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The Role of Willebrand Factor in Platelet-Blood Vessel Interaction, Including a Discussion of Resistance to Atherosclerosis in Pigs with von Willebrand's Disease [and Discussion]

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The role of Willebrand factor in platelet – blood vessel interaction, including a discussion of resistance to atherosclerosis in pigs with von Willebrand's disease

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Von Willebrand pigs have all the manifestations of the severe human disease. The role of Willebrand antigen (VIII R:AG) and ristocetin cofactor (VIII: RWF) was assessed in these pigs by (1) transfusion and (2) '*in vitro*' bleeding time assay. The skin bleeding time became normal when the level of transfused Willebrand factor (VIII R:AG/RWF) was raised in the plasma above 30 U/dl. After single or repeated transfusions, skin capillary endothelium and platelets were still distinguished from normal by VIII R:AG deficiency. When incisions in excised porcine skin ('*in vitro*' bleeding time) were perfused with blood and plasma fractions, haemostasis occurred when plasmatic Willebrand factor exceeded 30 U/dl whether the skin or platelets came from normal or from von Willebrand pigs. The platelet plug occluding the skin incision contained VIII R:AG by immunofluorescence. Willebrand factor appears to coat surfaces and to serve as a platelet attachment protein.

These bleeder pigs are resistant to atherosclerosis. If platelets are involved in early atheroscolerotic lesions, the role of Willebrand factor in platelet – blood vessel interaction may be important.

Von Willebrand's disease

In 1926, Professor Erik von Willebrand described a bleeding disease (von Willebrand 1926) and found that prolongation of the bleeding time was the main haemostatic abnormality. Since that time, a variety of haemostatic defects have been described in his eponymous disease and it has been the subject of research by many groups in many countries. The disease affects not only the coagulation portion of the haemostatic mechanism but also the interaction of the platelet and the blood vessel wall. Research into this disease is leading to a greater understanding of the haemostatic defect and is also making fundamental contributions to our knowledge of the haemostatic mechanism. More recently, the disease has assumed even greater importance because of the observation that severely affected pigs are resistant to the development of atherosclerosis.

Patients with von Willebrand's disease, like patients with classic haemophilia, have a decrease factor VIII coagulant activity; but they differ from haemophiliacs because their bleeding time is prolonged. Von Willebrand himself suggested that the abnormality in the disease was related to the capillary and the platelet. Subsequent experiments with the use of an instrument known as a capillary thrombometer showed that the thrombosing time, a correlate of platelet function was abnormally long (Morawitz & Jurgens 1930). Further evidence of a functional abnormality of platelets was proposed by Borchgrevink, who showed that considerably more platelets escape from skin incisions in von Willebrand's disease than in normal people (Borchgrevink 1961). An *in vitro* test was then developed using glass bead columns, the 'platelet

retention' test. It was found that, in von Willebrand's disease, fewer platelets were retained in the column (Salzman 1963; Zucker 1963; Bowie *et al.* 1959). A further abnormality of platelet function was demonstrated by the use of the antibiotic ristocetin. Ristocetin was shown to aggregate platelets in normal platelet-rich plasma but failed to aggregate them in the platelet-rich plasma of patients with von Willebrand's disease (Howard & Firkin 1971).

Testing of platelet function and electron microscopy failed to produce any convincing evidence of an intrinsic abnormality of the platelet. There was suggestive evidence that the abnormality of platelet function in von Willebrand's disease was due to the decrease or abnormality of some factor in the plasma, because the platelet retention test was corrected by the addition of normal plasma (Bowie *et al.* 1969; Meyer & Larrieu 1979). Our group then showed that the abnormality of platelet retention could be corrected by the addition of concentrates of factor VIII (Bowie *et al.* 1959); this was the first demonstration that the functional abnormality of the platelet and the decrease of factor VIII were in some way connected (Bowie *et al.* 1969). These findings were subsequently confirmed by Weiss & Rogers (1972) and Bouma *et al.* (1972).

The next observation about platelet function was that normal or haemophilic plasma and factor VIII preparations would allow ristocetin to produce aggregation of von Willebrand platelets (Firkin & Howard 1971; Howard & Firkin 1971; Howard *et al.* 1973; Weiss *et al.* 1973; Meyer *et al.* 1974; Olson *et al.* 1975).

Subsequently, it was found that an antibody made against factor VIII detected a protein that was present in normal quantities in patients with haemophilia and absent, or decreased, in patients with von Willebrand's disease (Zimmerman *et al.* 1971). Further observations of the chemistry of factor VIII have demonstrated that the factor VIII molecule is a complex structure composed of two non-covalently linked proteins, one of which has the coagulant property and is missing or abnormal in haemophilia and the other a large polymeric protein that is missing or abnormal in von Willebrand's disease. It is this latter protein that can be detected immunologically by precipitating rabbit antibodies (the so-called factor VIII related antigen). This protein is also probably responsible for the ability of ristocetin to agglutinate platelets (the ristocetin Willebrand factor or the ristocetin cofactor).

This introduction has been a brief summary of the complex series of investigations that led to the discovery of Willebrand factor. At this point the disease can be even more concisely summarized by stating that it is due to the decrease or abnormality of a protein in the plasma (Zimmerman *et al.* 1971), which can be detected immunologically by immunoelectrophoresis (Zimmerman *et al.* 1971) or immunoradiometric assay (Ruggeri *et al.* 1976) and functionally by its ability to allow ristocetin to cause aggregation of washed platelets (Howard *et al.* 1974; Olson *et al.* 1975).

Willebrand factor in haemostasis

Studies in humans

Willebrand factor appears to be synthesized in the endothelial cell (Jaffe *et al.* 1973, 1974; Booyse *et al.* 1977) and perhaps also in the megakaryocyte (Nachman *et al.* 1977). The protein is also present in the platelet (Glagov 1973) and, of course, it circulates in the plasma. In cultures of endothelial cells from pig aortas, the protein is to some extent extracellular and it may be fibrillar or associated with a fibrillar protein (Booyse *et al.* 1977).

Examination of partly purified and highly purified preparations of Willebrand factor by

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electrophoresis in sodium dodecyl sulphate (SDS) suggest that the protein circulates in plasma as a series of polymers (van Mourik *et al.* 1974; Counts *et al.* 1978; Fass *et al.* 1978; Perret *et al.* 1979; Weinstein & Deykin 1979; Ruggeri & Zimmerman 1980). The protein is extremely large and in the pig the polymers range in relative molecular mass from 10^6 to 21×10^6 (Fass 1978) and in the human from 0.86×10^6 to 9.9×10^6 (Ruggeri & Zimmerman 1980). This large polymeric protein therefore exists in a series of biological compartments; one of the important investigations in the physiology of haemostasis is to elucidate the role of Willebrand factor in these various areas.

There is increasing evidence that the level of plasmatic Willebrand factor plays an important role in platelet – blood vessel interaction and the eventual production of haemostasis. Platelets from patients with von Willebrand's disease adhered poorly when exposed to the subendothelium of the de-endothelialized everted segments of rabbit aorta in a perfusion chamber (Tschopp *et al.* 1974; Weiss *et al.* 1975; Fuster *et al.* 1980). The type of anticoagulant used in these experiments is important and when higher citrate concentrations were used, the adhesion defect was more pronounced (Weiss *et al.* 1977). When an antibody to human factor VIII (Baumgartner *et al.* 1977) was used to deplete normal plasma of Willebrand factor, there was also defective adhesion of platelets to subendothelium.

An interesting finding in all of these studies was that the difference between normal and von Willebrand blood depended upon the shear rate, and was greater at higher shear rates. In more recent studies, the perfusion system has been modified to allow the examination of native human blood, and subsequent studies have confirmed the shear rate dependent decrease of adhesion in von Willebrand's disease. These experiments have been criticized because they were not done with tissue from the same animal species; human blood was used in conjunction with rabbit aorta. Sakariassen and colleagues, however (Sakariassen *et al.* 1979), have now adapted the Baumgartner perfusion chamber to use human tissues only by substituting renal arteries, obtained at post-mortem, for the rabbit aortas. These workers labelled platelets and Willebrand factor radioactively and their study suggested that Willebrand factor bound to the subendothelium induced platelet adhesion and that it was the plasma protein alone that was necessary for normal platelet adhesion. Recent work in our own laboratory, with the use of a porcine system, is entirely consistent with these results (Bowie *et al.* 1978; Kimura *et al.* 1979).

Studies in pigs

At the Mayo Clinic, we have maintained for several years a breeding colony of pigs with von Willebrand's disease (Bowie *et al.* 1973). The term for a collection of pigs is actually a 'sounder' – a particularly appropriate designation, as pigs are rather vocal animals. The Mayo Clinic 'sounder' shares the observed impairment of primary haemostasis and other haemostatic abnormalities of the severe form of the disease in humans (Mannucci *et al.* 1976; Bowie & Owen 1979). The animals are afflicted with a serious bleeding tendency (Bowie *et al.* 1973; Fass *et al.* 1976*a*) that is autosomally transmitted (Owen *et al.* 1974; Fass *et al.* 1979) and is manifested by a long bleeding time, reduced platelet retention, an almost complete absence of factor VIII related antigen (0.025% by immunoradiometric assay) and a lack of Willebrand factor in the plasma. Like the human protein, porcine Willebrand factor purifies with and is probably identical to the factor VIII related antigen (Fass *et al.* 1976*b*).

The availability of such an animal model has allowed us to conduct experiments on the role of Willebrand factor in haemostasis, with tissues from the same animal species. We have de-

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veloped a technique known as the '*in vitro*' bleeding time. A small piece of excised porcine skin is attached to the channel in the base of a flow cube, and heparinized porcine blood or plasma is allowed to flow through a 5 mm incision in the skin at a constant hydrostatic pressure The technique is described in detail by Kimura *et al.* (1979). The volume of blood or plasma exuding from the incision is measured and the incision site examined microscopically after haemostasis has occurred.

When normal blood or platelet-rich plasma was allowed to flow through the skin incision, there was a gradual decrease in the volume of blood and the bleeding eventually stopped whether the skin came from a normal or a von Willebrand pig. When the incision in the skin was examined microscopically, a platelet clump was found to occlude the epidermal end of the incision, and it was shown to stain positively for Willebrand factor by immunofluorescence. If blood or platelet-rich plasma from a pig with von Willebrand's disease was used, the bleeding continued and haemostasis did not occur. The delayed haemostasis was corrected to normal by the addition of normal plasma or cryoprecipitate. The addition of an antibody to Willebrand factor to normal pig blood caused a gradual increase in the bleeding time, which became indefinite when the Willebrand factor had been completely inhibited by the antibody. From these experiments, it was concluded that plasmatic Willebrand factor played an essential role in haemostasis in this *in vitro* system.

Transfusions of plasma and concentrates of Willebrand factor into the living animal have shown a remarkable correlation with these *in vitro* experiments. Haemostasis in these studies was evaluated by a new technique, the 'ratio bleeding time' developed by one of us (D.N.F.), which is fully described elsewhere (Bowie *et al.* 1980). In a series of experiments involving the administration of 20 transfusions of plasma cryoprecipitate and a partly purified preparation of Willebrand factor, the role of Willebrand factor in the plasma, platelet and endothelial cell was evaluated. Platelets and endothelial cells (skin biopsies) were examined at intervals after the transfusion of a large quantity of cryoprecipitate. Although the ratio bleeding time was completely corrected, there was never any evidence of Willebrand factor in the platelets or in the endothelial cells. In these studies, the bleeding time was found to be normal when the level of Willebrand factor in the plasma was above 30 %, about the same level as was found with the '*in vitro*' bleeding time.

Although the brief correction of the bleeding time after transfusion can be correlated with the brief residence of Willebrand factor in the plasma, there is evidence that the level of plasmatic Willebrand factor is not the whole story. One line of evidence comes from studies on the bleeding time. The Duke bleeding time may be normal in some patients with von Willebrand's disease in whom the Ivy bleeding time is prolonged (Nilsson *et al.* 1959; Cornu *et al.* 1963; Larrieu *et al.* 1968). Moreover, after transfusion the Duke bleeding time is more easily corrected than the Ivy bleeding time (Nilsson *et al.* 1959; Cornu *et al.* 1964; Silwer & Nilsson 1964; Larrieu *et al.* 1968). Capillary pressure is increased in the Ivy bleeding time test, in contrast to the Duke bleeding time, and the consequent increased shear rate may be enough to impede haemostasis in the Ivy test when compared with the Duke test (Jaffe *et al.* 1974). After transfusion, the level of plasmatic Willebrand factor in the Duke and Ivy tests would be the same, which suggests that for completely normal haemostasis, Willebrand factor must be present in the platelets and endothelial cells as well as in the plasma.

Another line of evidence comes from the study of patients with variant forms of von Willebrand's disease. It is becoming clear that von Willebrand's disease is a heterogeneous group of

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diseases, and in one type of the disease, normal Willebrand factor may be decreased or absent in the plasma, platelet and endothelial cell. In a second type the Willebrand factor may be abnormal (Mannucci 1977; Ruggeri & Zimmerman 1980). In a third type the level of Willebrand factor in the plasma may be extremely low, but there are normal or increased levels in the platelet and endothelial cells. In this last type of the disease, it would appear that the Willebrand factor is not being released from its sites of synthesis; it is this variant form of the disease that may give us a clue as to the role of Willebrand factor in haemostasis. Pigs and people who have no Willebrand factor in plasma or platelets or endothelial cells have a severe haemorrhagic diathesis. As we shall point out later, our pigs periodically bleed to death. On the other hand, humans with the third variant type of von Willebrand's disease have a mild bleeding diathesis although they have extremely low levels of plasmatic Willebrand factor. One explanation for their haemostatic competence would be the presence of Willebrand factor in the platelets and endothelial cells. It is, of course, possible that we are dealing with another factor – a bleeding time factor – which is hinted at in earlier work summarized by Blombäck *et al.* (1964). The bleeding time factor, however, has not been isolated and has not been clearly differentiated from Willebrand factor.

From these studies, we can conclude that plasmatic Willebrand factor plays an essential role in the haemostatic mechanism but that for entirely normal haemostasis Willebrand factor must be present both in the platelet and endothelial cell. It seems likely that plasmatic Willebrand factor coats surfaces and serves as an attachment protein for the platelet (Bowie *et al.* 1978; Nyman 1980).

Resistance to atherosclerosis in porcine von Willebrand's disease

Our current understanding of the haemostatic mechanism gives a central role to the platelet, which forms the haemostatic plug and also makes an essential contribution to the coagulation mechanism and the subsequent formation of fibrin. Furthermore, the platelet may be the main reason why the haemostatic mechanism is localized to an injured area because all the reactions involved in haemostasis may take place on its surface membrane.

It is paradoxical that the platelet, which may preserve life by staunching bleeding, may cause death by initiating atheroma. It has been suggested by many investigators (Stemerman & Ross 1972; Ross *et al.* 1974; Fishman *et al.* 1975; Mustard 1975; Harker *et al.* 1976; Lewis & Kottke 1977) that the platelets adherent to the damaged endothelium may promote the intimal hyperplasia seen in the early atherosclerotic plaque. The early lesion of atherosclerosis is a nodule of hyperplastic smooth muscle in the intima as a result of the hyperplasia of medial smooth muscle that has migrated into the intima through fenestrae in the internal elastic lamina. Platelets are believed to release a mitogenic factor or factors – the platelet-derived growth factor – that stimulate the proliferation of smooth muscle cells and, in fact, smooth muscle cells cannot grow in tissue culture when the serum is derived from platelet-free plasma. Serum derived from fatty platelet-rich plasma is effective in supporting cell growth (Ross *et al.* 1974).

Harker *et al.* (1976) have observed that in experimental homocystinaemia, platelet consumption correlated with the formation of intimal lesions. When platelets were inhibited by the administration of dipyridamole, the increased platelet consumption was prevented as well as the formation of the intimal lesions. The studies of Moore *et al.* (1976) and of Friedman *et al.*

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(1977) showed that thrombocytopenia prevented the arteriosclerosis induced by aortic catheters. Cohen & McCombs (1968) found that ^{32}P-induced thrombocytopenia prevented arteriosclerosis in rabbits fed on an egg yolk diet, but they showed increased arterioclerosis in rabbits with thrombocytosis induced by phlebotomy (Cohen & McCombs 1967). More recently Pick *et al.* (1979) have found that the development of coronary atherosclerosis in cynomologus monkeys (*Macaca fascicularis*) fed on an atherogenic diet was inhibited by the administration of aspirin.

In the light of these studies, therefore, the demonstration that von Willebrand pigs may be resistant to atherosclerosis (Fuster *et al.* 1980) was extremely interesting because of the possibility that it was related to the abnormality of primary haemostasis in these animals. The observation assumed even greater importance because the pig appears to be an ideal animal model of human atherosclerosis. Porcine atherosclerotic lesions are similar in distribution and development to the human lesions, and they are big enough to be easily measured macroscopically and microscopically. In addition, porcine atherosclerosis may be induced by a high cholesterol diet in less than 6 months. A number of studies were therefore initiated to compare the development of atherosclerosis in von Willebrand pigs with that in normal pigs.

As already mentioned, the von Willebrand pigs have a severe bleeding diathesis and bleed to death periodically from exsanguinating gastrointestinal haemorrhage and severe epistaxis. For unknown reasons, the epistaxis always starts in the posterior nose, and because porcine turbinates are convoluted, the bleeding site cannot be visualized. At post-mortem, it was observed that there was negligible aortic atherosclerosis (Bowie *et al.* 1975). This observation was interesting and unexpected because, as already mentioned, pigs have an arterial system closely resembling that of man (French *et al.* 1965), and they usually develop atherosclerotic lesions early in life (Getty 1965). Following the post-mortem observations, a retrospective study of spontaneous atherosclerosis was started in 1974. The incidence of spontaneous aortic atherosclerosis in von Willebrand pigs was compared with the incidence in normal pigs obtained from the slaughterhouse. The normal pigs were of the same breed as the von Willebrand pigs – a cross between Poland–China and Yorkshire–Hampshire. The aortas of 11 normal pigs were examined, and 6 were found to have multiple raised fatty or fibrous atherosclerotic plaques (Fuster *et al.* 1978). Measurement of the intima showed an increased thickness over the plaques ranging from 63 to 130 μm in contrast to the normal areas, where the intima measured 20–64 μm. In the 11 von Willebrand pigs very little atherosclerosis was seen. None of the aortas had multiple plaques, four had single plaques, but only one was more than 2 mm in diameter.

Although the von Willebrand pig aortas appeared to be macroscopically normal, seven showed evidence of extensive fat deposition after staining with Sudan IV. Subendothelial infiltration of fat in the intima was found on microscopic examination. Measurement of the intima showed no evidence of significant thickening and ranged from 27 to 81 μm. Areas of the aorta that were influenced with fat were called non-atherosclerotic flat fatty lesions.

This first study therefore showed that there was a striking difference between the normal pig and pigs with von Willebrand's disease in the incidence and extent of atherosclerotic lesions in the aorta. However, the study had a number of shortcomings: it was retrospective, the body mass of the controls was higher than that of the pigs with von Willebrand's disease, and we did not have exact information about the diets of the normal pigs. We therefore decided in the following year to initiate a prospective study in which the pigs were matched for breed, age and sex and the diet was strictly controlled (Fuster *et al.* 1978): 18 newborn pigs were fed with

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maternal milk supplemented with cows' milk until 3 months of age at which time they received an atherogenic high-cholesterol diet (approximately 500 g/40 kg body mass, containing 2 % cholesterol) for a period of 6 months. Seven pigs (four male and three female) had homozygous von Willebrand's disease and there were eleven normal control pigs (five male and six female). At 3 and 4 months, respectively, two pigs bled to death and control animals were killed. At autopsy, the mean body mass was 94 kg (± 21 kg s.d.) for the von Willebrand pigs and 105 kg (± 30 kg s.d.) for the normal pigs. The 11 control pigs all developed raised fatty or fibrous atherosclerotic plaques on the aorta. Between 13 and 34 % of the aortic surface was involved in 9 of the pigs, and in the remaining 2 pigs the involved areas measured 2 and 5 %. The plaques were located mainly in the aortic arch and at the trifurcation of the aorta. Measurement of the intimal thickness showed a range of 50 to 390 μm over the plaques in contrast to the intima in the normal areas, which ranged from 18 to 40 μm. In the von Willebrand pigs there was a striking contrast: four pigs developed no atherosclerotic lesions at all, two developed atherosclerotic lesions effecting 6 and 7 % of the aortic surface, and in the seventh, 13 % of the aortic surface was involved.

It was again noted that on staining with Sudan IV, the aortas of the pigs with von Willebrand's disease showed large areas of non-atherosclerotic flat fatty lesions. Six of the von Willebrand pigs had 2–23 % of the aortic surface involved with these lesions. In these areas, it was again found that there was subendothelial infiltration of fat without intimal thickening.

Dietary-induced atherosclerosis may, of course, be different from the spontaneously occurring variety, so a prospective study of spontaneous atherosclerosis was also undertaken (Fuster 1979*a*). Twelve newborn pigs were fed with maternal milk supplemented with cows' milk until 3 months of age, at which time they received a non-atherogenic diet for a period of 4 years. Six pigs (four male and two female) had homoxygous von Willebrand's disease and six were normal control pigs (all female). At 5 and 6 months, two von Willebrand pigs bled to death and were excluded from the study. The other two von Willebrand pigs bled at 23 and 26 months and at these times control animals were also killed. The mean body masses at the time of death were 261 kg (± 94 kg s.d.) for the four pigs with von Willebrand's disease and 284 kg (± 68 kg s.d.) for the six normal control pigs.

On macroscopic examination, the aortas of five of the six control pigs showed raised atherosclerotic plaques involving 2–8 % of the surface. The distal trifurcation was primarily involved, and the lesions were large, irregular and elongated. In contrast, two of the four pigs with von Willebrand's disease had no atherosclerotic lesions, one had 1 % of the aortic surface involved and the other 2 %. When the aortas were stained with Sudan IV, again non-atherosclerotic flat fatty lesions were seen in three of the von Willebrand pigs.

These three studies, therefore, are evidence that pigs with von Willebrand's disease are resistant to the development of atherosclerosis. There are a number of possible explanations for these findings, and one of them may lie in the abnormal platelet – blood vessel interaction seen in von Willebrand's disease (Booyse *et al.* 1977). The explanation that we shall now develop is speculative, but it is supported at many of the steps by experimental observation.

The explanation starts at the endothelial cell which, in normal pigs, is known to be damaged by haemodynamic factors (Wesolowski *et al.* 1965; Fry 1969; Caro *et al.* 1971; Somer *et al.* 1972; Texon 1972; Glagov 1973; Cornhill & Roach 1976; Reidy & Bowyer 1977) and also by diets high in cholesterol (Imai & Thomas 1968; Frost 1969; Shiamoto *et al.* 1971; Chvapil *et al.* 1976; Ross & Marker 1976). Following the endothelial injury, the platelet then adheres to the damaged

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endothelial surface or the exposed subendothelial tissue (Stemerman & Ross 1972; Fishman *et al.* 1975; Lewis & Kottke 1977). This platelet – arterial wall interaction is enhanced by the von Willebrand factor in the plasma (Fass *et al.* 1976*a*; Bowie *et al.* 1978), platelets and the arterial wall (Jaffe *et al.* 1974). The earlier discussion of our studies with the use of transfusions and the *in vitro* bleeding time technique suggests that the level of Willebrand factor in the plasma plays an important role in this platelet – blood vessel interaction (Bowie & Owen 1979; Kimura *et al.* 1979). There is also evidence that adherence of platelets to the arterial surface in the normal animal may help to repair the endothelium and reduce its permeability (Gimbrone *et al.* 1969; Wojcik *et al.* 1969; Fry *et al.* 1976; Reidy & Bowyer 1977). In a normal pig, therefore, the continual endothelial damage results in platelet blood vessel adherence mediated by Willebrand factor and the restoration of endothelial integrity. In addition, the platelet-derived growth factor may, in some instances, result in intimal smooth muscle hyperplasia and the beginning of atherosclerosis.

In the von Willebrand animal, in contrast, although endothelial damage occurs as in the normal animal, the platelet cannot adhere because of the lack of Willebrand factor. Endothelial integrity is therefore not restored, and there is increased endothelial cell permeability. This increase of permeability allows fat to accumulate in the intima and results in the non-atherosclerotic flat fatty lesions. The intima in the areas of fatty infiltration have increased permeability, because intravenous injection of Evans blue dye 3 h before killing resulted in an intense blue staining of these areas. Scanning electron microscopy showed that the blue-stained areas were completely denuded of endothelium (Fuster *et al.* 1978). Some of the aorta was stained less intensely; in these areas, scanning electron microscopy showed endothelial damage. In pigs with von Willebrand's disease, platelets cannot adhere effectively to the subendothelial surface (Weiss *et al.* 1975; Baumgartner *et al.* 1977) so that the animals are functionally thrombocytopenic like the animals that are resistant to atherosclerosis after the experimental induction of thrombocytopenia. Furthermore, if such adherence is a necessary first step in the generation of atherosclerosis (Stemerman & Ross 1972; Ross *et al.* 1974; Harker *et al.* 1976), the life-threatening haemostatic defects of these pigs may be a life-preserving vascular phenomenon.

It is important to recognize that there may be explanations other than impaired platelet function for the resistance to atherosclerosis described in von Willebrand pigs. We considered the possibility that the level of platelet-derived growth factor in von Willebrand platelets might be decreased. Fass *et al.* (1977, 1981) showed that porcine von Willebrand's platelets contain the same amount of factors mitogenic for 3T3 cells as normal platelets. They also have shown for the first time that normal and von Willebrand porcine aortic endothelial cells contain a potent mitogenic activity that appears to be different from the mitogenic activity in platelets. The description of a mitogenic activity in endothelial cells is an observation of great significance and suggests new areas for investigation in the initiation of the early arteriosclerotic lesion.

It was also noted that the level of serum cholesterol was slightly higher in the control pigs than in the von Willebrand pigs (Fuster *et al.* 1978). During the time of administration of the high cholesterol diet, however, the levels of serum cholesterol were not different in the two groups of animals. There was also no significant difference in blood sugar and the haematocrit. We have no observations on blood pressure because, for accurate readings in pigs, it is necessary to cannulate the arteries; this would be a hazardous undertaking in our bleeder animals

We have also considered the possibility that the aortas of the pigs are genetically less re-

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sponsive to the development of atherosclerosis than the aortas from normal pigs. The cross-aortic transplantation study was, therefore, begun to test this hypothesis (Fuster *et al.* 1979*b*) and 5 cm segments of abdominal aorta, 1 cm proximal to the aortic trifurcation, were excised from each pig. Aortic segments of two normal control pigs were cross-transplanted with the aortic segments of two von Willebrand pigs. The appropriate control operations were also done, and we have actually performed 18 such procedures. The von Willebrand pigs were infused with 60 ml of normal pig cryoprecipitate about three hours before the procedure, to improve haemostasis. Ten days after operation, at which time the effect of cryoprecipitate had disappeared, the pigs began to receive an atherogenic high-cholesterol diet. The diet was continued for 6 months, the pigs were killed, and the aortas were removed, examined and measured for gross atherosclerotic plaques. It was found that atherosclerosis developed in the aortic segments from the von Willebrand pigs that were transplanted into normal control pigs. Endothelial von Willebrand factor was identified by immunofluorescence in the vasa vasorum of these transplanted von Willebrand aortic segments, although, of course, it was originally absent. The aortic segments from control pigs that were transplanted into von Willebrand pigs did not develop atherosclerotic plaques; in addition, it was shown by immunofluorescence that they lost their Willebrand factor (Fuster *et al.* 1979*b*). By transplanting aortas from pigs of opposite sexes, we are attempting to discover whether the transplanted segment becomes covered with the endothelial cells from the host or whether it retains its own. The study is turning out to be more complicated than we surmised because the sex chromosomes of pigs do not fluoresce, and it is necessary to make cultures of the endothelial cells to identify their sex. At the moment, however, we can say that the development of atherosclerosis seems to be related to the presence of Willebrand factor in the transplanted segments.

Finally, we should consider how these studies may relate to the incidence of atherosclerosis in von Willebrand's disease in man. There have been few studies on this subject apart from the paper published by Silwer *et al.* (1966), who found that the incidence of atherosclerosis in von Willebrand's disease in man was the same as in normal subjects. Silwer's findings are, perhaps, not surprising because the severe type of von Willebrand's disease that is found in our pigs is extremely rare in humans (Mannucci *et al.* 1976). Most humans with von Willebrand's disease have detectable levels of normal or abnormal Willebrand factor in their plasma, and the levels may be high enough to cause a sufficient platelet – endothelial cell interaction to initiate atherosclerosis. The heterozygous pigs that carry von Willebrand's disease have approximately 40 % of Willebrand factor in their plasma, and our recent study of the incidence of diet-induced atherosclerosis in these carrier animals has shown that they do not appear to be resistant to the development of atherosclerosis (Fuster *et al.* 1979*c*). There is also a form of variant von Willebrand's disease in our pigs, and we are about to initiate a study of diet-induced atherosclerosis in these animals. This last study may be of more relevance to the incidence of atherosclerosis in human von Willebrand's disease than the carrier studies described.

The study of von Willebrand's disease has given us important new information about the physiology of haemostasis as well as a new avenue for investigation of atherosclerosis. There is a great deal of ambivalence nowadays about the importance of animal models in investigation of human disease. In our view, animal experimentation has provided information about human physiology and pathology that could not have been discovered by other means. Von Willebrand's disease in pigs is an excellent example of how an animal model can lead to important advances in our understanding of human disease.

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Discussion

J. McMichael, F.R.S. (2 *North Square, London, U.K.*) In rabbits, fibrous plaque produced by mechanical injury can form a lipid-rich lesion in the presence of low levels of cholesterol levels (Moore, 1973). Does Dr Bowie find it necessary to add cholesterol to get fatty plaques in his experimental atheroma in pigs?

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E. J. W. Bowie. The atherosclerotic lesions discussed were induced by a 2% cholesterol high-tallow diet. We do, however, see lipid in the spontaneously developing atherosclerotic lesions in animals that have a normal level of cholesterol.

C. R. W. Gray (*Thoracic Unit, Westminster Hospital, London, U.K.*). Dr Bowie's results show a dramatic difference in the distribution of fats in aortas from normal and von Willebrand pigs on a high-fat diet. Has he considered the possibility that the aortic fat deposits are derived from platelet lipids and not from plasma lipids?

E. J. W. Bowie. We have, of course, considered that possibility. We are currently studying the intimal, fatty depositions in the von Willebrand pigs.

Elspeth B. Smith (*Department of Chemical Pathology, University Medical Buildings, Foresterhill, Aberdeen, U.K.*). We have been measuring factor VIII-related antigen (VIII RA) in human aortic intima and lesions by using an immunoelectrophoretic assay, and have obtained very odd results. A large variety of plasma proteins in intima is invariably present in concentrations that are a function of their concentration in the patient's plasma and of their molecular mass; large molecules are retained to a much greater extent than small ones. However, factor VIII RA is an exception and is unpredictable; it was present in high concentration in some samples but in others that appeared morphologically similar we failed to recover any, despite normal concentrations in the patient's plasma. This variability was not confined to the endothelialized surfaces of intima; the concentration in the centres of plaques was actually twice the concentration in the caps but half appeared to contain no factor VIII RA at all, although all samples contained high concentrations of prothrombin.

I wonder if Dr Bowie has any explanation for this anomalous observation.

E. J. W. Bowie. I think that these results are very interesting, although I have no explanation for them. Factor VIII-related antigen exists in plasma as a series of polymers, the largest of which have extremely high relative molecular masses up to 20×10^6. Each polymer would diffuse at a different rate and this may account for the variability in the results. I certainly think that it is worth pursuing further.

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