Recent Progress in the Role of Platelets in Occlusive Vascular Disease

AARON J. MARCUS, M.D.

Platelets are derived from bone marrow megakaryocytes by extension of cytoplasmic processes which undergo attenuation, develop constrictions at the distal ends, and then rupture, thereby releasing platelets. During its life span of approximately 10 days the unstimulated platelet functions in some unknown manner to maintain vascular integrity. The platelet is also the only blood cell component involved in the uptake and transport of serotonin (5-HT). If blood vessel continuity is interrupted, the vessel contracts and concomitantly platelets at the site are activated. Activation can also take place intravascularly by pathological stimuli such as endotoxin and immune complexes generated in certain disease states. Adhesion of platelets in proximity to the subendothelium occurs and this is accompanied by aggregation (cohesion) of additional platelets which have been "recruited" into the microenvironment. This sequence (primary hemostasis) is modulated by adhesion of platelets to subendothelial collagen, formation of thromboxane A2 (TXA2), mobilization of intraplatelet calcium, release of adenosine diphosphate (ADP) and 5-HT (fig. 1). Platelet activation also results in exposure of specific surface receptors which bind fibrinogen and this bound fibrinogen is a cofactor for aggregation. Such exposure is inhibited when platelet cAMP levels are elevated by agents such as prostacyclin (PGI2).3

The stimulated platelet undergoes a unique morphological change from that of a disc to a spiny sphere* and the surface membrane phospholipoprotein develops the capacity to catalyze interactions between activated coagulation proteins, culminating in thrombin formation and fibrinogen polymerization (secondary hemostasis). The process of clot retraction is initiated when platelets form pseudopodia which adhere to fibrin strands at points where the strands cross one another. The platelet pseudopodia then contract and draw the sides of injured vessels together. Clot retraction requires ATP, glucose, calcium and normal fibrin formation. Formation of the hemostatic platelet plug is then complete.

Stimulated platelets also secrete proteins which were originally synthesized in the megakaryocyte. Among these are platelet factor 4 (PF-4), which has anti-heparin properties and can react with heparan sulfate in the vessel wall. The platelet-derived growth factor (PDGF) which stimulates smooth muscle cell proliferation is also released. PDGF has been implicated in the atherosclerotic process.4

Platelets and Thrombosis

Arterial thrombi resemble hemostatic plugs that they form via an interaction of platelets with an injured vascular surface. Morphologically such thrombi contain mainly adherent platelets at the interface and a mixture of leukocytes, fibrin and erythrocytes in the distal portion. These observations prompted the use of pharmacologic agents capable of suppressing platelet aggregation, release and adhesion.3 In the early 1970's clinical trials were initiated with the overall goal of attempting to prevent or reduce platelet accumulation in diseased vessels of the heart, brain, extremities and on vascular prostheses.5-8 To date analyses of almost every clinical trial have been fraught with interpretive difficulties.5-8

Attempts were initially made to prolong platelet survival with platelet inhibiting drugs plus anticoagulants in patients with valve prostheses and with aortocoronary bypass grafts, since shortened platelet survival as measured by isotopic techniques correlated with malfunctioning prostheses and thromboembolic phenomena.5 In arterial thromboembolism, increased con-
Thrombin - Collagen - Epinephrine

FIGURE 1. Simplified scheme of events following platelet stimulation. Platelet stimuli vary in their ability to initiate the process of TXA₂ synthesis. Formation of TXA₂ is followed by calcium mobilization, and the presence of free calcium inhibits platelet adenylate cyclase. Such inhibition initiates events leading to aggregation. Free calcium also stimulates dense granule secretion. Among the most important secreted products are fibrinogen (which is a co-factor for aggregation), serotonin and ADP. Released thromboxane and ADP then serve to "recruit" other platelets into the hemostatic plug or thrombus — as the case may be. In contrast, platelet aggregation and release are inhibited by prostaglandins I₂, D₂ and E₇ (which is not a natural product). The latter prostaglandins stimulate adenylate cyclase to varying degrees which results in elevations in platelet cAMP, consequences of which include inhibition of both phospholipase A₂ and calcium mobilization. The sequence shown here is mainly hypothetical because the events following exposure to a platelet stimulus are so rapid that currently available techniques cannot monitor them accurately. (Courtesy of the Upjohn Co.)

The purpose of this review is to summarize current concepts of arachidonic acid (20:4) metabolism in platelets and neutrophils and to discuss new information on interactions between different cell types involving this pathway. Some of these phenomena may be directly applicable to problems in pharmacologic modification of platelet function.

Mobilization and Metabolism of Arachidonic Acid

The compounds depicted in figs. 2 and 3 are derived from oxygenation of arachidonic acid. They are formed mainly via enzymatic pathways and the major end products are known as "eicosanoids." These include "classical" prostaglandins (PGD₂, PGE₂, PGF₆, hydroxy acids, thromboxanes, leukotrienes and prostacyclin. Since there is no free arachidonate in cells, it must initially be released from cell phospholipids by the action of phospholipase(s). In prevention and treatment of thrombosis, a phospholipase inhibitor might be useful for blocking this initial step in the arachidonic acid cascade. In cells which synthesize protein (in contrast to platelets), steroids induce synthesis of macrocortin — a polypeptide which inhibits phospholipase activity. As will be mentioned subsequently, more platelet arachidonate is released than is utilized for eicosanoid formation and some of this released 20:4 can be taken up and metabolized by other stimulated cells such as neutrophils.

The Cyclooxygenase Reaction

Cyclooxygenase is a particulate enzyme which is preferentially activated by free arachidonate. Endoperoxides — the initial oxygenation products are formed

The consumption of platelets as measured by rapid platelet turnover is an accurate parameter for monitoring thromboembolic events. Unfortunately, use of this sensitive and specific technique is not practical in most clinical situations because it involves labeling of the patient's platelets with radioactive elements. Alternative tests currently in use are those which detect circulating platelet aggregates, spontaneous platelet aggregation, increased sensitivity to aggregating agents and measurements of secreted platelet proteins. These procedures present at least two difficulties in interpretation: first they may reflect "epi-phenomena" and second they do not directly tell us whether the platelets have aggregated and then disaggregated. They do however alert the physician to the presence of stimulated platelets in the circulation. Thus there are detectable platelet functional abnormalities (in addition to increased platelet turnover) in thromboembolic diseases, but whether they can serve as accurate guides to diagnosis, localization and therapy is not entirely clear.

Unfortunately clinical assessment of the therapy of occlusive vascular diseases is not highly accurate either and has rendered evaluation of therapeutic trials difficult. However, incomplete knowledge of the basic pharmacology of the drugs employed on the metabolism of platelets and other tissues is, in our opinion, an even greater obstacle in evaluating their efficacy. This is particularly pertinent in the case of aspirin and sulfipyrazone as well as dipyridamole. In many cases it is not clear which action of the drug was responsible for beneficial effects observed or why no benefit was obtained in instances where improvement should theoretically have occurred.
rapidly (platelet oxygen burst), and in platelets, endothelial cells and other tissues are the precursor molecules for prostaglandins, TXA₂, and PGI₂. Since non-steroidal antiinflammatory agents such as aspirin inhibit cyclooxygenase the reaction is of great clinical significance. In platelets the inactivated enzyme is not replaced because of the absence of protein synthesis. Inhibition of platelet cyclooxygenase by aspirin and its consequent clinical effects on platelet function due to the absence of thromboxane formation was the major justification for initiating clinical trials in thrombosis prevention. In re-evaluating results of most clinical trials to date, the question has arisen as to whether beneficial effects observed with aspirin were due to effects other than cyclooxygenase inhibition. The latter also applies to sulfinpyrazone. For example in some studies women did not benefit from aspirin administration. This observation is difficult to comprehend if only cyclooxygenase inhibition was involved. It would have been of interest to know whether the females who did not respond demonstrated any alterations in bleeding time after aspirin ingestion. Sulfinpyrazone is a weak, reversible cyclooxygenase inhibitor and its beneficial effect may be in preventing endothelial injury and cardiac arrhythmia. Aspirin therapy was effective in venous thrombosis — which is also difficult to explain since platelets are not the major component of venous thrombosis and thrombin-fibrin formation is involved.

In human vascular endothelium endoperoxides are converted mainly to PGI₂ which induces strong vasodilation and inhibits platelet aggregation via elevation of cAMP. Under conditions which favor close cell contact, endothelial cells can utilize platelet endoperoxides for PGI₂ production. Some investigators have proposed that a balance may exist between thromboxane and prostacyclin production in the maintenance of blood fluidity, but this has become controversial. Although aspirin-treated endothelium can renew PGI₂ production via protein synthesis, low doses of aspirin have been recommended in order to inhibit only the more sensitive platelet cyclooxygenase. Against this hypothesis are several instances in which aspirin in doses of 1 gm or more per day were utilized with successful results.

The Lipoxygenase Reaction

In contrast to cyclooxygenase, the initial products of which (endoperoxides PGG₂ and PGH₂) are common to all tissues, lipoxygenases are tissue-specific with regard to positional specificity of the oxygenation step. Platelets contain a 12-lipoxygenase which catalyzes insertion of a hydroperoxy group at position 12 of arachidonate followed by reduction by a peroxidase to a hydroxyl group. The platelet lipoxygenase can be substrate-activated in the absence of other stimuli. In contrast neutrophils contain a 5-lipoxygenase which is not substrate-activated and requires cell stimulation leading to formation of eicosanoids such as leukotrienes and other hydroxy acids (figs. 2 and 3). Although platelet 12-HETE (fig. 3) is chemotactic for other cells, a direct effect on platelet function by its lipoxygenase products has not as yet been demonstrated. However lipoxygenases are not inhibited by aspirin and platelet lipoxygenase products have been found to interact with leukocytes by various mechanisms. Such information on cell-cell interactions was not available when aspirin trials were initiated, and we do not know whether these phenomena result in beneficial or detrimental effects, if any.

Recently Borgeat and associates isolated a new metabolite of arachidonic acid which formed following addition of 12-HETE to leukocytes which were stimulated with ionophore A23187. This product was identified as 5 S, 12 S-diHETE which is a stereoisomer of leukotriene B₄. The full spectrum of biological activity for diHETE has not as yet been determined, but it appears to be a major product in platelet-neutrophil interactions.

In an extension of previous studies of cell-cell interactions we directly demonstrated that aspirin-treated platelets provided precursors for the formation of neutrophil leukotrienes and diHETE. Platelets radiolabeled with arachidonic acid were stimulated with ionophore A23187 in the presence of unlabeled neutrophils. Several radiolabeled products not produced by platelets alone were detected. These included: LTB₄, diHETE, and 5-HETE. The results indicated that platelet-derived arachidonate served as precursor for LTB₄ and 5-HETE synthesized by the neutrophil. Platelet-derived 12-HETE was converted to diHETE by the neutrophil.

Thus, stimulated platelets from a subject who has ingested aspirin (or other non-steroidal anti-inflammatory agents) remain capable of serving as sources of compounds synthesized by other cells. These substances possess biological activities such as chemotaxis, neutrophil activation and smooth muscle contractibility. Perhaps previously unexplained beneficial effects of aspirin on ischemic vascular diseases may eventually be attributable to products produced in cell-cell interactions from precursors which are quantitatively increased due to diversion of arachidonate away from the cyclooxygenase pathway.

Concluding Remarks

The clinical trials involving aspirin and sulfinpyrazone were devised because of the ability of these compounds to induce a platelet functional defect. Clinically, the aspirin defect is manifested as a prolongation of

Precursors for prostaglandins, TXA₂, and PGI₂.
the bleeding time and the corollary assumption was that platelets would be less effective as participants in thrombotic diatheses. Subsequently it was shown that aspirin permanently acetylated and inactivated platelet cyclooxygenase (fig. 4). However aspirin may also affect additional platelet proteins as well as those in other tissues. Furthermore sulfinpyrazone may exert a beneficial protective effect on the vasculature. The lipoxygenase step results in formation of 12-HPETE and 12-HETE from arachidonate and is slightly delayed but not inhibited by aspirin. (Courtesy of the Upjohn Co.)

**Figure 2.** Pathways of arachidonic acid metabolism in human platelets and endothelial cells. Free arachidonate is metabolized by two different enzymatic mechanisms. Formation of the endoperoxides PGG$_2$ and PGH$_2$ is catalyzed by cyclooxygenase. The endoperoxides are pivotal compounds in all tissues in which they form. Thus, in platelets they are converted to thromboxane A$_2$ and in the endothelial cell PGI$_2$ is formed. In addition endothelial cells synthesize PGF$_{2\alpha}$, PGE$_2$ and PGD$_2$. The cyclooxygenase pathway is inhibited irreversibly by aspirin and reversibly by sulfinpyrazone. The lipoxygenase step results in formation of 12-HPETE and 12-HETE from arachidonate and is slightly delayed but not inhibited by aspirin. (Courtesy of the Upjohn Co.)

**Figure 3.** Leukotriene synthesis from arachidonic acid. In leukocytes there is a 5-lipoxygenase which converts arachidonic acid into 5-HPETE which can then be converted to LTA$_4$ a common precursor of leukotrienes B$_4$, C$_4$, and D$_4$. Leukotrienes possess numerous biological properties which may play an important role in allergic and inflammatory diseases. Neutrophils process LTA$_4$ mainly to LTB$_4$ which appears to be a regulator of neutrophil function. In mononuclear cells and basophils LTA$_4$ combines with glutathione in a reaction catalyzed by glutathione-S-transferase which results in the formation of leukotrienes C$_4$ and D$_4$. The latter are components of the slow-reacting substance of anaphylaxis (SRS-A) and have the ability to induce smooth muscle contraction and increase vascular permeability in small blood vessels. The lipoxygenase pathway described here is not affected by aspirin and may account for some of the side-effects of aspirin ingestion. Neutrophils can utilize metabolic intermediates from aspirin-treated platelets for the production of leukotrienes and other hydroxy acids. (Courtesy of the Upjohn Co.)

**Figure 4.** Metabolic fate of released arachidonate as currently understood. Following its release from phospholipid, some arachidonate can leave the cell. It can then be processed by a cell in the immediate microenvironment. In the absence of aspirin a portion of the 20:4 is rapidly converted to endoperoxide by cyclooxygenase. The lipoxygenase also acts on arachidonate and continues to do so until the substrate is no longer available. In the presence of aspirin, arachidonate which would ordinarily be available to cyclooxygenase is diverted toward hydroxy acid production. The precise role of reacylation and hydrophobic binding by albumin has not been determined but may play a role in the modulation of platelet function by therapeutic agents currently in use or those which may be developed in the future.
ronment. We do not know the effects of excess free arachidonate in the aspirin-treated platelet on reactivation reactions or binding to albumin. On theoretical grounds a platelet phospholipase inhibitor might be effective, since all pathways of arachidonate metabolism would be blocked. Thromboxane synthetase inhibitors are now under study since they may also promote $\text{PG}_2$ production in the absence of $\text{TXA}_2$ via endoperoxide transfer from platelets to endothelial cells.

Unfortunately this report must be concluded in a paradoxical and rather unscientific manner. We still recommend low-dose aspirin and dipyridamole as a therapeutic modality for patients with ischemic vascular diseases, but only because they appear to be better than nothing and may not be harmful. Nevertheless one should keep in mind the statement by Sir Karl Popper: “Science really is nothing more than a continuous abandonment of ideas shown by objective, scientific inquiry, to be untenable.” Thus, further clinical trials at this time would not seem to be indicated because we do not know: a) which patient subgroups to select, b) which mechanisms of thrombosis predominate in a given patient — thrombin formation or aggregation as induced by ADP and thromboxane, c) the optimal dose to employ, and d) the implications of cell-cell interactions via the arachidonate pathway.

References

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A J Marcus

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