EXPERIMENTAL CORONARY ARTERY OCCLUSION AND REPERFUSION.

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Cover: *Scanning electron microscopy (700x) of experimentally induced coronary artery thrombosis.*

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EXPERIMENTAL CORONARY ARTERY OCCLUSION AND REPERFUSION

EXPERIMENTELE AFSLUITING EN REKANALISATIE VAN CORONAIR ARTERIEN

PROEFSCHRIFT

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Chapter 1

INTRODUCTION

1.0 INTRODUCTION

1.1 Clinical coronary artery occlusion and reperfusion

1.1.1 Clinical coronary artery occlusion

Some epidemiological data

Although a decline in the mortality of coronary artery disease has been reported, this disease continues to be the leading cause of death in the United States and Europe.[1,2,3] Approximately one third of all deaths in industrialized countries are due to cardiovascular diseases. However, considerable geographical differences in its incidence are reported.[4,5] In the Netherlands 42% of the overall mortality in 1988 was attributable to cardiovascular diseases. Approximately 50% of this is due to acute myocardial infarction or sequelae of ischemic heart disease.[6] One half of the deaths of the patients with acute myocardial infarction occur within one hour after the onset of symptoms, presumably due to lethal arrhythmias.[7] Most of the remaining patients are admitted to hospitals. The mortality rates within the hospital vary from 2-30% depending on the size of the infarct, its complications and the patient's age. Another 10% of the admitted patients die during the first year following acute myocardial infarction.[8,9] In addition to this early mortality, the surviving patients face a three-to fourfold excess in the risk of death during the next 10 years, due to recurrent myocardial infarction, left ventricular failure or sudden death.

Pathogenesis of coronary artery occlusion

Ischemic heart disease is caused, almost exclusively, by atherosclerotic coronary arterial disease. Turbulent blood flow, endothelial injury, monocyte invasion, platelet adhesion and proliferation of smooth muscle cells under the influence of platelet products, infiltrated lipids and lipoproteins, chemotactics and growth factors may all be involved in the genesis and progression of this lesion.[10-15] Two time constants of atherosclerosis progression have been postulated[16]:

1) gradual progression of early coronary atherosclerotic lesions (raised fatty streaks) during several decades to hemodynamically and clinically relevant advanced lesions (fibrous or fibromuscular plaques).[17,18] The rate of this progression is influenced by the so-called risk factors of atherosclerotic disease. However, cause and effect relationships have not been demonstrated for the vast majority of all suggested risk factors.[19] Also local abnormalities in coronary blood flow may independently influence the progression of minimal lesions.[20,21]

2) at some point in time coronary lesions may progress rapidly, probably influenced by mural thrombus incorporation, fissure of the fibrous cap overlying the lesion, ulceration or hemorrhage within the lesion, resulting in temporary or

permanent thrombotic occlusion. This second type of progression is associated with the acute coronary event.[22] The prevailing concept of how coronary arterial disease can become the harbinger of acute life-threatening cardiac syndromes focusses on a specific phenotype of the coronary atherosclerotic lesion. Angiographic, post-mortem angiographic and histologic techniques have identified the typical morphology of these coronary lesions.[23-26]
In unstable angina non-occlusive thrombus material covers the often ulcerated plaque[27], while in acute myocardial infarction and sudden death plaque fissuring and an occlusive coronary thrombus have been observed in the majority of cases.[28,29] In contrast, intracoronary platelet activation is probably not operative in stable angina pectoris.[30]
The role of coronary thrombosis in the development of incomplete or total coronary occlusion has been the subject of vigorous debate during the largest part of this century. Evidently this has delayed the use of logical treatment (i.e. thrombolysis). In the late 1970's the valiant work of Rentrop, using intracoronary streptokinase[31] , received its pathophysiological basis by the work of DeWood.[28] Since then the concept that thrombosis superimposed on more or less advanced coronary lesions plays a critical role of the pathogenesis of transmural myocardial infarction has been amply confirmed by others, and received widespread acceptance. As the degree of encroachment of the coronary lumen caused by the atherosclerotic lesion per se does not differ importantly between stable and unstable coronary syndromes,[32,33] the local coronary resistance and resulting turbulence are not the only factors responsible for thrombosis and occlusion. Not only plaque morphology, but also circulating catecholamines,[34] local production of proaggregatory and antiaggregatory autacoids (thromboxane A_2, prostacyclin)[35,36], endothelium derived relaxing factor vs endothelin[37], local production of tissue plasminogen activator and plasminogen activator inhibitors[38-40], vasoactive effects of growth factors[41] and receptor density may all influence the ultimate behavior of the coronary atherosclerotic lesion.

1.1.2 Prevention and treatment of acute coronary syndromes

Primary prevention
Given the considerations enumerated in the previous pages, primary prevention must be targeted at preventing or slowing the progression of atherosclerosis.[42] While interventions which favorably alter the absolute or relative levels of serum lipids and lipoproteins have been successful in decreasing coronary morbidity and mortality,[43-47] these results were however obtained in highly selected and high risk patient groups. Furthermore, overall mortality was not reduced in these studies.[48] The efficacy of aspirin for the primary prevention of cardiovascular mortality caused by the thrombotic component has been studied in two large scale human trials, one in the U.S. and the other in Britain. Both studies,

although different in design and the dose of aspirin used, could not demonstrate a reduction in this primary end-point.[49,50]
It is also evident that with the modest impact of primary prevention, once significant atherosclerotic lesions have developed other measures have to be taken. Current research in regression of atherosclerosis has not (yet) provided a solution, although several studies have reported angiographic evidence of slowing the progression of lesions in subgroups of patients. True regression has thusfar not been substantiated.[51,52] Preventing the occurrence of platelet aggregates or thrombi superimposed on the arterial lesion then seems the logical approach at present. In unstable or preinfarction angina the administration of aspirin or the calcium antagonist nifedipine, added to conventional anti-anginal medication, seems promising.[53-55] However, the efficacy of this treatment is still less than optimal, as major cardiac events could only be reduced by 30-50%, while a decrease in symptoms could not be demonstrated.[56]

Treatment of acute coronary artery occlusion
Acute coronary artery occlusion will ultimately lead to myocardial infarction or sudden death, assuming that no early (within hours) spontaneous recanalization occurs and the jeopardized myocardium does not receive an adequate collateral perfusion.[57,58] If this is not the case then the only means to prevent necrosis is early recanalization by the administration of thrombolytic agents and/or mechanical desobstruction (Percutaneous Transluminal Coronary Angioplasty: PTCA). Acute bypass surgery may form an alternative approach, but logistical and economic reasons limit its use.[59-61] Administration of thrombolytic agents in the first (4-6) hours of acute myocardial infarction is the treatment of choice at this moment.[62] By this approach infarct size and mortality can be reduced.[63-73] However, this treatment is still not optimal, as even with very early administration 20-35% of occluded vessels still do not reopen.[74] Furthermore, reocclusion of recanalized vessels (15%) and bleeding complications (0.3-17%)[64,66,67,75-77] limit the efficacy and safety of thrombolysis. New developments in molecular biology and protein engineering may provide the clinician with safer and more effective plasminogen activators in the future.[78,79] However, combining thrombolytic therapy with other classes of drugs may have additional advantages.

Treatment of reocclusion
Reocclusion of successfully recanalized coronary arteries occurs in a considerable number of cases. Also during the acute phase of myocardial infarction spontaneous intermittent recanalization and reocclusion resulting from a variable combination of thrombosis and vasoconstriction are frequent.[80] Adjunctive therapy with vasodilators and/or antiplatelet agents thus may be of benefit,[80,81] but even then a significant percentage of patients face reocclusion or restenosis.

After PTCA for stable or unstable angina, acute occlusion has been reported to occur in 2-5% of dilated arteries,[82,83] while late restenosis occurs in about 30% of the dilated vessels.[84-89] Studying those patients or specific lesions prone to acute or subacute complications has provided us with some general ideas[89], but prediction of a subsequent occlusion or restenosis in individual cases is not yet possible. Medical treatment with various agents has not provided a solution to the restenosis-problem,[91-98] although one trial that used fish oil preparations has suggested benefit.[99] The employment of a vascular endoprosthesis, introduced at the site of the reopened artery, is currently under investigation.[100-102] Although the efficacy of vascular endoprostheses to ensure patency of the coronary vessels after thrombolysis has not been investigated yet, and may be an unnecessary measure as a routine, subgroups of patients with very large infarcts (cardiogenic shock) may be identified in the future who may benefit from this procedure. Also the magnitude of the restenosis-problem after PTCA requires a solution. In the setting of acute occlusion after PTCA the employment of stents when used as a "bail-out"-device has been successful. However, the present generation of metallic stents will not likely be the ultimate treatment, due to their thrombogenicity.[103-105]

1.2 Experimental coronary artery occlusion and reperfusion

1.2.1 Coronary artery occlusion in experimental animals

Models of coronary artery occlusion

Investigations of the effects of coronary artery occlusion in experimental animals were initiated more than a century ago.[106] During these early years phenomena such as the loss of regional myocardial function and lethal arrhythmias were already described in detail. Paradoxically, at the moment when Herrick proposed coronary thrombosis as the initiating event in clinical myocardial infarction,[107] most of the used animal models were those with sudden coronary ligation or clamping. On the other hand when coronary artery thrombosis was questioned as the ultimate event in the 50'ties and 60'ties of this century, the first animal models of coronary artery thrombosis were tested.[108,109] Whatever factors or attitudes caused this discrepancy may form an interesting chapter in the history of cardiology, but is beyond the scope of this thesis. However, both directions in animal research have contributed to our knowledge of clinical coronary syndromes. Available animal models of myocardial ischemia, including models of coronary artery thrombosis are discussed in chapter 2.

1.2.2 Prevention and treatment of experimental coronary occlusion

Prevention of occlusive thrombosis

Arterial thrombosis is considered the consequence of a dynamic interaction among functional and structural arterial defects, spasm, platelet activation and initiation of the coagulation cascade.[110] While in the healthy situation the delicate balance between bleeding and hemostasis remains undisturbed, a defect at any of the above mentioned levels can initiate thrombosis. As already described above the efficacy of clinical measures aimed to prevent coronary arterial thrombosis is limited. Appropriate animal models of coronary thrombosis may be helpful in identifying new drugs, which are efficacious in preventing or slowing the occurrence of thrombotic coronary occlusion. The first aim of this thesis was the development and use of an animal model of coronary thrombosis, to test the efficacy of new drugs to prevent thrombotic coronary occlusion. In chapter 3 a porcine model of coronary thrombosis is described, which has been developed at the Laboratory for Experimental Cardiology at Erasmus University, Rotterdam.

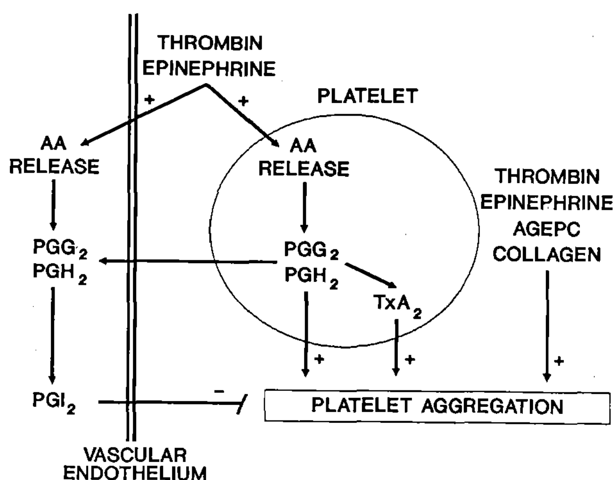

Fig. 1. Actions of prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) on platelet aggregation. AA:arachidonic acid ; AGEPC: platelet activating factor ; PGG_2 and PGH_2 are unstable endoperoxydes of arachidonic acid. + : stimulation ; - : inhibition.

Chapters 4 and 5 describe two studies using this model for the identification of efficient antithrombotic drugs in the coronary arterial tree. The concept underlying the choice of these two specific drugs is based on the hypothesis that

the balance between two metabolites of arachidonic acid, the prothrombotic thromboxane A_2 and the antithrombotic prostaglandin I_2, is of importance in arterial thrombosis.[35,36,111-113]

Myocardial reperfusion in experimental animals
The second aim of this thesis was to study the possibility to improve the efficacy of early thrombolysis and prevent reperfusion damage. To this end the combination of a thrombolytic agent with a calcium antagonist was studied (chapter 6).
Two related issues have been addressed in chapters 7 and 8. The former dealing with the possibility to improve the recovery of post-ischemic myocardium by a pharmacologic approach. It may well be that patients with large areas of reperfused, but still not contracting (so called "stunned") myocardium can benefit from such an intervention. The latter concerns the clinically very relevant question: is it possible to obtain a diagnostic marker discriminating between reversibly and irreversibly damaged myocardium? Such a marker, if readily available to the clinician, may strongly influence the decision making process around the patient presenting with acute coronary occlusion.

Reocclusion and restenosis after coronary recanalization in experimental animals
As has been stated above, early reocclusion and late restenosis limit the efficacy of thrombolytic therapy in acute myocardial infarction and coronary angioplasty in unstable or stable angina pectoris. Experimental studies have shown that anatomical as well as functional factors are involved which promote reocclusion.[114] Firstly, there is the pathologic substrate of deep injury of the ruptured atherosclerotic plaque with exposure of collagen (and especially the more thrombogenic types I and III predominantly present in the arterial media, as opposed to the less thrombogenic types IV and V of the intima), and lipid gruel.[115] During thrombolytic therapy also the thrombogenic surface of the residual thrombus, causing thrombosis coincident with thrombolysis to occur, seems to be a specific complicating factor. After PTCA the presence of intimal flaps may limit the ultimate result. Secondly, there is the pathologic substrate of a high shear rate and turbulent blood flow in the presence of a residual stenosis. This may occur after thrombolytic therapy, as well as after incomplete coronary angioplasty. Blood flow through a significant stenosis is suddenly accelerated, promoting flow separation and activation of blood platelets to form aggregates and a (predominant) platelet thrombus. Downstream the stenosis, recirculation with eddy currents will be present. This low shear distally leads to disposition of fibrin which in turn traps red blood cells. This confirms clinical observations at angioscopy and autopsy of platelet thrombus within the stenosis and red thrombus distal to the stenosis. In addition the proportion of reocclusion

in coronary arteries after angioplasty or thrombolytic therapy has been directly related to the minimal cross-sectional area after recanalization.
Functionally, the absence of normal endothelium predisposes to thrombosis and vasoconstriction.[116] Normal endothelium produces prostacyclin, endothelium derived relaxing factor, plasminogen activator and protein C which inactivates thrombin.[117] The absence of endothelium may cause vasoconstriction by thrombus originating substances like thrombin, ADP, serotonin and thromboxane A_2. Therefore, the persistence of pro-occlusive factors during thrombolysis or after successful angioplasty, is a dynamic process with many simultaneously occurring stimuli promoting reocclusion. Thus instead of the administration of a single antiplatelet or anticoagulant agent, a combined approach of mechanical dilatation and a pharmacological "protection" against reocclusion may theoretically be the more effective. The employment of endoluminal vascular endoprostheses, preferably with drug eluting properties, provides such an approach.[118] However, currently available metal stents are thrombogenic in itself.[100,119,120] Therefore, the improvement of the surface-characteristics of one of the stents, which is currently available for clinical evaluation, was the object of chapter 9 of this thesis.
In closing, a discussion of these approaches and their promises as well as lack of solution, is provided in chapter 10. For the reader with limited time, therefore perusal of chapter 1 and 10 would suffice.

Chapter 2

EXPERIMENTAL MODELS OF MYOCARDIAL ISCHEMIA

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Experimental Models of Myocardial Ischemia

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Historical Background

One of the first references to the use of animals by man for the advancement of knowledge can be found in Jewish history. The Jewish community in Babylon collected vast amounts of data concerning animal anatomy, physiology, pathology and veterinary medicine for the purpose of the interpretation of their dietary laws (1). Unfortunately this derived knowledge did not reach the non-Jewish part of the population and most of it was consequently lost, but obviously one, if not the first attempt at comparative biology had been made.
In all likelihood Hippocrates (460–377 BC) did not use animals in his studies and it is thought that Galen (130–200 AD) introduced the dissection of primates and lower animals and applied his findings to advance the knowledge of human anatomy and physiology. However, he did not suggest using animals as models for human disease. After the Middle Ages it is odd that diseased animals were often treated as if they were human beings (2).
The example of experimenting on animals to elucidate the genesis of human disease was probably set by Jenner in the early 18th century. While studying cows suffering from

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cow-pox on their teats and mammae, he performed one of the most successful exploits with animal models in medicine: the eradication of small-pox. The finding of animals highly susceptible to human pathogens established the use of animal models in medical research. Koch's postulates concerning infectious diseases could not have been confirmed without a susceptible animal model.

The structure of the heart and the coronary circulation was described early on by Andreas Vesalius in his classical work (3), and William Harvey elucidated much of the function of the heart at the beginning of the 17th century (4). English clinicians of the nineteenth century defined coronary heart disease. Its clinical picture was drawn by Heberden (1818) and these symptoms were correlated with autopsy findings by Parry (1825). Mention should also be made of Allan Burns' (1781–1813) book, published in 1809, «Observations on some of the most frequent and important diseases of the heart» containing a detailed and remarkably accurate description of the pathophysiology of effort angina. Pierre Chirac and Raymond de Vieussens were among the first to study the effect of ligation of the coronary arteries in animals (5). But the beginnings of experimental pathology in a systematic fashion can be ascribed to Marshall Hall and John Erichsen of Edinburgh University. To study sudden death they ligated the coronary arteries of the dog. These experiments were expanded by the German School of experimental pathology, with Rokitansky and Virchow as forerunners. Von Bezold and Panum investigated the influence of coronary ligation in more detail and were the first to use physiological measurements of cardiac activity. Panum's work is of particular interest to the scope of this review, as he applied techniques other than ligation to occlude coronary arteries. In fact he embolized coronary arteries by injection of powder, wax, oil and india ink (6). Moreover these workers even examined the possiblity that the consequences of ligating a coronary artery arose from a lack of oxygen. Thus Panum and his group became the founders of the modern studies of the pathophysiology of myocardial ischemia.

Today inbred strains of spontaneous animal models are kept and studied with evident success, not only in the field of infectious disease but also of genetic and immunologic diseases and of disorders of thrombosis and hemostasis. Unfortunately only a few spontaneous models of atherosclerosis and myocardial ischemia are known (7), so most of the models discussed will be induced models and suite only for the study of pharmacological approaches or for the study of sequels of myocardial ischemia.

Definitions and Criteria

The use of models has taken an increasingly central position, because it often fits extremely well in a modern research strategy. It is an attempt to compare: can we get more insight in the unknown by studying the known? In a general sense a definition of a good model is: «If one uses a known system A, independent from H, to get information about H, then A is a model of H.» This definition stresses one of the major points in the use of models: the model should be very well defined and «known» at the point where the investigator is planning an intervention. An animal model of human disease has

been defined as: «a living organism with an inherited, naturally aquired or induced pathological process that in one or more respects closely resembles the same phenomenon occuring in man» (8). The Institute of Laboratory Animal Resources (ILAR) of the National Academy of Sciences of the U.S.A. modified this definition to: «an animal model is a living organism in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other animal species» (9). The primary criteria for the use of animal models for a disorder of the cardiovascular system are: a) spontaneous or induced animal disease that closely mimics the human condition, b) a host whose cardiovascular system resembles that of man in structure and function, c) availability, either as large numbers of the naturally occurring disease, or as reproducible models.
For the human condition to be mimicked, some criteria must be defined for the human disease under study: a) documentation of its pathological features, and b) identification of the human disorder as a single disease or as including conditions that are different in cause and pathogenesis, although they share similar pathophysiological features, e.g. the same endresult may arise from different pathogenetic pathways, and a given pathway may be triggered by different causes.

Concept of Myocardial Ischemia

For decades myocardial infarction was synonymous with coronary thrombosis, based on the pioneering work of Herrick on the cause of ischemia (10). This concept was challenged by Branwood and Montgomery (11) in the nineteen fifties and the debate has continued since then. Current belief is that myocardial ischemia is the consequence of a mismatch between the demand for oxygen and substrates and its supply through the coronary arteries. In patients with either angina pectoris or myocardial infarction the ischemia-related coronary artery is almost invariably significantly narrowed (> 50 % diameter stenosis) by an atherosclerotic lesion (12). In acute transmural myocardial infarction total coronary occlusion was observed in 87 % of cases by coronary angiography in the early hours after symptoms developed (13). In the first few weeks after infarction frequencies of 86–96 % were observed at autopsy (14–17), although this incidence was slightly lower (18) as the time after infarction increased. In a recent report Davies and Thomas (19) observed coronary artery thrombi in 96 out of 100 cases of acute cardiac ischemic death, and these thrombi were occlusive in 74 of the cases. All studies established an excellent spatial relationship between coronary thrombi and transmural infarction. A temporal relationship is suggested but other techniques will have to be applied to prove this.
In a minority of patients coronary vasospasm has been documented as the cause of coronary occlusion (20). Furthermore it has been shown that in the first few days following an acute infarction coronary vasospasm occurs spontaneously or can easily be provoked by administration of ergonovine (21).
Other mechanisms probably dominate (22) in subendocardial infarction or recurrent

myocardial ischemia of angina pectoris. The frequency of coronary thrombosis is low in cases of subendocardial infarction and is unknown in angina occurring in the presence of advanced coronary sclerosis. The latter disorders can result from a relative lack of blood supply to the dependent part of the myocardium, because of either suddenly increased work by the heart or decreased perfusion. Current reports suggest that a dynamic interaction between damaged endothelium, platelet aggregates and coronary vasospasm may occur in recurrent myocardial ischemia although more evidence is needed and the exact sequence of the events remains to be established.

Cardiovascular System of the Host

Much of our current thinking regarding myocardial ischemia has been based upon preparations in larger mammals such as the dog, pig and baboon. None of these species resembles man completely. Depending on the main feature of the studies, a sensible choice can nearly always be made. Excellent reviews regarding the comparative biology are available of these species (23–28).
Available animal models roughly cover three distinct areas of the afore mentioned concept of disease. They are used for: a) the search for the etiologic agent, b) the search for the pathogenetic mechanism, and c) advancing knowledge about the sequels of ischemia. As myocardial ischemia may be a sequel of many diseases, option a) and b) remain limited to animal models of those specific diseases. No mention will thus be made here of ischemia caused by congenital coronary abnormalities, coronary vasculitis or hypertrophic cardiomyopathy. Models of myocardial ischemia related to valvular heart disease will also be omitted. The review will be limited to: 1. models of coronary atherosclerlosis, 2. models of acute or chronic coronary occlusion, 3. models of coronary thrombosis or spasm.

Animal Models of Atherosclerosis

Most of the existing information on which we base our understanding of the atherosclerotic process has been acquired through animal models. Atherosclerosis has been induced successfully in several species of animals, including rats, rabbits, chicken, pigeons, pigs, dogs and non-human primates (29–31). Originally, rodents and especially rabbits were widely used as experimental animals. No naturally occurring lesions are observed in this species but upon feeding diets rich in cholesterol, this substance accumulates in various tissues. The thoracic aorta will be affected predominantly but with more diffuse involvement the coronary arteries will also contain lesions, although mainly at the intramyocardial branches. These lesions comprise mainly massive foam-cell accumulation. More complicated lesions can very rarely be produced, unless a high-cholesterol diet is combined with coconut oil or allergic injury to the endothelium.

Avian species e.g. the chicken and the pigeon have the advantage that the naturally occurring diesease is amenable to study. In the chicken the lesions are mainly located at the intima, and are cellular to fibrous, while lipid is absent initially. In the pigeon, with its natural hypercholesterolemia (350 mg/100 ml), the lesions show a remarkable similarity to those in man, and even progress to myocardial infarction in older birds. Breeds such as the White-Carneau are highly susceptible to this disorder while the Show-Racer is relatively resistant. Upon cholesterol feeding new lesions can be produced and naturally occurring ones are aggravated. In the chicken and the Japanese quail the disease is mainly located in the thoracic aorta and the coronary arteries, while in the pigeon lesions are found primarily in the abdominal aorta (as in man) with a predilection site at the bifurcation of the celiac artery. These severe lesions show all the complications present in man: calcification, ulceration, hemorrhage and mural thrombi. The potential for regression of lesions in avian species is very great, making them highly attractive for interventions aiming at that end result. The significance of this finding with respect to human lesions has still to be established.
Dogs are resistant to both naturally occurring and induced atherosclerosis. High dietary cholesterol combined with either thyroidectomy or endothelial damage is required to produce lesions. Yet rather large individual differences in the extent of lesions exist; the media shows more extensive involvement than the intima. These factors make the dog less suitable for the study of atherosclerosis.
Both naturally occurring and experimentally induced atherosclerotic lesions can be studied in the pig. The abdominal aorta predominates initially as site of these lesions. Musculo-elastic lesions develop easily upon cholesterol feeding, but complicated lesions (as in the avian species or man) require endothelial damage also. At that stage complications like myocardial ischemia can also be observed. The potential problems of handling these fast growing animals can be avoided by using the equally susceptible miniature swine as a model.
In non-human primates naturally occurring atherosclerotic lesions remain limited to small areas of intimal thickening. Also considerable species variability exists regarding the response to dietary cholesterol and the extent and severity of the lesions thus produced. However upon cholesterol feeding susceptible species show complicated lesions in the aorta and coronary arteries although not so severe as can be observed in swine. Occlusive coronary disease can nevertheless be produced. A further advantage of primates is the sharing of personality characteristics with human victims of the disease (competitive males). Also baboons can be taught to smoke tobacco (32).

Animal Models of Coronary Occlusion

The models now in use can be divided into those employing acute, chronic or gradual coronary occlusions in anesthetized or conscious animals. The most widely used way to induce coronary occlusion is sudden ligation of the vessel in open-chest, anesthetized animals. The possibility of exact timing of the onset and duration of ischemia and the

complete cessation of antegrade blood flow are attractive features of this method. It is also possible to adjust the level of flow reduction accurately by placing flowmeters around the coronary artery. Furthermore, releasing the ligature or clamp makes it feasible to study myocardial reperfusion. A disadvantage of acute occlusion will be the fact that previously healthy myocardium abruptly becomes ischemic, not allowing any adaptation to ischemia. Pathological and angiographic studies in man suggest a more gradual or repetitive type of narrowing of the vessel before the ultimate occlusion.
Using coronary ligation in the conscious animal avoids the suppression of cardiovascular reflexes and the frequent negative inotropic effect of anesthesia. However the possibilities for manipulating flow-reduction will be limited to the 0 % and 100 % level, as the need for frequent zerosetting of the implanted flowprobes will be the limiting factor. Compressing the coronary artery by inflating an implantable balloon occluder seems superior to the ligation technique in this respect. Although successes have been reported (33), this method is only reproducible in experienced hands.
After acute ligation the incidence of myocardial ischemia will be 100 % in both dogs and pigs. The size of the resulting ischemic zone will be between 60 and 80 % of the perfusion area after occlusion of the left anterior descending coronary artery (LAD) in the dog, and between 90 and 100 % in the pig. This difference can be explained by the pronounced collateral flow in dogs (28). After permanent LAD occlusion subendocardial necrosis will become demonstrable as early as 45 minutes after occlusion in the dog, reaching its ultimate transmural size after 4 to 6 hours (34, 35). Reperfusing the myocardium within this period of occlusion will prevent tissue from becoming necrotic to an extent related to the duration of ischemia. After 1 to 3 hours of ischemia and subsequent reperfusion regional myocardial function will be moderately to severely depressed for more than 4 weeks (36, 37). An occlusion period of only 15 minutes in conscious dogs requires up to 6 hours of reperfusion for complete recovery (38). In the pig coronary occlusion will even be less well tolerated. No recovery of function can be demonstrated in this species after a 30 minute LAD occlusion followed by one hour of reperfusion (39), whereas abnormalities of wall motion can still be shown after 2 weeks of reperfusion (unpublished results from our laboratory). As concerns non-human primates, an established model of coronary ligation has been developed in the baboon (24).
This model being phylogenetically closer to man may offer advantages with respect to studies employing immunological techniques or work requiring a close molecular resemblance of cardiac or extracardiac receptors. Like the pig, the baboon lacks a coronary collateral circulation. After acute coronary occlusion a clearly defined area of transmural myocardial ischemia will be generated with accompanying abrupt ST segment changes and marked metabolic alterations (32). Although some are difficult to handle conscious primates have been studied successfully (40).
For the evaluation of an intervention intended to modify the consequences of myocardial ischemia, a control group of normal or «control-occluded» hearts will be needed. The occlusion in sequence of two small to medium sized branches of the same coronary artery can be compared within the same heart (41). This technique combines the advantages of nearidentical hemodynamic variables during the two occlusions and of not needing a control group of animals. However, recent evidence suggests that

biochemical alterations occur in myocardial tissue adjacent to an area of ischemia. The in vitro phosphorylation of sarcolemmal membrane components reaches significantly higher values when the samples are obtained from a part of the myocardium adjacent to an area of ischemia (42).

The production of a fixed coronary artery stenosis, which is not critical at rest but becomes flow-limiting under stress, offers additional information. Models have been described that use the closed-chest animal (43, 44) but for which the open-chest preparation can also be used. In the former a small plastic truncated cone with a narrow, central internal lumen (diameter 0.625 mm) is passed over a guide wire into the LAD of a closed-chest pig. In the open-chest preparation the proximal LAD is secured through a slit in a small plastic ring (internal diameter 2.0 mm). Models like these show normal perfusion distal to the stenosis under baseline conditions, but flow as well as function declines markedly following atrial pacing at higher rates. A disadvantage of the intraluminal placement of devices is that a small side-branch of the artery into which the device is inserted can become occluded. Furthermore the precise location can often not be controlled, as the dimensions of both the cone and the vessel dictate the site of fixation. Moreover, the device cannot be removed or its site of placement adjusted.

The application of these techniques in chronic animals allows the study of the formation of a collateral circulation, the effects of exercise and long-term pharmacological interventions (45, 46).

Ameroid constrictors have been used for the same purposes (28). These cylindrical, hygroscopic plastic rings are encased in a steel ring to prevent outward swelling after placement around a coronary artery. The Ameroid constrictor will gradually narrow the vessel and about 50 % of the arteries will be completely occluded after 2 to 3 weeks. An advantage of this method is that the growth of coronary collaterals could be stimulated in dogs and pigs; rhesus monkeys however did not respond (47). A disadvantage of using Ameroid constrictors or critical stenoses is that one never knows whether the desired occlusion has been accomplished.

Animal Models of Coronary Spasm

The search for animal models of coronary artery spasm is only a few years old. In the dog, the administration of ergonovine can provoke coronary artery constriction at the site of experimentally induced atherosclerotic lesions, whereas unaffected sites do not respond (48). However the maximal response in these experiments is a constriction of only 20 %, without the occurrence of ischemic ECG changes. In miniature pigs with experimentally induced atherosclerotic lesions in the coronary arteries, the same group of investigators were able to provoke spasm of the left circumflex coronary artery (LCX) with a reduction of over 75 % in coronary diameter with accompanying ST segment changes (49). But these episodes could only be provoked after administration of histamine into arteries with endothelial denudation. Ergonovine was ineffective in the pig model but this agent is frequently used to provoke coronary artery spasm in man.

Models with Cyclical Reductions in Coronary Flow

An interesting approach used in one of these models is the placement of a plastic cylinder 2.5 mm in length, designed to produce 60–80 % narrowing, around a coronary artery in dogs (50). This type of narrowing will abolish the hyperemic response to complete occlusion but under basal conditions mean coronary flow should be at near normal level. The phasic pattern of coronary flow will be largely absent, however. In the first two hours of coronary stenosis cyclical reductions in coronary blood flow and distal coronary artery pressure occur in many of the dogs. During these episodes coronary blood flow decreases gradually to about 50 % of baseline, subsequently followed by a spontaneous, sudden recovery. While flow is reduced the epicardial ECG shows ST segment depression suggestive of ischemia. Histologic examination of the arteries sampled at the time of obstruction reveals platelet aggregates. Cigarette smoke, nicotine and indomethacin all result in an aggravation of this phenomenon (51), while aspirin, prostacyclin, inhibition of thromboxane synthesis, phentolamine and papaverine inhibit or completely abolish the cyclical reductions (52). The exact mechanism of these episodes of flow reduction is not yet clear. Although coronary vasospasm or platelet aggregation and subsequent embolization have been held responsible, current evidence favours a primary thrombotic cause. However all active pharmacological compounds also have vasodilating properties in this model and it has been shown recently that only very slight reductions in luminal diameter may cause endothelial damage and secondary thrombus formation (53).

Models of Coronary Thrombosis

Numerous methods are available to induce arterial thrombosis in laboratory animals (see 54 and 55). Only a minority of these methods fit the above described concept of the pathogenesis of myocardial ischemia. Experimental models based on the intravascular injection of proaggregatory substances produce multiple platelet aggregates and small thrombi «plugging» the myocardial microvasculature (56). Although it is not an animal model of thrombosis, the in vitro production of a white platelet thrombus using the Chandler rotating loop technique (57) with subsequent intracoronary placement of that thrombus has been used for studying intracoronary thrombolysis. The current concept that coronary thrombosis develops upon a preexistent endothelial lesion limits the use of these two models.
Currently used animal models of coronary thrombosis involve either the intracoronary placement of a thrombogenic device or production of a controllable thermal injury to the coronary vessel wall by an electrical current, laser injury or freezing. In a model using anesthetized dogs a Teflon-coated stainless steel electrode exposed only at its tip is advanced through a catheter under fluoroscopic guidance in either the LAD or the LCX (58). A direct current of between 100 and 900 microamperes is applied through this

electrode to the vascular wall and causes thrombotic occlusion of the coronary artery between 18 and 93 minutes of current flow. An advantage of this method is its reproducibility by other investigators (59, 60). A disadvantage may be the need for fluoroscopic facilities and the variability in the sites where the catheter tip will be in contact with the vessel wall. Also the possible obstruction of coronary flow through the coronary artery or its side-branches by the guidance catheter may cause myocardial ischemia or serious arrhythmias. The latter may be rather well tolerated by dogs, but are frequently lethal in pigs (60). To improve control of the moment of occlusion by an intracoronary thrombus, a catheter technique for placing a thrombogenic device has been developed. A helically shaped thrombogenic device (copper or magnesium alloy wire) can be inserted into the lumen of the LAD or LCX of closed-chest, anesthetized dogs by placing it on the tip of a catheter. The timing of thrombotic occlusion can be controlled by altering the shape and length of the device (61, 62). A further advantage is that coronary thrombolysis can be studied in the closed-chest animal. In our laboratory, the placement of an intracoronary copper wire in the open-chest pig resulted in instantaneous coronary vasospasm at the site of the device, with the resultant formation of a red, fibrin-rich clot. Applying magnesium alloy devices will produce typical platelet-rich thrombi, although the time to complete occlusion remains short.

An electrical method, using a needle-tipped electrode inserted through the wall of the LCX so that approximately 2–3 mm of the tip of the electrode is within the vessel lumen and in contact with the intimal lining, was developed for the anesthetized, open-chest as well as the conscious ambulatory dog (63, 64). This method using currents of 50–150 microampere has proven its reproducibility in several experimental series. Furthermore the damage to the coronary artery will be limited to the intimal lining, leaving the media intact, thus probably limiting the incidence of spasm to the site of stimulation. A disadvantage of this model is the incidence of embolisation of thrombotic material, which occurs more frequently in the pig when currents less than 200 microampere are used (unpublished results). Furthermore, leakage of current to the bloodstream limits the controlability of the electrical stimulus particularly in the closed-chest animal. This will be a feature of all methods involving an electrode in direct contact with the bloodstream, as the build-up of electrical resistance by the damaged vessel wall will be fast. A further disadvantage of all the models of coronary thrombosis just discussed is that spasm cannot be excluded as the cause of occlusion.

A model using the open-chest pig (65) was therefore developed in our department. Using a circumferential electrode around the electrically isolated LAD, a well controllable DC current of 1600 microampere is applied. An occluding thrombus was produced in 80–100 % of the experiments within 20 to 45 minutes of stimulation. Coronary vasospasm preceded thrombosis as the cause of obstruction in about 30 % of the experiments, but could be excluded by local application of nitroglycerine. Testing of the antithrombotic efficacy of drugs can be done reliably in this model, as has been shown recently for the stable prostacyclin analogue ZK 36374 (66). A disadvantage of this model is that extensive damage occurs in all layers of the vessel wall (65), thereby inhibiting the synthesis of locally produced antithrombotic substances.

Conclusion

All the animal models discussed above have a feature in common: they change the variable clinical entity of myocardial ischemia to an enumeration of different disorders. No single animal model will give definite answers but each one provides new insights or contributes to the study of a particular problem concerning the cause, the pathogenesis or the consequences of myocardial ischemia.

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Chapter 3

A NEW MODEL FOR CORONARY THROMBOSIS IN THE PIG: PRELIMINARY RESULTS WITH THROMBOLYSIS

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A new model for coronary thrombosis in the pig: preliminary results with thrombolysis

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KEY WORDS: Pig, coronary thrombosis, experimental myocardial infarction, spasm, thrombolysis, regional wall function, microspheres.

A new model for the electrical induction of coronary thrombosis in the pig is described. In 35 out of 47 experiments (74%) a 100% occluding thrombus developed. In 16 experiments (34%), at least one episode of coronary spasm occurred at the site of electrical stimulation. Flow in the region of the left ventricle supplied by the thrombosed coronary artery decreased from 1·04±0·21 to 0·09±0·04 ml/min/g tissue (mean±SD). Histological examination showed that the lesion caused by the electrical current occurred through the arterial wall and was quite discrete, measuring a few millimetres in length. Scanning and transmission electron microscopy revealed that the occlusions were caused by typical platelet thrombi. Lysis of the thrombi by the intracoronary administration of porcine plasmin was successful in 15 out of 19 animals (79%). Preliminary results show that thrombolysis after 40 min occlusion restores flow to nearly base-line values, but does not improve regional function in the first 3 h.

The management of acute myocardial infarction by the intracoronary administration of thrombolytic agents, such as streptokinase, is a very recent and promising development in clinical cardiology. Yet the ultimate benefit of this treatment for the patient still remains an open question. In order to evaluate the effect of thrombolysis in acute myocardial ischemia, the design of a reliable model for coronary thrombosis in the laboratory animal seems an appropriate aim.

Numerous methods for inducing arterial thrombosis are available[1–6]. Since the aetiology and pathogenesis of arterial thrombosis is still largely obscure, every justification of a new model is still based on presumptions. Ours are that (a) coronary thrombosis develops upon a pre-existent endothelial lesion; (b) the various stages of thrombogenesis require a certain amount of time, since methods of producing experimental thrombi within a few minutes lead to blood stasis which bears little resemblance to thrombosis; (c) methods which utilize foreign bodies within the arterial lumen cannot be employed since they are prone to reduce or disturb coronary flow or induce occlusion by spasm instead of thrombosis.

In the present paper we describe the development of a new model for experimental coronary thrombosis, which fulfils most of these criteria.

Methods and materials

Experiments were performed on 47 Yorkshire pigs. Anaesthetic and catheterization procedures have been described elsewhere in detail[7,8]. Briefly, after sedation with 120 mg azaperone i.m., anaesthesia was induced by i.v. administration of 150 mg metomidate. The animals were intubated and connected to a respirator for artificial ventilation (O_2: $N_2O = 1{:}2$). Catheters were placed in the superior vena cava (7F Cournand) for the administration of pentobarbital to maintain anaesthesia, and 0·9% saline to correct for fluid losses, in the ascending aorta (7F Millar) for the measurement of mean aortic pressure, in the abdominal aorta (8F Cour-

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nand) for the collection of blood samples for determination of blood gases (ABL 1, Radiometer, Copenhagen, Denmark) and for withdrawal of reference samples during the injection of radioactive microspheres. A peripheral ECG-lead was monitored throughout the experiments.

The heart was exposed by means of a midsternal thoracotomy, and suspended in a pericardial cradle. Parts of the fourth and fifth ribs of the left hemithorax were removed to get easy access to the left descending coronary artery (LAD) and its accompanying vein. An electromagnetic flow probe, 14–17 mm in diameter, was placed around the ascending aorta for measurement of cardiac output. The LAD was prepared free from its first to its second diagonal branch. An electromagnetic flow probe (Skalar, Delft, The Netherlands, 2·0–2·5 mm diameter) was placed just distal to the first diagonal branch around the artery. A polyethylene strip (40×5×0·5 mm) was placed under the LAD, just distal to the flow probe, to isolate the artery from the myocardium. At this point, a stainless steel wire electrode (diameter 0·3 mm) was coiled around the LAD without impeding its flow and yet so tight that it made contact with the adventitia at all points in the circumference of the vessel. The electrode was connected to the anodal side of a direct current source adjustable from 0 to 3 mA, delivering a maximal output level of 30 V. The cathodal side was connected to the abdominal fat of the pig. The accompanying vein of the LAD was cannulated and a polyethylene catheter was inserted with its tip in the area to become ischemic. A 5 MHz ultrasonic transducer (Krautkramer-Branson, Lewistown, PA, USA) was sutured on the epicardium of the same area, for the measurement of regional wall motion. A 2F polyethylene catheter was positioned through the wall of the left atrium for the injection of radioactive labelled microspheres.

PREPARATION OF PORCINE PLASMIN

Neither streptokinase nor urokinase can be used in the pig to achieve fibrinolysis[9], so porcine plasmin was used as the thrombolytic agent. Plasminogen was isolated from porcine plasma as described by Deutsch and Mertz[10]. The pure plasminogen was subsequently activated to plasmin by immobilized urokinase (Laboratoires Choay, Paris, France), according to Wiman and Wallén[11] and as modified by Cederholm-Williams *et al.*[12]. The plasmin was stored at −30°C until the time of infusion. Activity of pure porcine plasmin was measured spectrophotometrically with S2251 as substrate (Kabi Vitrum, Amsterdam, Holland). One unit is defined as the plasmin activity measured in 1 ml porcine plasma after activation by a mixture of streptokinase and urokinase.

MEASUREMENTS

Electrocardiogram (ECG), mean arterial pressure (MAP), cardiac output (CO) and LAD-flow were continuously written on a Gould Brush 480 recorder. Arterial (ASO_2) and local coronary venous oxygen saturation ($CVSO_2$) was measured by a MSO_2 hemoximeter (Radiometer, Copenhagen, Denmark). Regional myocardial blood flow was measured with radioactive labelled microspheres (^{141}Ce, ^{51}Cr, ^{113}Sn, ^{103}Ru, ^{95}Nb)[13], while regional systolic wall thickening (SWT) was determined from the echographic tracings[8]. No pharmacological adjuvants were used. Ventricular tachycardia (VT) or fibrillation (VF) were treated by DC-counter-shock only.

EXPERIMENTAL PROTOCOL

After the preparation had been stable for at least 30 min the experiments were performed according to the following steps.

(1) Baseline recordings were made of: heart rate (HR), MAP, CO, LAD-flow, ASO_2 and $CVSO_2$, injection of the first microsphere.

(2) Start of electrical stimulation at 800–1600 uA for 90 min, unless a thrombus developed within that time. In the animals who did not develop a thrombus within 90 min, any further thrombus formation was spontaneous. Before removal of the electrode and isolation material, the second microsphere was injected.

(3) When the vessel was completely occluded (determined by LAD-flow, the absence of SWT and visual inspection) nitroglycerin (NTG) was dripped locally on the vessel wall to exclude spasm as the cause of occlusion. When spasm was present, the use of NTG was repeated at each following occlusion.

(4) When no reaction to NTG was observed, thrombus formation was supposed to be complete and all measurements as described under (1) were repeated, and the third microsphere was injected.

(5) In 32 experiments, only the formation of thrombosis was studied. After 30 min occlusion, 2 U/min of porcine plasmin was infused in the LAD in 14 of these, solely for the assessment of the success rate of thrombolysis. These 32 experiments were terminated within an hour after occlusion. In 5 animals, in which thrombus formation was considered to be complete, the LAD was processed for

Table 1 Incidence of occluding coronary thrombosis and/or spasm in anaesthetized pigs

		Thrombus	No thrombus
Experiments	47	35 (74%)	12 (26%)
Spasm	16 (34%)	11	5

Table 2 Regional myocardial blood flow (ml/min/g) during thrombus development in 10 anaesthetized pigs

	Base line	90 min	Thrombus
Control area	1·10±0·23	0·94±0·26	1·09±0·35
Area at risk	1·04±0·21	1·03±0·34	0·09±0·04*

*$P<0{\cdot}05$ from base line and 90 min.

microscopical and ultrastructural evaluation. Finally, the animals who did not develop a thrombus were followed for at least 240 min. In seven of these 'failures' the LAD was processed for microscopy at the end of the experiment.

(6) In 15 experiments the observation period was extended up to 3 hours after occlusion. In five of these animals 2 U/min of porcine plasmin was infused in the LAD after 30 min of occlusion. No intervention was done in the 10 other experiments which served as controls. Recordings as described under (1) were repeated every hour.

Results

A. THROMBUS DEVELOPMENT

In 35 out of 47 experiments (74%) an occluding thrombus developed, with the occlusion time ranging from 22–225 min (122±54 min. mean±S.D.) (Table 1).

At least one period of spasm, as confirmed by the local application of NTG, occurred in 16 experiments (34%). In five of these, no thrombus developed (Table 1). In the 11 animals in which spasm preceded thrombosis, the time between the two events ranged from 2 to 32 min (median 12 min).

Regional myocardial flow data at the beginning of electrical stimulation, at 90 min, and at the time of complete occlusion are grouped in Table 2. Arrhythmias were analysed in 11 experiments, in which an occluding thrombus developed. During the first 15 min after occlusion, a relatively large number of arrhythmias were observed, but the incidence of VF was low (Table 3). In the following 15 min ventricular ectopic activity decreased, but VF was observed in the majority of the animals.

Table 3 Incidence of ventricular arrhythmias during first 30 min after thrombus development in 11 pigs

	Period	
	0–15 min	15–30 min
PVCs/min		
Range	0–14	0–8
Median	3·3	0·15
VT	4	1
VF	1	7

PVC, premature ventricular contraction; VT, ventricular tachycardia; VF, ventricular fibrillation

B. MORPHOLOGICAL FINDINGS

At the site of the electrical wire, the arterial wall was severely damaged. On gross inspection, a mural thrombus was found here, totally or subtotally occluding the vascular lumen. This was confirmed by light microscopy (Fig. 1b). Scanning electron microscopy from this area showed a platelet thrombus with fibrin strands and adhering leucocytes (Fig. 2c). Occasional platelets and leucocytes were found adhering to the intimal surface in the immediate vicinity of the lesion (Fig. 2b). A few millimetres away, the intimal surface appeared normal (Fig. 2a). Light microscopical sections through the arterial wall at the site of the wire showed coagulation necrosis of the media, whilst the elastic skeleton remained more or less intact (Fig. 1b and 1d). The intima was absent at this site. This lesion was quite small and discrete, measuring a few millimetres in length, and occupying the whole or part of the vessel's circumference (Fig. 1b). Transmission electron microscopy showed severe degenerative changes of the medial smooth muscle cells (Fig. 3a). Instead of the intima, which was destroyed, accumulations of amorphous or granular material were found, probably representing denaturated cellular debris (Fig. 3b). Adhering to this material, the thrombus, consisting of a dense aggregate of platelets, with fibrin strands and polymorphonuclear leucocytes, was found (Fig. 3c).

In all but one of the vessels in which no occluding thrombus developed, a circumferential endothelial lesion was seen on gross inspection. A smaller mural thrombus in those vessels was the only difference with the others.

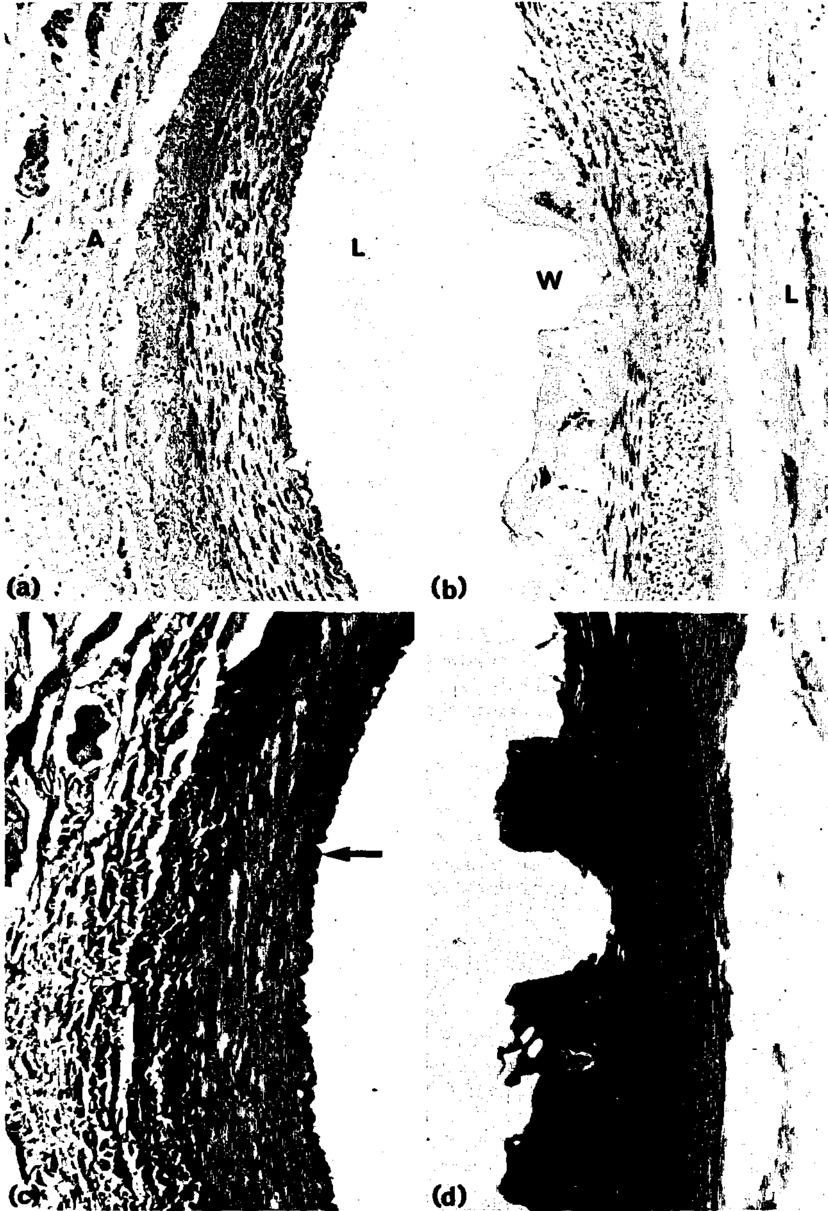

Figure 1(a) Transverse section of normal coronary artery (Hematoxylin and Eosin). Note the clearly distinguishable adventitia, A, media M, and the thin intima. The lumen, L, appears empty due to the perfusion-fixation used, which washed out the blood. The nuclei of the medial smooth muscle cells are clearly seen as elongated blue dots, whilst the endothelial nuclei are just discernible. (Light microscopy; magnification × 100.) (*b*) Longitudinal section of a coronary artery at the site of the lesion (Hematoxylin and Eosin). The lumen, L, is situated at the right. The site where the electrical wire was placed is clearly seen: W, The adjacent medial smooth muscle cells, show blurring of their nuclei, whilst the intima is no longer recognizable. The lumen is partly filled with an amorphous eosinophilic mass, representing a large platelet thrombus and denaturated cellular debris (compare with Fig. 3c). (light microscopy × 100.) (*c*) As Figure 1a, stained with van Gieson's elastic stain. The elastic fibres are stained black, showing thick adventitial elastic fibres, delicate fibres within the media, and a prominent inner elastic lamina (arrow) (light microscopy × 100.) (*d*) As Figure 1b, stained with van Giesson's elastic stain. The medial elastic fibres and inner elastic lamina are clearly identifiable, also at the site of the lesion. (Light microscopy × 100.)

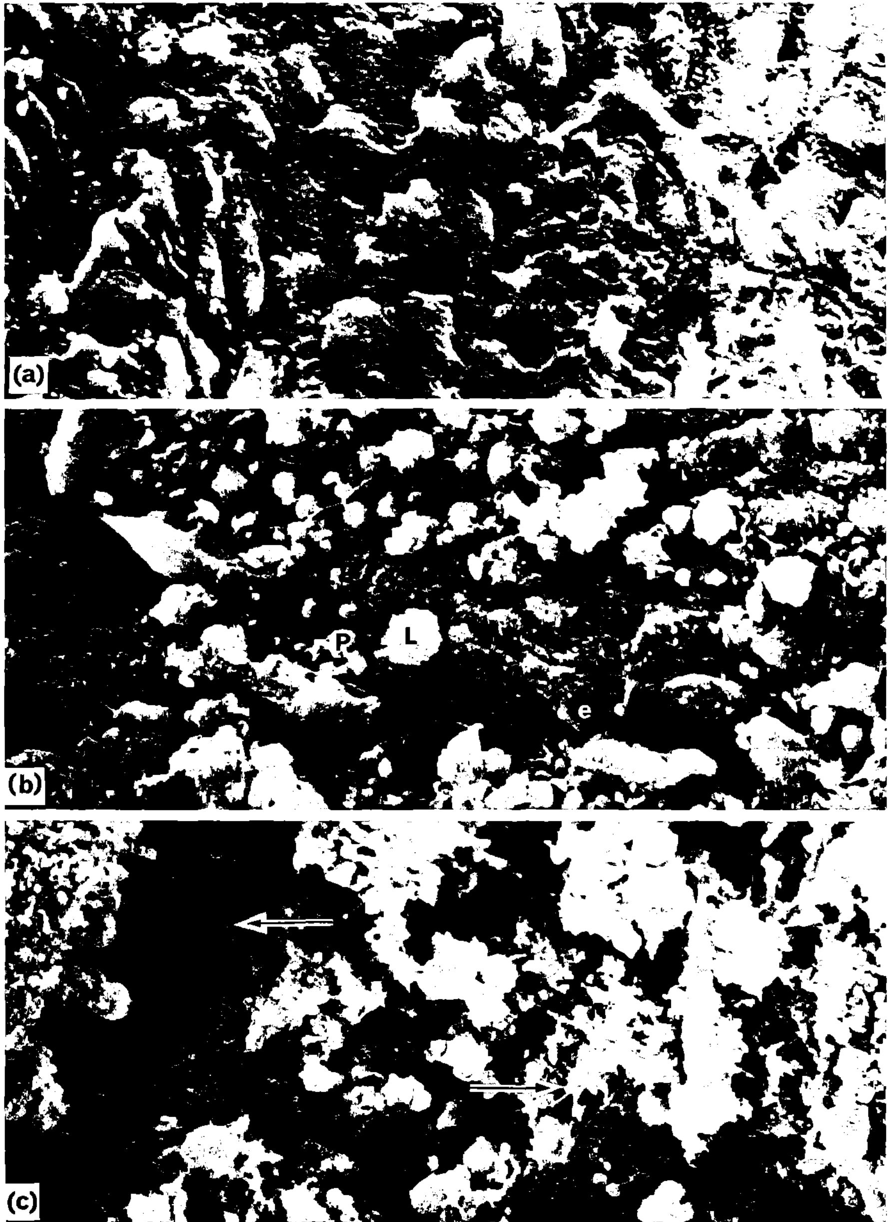

Figure 2(a) Normal coronary artery. The luminal surface shows the endothelial cells bulging into the lumen. (Scanning electron microscopy×700.) (*b*) Coronary artery in the direct vicinity of the lesion. Many platelets P, and leucocytes, L, are adhering to the luminal surface. Individual endothelial cells, e, are still easily identifiable. (Scanning electron microscopy×700.) (*c*) Surface of the thrombus at the site of the lesion. Aggregates of blood cells and platelets, intermingled with strands of fibrin (arrows), produce a highly irregular surface. Such a thrombus either totally or subtotally occluded the vascular lumen. (Scanning electron microscopy×700.)

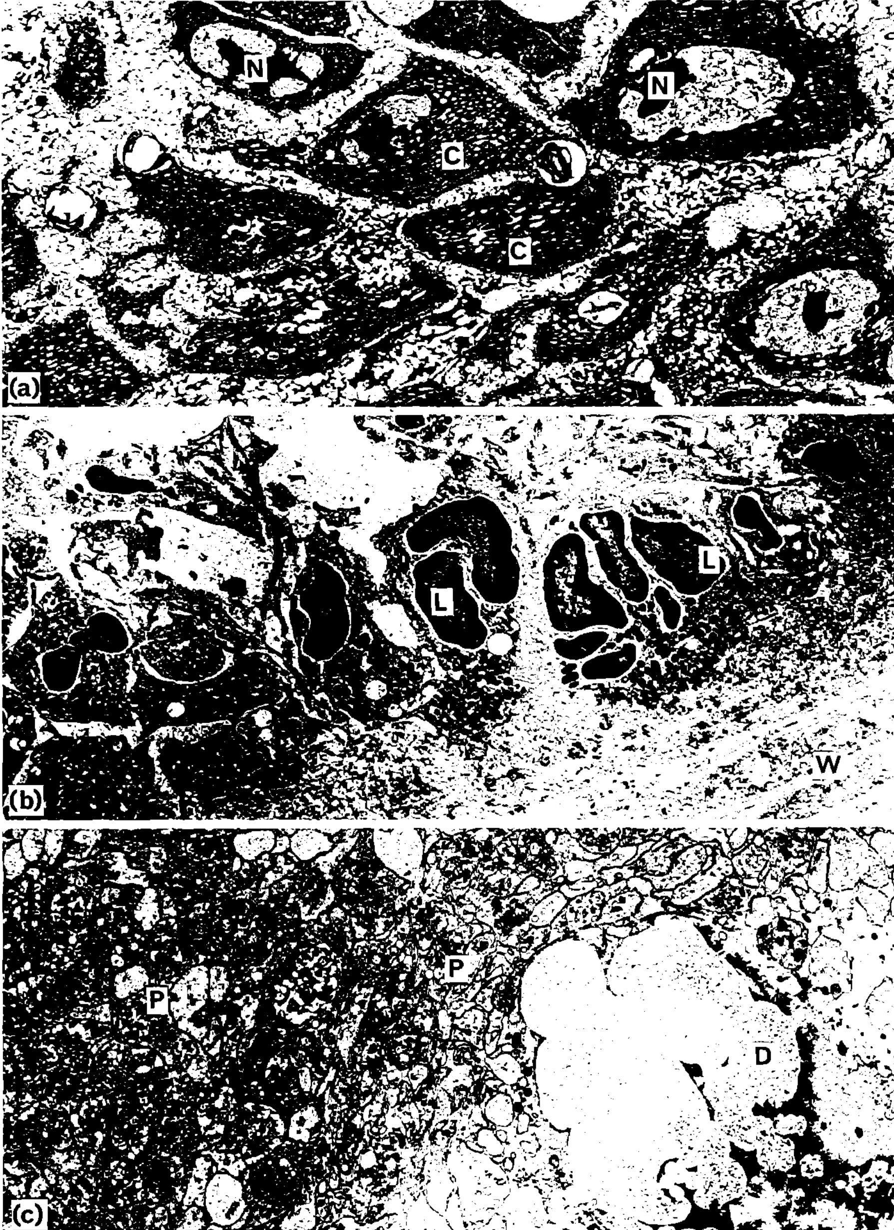

Figure 3(a) Media at the site of the lesion. Smooth muscle cells show extensive damage: the nuclei, N, contain irregular large clumps of chromatin, and are grossly distorted; the cytoplasm, C, has lost its normal ultrastructure and appears as an amorphous or vacuolated grey substance. (Transmission electron microscopy × 7300.) (*b*) Luminal surface at the site of the lesion. Polymorphonuclear leukocytes, L, are adhering to the luminal surface of the arterial wall, W, where the intimal cells seem to be absent. (Transmission electron microscopy × 5000.) (*c*) Thrombus. A mass of closely packed platelets, P, is seen adjacent to an amorphous mass of material, probably representing denaturated cellular debris, D. This material is found in large amount, adjacent to the vessel wall. (Transmission electron microscopy × 5000.)

Table 4 *Size of central core and borderzone of the area at risk in plasmin treated and control animals*

	Group I (plasmin)	Group II (control)
Central core		
Grams	8·5±5·4 (n=5)	10·2±3·0 (n=10)
% LV mass	12·3±6·5	16·0±4·3
Borderzone		
Grams	3·5±0·7 (n=4)*	3·9±1·8 (n=7)*
%LV mass	4·8±1·0	6·0±2·7

Data are expressed as mean±S.D.
%LV percentage of left ventricle.
*No borderzone could be detected in one animal in group I and three in group II.

C. THE EFFECTS OF INTRACORONARY PLASMIN

In 19 experiments, thrombolysis via the intracoronary administration of porcine plasmin was attempted. In 15 animals (79%) thrombolysis was achieved, as measured by LAD-flow and visual inspection, after 4 to 20 min of infusion. In 5 out of these 15 experiments, the observation period was extended to 180 min after occlusion (Group I). No differences in HR, MAP and CO between these and the control animals were present. Data comparing the two groups with respect to the sizes of the area of infarction and the presence and size of a borderzone are comprised in Table 4.

Regional flow in the area at risk increased significantly ($P<0·05$) after thrombolysis (Figs 4a and b), but no significant differences in systolic wall thickening were demonstrable between the treated and control groups (Figs 4c and d). However oxygen-extraction decreased during the three hours of reperfusion from 80±6% to 26±14% ($P<0·001$) in the plasmin treated group, whereas it remained stable in the control group.

Discussion

Current knowledge regarding the importance of coronary thrombosis as a cause of myocardial infarction is still limited. Much has also to be learned about the time course and stages of thrombus development in the coronary arterial system. In the model described above, an occluding thrombus developed in 74% of the animals. The finding that

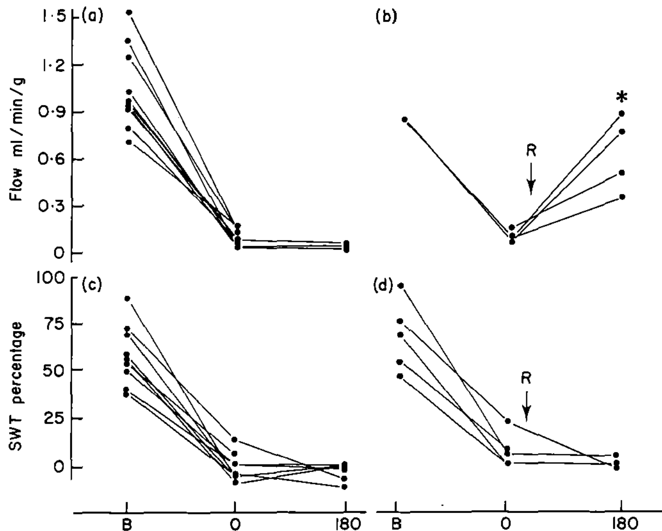

Figure 4 Regional myocardial blood flow (top), and systolic wall thickening (SWT) (bottom), in the control (left) and treated (right) group. B, base line; O, occlusion, 180 = 180 minutes after occlusion; R, reperfusion. *$P<0·05$ vs control group.

in 34% of the cases the thrombus was preceded by vascular spasm underscores the importance of testing the presence of this phenomenon by the topical application of nitroglycerine. Since relief of spasm often occurs spontaneously the relevance of this model for the investigation of spasmolytic agents is not clear. Its usefulness as a model for the study of antithrombotic agents and possibly in studies involving limitation of infarct size is more evident[6].

In open-chest animal preparations, the choice of the anaesthetic regimen requires special attention, as some anaesthetic agents may exert an antithrombotic action by themselves[14]. We used the open-chest rather than the closed-chest pig model, although the latter seems to be more attractive. Placement of intracoronary devices induces a high incidence of ventricular fibrillation before thrombus formation occurs and results in an unacceptably high loss of animals. Furthermore, the induction of intractable coronary artery spasm by the placement of a copper wire is an additional cause for concern.

The preliminary results in the present study indicate that thrombolysis with plasmin after an occlusion of approximately 40 min is capable of reinstating perfusion of the jeopardized segment. The data also show that a complete or even partial recovery of regional function is not achieved within 3 h after reperfusion. It must be stressed that these data are obtained in a model with virtually no collateral flow. The presence of such a flow could provide additional time before the institution of thrombolysis becomes futile in terms of the limitation of necrosis. However the gradual decrease in oxygen extraction in our experiments suggests that substantial damage has already occurred. The lack of recovery of regional function in our model is consistent with observations during reperfusion after acute ligation of 30 min in the same species[15]. Another factor which could play a role is the observation that in dogs at least six hours of reperfusion are needed to achieve complete recovery after an occlusion of only 15 min[16]. Obviously, variations in the occlusion period and various cardioprotective agents must be studied before final conclusions about the relevance of human coronary desobstruction can be drawn.

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Chapter 4

THE EFFECT OF THE STABLE PROSTACYCLIN ANALOGUE ZK 36374 ON EXPERIMENTAL CORONARY THROMBOSIS IN THE PIG

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THE EFFECT OF THE STABLE PROSTACYCLIN ANALOGUE ZK 36374 ON EXPERIMENTAL CORONARY THROMBOSIS IN THE PIG

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ABSTRACT

The hemodynamic and antithrombotic action of ZK 36374, a stable carbacyclin derivative of prostacyclin, was studied during electrically-induced coronary artery thrombosis in the open chest anesthetized pig. Infusion of ZK 36374 (100 ng/kg/min, n=6) had no effect on heart rate and cardiac output, but caused a 20% reduction in mean arterial blood pressure by peripheral vasodilation. In animals receiving solvent or no drug prior to thrombosis induction, the time to occlusive coronary artery thrombosis (TOT) was 30 ± 2 minutes (mean ± SEM, n=17). Pretreatment with an i.v. infusion of ZK 36374 (100 ng/kg/min) prolonged TOT by 50% to 47 ± 7 minutes ($p < 0.005$, n=6). This prolongation of TOT was not due to the lower blood pressure in the ZK 36374 group, as dihydralazine in a dose that lowered arterial blood pressure to the same extent had no effect on TOT (32 ± 4 minutes, n=4). The results indicate that ZK 36374 may be useful in delaying (or preventing) occlusive coronary artery thrombi.

INTRODUCTION

Thrombosis of a coronary artery is involved in most acute myocardial infarctions (1). Prophylactic treatment with

Key words: Prostacyclin, ZK 36374, Experimental coronary thrombosis, Coronary vasospasm, Antithrombotic agents.

antithrombotic agents appear therefore to be an attractive approach in preventing these events. Although prostacyclin is effective against experimentally-induced coronary artery thrombosis in the dog (2), the instability of the compound at physiological pH and temperature limits its clinical use. The carbacyclin derivative ZK 36374, 5-{(E)-(1S, 5S, 6R, 7R)-7-hydroxy-6-|(E)-(3S, 4RS)-3-hydroxy-4-methyl-oct-1-en-6-yn-yl|-bicyclo-|3.3.0|-octan-3-yliden}-pentanoic acid, is not only equipotent to prostacyclin in its antiaggregatory effects in man (3), but is more stable. In this study we describe the hemodynamic and antithrombotic effects of ZK 36374 in experimentally-induced coronary thrombosis in the open-chest, anesthetized pig.

METHODS

Experiments were performed in 27 young Yorkshire pigs (22-30 kg). After sedation with 120 mg azaperone and induction of anesthesia by intravenous administration of 150 mg metomidate (both from Janssen Pharmaceutica, Belgium), anesthesia was maintained by i.v. infused pentobarbital (6-20 mg/kg/h). After insertion of an endotracheal tube, the animals were ventilated by a respirator pump under frequent control of arterial blood gases (ABL 3, Radiometer Copenhagen, Denmark). Surgical procedures and recording of hemodynamic variables have been described elsewhere (4-6). Solvent or ZK 36374 was infused via a catheter placed in the superior caval vein. No heparin was used during the experiments. A detailed description of the method to induce coronary artery thrombosis has been reported earlier (6). Briefly, after a midsternal thoracotomy and opening of the pericardium, the left anterior descending coronary artery (LAD) was prepared free between its first and second diagonal branch. A stainless steel electrode, isolated from the surrounding myocardium by a polyethylene strip, was coiled around the artery so that it was in contact with the vessel without impeding LAD-flow (electromagnetic (EM) flow-meter reading). The electrode was connected to the anodal side of an adjustable current source, and the circuit was completed by an electrode attached to the abdominal fat. Electrical stimulation occurred at 1.6 mA. After the animals had been stable for at least 30 min, base-line recordings were made of heart rate, mean arterial pressure (from a catheter in the ascending aorta), cardiac output (through an EM flow-probe around the ascending aorta), LAD-flow and regional systolic wall thickening of the area of the left ventricle supplied by the distal LAD (from a 5MHz ultrasonic transducer sutured on the epicardium). The animals were divided in five groups. In 2 groups an infusion of either solvent (group 1, n=6) or 100 ng/kg/min ZK 36374 (group 2, n=6) was started 10 minutes before electrical stimulation and continued throughout the experiments. In 2 other groups the infusion of solvent (group 3, n=5) or 100 ng/kg/min ZK 36374 (group 4, n=6) was started 10 minutes after occlusion of the LAD, and after spasm of the vessel as the cause of obstruction was excluded. In 4 animals (group 5) an infusion of dihydralazine was given before electrical stimulation. The dose (100-500 µg/kg) was adjusted

to obtain the same decrease in mean arterial pressure as in group 2. Electrical stimulation in group 3 was applied for only 15 minutes. The vessel was thought to be occluded when continuously recorded LAD-flow had decreased, regional systolic wall thickening of the area perfused by the distal LAD became negligible and stasis of blood was observed. At that time the electrode was removed (duration of electrical stimulation was 32 ± 6 minutes in group 2, and 26 ± 2 minutes in the pooled data of groups 1 and 4) and nitroglycerine (NTG) was dripped on the vessel to exclude coronary artery spasm as the cause of obstruction. When the vessel reopened, a spasm of the LAD was thought to have been responsible for the obstruction. With every next occlusion the application of NTG was repeated. An occluding thrombus was considered to be present when no reaction to NTG occurred. The observation period was extended until the occurrence of ventricular fibrillation or the animals survived 90 minutes of occlusion. Then the LAD was excised and placed in buffered glutaraldehyde for microscopical confirmation of the presence of the thrombus and for the measurement of the internal diameter of the vessel at the site of the electrode. In vitro platelet aggregation induced by collagen (5 µg/ml) was measured in platelet rich plasma (PRP), adjusted to a platelet count of 300.10^9 per l, using a Payton aggregometer. PRP was prepared from arterial blood, withdrawn in a syringe containing sodium citrate (final concentration 3.8%) at base line and after TOT. Values are expressed as mean ± standard error of the mean unless otherwise stated. Only $p<0.05$ (two-tailed) was considered statistically significant.

RESULTS

Systemic hemodynamic values are detailed in TABLE 1. The infusion of solvent had no effect on heart rate (HR), mean arterial pressure (MAP), cardiac output (CO) and systemic vascular resistance (SVR). ZK 36374 did not affect HR, but caused a 20% decrease in MAP. Since CO was not affected, the fall in blood pressure was caused by a peripheral vasodilatory effect. This was represented by a 15% decrease in SVR. The dose of dihydralazine was chosen such that at the time of electrical stimulation MAP was similar to that of the ZK 36374-pretreated group (60 ± 1 mmHg). No significant changes in platelet count were observed between the groups. In all animals of the ZK 36374-pretreated group platelet aggregation decreased (-20 ± 4%), but in the solvent group or group 4 the results were variable: in 8 animals there was an increase (37 ± 15%), while in 4 animals there was a substantial decrease (-50 ± 5%, TABLE 2). At least one episode of coronary artery spasm occurred (after 32 ± 6 minutes) in the ZK 36374-pretreated animals. This incidence was considerably lower (7 out of 17, after 24 ± 3 minutes) in the untreated or with solvent pretreated group ($p<0.005$), Fisher exact test). Time to occlusive coronary thrombosis (TOT) for the animals of groups 1 and 4 was 30 ± 2 minutes, which was nearly identical for the animals which were stimulated for 15 minutes (30 ± 4 minutes), but was significantly prolonged for the ZK 36374-pretreated group (47 ± 7 minutes; $p=0.0023$; Wilcoxon rank

TABLE 1

Systemic hemodynamic variables.

		Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=5)	Group 4 (n=6)	Group 5 (n=4)
	a	85 ± 4	83 ± 2	91 ± 1	82 ± 4	87 ± 2
HR	b	87 ± 4	86 ± 2	90 ± 1	82 ± 3	92 ± 7
	c	84 ± 3	84 ± 1	86 ± 2	83 ± 5	91 ± 10
	a	77 ± 2	75 ± 3	83 ± 3	81 ± 2	95 ± 4
MAP	b	80 ± 3	59 ± 2*	82 ± 3	81 ± 2	60 ± 1*
	c	71 ± 2	55 ± 3*	74 ± 3	75 ± 3	59 ± 1*
	a	2.2 ± 0.3	2.4 ± 0.3	2.6 ± 0.1	2.4 ± 0.1	2.3 ± 0.3
CO	b	2.3 ± 0.3	2.2 ± 0.3	2.5 ± 0.1	2.3 ± 0.1	2.2 ± 0.2
	c	2.1 ± 0.3	2.0 ± 0.3	2.2 ± 0.1	1.9 ± 0.1	2.1 ± 0.2
	a	37 ± 5	34 ± 5	33 ± 2	34 ± 2	44 ± 6
SVR	b	37 ± 5	29 ± 4*	33 ± 2	36 ± 2	27 ± 2*
	c	38 ± 5	29 ± 4*	34 ± 2	40 ± 3	29 ± 3*

Abbreviations: HR = heart rate (beats.min^{-1}); MAP = mean arterial blood pressure (mmHg); CO = cardiac output (l.min^{-1}); SVR = systemic vascular resistance (mmHg.min.l^{-1}); Systemic hemodynamic variables measured at a (= base line); at b (= after infusion of drug or solvent, immediately before the start of electrical stimulation) and at c (= at the time of occlusion by the thrombus); values are expressed as mean ± SEM; * = $p<0.05$, analysis of variance.

sum test, FIG. 1). The data for the four dihydralazine pretreated animals were not different from those of groups 1, 3 and 4 (32 ± 4 min). Analysis of covariance showed no relation between TOT and MAP, CO, SVR, LAD-flow or platelet aggregation. The linear relation between TOT and the internal luminal diameter (ILD) of the LAD at the side of the electrode could be described by the equation: $TOT_{(min)} = 16 + 26 \times ILD_{(mm)}$, for the ZK 36374-pretreated group, and by: $TOT_{(min)} = -2 + 26 \times ILD_{(mm)}$, for all other groups. The common slope (i.e. 26) was significantly different from zero ($p<0.05$), also the intercepts (16 and -2, resp.) were significantly different ($p=0.003$). The difference between the intercepts (18 = 16-(-2)) is the average difference in TOT (adjusted for ILD) between group 2 and all others. Obviously these linear relations are only valid for the interval

of ILD-values in our experiments: 1.0-1.7 mm (group 1: 1.25 ± 0.07 mm; group 2: 1.22 ± 0.11; group 3: 1.20 ± 0.08; group 4: 1.30 ± 0.07; group 5: 1.22 ± 0.05). In all animals the existence of an occlusive coronary artery thrombus was confirmed by microscopy.

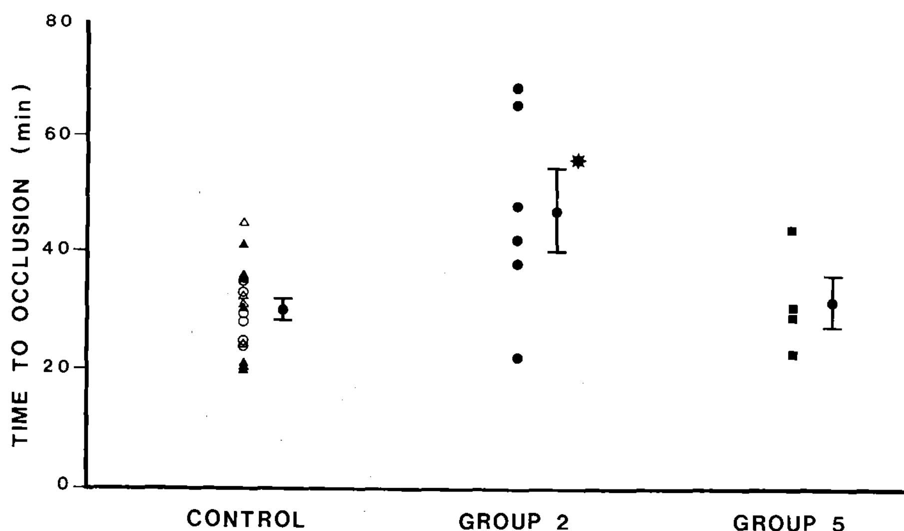

FIG. 1

Time to occlusive coronary thrombosis. Control: values of group 1 (▲), group 3 (△) and group 4 (○) together. Group 2 (●) received ZK 36374 before thrombus induction. Group 5 (■) received dihydralazine. ✱ = $p<0.005$, compared to all other groups.

TABLE 2.
Collagen induced plated aggregation (PA, Δ% from baseline) and occlusion times (TOT, minutes).

Animal	Group 1		Group 4		Group 2	
	PA	TOT	PA	TOT	PA	TOT
1	-36	20	+7	29	-19	48
2	+5	41	-46	24	-17	38
3	+76	20	+3	25	-21	66
4	-57	35	-59	35	-24	68
5	+20	30	+9	28	-6	42
6	+1	36	+95	32	-36	22

DISCUSSION

In this model ZK 36374 prolonged the time to occlusive coronary thrombosis by 50%. Surprisingly no correlation existed between this effect and the decrease in platelet aggregability caused by the compound. On the other hand, the effects of ZK 36374 on platelet aggregation in man (7) and of the solvent in our study show rather large individual differences. The results in the dihydralazine-treated animals, and the negative result of the analysis of covariance prove that the effects of ZK 36374 on thrombus formation in this model cannot be attributed to its vasodilatory properties. Probably, the efficacy of the compound is caused by multiple factors: inhibition of platelet adhesion to the vessel wall and inhibition of aggregability (8). The significance of the relation between the time to occlusion by the thrombus and the internal luminal diameter of the LAD implies that in comparable models of experimental thrombosis this parameter must be included in the analysis. In addition, the finding that occlusion by a thrombus is preceded in about 40% of the experiments by at least one episode of coronary artery spasm also necessitates the exclusion of this phenomenon. The last argument may also limit the usefulness of this model in the conscious animal. The higher incidence of spasm in the ZK 36374-pretreated group could be a cause of concern. However, we must realize that spasm in these animals occurred at a time (32 $\pm$ 6 minutes) that the coronary artery in the untreated animals already was occluded by the thrombus. This frequent occurrence of coronary artery spasm could be due to a ZK 36374-induced release of 5-hydroxytryptamine from the thrombocytes, which occurs with ZK 36374 as well as prostacycline (8).

The potential usefulness of ZK 36374 in clinical practice may be limited by its hypotensive action. The combination of a lower dose of ZK 36374 and a phosphodiesterase inhibitor as shown for prostacyclin and aminophylline in the dog (9), may therefore be a more attractive combination.

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Chapter 5

THE EFFECT OF THE THROMBOXANE RECEPTOR ANTAGONIST BM 13.177 ON EXPERIMENTALLY INDUCED CORONARY ARTERY THROMBOSIS IN THE PIG

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The effect of the thromboxane receptor antagonist BM 13.177 on experimentally induced coronary artery thrombosis in the pig

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We studied the effect of pretreatment with two doses of the thromboxane antagonist BM 13.177 and its solvent on the development of electrically induced coronary artery thrombosis in pigs. Results were compared with those obtained in animals pretreated with intravenously administered acetylsalicylate and its solvent. The effects of both compounds on the overall cardiovascular performance (heart rate, mean arterial blood pressure, cardiac output, systemic vascular resistance) were minimal. In the animals receiving solvent or acetylsalicylate the time to occlusive coronary thrombosis was 33 ± 4 and 32 ± 6 min, respectively. BM 13.177, in a dose of 5 $mg \cdot kg^{-1}$, did not modify the time to thrombotic occlusion (35 ± 7 min), but in six of the eight animals that had received 10 $mg \cdot kg^{-1}$ BM 13.177, there was no occlusion within 120 min. In the acetylsalicylate-treated animals, collagen-induced platelet aggregation and plasma thromboxane B_2 declined by 72 and 82%, respectively. The decreases were 46 and 20%, respectively, with the higher dose of BM 13.177. It is concluded that, in this porcine model of coronary artery thrombosis, the thromboxane antagonist BM 13.177 effectively suppressed formation of occlusive thrombi whereas acetylsalicylate was ineffective at a dose that lowered arterial thromboxane levels.

Coronary thrombosis (experimental); Thromboxane antagonist; BM 13.177; Acetylsalicylate; Antithrombotic agents; (Pig)

1. Introduction

Coronary thrombosis often leads to acute myocardial infarction and sudden death (DeWood et al., 1980; Davies and Thomas, 1984). Antithrombotic agents could therefore be useful in helping to avoid these clinical situations. An important factor in thrombogenesis in vivo is the balance between the antithrombotic action of prostacyclin, released by the vessel wall, and the pro-aggregatory action of thromboxane A_2, released by stimulated platelets (Moncada and Vane, 1978). The thromboxane antagonist BM 13.177 (4-[2-benzenesulfonamido)-ethyl]phenoxyacetic acid) has been shown to be an effective inhibitor of platelet aggregation in blood obtained from both healthy human volunteers (Patscheke and Stegmeier, 1984; Gresele et al., 1984) and from patients with atherosclerotic disease (Riess et al., 1984). In the present investigation we evaluated the effect of BM 13.177 on the development of experimentally induced coronary thrombosis in open-chest anaesthetized pigs.

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2. Materials and methods

2.1. Animal model

Experiments were performed in 32 young Yorkshire pigs (23-32 kg). The animals were sedated with an intramuscular injection of 120 mg azaperone (Stresnil®, Janssen Pharmaceutica, Beerse, Belgium) and anaesthetized with 150 mg metomidate (Hypnodil®, Janssen Pharmaceutica) administered via a dorsal vein of the ear. After endotracheal intubation, the pigs were ventilated with a mixture of O_2 and N_2O by a volume cycled, positive-pressure-limited (range 10-30 cm H_2O) Bennett BA4 respirator; ventilation was controlled by frequent measurement of the arterial blood gases (ABL-3, Radiometer, Copenhagen, Denmark). Anaesthesia was maintained by the continuous infusion of sodium pentobarbitone (Nembutal®, Sanofi, Paris, France, 12 $mg \cdot kg^{-1} \cdot h^{-1}$). Standard cardiovascular parameters were recorded from peripheral ECG leads, a 7F catheter positioned in the ascending aorta and an electromagnetic flow probe placed around the ascending aorta after a midsternal thoracotomy was performed. A 7F catheter was also placed in the superior vena cava for the administration of drugs or solvent. Detailed descriptions of the surgical procedures, the recording of haemodynamic variables and the method used to induce coronary artery thrombosis in pigs have been reported earlier (Verdouw et al., 1981; Van der Giessen et al., 1983). Regional myocardial wall thickness was recorded with a small ultrasound-transducer (5 MHz, Krautkramer-Branson, Lewistown, PA, USA) sutured on a segment of the left ventricle that is supplied by the distal left anterior descending coronary artery. After this coronary artery was prepared free between its first and second diagonal branch, a stainless steel wire electrode, isolated from surrounding myocardium by a polyethylene strip, was coiled around the vessel. Extreme care was taken that this procedure did not impede the coronary flow, as verified by flowmeter readings. The electrode was connected to the anodal side of an adjustable current source and the circuit was completed by an electrode connecting the cathodal side to the abdominal fat.

2.2. Experimental procedures

2.2.1. Experimental protocol

After the preparation had been stable for at least 30 min, basal recordings were made of the heart rate, the mean arterial blood pressure, the aortic blood flow, the coronary blood flow and the regional systolic wall thickening of the area perfused by the left anterior descending coronary artery.

Thrombus induction was initiated by passing a continuous current of 1.6 mA through the stainless steel electrode until the coronary artery was considered to be occluded. The latter was assumed to have occurred when the continuously recorded coronary blood flow had decreased to almost zero, and when the regional systolic wall thickening of the segment perfused by the left anterior descending coronary artery had become negligible, and when stasis of the blood was observed behind the electrode. These criteria have been validated earlier (Van der Giessen et al., 1984). The electrode was then removed and nitroglycerine (total dose 250 μg) was dripped locally onto the vessel to exclude the possibility that a coronary artery spasm was the cause of obstruction.

When the vessel reopened, vasospasm was considered to be responsible for the occlusion and the formation of thrombus was awaited without further electrical stimulation. The application of nitroglycerin was repeated with every further occlusion. A thrombus was considered to be the cause of occlusion when there was no reaction to nitroglycerin. Endpoint of the study was either occlusion by a thrombus or 2 h of observation after initiation of electrical stimulation. The left anterior descending coronary artery was then excised and placed in buffered glutaraldehyde. Light microscopy was performed on all occluded arteries in order to confirm the presence of an occlusive thrombus.

2.2.2. Study groups

Five groups of animals were studied (table 1). BM 13.177 and its solvent (NaCl 57 mg, trometamol 1.25 g, H_2O ad 10 ml, NaOH at pH 9.0-9.5) were administered i.v. 15 min before the start of thrombus induction. The BM 13.177 doses

were comparable to those used in man (Gresele et al., 1984; Riess et al., 1984), and the rate of infusion was adjusted so as to compensate for the elimination of the drug, as determined in dogs (personal communication, Dr. K. Stegmeier). Acetylsalicylate as well as its solvent (H_2O) was administered as a single bolus injection 45 min before the start of thrombus induction. Heparin was not infused during the experiments.

2.2.3. Haematological measurements

Ten milliliters of aortic blood were withdrawn in a syringe containing heparin and indomethacin (final concentration 10 $\mu mol \cdot l^{-1}$) before the infusion of the drugs, at the start of the induction of the thrombus and at the end of the experiment. The blood was placed immediately on ice for 20 min and centrifuged subsequently for 10 min ($2000 \times g$). The plasma was removed and stored at $-70\,^{\circ}C$ for the measurement of thromboxane B_2 with the radioimmunoassay method of Zijlstra et al. (1983). Platelet aggregation induced by collagen (1 $\mu g \cdot ml^{-1}$) in vitro was measured in platelet-rich plasma that had been adjusted to a platelet count of $300 \cdot 10^9 \cdot l^{-1}$ with a Payton aggregometer. Platelet-rich plasma was prepared from arterial blood, withdrawn in a syringe containing sodium citrate (final concentration 0.38%).

2.2.4. Statistical analysis

Values are expressed as means ± S.E.M. The Wilcoxon rank sum test was employed to determine the statistical significance of the differences in the occurrence of thrombotic occlusion between the various groups. Comparisons were made for the other parameters with a two-way analysis of variance (ANOVA), and the significance of the differences between treatment groups and time points was evaluated by Duncan's new multiple range test after ANOVA had revealed that the samples represented different populations. $P < 0.05$ (two-tailed) was considered to be statistically significant.

3. Results

The data of the two solvent groups were pooled because they were not different under basal conditions or during the course of the experiments.

3.1. Duration of electrical stimulation and incidence of vasospasm

Electrical stimulation was terminated when the coronary artery blood flow (basal flow: 30-40 $ml \cdot min^{-1}$) had decreased to almost zero and the systolic wall thickening (basal value 24-35%) was abolished, due to either a spasm or an occlusive thrombus. The durations of electrical stimulation for the different treatment groups are summarized in table 1. This duration was longer for the animals treated with 10 $mg \cdot kg^{-1}$ of BM 13.177 than in the other treatment groups. Nitroglycerine was then applied to all animals to exclude spasm as the cause of occlusion.

TABLE 1

Experimental groups, duration of electrical stimulation and incidence of vasospasm.

	Number of animals	Dose		Duration of electrical stimulation (min)	Vasospasm (n)
		$mg \cdot kg^{-1}$	$+ mg \cdot kg^{-1} \cdot min^{-1}$		
(1) BM 13.177	3	5	0.15	30 ± 4	1 (33%)
(2) BM 13.177	8	10	0.3	38 ± 5	6 (75%)
(3) Solvent of BM 13.177	6			32 ± 7	4 (66%)
(4) Lysine acetylsalicylate	8	25		31 ± 5	2 (25%)
(5) Solvent of acetylsalicylate	7			26 ± 4	3 (43%)

TABLE 2

Systemic haemodynamic effects of BM 13.177 and acetyl salicylate (ASA). b = basal, s = start of electrical stimulation, o = occlusion by the thrombus.

	Solvent n = 13	ASA ($25\ mg \cdot kg^{-1}$) n = 8	BM 13.177 ($5\ mg \cdot kg^{-1}$) n = 3	BM 13.177 ($10\ mg \cdot kg^{-1}$) n = 8
Heart rate (beats · min⁻¹)				
b	85 ±4	93 ±7	71 ±13	78 ±5
s	84 ±4	87 ±6	68 ±13	73 ±4
o	85 ±4	82 ±7	79 ±14	73 ±6
Mean art. pressure (mm Hg)				
b	83 ±3	82 ±6	82 ±10	89 ±6
s	83 ±4	90 ±4	83 ±10	92 ±5
o	77 ±4	82 ±4	77 ±11	88 ±5
Aortic flow (l · min⁻¹)				
b	2.3±0.1	2.4±0.2	1.8± 0.3	2.2±0.2
s	2.3±0.1	2.2±0.2	1.7± 0.3	2.1±0.2
o	2.0±0.2	1.7±0.2	1.5± 0.2	2.0±0.2
Syst. vasc. resistance (mm Hg · min · l⁻¹)				
b	37 ±3	36 ±4	48 ±12	41 ±4
s	38 ±3	44 ±6	52 ±14	45 ±4
o	42 ±3	53 ±8 [a]	52 ±12	43 ±4

[a] $P < 0.05$.

3.2. Effect of acetylsalicylate and BM 13.177 on systemic haemodynamic variables

The infusion of both doses of BM 13.177, or acetylsalicylate had no effect on heart rate, mean arterial blood pressure, ascending aortic blood flow and systemic vascular resistance (table 2). The systemic vascular resistance increased during the course of the experiments in animals receiving acetylsalicylate, but the cardiovascular parameters of the animals treated with the higher dose of BM 13.177 remained stable during the further course of the experiments.

3.3. Effect of acetylsalicylate and BM 13.177 on plasma thromboxane B_2

Forty-five minutes after the bolus injection of acetylsalicylate, arterial TXB_2 levels had decreased from 73 ± 4 to $13 \pm 3\ pg \cdot ml^{-1}$ ($P < 0.05$, fig. 1), but did not change further since values of $14 \pm 6\ pg \cdot ml^{-1}$ were found at the time of the thrombotic occlusion. Infusion of the higher dose of BM 13.177 moderately decreased TXB_2 levels from 59 ± 12 to $45 \pm 13\ pg \cdot ml^{-1}$ ($P < 0.05$), and

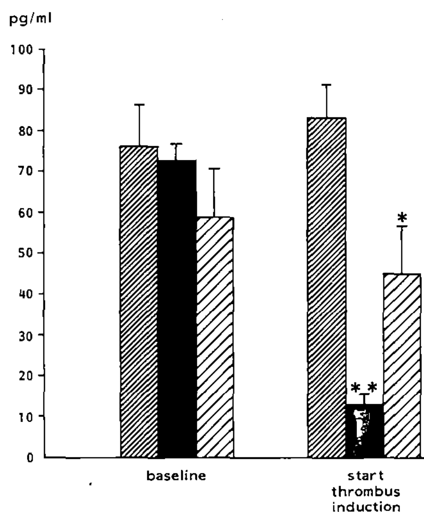

Fig. 1. Effect of BM 13.177 ($10\ mg \cdot kg^{-1}$) and acetylsalicylate (ASA, $25\ mg \cdot kg^{-1}$) on arterial plasma levels of thromboxane B_2. (▨) solvent (n = 7), (▨) BM 13.177 (n = 7), (■) ASA (n = 5). * $P < 0.05$ vs. solvent; ** $P < 0.05$ vs. solvent and BM 13.177.

these levels remained stable (46 ± 11 pg · ml^{-1} after 120 min).

3.4. Effect on platelet aggregation

Acetylsalicylate caused a 72% decrease ($P < 0.05$) in platelet aggregation, which was sustained during the experiments. Both doses (5 and 10 mg · kg^{-1}) of BM 13.177 caused similar decreases in platelet aggregation during infusion (41 and 46%, respectively, $P < 0.05$), although the decreases were smaller than those observed for acetylsalicylate.

3.5. Effect on occlusive coronary thrombosis

Occlusive coronary thrombi, as confirmed by microscopical examination, occurred in all 13 animals of the solvent-treated groups (time to occlusion = 33 ± 4 min, fig 2). One of the eight animals treated with acetylsalicylate did not show a thrombotic occlusion, but for the seven other animals the time to thrombotic occlusion was 32 ± 6 min. The low dose of BM 13.177 had no effect on the occlusion time (35 ± 7 min), but the high dose of BM 13.177 was effective in preventing coronary thrombosis ($P < 0.02$), as in six of the eight animals occlusive thrombi did not develop within 2 h of electrical stimulation. The time to occlusion of the two animals in this group in which the coronary artery had become occluded by the formation of a thrombus (after 30 and 32 min, respectively) were comparable to the occlusion time of the solvent-treated groups. In all but one of the animals in which the coronary artery remained patent, blood flow and systolic wall thickening after 120 min were not different from basal values. In one animal the flow value was not different but systolic wall thickening was negligible due to two periods of coronary artery spasm which lasted 6 and 8 min, respectively.

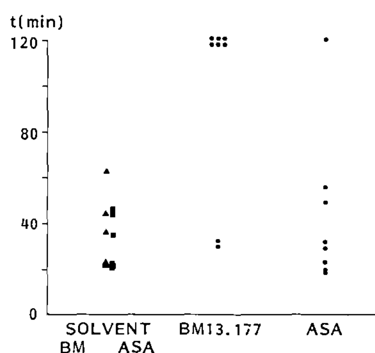

Fig. 2. Time to occlusive coronary thrombosis for the individual experiments. The solvent group was divided into 2 groups of animals receiving the vehicle of either of the drugs. BM 13.177 = 10 mg · kg^{-1}, ASA = acetylsalicylate 25 mg · kg^{-1}.

4. Discussion

BM 13.177, in a dose of 10 mg · kg^{-1} (followed by a continuous infusion of 0.3 mg · kg^{-1} · min^{-1}) but not in a dose of 5 mg · kg^{-1} (followed by a continuous infusion of 0.15 mg · kg^{-1} · min^{-1}), proved to be effective in preventing the formation of occlusive coronary thrombi. In fact, occlusion did not occur in 75% of the animals during infusion of the highest dose. The animals which received the lower dose of BM 13.177 or the solvent all had occlusive thrombi at times similar to those reported for an untreated group of 17 animals which were exposed to identical procedures (Van der Giessen et al., 1984). Inhibition of silver nitrate-induced thrombosis in the rabbit aorta has also been demonstrated (Stegmeier et al., 1984), although in that study the effective dose of BM 13.177 (20 mg · kg^{-1}) was higher. This difference could be due to the fact that rabbit platelets are, unlike other species, unresponsive to certain agonists and antagonists of thromboxane, such as carbocyclic thromboxane and pinane thromboxane (Burke et al., 1983).

In the present study nitroglycerine was topically administered to exclude coronary vasospasm as the cause of occlusion. Some studies have demonstrated that this compound enhances vascular PGI_2 formation (Levin et al., 1981; Schrör et al., 1981) and prolongs bleeding time (Ring et al., 1983). However, other studies were unable to confirm this (De Catarina et al., 1985; Neichi et al., 1980). Although we cannot exclude a similar effect of nitroglycerine in this study, it should be noted that the group receiving the high dose of BM

13.177 received the drug for the first time after 38 min. At this time 80% of the animals of the acetylsalicylate-treated and solvent groups already had a thrombotic occlusion. However, only the combination of nitroglycerin and BM 13.177 was effective in preventing both vasospasm and coronary thrombosis.

Our observation that BM 13.177 slightly lowered the content of thromboxane in arterial blood is in agreement with the results reported for human platelets in vitro and in vivo (Patscheke and Stegmeier, 1984; Riess et al., 1984). Verheggen and Schrör (1986) showed that higher, but not lower, doses of BM 13.177 influenced thromboxane biosynthesis in vitro, which has been confirmed in vivo (Schrör and Thiemermann, 1986). A similar effect has also been reported for the thromboxane antagonist SQ 29,548 (Mehta et al., 1986; O'Keefe et al., 1985). The mechanism responsible for this decrease is unknown, since no direct inhibitory effect on the activity of either cyclooxygenase or thromboxane synthetase could be demonstrated (O'Keefe et al., 1985). It is also unlikely that this decrease is due to interference of BM 13.177 with the measurement of thromboxane B_2 by radioimmunoassay, as only serum concentrations higher than 12.5 $\mu g \cdot ml^{-1}$ of BM 13.177 caused a disturbance of the assay in our laboratory.

Acetylsalicylate, in a dose that dramatically lowered arterial thromboxane levels, was not effective in our model, although inhibition of ex vivo platelet aggregation by this dose of acetylsalicylate was more pronounced than that produced by BM 13.177. An explanation of this phenomenon could be that acetylsalicylate also inhibited vascular prostacyclin production. This has been demonstrated for lower doses in man (Weksler et al., 1985). Moreover, pretreatment for several days with very low doses of acetylsalicylate effectively inhibited platelet function in other studies (Patrignani et al., 1982; Weksler et al., 1983). Although it has also been shown that such low doses simultaneously block thromboxane as well as prostacyclin production in healthy males (Preston et al., 1981), one might argue based on these studies in man, that the dose of acetylsalicylate used in the present study was too high. However, inhibition of both thromboxane and prostacyclin production has only been demonstrated after much higher doses in animals (Kelton et al., 1978; Wu et al., 1981). Acetylsalicylate has been reported to be thrombogenic in animal models at doses which were several times higher than those used in the present study (Kelton et al., 1978; Korbut and Moncada, 1978). In these models 10 $mg \cdot kg^{-1}$ of acetylsalicylate had no thrombogenic effects. The effect of different doses of acetylsalicylate on platelet and vascular cyclooxygenase in our pig model is unknown, but could be clarified by the use of acetylsalicylate in a dose which would irreversibly block platelet cyclooxygenase, but which would not affect the vascular enzyme. Animals should therefore be pretreated several days before the experiments take place. This type of experiment would also be helpful for a better understanding of the mechanism of action of BM 13.177.

The ineffectiveness of the lower dose of BM 13.177 was another example of the discrepancy between the effect on platelet aggregation in vitro and the antithrombotic effect in vivo. Schmitz et al. (1985) have reported that the increased production of thromboxane at the site of the damaged arterial wall is a very potent trigger for irreversible platelet aggregation and thrombus formation. The higher, but not the lower dose of BM 13.177, was apparently able to block the actions of thromboxane effectively during this process.

BM 13.177, in the doses used in this study, did not influence systemic haemodynamic parameters. This could prove to be an advantage in comparison with the use of prostacyclin or its analogues, which also inhibit thrombosis in this model (Romson et al., 1981; Van der Giessen et al., 1984), but cause a 20% decrease in arterial blood pressure in the effective doses. Additional anti-ischaemic effects, as demonstrated for prostacyclin (Lefer et al., 1978; Melin and Becker, 1983) or its analogue Iloprost (Schrör et al., 1981; Van der Giessen et al., 1986), have also been described for BM 13.177 (Brezinski et al., 1985; Schrör and Thiemermann, 1986). Furthermore, because of the absence of haemodynamic effects, the use of a higher dose of the compound could be feasible and even more effective.

In conclusion, the results of the present study indicate that BM 13.177 is capable of preventing experimentally induced coronary artery thrombosis in swine, but further studies are needed to elucidate the mechanism of its antithrombotic action.

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Chapter 6

CORONARY THROMBOLYSIS WITH AND WITHOUT NIFEDIPINE IN PIGS

Basic Research in
Cardiology

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Coronary thrombolysis with and without nifedipine in pigs*)

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Summary: To investigate whether addition of Ca^{2+} antagonists adds to the beneficial effects of thrombolysis we studied recovery of regional myocardial performance in pigs, in which occlusive thrombi were induced by electrical stimulation, with and without addition of nifedipine to the thrombolytic agent. To this end, four different groups of animals with thrombotic coronary occlusion were studied. Groups 1 and 2 received either saline or intracoronary nifedipine ($0.1\ \mu g \cdot kg^{-1} \cdot min^{-1}$) 15 min after coronary artery occlusion. Groups 3 and 4 were treated with the thrombolytic agent plasmin which was infused directly into the left anterior descending coronary artery (LADCA) at a rate of 2 $U \cdot min^{-1}$. The animals in group 4 also received intracoronary nifedipine. 4 h after thrombus formation the animals were sacrificed. No important differences in systemic hemodynamics were observed between the four groups of animals. Reperfusion occurred only in the animals which received plasmin, with or without nifedipine. After intracoronary plasmin regional blood flow increased from 7 ± 2 to $40 \pm 7\ ml \cdot min^{-1} \cdot 100\ g^{-1}$ in the LADCA-perfused subepicardial and from 9 ± 2 to $30 \pm 6\ ml \cdot min^{-1} \cdot 100\ g^{-1}$ in the LADCA-perfused subendocardial layers. The combination of plasmin and nifedipine increased flow to the LADCA-perfused subepicardial layers from 8 ± 2 to $74 \pm 21\ ml \cdot min^{-1} \cdot 100\ g^{-1}$ and that to the subendocardial layers from 8 ± 2 to $57 \pm 16\ ml \cdot min^{-1} \cdot 100\ g^{-1}$ (in both cases: $p < 0.05$ vs. plasmin alone). However, addition of nifedipine did not enhance recovery of regional myocardial function or high-energy phosphate metabolism. Because reperfusion was accompanied by a high ventricular ectopic activity, the question may be raised of whether reperfusion of ischemic myocardium which does not result in functional recovery could be deleterious.

Key words: coronary thrombosis; thrombolysis; nifedipine; regional myocardial blood flow; myocardial function; adenine nucleotides

Introduction

Coronary thrombolysis has been widely advocated as a therapeutic modality to reduce the size or even prevent the development of transmural myocardial infarctions. Recently two large randomized clinical trials showed indeed a marked reduction in mortality by this therapy (6, 19). Experimental and clinical studies also demonstrate a beneficial action of calcium entry blockers on ischemic myocardium (7, 9, 24, 28). These drugs are most effective when administered before the occurrence of ischemic events (4), but nifedipine also improved recovery of regional myocardial function during reperfusion when the drug was administered after induction of mild ischemia (24). Furthermore, administration of nifedipine upon reperfusion reduced the rate of intracellular Ca^{2+} accumulation (14).

Because reperfusion during thrombolysis occurs less abruptly than after release of a coronary artery occlusion (11), the rate of intracellular Ca^{2+} accumulation may also be reduced during the earliest reperfusion phase. Under these circumstances, administration of Ca^{2+} antagonists after the onset of the ischemic event may be more effective. We therefore studied the effects of the combination of thrombolysis and intracoronary infusions of nifedipine in an experimental model of coronary artery thrombosis.

Methods

Anesthesia and catheterization

In 38 young pigs (21–38 kg) electrical induction of occlusive coronary thrombosis was attempted as described earlier (21). Briefly, the animals were sedated with 120 mg azaperone i.m. and anesthetized with 150 mg metomidate i.v. After endotracheal intubation, the pigs were connected to a respirator for mechanical ventilation with a mixture of oxygen and nitrous oxide (1:2). Acid-base balance and oxygenation were controlled by frequent measurement of arterial blood gases (ABL3, Radiometer, Copenhagen). Anesthesia was maintained with pentobarbital ($12\ mg \cdot kg^{-1} \cdot h^{-1}$), infused via a catheter placed in the superior vena cava. Catheters were placed in the ascending aorta (7F Millar) for measurement of mean arterial blood pressure, and in the abdominal aorta (8F Cournand) for withdrawal of arterial reference samples for calibration of the radioactive microsphere method (see below).

Thoracotomy

4 mg of the muscle relaxant pancuronium bromide was administered before the thorax was opened via a midsternal split. The tip of a 2F catheter was located in the left atrial appendage for injection of radioactive microspheres ($15 \pm 1\ \mu m$; ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb), while electromagnetic flow probes were placed around the aortic root and the left anterior descending coronary artery (LADCA). A 5 MHz ultrasound transducer (Krautkramer-Branson, Lewistown, PA, U.S.A.) was sutured onto the epicardium of the area of the left ventricle supplied by the LADCA.

Induction of thrombus

For the electrical induction of occlusive thrombosis the LADCA was prepared free distal to its first diagonal branch. A circumferential stainless steel electrode was placed at that point around the vessel and isolated from surrounding tissue by a polyethylene strip. The electrode was connected at its anodal side with a direct current source, while the cathodal side was connected to the abdominal fat to complete the circuit (20, 21).

Regional myocardial function, perfusion and ATP content

From the ultrasound tracings, the myocardial thickness at end-systole (EST) and end-diastole (EDT) was determined for calculation of systolic wall thickening (SWT) as $SWT = (EST - EDT)/EDT \times 100\,\%$. Regional myocardial blood flow was determined 10 min after thrombus formation, and also 4 h later. The withdrawal of the arterial reference sample (10 ml/min) was started before the injection of the spheres and continued until 90 s after the injection was completed. Then the heart was excised, the myocardial tissue divided into epicardial- and endocardial layers and the radioactivity counted with a Hewlett-Packard gamma counter (16).

At baseline, after thrombotic occlusion, and again at the end of the experiment, transmural myocardial biopsies were taken from the LADCA-perfused and an adjacent control area using a Trucut Travenol needle. The specimen were immediately placed in liquid nitrogen and stored for later measurement of adenine nucleotides using HPLC (8).

Experimental protocol

After the preparation had been stable for 30 min, pre-occlusion recordings were made of heart rate, mean arterial blood pressure, cardiac output, LADCA-flow and left ventricular wall thickness.

Subsequently, thrombosis of the LADCA was started by passing a 1.6 mA current through the electrode. Occlusion by the thrombus was thought to be present when antegrade flow became zero, systolic wall thickening was abolished and visible stasis of blood occurred. Zero antegrade flow was thought to be present when the flowmeter reading had decreased to the residual flow which remained during a temporary occlusion of the LADCA at the site of stimulation prior to the recording of the pre-occlusion data. These indications have been validated by microscopy in earlier studies (20, 21). In 31 of the 38 animals (82 %) an occluding coronary thrombus occurred. Seven animals were withdrawn from further study because the occlusion criteria were not fulfilled.

Median time to occlusion for the 31 animals, in which these criteria were fulfilled, was 36 min (range 16–83 min). At least one episode of coronary vasospasm (confirmed by the topical application of nitroglycerin) preceded the occlusion in 16 animals (52 %). Occlusion by thrombus or vasospasm were fast phenomena, as in both instances coronary blood flow decreased from basal values to "zero", and systolic wall thickening was abolished within 2 min.

15 min after thrombolytic occlusion the animals were randomly assigned to four groups which received intracoronary infusions ($1\ ml \cdot min^{-1}$) of (1) saline ($n = 5$, control group); (2) nifedipine ($0.1\ \mu g \cdot kg^{-1} \cdot min^{-1}$, $n = 9$); (3) porcine plasmin ($2\ U \cdot min^{-1}$, $n = 10$); (4) a combination of porcine plasmin ($2\ U \cdot min^{-1}$) and nifedipine ($0.1\ \mu g \cdot kg^{-1} \cdot min^{-1}$, $n = 7$). Infusions were administered proximal to the flow probe via an 18-gauge needle attached to the tip of a 2F cannula, punctured through the wall of the LADCA. Preparation of porcine plasmin and the dose needed to achieve thrombolysis have been described earlier (21). The intracoronary dose of nifedipine has a pronounced effect on coronary hemodynamics, but affects systemic hemodynamics only minimally (23). Heparin was not administered until 30 min after reperfusion ($1\ mg \cdot kg^{-1}$). At that time the plasmin infusion was stopped, but that of nifedipine was continued throughout the experiment.

Ventricular arrhythmias

When the incidence of ventricular arthythmias was higher than 10 premature ventricular beats per min, the animals were treated with a single dose of lidocaine ($1\ mg \cdot kg^{-1}$). Animals which had a ventricular fibrillation were promptly (< 1 min) defibrillated.

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether the differences between the groups were statistically significant. All data are expressed as means ± SEM, unless otherwise stated. Only $p < 0.05$ (two-tailed) was considered to be statistically significant.

Results

Incidence of reperfusion and duration of ischemia

Reperfusion did not occur in any of the animals of the saline- or nifedipine groups. In nine of the ten (90 %) plasmin-treated animals, reperfusion occurred after a median infusion time of 18 min (range 3–65 min). Consequently in the nine animals in which reperfusion was established, the median interval between the onset of ischemia and the start of reperfusion was 33 min (range 18–80 min). In the pigs in which the combination of plasmin and nifedipine was used reperfusion occurred in six of the seven animals (86 %) after a median time of 13 min (range 9–69 min). So, in the six animals with reperfusion, the median interval between the onset of ischemia and the start of reperfusion was 28 min (range 24–84 min).

Systemic hemodynamics

The differences in systemic hemodynamics of the four groups were minimal during the course of the experiments (Table 1). 4 h after thrombus formation, mean arterial blood pressure was 14 % higher ($p < 0.05$) in the saline-treated group compared to the animals which received nifedipine alone or in combination with plasmin.

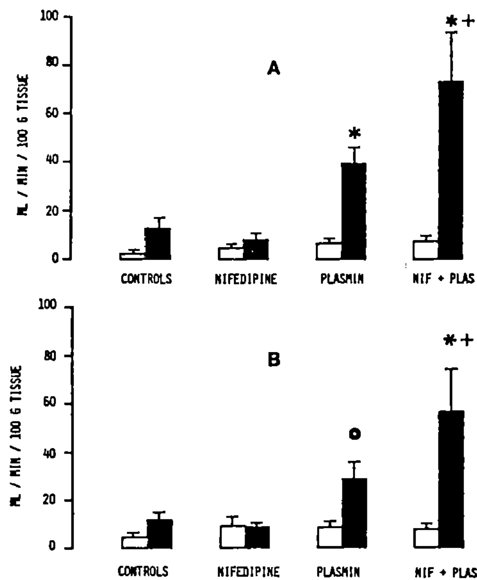

Fig. 1. Subepicardial (A) and subendocardial (B) flow of the LADCA-perfused area after occlusive thrombosis (□) and four hours after occlusion (▥) with and without reperfusion. * $p < 0.05$ versus controls and nifedipine group. ✪ $p < 0.05$ versus nifedipine group. $^{+}p < 0.05$ versus plasmin alone.

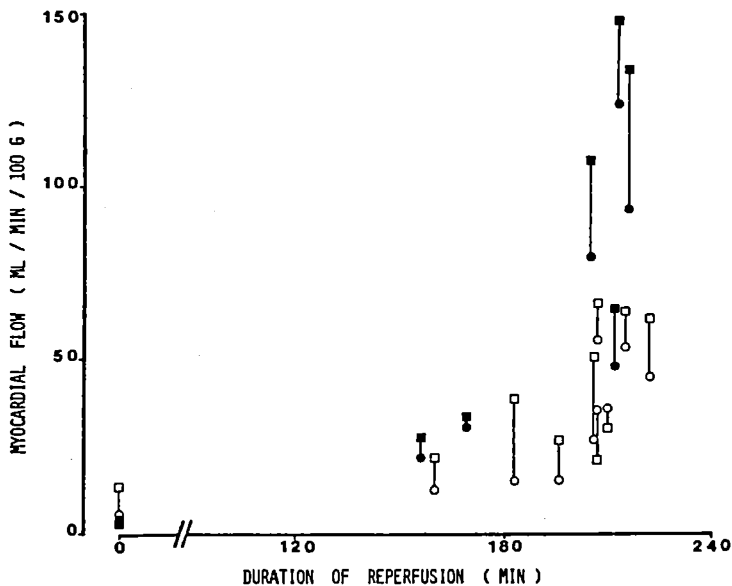

Fig. 2. Individual values of regional subepicardial (□) and subendocardial (○) flow for the experiments which received plasmin alone (open symbols) or in combination with nifedipine (closed symbols). Data were obtained 4 h after thrombotic occlusion.

Regional myocardial function

Before occlusion, systolic wall thickening of the LADCA-perfused area was similar for all four groups (44 ± 4 %). After occlusion, systolic wall thickening was completely abolished. No signs of functional recovery were observed in any of the groups (Table 1).

Regional myocardial blood flow

Thrombus formation caused an almost complete cessation of regional myocardial blood flow in all groups. 4 h after occlusion, flow had not improved in the saline and nifedipine groups (Fig. 1). However, when plasmin was infused regional perfusion increased from 9 ± 2 to 30 ± 6 $ml \cdot min^{-1} \cdot 100 g^{-1}$ in the subendocardial layers, and from 7 ± 2 to 40 ± 7 $ml \cdot min^{-1} \cdot 100 g^{-1}$ in the subepicardial layers. The combination of plasmin and nifedipine increased the flow even more (from 8 ± 2 to 57 ± 16 $ml \cdot min^{-1} \cdot 100 g^{-1}$ in the subendocardial layers and from 8 ± 2 to 74 ± 21 $ml \cdot min^{-1} \cdot 100 g^{-1}$ in the subepicardial layers, in both cases $p < 0.05$). Thus in both groups thrombolysis resulted in only a gradual return of myocardial blood flow, but with the addition of nifedipine, the flow was higher in all animals for the same duration of reperfusion (Fig. 2).

Adenine nucleotides

Before induction of the thrombus, the ATP, ADP, AMP and energy charge data of the LADCA-perfused and adjacent myocardium were the same for all four groups of animals (only the ATP data are shown in Table 2). ATP content had decreased similarly in all four groups at the start of treatment. No recovery was seen with successful thrombolysis. The energy-rich phosphate content of adjacent myocardium remained stable throughout the experiments (highest value for ATP 26 ± 1 $\mu mol \cdot g^{-1}$ protein in control animals, lowest value 24 ± 2 $\mu mol \cdot g^{-1}$ protein in nifedipine-treated animals).

Arrhythmias during ischemia and reperfusion

During occlusion, ventricular arrhythmias were present in approximately 50 % of the animals of each group (Table 3). Ventricular fibrillation occurred in 13 of the 31 animals (42 %, median time 24 min after occlusion). Defibrillation was successful in all but one of these animals. Opening of the vessel lumen was accompanied by a high incidence of ventricular fibrillation (60–70 %), which occurred exclusively during the first 15 min of reperfusion.

Discussion

Spontaneous reperfusion did not occur in this experimental model of coronary artery thrombosis, but reperfusion could be produced with a thrombolytic agent. The addition of intracoronary nifedipine had no effect on lysis of the thrombus itself. No important hemodynamic changes occurred in any group of animals after formation of the thrombus. Although the 10–15 % lower mean arterial blood pressure after 4 h of reperfusion very likely contributes to the incomplete reperfusion in the plasmin group (normal flow values: 80–120 $ml \cdot min^{-1} \cdot 100 g^{-1}$), it is also feasible that platelet aggregates dislodge from growing thrombi and embolize in the myocardial microvasculature (12). A third hypothesis is that ischemic injury of 20–80 min duration causes a no-reflow phenomenon (10, 27) in pigs, a species without a significant collateral circulation (18). The regional flow data demonstrate that addition of a low dose of nifedipine to the plasmin infusion results in a more complete

Table 1. Systemic hemodynamics and regional myocardial wall thickening of the segment nourished by the thrombosed coronary artery of anesthetized pigs with and without thrombolysis (2 U/min plasmin i.c.) and nifedipine (0.1 µg/kg/min i.c.). Treatment started 15 min after thrombus formation.

	Saline (n = 5)	Nifedipine (n = 9)	Plasmin (n = 10)	Plasmin + Nifedipin (n = 7)
15 min after thrombus formation				
HR (beats/min)	88 ± 8	81 ± 5	83 ± 5	86 ± 6
MAP (mm Hg)	64 ± 3	68 ± 3	69 ± 5	67 ± 4
CO (l/min)	2.9 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.6 ± 0.3
SWT (%)	−2 ± 3	−3 ± 3	3 ± 2	−3 ± 2
4 h after thrombus formation				
HR (beats/min)	86 ± 10	91 ± 6	87 ± 9	85 ± 6
MAP (mm Hg)	66 ± 4	59 ± 2*	59 ± 3	57 ± 2*
CO (l/min)	2.2 ± 0.3	1.9 ± 0.1	2.0 ± 0.2	2.0 ± 0.2
SWT (%)	−3 ± 3	−2 ± 2	4 ± 4	0 ± 3

Abbreviations: HR = heart rate; MAP = mean arterial blood pressure; CO = cardiac output; SWT = regional systolic wall thickening of the area perfused by the thrombosed coronary artery. All data are presented as means ± SEM. * $p < 0.05$ versus saline. Note that negative values for SWT indicate thinning of myocardium.

Table 2. Adenosine triphosphate content (μmol/g protein) of myocardium nourished by the thrombosed coronary artery of anesthetized pigs with and without thrombolysis (2 U/min plasmin i.c.) and nifedipine (0.1 μg/kg/min i.c.). Treatment started 15 min after occlusion.

	n	baseline	15 min after thrombus formation	4 h after thrombus formation
Saline	5	26 ± 3	5 ± 2*	2 ± 1*
Nifedipine	9	22 ± 3	4 ± 1*	3 ± 1*
Plasmin	10	28 ± 4	5 ± 2*	8 ± 4*
Plasmin + Nifedipine	7	22 ± 2	5 ± 1*	5 ± 2*

No thrombolysis occurred in the saline- and nifedipine-treated groups. Reperfusion occurred 3–69 min after the start of thrombolytic treatment in all but one animal of each of the other two groups. All data have been presented as means ± SEM; * $p < 0.05$ versus baseline value.

Table 3. Ventricular arrhythmias during occlusion and reperfusion in anesthetized pigs with and without thrombolysis (2 U/min plasmin i.c.) and nifedipine (0.1 μg/kg/min i.c.). Treatment started 15 min after thrombus formation.

		Occlusion			Reperfusion		
	n	PVC's/min > 10	VF	episodes of VF	PVC's/min > 10	VF	episodes of VF
Saline	5	2	3	5			
Nifedipine	9	3	4	12*			
Plasmin	10	6	4	4	8	5	7
Plasmin + Nifedipine	7	4	2	4	6	4	8

No thrombolysis (reperfusion) occurred in the saline- and nifedipine-treated groups. Reperfusion occurred 3–69 min after the start of thrombolytic treatment in all but one animal of the other two groups. n = number of animals; PVC = premature ventricular contraction; VF = ventricular fibrillation; * one animal had six episodes of VF.

reperfusion, as flow values resembled more closely those reported for the same species without coronary artery obstruction (17, 24, 25). However, during the first 4 h of reperfusion, this increase in regional myocardial blood flow failed to enhance recovery of either ATP content or regional systolic wall thickening. The latter observation is not surprising, as in the same species 2 h of complete reperfusion after 30 min of ischemia does not result in any sign of functional recovery (15). Murphy et al. have suggested that 30 min of ischemia followed by reperfusion results in calcium overload and irreversible cell damage in pigs (13). Our own results in conscious pigs do not support this hypothesis, as regional myocardial wall thickening, absent after 2 h of reperfusion, had normalized after 2 weeks following 30 min of coronary artery occlusion (15). ATP levels were still low at the end of the experiments. This does not exclude ultimate recovery of function because myocardial ATP content is of limited value in predicting the recovery of function of reperfused myocardium (22). In the present model, however, the exact timing of the duration of ischemia remains an uncertain factor. The onset of ischemia can be determined within narrow limits, but that of reperfusion is more difficult to ascertain. The reopening of the vessel can be monitored by electromagnetic flow meter, but in view of the present data and those reported by others (11), reperfusion occurred gradually, and therefore a not unimportant episode of hypoperfusion may prolong the actual ischemic period.

The pig is very susceptible to ventricular fibrillation following coronary artery occlusion (1, 2, 26). The results of the present study support these findings. Abrupt reperfusion of ischemic myocardium is frequently accompanied by ventricular tachycardia and fibrillation, particularly when the duration of ischemia varies between 10 and 30 min (2, 26). Reperfusion arrhythmias are usually less severe when the duration of ischemia is longer or the release of the obstruction more gradual (see ref. (26)). With thrombolysis, reperfusion is very likely reinstated more gradually (Fig. 2) and, because the duration of ischemia exceeds 30 min in 50 % of the animals, the incidence of reperfusion arrhythmias may be lower. Nevertheless, the occurrence of arrhythmias during thrombolysis has been used as a measure of the success of the procedure (5), although it may also be a sign of incomplete reperfusion (3). In the present study, the incidence of arrhythmias during reperfusion was unexpectedly high (60–70 %) in both groups which received plasmin. All these arrhythmias occurred during early reperfusion and may be indicative of incomplete reperfusion, as our flow data were below normal. It is, however, of interest that thrombolysis resulted in new episodes of ventricular ectopic activity, whereas in the animals in which no reperfusion occurred there was a quiescent period as far as ectopic activity was concerned. In view of these observations the question may be raised of whether attempts to reperfuse ischemic myocardium could be detrimental when ultimate recovery of function cannot be achieved.

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Chapter 7

ILOPROST (ZK 36374) ENHANCES RECOVERY OF REGIONAL MYOCARDIAL FUNCTION DURING REPERFUSION AFTER CORONARY ARTERY OCCLUSION IN THE PIG

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Iloprost (ZK 36374) enhances recovery of regional myocardial function during reperfusion after coronary artery occlusion in the pig

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1 Ligation of the left anterior descending coronary artery in open-chest pigs for 20 min caused a complete loss of regional myocardial function, which did not recover during the first two hours of reperfusion.

2 Infusion of the stable prostacyclin analogue Iloprost (100 ng kg^{-1} min^{-1}) did not prevent the loss of systolic wall function during ischaemia.

3 Recovery of regional myocardial function during the first two hours of reperfusion was enhanced to 40% of baseline by Iloprost.

4 This effect of Iloprost cannot be explained by a decreased O_2-demand during ischaemia or an enhanced recovery of myocardial ATP content.

Introduction

In the experimental animal, prostacyclin exerts a beneficial effect on acutely ischaemic myocardium (Lefer *et al.*, 1978; Melin & Becker, 1983). However, its chemical instability limits its use. Iloprost ZK 36374; 5-{(E)-(1S, 5S, 6R, 7R)-7-hydroxy-6-|(E)-(3S, 4RS)-3-hydroxy-4-methyl-oct-1-en-6-yn-yl | -bicyclo- | 3.3.0-octan-3-yliden}-pentanoic acid) a more stable analogue of prostacyclin, has been shown to reduce ST-segment elevation and myocardial creatine kinase depletion after coronary artery occlusion in the cat (Schrör *et al.*, 1981) and to possess a pronounced antithrombotic effect in the pig (van der Giessen *et al.*, 1984). We now describe the effect of Iloprost on regional myocardial function in a model of coronary artery occlusion followed by reperfusion.

Methods

General

Studies were performed in young Yorkshire pigs (22–30 kg). The animals were sedated with azaperone (120 mg i.m.) and anaesthesia was induced with 150 mg metomidate, administered via a dorsal ear vein. Subsequently the animals were intubated and connected to a respirator for artifical ventilation with a mixture of O_2 and N_2O (1:2). Pentobarbitone (6–12 mg kg^{-1} h^{-1}) was administered intravenously for maintenance of anaesthesia. Iloprost or its solvent was infused via a catheter in the superior caval vein. Central aortic pressure was obtained via a 7F Cournand catheter. Left ventricular pressure was measured via an 8 F Millar catheter. A standard lead ECG was recorded continuously.

After exposure of the heart, an electromagnetic flow probe (Skalar, Delft) was placed around the ascending aorta for the measurement of aortic blood flow. Stroke volume was computed from the integral of this flow signal.

Myocardial wall thickness was monitored with a 5 MHz ultrasonic transducer (Krautkramer-Branson, Lewistown, Pa, USA) sutured onto the epicardial surface of that part of the left ventricle perfused by the left anterior descending coronary artery (LAD). From the tracings, the local wall thickness was measured at end-diastole (EDT) and end-systole (EST), while systolic wall thickening (SWT) was calculated as:

SWT = (EST − EDT)/EDT (Verdouw *et al.*, 1980)

Transmural myocardial biopsies were taken from control and ischaemic areas with a Trucut Travenol needle and immediately placed and stored in liquid nitrogen until analysis. The biopsies were homogen-

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Table 1 Systemic haemodynamic values before and after a 10 min infusion of Iloprost and vehicle; during occlusion and after reperfusion

		Baseline	*Start occlusion*	*End of occlusion*	*Reperfusion*	
		– 10 min	*0 min*	*15 min*	*30 in*	*120 min*
Heart rate (min^{-1})	Iloprost	90 ± 3	93 ± 4	94 ± 4	102 ± 6^{f}	99 ± 6^{f}
	Solvent	84 ± 4	82 ± 4	85 ± 4	83 ± 4	84 ± 4
LV-syst.pr (mm Hg)	Iloprost	106 ± 3	97 ± 4$*	87 ± 4*	84 ± 4†	78 ± 3
	Solvent	102 ± 4	101 ± 3	90 ± 3*	86 ± 5	83 ± 5
Mean art.pr (mm Hg)	Iloprost	92 ± 3	82 ± 3$*	73 ± 3*	68 ± 4†	62 ± 4
	Solvent	85 ± 3	85 ± 3	76 ± 3*	73 ± 4	70 ± 4
Aortic flow ($l\ min^{-1}$)	Iloprost	2.5 ± 0.1	2.3 ± 0.1†	2.1 ± 0.1*	2.1 ± 0.1	2.2 ± 0.2
	Solvent	2.4 ± 0.2	2.4 ± 0.2	1.9 ± 0.1*	1.9 ± 0.2	1.7 ± 0.2
Systemic vasc. resist.	Iloprost	37.8 ± 2	36.3 ± 2	35.6 ± 2	33.7 ± 3	29.6 ± 3
(mm Hg $l^{-1}\ min^{-1}$)	Solvent	36.8 ± 3	37.3 ± 2	40.6 ± 2	41.0 ± 3	46.2 ± 6

Data shown are means ± s.e.mean.
$^{f}P < 0.05$ vs solvent; $^{\$}P < 0.01$ vs solvent (change 0 vs − 10); * $P < 0.01$ vs previous data point; † $P < 0.05$ vs previous data point.

ized at liquid nitrogen temperatures in 4% perchloric acid. After neutralization the high energy phosphates of adenosine, adenosine 5′-triphosphate, ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-phosphate (AMP)) and creatine phosphate (CP) were measured using high-pressure liquid chromatrography as described by Harmsen *et al.* (1982). Protein content was estimated according to the method of Bradford (1976) using a Bio-Rad protein assay (Bio-Rad, München, FGR).

Experimental procedure

Baseline measurements were recorded after the preparation had been stable for at least 30 min. Two groups of animals were studied. One group of 16 animals received 100 ng $kg^{-1}\ min^{-1}$ Iloprost (supplied by Schering Chemicals, Ltd, UK) during the entire course of the experiment, while 12 other animals received the solvent. This dose of Iloprost was chosen because it caused an acceptable decrease in blood pressure (15–20%) and the haemodynamic changes would be comparable with other data on the compound (Coker & Parratt, 1983). Furthermore, this specific dose has proved to be effective against experimental coronary thrombosis (van der Giessen *et al.*, 1984). Ten minutes after the start of the infusions, the LAD was completely occluded distal to its first diagonal branch using a microsurgical clamp. Twenty minutes later the clamp was released and the ischaemic myocardium abruptly reperfused for two hours.

In the solvent group one animal was withdrawn from the protocol due to sustained ventricular tachyarrhythmias in the early reperfusion phase. In the Iloprost group, 6 animals did not complete the entire protocol as 4 of these had persistent ventricular fibrillation and two others were withdrawn because their mean arterial blood pressure had declined below 50 mmHg. Of the four pigs with ventricular fibrillation, in three this occurred during collection of a biopsy. This procedure may have triggered the arrhythmia. Data obtained from these animals were included until the time of withdrawal.

Statistical analysis

Student's *t* test was employed to determine whether the Iloprost-induced haemodynamic changes were statistically significant ($P < 0.05$). The same test was used to assess changes in ATP or CP. Since repeated testing occurred for the assessment of changes in SWT, only $P < 0.01$ was considered statistically significant. All data are expressed as means ± s.e.mean.

Results

Infusion of the solvent did not cause changes in cardiovascular performance (Table 1). Iloprost did not affect heart rate, but produced a 9% decrease in left ventricular systolic pressure (from 106 ± 3 to 97 ± 3 mmHg, $P < 0.01$) and mean arterial pressure (from 92 ± 3 to 82 ± 3 mmHg, $P < 0.01$), mainly due to systemic vasodilatation as cardiac output (from 2.5 ± 0.1 to 2.3 ± 0.1 $l\ min^{-1}$) only slightly decreased. Occlusion of the LAD resulted in similar decreases in blood pressure in both groups: 11 ± 1% in the Iloprost group and 10 ± 2% in the solvent group (Table 1).

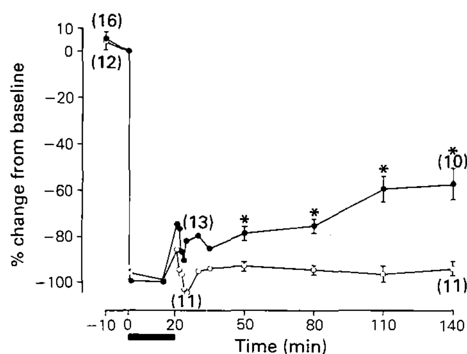

Figure 1 Recovery of systolic wall thickening after 20 min of left anterior descending coronary artery occlusion (solid bar) in (●) Iloprost (100 ng kg^{-1} min^{-1}) and (○) solvent-treated pigs. Data are expressed as percentage change from baseline (0.40 ± 0.02 for the Iloprost and 0.35 ± 0.03 for the solvent group). Each point represents the mean, and vertical lines s.e.mean, of the number of animals in parentheses. *$P < 0.01$.

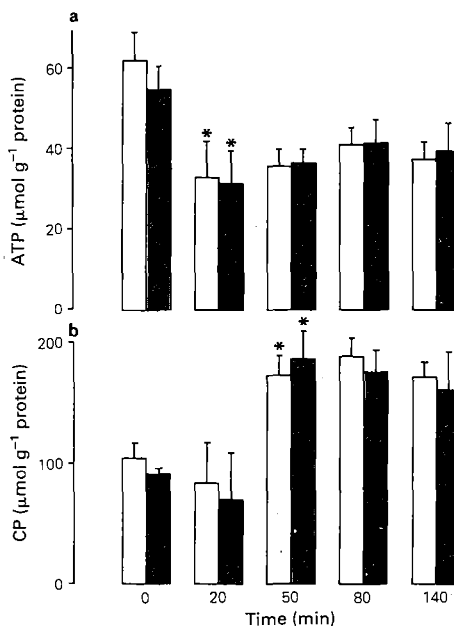

Figure 2 The amount of (a) ATP and (b) creatine phosphate (CP) in the area of the left ventricle perfused by the left anterior descending coronary artery (LAD) in Iloprost (shaded columns)- and solvent (open columns)-treated pigs. Biopsies were obtained before (0) and after (20) occlusion, and after 30 (50), 60 (80) and 120 (140) min of reperfusion. *$P < 0.02$ vs previous data point.

This decline in blood pressure was caused by a decrease in stroke volume (17 ± 3% for the solvent-group versus 10 ± 2% for the Iloprost group), while heart rate remained unchanged. After removing the obstruction, heart rate increased slightly (from 94 ± 4 to 102 ± 6 beats min^{-1}, $P < 0.05$) in the Iloprost group, while other haemodynamic parameters remained relatively similar between the groups.

Systolic wall thickening of the area perfused by the LAD (0.40 ± 0.02) was not affected by either Iloprost or the solvent (Figure 1). Immediately following LAD occlusion, SWT was completely abolished in both groups of animals. During the first two hours of reperfusion, no recovery of function of the ischaemic zone was observed in the solvent group. Immediately after reperfusion there was a slight recovery in SWT in the Iloprost-treated group which was maintained for the first hour. Subsequently, there was a further increase and after two hours of reperfusion SWT had returned to about 40% of its pre-occlusion value.

Animals not completing the protocol had a slightly lower SWT before occlusion (0.37 ± 0.02, compared to 0.42 ± 0.02 for the survivors), but shortly before the start of the fatal arrhythmia SWT in these animals was at least as good (0.11 ± 0.04 compared to 0.04 ± 0.01 in the survivors).

During occlusion ATP levels in the ischaemic area declined from 62 ± 7 to 33 ± 9 μmol g^{-1} protein and from 55 ± 6 to 32 ± 8 μmol g^{-1} protein in the vehicle- and Iloprost-treated groups, respectively (Figure 2). After reperfusion ATP recovered slightly, but did not reach pre-ischaemic values in either group (Figure 2). In the non-ischaemic tissue ATP remained constant (60 ± 5 μmol g^{-1} protein) during the experiments in both groups. The energy charge decreased slightly but not significantly during ischaemia and returned to pre-ischaemic values after reperfusion (Table 2). In the ischaemic area CP decreased during and just after occlusion from 104 ± 12 to 84 ± 33 and from 90 ± 6 to 70 ± 38 μmol g^{-1} protein in the solvent and drug-treated groups, respectively (Figure 2). After the ischaemic period CP had increased to a significantly higher level than that in the pre-ischaemic period ($P < 0.02$; Figure 2) in both groups. However, there were no significant differences in the biochemical changes between the groups.

Discussion

No recovery in regional function was observed in the solvent-treated animals during the first two hours of

Table 2 Adenylate energy charge (ATP + 0.5 ADP/ATP + ADP + AMP) in transmural biopsies obtained from the occluded segment

	Baseline	*End of occlusion*	*Reperfusion*	
	− 10 min	15 min	30 min	120 min
Treatment				
Solvent	0.85 ± 0.01	0.78 ± 0.03	0.83 ± 0.01	0.85 ± 0.01
Iloprost	0.86 ± 0.01	0.79 ± 0.02	0.85 ± 0.01	0.85 ± 0.01

Data shown are means ± s.e.mean.

reperfusion following 20 min of coronary artery occlusion. This finding is consistent with that reported by Murphy *et al.* (1982) in the same species. In the Iloprost-treated animals, regional function showed a partial recovery. The mechanism by which Iloprost improved regional function during reperfusion is unclear, although a number of factors can be eliminated. A lowered myocardial O_2 demand at the time of occlusion is an unlikely factor as the double product of heart rate x left ventricular systolic pressure was very similar for both groups. The biochemical parameters ATP, energy charge and CP were also not significantly affected by the Iloprost treatment. Our data also showed that inhibition of ATP depletion during ischaemia or an enhanced recovery during reperfusion by Iloprost was not an important factor. This mechanism of action is possibly operative in experimental hypothermic cardiac arrest in the rat (van Gilst *et al.*, 1983). The CP level overshoot and the lower level of ATP, compared to pre-ischaemic values, occurring during reperfusion is consistent with results obtained by others, as summarized by Ichihara & Abiko (1984).

Schrör *et al.* (1982) have suggested that prostaglandin I_2 may exert a beneficial action via structural preservation of myocardial membrances or adrenergic nerve endings. We cannot exclude the possibility that such a mechanism is also effective for Iloprost.

The favourable effect of Iloprost demonstrated in this study may be of value to certain patients, but the greater vulnerability of the heart to reperfusion arrhythmias, an observation previously made in the dog (Coker & Parratt, 1983), might pose a serious problem. We observed ventricular tachyarrhythmias (VT,VF) in 42% of the solvent-treated and in 56% of the Iloprost-treated animals during the first 15 min after reperfusion. Most of the ventricular fibrillations occurred during collection of the biopsies and mechanical irritation of vulnerable myocardium may have triggered those fatal arrhythmias. The higher incidence of ventricular fibrillation in the Iloprost-treated animals could indicate that the hearts of these animals were electrically more unstable, but the design of the protocol (reperfusion combined with needle biopsies) is not really suited to draw any definite conclusions.

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Chapter 8

IN VITRO CYCLIC AMP INDUCED PHOSPHORYLATION OF PHOSPHOLAMBAN: AN EARLY MARKER OF LONG-TERM RECOVERY OF FUNCTION FOLLOWING REPERFUSION OF ISCHAEMIC MYOCARDIUM?

Cardiovascular Research, 1988, **22**, 714–718

In vitro cyclic AMP induced phosphorylation of phospholamban: an early marker of long-term recovery of function following reperfusion of ischaemic myocardium?

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ABSTRACT Changes in myocardial membrane biochemistry and ultrastructure, determined shortly (2 h) after reperfusion of ischaemic myocardium, were compared with the long term (4 wk) recovery of regional myocardial function. Anaesthetised pigs were subjected to 30 min (n=14, group I) or 60 min (n=14, group II) of left circumflex coronary artery occlusion. Seven animals of each group were studied 2 h and the others 4 weeks after flow was reinstated. After 2 h of reperfusion, regional myocardial function was absent in both groups. At 4 weeks regional function had returned to normal in group I, but was still significantly depressed in group II. Biochemical studies after 2 h of reperfusion showed that a functional index of the cardiac membrane, the in vitro cyclic AMP dependent ^{32}P incorporation into phospholamban, was 71 (SEM 9)% compared to non-ischaemic myocardium in group I and 31 (6)% in group II ($p<0.05$). After 4 weeks this index had completely recovered in group I, 114 (13)%, but a significant decrease to 79 (2)% could still be observed in group II ($p<0.05$). After 2 h of reperfusion as well as after 4 weeks of recovery the myocytes in group II were more severely damaged than in group I.

This study suggests that determination of in vitro phosphorylation of phospholamban shortly after reperfusion of ischaemic myocardium may be of value in the prediction of long term recovery of regional myocardial function.

Cyclic AMP induced ^{32}P incorporation into phospholamban, which regulates Ca^{2+} transport in membranes of sarcolemma and sarcoplasmic reticulum, decreases during myocardial ischaemia.[1–4]

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This decrease coincides with the appearance of ultrastructural damage and may therefore reflect a loss of functional integrity of the myocardial cell.[3 5–8] It is not known whether the in vitro phospholamban phosphorylation recovers during reperfusion and, if this occurs, whether it reflects recovery of myocardial ultrastructure and contractile function. Therefore we studied these factors in 30 min or 60 min ischaemic myocardium, which was subsequently reperfused for 2 h or 4 weeks. Because the development of myocardial infarcts not only depends on the time of occlusion but also on the available collateral circulation, we used domestic pigs. This species has a coronary artery pattern and a distribution of blood supply remarkably similar to that of humans, and is known for its lack of collaterals.[9–11]

Materials and methods

ANIMAL PREPARATION AND PROTOCOL

Acute experiments — In a first series 14 Yorkshire pigs (11-18 kg) were anaesthetised and instrumented with central arterial and venous catheters as described earlier.[12] After thoracotomy the pericardium was opened, and a 5 MHz ultrasound crystal (Krautkramer-Branson, Lewistown, Pa, USA) was placed on to sections of the left ventricle supplied by the left circumflex coronary artery (LCXCA) for the measurement of regional systolic wall thickening.[13] After a stabilisation period of 30 min, baseline measurements of heart rate and mean arterial pressure were recorded and the LCXCA was proximally occluded with a microsurgical clamp for 30 (group Ia, n=7) or 60 (group IIa, n=7) min. After the myocardium had been reperfused for 2 h, transmural biopsies for electron microscopy and biochemical analysis were obtained.

Chronic experiments — In a second series of 14 animals, thoracotomy was performed under sterile conditions.[14] After a stabilisation period of 30 min the LCXCA was occluded proximally for 30 (Group Ib, n=7) or 60 (Group IIb, n=7) min, and the myocardium was then reperfused by declamping the vessel. The thorax was closed after 2 h and the animals were permitted to recover from surgery.

In these animals regional myocardial function was assessed by two-dimensional echocardiography (2-DE) on the day before surgery, and 2 and 4 weeks thereafter. The 2-DE images were obtained with a 5 MHz transducer of 32 elements using a commercially available 2-DE apparatus (Hewlett-Packard). Recordings were made after the animals had been sedated with 30 $mg \cdot kg^{-1}$ ketamine intramuscularly and placed with the right chest wall over a window in the examination table.[15] The leading edge method was used to trace epi- and endocardial interfaces in all short axis left ventricular images.[16] The circumference of each contour was subdivided in 12 radii of 30 degrees increment and systolic wall thickening was calculated for each of the twelve radii. After the measurements at 4 weeks, the animals were anaesthetised and the hearts excised for ultrastructural and biochemical analysis.

In case of ventricular fibrillation, the animals were promptly defibrillated. When sinus rhythm was not restored within 1 min the animal (one in each group) was excluded from further study. Anti-arrhythmic drugs were not administered.

MORPHOLOGICAL ANALYSIS

At least four needle biopsies were taken from the subepicardial layers of the central parts of the ischaemic and non-ischaemic regions of the beating hearts. The biopsies were immediately fixed in ice cold glutaraldehyde, postfixed with osmium tetroxide and embedded in Eppon.[17] Ultrathin sections from artefact free areas were examined in a Philips EM 201 (Eindhoven, The Netherlands).

BIOCHEMICAL ANALYSIS

Transmural biopsies (5 g) were collected from the area perfused by the LCXCA (ischaemic segment) and the left anterior descending coronary artery (LADCA) (non-ischaemic segment) and homogenised in an ice-cold buffer solution. Membrane fractions, enriched in sarcolemmal vesicles, were isolated from the homogenates by differential centrifugation as previously described.[2,18] Small fractions of 100 μl of the final membrane preparations (containing about 3 mg membrane protein per ml) were rapidly frozen in liquid N_2 and stored at −80°C. Incorporation of ^{32}P was measured by incubating membrane vesicles with [^{32}P]-ATP, cyclic-AMP and exogenous cyclic AMP dependent protein kinase followed by sodium dodecylsulphate polyacrylamide gel electrophoresis of solubilised membrane proteins.[2,18] The ^{32}P-labelled material at the 9000 dalton mobility region of the vacuum dried gel (phospholamban), identified by its characteristic mobility shift after boiling membranes in sodium dodecylsulphate[18]), was cut out and counted by liquid scintillation. Protein concentrations of membrane fractions were determined by the Lowry procedure with bovine serum albumin as the standard.[19] Relative ^{32}P incorporation (%) was determined by dividing the incorporation per mg membrane protein observed in the ischaemic (LCXCA) region by that found in the non-ischaemic (LADCA) region.

STATISTICAL ANALYSIS

All values are expressed as means (SEM). Comparisons were made with a two way analysis of variance (ANOVA), and the significance of the differences was evaluated by Duncan's new multiple range test after ANOVA had revealed that the samples represented different populations. P values <0.05 were accepted as statistically significant.

Results

HEART RATE AND ARTERIAL BLOOD PRESSURE

Immediately before occlusion of the LCXCA, heart rate was 102(3) $beats \cdot min^{-1}$ in group I and 100(6) $beats \cdot min^{-1}$ in group II. Mean arterial blood pressure in these groups was 82(5) and 81(3) mm Hg respectively.

REGIONAL MYOCARDIAL FUNCTION

Acute experiments — Regional systolic wall thickening (RSWT) of the LCXCA-perfused area

decreased from pre-occlusion values of 42 (7)%, to 4 (2)% ($p<0.05$) during ischaemia in both groups and did not recover during the first 2 h of reperfusion.

Chronic experiments — Before thoracotomy RSWT varied between 37 (10) and 99 (45)% in the 12 segments. After 2 weeks, RSWT was normal in the animals in which the LCXCA had been occluded for 30 min. However, in the animals in which the vessel had been occluded for 60 min, RSWT was still absent after 4 weeks reperfusion (fig 1).

MYOCARDIAL ULTRASTRUCTURE

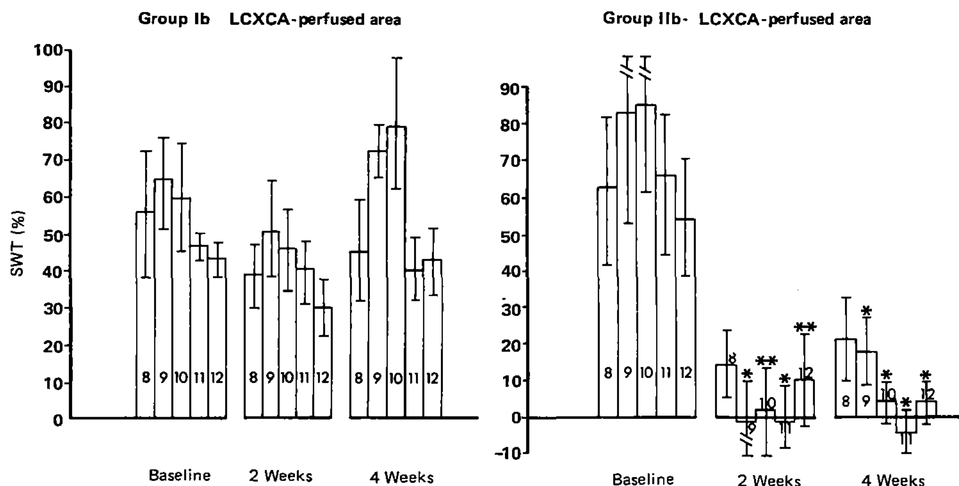
After 2 h reperfusion, mitochondrial swelling with minimal or no loss of christae was observed in the animals subjected to 30 min ischaemia. Severe loss of christae, the presence of many electron-dense bodies, clumping and margination of nuclear chromatin, and disruption of the sarcolemma indicated irreversible ultrastructural damage in the myocardium which had been ischaemic for 60 min.

After 4 weeks recovery the myocytes of the 30 min ischaemia animals appeared normal, but in the 60 min ischaemia group there was a loss of mitochondrial christae and occasional lipid droplets.

^{32}P INCORPORATION INTO PHOSPHOLAMBAN

Cyclic AMP dependent ^{32}P incorporation into phospholamban measured in membranes isolated after 2 h reperfusion of 30 min ischaemic tissue was reduced by 29% (table 1). In myocardium which had been ischaemic for 60 min this reduction was more pronounced (69%).

After the 4 week recovery period no reduction in in vitro phosphorylation in the myocardium which had been ischaemic for 30 min could be shown. However, a significant depression of 21% could still be observed in the 60 min ischaemia group.



*Systolic wall thickening (SWT) of the five radii (8-12) in the LCXCA perfused myocardium (see methods) subjected to 30 min (group Ib, n=6) or 60 min (group IIb, n=6) of ischaemia. Measurements were made before ischaemia (baseline) and after 2 and 4 weeks of reperfusion. **p<0.05 v baseline; *p<0.05 v baseline and group.*

TABLE *^{32}P incorporation into phospholamban like protein of sarcolemma. Values are mean(SEM)*

Duration of ischaemia	*Duration of reperfusion*	*n*	*^{32}p incorporation (pmol·mg^{-1} protein)*		*Ischaemic area / Control area* × *100(%)*
			Control area	*Ischaemic area*	
30 min	2 h	7	738(76)	528(95)*	71(9)
60 min	2 h	7	659(68)	212(53)*†	31(6)
30 min	4 wk	6	730(117)	768(89)	114(13)
60 min	4 wk	6	642(51)	501(43)*†	79(2)

*p<0.05 *v* control area. †p<0.05 *v* 30 min ischaemia.

Discussion

Although the 2-DE method is limited by its interobserver variability,[20] it is capable of discriminating between contractile and non-contractile myocardium. Using this method and morphological changes in combination, this study confirms earlier reports that recovery from an ischaemic insult is still possible when its duration does not exceed 30 min.[21 22] Recurrence of function in the 60 min ischaemia group after longer than 4 weeks is unlikely.[23 24]

The present data show that recovery of in vitro phosphorylation of phospholamban, which decreases during ischaemia,[1-4] is possible, but depends on the duration of ischaemia and reperfusion (table 1). After 4 weeks of reperfusion this index had returned to normal in the myocardium which had been ischaemic for 30 min. Although less so than in the early reperfusion phase, it was still depressed in the animals which had been ischaemic for 60 min. The present experimental design did not allow the measurement of ^{32}P incorporation in LADCA and LCXCA perfused myocardium before coronary artery ligation. Therefore it could be argued that the observed differences in phospholamban phosphorylation in the ischaemic myocardium were due to biological or analytical variability. However, in the control area the variability was small (table 1). Moreover, in sham operated animals we showed that the ^{32}P incorporation did not vary between several left ventricular sampling sites.[2]

In an earlier study we reported that the ^{32}P incorporation into phospholamban had decreased by 40% at the end of an ischaemic period of 60 min.[2] The 70% decrease in phosphorylation after 2 hours of reperfusion may point towards a detrimental effect during early reperfusion. Whether in vitro ^{32}P incorporation will recover completely later than 4 weeks remains unanswered. Reversing the argument, we suggest that when recovery of ^{32}P incorporation is not complete the contractile function of the myocardium remains absent.

Phosphorylated phospholamban is believed to play a role in the second messenger mediated control of sarcolemmal and sarcoplasmic reticulum Ca^{2+}-pumping ATPases.[25 26] The decreased in vitro phosphorylation of the Ca^{2+} transport regulating phosphoprotein from the ischaemic reperfused heart may therefore reflect a loss of functional integrity of cardiac membranes. We and others found a reduction in Ca^{2+} pumping ATPase activity of both sarcolemma[27 28] and sarcoplasmic reticulum membranes[4 29-31] obtained from ischaemic heart. A change of second messenger control of the Ca^{2+} pump due to modification of the properties of phospholamban may be a factor involved in the development of Ca^{2+} pump deficiency. Phospholamban in cardiac membranes may be either partially removed from the membrane or partially degraded by Ca^{2+} dependent protease during myocardial ischaemia.[1 3 26] The accumulation of amphiphilic lipid intermediates, eg long-chain acylcarnitine and free fatty acids, membrane constituents potently interacting with bilayer components, may also play a role.[26 32]

A deficient phospholamban phosphorylation may be responsible for a reduced rate of Ca^{2+} removal from the cytosol and thereby contribute to the production of cellular Ca^{2+} overload, particularly in the early reperfusion phase when Ca^{2+} influx returns to normal.[3-5 7 26 33 34] Such an hypothesis is consistent with earlier data showing that a reduced activity of the sarcoplasmatic reticulum Ca^{2+} pump coincides with Ca^{2+} overload and a lack of recovery of ischaemic reperfused porcine myocardium.[31] The present study also shows that a mild decrease (30%) in phospholamban phosphorylation is compatible with long term functional recovery.

The present preliminary data show that if coronary flow is restored 30 min after occlusion of a coronary artery, recovery of regional myocardial function (absent after 2 h) is complete after 2 weeks. Functional recovery in the 30 min ischaemia group of animals is accompanied by restoration of in vitro phospholamban phosphorylation of isolated membranes to normal, suggesting a critical role of this Ca^{2+} transport regulating phosphoprotein.

Further studies are needed to delineate the rate of recovery of in vitro phospholamban phosphorylation in relation to other functional measures. Perhaps a critical threshold of membrane function can be defined below which recovery may no longer be possible.

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Chapter 9

ENDOTHELIALIZATION OF INTRAVASCULAR STENTS

Endothelialization of Intravascular Stents

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Wide clinical application of intravascular stenting devices is currently limited by occlusion or intraluminal narrowing caused by thrombosis and neointimal thickening in a considerable percentage of implantations. We studied the possibility of seeding one of the currently availiable stents, a stainless steel, self-expandable wire-mesh, with endothelial cells in vitro. Endothelial cells, derived from human umbilical cord veins, could be successfully attached to stent filaments. In vivo stent implantations in porcine femoral arteries showed complete covering of stent wires by endothelium after 1 week. We conclude that coating of stents with autologous endothelial cells prior to implantation might protect against early thrombosis during the period in which a neointima is formed. (J Interven Cardiol 1988:1:2)

Introduction

Invasive therapy for atherosclerotic vascular disease has gradually shifted from surgery to a choice between surgery and angioplasty. Intraluminal stenting with vascular endoprostheses was already attempted in the early days of balloon angioplasty for the treatment of procedure-related complications and the prevention of restenosis.[1] Narrowing of the stented segment caused by neointimal hyperplasia limited the use of these devices even in the larger (peripheral) arteries. Changes in design, the use of other metal alloys, and miniaturization of the endoprostheses now yielded several stents for experimental and clinical evaluation.[2–6] Studies of peripheral and coronary arteries in sheep and dogs have, however, shown that thrombosis still occurs in 20%–30% of the stents, in particular during the first 14 days after implantation.[6,7] This may be related to the lack of a protective endothelial covering. Thrombosis occurs in these animals between 2 weeks and 3 months after implantation. Effective measures should be taken during the period between implantation and neointimal covering to prevent the incidence of thrombosis. In the present study we show that seeding stents with endothelial cells may be such a measure.

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Materials and Methods

In Vitro Endothelialization of Stent

Harvesting endothelium from human umbilical veins. Immediately after delivery the umbilical cord vein was cannulated and flushed with Hepes-buffered saline. Thereafter the vein was filled with medium containing 0.1% collagenase and incubated for 20 min at 37°C. Then the vein was flushed with medium and the cell-suspension collected in plastic tubes. After centrifugation for 5

min at 125 g, the cell pellet was resuspended in Medion M199 (Flow Laboratories Ltd, Irvine, Scotland) supplemented with serum and growth factors and cultured in a flask coated with fibronectine (10 μg/cm^2) for 2 days.[8,9]

Description of the Stent. The stent used (Wallstent^R, Medinvent SA, Lausanne, Switzerland) was a stainless-steel, open-weave wire-mesh (Fig. 1). The prosthesis is self-expanding and its elastic properties are such that its diameter can be substantially reduced by elongation. It can thus be constrained on a small-diameter delivery catheter, which consists of two coaxial catheters, the proximal regions of which are joined by an invaginated rolling membrane, which effectively retains the prosthesis. Withdrawal of the outer catheter rolls back the membrane progressively, thus releasing the stent, which tends to return to its orginal diameter, thereby anchoring itself against the arterial wall. The unconstrained diameter of the stent used in this study was 3.5 mm and the length 15 mm.

Coating the Stents with Endothelium. The unconstrained stents were incubated with fibronectine (100 μg/mL) for 15 min at 37°C for support of endothelial coverage. Endothelial cell suspensions (derived as above) were brought together with the stent in a small siliconized glass tube and rotated (2 rpm) for 2 hours at 37°C. Subsequently, the stent with cells were transferred to a petri dish, covered with medium and cultured overnight. For the coating of 1 cm stent (diameter 3.5 mm) about 50,000 endothelial cells were used. The cells on the stent were stained with the vital DNA-stain Hoechst 33342 (Hoechst AG, Frankfort, FRG) (10 uM; 1 hour; 37°C) or with the vital dye fluorescence diacetate (Serva, Heidelberg, FRG) (0.1 μg/mL, 10 min; 37°C). Other stents were fixed with buffered glutaraldehyde (3%) and stained

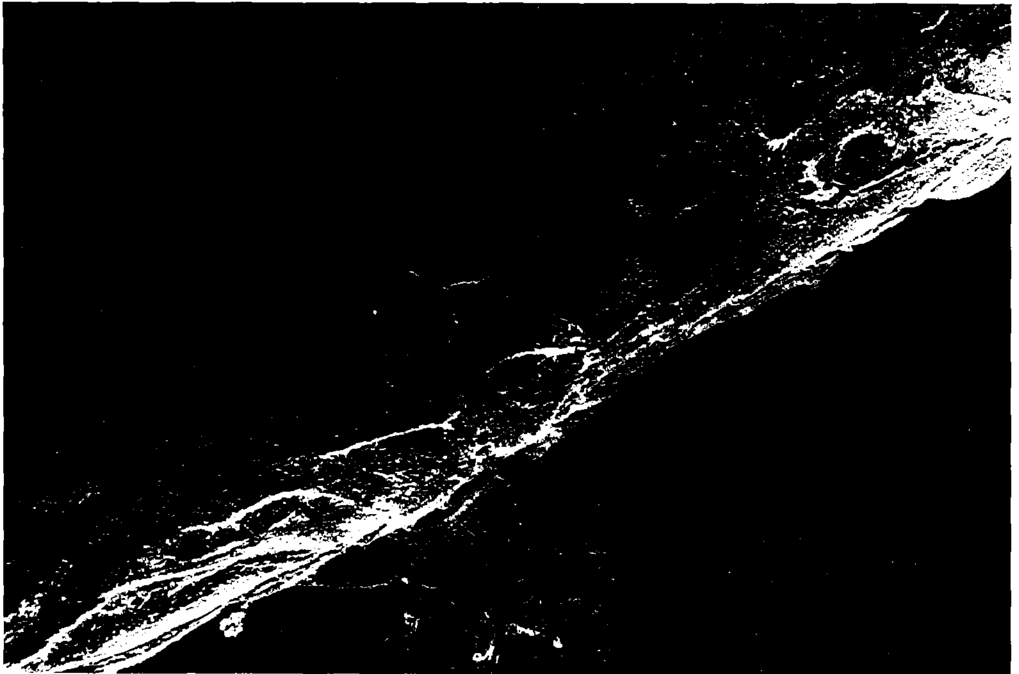

Figure 1. Scanning electron microscopic picture (magnification 550×) of stent wires seeded in vitro with cultured endothelial cells derived from umbilical cord veins. The flattened cells are in close contact.

with the nuclear stain propidium iodide (Sigma, St Louis, MO, USA) (10 μg/mL). The cell-covered stents were observed with a fluorescence microscope with epi-illumination.

Coating the Stents Mounted on the Delivery Catheter. First the fibronectine solution and then the endothelial cell suspension was drained by applying a minimal suction at the central catheter of the stent-loaded delivery system. After sealing the tip of the stent-delivery catheter by a sterile glove covered finger the suspension was advanced through the pores of the central catheter until it had replaced the air around the stent filaments. Then the mounted stent was incubated overnight. The next day the stent was released from the catheter and placed in fluorescent dye (propidium iodide, 10 μg/mL) after fixation in phosphate buffered 3% glutaraldehyde.

In Vivo Endothelialization of Stents. In 6 young Yorkshire swine (18–25 kg) a total number of 18 stents were implanted. After sedation with 500 mg of ketamine hydrochloride (Aescoket,^{R} Aesculaap BV, Boxtel, Holland) the animals were connected to a respirator for artificial ventilation with a mixture of oxygen and nitrous oxide, after endotracheal intubation. Anesthesia was maintained with 1–4 vol% enflurane (Ethrane,^{R} Abbott BV, Amstelveen, Holland) while pancuronium bromide (Pavulon,^{R} Organon, Oss, Holland) was used as a muscle relaxant.

An 8F introduction sheath was placed via the left carotid artery in the descending aorta. After

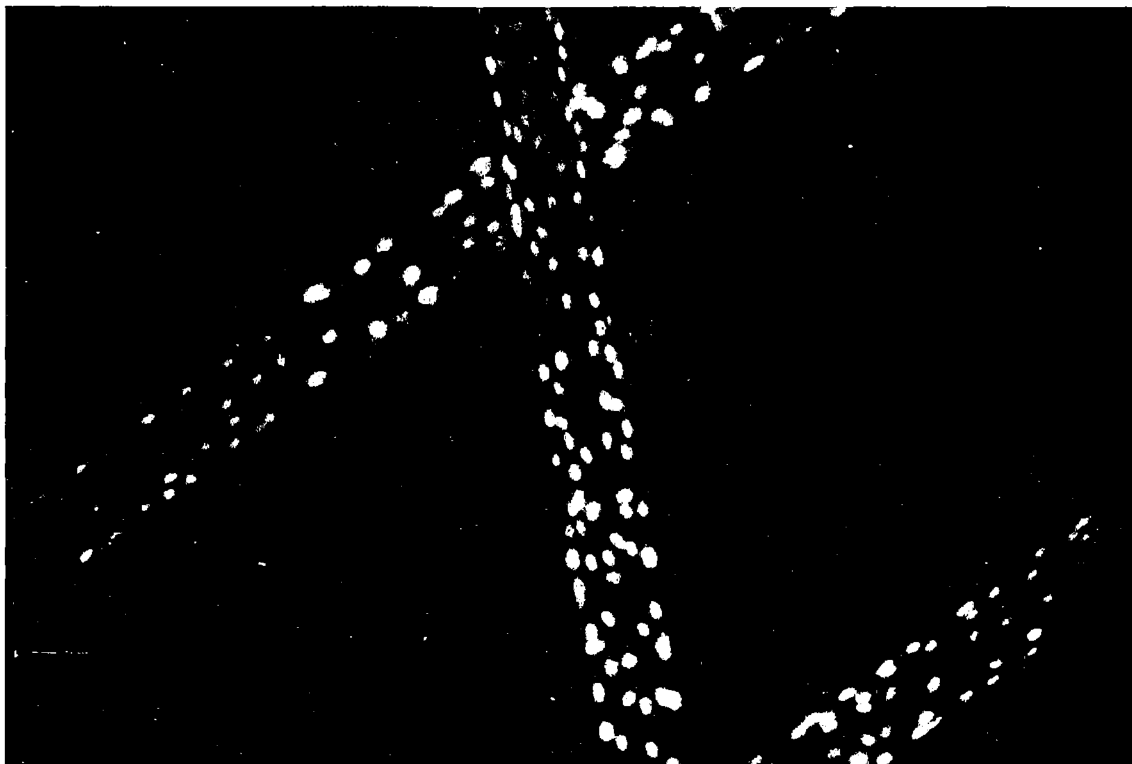

Figure 2. Fluorescence microscopy of stent wires coated with endothelial cells stained with the vital DNA stain Hoechst 33342. The nuclei of the umbilical cord vein derived cells are uniformly distributed and abundant on the metal alloy filaments (magnification 200×).

intravenous administration of 5,000 IU heparin and 100 mg aspirin (Aspégic.[R] Lorex, Weesp, Holland), an 8F guiding catheter was advanced to the aorto-iliac bifurcation.

After baseline angiography of both femoral arteries a 300 cm, 0.014 inch long guidewire was placed in the left femoral artery. Over the guidewire an angioplasty balloon catheter (balloon size 3.5 mm) was advanced. From the angiograms, and using the diameter of the guide catheter as a reference, a segment of the left femoral artery was chosen with a diameter of 3.0 mm. At that point the angioplasty balloon was inflated twice for 60 sec and 10 Atm inflation pressure. Then the angio-

Figure 3A. X-ray of the stent (arrow) unplanted in the left femoral artery.

plasty catheter was withdrawn, and angiography was repeated. The stent constrained delivery catheter, with an endoprosthesis mounted on its tip was positioned over the long guidewire at the dilated arterial segment. After the stent was released, catheters and guidewire were withdrawn to the aortic bifurcation, and repeat angiography was performed. The same procedure was followed for placement of a stent in the right (contralateral) femoral artery, except that at this site angioplasty was not performed. The catheters were removed, and the animals allowed to recover.

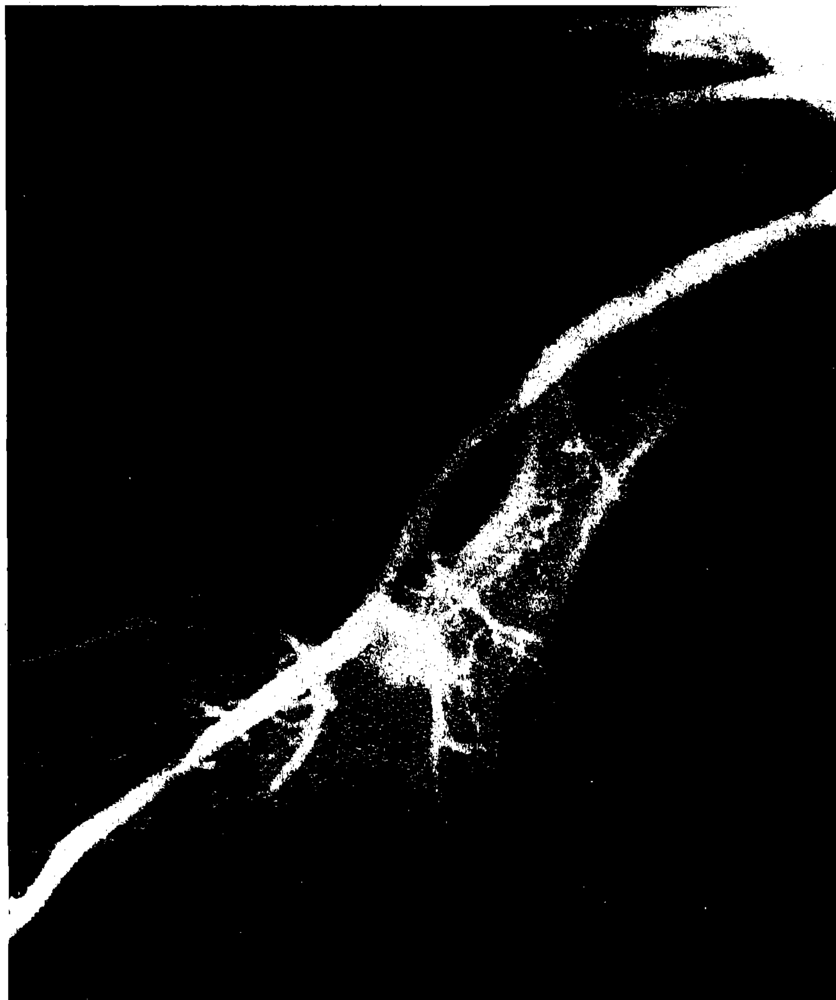

Figure 3B. Angiogram 10 days after implantation. The stent related artery is patent without signs of intraluminal defects.

Starting the day of the procedure animals 1 and 2 received calciparin 25,000 IU subcutaneously daily until acenocoumarol had prolonged the prothrombin time three-fold. Aspirin 100 ml/24 h intravenously was also added. The other animals received only 100 mg of aspirin in one daily oral dose. After 6 days (animals 1 and 2; 8 stents), 8 days (animals 3–5; 6 stents) and 10 days (animal 6; 4 stents), the animals were again anesthetized, and angiography of the stent-related arteries was performed. Immediately thereafter the stent-containing arterial segments were dissected free, perfused in vivo with saline (perfusion pressure 75 mmHg) and removed. Thereafter the animals were sacrificed with an overdose of pentobarbitone sodium. The stent-containing arterial segments were placed in buffered glutaraldehyde for subsequent electron microscopy.

Electron Microscopy. After fixation in 4% formaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for at least 48 hours, the stent containing arterial segments were washed in cacodylate buffer and divided lengthwise into two equal parts using a pair of fine scissors. One half of the stent underwent postfixation for 6 hours in 1% osmium tetroxide, and washing overnight in distilled water, and dehydration in graded ethanol and critical point drying with liquid $C0_2$. This part of each vessel was mounted and sputtercoated with gold before examination in a scanning electron microscope (Cambridge 180 steroscan, Cambridge, U.K.).

From the other half of each vessel the stent wires were removed and the tissue was prepared for light microscopy.

Hematologic Measurements. Before administration of the anticoagulants, at the end of the implantation procedure and again after 2 and 6 days of recovery, 10 mL arterial blood was collected in a syringe containing sodium citrate (final concen-

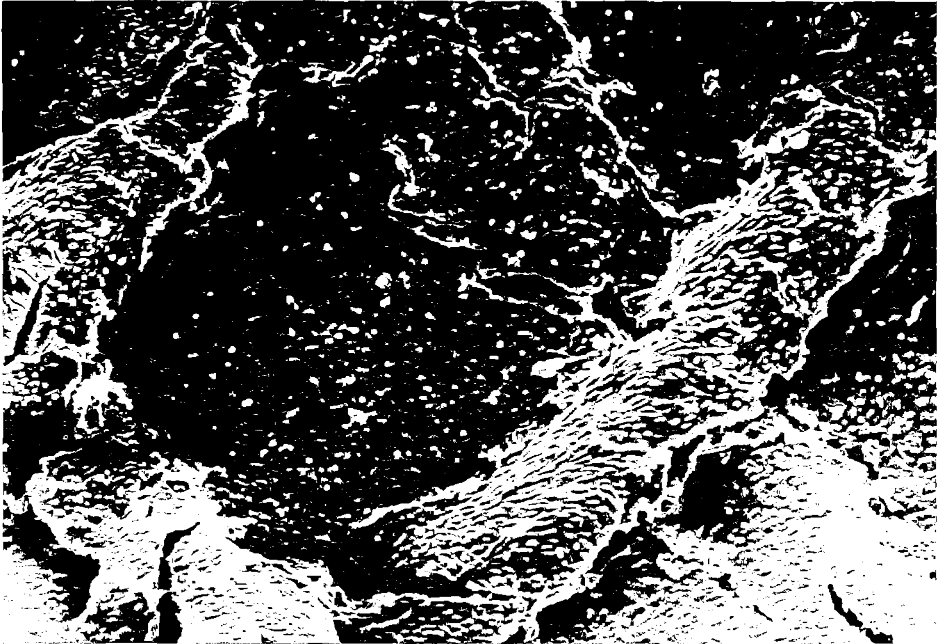

Figure 4. Scanning electron micrograph (magnification 1,250×) of porcine femoral arterial segment 5 days after stent implantation. The wires are covered by a neointima with spindle shaped endothelial cells on the surface. Fissures in the endothelial layer are caused by this fixation procedure.

tration 11 mM) for determination of the platelet count, recalcification time, and prothrombin time.

Results

In Vitro Endothelialization. Harvesting endothelium from human umbilical cord veins yielded approximately 2.10^5 cells per vein (10–15 cm). After culturing these cells for 4 days about 10^6 cells were available for seeding the stent. Incubation of these cells with the isolated stent for 2 hours and subsequent culturing resulted in an almost complete blanketing of the inner surface of the stent wires by flattened endothelial cells (Fig. 1).

Fluorescence staining of endothelial nuclei with Hoechst 33342 showed a uniform distribution of the cells on the stent wires (Fig. 2). Incubation of the stent mounted on the delivery catheter with isolated endothelium for 2 hours resulted in a similar endothelial lining. However, the inner surface proved to be covered for only approximately 50% after the stent had been released.

In Vivo Endothelialization in Porcine Femoral Arteries. All implanted stents remained patent during the period after implantation (6–10 days). Angiograms obtained at the end of the observation period showed no signs of stent-displacement, while stent-related intraluminal defects (considered to be caused by thrombus) could not be demonstrated in any of the implants (Fig. 3). Scanning electron microscopy showed that the stents were completely covered by an endothelial lining as early as 6 days after implantation (Fig. 4). No deposition of platelet aggregates or thrombus material was observed on the neointimal lining. Trans-

Figure 5A. Light microscopy of stented porcine femoral artery 3 days after implantation (magnification 190 ×). The empty spaces correspond with the stent wires. At the site of the stent filaments the media is considerably compressed, while the internal elastic lamina is interrupted.

mission microscopy showed that the stent filaments compressed the arterial media (Fig. 5A), with disruption of the internal elastic lamina (Figs.5A and 5B). A neointima of varying thickness covered the stent wires. While normal femoral arteries showed a thin intimal layer (5 μm), the median thickness of the stent covering neointima was 80 μm (range 60–125 μm). The neointima was covered with endothelial cells (Fig. 5C). In the neointima directly underneath the endothelium a

Figure 5B. (detail of 5A, magnification 510×) In the neointima myofibrillar cells and macrophages are abundant. In the vicinity of this stent filament many trapped erythrocytes can be seen.

Figure 5C. (detail of 5A, magnification 510×) The neointima is covered by a continuous layer of endothelial cells. Between the endothelium and the stent many fibroblast-like cells can be seen, together with trapped erythrocytes and fibrous material.

mixture of fibers, trapped red blood cells and macrophage-type cells were present (Figs. 5B and 5C). Fibrous material, probably consisting of fibrin, occupied small areas between the stent filament and new intima. The presence of this fibrous material and the entrapped cells suggests that some deposition of thrombotic material might have occurred before the development of the neointima was completed.

No differences in ultrastructural appearance could be demonstrated between arterial segments that had been subjected to balloon angioplasty and those segments which were not dilated.

Hematological Data. The results of the hematological measurements are summarized in Table 1. During recovery prothrombin time and recalcification time were significantly prolonged in the animals receiving extensive anticoagulant therapy. In the animals receiving only aspirin, these values were normal again 2 days after implantation.

Dissussion

The application of percutaneous transluminal coronary angioplasty (PTCA) for the treatment of coronary artery disease has increased considerably in recent years. The initial success rate is high,[10,11] with only a small incidence of acute or subacute occlusion at the site of angioplasty.[12,13] However, restenosis several months after the procedure remains an unsolved problem with an incidence of 20%–40%.[14–16] The effect of pharmacological therapy on acute complications[17–20] and late restenosis[20] is still unclear. The implantation of vascular endoprostheses after angioplasty to prevent these

Table 1. Hematological Parameters of Pigs with Stents Implanted in the Femoral Arteries

		Prothrombin Time (s)	Recalcification Time (min)	Platelet Count (mm-3)
Baseline	group 1	25 ± 3	2.5 ± 0.3	255 ± 32
	group 2	25 ± 1	2.3 ± 0.3	305 ± 44
End implantation	group 1	>180	>15	297 ± 51
	group 2	30 ± 2	>15	266 ±43
Day 2	group 1	>180	>15	261 ± 7
	group 2	25 ± 1	1.5 ± 0.3	314 ± 52
Day 6	group 1	>180	>15	288 ± 28
	group 2	24 ± 1	1.6 ± 0.3	286 ± 21

Group 1 (animals 1 and 2) received the extensive anticoagulant therapy; group 2 (animals 3–6) received only aspirin after implantation. All data have been presented as mean ± SEM.

sequelae in patients with coronary atherosclerosis has proven feasible.[21] Whether this technique actually reduces the incidence of restenosis is as yet unknown. Observations in a small number of patients show that the implantation of an endoprosthesis after balloon angioplasty causes an immediate slight further dilatation of the arterial segment.[22] However, after 3 months a small but significant diffuse narrowing of the stented artery was observed.[23]

Implantations of such endoprostheses in peripheral and coronary arteries in sheep and dogs showed early thrombotic complications (< 14 days) in 20%–30%.[6,7] This percentage can probably be reduced by avoiding some "thrombogenic" implantation parameters such as diameter mismatch between stent and receiving artery, stent margin at side-branch, low flow through the stented vessel and administration of low doses of aspirin (5 mg/kg) after implantation as in the present study. In vascular surgery comparable problems are encountered. Proposed solutions by this discipline comprise the design of nonthrombogenic vascular grafts, or seeding the graft surface with autologous endothelial cells. Cell-seeding might be the better solution, as this provides an active antithrombotic graft surface.[24] Metal-alloy vascular endoprostheses currently available share the advantage, contrary to polymer surface vascular grafts, of macroporosity, covering up to 20% of the vascular surface area. This will allow earlier covering of the stent filaments with native endothelium. Endothelial covering was reported after 10 days to 8 weeks dependent upon the species, type of stent, size of receiving vessel and whether or not atherosclerotic lesions were present.[3-6,25-27] In the present study it was shown that stents placed in porcine femoral arteries were already covered with neointima after 6 days. This faster rate of neointimal generation may be related to age and species of the animals.[28] Prior balloon angioplasty, which has been shown to cause effective endothelial denudation in the same species,[29] did not seem to delay this process. The thickness of the neointima in the present study (80 μm) is comparable to data reported by others in the same species 8 weeks after implantation.[25] Data reported for dog implantations seem more variable. After 1 to 3 weeks mean neointimal thicknesses of 80 to 300 μm have been reported.[6,26,27] After 6–12 months these values were 270–450 μm.[7,27] Although one should be cautious in comparing results obtained in different species with different types of stents, and while most authors do not specify their measurements, the conclusion seems justified that neointimal thickening progresses gradually.

In man endothelial covering of vascular prothesis occurs much slower than in the pig.[28] Accordingly the prosthesis is subjected to prothrombotic forces for a longer duration. This might imply the need for chronic antithrombotic treatment or other alternatives. Cell-seeding the stents might be that alternative. In the present study one of the clinically most relevant stents can be covered by endothelial cells in vitro, after relatively simple and nontime consuming measures. Scanning electron microscopy shows that the endothelial cells are flattened with close cell-to-cell contact.

Whether these cells remain attached to the stent after intra-arterial placement depends upon the shear resistance, which has to be studied in further experiments. Furthermore it has to be established whether the cells remain viable and metabolically active, considering the dependence of the cells on endothelial cell growth factor. This dependence might be related to cell density in culture, but more data are needed to establish this. The use of autologous endothelial cell suspensions harvested from subcutaneous fat biopsies, avoids the need for tissue typing and the risk of cell rejection.

In conclusion, our study showed an early time-span wherein endovascular stents were covered by a neointima. Present data suggest that stents are at risk for partial or complete thrombotic occlusion during at least the first 6 days after implantation. Cell-seeding of the stent filaments, to obtain an active antithrombotic stent surface, seems an attractive method to reduce this risk. In vitro experiments demonstrated that stents can be covered by a substantial number of endothelial cells within hours. These data strongly suggest further in vivo experiments to investigate whether this approach may contribute to the solution of a major problem in current cardiologic interventions.

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Chapter 10

GENERAL DISCUSSION

10.0 GENERAL DISCUSSION

10.1 Coronary occlusion

Currently there is little doubt that the common final pathway of coronary occlusion is thrombus formation. Clinical evidence for this phenomenon has been obtained in thousands of patients responding favorably to thrombolytic therapy. Yet, prevention of thrombotic occlusion of narrowed coronary arteries in patients is generally limited by two factors. Firstly, most patients eventually suffering from acute coronary occlusion (myocardial infarction) had previously no symptoms directing towards an imminent coronary event. Secondly, the mechanisms leading to the final occluding thrombus are poorly understood at best, and several factors seem to be involved.[121] Therefore, a single intervention or drug therapy in those patients who present themselves with symptoms of an impending infarction is likely to fail. Several leaders in the field have therefore proposed a "blunderbuss"-approach towards unstable angina and impending myocardial infarction. This approach exemplifies our incomplete understanding of the predominant mechanisms playing a role in the individual patient and underscores the complex pathophysiology of acute coronary occlusion.
Experimental models have been employed to gain more insight, and attack the problem with a more rational (but necessarily more simplistic) attitude. The models used differ in the initiation of the thrombotic event and the composition of the occluding thrombus achieved. For example, those models employing metal coils in the coronary arterial tree are characterized by a reproducible, but short time until complete occlusion, while the thus achieved thrombus will be rich in fibrin.[109,122,123] Alternatively, those models using a critical coronary stenosis with superimposition of a subsequent insult to the endothelium are characterized by long periods of intermittent reductions in coronary blood flow, and by thrombi rich in platelets.[124,125] Most experimental studies are carried out using the latter type of model, and it is therefore not surprising that several antiplatelet drugs have been proposed to be beneficial for the prevention of thrombotic coronary occlusion.[126-130] Also in the model described in this thesis a coronary occlusion is caused by a thrombus containing a significant amount of platelets. This explains the efficacy of antiplatelet drugs to prevent or delay occlusion (chapters 4 and 5). However, in both interventional studies described, the response was not optimal, perhaps pointing out that this model may be representative of the "kaleidoscopic" clinical situation. Accepting the histological data that the coronary thrombus achieved in this model is of the mixed type, i.e. a significant amount of platelets and fibrin is present, may underscore the value of this model for testing proposed antithrombotic drugs. Future diagnostic techniques in patients, which allow for the characterization of the composition

of the occlusive thrombus and its initiating mechanisms, have to be awaited before an individualized recanalization strategy can be implemented in clinical practice.

10.2 Myocardial reperfusion

Controlled, randomized, clinical studies have taught us that the maximal benefit from coronary recanalization in the setting of acute myocardial infarction is dependent on initiating reperfusion as soon as possible.[65,68,69] In limited numbers of patients presenting with a complete obstruction of a coronary artery, where thrombolytic therapy could be instituted very early by the intracoronary administration of streptokinase the actual development of myocardial necrosis was prevented.[131] However, variability exists between individual patients in the onset of irreversible myocardial damage. This is usually attributed to differences in the extent of collateral vessels and the hemodynamic responses to coronary occlusion. The experimental data pointing towards an increased incidence of life-threatening arrhythmias after early reperfusion, seem not to be representative of clinical practice, where these arrhythmias are rare. Maybe the occurrence of earlier ischemic periods, or the presence of an adequate collateral circulation plays a role in this respect. Notwithstanding such discrepancies between clinical and experimental data, there may still be a role for experimental myocardial reperfusion studies. First of all effective regimens will have to be developed which accelerate the thrombolytic process. Thus far, all pharmacologic attempts to achieve this goal once an occluding thrombus has been formed were unsuccessful.[132-134] Probably, the most promising approach lies in the pretreatment with agents antagonizing the effects of thrombin, and preventing the several steps in the cross-linking of fibrin.[135] The administration of higher doses of fibrinolytic agents may be effective, but probably at the expense of more bleeding complications.
Another approach may be the employment of drugs, which limit irreversible reperfusion injury but do not enhance myocardial reperfusion,[136] or drugs that enhance the contractile recovery of reperfused myocardium (chapter 7). This latter effect might be achieved at the cost of an increased incidence of reperfusion arrhythmias, but this observation has to be substantiated in the clinical setting. However, all these considerations open new perspectives in the development of effective drugs for human use.

10.3 Coronary artery reocclusion and restenosis

(Repeated) reocclusion after successful thrombolysis limits its beneficial effects on myocardial cell survival. In 50% of a selected group of patients with acute myocardial infarction the addition of intracoronary bolus doses of isosorbide dinitrate to the intracoronary infusion of streptokinase was able to reestablish

patency of the coronary artery within one to two minutes after angiographically documented reocclusion.[80] It has been also shown that the addition of sufficient doses of heparin to the thrombolytic agent rt-PA increases coronary patency rates.[137] The use of the calcium-antagonist nifedipine may be effective as well, as this agent improves reperfusion after experimental thrombolysis (chapter 6). However, several other approaches may be useful, as in experimental studies also thromboxane antagonists as well as specific inhibitors of platelet receptors have proved beneficial in limiting the decrease in myocardial blood flow after successful thrombolysis.[132,138,139] From clinical and experimental studies several mechanisms have been proposed which likely optimize and maintain the extent of reperfusion after coronary recanalization by thrombolysis. Acutely, endothelium-independent vasodilators, like nitrates or calcium antagonists may oppose the vasoconstrictive factors originating from the occlusive thrombus. If lysis has established a sufficient rate of antegrade flow, antagonists of these vasoactive substances like thromboxane receptor blockers or serotonin antagonists may become effective. Alternatively, several agents have become available now, which are directed against the prothrombotic forces still operative during thrombolysis. Examples of the latter group of drugs are thromboxane synthetase inhibitors, specific thrombin antagonists or monoclonal antibodies against platelet receptors.[140,141] Several of these agents are now undergoing clinical investigation, and results are awaited within the next years. Animal experiments may be useful in studying possible synergistic drug combinations in models of arterial thrombosis.[142]

Restenosis remains a clinical (and economic) limitation of PTCA since this technique was first introduced more than 10 years ago.[143] The main factors promoting restenosis after successful angioplasty are the extent of vascular damage, platelet accumulation at the site of the lesion, and the activation of smooth muscle cells.[144] Recently developed pharmacologic tools are evaluated in experimental models (e.g. recombinant hirudin, 7E3).[145] Next to these essentially antithrombotic drugs, agents inhibiting the proliferation of smooth-muscle cells, which have shown experimental benefit such as the ACE-inhibitor cilazapril,[146] are studied in several major multicenter clinical trials of restenosis. As growth factor research is advancing rapidly, specific inhibitors or antibodies to , for instance, platelet derived growth factor, a substance promoting migration as well as proliferation, and specifically enhanced in human atherosclerotic plaque, are expected to become available in the near future.[147,149]

The application of cytostatic agents in the prevention of restenosis has found only limited experimental application (D. Holmes, personal communication), due to expected side-effects. However, the introduction of microporous balloon catheters, able to infuse drugs locally into the arterial wall, may revive the

investigation of specific anti-tumor agents.[150,151]

An alternative approach in the battle against restenosis is the utilization of several mechanical devices.[152] In general these devices are aimed to either debulk the coronary lesion (directional atherectomy, rotational atherectomy, laser) or splint the stenosed artery, thereby spreading the plaque over and into the vascular wall (stents). We have studied several stent devices experimentally, with variable results.[105] Currently available stents are constructed of metal wires, which by itself serve as a thrombogenic surface. Furthermore, limited clinical experience has revealed several cases of restenosis within the stented arterial segment.

Current understanding is that the next generation of coronary stents will have to be a combination of a biodegradable device with either drug eluting properties or a device seeded with normal or by DNA-technology transformed endothelial cells.[153] We showed (chapter 9) that cell-seeding onto metal stents is possible. Others have extended this observation using genetically engineered endothelial cells, capable of enhanced t-PA production.[154] The next years will learn which approach, drug eluting biodegradable stents or genetically engineered cell-seeded stents will prove to be most effective. The current status of polymer-chemistry even makes a synthesis of both approaches feasible.[155,156] The production of block-copolymers with variable rates of biodegradation, and containing different antithrombotic or antiproliferative drugs, each with distinct periods of diffusion into the coronary segment after stenting is already possible.

The current explosion of new pharmacologic agents and mechanical devices, with ongoing basic research in DNA-technology, signal transduction of growth factors and local delivery systems may even exceed our imagination by producing an effective solution. It will, however, call for a concerted research effort between basic scientists, clinical cardiologists and the industry in the evaluation of the optimal approach in each of the acute coronary syndromes, for the ultimate benefit of large groups of patients.

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SUMMARY

Unstable angina pectoris, acute myocardial infarction, sudden coronary death, but also the application of coronary angioplasty, are all characterized by acute coronary arterial damage, activation of platelets and the coagulation cascade, liberation of vasoactive substances and growth factors. During the last decade, the increased application of thrombolytic therapy and percutaneous coronary angioplasty have provided us with better clinical results, but also with new problems. The prevention of thrombotic occlusion, the efficacy of arterial recanalization, as well as the prevention of early reocclusion and later restenosis are all subject to increased research efforts.

In the present thesis a model of thrombotic coronary occlusion has been described. In this model it was demonstrated that two experimental drugs, the stable prostacyclin analogue iloprost and the thromboxane receptor blocker solutroban, may be useful for clinical testing to prevent this potentially life-threatening event. Using the same animal model for coronary thrombosis, it was shown that the addition of the calcium antagonist nifedipine to a thrombolytic agent, improves myocardial perfusion in the acute phase of thrombolytic coronary recanalization.

Employing a porcine model of coronary ligation it was shown that iloprost, administered at the time of reperfusion, enhances left ventricular performance after flow had been reestablished. This finding may have clinical relevance in those patients with large infarcts and severely compromised left ventricular systolic function. In a next chapter we demonstrated that the in vitro phosphorylation of an essential protein in the excitation-contraction coupling in myocardial cells, the second messenger phospholamban, may be a marker of functional recovery of ischemic myocardium. This finding, if confirmed, may be a first step in the development of a clinical laboratory test, which may help the clinician to decide whether recanalization of the occluded coronary artery is still of value.

Finally, the last experimental study in this thesis describes the improvement of the surface characteristics of a coronary endoprosthesis. This device is currently under investigation in the treatment of acute reocclusion and the prevention of late restenosis after coronary angioplasty. As these endoprostheses proved to be thrombogenic, and normally functioning endothelium is important in the prevention of local thrombosis as well as smooth muscle cell proliferation, we studied the interaction between these metal devices and the native and seeded endothelial cells. Covering the stents with healthy endothelium proved to be possible. This means a distinct, albeit modest step in the prevention of acute occlusion and restenosis after recanalization of coronary arteries by angioplasty.

SAMENVATTING

Het bijna of geheel afgesloten raken van een kransslagader, vaak leidend tot een hartinfarct of plotse hartdood, maar ook het therapeutisch verwijden van een vernauwde kransslagader door middel van een balloncatheter (PTCA), gaan gepaard met een ernstige beschadiging van de wand van dit bloedvat. Dit resulteert in de aktivatie van bloedplaatjes en bloedstolling, en de vrijmaking van vaatvernauwende en groei stimulerende factoren. De laatste tien jaar hebben bloedstolseloplossende geneesmiddelen (thrombolytica) en kransvatverwijding door middel van PTCA, ons de mogelijkheid gegeven kransvatafsluiting beter te behandelen. Beide behandelingen hebben echter ook nieuwe problemen aan het licht gebracht. Het voorkomen van kransvatafsluiting, de effectiviteit van het heropenen van het bloedvat, alsmede het tegengaan van vroege en late herafsluiting worden derhalve momenteel intensief onderzocht.

Dit proefschrift beschrijft een diermodel voor kransvatafsluiting door een bloedstolsel. In dit model bleken twee experimentele geneesmiddelen, te weten het stabiele analogon voor prostacycline iloprost en de thromboxane receptor blokkerende stof solutroban, effectief om zo'n potentiëel levensbedreigende afsluiting te voorkomen. Experimenten in hetzelfde model resulteerden in de bevinding dat dat toevoeging van het calcium instroom blokkerende geneesmiddel nifedipine aan een stolseloplossend middel, de bloedvoorziening van de hartspier direct na het weer open maken van het afgesloten kransvat verbetert.

In een ander proefdiermodel werd aangetoond dat de toediening van iloprost juist voordat het kransvat weer geopend werd, een sneller herstel van de functie van de linker hartkamer tot gevolg heeft. Deze bevinding kan in de praktijk van belang zijn voor die patiënten met een beperkte hartfunctie.

In hoofdstuk 8 wordt een studie beschreven naar de levensvatbaarheid van het hartspierweefsel na een kransvatafsluiting. Er werd gevonden dat de aktivatie in een reageerbuis van het hartspiereiwit phospholamban, dat betrokken is bij de aanzetting van de spiercel tot samentrekking, geassocieerd is met het herstel van spierfunctie na heropenen van het kransvat. Deze bevinding is mogelijk een eerste stap tot het ontwikkelen van een laboratoriumtest, die aan kan geven of heropenen van het afgesloten kransvat nog wel zinvol is.

Tenslotte wordt in hoofdstuk 9 van dit proefschrift een onderzoek beschreven waarin gepoogd werd om de oppervlakte-eigenschappen van een intracoronaire veerspiraal te verbeteren. Deze zogenaamde "stent" wordt momenteel bij patiënten onderzocht ter

behandeling van plotse afsluiting of ter voorkoming van latere vernauwing na ballonverwijding. Voorlopige gegevens wijzen echter op het feit dat op de draden van deze veerspiralen gemakkelijk bloedstolsels ontstaan. In een gezond bloedvat zorgen de cellen die de vaatwand bekleden voor een natuurlijke afweer tegen de vorming van bloedstolsels. Het bleek mogelijk om in het laboratorium dit type cellen op de draden van zo'n veerspiraal te hechten. Dit laatste betekent mogelijk een stap verder op weg naar het voorkomen van plotse afsluiting of latere vernauwing na het openen van kransslagaders door middel van ballonverwijding.

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LIST OF PUBLICATIONS

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