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Integrins and cardiovascular disease

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Abstract. Cardiovascular diseases involve abnormal cell-cell interactions leading to the development of atherosclerotic plaque, which when ruptured causes massive platelet activation and thrombus formation. Parts of a loose thrombus may detach to form an embolus, blocking circulation at a more distant point. The integrins are a family of adhesive cell receptors interacting with adhesive proteins or with counterreceptors on other cells. There is now solid evidence that the major integrin on platelets, the fibrinogen receptor $\alpha_{IIb}\beta_3$, has an important role in several aspects of cardiovascular diseases and that its regulated inhibition leads to a reduction in incidence and mortality due to these disorders. The development of $\alpha_{IIb}\beta_3$ inhibitors is an important strategy of many pharmaceutical companies which foresee a large market for the treatment of acute conditions in surgery, the symptoms of chronic conditions and, it is hoped, maybe even the successful prophylaxis of these conditions. Although all the associated problems have not been solved, the undoubted improvements in patient care resulting from the first of these treatments in the clinic have stimulated further research on the role of integrins on other vascular cells in these processes and in the search for new inhibitors. Both the development of specific inhibitors and of mice with specific integrin subunit genes ablated have contributed to a better understanding of the function of integrins in development of the cardiovascular system.

Key words. Integrins; inhibitors; thrombosis; atherosclerosis; platelets; endothelial cells; smooth muscle cells; leukocytes.

Introduction

Although the last few years have seen much progress in understanding the pathophysiology of cardiovascular diseases, there is still considerable controversy about their origins. As the major cause of death and disablement in developed countries, they have received surprisingly little attention compared with other important health problems such as cancer. In many less-developed, countries, cardiovascular diseases are overshadowed by parasitic diseases such as malaria. However, it is noticeable that as soon as the worst aspects of Western lifestyles are adopted, fatty foods, reduction in intake of traditional unprocessed foods, lack of exercise and smoking, which is the case in many such countries, cardiovascular problems immediately become a cause for concern. In Europe there is a remarkable gradient in the incidence of these disorders from south to north, with the relatively low incidence in the south being attributed to the 'Mediterreanean' diet, rich in vitamins, fruits and vegetables as well as garlic and red wine, all containing substances which have been shown to have beneficial effects, as well as the milder climate and increased sunshine encouraging a more active outdoor life with a lower energy input. The possible role of genetic factors, which may have conveyed a selective advantage to life in the cold grey north in other epochs when survival depended on using every available calorie, can also not be ignored. In the absence of a miracle inducing the great majority of the population to adopt a healthier lifestyle can these problems be alleviated by a pharmacological approach? As adhesive receptors, integrins have both well-defined and less clear roles in

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the origins as well as the complications of cardiovascular diseases. Models for the origin of the vascular lesions leading to plaque, which are so typical for these disorders, include the tumour model, implying that environmental or genetic factors cause a clonal amplification of vascular wall cells; the inflammatory model, implying that a local hyperinflammatory situation is the origin, and the related infectious model, which suggests that local bacterial or viral infections are the origin of a persistent inflammatory situation. The real situation may involve aspects of all of these or other causes still unrecognized. Many cell types are involved in these processes; but the main types which will be considered here are platelets, endothelial cells, smooth muscle cells, monocytes and leukocytes. All of these cells contain a variety of integrins on their surfaces which are either expressed constitutively in an active form, become active following cell activation or are expressed de novo following cell activation. Because of the established or suspected roles of these receptors in the processes leading to cardiovascular diseases, the last decade has seen a determined effort by pharmaceutical companies to prepare specific inhibitors for the treatment and prophylaxis of these disorders. Some of these will be dealt with here together with an overview of the directions in which the newer developments are leading.

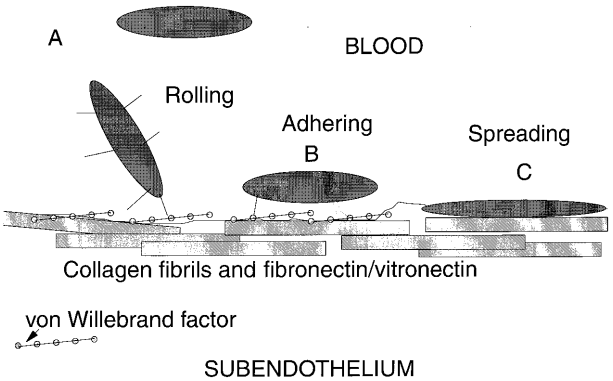

Figure 1. The various stages in resting platelet interaction with injured vessel wall. (*A*) Rolling interactions between GPIb-V-IX on the platelet, and vWf bound to collagen on the subendothelium, slows the platelet down so that other receptors can participate and starts the platelet activation process. (*B*) Other major platelet receptors such as those for collagen (including the $\alpha_2\beta_1$ integrin), for fibronectin ($\alpha_5\beta_1$ integrin), vitronectin ($\alpha_v\beta_3$ integrin) as well as laminin ($\alpha_6\beta_1$ integrin) come into play, and major activation occurs leading to release of granules. (*C*) The major platelet integrin $\alpha_{IIb}\beta_3$ also is activated and interacts with surface adhesive proteins as well as participating in the cytoskeletal reorganization, leading to spreading on the subendothelial surface.

Platelets

A major role for platelets in cardiovascular diseases was generally excluded until about 15 years ago. It was well established earlier that the thrombus which forms after rupture of an atherosclerotic plaque or the embolus which blocks an artery is made up of aggregated platelets, but many researchers felt that the platelets were simply innocent bystanders that had no active role in these processes. With the observation that aspirin, which inhibits platelet cyclooxygenase fairly specifically at low doses, reduces fatalities and complications due to cardiovascular disorders [1], as well as indications of a more basic involvement of platelets [2], a much more positive attitude prevailed, and the search for 'a better aspirin' had started. Although there are many target receptors in platelets, the major platelet integrin, $\alpha_{IIb}\beta_3$, which as fibrinogen receptor is essential for linking platelets in aggregation, soon became the main focus of interest. To understand the reasons for this, a brief overview of the current model of platelet activation is necessary. Platelets contain integrins of the β_1 and β_3 families, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, whose struc-

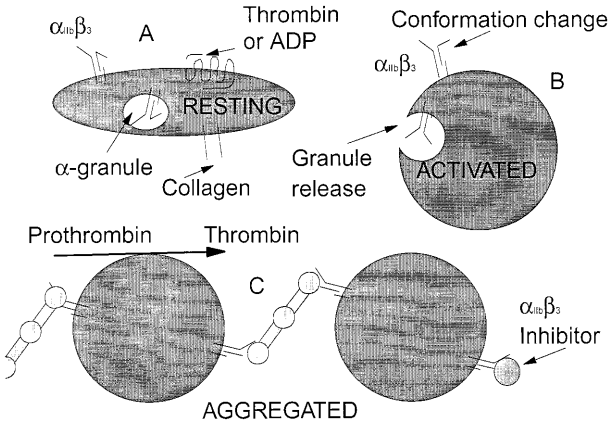

Figure 2. The various stages in platelet activation showing the changes in the status of the major integrin, $\alpha_{IIb}\beta_3$. (*A*) A resting platelet with $\alpha_{IIb}\beta_3$ in the inactive state. Platelet α-granules also contain $\alpha_{IIb}\beta_3$ in their membranes. Platelets are activated by various agonists over membrane receptors. ADP and thrombin act via different types of seven transmembrane receptors, while collagen acts via a number of receptors leading to activation of tyrosine kinases. (*B*) In the activated platelet, shape change and granule release have occurred and $\alpha_{IIb}\beta_3$ has become activated, changing conformation so that it can bind fibrinogen. Release of $\alpha_{IIb}\beta_3$ from a-granules increases the number of these receptors on the platelet surface. (*C*) Fibrinogen can now interact with $\alpha_{IIb}\beta_3$, causing platelets to link together and leading to further signal transduction events. One consequence of these is the development of procoagulant activity on the platelet surface caused by exposure of negative phospholipid and leading to conversion of prothrombin to thrombin. Inhibition of $\alpha_{IIb}\beta_3$ by specific inhibitors (which can also bind to the nonactivated state of this integrin) blocks both aggregation and these later events.

tures and functions are given in detail in the other reviews. In brief, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ act as constitutively active receptors for collagen, fibronectin and laminin, respectively, and are present at 1000 to 2500 copies per platelet; $\alpha_v\beta_3$ is a vitronectin receptor present in a few hundred copies per platelet; and $\alpha_{IIb}\beta_3$ is an activatible receptor principally for fibrinogen, but also for fibronectin, von Willebrand factor (vWf) and vitronectin [3]. This latter major receptor is present in 50,000 to 80,000 copies per platelet, depending on the method of measurement and whether molecules present in α-granules released on activation are included or not. When platelets are activated either via adhesion to exposed subendothelium (Fig. 1) or by exposure in the circulation or in a growing thrombus to a variety of agonists (Fig. 2), one of the consequences is that $\alpha_{IIb}\beta_3$ is activated by a complex process of downstream signalling [4, 5] (see also review by Longhurst and Jennings, ref. [6]) which allows fibrinogen to bind and to form cross-links to other platelets. Events following this bridging include further platelet activation (Fig. 3) as well as polymerization of fibrinogen to fibrin, necessary for consolidation of a thrombus. Thus, activation of $\alpha_{IIb}\beta_3$ is an unavoidable consequence of platelet activation by most if not all physiological agonists, and as such is an attractive target for a general approach to platelet inhibition. This is supported by the existence of patients with the bleeding disorder Glanzmann's thrombasthaenia, who either lack $\alpha_{IIb}\beta_3$ on platelets completely or partially or have molecules which are functionally defective [7, 8]. The attractiveness of $\alpha_{IIb}\beta_3$ as a target was also enhanced by the discovery that monoclonal antibodies [9] as well as small, sterically constrained peptides [10] could act as efficient inhibitors. More recently, a range of peptidomimetics have been prepared for use both intravenously in acute situations and in forms which are orally available and can be used for long-term prophylaxis. These are currently being tested clinically.

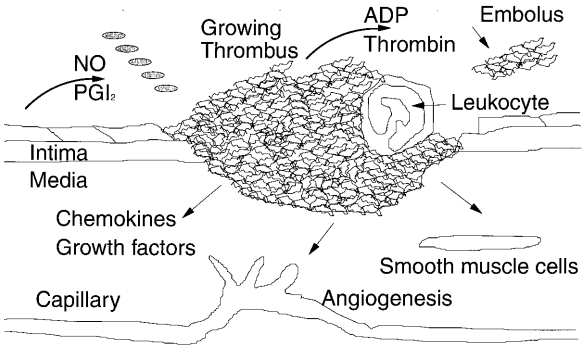

Figure 3. The first stages in the repair of damaged vessel wall. Platelets are brought into contact with the exposed subendothelium, and a thrombus is rapidly formed which often includes trapped leukocytes. The size reached by the thrombus depends on a complex equilibrium between feedback factors such as ADP and thrombin produced by the activated platelets and factors from the surrounding endothelial cells such as NO and PGI_2, which have a relaxing effect. Eventually, newly arriving resting platelets cease to interact with the thrombus, and it ceases to grow. This is probably dependent on the degree to which $\alpha_{IIb}\beta_3$ is activated in the outer layers of the thrombus. Normally, the thrombus partially dissociates at this point, and only the necessary platelet mass to leave a nonthrombogenic surface is left. Parts of a loose thrombus may break off and be swept away by the circulation as an embolus. Normally, these rapidly dissociate into single platelets, but under pathological conditions they can block smaller vessels. The platelets activated in the thrombus release their granule contents, including chemokines and growth factors which influence the healing of the wound by attracting endothelial and smooth muscle cells from the surrounding tissue as well as monocytes and leucocytes to clear up the debris. The blood supply to the underlying tissue is increased by angiogenesis – the extension of new blood vessels in response to growth factors from the wound.

In general, what has the use of these substances in animal models, in clinical trials or in clinical situations told us about the role of $\alpha_{IIb}\beta_3$ in cardiovascular disorders? The main sources of data which are available come from the treatment of acute situations where tissue damage and platelet activation lead to stenosis of vessels which need to be kept open. At present, classical therapy involves fibrinolytic treatment with a variety of enzymes as well as with heparin or low molecular weight heparin to reduce thrombotic complications. It is well known that clots contain active thrombin associated with fibrin and, on fibrinolytic treatment, this is released and can restart a cycle of platelet activation and restenosis. This can be reduced or avoided by the presence of thrombin or platelet inhibitors. The humanized chimeric Fab fragment of anti-$\alpha_{IIb}\beta_3$ antibody, ReoPro (abciximab), has been used extensively in such situations and has been shown to decrease death and reocclusion considerably [11,12]. Unlike more specific peptides and peptidomimetics, ReoPro recognizes other integrins as well as $\alpha_{IIb}\beta_3$, including $\alpha_v\beta_3$ in platelets, endothelial cells and smooth muscle cells, as well as $\alpha_M\beta_2$ in leukocytes [13], which may play a role in its effectiveness. In searching for inhibitory peptides and peptidomimetics much stress was laid upon their specificity for $\alpha_{IIb}\beta_3$ vs. $\alpha_v\beta_3$, obtained in the case of cyclic peptides by a sterically constrained Lys-Gly-Asp (KGD) rather than Arg-Gly-Asp (RGD) sequence [14, 15]. It was feared that, for example, $\alpha_v\beta_3$ inhibitors might detach endothelial cells from the subendothelium, which was not the object of the exercise. Although such peptides and peptidomimetics do show valuable improvements in patient survival and vessel patency in treating major cardiovascular disorders, there are a number of problems associated which suggest that progress can still be made in this direction. One of these controversial points is the extent to which

control of platelet aggregation can be dissociated from increased bleeding in general, and in particular, major bleeding incidents, which may be life-threatening [16]. A minor additional complication with ReoPro is a rare incidence of acute thrombocytopenia, which can be treated by platelet transfusion and which is presumed to be caused by an immunological response to the antibody [17,18].

Platelets acquire fibrinogen in their α-granules by an active transport process from plasma which may already function in megakaryocytes [19]. This involves $\alpha_{IIb}\beta_3$, since in Glanzmann's thrombasthenia platelets fibrinogen is absent or levels are reduced depending on the severity of the case. In addition, in animal models, snake disintegrins or antibodies to $\alpha_{IIb}\beta_3$, which block this receptor, prevented the transport of biotinylated fibrinogen to α-granules. However, labelled anti-$\alpha_{IIb}\beta_3$ antibodies were transported from the platelet surface to the inner surface of α-granule membranes [20]. Thus, this is a side-effect of treatment with $\alpha_{IIb}\beta_3$ inhibitors that needs to be taken into consideration. On the one hand, they may reduce or even abolish the normal accumulation of fibrinogen in α-granules; on the other, they may be stored there in its place. Both these possibilities may have consequences for the dosage and longer-term effects of these inhibitors. Studies with Glanzmann's thrombasthenia patients, with defects either in α_{IIb} or in β_3 expression, which either do not affect or do affect, respectively, the expression of the $\alpha_v\beta_3$ receptor, suggest that this receptor is responsible for transport of vitronectin in the opposite direction, namely from the α-granules to the platelet exterior [21]. Despite the presence of two subtypes of fibrinogen in plasma γA dimer and $\gamma A\gamma'$, only γA dimer is endocytosed and occurs in platelets [22], suggesting that only this type is recognized, or perhaps induces activation, by the $\alpha_{IIb}\beta_3$ involved in transport to the α-granules. Since both types are bound to $\alpha_{IIb}\beta_3$ when platelets are activated, this suggests that either resting $\alpha_{IIb}\beta_3$ or some intermediate state is involved in this uptake process. Are small molecular mass $\alpha_{IIb}\beta_3$ inhibitors, peptides or peptidomimetics capable of activating this uptake process and of being transported into α-granules? This is not an easy process to follow, because unlike large molecules such as antibodies or fibrinogen, it is difficult to label peptides or peptidomimetics other than by radioactive methods without affecting their binding properties. This coupled, with their diffusion rates during sectioning, makes histology problematic. Separation of α-granules and demonstration of enrichment remains one possibility, as does measuring a linear uptake from plasma after exposure for different times of platelets to the inhibitor by inducing granule release after washing the platelets to remove surface associated inhibitor. There still remain many unanswered questions in this area, such as whether the dimeric structure of fibrinogen (or antibodies) is necessary to induce transport and whether the on/off rate between the inhibitor and $\alpha_{IIb}\beta_3$ is such as to permit any transported inhibitor to be left behind in the α-granule when the $\alpha_{IIb}\beta_3$ recycles to the platelet surface. All of these parameters may vary considerably between different inhibitors. A further point which may cause functional differences between inhibitors (and this applies to all integrins) is that binding of inhibitors may not just be passive. Because fibrinogen and other adhesive proteins induce outside-in signalling (see review by Longhurst and Jennings, ref. [6]), some inhibitors may also affect the conformation of the integrin or may even induce signals which can have consequences in terms of either immunological complications (the inhibitor-integrin complex is recognized as foreign) or cellular activation (caused by the signalling) [23].

There has been a marked effort in recent years to relate polymorphisms in proteins involved in fat metabolism or coagulation cascades to increased or decreased tendencies to develop cardiovascular disorders, and many well-documented links have been found. It is not surprising, therefore, that the existence of a polymorphism in platelet GPIIIa (β_3) should have incited several groups to investigate if there was any effect on disease incidence or severity. The first report suggested that there was indeed an overrepresentation of the Pro vs. Leu33 form (PLA2 or HPA-1b) among patients with cardiac infarction [24]. However, this is still controversial, because other groups have either found no relationship between the polymorphic type of the patient and disease status [25, 26] or do support these findings [27]. This is clearly a problem that will require much greater numbers of patients as well as a stricter definition of disease status to reach a conclusion about significance, and such studies are in progress. Although there has been much speculation about possible effects of the polymorphic forms on the avidity of fibrinogen binding or on downstream signal transduction events, there still are no biochemical or biophysical data to support such differences. However, a recent publication suggests that the presence of the PLA2 allele gives platelets that respond more weakly to thrombin receptor peptide as agonist [28], which is the reverse of what was expected. It should be pointed out here that other major platelet glycoproteins, in particular GPIbα, also contain polymorphisms which might possibly affect platelet response levels as well. There is also interest at the moment in investigating whether any statistically significant relationship exists [29]. Clearly, if any is found, overall effects in the general population will be related to the combination of the polymorphisms on the different receptors.

Another platelet integrin which shows interesting differences is $\alpha_2\beta_1$ [30]. Here the changes seem to be related to levels of expression on the platelets of an individual rather than to amino acid polymorphisms in the cases mentioned above [31]. It is still unclear what the factors are that affect the levels of expression, but they could be very indirect, such as levels of hormones affecting transcription factor levels or activity or could be related to differences in DNA in promotor regions of the gene for one or other subunit affecting transcription rates. This explanation is supported by the strange cases of two women with less than 20% of normal levels of $\alpha_2\beta_1$, leading to mild bleeding problems. The older of these showed a reversion to normal and loss of symptoms on reaching the menopause, as was reported earlier [32], and the younger one [33] apparently also now shows the same normalization phenomenon in reaching that age (J. J. Sixma, personal communication). Thus, hormonal control of integrin expression may also be important for regulating platelet adhesiveness and hence cardiovascular health.

What is the role of the β_1 integrins in platelet function and in possible contribution to cardiovascular diseases? $\alpha_2\beta_1$ is probably the most studied of these, not only in platelets but also in other cells [34]. In platelets its role is seen as being critical to the adhesion process under both low and high shear conditions. Under high shear conditions, additional platelet interactions with the subendothelium via GPIb and vWf are necessary to slow down the platelets so that $\alpha_2\beta_1$ can bind to collagen. While the absence of $\alpha_2\beta_1$ as well as specific antibodies and peptides clearly prevent platelet aggregation and adhesion to collagen, little is known of possible effects of such agents in in vivo situations.

Although both $\alpha_5\beta_1$ [35] and $\alpha_6\beta_1$ [36] are known to have ancillary roles during platelet adhesion to subendothelium under static conditions, little is known about the extent to which they are involved in physiological (or pathological) haemostasis.

Development of inhibitors targeted at platelet integrins as putative modifiers of thrombosis

The receptor on platelets which is missing or defective in the bleeding disorder Glanzmann's thrombasthenia was shown to be GPIIb-IIIa, which was afterwards recognized as an integrin and given the nomenclature $\alpha_{IIb}\beta_3$. Once this had become clear, it occurred to several talented investigators that here might be a way of modifiying the function of normal platelets to give an artificial Glanzmann's thrombastenia-like state and so reduce thrombosis in patients at risk. The first approach was to prepare monoclonal antibodies to platelets and select for those that inhibited platelet aggregation in specific assays [37]. The technology had just been developed, and several monoclonal antibodies to platelets had already been prepared. The first screenings for such antibodies gave rise to two which turned out to be against GPIIb-IIIa [9] and GPIb [38], respectively. Because the antibody against GPIb did not cross-react with animal platelets, whereas that against GPIIb-IIIa did, and could be tested in animal models [39], it was this that was developed in various stages. First of all it was demonstrated that the mouse antibody was effective in animal models of thrombosis in reducing restenosis. The next step was humanization of the antibody by molecular biology methods by swapping the Fc domain for a human sequence to reduce the risk of immune reactions when used in humans. Then followed a long period of clinical testing, which ended by showing that this antibody is an effective supplementary treatment to reduce acute thrombotic events associated with surgery or in prevention of restenosis after treatment such as percutaneous transluminal coronary angioplasty (PTCA) to open a blocked vessel. A humanized chimeric Fab fragment derived from this antibody is now in clinical use, so far mostly in North America, under the name ReoPro (abciximab).

Other investigators, inspired by the Glanzmann's thrombasthenia model and by the promising effects of anti-GPIIb-IIIa antibodies, have sought alternative approaches. It was known for many years that venom from the families of snakes which cause major haemorrhage contains components which inhibit platelet aggregation. In the mid-1980's it was shown that GPIIb-IIIa is a major target for many such components, and the sequence of these proteins from several snakes was determined (see review by T. F. Huang, ref. [40]). It was noted that they contain typically RGD or related sequences in the binding domain. At about the same time it was discovered that fibronectin contains an RGD sequence and that peptides with this sequence inhibit fibronectin binding to its cellular receptor [41] (which was the first integrin to be described and named as such [42]) as well as fibrinogen to $\alpha_{IIb}\beta_3$ on platelets. Based upon these findings it was rapidly concluded that small peptides containing the RGD sequence could constitute an alternative strategy to antibodies for preventing aggregation of activated platelets by blocking $\alpha_{IIb}\beta_3$. The next step was to improve the avidity of the peptides for integrins, and this was achieved by including the RGD sequence in a small cyclic peptide [43]. A remaining hurdle was the question of the specificity for $\alpha_{IIb}\beta_3$ compared with other integrins, since many of these RGD peptides, whether linear or cyclic, interact to varying degrees with a wide range of integrins and this could provoke side-effects on cells other than platelets. One solution to this problem came from screening the $\alpha_{IIb}\beta_3$ blocking proteins (now called disintegrins, see the

review by T. F. Huang, ref. [40] for a more detailed account) from different snakes for their specificity for $\alpha_{IIb}\beta_3$ compared with other integrins, in particular $\alpha_v\beta_3$, also present on platelets as well as other cells. In this way it was discovered that cyclic peptides containing KGD have a high degree of specificity for $\alpha_{IIb}\beta_3$ [14], which laid the groundwork for the development of the cyclic KGD-containing peptide Integrilin (eptifibatide) as a clinically useful drug [44]. Since it is a peptide it suffers, as does ReoPro, from the problem that it can only be administered intravenously, which restricts its use to acute situations. There was therefore considerable interest in the development of nonpeptide compounds which retain the specificity of cyclic peptides like Integrilin but can be administered orally for treatment not only of acute but also chronic disorders. The first step was the development of peptidomimetic structures that resemble the peptides mentioned above by a combination of molecular modelling and screening approaches. Many such compounds with a variety of structures are now known [45], and many of them are in various stages of clinical testing. A characteristic that seems to be essential for both specificity and avidity is the doubly charged zwitterionic structure found in RGD (or KGD) with the positive charge of the arginine (or lysine) and the negative charge of the aspartic acid separated by a specific distance. However, such highly charged species are very poorly absorbed through the gut, and orally available versions are being tested as prodrugs which have hydrophobic blocking groups on these charged moieties which can be removed by the enzymes of the digestive tract [46]. The results of testing of these prodrugs, as well as maintaining the efficacy and specificity of their precursors, which seem to have a good oral availability in clinical situations, are eagerly awaited.

All of these anti-$\alpha_{IIb}\beta_3$ blockers are limited by the degree to which they have bleeding complications as a side-effect, which is related to the fact that they often have a relatively steep dose-response curve, having little effect at one dose and causing almost complete inhibition at a somewhat higher dose. In addition, body weight dosing was first established in animal models and can also be influenced by the anticoagulants (such as citrate) used for in vitro titration to establish the degree of saturation of $\alpha_{IIb}\beta_3$. This is because the presence or absence of calcium can influence the avidity of the integrin ($\alpha_{IIb}\beta_3$) for the inhibitor and therefore change the actual dose requirement [47]. A major practical problem is establishing this degree of saturation of the receptor on the platelets in the patient and monitoring this during treatment [48]. Many pharmacokinetic parameters of how these inhibitors behave in vivo in humans will only become apparent with substantial experience in their use and improved monitoring methods. Considerable thought has also been given to how to handle those rare cases with these inhibitors where a major bleeding incident occurs. In the case of small molecular mass inhibitors which are rapidly cleared from circulation, treatment may simply consist of stopping infusion. However, some have relatively long lifetimes in vivo because of their high avidity, the possibility of being handed on from one platelet to another (as in the case of ReoPro) and the not yet established possibility of storage in α-granules. In such cases platelet transfusions may provide a first emergency treatment. Alternative strategies are being actively investigated. With the advent of effective oral prodrugs used prophylactically to avoid cardiovascular disease, it is not yet clear whether emergency wards will be faced with accident patients with prolonged bleeding and, if so, how they can be alerted to such problems and trained to recognize and treat them.

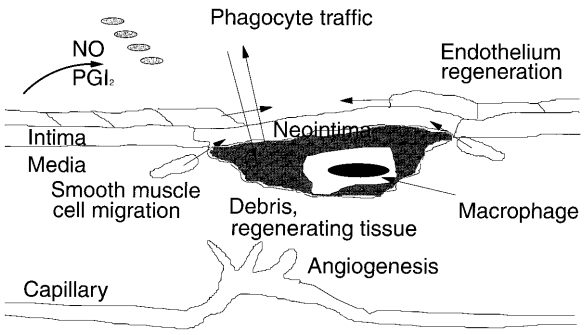

Figure 4. And the healing of the wound is nearly completed. Endothelium regrowth has occurred and smooth muscle cells have migrated from the media to form a neointima and strengthen the vascular wall. Macrophages have been attracted in to remove the debris, and other phagocytic cells are entering and leaving the wound area. It is the repetitive injury and healing process under poor physiological conditions which is thought to lead to an atherosclerotic lesion at such a site.

Because inhibitors of $\alpha_{IIb}\beta_3$ have now been demonstrated to have useful effects in patients, a considerable effort is under way to make specific inhibitors of other integrins [49]. A major target is $\alpha_v\beta_3$ because of its role in angiogenesis and because it is hoped that inhibitors will prevent tumour development (see review by P. Clezardin, ref. [50]) and could be used together with cytotoxic drugs in cancer therapy. However, as noted below, tissue reorganization following vessel wall damage also involves movement of endothelial and smooth muscle cells (Figs 3 and 4) and could be modulated by such inhibitors. Looking far ahead, it is possible that we will see treatment regimes involving variable doses of specific inhibitors to given integrins being used to control tissue regeneration during the healing process. In-

hibitors of $\alpha_{IIb}\beta_3$ were selected for development to control thrombosis largely because activation of $\alpha_{IIb}\beta_3$ is a common event to which many pathways lead (Fig. 2). As such it was felt that this would be a broad spectrum approach and effective regardless of the agonist(s) or situation leading to platelet activation. This has so far been justified in terms of the effectiveness of these substances in inhibiting platelet aggregation. Nevertheless, in looking ahead to how future generations of platelet inhibitors may change, it is worth comparing the effects of direct inhibition of $\alpha_{IIb}\beta_3$ with that of inhibition of receptors or pathways leading to activation of $\alpha_{IIb}\beta_3$. While these may have the apparent disadvantage of not covering all pathways which may be invoked leading to platelet aggregation, they may have less obvious advantages in terms of reducing overall activation of platelets. Inhibiting an activation pathway can at the same time prevent not only the involvement of $\alpha_{IIb}\beta_3$ but also the release of storage granules containing a wide variety of growth factors and hormones as well as exposure on the platelet surface of granule membrane glycoproteins which are involved in adhesion to other cells such as monocytes and leukocytes. Thus, by reducing repetitive and exaggerated activation of platelets at sensitive sites, which seems to be requisite for the development of atherosclerosis it may be possible to slow or prevent the progress of this disease.

What approaches have been used or are feasible to inhibit platelet activation in this way? Methods that have been used include aspirin, a tried and tested standard, which inhibits thromboxane synthesis relatively specifically at appropriate doses and thus prevents thromboxane feedback to a primary stimulus, reducing the overall activation of platelets, including $\alpha_{IIb}\beta_3$ [51]. More recently, ticlopidine and clopidogrel, which probably function by blocking adenosine diphosphate (ADP) receptor biosynthesis, have been shown to be effective clinical agents [52, 53]. This ADP receptor is thought to act principally in feedback via ADP released from platelet dense granules (but also from erythrocytes) and signals both amplified release from granules as well as activation of $\alpha_{IIb}\beta_3$. The combination of these agents together with aspirin, blocking two important feedback mechanisms, looks particularly promising [54]. Small molecule direct inhibitors of this major ADP receptor (there are thought to be two or three types of ADP receptors on platelets) also have a dramatic effect on activation of $\alpha_{IIb}\beta_3$ under conditions similar to those often found in vivo [55]. PAF (platelet activating factor) receptor inhibitors may also be useful for this purpose. Ginkgo preparations which contain the PAF receptor inhibitor ginkgolide B are used extensively for self-medication, principally to improve cerebral circulation in old age [56].

Attention has also been directed to other platelet adhesion receptors that are thought to act upstream of $\alpha_{IIb}\beta_3$ to activate this integrin as a normal physiological reponse (Fig. 1). These include the von Willebrand factor receptor, GPIb-V-IX [57, 58], and various collagen receptors including $\alpha_2\beta_1$ [34] and GPVI [59, 60]. The interaction between platelets and collagen under high shear conditions can also be inhibited by blocking the binding site for vWf on collagen. There are already some interesting data suggesting that inhibition of these receptors can be effective in reducing thrombosis in experimental animal models, but a major handicap so far is the lack of efficient pharmacological inhibitors for testing under clinical conditions.

Endothelial cells

Since endothelial cells have a major role in maintaining a nonthrombotic surface on blood vessels, by acting as a barrier between cells in the circulation and the subendothelial tissue, they obviously also have an important role in the pathology of atherosclerosis by the way in which they respond to injury or chronic insult. Since endothelial cells show differences between basal and apical faces, the expression of adhesive molecules (including integrins) on these two faces also influences their function. The major integrins expressed on endothelial cells are $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_5\beta_1$ [61]. There have been reports of the presence of $\alpha_1\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_5$ on the endothelium of some tissues. Endothelial basal integrins act as receptors for collagen, laminin, fibronectin and thrombospondin. Specific proteoglycans present in subendothelial tissue may also interact with these integrins. The expression of integrins on endothelial cells is up- or downregulated by various factors such as tumour necrosis factor α or transforming growth factor β. Both $\alpha_v\beta_3$ and $\alpha_2\beta_1$ as well as $\alpha_1\beta_1$ are known to be involved in angiogenesis [62], and because of this $\alpha_v\beta_3$ has been actively investigated as a target for inhibitors [63], in particular for treatment of tumours (see review by P. Clezardin, ref. [50]). However, both endothelial and smooth muscle cells are involved in tissue remodelling and repair after vascular damage and in the changes occurring in atherosclerosis. One of the main reasons why $\alpha_v\beta_3$ rather than $\alpha_2\beta_1$ has attracted attention in this context is that, like $\alpha_{IIb}\beta_3$, is also inhibited by fairly simple peptides containing RGD, so that peptidomimetics have also been developed [64]. The main difference in preference for the two integrins is the conformation of the peptide around the glycine residue. Until recently, specific inhibitors of $\alpha_2\beta_1$ (other than antibodies) had not been developed, and so little work has been done yet on their effects in vivo. The fact that this integrin is also a major collagen receptor on

platelets complicates the possible use of inhibitors in cancer therapy. An additional problem is that no small peptides or peptidomimetics have yet been found to inhibit $\alpha_2\beta_1$ efficiently, perhaps because the binding site is more complex than on the non-I-domain class of integrins (see review by Dickeson and Santoro, ref. [65]).

It seems likely that activated endothelial cells at the edge of a wound, which are spreading and dividing to cover the exposed subendothelial surface after haemostasis as it is cleaned up by phagocytes (Fig. 4), express higher levels of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ because of exposure to vascular endothelial growth factor [62]. A similar phenomenon is seen at the tips of growing neonatal blood vessels [66]. One possible reason for the effectiveness of ReoPro vs. more specific peptidomimetics in preventing restenosis may be that it blocks $\alpha_v\beta_3$ on endothelial and/or smooth muscle cells as well as $\alpha_{IIb}\beta_3$ on platelets. In order to investigate whether this is really the explanation, a possible approach might be to use combinations of specific $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ inhibitors; however, there have not yet been any reports of such trials. Another factor to take into consideration is that the size of ReoPro may limit access to the basal endothelial cell/subendothelium surface, protecting it from unwanted effects; but nevertheless ReoPro should be able to reach endothelial cells and smooth muscle cells present on the edge of an injury which are activated by growth factors and hormones generated at the wound site. On the other hand, small molecular mass inhibitors may have access to the basal surface of the endothelial cells and could possibly loosen their adhesion and therefore aggravate the situation. It will be interesting to see whether the use of $\alpha_v\beta_3$ inhibitors in cancer treatment to inhibit angiogenesis has such side-effects.

Smooth muscle cells

One of the main causes of restenosis after vascular clearance or manipulation is neointimal hyperplasia, due to smooth muscle cell migration from the media into the neointima, affecting blood flow. In a migration assay model it was demonstrated that both antibodies to $\alpha_v\beta_3$ as well as specific peptide inhibitors inhibited smooth muscle cell migration [67]. The conclusion was drawn that this integrin is also important in neointimal hyperplasia in vivo.

Monocytes/macrophages

Attraction of monocytes and macrophages to clean up damaged tissue during wound healing and the formation of typical foam cells are a part of the atherosclerotic process. Integrins play a role at various stages in this process. The $\alpha_D\beta_2$ integrin is expressed strongly on foam cells and interacts with ICAM3 as ligand [68]. Monocyte transmigration through endothelium was also related to integrin expression [69, 70]. It is known that monocytes and macrophages leave atherosclerotic lesions less as these develop, and this may be related to the decreased expression of the integrin $\alpha_D\beta_2$ [71].

Leukocytes

Leukocytes such as neutrophils are regularly found associated with the thrombus formed after vessel injury and are thought to play important roles in the scavenging processes involved in tissue repair. However, they are also involved in inflammation, and there may again be a delicate balance [72]. They can produce both coagulant and anticoagulant molecules and may help to regulate haemostasis. The activation of the leukocyte integrin $\alpha_M\beta_2$ mediated by antigen exposure leads to a higher avidity for endothelium and to leukocyte adhesion [73]. On the other hand, it is becoming clearer that activated platelets help to attract and hold the leukocytes in the thrombus. Platelets contain several chemokines in their α-granules, including NAP-2, RANTES, MIP-1α and ENA-78 [74–77] that may attract leukocytes to these sites. Adhesion molecules expressed on activated platelets such as P-selectin may be important for retaining the leukocytes. A role for integrins on both platelets and leukocytes in these interactions still remains unclear. Interactions between leukocytes, platelets and endothelial cells leading to migration of leukocytes to inflammatory sites is also thought to involve integrins [78]. The leukocyte integrins are dealt with in detail in the review by Gahmberg et al. [79].

Gene deletion

A modern approach used to try to unraveling the physiological role of the various integrins has been the use of specific 'knockout' mice. This area has recently been reviewed [80], and the insights that this approach offers were discussed. Elimination of several integrin subunits that have been discussed above, including β_1, α_5, α_6 and α_v, lead to embryonic or perinatal lethality. On the other hand ablation of β_3 or α_1 does not affect development or viability, and mice lacking α_2 or α_{IIb} have still not been bred. Despite the lethality of some of these knockouts, some fetuses develop to term and die soon afterwards, which allows their effects on organ development to be examined. Thus, α_v-null animals develop heart and vasculature but bleed in the brain and intestine. While depletion of fibrinogen and its receptors was fatal, there were suggestions that integrins can substi-

tute for one another such as $\alpha_v\beta_1$ for $\alpha_5\beta_1$, and that such substitutions account for the relatively mild effects of these 'knockouts' on organ development. The next step in these studies once knockouts for all integrin subunits have been developed will be to look at the combinations between these leading to double knockouts. Undoubtedly, the lethal 'knockouts' will, in the long run, provide valuable information about the role of these integrin subunits in tissue development. It is difficult, however, to see how these knockouts can provide much insight into their role in cardiovascular diseases. The situation is of course different for those integrin subunit knockouts that are viable. For example the availability of the β_3 knockout mouse should enable clear experimental results to be obtained concerning the role of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ in the development of atherosclerosis by examining changes in the vascular system of such mice on control and on atherosclerotic diets. Another integrin subunit knockout mouse that should be of great interest for such studies, if it is viable, is the α_2 because of its role in the collagen and laminin receptor $\alpha_2\beta_1$. Although the use of such gene knockouts can also be simulated by giving specific inhibitors, available in oral form, over the lifetime of the animal, this is nevertheless a more complicated procedure and requires extensive controls on the efficiency of the inhibition. Earlier there was considerable interest in whether patients with natural knockouts such as in α_{IIb} or β_3, causing Glanzmann's thrombasthenia [7, 8], or in GPIb-IX subunits, causing Bernard-Soulier syndrome [81], or heterozygotes of these, would show decreased atherosclerosis. However, the rarity of these disorders, the normally long human life-span associated with the difficulty in obtaining autopsy material in normal deaths, the various genetic backgrounds and the effects of different, uncontrolled normal diets make this an undertaking which is hardly likely to yield statistically significant results. Thus, mouse knockouts offer all that the humans lack – controlled diets, short life-spans and adequate numbers of genetically identical animals with the identical defect plus the possibility of having controls with the same genetic background.

Role of infections

There has been considerable interest in the last few years in a possible role of bacterial and viral infections in the pathogenesis of cardiovascular disease [82, 83]. Unfortunately, although bacteria, in particular *Helicobacter pylorii* and *Chlamydia pneumonia* [84] as well as viruses such as cytomegalovirus and *Herpes simplex* [85, 86], have often been found associated with plaque, it is not easy to determine whether they have a role in the origins of the disease or are simply associated with the plaque structure after it has formed. Some evidence does point to a possible initiation role. It has been demonstrated that bacteria can act as an inflammatory focus by releasing toxins causing endothelial cells to detach from the subendothelium [87] and providing a thrombotic lesion and indirectly a protected environment for further bacterial growth [88]. The effects of the toxins on adhesion are probably mediated by integrins, but relatively little is known in detail. It is known that several toxins act on G proteins, preventing or inducing signalling via seven transmembrane receptors, and that others can inactivate or activate important signalling pathway molecules. Toxins may also affect expression of adhesion molecules on endothelial cells, allowing direct interactions between the endothelial cells and lymphocytes and granulocytes in the blood-stream. Such adhesion is thought to involve integrins on the immune cells and Ig superfamily receptors on endothelial cells, as mentioned above.

Conclusions

Sufficient evidence has now been accumulated to support a major role for integrins, particularly on platelets, in the origins and development of cardiovascular diseases. However, many unanswered questions about their role on other cells remain. There is considerable interest at present in investigating the function of integrins in both physiology and pathology, and important tools are being developed, specific inhibitors as well as 'knockout' mice, to analyse the function of the individual integrins. The next few years will undoubtedly bring many advances in this area which can be used to improve prophylaxis and therapy.

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