PLATELETS AND VASCULAR OCCLUSION

Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view

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Platelets in hemostasis

When blood vessels are injured so that they bleed, circulating platelets adhere to the damaged vessel walls and aggregate, so diminishing or arresting the hemorrhage. This interaction between platelets and vessel walls therefore has an easily demonstrable physiological function. There is much clinical and experimental evidence that a deficiency or defect in circulating platelets is associated with "spontaneous" hemorrhages from small vessels. This suggests that platelets are somehow essential for the functional integrity of these vessels, but if there is such a mechanism it has yet to be established.

Platelets in atherogenesis

The old thrombogenic hypothesis of atherosclerosis has recently reappeared in modern costume as claims that platelets contribute to atherogenesis in three ways: first, through damaging arterial endothelial cells by releasing injurious agents, presumably where circulating platelets adhere; second, through the release in such situations of a factor responsible for smooth muscle proliferation in the arterial wall; and third, through the formation of persistent mural thrombi, which are organized into intimal thickenings. Such evidence as there is for these propositions fails to establish any of them as relevant to atherosclerosis in animals or human beings. Underlying all three claims is the assumption that some normal circulating platelets settle on arterial walls long enough to release some of their contents. There is no observational basis for this assumption in normal arteries, although quantitative observations should now be possible with modern imaging techniques.

Because the assumption is essential for the various claims referred to above, we have begun an investigation designed to quantify the adhesion of circulating platelets in different types of blood vessel. So far, the observations have been mainly on small veins. However, some observations were made also on small arteries for comparison. The venous vessels were chosen for quantification in preference to arteries because the comparative slowness of blood flow made it easier to develop and control the new methods required.

The results showed that the injection of hamster platelets, made fluorescent by labeling in vitro with fluorescein isothiocyanate, into the circulation of other hamsters makes it possible to quantify the flowing and sticking of platelets in vivo. The experiments provide evidence that there is very little tendency for circulating platelets to stick to the walls of small veins and arterioles, even under conditions in which the adhesion propensities of both platelets and vessel walls might be expected to be increased by preparative manipulations. In venules such sticking as there was affected only a very small proportion of circulating platelets and those only for very short periods. In arterioles, sticking appeared to increase somewhat under conditions in which the blood flow could be assumed to be nonlaminar. If the sticking behavior of platelets is at all similar in larger arteries, it seems unlikely that it would permit the release of platelet constituents capable of affecting the arterial walls, except just possibly under hemodynamic conditions such as occur at branches or other major unevennesses in the walls; but platelets are not normally found even there.

It is assumed further that arterial endothelium is continuously subject to "damage" or "injury" of some kind as a precondition for the adherence of platelets. There is no convincing evidence for this generalization, especially not in human beings. The first lesions of atherosclerosis, the fatty streaks, commonly begin during childhood or adolescence. It seems most improbable that these could be caused by any conceivable "injury." Specifically, the common assertion that damage is produced by shear stresses in the high-pressure blood system is inherently improbable because of
functional adaptations in the course of evolution. The only finding that could conceivably apply to human arteries is that guinea pig aorta has a higher replacement rate of endothelium around the openings of branches than elsewhere. This is most simply explained by assuming that endothelial turnover depends, inter alia, on hemodynamic effects of nonlaminar blood flow over such areas. But this should be thought of more correctly as a quasiphysiologic effect and, even there, platelets are rarely if ever seen adhering to the walls. The turnover rate of endothelium is increased in experimental hypertension. This is compatible with hypertension as a "risk factor" for coronary heart disease. It seems more likely that this is caused by an accelerating effect of interendothelial gaps on plasma lipoprotein accumulation than to an increase in the indiscriminate or even selective deposition of platelets on arterial walls.

**Platelets in thrombogenesis**

There is conclusive evidence that occlusive thrombi in arteries damaged by atherosclerosis contain platelets as a major, if not the main, component. The formation of platelet thrombi appears so similar to that of hemostatic plugs of platelets that analysis of the mechanism of the latter is likely to provide an understanding of the former. This article poses questions about how the thrombogenic mechanism depends on the hemodynamic and chemical environment in which platelets aggregate on acutely damaged vessel walls.

Both the gross and the histologic appearance of arterial thrombi establish that their central mass consists mainly of aggregated platelets. What, therefore, is the mechanism responsible for rapid and extensive platelet aggregation in an artery as an apparently random event in time? Close serial sectioning of obstructed coronary arteries has established that the platelet thrombus responsible is invariably associated with recent hemorrhage into an underlying atherosclerotic plaque. The hemorrhages occur through fissures or fractures in the plaque, and the sudden appearance of such a fissure or fracture may well be the random, individually unpredictable event affecting coronary arteries that has to be assumed to occur to account for the clinical onset of acute coronary thrombosis.

How does hemorrhage into a ruptured plaque start off platelet thrombogenesis? This can be regarded as part of the general question of how platelets are caused to aggregate through hemorrhage, and most effectively through hemorrhage from arteries. Until recently this question was commonly answered by assuming that the process depends on the adhesion of platelets to collagen, which is exposed where damaged vessel walls are denuded of endothelium. Adhering platelets then release other agents, including thromboxane A₂ and ADP, which in turn are responsible for the adhesion of more platelets as growing aggregates. This explanation is unlikely to be correct for the following reasons. First, hemostatic and thrombotic aggregates of platelets grow without delay and very rapidly. For example, when an arteriole 200 μm in diameter is cut laterally, the rate of accession of platelets to the hemostatic plug is of the order of 10⁴/sec. In contrast, although the adhesion of platelets to collagen itself is almost instantaneous, the subsequent aggregation of platelets, even under optimal conditions for their reactivity, begins only after a delay or lag period of several seconds. Second, platelets tend to aggregate as mural thrombi when anticoagulated blood flows through the plastic vessels of artificial organs such as oxygenators or dialyzers that contain no collagen or anything else capable of activating platelets similarly. This implies that there are conditions under which platelets are activated in the blood by something other than collagen or other constituents of the walls of living vessels.

The plaque on which a thrombus grows has usually narrowed the arterial lumen. At constant blood pressure the flow of blood is faster through the constriction than elsewhere in the artery. Therefore high flow and wall shear rates do not prevent the aggregation of platelets as thrombi. Indeed, the question arises of whether the activation of platelets that precedes their aggregation depends in some way on such abnormal hemodynamic conditions.

The effectiveness of platelet aggregation in plugging a leak is at least as effective in arterioles as in venules. Because the hemodynamic situation should be more unfavorable to the formation of aggregates in arterioles than in venules, an explanation of arteriolar hemostasis is likely to account in principle also for that in venules. For that reason, the following considerations are limited to arterioles.

When an arteriole is cut, platelets are seen to adhere with great rapidity to the damaged vessel wall, while the red cells continue to rush by. This high flow velocity in relation to the small size of the vessels implies the presence in the fluid of strong mechanical forces acting normally and tangentially on and near the vessel walls. The cut causes peripheral resistance to the flow to diminish suddenly, and if the inflow pressure remains constant the mean flow velocity increases. Thus the fluid-mechanical forces on platelets adhering and aggregating on the vessel wall becomes greater still. With increasing size the platelet aggregates tend to
constrict the cut, causing a further, although usually temporary, increase in flow velocity.

The blood-flow velocities that would be experienced by platelets closest to the vessel wall, and therefore with the highest probability of colliding with the sites of damage, can be calculated. Human platelets have a major diameter of about 1.5 μm. In an arteriole of medium size, the flow velocity of plasma and of any cells in it at a distance of 1 μm from the wall is of the order of 10 to 100 μm/msec. Therefore a platelet flowing within a distance no greater than its own diameter would pass an injury site 100 μm long in at most 10 msec. In the absence of other influences, this would seem to be the time available for such a platelet to adhere to the damaged wall.

The time just calculated as available to circulating platelet “at risk” for adhering to a wall lesion has to be compared with what is known about the time required for platelets to be activated into a condition in which their collision with such a lesion would very probably result in adhesion. That a process of activation is an essential prerequisite for adhesion and aggregation is implied by the nonreactivity of normal circulating platelets. Presumably, activation consists of a sequence of physical and biochemical events analogous to the activation of muscle. The sequence is still being worked out, but several similarities with muscle are already established. Thus it is known that in platelets an early event is an increased influx of sodium unaccompanied by an equivalent amount of chloride and that activation is associated with an increase of free calcium in platelets.

Because activation is indicated by adhesiveness, the change must involve one or more constituents of the outer surface of platelets. There is evidence that the essence is the exposure of surface receptors for fibrinogen, which has long been known to be an essential and specific plama cofactor for platelet aggregation. The activation time of platelets may then be defined as the interval between the encounter of platelets with an activating agent such as ADP and their ability to react with plasma fibrinogen.

That circulating platelets can be activated to adhere in much less time than that required by their gross changes in shape, i.e., t1/2 = 1 to 2 sec at 37°C, is indicated by direct experimental observations. An arteriole can be irradiated by a laser in such a way that damage is limited to a few square micrometers of endothelium. The site of damage is covered almost immediately with platelets that must have been activated in small fractions of a second.

Very similar events follow the application of the activating agent ADP by microiontophoresis to the outside of an arteriole or venule under conditions in which appropriate controls indicate that there is no evidence at all of damage to the endothelial layer. Platelet aggregates grow in the vessel exactly opposite the tip of the micropipette, while the blood continues to flow rapidly and without noticeable disturbance over the site. This is explained most simply by assuming that sufficient ADP diffuses between the endothelial cells into the blood to reach platelets passing close to the wall and that this ADP activates them very rapidly indeed.

An extension of this technique has provided a basis for calculating an average activation time for circulating platelets. It was found that the size of platelet aggregates produced by the iontophoretic application of ADP increases exponentially. The rate constant of this increase depended on the mean blood flow velocity, determined in the same vessels at the same time. The shape of the experimentally determined curve was simulated closely by a theoretical curve that was derived on the single assumption that platelets require an activation time of about 100 to 200 msec. This time is still one to two orders of magnitude greater than that indicated by the earlier theoretical considerations, so either this experimental derivation overestimates the true activation time or the earlier considerations failed to take something into account that would allow flowing platelets more than a few milliseconds for activation. For example, more time would be available if the blood flow near the vessel wall were nonlaminar, so that platelets caught up in vortices, however small, might be exposed to localized activating conditions for longer than they would otherwise be. When branching vessels of the microcirculation are observed microscopically, platelets can often be seen trapped in vortices for variable times of up to several seconds. Such delays may occur in the immediate vicinity of major vessel wall lesions, whether caused by disease such as the sudden rupture of an atheromatous plaque or by traumatic injury such as a puncture or transection. However, there is no evidence of even the smallest disturbances in the flow of blood in a normal vessel in which platelets are caused to adhere by iontophoretically applied ADP. Moreover, it seems most unlikely that any endothelial unevenness produced by laser injury would give rise to flow disturbances large enough to delay the passage of platelets.

The chemical environment of platelets aggregating in vivo has been in doubt until recently. Platelets are activated by various chemical agents in vitro, and it is uncertain which if any of these are responsible for the

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hemostatic aggregation of platelets in living blood vessels. Recent evidence\textsuperscript{29} suggests that ADP is involved in activating platelets in vivo. Novel techniques were developed for the reproducible determination of bleeding times from small arteries of rats and rabbits in the territory supplied by the superior mesenteric artery. One of its main branches was cannulated and infusions were made into the mesenteric circulation of one or other of two enzyme systems that are specific for removing ADP. The reactions catalyzed by these enzymes are as follows:

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\begin{align*}
\text{creatine phosphokinase} & : \quad \text{ADP} + \text{creatin phosphate} \xrightarrow{\text{Creatine kinase}} \text{ATP} + \text{creatin} \\
\text{Pyruvate kinase} & : \quad \text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{ATP} + \text{pyruvate}
\end{align*}
\]

For both reactions, the equilibrium at physiologic pH is far to the right. In both species these infusions increased the bleeding times very significantly. This suggests that the observed increases in bleeding time are caused by removal from the blood plasma of ADP, the presence of which makes a major contribution to the activation and hemostatic aggregation of arriving platelets. This conclusion is supported by control experiments in which creatine phosphate was infused alone, without creatine phosphokinase. We have shown this enzyme to be present in blood plasma of both rats and rabbits, in concentrations somewhat higher than in human plasma.\textsuperscript{30} Completion of the ADP-removing system by the naturally occurring plasma enzymes could therefore account for the observed increases in bleeding time in these control experiments. In each experiment, control bleeding times were measured before and after infusions of the enzyme systems. Changes in general factors that could be expected to influence bleeding time values, namely the moderate decreases in blood pressure, blood platelet concentration, and hematocrit that were observed, were thereby taken into account. There is other evidence\textsuperscript{31} that the bleeding time is not affected by moderate decreases in blood platelet concentrations, i.e., as long as they remain above about $5 \times 10^7$ platelets/ml.

One reservation about the conclusion arises out of the presence of nucleotide-dephosphorylating enzymes in normal blood, both in plasma\textsuperscript{32} and as ectoenzymes on the surfaces of different types of circulating cells.\textsuperscript{33, 34} These enzymes hydrolyze the $\gamma$-phosphate of ATP several times more rapidly than $\beta$-phosphate of ADP. Both of the infused enzyme systems convert ADP to ATP, which the dephosphorylating enzymes presumably reconvert into ADP. It must be assumed, therefore, that the overall balance between the reaction rates in both directions is sufficient to diminish the concentration of ADP.

Another reservation comes from recent observations showing that creatine phosphate plus creatine phosphokinase inhibits platelet aggregation induced by the stable thromboxane analogue U46619\textsuperscript{35} under conditions in which the concomitant presence of ADP is unlikely.\textsuperscript{36} More evidence in support of an essential role of ADP in platelet hemostasis will therefore depend on substituting for the above enzyme systems one, such as apyrase, which only dephosphorylates ADP to AMP. Apyrase has already been shown to increase the bleeding time from transected vessels in isolated rabbit mesenteries\textsuperscript{31} and from punctures or cuts into artificial vessels.\textsuperscript{37} Apyrase also prevents the activation of platelets during their preparation.\textsuperscript{38} All this is in vitro or ex vivo evidence for the dependence of the hemostatic aggregation of platelets on free ADP in the blood. The results reported here are the first to provide similar evidence in vivo, as far as the primary physiologic function of platelets is concerned. Intravascular coagulation associated with the generalized Schwartzman phenomenon in rabbits can be prevented by the infusion of creatine phosphate with creatine phosphokinase,\textsuperscript{39} but that is under pathologic conditions in which damaged vascular or blood cells, possibly including platelets, can be expected to release ADP among other agents into the circulating blood.\textsuperscript{40}

Our observations increase current interest in the following questions. First, is it possible to demonstrate plasma ADP directly in blood at sites of hemostasis or thrombosis at concentrations required to activate platelets? Second, what is the cellular source (or sources) of the ADP? And third, how does this evidence affect therapeutic possibilities for the prevention or reversal of intravascular, particularly arterial, thrombosis?

It has recently become possible to provide more direct evidence for the presence of ADP in blood emerging from vascular injuries in experimental animals and human subjects.\textsuperscript{41, 42}

A new technique was developed for the measurement of extracellular free ATP in very small samples of whole blood with the luciferin-luciferase enzyme system. The method had a very low background corresponding to approximately $10^{-10}$ moles of ATP. ATP was measured in blood as it emerged during hemostasis after precise puncture of rat and rabbit arteries and after standardized incisions of human skin by the Simple device.

The initial concentration of free ATP in blood emerging 2 to 4 sec after vascular injury was about $2 \times 10^{-7}$ M in rats and rabbits and about $2 \times 10^{-6}$ M in...
humans. The free ATP concentration increased to about $10^{-5}$ M to 5 min after injury, and these increases could be prevented by heparin (20 U/ml).

The source of the initial free ATP was identified as damaged cells in the injured vessel walls. It could be inferred that sufficient ADP, both released as such with ATP and generated by the rapid enzymic dephosphorylation of ATP, is present at the site of injury to initiate the hemostatic aggregation of platelets. The results confirm a long-standing proposition\(^\text{13}\) that at sites of vascular injury, enough ADP is rapidly released from damaged cells into the blood to account for the hemostatic aggregation of platelets.

Whatever the source of hemostatic ADP, its demonstration suggests novel therapeutic possibilities in the prevention of arterial thrombosis when it is initiated by platelet aggregation.\(^\text{12}\) One such possibility would turn on the feasibility of infusing enzyme systems that remove free ADP from the blood. In spite of immunologic and other problems, it is conceivable that one or other enzyme with this specificity and a sufficiently long half-life in blood could be investigated for this purpose in appropriate clinical conditions. Furthermore, on the basis of our demonstration that bleeding times were increased also by infusing substrates only, with the implication that the systems are completed by the endogenous plasma enzymes, it is conceivable that therapeutic removal of ADP can be achieved by administering creatine phosphate or phosphoenolpyruvate in a way that provides adequate concentrations in the circulating blood.

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Melvin Paul Judkins (1922–1985)

Melvin Paul Judkins died on January 28, 1985, after a day of strenuous exertion on behalf of his father-in-law, who preceded him in death by a few hours. Dr. Judkins’ freely bestowed gifts reflected dedicated excellence — as with his exquisite method for in vivo visualization of the coronary circulation, which is today the one most used throughout the world. It brought the “greats” and the “nameless foot soldiers” in cardiology and radiology to his laboratories — a tacit tribute to his preeminence in both fields. He also shared in the development of transluminal angioplasty, which today has ever-increasing coronary application. He has literally entered the lives of thousands upon thousands, with an immensely beneficial impact. Dr. Judkins was a giant in modern cardiovascular diagnosis, a creative genius, a master teacher, and a tireless worker. He was folksy, artfully humorous, intensely practical, and a marvel in making the complex simple. In all labors, his wife, Eileen, fully shared round the clock.

From 1970 to 1978, Dr. Judkins was Professor and Chairman of the Department of Radiation Sciences at his alma mater, The Loma Linda University School of Medicine. In two decades he authored more than 150 major scientific papers, books, chapters, and exhibits. He presided over The Society for Cardiac Angiography et al., and led or served numerous national and international committees. He also served on the editorial boards of The American Journal of Cardiology and Applied Radiology and as editorial consultant to Chest, Circulation, Radiology, and Cardiovascular and Interventional Radiology.

To his many admirers, Mel was a towering, yet simply based gentle man who left us much too soon. Robert Frost’s verse speaks for him in a way that even his modesty might permit him to accept: “Two roads diverged in a wood, and I— I took the one less traveled by, and that has made all the difference.”

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