aspirin and dipyridamole\cite{4-15}.

A well-documented example of this intercellular interaction is the transcellular metabolism of eicosanoids, which occurs among different blood cells, and between blood and endothelial cells\cite{16,17}. In this process, arachidonic acid, compounds of their intermediate metabolism or the specific final products of the metabolism of one cell are changed by another one into specific products of the second, or into new compounds which neither cell can synthesize in an independent manner\cite{16}. Metabolic cooperation in the synthesis of
lipid mediators occurs through various cellular interactions, including interactions between platelets and endothelial cells, platelets and neutrophils, platelets and erythrocytes, endothelial cells and neutrophils, and neutrophils and erythrocytes[18-23].

**Platelet–neutrophil interaction:** Experimental studies suggest that polymorphonuclear neutrophils (PMNs) play a role in thrombosis[24]. It is thought that PMNs play a role in vascular disease through their capacity to adhere to endothelial cells and aggregate during acute inflammatory processes[25,26]. Activated PMNs release into the micro environment azurophilic granules, rich in proteases, and oxygen radicals, which may damage endothelial cells, disrupting their thromboregulating function[27-29]. Moreover, PMNs participate in thrombotic events by contributing to coagulant processes and by modulating different stages of the process of thrombosis[30].

There are conflicting reports on the role of PMNs in the process of thrombosis[31]. Some authors report that PMNs stimulate platelet activation, whereas others report inhibition by PMNs[6,12,32-38]. These conflicting results may arise from differences in experimental conditions between studies, in particular whether PMNs are studied at rest or after stimulation and whether whole cells or isolated compounds derived from stimulated leukocytes, such as the cathepsin G or oxidant products, are assessed. Various mechanisms by which PMNs might inhibit platelet function have been suggested: Synthesizing nitric oxide (NO), modulating synthesis of eicosanoids, modifying glycoproteins of the platelet membrane, generating H₂O₂ and other oxidants, membrane ADP-arg, or other mechanisms[6,12,34-42]. Physical proximity between cells is favored by P-selectin, an adhesion protein exposed on the membrane of activated platelets, which binds to a specific receptor in the PMNs, PSGP-1, and between the GP IIb IIIa on platelets and β² integrin on PMNs[33,43,44].

Aspirin amplifies the inhibiting effect of PMNs on thrombocytic reactivity, although it does not change the capacity of the platelets to expose P-selectin[6,12,37,45-47]. Thus, aspirin may not modify other processes depending on P-selectin that are important in the PMN-platelet interaction, such as the recruitment of PMNs by activated platelets to the forming thrombus in the area of vascular damage, or fibrin formation mediated by platelet P-selectin leading to the expression of tissue factor in leukocytes[30,44,48].

**Role of erythrocytes in hemostasis and thrombosis:** Erythrocytes (RBCs) also play a role in thrombogenesis[10,49]. This was first reported by Duke in 1910, who found that the prolonged bleeding times in anemic patients became normal after the hematocrit was increased[50]. This clinical association between bleeding times and the hematocrit were confirmed by other authors[51-54]. Erythrocytes have also been involved in atherothrombotic processes and associated with established risk factors such as diabetes, hypercholesterolemia, and hypertension[51,55-63].

Erythrocytes may participate in thrombus formation, changing the hemorrheologic properties of blood, modulating the antiaggregating action of the blood vessels, inactivating prostacyclin, eliminating NO (EDRF) and influencing thrombocytic function, although it has no effect on the ecto-ADP loops of the vascular endothelium[64-72].

**Effect of the erythrocytes on thrombocytic reactivity:** Erythrocytes act on platelets by physical and biochemical mechanisms[73,74]. Hellem and Gaarder reported for the first time in 1961 on the capacity of erythrocytes to increase thrombocytic function; in this study, platelet adhesiveness to glass increased in the presence of erythrocytes[75]. These authors suggested that ADP released by lysis or cellular microlysis of erythrocytes could be the biochemical factor responsible for increased adhesion-aggregation of platelets in the presence of erythrocytes[76]. It was later shown that erythrocytes increase platelet adhesion and thrombus formation in damaged endothelium[77]. They correct adhesion defects in patients with “storage pool deficiency (SPD)” depending on the hematocrit, they increase spontaneous platelet aggregation, enhancing platelet aggregation due to collagen, they increase platelet activation and recruitment and they shorten the formation time of the thrombus in the platelet function analyzer PFA-100[4,5,14,63,78-82].

The biochemical mechanisms that regulate the effects of erythrocytes on platelet function are not well known. We have shown that erythrocyte–platelet interaction increases in the presence of cellular stimulation, both by ADP concentration (7 times) and ATP (5 times); this occurs without erythrocyte lysis[55]. It is
worth mentioning that the potentiating effect of erythrocytes on the erythrocyte reactivity requires the simultaneous presence of activated platelets and intact erythrocytes[4,5]. The prothrombotic effect of erythrocytes does not occur in the presence of platelet stimulation or erythrocyte lysis. This suggests that, under experimental conditions, the effect of erythrocytes is related more to metabolic than physical factors, requiring the cellular integrity of the erythrocyte.

Erythrocyte–platelet interaction increases hydrolysis of the arachidonic acid of the phospholipids, as well as the metabolism of the platelet enzymes cyclooxygenase and lipoxygenase[4]. This interaction increases the release of eicosanoids and arachidonic acid to the cellular microenvironment, which could stimulate other platelets and provide the substratum needed for the transcellular metabolism with other blood cells. An example of this effect may be how the PMNs use the arachidonic acid in a thrombus formation to generate LTB$_4$ from the arachidonic acid released by the platelets[83].

Erythrocyte–platelet interaction also leads to a significant increase in release of TXA$_2$ and 12-HETE and generates proteolytic activity, which may participate in the erythrocyte effect on platelet activation[4,84,85]. This interaction also releases free radicals and iron, which may favor increased platelet activation[86,87]. More recently, we have shown that erythrocytes increase the activation of platelet glycoprotein IIb IIIa, playing a regulatory role in the intracellular biochemical mechanisms of platelet signaling[88,89]. Nevertheless, our knowledge of platelet-erythrocyte interactions remains incomplete, since not only do erythrocytes modify the biochemical and functional responses of platelets, but products released by platelets, such as PGE$_2$ or serotonin, may induce changes in erythrocyte metabolism, fragility and deformability[90-93].

Erythrocyte–platelet interaction: Effect of aspirin:

In high-risk patients, cardiovascular or neurological thrombotic episodes may be favored by increased platelet activation in areas of endothelial damage or plaque rupture[94]. Thus, inhibition of platelet function is important in the prophylaxis and treatment of ischemic and thrombotic processes in these patients. The drug of choice in these cases, aspirin, reduces morbidity due to vascular causes in about 15% of patients, and new non-fatal accidents in 30% of patients[95]. Treatment with aspirin is not protective in a high proportion of patients, however, and new therapies are needed to reduce thrombotic risk in these patients.

Aspirin inhibits synthesis of TXA$_2$ in an irreversible manner[96]. Thrombotic events in patients treated with aspirin could be due to biochemical mechanisms of platelets that surpass the aspirin effect. This might occur because of exposure of platelets to high concentrations of collagen or thrombin, or possibly ADP. Under these conditions, platelets do not require the amplification action of thromboxane to produce a proaggregation response, i.e., platelet activation is stimulated by cyclooxygenase 1 (COX-1) independent mechanisms[97,98].

We have found experimental evidence that aspirin not only acts on platelets by inhibiting COX-1, but also exerts a direct effect on erythrocytes, reducing their prothrombotic potential under certain conditions[5,14]. This newly identified antithrombotic effect of aspirin occurs when platelets of patients treated with aspirin (500 mg) are stimulated with low doses of collagen (0.5-1 µg/mL). Under these conditions, the platelets alone are completely inhibited and the erythrocytes of the same donor, also treated with aspirin, produce no increase in platelet stimulation. Conversely, erythrocytes obtained from the same patients before aspirin administration can significantly stimulate the aspirinized platelets[5]. Thus, it seems reasonable to suggest that a greater beneficial clinical effect could be achieved by blocking the prothrombotic effect of erythrocytes with appropriate doses of aspirin.

Experimental data indicate that the prothrombotic effect of erythrocytes is inhibited, in a dose-dependent manner, by 50–500 mg of aspirin; nearly-complete inhibition occurs after two hours of a single dose of 500 mg[14]. Moreover, the antithrombotic effect of 500 mg of aspirin on erythrocytes is reversible after some time and disappears within few days[14]. Therefore, we assessed whether a low daily dose of aspirin could maintain the effect of the initial dose of 500 mg for a longer time. To evaluate this possibility, normal donors were given an initial dose of 500 mg of aspirin, followed by 50 mg/day. This dosage reduced the prothrombotic effect of erythrocytes for 2–3 weeks, but normal conditions were restored after 3–5 weeks[14]. Thus, to control simultaneously the prothrombotic activity of platelets and erythrocytes in normal subjects, a dose of 500 mg
every 15 days along with a daily dose of 50 mg seems to be appropriate[14].

We also assessed to what extent the usual doses of aspirin given in our medio to patients with cardiovascular or cerebrovascular disease (200–300 mg/day) were appropriate to inhibit simultaneously the prothrombotic actions of platelets and erythrocytes. Eighty patients (62 with cardiovascular pathology and 20 with stroke) were assessed. The results showed that these doses of aspirin inhibited TXA2 synthesis in more than 94% of patients[81]. Nevertheless, when platelet recruitment was assessed, we found that 39% of patients showed complete inhibition, both in the platelets alone and in presence of erythrocytes. In 45% of patients, the response of isolated platelets was completely inhibited, but the inhibition was not enough in the presence of erythrocytes. Finally, suboptimal inhibition of platelet activity was observed in the remaining 16% of patients, in the platelets alone and in the presence of erythrocytes. Therefore, we conclude that, in almost 60% of the patients treated with 200–300 mg aspirin/day, aspirin was not sufficient to block the platelet reactivity in the presence of erythrocytes, despite the inhibition of TXA2 synthesis. Another finding of interest is that, in aspirin treatment, a larger proportion of cerebrovascular compared with cardiovascular patients show suboptimal control of platelet function when whole blood is assessed. This may indicate that aspirin exerts a different effect on the platelet response in different vascular areas, which could be stronger in coronary than brain areas[99]. Moreover, we have observed that the effect of aspirin is not homogeneous in all patients, consistent with other reports; drug resistance has been reported in some patients[100,101].

In further studies, we proved that 500 mg of aspirin can inhibit the prothrombotic effect of erythrocytes in different groups of patients, as has been observed in normal subjects[14]. In contrast the daily dose of 50 mg/day is clearly not enough in vascular patients, especially in the presence of erythrocytes, although TXA2 synthesis is inhibited[14,102]. Thus, at present we are assessing the efficacy of a dose of 500 mg every 15 days, and higher daily doses of 100 and 200 mg given once daily, or the same dose divided in two intakes every 12 hours. Preliminary results of this on-going study suggest that better control of platelet recruitment in whole blood is achieved with a loading dose of 500 mg per day every 15 days, with a daily maintaining dose of 100 or 200 mg/day, compared with a dose of 200–300 mg/day given in a continuous manner[103]. These data show that the dose of aspirin is important for the control of the prothrombotic effect of erythrocytes. Furthermore, we have found that the same dose of aspirin given every 12 hours significantly increases the inhibition of platelet recruitment in whole blood. We believe that this could be related to platelet turnover. The daily platelet turnover in normal subjects is about 10%, and turnover increases in patients with atherosclerosis[104-106]. Four hours after aspirin administration, platelets with active cyclooxygenase can be found in the blood circulation[107]. From a functional point of view, it is known that platelet activation can be maintained by a residual capacity of TXA2 synthesis of 10% in aspirin-treated platelets; among platelets treated with aspirin only, a small number of functional platelets exhibit increased reactivity, both in vitro and in vivo, in thrombus formation[108,109]. We have also shown that, when only 7% of the platelets are left untreated with aspirin, platelet recruitment occurs. Thus, the effects mentioned above account for better control by aspirin given every 12 hours, since it reduces the number of platelets not treated with aspirin.

At present, it is known that aspirin is effective in a wide range of doses from 50 to 1500 mg/day; in high-risk patients, any of these doses reduces vascular events to a similar extent[95,99]. This is consistent with the hypothesis that the effect of aspirin on platelet COX-1 is saturable and shows no dose-dependence[99]. Nevertheless, the optimal dose of aspirin is still a controversial issue. Since there are gastrointestinal side effects (which are dose-dependent) and risk of hemorrhage (not dose-dependent), it seems advisable to recommend the minimal effective dose in each type of vascular pathology[99,110,111]. There are other effects of aspirin which are not associated with TXA2 synthesis, such as effects on coagulation, fibrinolysis, trombin generation, or the prothrombotic effect of erythrocytes mentioned above[99]. These effects of aspirin are not related to the synthesis of eicosanoids and are less characterized than the effects of aspirin on COX-1 but probable play also a role on thrombogenesis. The average doses recommended by different scientific consensus inhibit COX-1 in platelets. However as suggested by our data, some of aspirin’s mechanisms of action, such as the effects on erythrocytes or leukocytes, may play also an
important role in the occurrence of ischemic and thrombotic events. Thus, to allow prescription of the minimal effective dose, we advise that the antithrombotic effect of aspirin should be assessed in each patient in platelets and in whole blood.

REFERENCES


57. Tohgi H, Yamanousi H, Murakami M, Kameyama M.


73. Reimers RC, Sutera SP, Joist JH. Potentiation by red blood cells of shear-induced platelet aggregation: Relative importance of chemical and physical mechanisms.


Address for Correspondence:
Justo AZNAR, MD
Departamento de Biopatología Clínica
Hospital Universitario La Fe
Avda. Campanar 21
46009, Valencia, SPAIN

Turk J Haematol 2002;19(2):103-111
Cell-Cell Interactions in Thrombosis: Modulation of Platelet Function and Possibilities of Pharmacological Control with Aspirin Santos MT, Vallés J, Aznar J.