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Eingereicht über das
Institut für Veterinär-Anatomie, -Histologie und -Embryologie
der Justus-Liebig-Universität Gießen

Importance of $\alpha_v\beta_3$ integrin in arteriogenesis in the peripheral circulation of the rabbit

Inaugural-Dissertation
zur Erlangung des Doktorgrades
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

Eingereicht von
Kerstin Broich

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Tierärztin aus Rheinhausen

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Dekan: Prof. Dr. Dr. h.c. B. Hoffmann

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to everybody who loves me

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I. INTRODUCTION

1. Background of the study

In spite of increasing prevention efforts, cardiovascular diseases (CVD) remain the most common cause of death and major morbidity in the world. In 2001 CVD contributed to nearly one third of global deaths [1]. In Europe, about half of all deaths caused by CVD were derived from coronary heart disease (CHD) [1]. In Germany, 92,775 patients died of chronic ischemic heart disease and 65,228 patients died of acute myocardial infarction in 2001, which together represent 19.1 % of all deaths in that year [2]. In the developing countries, economic transition, urbanization, industrialization and globalization lead to lifestyle changes that promote heart disease [1]. The aging population in the industrialized countries and the increased survival of patients with acute coronary syndromes achieved by medical or revascularization treatments are leading to increasing numbers of patients with chronic arterial disease [3].

Coronary heart disease is characterized by myocardial ischemia as a consequence of coronary stenosis or occlusion, which is usually caused by atherosclerosis. CHD mainly becomes manifest in angina pectoris and myocardial infarction [4].

In the peripheral circulatory system, vascular disease (PVD) often manifests with the symptom of intermittent claudication, when the limited arterial supply of the lower limb cannot meet the dynamic metabolic demand of the muscles. PVD is associated with limitation of walking ability and potentially leads to severe necrosis and the need of amputation of the leg [5]. In addition, patients suffering from PVD are at higher risk of death from heart or brain infarction [1].

Current treatments for occlusive arterial disease include revascularization techniques such as percutaneous transluminal angioplasty (PTCA) and bypass surgery. Even though these techniques have been highly refined over the past decade [6], a large number of patients are not eligible for any of these therapies because of severe global coronary and peripheral disease or because it is unaffordable for them. Furthermore, surgery implicates high neurological risks for those patients, who need cardiopulmonary bypass for it [7], and the success of surgery is limited by the frequent development of restenoses [8,9].

It has been known for a long time that patients with ischemic vascular diseases can develop natural bypasses [10,11]. These bypasses arise from preexisting interconnecting arteriolar vessels called collateral arteries, which grow in response to shear stress after stenosis or occlusion of a major artery and develop

to functional, larger arteries [10,12]. This process of transformation of a small arteriole into a large conductance artery is termed arteriogenesis [13,14]. Collateral vessels are found not only in the heart but in the peripheral vasculature of patients with PVD as well [9,15]. The extent of angiographically visible collaterals seems to be related to the severity of arteriolar narrowing [11]. These growing collateral arteries can prevent death and limit the damage to the heart muscle in case of coronary occlusion. Thus, scientists are currently trying to understand the mechanisms that control collateral growth in order to develop alternative therapies, e.g. gene therapy, which increase arteriogenesis and therefore could minimize the consequences of arterial occlusion.

In the context of proliferative vascular diseases and angiogenesis, integrins and especially $\alpha_v\beta_3$ integrin have been shown to play an important role. Integrins are a family of cell surface glycoprotein receptors that mediate cell-to-cell and cell-to-extracellular matrix (ECM) interactions [16]. The $\alpha_v\beta_3$ integrin is expressed by several, especially vascular cells including endothelial cells (ECs) and smooth muscle cells (SMCs), and serves important cellular and vascular functions [17,18]. It plays a role in capillary growth or angiogenesis [19] and is used by ECs and SMCs for migration and formation of a neointimal layer, which is a characteristic process frequently observed in proliferative vascular diseases such as restenosis and atherosclerosis [20-24]. The main ligand of $\alpha_v\beta_3$ integrin is vitronectin, which is known to promote adhesion, spreading, and migration of vascular cells through $\alpha_v\beta_3$ integrin binding [25,26].

Over the last years, scientists have developed new techniques directed at the reduction of intimal thickening and the increase of lumen size of diseased vessels using $\alpha_v\beta_3$ integrin antagonists or blockers [27-36], and integrins have attracted attention as targets for anti-angiogenic therapy. However, no efforts have been made in the other direction: to increase growth by stimulating $\alpha_v\beta_3$ integrin.

Even though $\alpha_v\beta_3$ integrin has been well investigated in the area of angiogenesis, only very few studies have been conducted in the scenario of arteriogenesis [37,38].

2. Objectives of the study

The objective of this study was to investigate the role of $\alpha_v\beta_3$ integrin in arteriogenesis. For this purpose, the expression of $\alpha_v\beta_3$ integrin in collateral arteries of rabbits subjected to femoral artery occlusion or sham operation as well as the location and expression of its main ligand, vitronectin, was studied by means of immunohistochemistry.

II. REVIEW OF LITERATURE

1. Mechanisms of vascular growth

1.1 Vasculogenesis

The term vasculogenesis refers to the earliest form of vascular development, the formation of blood vessels during embryogenesis. It defines the coalescence, differentiation, and expansion of vascular endothelial cell precursors into the initial vascular network [39]. Under influence of different fibroblast growth factors (FGFs), the paraxial and lateral plate mesoderm forms bipotential precursor cells, so-called hemangioblasts [40,41]. These cells aggregate into clusters called blood islands. The inner cells of the blood islands differentiate into hematopoietic precursors while the outer cells flatten and develop to primitive endothelial cells (ECs) [40,42]. By coalescence, these ECs form a vascular network that consists of primitive endothelial tubes devoid of mural cells [40,43]. The primitive vascular plexus is further expanded into a complex, organized, and interconnecting network by two different angiogenic processes (see next chapter). [41,44,45].

At the same time, aggregating angioblastic precursors form solid endothelial strands, which later differentiate into the dorsal aorta, the cardinal veins and endocardial tubes. Furthermore, they develop the stems of the yolk sac arteries and veins [40,43,46,47].

When the heart starts beating, the blood cells derived from the blood islands enter the circulation. They only represent a transient population of blood cells because they are gradually replaced by definite blood cells [39,40].

However, vasculogenesis might not be restricted to embryogenesis. It has been shown that circulating endothelial progenitor cells can possibly induce vascular growth after birth [48].

1.2 Angiogenesis

The further expansion of the vascular system is called angiogenesis. This term refers to the development and growth of new capillary blood vessels from preexisting vessels [41,49]. It was first introduced by Hertig in 1935 to describe the growth of new blood vessels in the placenta [50]. Later, in 1971, it was used by Folkman to describe neovascularization in the growth of solid tumors [51].

There are two different kinds of angiogenic processes: first, new capillaries can form by sprouting from preexisting ones; secondly, non-sprouting angiogenesis, or intussuception, which is the splitting of preexisting vessels by formation of transcapillary pillars and invagination of pericytes or SMCs, or by posts of ECM [41]. The latter form was first described for the embryonic lung [52].

When an organ starts to grow, the type of angiogenesis probably depends on the number of vessels present in that particular organ or tissue [41]. In some organs, such as the yolk sac, both types can occur at the same time [40].

In the adult organism, angiogenesis is a component of several physiological conditions such as wound healing, ovulation, and pregnancy [53,54]. Although it is a tightly regulated process, angiogenesis can represent a significant factor in pathological conditions. Tumor growth and metastasis require large amounts of blood supply, which makes angiogenesis an important part of these processes. Furthermore, it plays an important role in inflammatory diseases such as rheumatic arthritis, retinopathies and atherosclerotic plaques [53].

The main stimulus for angiogenesis is hypoxia, which leads to enhanced expression and activation of the transcription factor hypoxia inducible factor-1 (HIF-1) [55-57]. HIF-1 in turn causes an increase in the transcription of several genes including vascular endothelial growth factor (VEGF) [56-58]. VEGF is a potent and specific angiogenic factor, which in addition functions as a vascular permeability factor and a cytokine that attracts monocytes [40,44,59,60].

In ischemic events, angiogenesis is induced and the tissue ischemia leads to the release of growth factors and inflammatory mediators that as a first response to tissue injury cause vasodilatation and increased vascular permeability [41,45,49]. Monocytes and macrophages accumulate and secrete growth factors and inflammatory mediators [61-63]. Then the matrix is dissolved by metalloproteinases released by inflammatory cells. Tissue hypoxia also sensitizes local endothelial cells to the effect of various growth factors by upregulation of receptors. The ECs form new vessels by detaching, migrating and proliferating [61,64]. Molecules that are involved in cell-cell or cell-matrix interactions, such as $\alpha_v\beta_3$ integrin, mediate endothelial cell spreading. This explains why $\alpha_v\beta_3$ integrin antagonists are able to inhibit angiogenesis [49].

1.3 Arteriogenesis

The third kind of vascular growth is arteriogenesis, which refers to the growth of preexisting collateral vessels into functional arteries [13]. Such collaterals are preexisting end-to-end anastomoses, which connect terminal branches of major arteries [11]. In case of chronic or acute occlusion of a major artery, these collaterals are recruited as natural bypasses to the occlusion site [15,49]. Figure 1 shows two angiograms of rabbit hind limbs before and after occlusion of the femoral artery as an example for arteriogenesis. This arteriogenesis is not a passive process of vasodilation as a reaction to the redistribution of blood flow, but it is a process of active proliferation and remodeling of the arteriolar vessel wall [15,65,66].

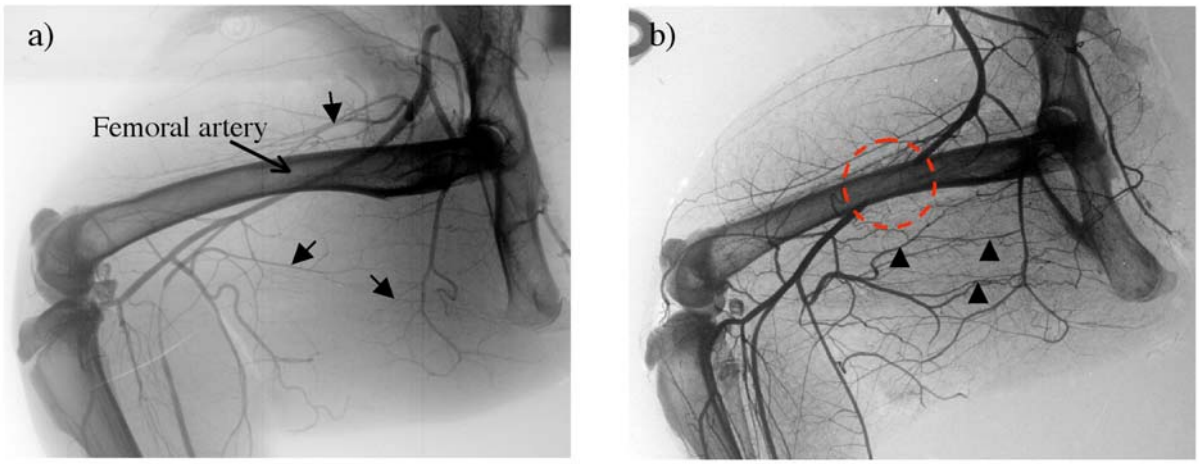


Fig. 1: Post-mortem angiograms of rabbit hind limbs a) before and b) 7 days after femoral artery occlusion.
Circle: occluded segment of the femoral artery
Arrows: collaterals before occlusion
Arrowheads: growing collaterals

1.3.1 Prevalence and morphology of collateral arteries

In 1669 the English anatomist Richard Lower was the first to describe the presence of collateral connections between the coronary arteries of the heart. He found that fluid injected in one of them also spreads into the other. Since he concluded that "there is a need of vital heat and nourishment, so deficiency of these is fully guarded against by such anastomoses", he not only precisely described the collateral vasculature, but he already realized the protective function that it can exert [67]. Later, in 1757, Albrecht von Haller confirmed Lower's findings proving that a system of interarterially connecting vessels exists at the site of high arterial pressure. He also described these connections as functional arteries, which are bigger than capillaries [68]. Despite these studies, in

the next century respected anatomists like Hyrtl [69], Henle [70] and Cohnheim [71] declared that the coronary arteries were "end" arteries. In the beginning of the 20th century, Spalteholtz [72] again demonstrated the existence of collateral vessels even in the healthy human heart by preparing postmortem angiograms.

The controversy finally ended when Longland in 1953 [73] and Fulton in 1965 [74] provided anatomical data that presented compelling evidence of the existence of coronary anastomoses in healthy human hearts as well as in patients with coronary heart disease (CHD).

Collateral arteries are present in both, the coronary and the peripheral circulation. Nevertheless, major individual and inter-species differences exist [15].

With regard to the coronary circulation, rabbits, pigs and rats rapidly develop infarcts of the complete myocardium after an acute coronary ligation, whereas dogs and cats only develop partial infarcts and guinea pigs do not develop infarcts at all. This is closely related to the different extent of the preexisting collateral vasculature as it is very well formed in guinea pigs [9,75,76]. In the dog heart predominantly epicardial collaterals can be found [10,77], whereas porcine hearts show smaller, thin-walled and mainly subendocardial and intramural collaterals [10,78]. Collaterals in the human heart are similar to those in the porcine heart located subendocardially and endomurally [10,74].

On one hand, clinical studies as well as a study of chronic coronary stenosis in dogs revealed that well-formed collaterals are associated with smaller infarcts and improved left ventricular function after acute myocardial infarction [13,64,79]. The natural variation of the collateral circulation was thus the major determinant of different responses to coronary artery stenosis [79]. On the other hand, the degree of angiographically visible collateral vessels correlates with the severity of coronary artery narrowing [11].

According to the definition that was established by Longland [73], the anatomy of collateral arteries can be divided into three segments: The *stem*, which is the branch proximal to the occlusion from which the blood flows into the collateral; the *midzone* as the middle segment of the collateral; and the *reentry*, which connects the distal stump of the occluded artery and the midzone. The collateral vessels are physiologically thin-walled arterioles and arteries composed of an endothelial lining, an internal elastic lamina and one or two layers of SMCs [73,80,81]. In a model of femoral artery occlusion in rabbits, the preexisting collaterals were found to have a diameter of around 50 μm [81]. In normal human hearts they were measured to have diameters of 50-200 μm and a length of 1-

3 cm [11,82]. By arteriogenesis, these collateral vessels develop to regular, functional arteries.

Since collaterals grow in width as well as in length and the additional length is arranged in loops and turns, they characteristically display a tortuous pattern [10,12,83]. The diameter of collaterals in the rabbit hind limb usually increases 4-5-fold, but can increase up to 12-fold [81]. In the dog heart the internal collateral diameter can increase 20-fold [13,66,84], and in the rat hind limb it usually doubles [83].

1.3.2 Mechanisms of arteriogenesis

The main elements of arteriogenesis include preexisting collaterals as substrates, endothelial activation by increased shear stress, basal membrane degradation, leukocyte invasion, proliferation of vascular cells, neointima formation, and changes of the ECM [85]. Thus, arteriogenesis is closely related to inflammatory features [80,86].

In case of a stenosis or occlusion of a major artery, the blood pressure behind the stenosis decreases and a steep pressure gradient develops along the shortest path within the interconnecting network. The blood takes the path of lowest resistance via preexisting collateral arteries. Consequentially, the blood flow velocity and hence fluid shear stress in those collaterals increase [12,87]. In 1893 the embryologist Thoma [88] already pointed out that there is a close relationship between the diameter of an artery and blood flow velocity. Any sustained deviation from the existing relation results in a process of either growth or atrophy. Thus, large vessels with lower blood flow will gradually decrease their diameter while small vessels with sustained higher blood flow tend to grow [88].

The sustained increase of shear stress in collateral arteries following a stenosis or occlusion leads to activation of the endothelium [12,57,81] and to increased expression of certain chemokines like monocyte chemoattractant proteine-1 (MCP-1) [89,90], the adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule (VCAM) [81,91], and growth factors [15,64,81,92,93]. The transcriptional activity of the corresponding genes is partially upregulated via a protein that binds to the shear stress responsive element (SSRE), which is present in the promoter of numerous genes (nitric oxide synthase (NOS); platelet derived growth factor (PDGF); MCP-1) [54,94-96].

Characteristically, activated ECs show a synthetic and proliferative phenotype with increased endoplasmic reticulum and free ribosomes. Chloride channels that

are responsible for volume control open, and the ECs loose volume control, appearing swollen in scanning electron microscopic images [10,12,97,98].

As a result of the endothelial activation and upregulation of MCP-1 as well as ICAM and VCAM, circulating monocytes are attracted, and adhere to and invade the collateral arteries. In turn, they themselves become activated, transform into macrophages [86], and produce more cytokines and growth factors [12,62,99,100]. Among these factors are MCP-1, which attracts more monocytes [62], tumor necrosis factor-α (TNF-α) that provides the required inflammatory environment and also attracts monocytes [57,99,101], and fibroblast growth factor-2 (FGF-2 or bFGF), which is a mitogen for endothelial as well as SMCs [57,102,103].

Platelets also adhere and produce growth factors and interleukin-4 (IL-4), which results in increased expression of adhesion molecules. Furthermore, matrix metalloproteinases (MMPs) remodel the old arteriolar structure and create space for the expansion of the growing collateral artery [62,101,104,105].

1.3.3 Phases of arteriogenesis in the peripheral circulation

The processes of endothelial activation, upregulation of different genes and adhesion and invasion of monocytes and platelets constitute the first stage of arteriogenesis, the *initiation*, which begins minutes after the occlusion of a major artery [81]. It is followed by proliferation of the endothelium, and later by proliferation of SMCs [12].

During this *proliferative* phase the mitotic activity in ECs, SMCs and fibroblasts is maximal [81,83,106].

The following *synthetic* phase is characterized by significant growth processes [81]. The lamina elastica interna, the main barrier to SMC migration is degraded and together with lysis of the ECM cell migration is facilitated [12,107]. At first, the smooth muscle cell layers slide under the influence of the increased blood pressure and the vessel is enlarged passively resulting in a vein-like appearance [12]. SMCs migrate from the media to the subendothelial space and form a neointima [54,85]. The new SMCs represent the dedifferentiated, synthetic phenotype, which has lost most of the differentiation markers and much of its actin apparatus [54,84,85]. They produce ECM, collagen, and elastin and build up a new internal elastic lamina [15,81]. In a model of femoral occlusion in rabbits, a neointima develops in the midzone of collaterals, first in the areas of SMC migration, and it correlates with the degree of disruption of the lamina elastica interna [81].

In the rat hind limb, collaterals double their diameter within the first 7 days after occlusion and assume a typical corkskrew appearance. After 7 days no further proliferation or increase in diameter can be detected, and only some further growth in length occurs [83].

The last phase of arteriogenesis in the rabbit model is *maturation*. In this phase most of the SMCs have been restored to the contractile phenotype, and proliferation, migration and proteolytic activity are low [81].

After several months (6 to 12 months in the canine heart model) the enlarged collateral artery shows almost no differences to normal arteries except for a slightly higher collagen content in between the SMC layers [15,98].

Initially, several collateral arteries start to proliferate but finally only a few (3 or 4) develop to large conducting arteries. The others regress, probably because they have lost in the competition for flow [98,108]. Those collaterals that grow faster than others are conducting more blood flow. Thus, blood flow, and hence, shear stress in other collaterals decreases and the induction of arteriogenesis by shear stress is reduced.

Canine coronary collaterals and collaterals in the rabbit hind limb particularly form a cell-rich neointima during arteriogenesis, which can finally be the largest part of the new arterial wall. Thus, it is assumed that the reduction of the number of growing collaterals over time is a result of excessive intimal proliferation, which leads to obliteration of the lumen and regression of the vessel to the advantage of a few remaining large vessels [15,57,98].

In spite of their remarkable growth, the collateral arteries only reach about one third of the conductance of the artery that they have replaced. The arteriogenic process stops prematurely, probably because shear stress falls under a critical value due to the collateral enlargement [12,15,109]. This leads to the possibility that arteriogenesis could be improved therapeutically either via exogenous protein application or local gene transfer [110].

1.3.4 Induction of arteriogenesis

While angiogenesis is initiated by ischemia, arteriogenesis is induced by shear stress [57,111]. Even though arteriogenesis is usually associated with ischemia after an arterial occlusion, it is not dependent on ischemia and collaterals often grow in oxygen-rich tissue [15,108,112].

Fulton already demonstrated in 1965 [113] that the stem zone of human epicardial collaterals is surrounded by non-ischemic muscle and it was also shown for

canine epicardial collaterals that they are never in contact with the ischemic endomyocardium [111].

Furthermore, in a rabbit model of femoral artery occlusion collaterals develop in the thigh, where neither the resting blood flow is decreased nor is adenosinetriphosphate (ATP) and phosphocreatinine (PCr) content changed or hypoxia inducible gene transcription activated [15,114-116]. The distance between the ischemic region and the location of arteriogenesis can be extremely large, e.g. 70 cm between a persons gangrenous toe and the collaterals bypassing the femoral or popliteal occlusion [111].

1.3.5 Modulation of arteriogenesis

1.3.5.1 Role of MCP-1 and GM-CSF

The concentration of blood monocytes is critical for enhancement of collateral growth [117]. This importance of monocytes can be utilized to stimulate arteriogenesis. It was shown in different animal models that the infusion of MCP-1, which attracts and activates monocytes, into the proximal stump of an occluded femoral artery significantly accelerates collateral growth and increases collateral conductance but does not change the final extent of growth after six months [90,116,118-121].

Apart from MCP-1 infusion, the infusion of granulocyte monocyte-colony stimulating factor (GM-CSF) stimulated arteriogenesis and increased collateral conductance [119]. GM-CSF prolongs the life span of monocytes by inhibiting apoptosis [122,123]. The simultaneous infusion of a combination of both factors had a synergistic effect and around 40% flow restoration was reached. Furthermore, it was shown that MCP-1 treatment alone lost effectiveness after the third week of occlusion. Together with GM-CSF the time window was widened and about 80% of the normal maximal conductance could be achieved, meaning that the combination can still increase the capacity of an already matured collateral circulation [119,124]. These effects are probably mediated by ICAM-1 as it was shown that antibodies against ICAM-1, which block monocyte adhesion and migration, are potent inhibitors of arteriogenesis [124].

1.3.5.2 Role of TGF-β

Transforming growth factor-β (TGF-β) is a cytokine expressed by different cells, especially ECs. It has been shown to be upregulated around growing collateral vessels in animal models as well as in humans [125-127]. It is a well-known

chemoattractant for monocytes that also enhances the expression of growth factors like IL-1, TNF-α, bFGF, and PDGF [128,129].

The local infusion of TGF-β into the collateral circulation of the rabbit hind limb after femoral occlusion has been shown to result not only in an increased number of visible collaterals, but also in an increase in collateral conductance [57,127,130]. Since TGF-β treated monocytes displayed enhanced endothelial adhesion and transmigration in migration assays [127], the arteriogenic potency of TGF-β is most likely monocyte-related [57].

1.3.5.3 Further growth factors

Different growth factors usually act by stimulating migration and proliferation of ECs and/or SMCs. As a consequence of the distribution of their receptors, those of the family of vascular endothelial growth factors (VEGFs) are more specific for ECs, whereas those of the family of fibroblast growth factors (FGFs) are more mitogenic. Both enhance the release of nitric oxide (NO), which causes vasodilation, which has to be clearly differentiated from true vessel growth [111,131,132]. Apart from causing vasodilation, VEGFs can increase vascular permeability, enhance leukocyte adhesion, stimulate proteases [133], and mobilize endothelial precursor cells from the bone marrow [134]. Even though VEGF is not a mitogen for SMCs, it has been shown to increase arteriogenesis in different animal models [120,135,136]. The infusion of high doses of VEGF over 7 days into the proximal stump of an occluded femoral artery resulted in significant augmentation of collateral blood flow [37] but less than the infusion of MCP-1. On the other hand, it was found that arteriogenesis is not associated with increased expression of VEGF and that VEGF does not induce arteriogenesis [116]. Closely related to VEGF is the placental growth factor (PlGF), which shares its receptor Flt-1 [59] and stimulates VEGF secretion by monocytes [63,137].

For PlGF it was shown that targeted disruption of its gene causes retardation of arteriogenesis but does not alter the final extent of the process compared to wild-type animals [138,139]. Thus, PlGF is a promoter of collateral growth [139]. Since both growth factors, VEGF and PlGF, have been shown to influence monocyte attraction via the VEGF receptor-1 [100], and VEGF increases endothelial adhesion and transmigration of monocytes [38], their positive effect on arteriogenesis is most probably monocyte related.

From the family of FGFs the basic fibroblast growth factor (bFGF or FGF-2), which is a mitogen for ECs and SMCs [102], has been found to increase angiogenesis as well as arteriogenesis [140,141] in experimental models. These results could

however not be confirmed in a placebo controlled trial in human patients, which were treated with an intracoronary bolus of bFGF. While anginal symptoms were reduced, the primary endpoint (exercise duration) remained unchanged [142].

In a model of femoral artery occlusion in rabbits it was shown that neither fibroblast growth factor-1 (FGF-1) nor FGF-2 transcript levels changed but during a critical brief window of time in arteriogenesis the FGF-1 receptor was upregulated [116,143]. This and the fact that most growth factors are already present in the tissue and are produced at low levels continuously, e.g. VEGF and FGF-1 and FGF-2, leads to the conclusion that arteriogenesis is regulated by receptor availability rather than ligands [13,144]. A recent study by Deindl et al. [143] showed that MCP-1 stimulated arteriogenesis was strongly reduced by application of an FGF inhibitor, indicating that FGFs also promote collateral growth via monocytes.

2. Integrins in general

Integrins are a family of heterodimeric cell surface glycoprotein receptors. They mediate cell-to-cell and cell-to-extracellular matrix interactions [16]. Their name, first established by Hynes in 1986, refers to their role in integrating the extracellular matrix (ECM) and the intracellular cytoskeleton [145,146].

Integrins are composed of an α- and a β-subunit, which are non-covalently bound. Currently, there are 18 different α- and 8 different β-subunits known in man, which form at least 24 different integrins [146]. The different types of α-subunits are highly homologous and vary in size between approximately 120 and 180 kDa. They contain seven 60 amino acid long tandem repeats. The β-subunits are also homologous to each other. They show high cysteine content and range from 90 to 110 kDa [145]. Each of the two subunits consists of a large extracellular domain, a single membrane-spanning domain and a short, non-catalytic cytoplasmic tail [147]. Both subunits are involved in ligand recognition [145] and the combination of subunits determines ligand specificity, but most integrins recognize more than one ligand and most ligands bind more than one integrin [18].

Generally, those integrins containing α_4-,α_5-, α_8-, α_{IIb}-, or α_v-subunits bind to ligands in the ECM that contain an RGD (Arg-Gly-Asp) sequence. Integrins containing the subunits α_3, α_6 or α_7 are known as laminin receptors, while those with α_1-, α_2-, α_{10}- or α_{11}-subunits recognize collagen. Those integrins that are expressed on hematopoietic cells usually bind to counter receptors on other cells, e.g. intracellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1), plasma proteins at sites of injury, or complement factors.

Inhibitors of platelet aggregation secreted by leeches and ticks, disintegrins from snake venoms, and even bacterial pathogens and viruses can also use integrins as receptors [146,148].

Thus, integrins are widely distributed, and many different cells like platelets, endothelial cells (ECs), smooth muscle cells (SMCs), monocytes and leukocytes contain integrins on their surface [17]. Sixteen of the 24 known integrins are involved in vascular biology with seven of them known to be expressed by endothelial cells [18]. The major integrins on ECs are $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ [149] but $\alpha_1\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_5$ can also be found [17,18].

Not all specialized functions of integrins are currently known. The $\alpha_{IIb}\beta_3$ integrin, for example, is the major platelet integrin, a fibrinogen receptor that is important in platelet aggregation [17]. Patients suffering from the human bleeding disorder Glanzmann thrombasthenia show defects in or lack of $\alpha_{IIb}\beta_3$ integrin [150].

The integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are both involved in angiogenesis since antagonists of these receptors block bFGF and VEGF induced angiogenesis, respectively [19]. $\alpha_v\beta_3$ integrin has attracted special attention because it can be inhibited by simple peptides containing an RGD sequence [151].

In this study, we are focusing on $\alpha_v\beta_3$ integrin and its main ligand vitronectin because it has emerged as an important component in vascular biology with diverse functions.

3. $\alpha_v\beta_3$ integrin

3.1 Expression and Ligands of $\alpha_v\beta_3$ integrin

$\alpha_v\beta_3$ integrin is expressed at highest levels in osteoclasts, but it is also expressed in platelets, megakaryocytes, monocytes, T-lymphocytes, macrophages, SMCs, ECs and fibroblasts [31,145].

Brooks et al. [152] have shown that $\alpha_v\beta_3$ integrin is abundantly expressed on activated ECs of blood vessels in granulation tissue. Several other studies in different animal models have demonstrated that $\alpha_v\beta_3$ integrin is strongly upregulated after vascular injury, especially in the regenerating endothelium and in the neointima [22-24,153,154].

$\alpha_v\beta_3$ integrin can be detected at high levels in the endothelium and at lower levels in the media of arteries showing diffuse intimal thickening or atherosclerotic plaque as well as in the microvessels of their adventitia and of the atherosclerotic

plaque. In the intima of such arteries, $\alpha_v\beta_3$ integrin expression colocalizes with SMCs. This suggests that $\alpha_v\beta_3$ integrin is expressed in normal arteries as well as in sites of SMC accumulation and of angiogenesis in atherosclerotic plaque [20]. Yet, in another study, $\alpha_v\beta_3$ integrin could not be found on SMCs in areas of recent plaque ruptures [155].

Upregulation of $\alpha_v\beta_3$ integrin expression has also been found in the media of arterioles in ischemic areas after middle cerebral artery occlusion [156] or in areas of myocardial ischemic injury after cardiac transplantation [157].

Vitronectin is the main ligand for $\alpha_v\beta_3$ integrin, which is also known as vitronectin receptor. However, vitronectin is not its only possible ligand. $\alpha_v\beta_3$ integrin can bind to osteopontin [158], fibrinogen, von Willebrand factor, thrombospondin [159], thrombin, fibronectin and proteolysed forms of collagen and laminin in the extracellular matrix [147,160]. Additionally, $\alpha_v\beta_3$ integrin has been shown to interact with counter receptors on other cells like platelet endothelial cell adhesion molecule (CD31/PECAM) [161].

The expression of $\alpha_v\beta_3$ integrin is upregulated generally after vascular injury. Senger et al. [162] found an upregulation of $\alpha_v\beta_3$ integrin on ECs in response to VEGF stimulation and Sepp et al. [163] showed regulation of $\alpha_v\beta_3$ integrin expression on ECs by bFGF. Additionally, SMCs show increased expression of the α_v-subunit after exposure to insulin-growth factor-I (IGF-I) [164] and of the whole integrin $\alpha_v\beta_3$ after induction by TGF-β, thrombin and platelet-derived growth factor-BB (PDGF-BB) [25,165]. Besides growth factors, hypoxia in general and nitric oxide, induced by hypoxic conditions, specifically lead to an increase in $\alpha_v\beta_3$ integrin expression [166,167]. Finally, the expression and activity of integrins is affected by other integrins in either way [146].

3.3 Functions of $\alpha_v\beta_3$ integrin

A large amount of studies has shown $\alpha_v\beta_3$ integrin to play key roles in cellular processes such as cell adhesion, migration, proliferation, differentiation, cell survival and apoptosis. $\alpha_v\beta_3$ integrin regulates diverse functions in SMCs. It controls SMC migration after arterial injury in rats by stimulating matrix metalloproteinase (MMP) production [168] and it is necessary for growth factor- and cytokine-induced SMC proliferation and fibronectin production [169]. Furthermore, $\alpha_v\beta_3$ integrin controls IGF-I-mediated [164], vitronectin-driven [25] and PDGF-induced SMC migration *in vitro* and in rabbits after carotid artery injury [27,170]. In addition, the interaction of $\alpha_v\beta_3$ integrin with osteopontin regulates adhesion, migration, ECM invasion and proliferation of coronary artery SMCs [171-173].

Apart from mediating actions of SMCs, $\alpha_v\beta_3$ integrin plays a role in EC adhesion and migration [174,175] and modulates $\alpha_L\beta_2$ integrin dependent monocyte adhesion to and migration on ICAM-1 containing substrates [176]. Furthermore, it mediates arteriolar vasodilation [29,30].

Stromblad et al. [177] showed that $\alpha_v\beta_3$ integrin promotes an adhesion-dependent cell survival signal by suppressing p53 activity, thus decreasing expression of p21WAF1/CIP1, a cell cycle inhibitor. In accordance with this, Stupack et al. [178] found that expression of unligated integrins or the use of integrin antagonists lead to apoptosis of adherent cells, which was confirmed by several other studies [32,33,179].

Being involved in all these cellular functions, $\alpha_v\beta_3$ integrin affects diverse biological processes in the vascular system. It is required for the survival and maturation of newly forming blood vessels during angiogenesis and vasculogenesis [19,180,181] and participates in neointima formation [31,34,36,182] and intimal thickening necessary for ductus closure after birth [183].

Thus, $\alpha_v\beta_3$ integrin plays an important role in pathological events concerning the vasculature such as atherosclerosis and restenosis (see below) and in neovascular diseases, but also in physiological conditions. Accordingly, antagonists of $\alpha_v\beta_3$ integrin have been shown to reduce arthritis [184,185], retinal neovascularization [186-188] and even tumor growth [180,189,190]. In a model of human breast tumors implanted on mice, $\alpha_v\beta_3$ integrin antagonist treated tumors were less invasive and contained fewer vessels. Furthermore, $\alpha_v\beta_3$ integrin functioned as a marker for tumor-associated blood vessels [191].

3.4 Integrin Antagonists and Restenosis / Neointima Formation

Several studies using different antagonists in different animal models have been conducted to investigate the role of $\alpha_v\beta_3$ integrin in the processes following vascular injury. Most of them were performed in a model of balloon angioplasty. Van der Zee et al. [32] used vitaxin, a humanized form of LM 609, a monoclonal antibody to $\alpha_v\beta_3$ integrin, to show that $\alpha_v\beta_3$ integrin antagonist treatment of rabbits after vascular injury limits intimal thickening and increases apoptosis of SMCs, but has no effect on re-endothelialization. They also proposed, that reduced intimal thickening is a result of inhibition of SMC adhesion, migration and survival signals. Coleman et al. [33] demonstrated a reduction in neointimal area, artery size, and arterial lumen after balloon injury in hyperlipidemic rabbits with vitaxin treatment. The suggested mechanisms for the reduction of

the neointimal area are to some extent direct inhibition of SMC migration, inhibition of TGF-β expression, and enhancement of apoptosis, which were also shown, but also inhibition of angiogenesis and modulation of the activation and localization of matrix metalloproteinase-2 (MMP-2). MMP-2 exerts proteolytic activities that can stimulate SMC migration through the ECM. The reduced artery size, which was found, could result from prevention of the vasodilatory responses that are mediated by $\alpha_v\beta_3$ integrin [29,30].

Bishop et al. [35] also found a smaller neointimal area in atherosclerotic rabbits after treatment with a non-peptide antagonist of $\alpha_v\beta_3$ integrin. In addition, they found less neo-vascularization, lower ICAM-1 and VCAM-1 expression and a significant decrease in macrophage cell density in the area of vascular injury together with a larger arterial lumen, leaving intimal and medial areas together the same as in controls. Furthermore, they showed a significant increase in $\alpha_v\beta_3$ integrin and vitronectin expression after injury, but no reduction of SMC content after antagonist treatment. Choi et al. [27] and Racanelli et al. [34] showed a reduction in neointima formation in rabbits, while Matsuno et al. [28,182] found the same in hamsters after carotid injury and antagonist treatment. Chico et al. [36] showed that a selective antagonist of $\alpha_v\beta_3$ integrin reduced SMC adhesion to vitronectin *in vitro* and neointima formation in pigs, but had no effect on thrombus formation. No significant differences in luminal or arterial areas were detected. Treatment with an $\alpha_{IIb}\beta_3$ integrin antagonist inhibited *ex vivo* platelet aggregation and reduced thrombus formation and neointima formation after vascular injury. They concluded that both, thrombus formation and $\alpha_v\beta_3$ integrin play a role in neointima development. Effects of $\alpha_v\beta_3$ integrin inhibition may be related to recruitment of SMCs to the thrombus, but also to effects on proliferation and apoptosis. Srivatsa et al. [31] found reduction of neointimal area and lumen stenosis in pigs after treatment with a selective $\alpha_v\beta_3$ integrin antagonist together with early upregulation of $\alpha_v\beta_3$ integrin at sites of cell accumulation. They postulated that this coordinated upregulation might serve to recruit different cells like ECs, SMCs, monocyte-macrophages and myofibroblasts to the neointima. In early neointimal development, thrombin generation and platelet adhesion mediated by $\alpha_v\beta_3$ integrin may be more important than osteopontin-$\alpha_v\beta_3$ integrin dependent cell migration. Wu et al. [192] and Slepian et al. [193] demonstrated limitation of neointimal hyperplasia and lumen occlusion in models of arterial injury and $\alpha_v\beta_3$ integrin antagonist treatment in rats.

Altogether, these studies demonstrate reduced neointima formation after vascular injury and $\alpha_v\beta_3$ integrin antagonist treatment in different models.

Some of the studies showed reduced SMC migration or adhesion, lower TGF-β and ICAM-1 and VCAM-1 expression, less neo-vascularization and macrophage cell density, reduced artery size and increased apoptosis. Coleman et al. [33] found a reduction in arterial lumen, whereas Bishop et al. [35] achieved a larger arterial lumen with $\alpha_v\beta_3$ integrin antagonist treatment. $\alpha_v\beta_3$ integrin, vitronectin and osteopontin were shown to be upregulated after vascular injury [23,35].

The suggested mechanisms, which are mediated by $\alpha_v\beta_3$ integrin, are SMC migration, adhesion and proliferation, modulation of activation and localization of matrix metalloproteinase-2, vasodilation, cell survival, cell recruitment in general, macrophage adhesion to the ECM and leukocyte adhesion to the endothelium.

Platelet aggregation and adherence is the first step in the development of a thrombosis and possible restenosis. Platelet activation includes activation of $\alpha_{IIb}\beta_3$ integrin [194] allowing fibrinogen to bind and cross-link to other platelets. This in turn is followed by further platelet activation and polymerization of fibrinogen [17]. Thus, $\alpha_{IIb}\beta_3$ integrin is a preferred target for restenosis prevention.

Currently, three integrin antagonists are in clinical use for restenosis prevention: eptifibatide, tirofiban and abciximab. Other integrin antagonists are in development. All three are inhibitors of the platelet integrin $\alpha_{IIb}\beta_3$ that functions as a fibrinogen receptor.

Eptifibatide is a cyclic heptapeptide derived from snake venom of the disintegrin family [195,196] and tirofiban is a non-peptide antagonist based on the disintegrin echistatin, which is also a snake-venom [197,198]. Both are specific antagonists of $\alpha_{IIb}\beta_3$ integrin [198].

Abciximab, though, is a chimeric 7E3 Fab monoclonal antibody fragment, that has been shown to interact with the leukocyte integrin $\alpha_M\beta_2$ [199] and to bind to $\alpha_{IIb}\beta_3$ integrin and $\alpha_v\beta_3$ integrin with equivalent affinity [200].

Wu et al. [192] have shown that abciximab inhibits neointima formation and lumen occlusion and reduces PDGF-BB in vessel lumen. SMCs and Txβ (2) plasma concentration in injured rat carotid arteries were also reduced. In a clinical study, it was shown that an abciximab bolus given at the time of coronary angioplasty followed by infusion improves outcomes as long as three years after the procedure [201]. In this regard, Baron et al. [202] found that abciximab reduces SMC adhesion and migration to vitronectin and osteopontin, but migration of cells lacking $\alpha_v\beta_3$ integrin is not reduced.

Bishop et al. [35] concluded from their results that blockade of $\alpha_v\beta_3$ integrin by abciximab may decrease inflammatory cell infiltration and neovascularization, thus improving plaque stability and reducing adverse cardiac events such as myocardial ischemia and infarction. Hence, some of the clinical benefits of abciximab might be due to inhibition of $\alpha_v\beta_3$ integrin functions.

3.5 Gene Ablation Studies

Apart from interventional studies with integrin antagonists, experiments with mouse strains lacking specific integrin subunits are another interesting possibility in research.

The majority of α_v-knockout mouse embryos dies by midgestation, probably due to placental defects, and about 20% die shortly after birth. Unexpectedly, they exhibit extensive vasculogenesis and angiogenesis in most organs and tissues. The processes of endothelial proliferation, migration, tube formation and branching all occur, and the embryos develop a normal yolk sac and early embryonic vasculature. Nevertheless, α_v-null mice show extensive cerebral and intestinal vascular abnormalities and hemorrhaging. Their cerebral vasculature becomes distended and eventually ruptures [203]. This seems similar to the defects found in PDGF-B-null embryos, which are thought to result from failure of pericytes to immigrate along the vessel [204]. Possibly, the α_v-null defects arise from a failure in pericyte recruitment, although this cannot be the only reason, because the defects are initiated earlier in the process than pericyte recruitment [159].

Antibodies to $\alpha_v\beta_3$ integrin, in contrast, disrupt regular vascular development and lumen formation, such as quail dorsal aorta formation [181] and angiogenesis on the chicken chorioallantoic membrane [152].

Furthermore, α_v-null mice do not display increased apoptosis of ECs as it is seen in blocking experiments with $\alpha_v\beta_3$ integrin antagonists [180].

Mice lacking the β_3 integrin gene serve as a viable and fertile model for Glanzmann thrombasthenia. The functions of both, $\alpha_{IIb}\beta_3$ integrin and $\alpha_v\beta_3$ integrin are disrupted, and these mice show prolonged bleeding time, cutaneous and gastrointestinal bleeding, and defects in platelet aggregation and clot retraction. Defects in the β_3-null placenta and gastrointestinal hemorrhaging lead to reduced survival. However, postnatal retinal angiogenesis does not show major defects [205], which is in contrast to inhibition studies (see 3.4).

The differences in the results of interventional and gene ablation studies might first be due to the different systems that are investigated. A second possibility is that in the absence of one integrin or subunit, the organism expresses or uses a different component that is able to compensate for the missing one. Additionally, functions of integrins overlap. Both would indicate an underestimation of integrin functions in gene ablation studies. Overestimation in blocking experiments is also possible as a result of cross-reactivities of antagonists or because of integrin cross-talk. [159].

4. Vitronectin

4.1 Introduction

Vitronectin is a multifunctional adhesive glycoprotein, which is present in blood and in the extracellular matrix of various tissues [206]. Initially, it was enriched from human serum by adherence to glass beads and defined as S-protein by Holmes in 1967 [207].

Vitronectin exerts regulatory functions in the blood coagulation, complement, and fibrinolytic systems and participates in cell attachment, differentiation, proliferation, and morphogenesis [208,209].

4.2 Structure of vitronectin

In human blood vitronectin circulates in a single chain form of 75 kDa and in a clipped two-chain form of 65 kDa and 10 kDa. In non-reducing conditions the two fragments are linked by a single disulfide bond [210]. The ratio between both forms of vitronectin is genetically determined but variable and depends on the presence of threonine or methionine at position 381. This difference in the amino acid sequence is responsible for the sensitivity of vitronectin to proteolytic cleavage between Arg 379 and Arg 380, which is favored in the presence of threonine [208,210,211].

Since the two forms of vitronectin are functionally different, the cleavage might have regulatory functions. Seger et al. [212] have shown that the cleavage probably occurs in the liver and is carried out by furin, a serine endoproteinase.

The human vitronectin gene consists of eight exons and seven introns from which a 1.7 kb pair transcript is derived. The cDNA encodes for 459 amino acids, which are preceded by a 19-amino acid signal peptide [208,210]. Human, mouse, rabbit, rat, and porcine vitronectin share more than 80% identity in their primary

structures, indicating that the protein has been well conserved during evolution [208,213].

The sequence of the amino-terminal domain of vitronectin is identical to that of somatomedin B and is involved in binding of the plasminogen activator inhibitor-1 (PAI-1) [208,214,215]. Following the somatomedin B domain is an Arg-Gly-Asp (RGD) sequence. This sequence is absolutely necessary for vitronectin to mediate cell attachment and spreading via specific integrin receptors [216,217]. Located c-terminally to the RGD sequence is a highly acidic region including two sulfatation sites, a putative cross-linking site, and a collagen-binding domain [208,218,219]. The acidic region also plays a role in vitronectin binding of thrombin-antithrombin III complexes (TAT complexes) [210].

The main part of the vitronectin molecule consists of seven hemopexin type repeats [220]. In the beginning of this segment, two regions with heparin-binding capacity have been identified [221]. The basic heparin-binding site with two consensus sequences for heparin binding is located further in the c-terminus and is also responsible for the binding of other glucosaminoglycans [222] and for the interaction of vitronectin-TAT-complexes with ECs [223]. In native vitronectin, this basic heparin-binding site is located in a cryptic area, whereas in extended vitronectin it is exposed, leading to high affinity binding of heparin [218,224]. Additionally, the carboxy-terminus of the molecule is involved in the binding of plasminogen [225] and PAI-1 [215,226].

Moreover, vitronectin possesses phosphorylation sites for different protein kinases, e.g. the cAMP dependent protein kinase, which modulates vitronectin conformation and function and leads to reduced binding of PAI-1, or the protein kinase C, which attenuates the plasmin cleavage of vitronectin and regulates plasminogen activation [227,228]. Phosphorylation of vitronectin by casein kinase is associated with cell adhesion and cell spreading [227].

Generally, changes in vitronectin conformation are closely related to differences in ligand and cell binding properties [208].

4.3 Distribution

Vitronectin is present in normal plasma at a concentration of 200-400 μg/ml [208,229,230]. This concentration does not significantly differ from that in serum [231]. Additionally, vitronectin can be found in seminal plasma [232], urine, amniotic fluid [229], cerebrospinal fluid, bronchoalveolar lavage fluid [208,233] and synovial fluid [234].

Hepatocytes have been shown to be the major vitronectin producing cell-type [213], and reduced levels of plasma vitronectin have been observed in patients with severe liver failure [208,235]. Furthermore, vitronectin can be synthesized and secreted by cultured mesothelial cells [206], smooth muscle cells [236], glioblastoma cells [237], and rheumatoid arthritic synovial cells [238]. An immunohistochemically identical protein was found in megakaryocytes [206,239] and monocytes and macrophages [240]. Plasma levels of vitronectin in patients that undergo elective orthopedic surgery and in rodents under acute phase conditions are elevated. The induction of the vitronectin gene seems primarily mediated by interleukin-6 [241].

Additionally, vitronectin is contained in platelets in a rapidly releasable form [242]. This platelet vitronectin is probably not synthesized by platelets but incorporated from plasma by endocytosis [243].

Apart from vitronectin in platelets and plasma, vitronectin depositions can be found in several tissues: in the connective tissue of the embryonic lung, smooth and skeletal muscle, kidney, and in the capsular surface of all viscera [209,244]. In the human skin, vitronectin is localized on dermal elastic fibers and on pathologically altered elastic material. It can also be found on keratin filament material and in abnormally thickened cutaneous blood vessels [245-247].

Generally, vitronectin depositions are localized in areas of fibrosis and necrosis and thus can be detected in several diseases like membranous nephropathy, arteriosclerosis [235], hepatitis, central nervous system disorders [208], and in kidney tissue in glomerulonephritis [248,249]. Vitronectin has been detected in areas of acute myocardial and kidney infarction [208] and in multiple sclerosis in dystrophic, demyelinated axons in active lesions, in the microvasculature and in few reactive astrocytes in active plaques [250]. In rheumatic disease, vitronectin levels are increased in the synovial fluid of the inflamed joint and in the synovial lining in rheumatoid arthritis [234].

With regard to the vascular system, vitronectin has been detected in association with elastin material in areas of pericytic matrices in arterial blood vessels [249,251,252]. Van Aken et al. [253] detected vitronectin in normal and atherosclerotic vessels in the lamina elastica interna and externa and in strand-like structures in the adventitia. In pulmonary arteries, they additionally detected vitronectin in the media. Generally, they found vitronectin in the proximity of elastin and increased levels of vitronectin in atherosclerotic arteries. Vitronectin could not be detected in the intima of vessels.

Contrarily, Stoop et al. [254] showed a colocalization of thrombin, PAI-1 and vitronectin in human neointimal atherosclerotic tissue. Dufourq et al. [236] found

strong expression of vitronectin and its mRNA in the intima and media of atherosclerotic plaques. In the intima, the mRNA was colocalized with SMCs, which synthesized vitronectin in vitro. The vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin were also colocalized with vitronectin in the intima. Moreover, Dufourq et al. [154] showed strong expression of vitronectin, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin in the early stage of neointima formation after balloon carotid injury and strong expression of vitronectin in migrating SMCs after injury *in vitro*.

While plasma vitronectin is mainly monomeric and lacks exposure of conformationally sensitive epitopes, platelet and tissue-associated vitronectin are conformationally altered and multimeric with different functions [208,235]. Most of the tissue vitronectin is probably derived from plasma [244,255], but extrahepatic cells also have the potential to synthesize vitronectin (see above) and vitronectin synthesis is regulated under inflammatory conditions [235,241].

4.4 Receptors for vitronectin

As mentioned above (chapter 4.2.) vitronectin can be a ligand for several different receptors, which mediate the cellular activities of vitronectin [208,255]. The recognition by these cellular receptors largely depends on the conformational state of vitronectin. Seiffert et al. [256] showed that native vitronectin is unable to bind to $\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$ integrin, while denatured vitronectin recognizes both of these receptors. Thus, the conformational state of vitronectin regulates its functions. Such changes in conformation are likely to occur in areas of tissue injury and thrombosis [256].

Vitronectin can bind to PAI-1, heparin and other glucosaminoglycans, plasminogen [225,257], osteonectin [208,258], collagen, TAT III complex [210], urokinase receptor (uPAR) [259], and IGF-II [260].

Vitronectin can bind to $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$, and $\alpha_{IIb}\beta_3$ integrin receptors. Although $\alpha_v\beta_3$ integrin also recognizes other proteins that contain an RGD-sequence, it is best known as "vitronectin receptor" [255]. The most selective vitronectin-binding integrin, however, is $\alpha_v\beta_5$. This integrin additionally interacts with viral proteins. It recognizes the basic domain in TAT protein of HIV and interacts with an adenovirus penton base protein [261,262].

It has been proposed for $\alpha_v\beta_3$ integrin and vitronectin that such multiple binding sites within one ligand recognized by the same or distinct domains within the integrin may stabilize the ligand-receptor interaction [263].

All four mentioned integrins recognize the RGD-motif in vitronectin [255]. Cherny et al. [217] showed that replacement of the RGD-sequence in vitronectin results in loss of all cell adhesion activity mediated by $\alpha_v\beta_3$, $\alpha_v\beta_5$, or $\alpha_{IIb}\beta_3$ integrin.

Some integrin receptors are closely related in their functions. Pijuan-Thompson et al. [264] showed that a signal from $\alpha_5\beta_1$ integrin is necessary for internalization of vitronectin by $\alpha_v\beta_3$ integrin whereas $\alpha_v\beta_5$ integrin internalizes vitronectin constitutively.

4.5 Functions of vitronectin

Due to its ability to bind to several different receptors and its widespread distribution, vitronectin exerts numerous diverse biological functions. It plays an important role in the immune defense system by interacting with bacteria and by regulating the complement system. Vitronectin acts as an inhibitor of cytolytic reactions of terminal complexes of complement and of perforin [210]. Additionally, vitronectin serves regulating functions in the coagulation, fibrinolysis, and plasminogen activation system and thus in hemostasis [206]. The deposition of vitronectin associated with PAI-1 alters the balance of the fibrinolytic system to favor inhibition of fibrinolysis and development of necrosis [210].

Since the focus of this study is on $\alpha_v\beta_3$ integrin and arteriogenesis, the following part is restricted to those functions of vitronectin that are associated with this integrin or the vessel wall.

By interacting with integrin receptors, vitronectin promotes cell adhesion, spreading, and migration [26]. Following vitronectin binding, integrins activate diverse signaling pathways and regulate cytoskeletal reorganization, intracellular ion transport, lipid metabolism and gene expression [265].

Generally, the α_v-subunit seems to mediate the attachment of various cells to different matrix proteins, whereas the β-subunit determines the kind of interaction between cells and matrix proteins [255]. The β_3-subunit has been shown to mediate cell migration on vitronectin and other RGD-containing substrates [160,266] while $\alpha_v\beta_5$ integrin promotes cell attachment to vitronectin [267].

Brown et al. [25] showed that vitronectin induces $\alpha_v\beta_3$ integrin mediated migration of human aortic SMCs and that the expression of $\alpha_v\beta_3$ integrin is induced by TGF-β and thrombin. Wilcox-Adelmann et al. [268] found that ligation of $\alpha_v\beta_3$ integrin or $\alpha_v\beta_5$ integrin is necessary for the localization of uPAR to areas of cell-matrix adhesion and suggested that vitronectin promotes cell migration by recruiting integrin receptors and components of the PAI- system to areas of cell-

matrix contact. PAI-1 on the other hand can inhibit cell migration *in vitro* by competing for vitronectin binding to integrins [269]. Hafdi et al. [270] demonstrated that adhesion of cultured human mesangial cells to vitronectin involves $\alpha_v ß_3$ integrin and $\alpha_v\beta_5$ integrin. Kanse et al. [271] showed that adhesion of ECs to immobilized vitronectin is integrin-mediated without involvement of the vitronectin-uPAR-system.

Furthermore, vitronectin plays a role in tumor growth and metastasis. It has been demonstrated that the attachment, spreading, and migration response of human melanoma cells on vitronectin correlates with expression levels of $\alpha_v\beta_3$ integrin and that these integrin levels are higher in cells from later stages of tumor progression [272].

Cultured glioblastoma cells do not interact with normal brain without vitronectin but they attach to biopsy material of glioblastoma cryostat sections at sites of vitronectin expression. This implicates a role for vitronectin in metastasis of this tumor [237].

Moreover, vitronectin has been related to atherogenesis in several studies. Schoppet et al. [273] showed that vitronectin and TGF-β are colocalized in atherosclerotic tissues and that the binding of TGF-β to vitronectin modulates either function. Naito et al. [274] suggested that vitronectin is involved in atherosclerosis by recruiting SMCs from the media to the intima. Dufourq et al. [154] recently found an upregulation of vitronectin after vascular injury and showed that $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin are required for SMC migration and that this migration as well as neointima formation can be inhibited by vitronectin blockade.

Diverse studies have additionally shown involvement of vitronectin and integrin ligation in the prevention of apoptosis. Isik et al. [275] found reduced apoptosis of microvascular ECs in the presence of vitronectin with the α_v-subunit apparently being the critical component. Human glioma cells are protected from $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin mediated, topoisomerase induced apoptosis by vitronectin [276]. Furthermore, Taga et al. [277] showed that an antagonist of $\alpha_v\beta_3$ integrin induces apoptosis of brain tumor cells by detaching them from vitronectin and tenascin.

Cell differentiation also seems to be partially regulated by vitronectin. The differentiation of ECs into capillary-like structures in response to vitronectin binding by $\alpha_v\beta_3$ integrin has been shown to involve protein kinase C and phosphorylation of the receptor [278]. Similarly, the binding of vitronectin by guinea pig megakaryocytes via $\alpha_v\beta_3$ integrin led to differentiation into pro-platelets [279].

A study by Gawaz et al. [280] suggests a contribution of vitronectin together with $\alpha_v\beta_3$ integrin to acute myocardial infarction and reperfusion injury.

In the vascular system, Wu et al. [281] showed that vitronectin and integrin binding maintains the barrier function of isolated porcine coronary venules and that the inhibition of integrin binding leads to increased venular permeability.

The role of vitronectin in atherosclerosis, neointima-formation and stenosis is controversially discussed. On one hand, Fay et al. [282] showed that after chemical injury, vitronectin -/- mice display significantly shorter occlusion times than wild-type mice and that vitronectin inhibits thrombin-fibrinogen interactions. In this way, vitronectin plays an anti-thrombotic role. In accordance, de Waard et al. [283] showed in a model of murine carotid artery ligation that vitronectin and PAI-1 protect against stenosis and neointima-formation as PAI-1 -/- and vitronectin -/- mice generate a larger intima and more extensive SMC proliferation than wild-type mice.

On the other hand, Eitzmann et al. [284] found that PAI-1 and vitronectin promote thrombosis in murine vessels after photochemical injury by regulating endogenous fibrinolysis. PAI-1 -/- and vitronectin -/- mice showed higher occlusion times than wild type. Similarly, Konstantinides et al. [285] demonstrated that PAI-1 and vitronectin stabilize arterial thrombi in mice after chemical vascular injury and that vitronectin is accumulated after vascular injury.

Peng et al. [286] showed promotion of neointima formation by vitronectin and PAI-1 in murine carotid arteries after ligation as well as after chemical injury. They suggested that this effect might be mediated by their capacity to promote fibrin deposition and to enhance SMC-fibrin interaction.

Although vitronectin exerts numerous functions, it seems not to be essential for cell adhesion and migration during mouse development and its role might overlap with other adhesive matrix components, because vitronectin-deficient mice show normal development, fertility and survival [287].

III. MATERIALS AND METHODS

1. The rabbit hind limb model

In the present study, an *in vivo* model of chronic ischemia in the rabbit hind limb was used. After skin and muscle incision of the medial thigh in anesthetized rabbits the femoral artery was dissected free and ligated. The study was performed with the permission of the County of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the *German Law for the Protection of Animals* (BGBl Jahrgang 1998, Teil I, Nr. 30, S.1106 ff.). The experiments were conducted in conformance with the guidelines of the GV SOLAS.

1.1 Animals

Seventeen specific pathogen free, male New Zealand White rabbits with an average weight of 2.5 kg were obtained from Charles River Laboratories, France. They were housed in standard cages with water and rabbit chow (Altromin®) ad libitum.

1.2 Surgical procedure

The animals were anesthetized with an intramuscular injection of a combination of 40 mg/kg body weight ketamine hydrochloride and 4 mg/kg body weight xylazine. Then the vena auricularis magna was cannulated with a venous catheter. Supplementary doses of the anesthetic combination (10-20% of the initial dose) were given intravenously, if necessary. The cornea was protected with dexpanthenol eye treatment and the area of the medial thigh was bilaterally shaved and desinfected.

After a longitudinal skin incision on the medial thigh the right and left femoral arteries were exposed by removal of the connective tissue. One femoral artery was then occluded proximally to the orifices of the arteria poplitea but distal to the branching-point of the arteria profunda femoris by two ligations about 1 cm apart from each other, leaving the arteria profunda femoris, the arteria circumflexa femoris lateralis and circumflexa abdominis patent. The wound was closed in one layer. For sham operations, the same procedure was carried out on the other femoral artery without tightening of the ligations. Figure 2 shows a schematic view of the occlusion site and the developing collaterals.

Even though animals subjected to the surgical procedures described above did not display any signs of pain or discomfort, all animals were treated with

50 μg/kg body weight buprenorphine injected intramuscularly. The animals received 5 mg/kg body weight enrofloxacine (Baytril®, Bayer) as antibiotic prophylaxis. After surgery, the animals were provided with an Elizabethan collar to protect the wound and housed individually with free access to water and chow. No gangrene or gross impairment of function could be observed after the femoral occlusion.

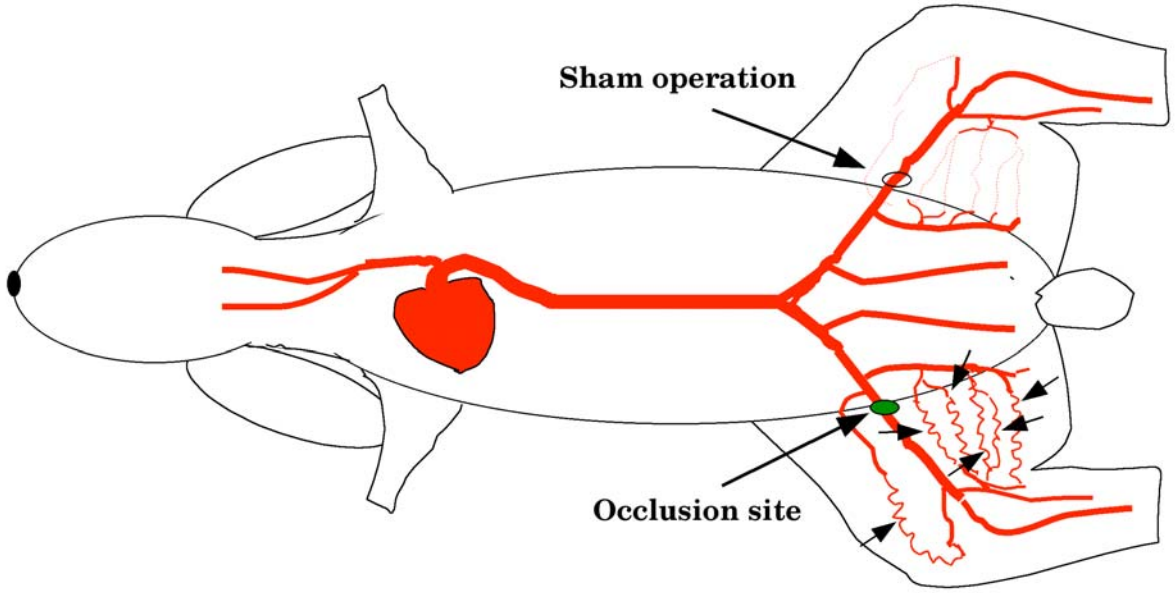


Fig. 2: *Schematic view of surgical occlusion of the rabbit femoral artery and the developing collaterals. Small arrows indicate growing collaterals.*

2. Acquisition and preparation of tissue

Femoral artery ligation was maintained for 1, 3, 7, 14, and 56 days. At least three rabbits per time point were operated. After completion of the protocols, the rabbits were anesthetized as described and received 2500 I.U. of Heparin-Na. Afterwards they were sacrificed by an overdose of pentobarbital intravenously. The following procedure was done as quickly as possible to prevent autolysis of the tissue. Usually, it did not take longer than 15 minutes.

After a skin incision on the medial thigh, the vastus intermedius of the quadriceps muscle, which is the red part of the muscle adjacent to the thighbone, was isolated and excised. This muscle is very suitable for quantitative studies in arteriogenesis because the area is physiologically supplied by the femoral artery and shows a constant distribution and localization of collateral arteries. The muscle was cleaned with a small amount of phosphate buffered saline (PBS, see next Chapter). Excess PBS was allowed to dry while two central pieces of the muscle were cut and each transversally oriented for mounting on a drop of water-

soluble tissue adhesive Tissue-Tec™ (O.C.T. Compound, Fa. Miles Inc., USA) placed on a piece of cork. Thus, two samples per hind limb and 4 samples per rabbit, respectively, were obtained. The samples were oriented leaning against two injection needles. Then they were covered thinly with Tissue-Tec™ and snap-frozen in methylbutane (-130 °C) cooled in liquid nitrogen (-196 °C). The proper temperature of the methylbutane is reached when a solid layer starts to form at the bottom of the jar. Finally the samples were stored at -80°C.

3. Histology

3.1 Cryostat Sectioning

Cryostat sectioning allows the preparation of thin sections of unfixed tissue, which reduces artifacts and conserves optimum antigenicity for immunohistochemistry.

3.1.1 Preparation of Slides

Thorough cleaning and coating of the slides is very important to prevent detaching of the sections during numerous washing and incubating steps of the labeling procedures. Thus, the glass slides (Menzel-Superfrost) were first washed in an Extran™ (Merck) solution and then thoroughly rinsed with tap water followed by distilled water (Aqua dest.). Afterwards they were dried in an incubator at 40°C over night. The following day the slides were coated with a 2% 3-aminopropyltriethoxysilane (Sigma) solution according to the table below.

Table 1: Coating of glass slides

1. Basin	196 ml Acetone + 4 ml 2% 3-Aminopropyl-triethoxysilane (Sigma)	Dip in each basin for approximately 5 sec
2. Basin	200 ml pure Acetone	
3. Basin	200 ml Aqua dest.	
4. Basin	200 ml Aqua dest.	
5.	Dry overnight at 40°C and store in an exsiccator	

3.1.2 Tissue preparation and sectioning

The tissue samples were transported to a Leica CM 3000 cryostat in liquid nitrogen and kept inside the cryostat (temperature –26°C to –30°C) for several minutes to equilibrate.

Large changes in temperature or repeated freezing and thawing cycles were carefully avoided to prevent destruction of the original structure of the tissue. Each sample was fixed on a labeled metal object carrier with Tissue Tec™. For better cutting conditions the object temperature was chosen slightly higher (from -20°C to -22°C) than the overall temperature inside the cryostat. The tissue was trimmed until a full section was obtained. Then every 100μm one section was stained with one drop of 1% toluidine-blue solution to select an adequate area for immunohistochemistry. Consecutive sections of the desired area were cut at 10μm thickness using an anti-rolling plate. They were each transferred to a warm (room temperature) slide by lowering the slide over the section, which will then easily adhere. After drying for 1-2 minutes, the sections were fixed for 10 minutes either in acetone at –20°C inside the cryostat and air-dried, or fixed in formaldehyde solution 4% (PFA4%) at room temperature and rinsed in PBS, depending on the staining protocol that followed. One section of each tissue sample was left unfixed for hematoxilin and eosin staining. In order to save tissue and avoid excessive trimming, each metal object carrier was marked in order to recognize the cutting direction.

3.2 Hematoxylin and Eosin staining

One section of each sample was stained with hematoxylin and eosin to provide an overview of the structures. With this standard method, the alkaline hematoxylin stains nuclear structures in blue. Other structures appear in different shades of red (eosin).

Table 2: Staining protocol for Hematoxylin / Eosin

1.	Stain in Hematoxylin without previous fixation	1 min
2.	Rinse in tap water	5 min
3.	Dip in Aqua dest.	5 times
4.	Stain in Eosin solution 2% (Chroma)	2 min
5.	Dip in alcohol 95%	5 times
6.	Dip in alcohol 100%	5 times
7.	Dip in alcohol 100%	5 times
8.	Clear in xylene	10 min
9.	Mount coverglass with Entellan (Merck®)	

3.3 Immunohistochemistry

3.3.1 Method

Immunohistochemical staining can be used to examine the location and distribution of antigens in tissue. Usually the protocols require multiple steps of incubation and washing.

Specific antibodies that bind to the desired antigen are used. In direct methods, these primary antibodies are conjugated to a marker, e.g. a fluorescent color that visualizes the bound complexes. Direct methods normally produce little background but require a large amount of marked antigen-antibody-complexes.

In indirect methods, a secondary antibody, which is conjugated to a marker, binds to the first one and detects the antibody-antigen complexes. These methods are usually more sensitive but less specific than direct stainings.

In enzymatic methods either the primary or the secondary antibody is conjugated to a marker enzyme. Different substrates (=chromogens) are used to visualize the

antigen-antibody binding. The enzyme is developed by the substrate to a colored product in the presence of a catalyst. Enzymatic methods are usually more sensitive than fluorescence methods and the results can be viewed with a regular light microscope.

In the present study the indirect enzymatic immunoperoxidase method was used (Figure 3). The primary antibodies were unconjugated. The secondary antibodies were immunoglobulins, which were conjugated to horseradish peroxidase as marker enzyme. The used substrate was 3,3´Diaminobenzidine (DAB). In the presence of H_2O_2 as a catalyst DAB reacts with the peroxidase to a brown result.

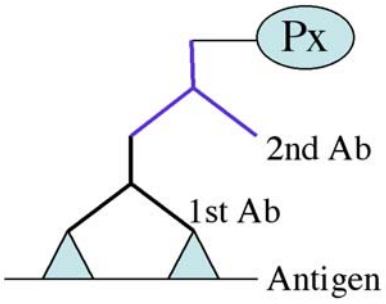


Fig. 3: *Indirect immunoperoxidase method. Px: Peroxidase*

3.3.2 Antibodies

3.3.2.1 Primary antibodies

In each experiment (staining batch) one section of each sample was stained with anti-Ki-67, an antibody that reacts with nuclear cell proliferation associated Ki-67 antigen. This marker is expressed in all active phases of the cell cycle and was used to identify growing collaterals.

The consecutive section was either stained with CD51/61 antibody, which specifically binds to $\alpha_v\beta_3$ integrin or with an antibody specific for vitronectin (Table 3). The exact staining protocols are described in chapter 3.3.4.

Table 3: Primary antibodies

Name	Company	Host	Clone	Fixation	Dilution
Ki-67	Dako Chemicals	Mouse monoclonal	Mib 5	PFA 4%	1:10
CD51/CD61	PharMingen	Mouse monoclonal	23C6	Acetone	1:20
Vitronectin 65	Santa Cruz Biotechnology, Inc.	Goat polyclonal		Acetone	1:50

3.3.2.2 Secondary antibodies

Secondary antibodies were horseradish peroxidase conjugated immunoglobulins from different hosts directed against the host species of the first antibody.

Table 4: Secondary antibodies

Name	Company	Host	Dilution
Anti-mouse IgG Horseradish peroxidase linked whole antibody	Amersham Life science	Sheep	1:100
Bovine anti-goat IgG Horseradish peroxidase conjugated, sc-2350	Santa Cruz Biotechnology, Inc.	Bovine	1:100

3.3.3 Further materials

Table 5: Phosphate buffered saline (PBS)

1.	Stock solution	397.40 g NaCl 10.00 g KC 10.00 g H_2PO4l 88.75 g $Na_2HPO4*H_2O$	Dissolve in 5 l Aqua dest.
2.	Ready-to-use PBS	500 ml stock solution 4500 ml Aqua dest.	Adjust pH carefully to 7.4

Table 6: 4% Formaldehyde solution

1. 2.	40g Paraformaldehyde (Merck) 2-3 cookies sodium hydroxide	Dissolve in 1 l of PBS
3.	Adjust pH to 7.4	

The ready-to-use solution was frozen in aliquots of 50 ml and thawed shortly before use.

Table 7: Mowiol

1.	50g Mowiol (Hoechst) + 200ml PBS	Stir for 16 hours
2.	Add 25ml dehydrated glycerin	Stir for 16 hours
3.	Centrifuge at 12000 rpm	15 min.
4.	Store pellet at 4°C	

Table 8: Citrate buffer

1.	2.1 g citric acid	
2.	1 l Aqua dest.	Dissolve and adjust pH to 6.0

Table 9: Blocking solution

1.	0.03 g BSA (0.1%)	
2.	0.12 g Glycine (0.4%)	Dilute in 30 ml of PBS

3.3.4 Staining protocols

Each staining protocol was developed during several preliminary experiments to carefully determine the optimum conditions. This includes optimum dilutions of each substance and most suitable times as well as optimum fixation and blocking substances.

At least one negative control was included in each batch of sections that was stained. This control section was treated exactly like the others except for the first incubation, in which the control sections were incubated in a solution without first antibody. Additionally, one section of a tissue sample with known positive result was used as positive control to check the quality of the staining.

Conditions were carefully kept the same to obtain comparable results.

Fixation
The fixative was chosen according to trial stainings. *Acetone* fixation leads to quick dehydration and is especially suitable for detection of proteins in cryo sections. Pure acetone was used for fixation of the sections inside the cryostat. Afterwards slides were air-dried.

Fixation with *Formaldehyde (PFA 4%)* causes polymerization and cross-bridging of amido-, amino- and other groups and offers good preservation of the tissue structure. PFA 4% was used at room temperature. Frozen aliquots were thawed shortly before use.

Table 10: Staining procedure for CD51/CD61 or Vitronectin

	Step	Substance	Temp.	Time
1.	Air-Dry		RT	1-2 min
2.	Fix	Acetone	–26°C to –30°C	10 min
3.	Air-Dry		RT	3-5 min
4.	Wash	PBS	RT	4 x 3 min
5.	Block	Blocking solution with BSA and Glycine	RT	30 min.
6.	Wash	PBS	RT	4 x 3 min
7.	1st Incubation	1. Ab=CD51/CD61 or Vitronectin	4°C	over night
8.	Wash	PBS	RT	4 x 3 min
9.	2nd Incubation	2. Ab=Peroxidase	RT	1 hour
10.	Wash	PBS	RT	4 x 3 min
11.	3rd Incubation	DAB	RT	Max. 16 min (see below)
12.	Wash	PBS	RT	3 min
13.	Rinse	Tap water	RT	3 min
14.	Wash	PBS	RT	4 x 3 min
15.	Mounting	Mowiol		

Washing

After each step of fixation, blocking, or incubation, the slides were washed carefully in PBS to take off residues of the previously used substance and to stop the ongoing reaction.

Blocking

Blocking non-specific binding of antibodies with BSA (Bovine Serum Albumine, Fluka Biochemicals, Germany) and Glycine (Fluka Biochemicals, Germany) saturates free reactive groups and reduces background staining.

If the peroxidase method is used, blocking endogenously existing peroxidases can prevent false positive reactions. For this purpose, a solution of PBS with 3% H_2O_2 was additionally used in the protocol for Ki-67.

Microwave treatment in citrate buffer

This process denatures DNA and is necessary for appropriate antigen reassessment for Ki-67 antibody. The slides have to be well covered with buffer throughout this step. They were heated in a microwave at 720 Watt for 15 minutes. Afterwards the sections were allowed to cool down to room temperature slowly to avoid detaching from the slides.

Incubation with 1st and 2nd antibodies

The primary antibodies were diluted to the desired concentration in blocking solution with BSA and Glycine.

The second antibody was diluted in PBS. Each section was covered with 30 μl of the appropriate dilution. The slides were kept in humid chambers during the first and second incubation.

Incubation with DAB

Since DAB is strongly carcinogenic, very strict safety precautions were taken. A 0.3% solution of DAB in PBS was freshly prepared each time. DAB is photosensitive, thus it was covered completely while stirring to dilute. Directly before use, 1 μl of 30% H_2O_2 per ml of solution was added. The sections were then incubated in the solution. Drying of the sections was carefully avoided. The process was stopped with PBS when the signal appeared to be strongest with minimum background. The maximum period of time is known to be around 20 minutes. After this time the signal does not increase anymore. The incubation time in this study ranged from 8 to 16 minutes.

Table 11: Staining procedure for Ki-67

	Step	Substance	Temp.	Time
1.	Dry		RT	1-2 min
2.	Fix	PFA 4%	RT	10 min
3.	Wash	PBS	RT	4 x 3 min
4.	1st Blocking	H_2O_2 3%	RT	15 min
5.	Wash	PBS	RT	4 x 3 min
6.	Cook	Citrate buffer	750 Watt	15 min
7.	Cool down		RT	1 hour
8.	Wash	PBS	RT	4 x 4 min
9.	2nd Blocking	Blocking solution	RT	30 min.
10.	Wash	PBS	RT	4 x 3 min
11.	1st Incubation	1st Ab=Ki-67	4°C	over night
12.	Wash	PBS	RT	4 x 3 min
13.	2nd Incubation	2nd Ab=Peroxidase	RT	1 hour
14.	Wash	PBS	RT	4 x 3 min
15.	3rd Incubation	DAB	RT	Max. 16 min (see above)
16.	Wash	PBS	RT	3 min
17.	Rinse	Tap water	RT	3 min
18.	Wash	PBS	RT	4 x 3 min
19.	Mounting	Mowiol		

Cover Glass Mounting

At the end of each staining procedure the sections were coverslipped for protection and conservation. A small drop of water-soluble prepared mounting media Mowiol™ was placed on the section. A clean cover glass was lowered over the section slowly from an angle and gently pressed on. Care was taken not to trap any air.

4. Western blot analysis

Commercially available antibodies binding to human vitronectin have either not been tested in rabbit tissue or have been shown not to detect rabbit vitronectin. Thus, western blot analysis was performed in the present study to investigate and verify the binding properties of two different anti-human vitronectin antibodies concerning rabbit vitronectin.

4.1 Tissue preparation

Western blot analysis was performed comparing human plasma (positive control), rabbit plasma, and rabbit aorta. The previously cryo-preserved tissue samples were cut in thin cryo-sections, which were collected in Eppendorf tubes, carefully weighed and kept frozen in liquid nitrogen. Cryo-sections were collected until 40-50 mg of the sample were obtained. The weight was recorded exactly and each tissue sample was thawed in 500μl of Ripa buffer and homogenized. Subsequently, the samples were incubated for 30 minutes on ice and centrifuged for 30 minutes at 14,000 rpm. The resulting supernatant fluid is clear cell lysate containing the total protein.

Rabbit and human plasma samples were directly diluted in Ripa buffer and incubated on ice.

The protein concentration in the lysate was determined by standard BCA (bicinchoninic acid)-method [288,289]. This method is based on the reduction of divalent copper (Cu^{2+}) by proteins in alkaline media. The formed complex reacts with BCA to a violet result, which can be photometrically measured to determine the protein concentration of the solution samples.

4.2 Gel-electrophoresis

Electrophoresis is a method that is used to separate different proteins from one sample according to their specific molecular weight. Briefly, proteins are resuspended in LDS sample buffer™ (Invitrogen, 4 times concentrated) and the negatively charged ion sodiumdodecylsulfate (SDS) binds to the hydrophobic part of the protein. Influenced by the resulting negative charge, the proteins move to the anode through an electric field. Small proteins move faster than the ones with a higher molecular weight and thus they separate. A standard electrophoresis unit (Invitrogen) was used. Protein samples were diluted in LDS buffer 4:1 and boiled for 10 minutes for denaturation. Unused samples were stored at -20°C.

Each well was loaded with 30μg of protein lysate for a 4-12% Bis-Tris gel of 10 mm thickness. The first well was filled with 5 μl of multimark molecular weight marker (Invitrogen) for comparison of molecular weights. Electrophoresis was performed for 50 min at 200 Volt and 110 mA.

4.3 Protein transfer

In order to be immunochemically detectable, the separated proteins were transferred from the gel to a nitrocellulose membrane from Invitrogen. A Novex apparatus was used for the transfer according to the manufacturer's protocol with Invitrogen transfer buffer at 170 mA and 30 Volt for one hour.

4.4 Immunochemical detection of protein

The following procedure was done to immunochemically detect and visualize the specific protein bands. Two different primary antibodies for vitronectin were used as shown in table 12.

The secondary antibodies were horseradish peroxidase conjugated. For Vitronectin 65 from Santa Cruz this was the same secondary antibody that was used for immunohistochemistry (table 13).

Table 12: Primary antibodies

Name	Company	Host	Clone	Dilution	kDa
Vitronectin 65	Santa Cruz Biotechnology, Inc.	Goat polyclonal		1:100	65-75
Anti-Vitronectin V7881	Sigma	Mouse monoclonal	Vit-2	1:100	65-75

Table 13: Secondary antibodies

Name	Company	Host	Dilution
Anti-mouse IgG Horseradish peroxidase linked whole antibody	Amersham Life science	Sheep	1:10000
Bovine anti-goat IgG Horseradish peroxidase conjugated, sc-2350	Santa Cruz Biotechnology, Inc.	Bovine	1:10000

An ECL western blotting kit from Amersham Biosciences, which is a light-emitting non-radioactive method, was used for visualization of the horseradish peroxidase conjugated secondary antibodies. Light emission is proportional to the amount of peroxidase-bound protein and can be caught on radio-sensitive film (Hyperfilm ECL, Amersham Biosciences).

The previously blotted membranes were incubated in 50 ml Falcon tubes during the following protocol (table 14):

Table 14: Immunochemical detection of protein in western blot

	Step	Substance	Temp.	Time
1.	1st Incubation	5% blocking solution	4°C	over night
2.	Wash	TBS-T	RT	3 x 15 min
3.	2nd Incubation	1st AB	4°C	over night
4.	Wash	TBS-T	RT	3 x 15 min
5.	3rd Incubation	2nd AB	RT	2 hours
6.	Wash	TBS-T	RT	3 x 15 min
7.	Detection	ECL-reagent	RT	1 min

Table 15: Preparation of TBS-T buffer

1	2.4 g Tris-HCL 0.5 M	Dilute in 1 l Aqua dest., pH 7.4
2	8.00 g NaCl 1 M	
3	1 ml Tween 20	

The used blocking solution contained 5% of non-fat dry milk in TBS-T buffer. It was applied to prevent non-specific binding of the antibodies to the membrane and other proteins. The primary as well as the secondary antibodies were used in previously tested appropriate dilutions in blocking solution according to tables 12 and 13.

After the procedure described in table 14, excess detection reagent was drained off, the membrane was wrapped in saran wrap, placed in an X-ray film cassette together with a sheet of Hyperfilm ECL™, and the film was exposed and developed.

5. Evaluation and documentation

5.1 Qualitative evaluation

A Leica DM-RB microscope was used for evaluations. First the section stained for Ki-67 of each sample was checked in order to differentiate between growing collaterals and non-growing arteries within the section. The corresponding arteries were localized in the consecutive sections that were stained for $\alpha_v\beta_3$ integrin or vitronectin, respectively. These arteries were first evaluated qualitatively. A picture of each artery was captured under standardized conditions using a Leica DC 200 camera system.

5.2 Quantification

The intensity of immunoperoxidase signal was quantified using NIH computer imaging software version 1.62 for MacIntosh. A similar method has been previously described by Dugar et al. [290]. Mean density with a maximum of 256 measurements, field width 9, and 4 digits right of decimal point were the chosen measuring options. Calibration was set to "uncalibrated object density" being:

$$Y=log10\ (255/(255-x)).$$

In this equation x accounts for the mean measured gray value of the area of consideration. Gray values range from 0 (white) to 255 (black). Three separate measurements were performed for each of the following: the arterial wall excluding the adventitia, the media, the endothelium, and the background. The neointima, if present, was considered to be part of the media. Measurements were expressed as optical densities in arbitrary units and Excel Version 2001 software was used for further evaluation. Three groups of arteries per time point were studied: growing collaterals and quiescent arteries after femoral occlusion

and arteries after sham operation (controls). The obtained optical densities were averaged across every three corresponding measurements and the mean background per picture was subtracted from the corresponding mean densities. The resulting values for each area measured (artery, media, and endothelium) were averaged across all arteries of one group and time point.

Within one experiment or time point, the conditions and the DAB exposure time were uniform, but the intensity of immunoperoxidase staining always varies slightly between experiments. Thus, the measurements had to be normalized to facilitate the comparison of the different time points. An additional staining procedure was done to compare controls from different time points. Since no significant differences were found, a factor for each time point was calculated that equalized the control values of all time points. This factor was then used to normalize the optical densities that were measured in growing collaterals and the described analysis was repeated. Optical densities were expressed in arbitrary units (A.U.) ranging from 0 to 0.2.

5.3 Statistical evaluation

Data are presented as mean ± standard error of the mean (SEM). All pictures are shown in the same magnification. A t-test was performed comparing the resulting means of growing collaterals to those of control arteries from tissue after sham operation to investigate statistical significance. In order to identify any significant differences between quiescent arteries after femoral occlusion and control arteries after sham operation, an additional set of t-tests was performed to compare these two groups. A probability-value (P) of ≤ 0.05 was considered statistically significant.

IV. RESULTS

1. Expression of $\alpha_v\beta_3$ integrin and Ki-67

1.1 One day after femoral occlusion

Evaluated were twenty-two arteries 1 day after femoral occlusion and 5 arteries after sham operation. At this early time point, Ki-67 positive cells were not detectable in the wall of arteries from the occlusion group. Thus, growing collateral arteries were selected by hypertrophy of the activated endothelium and their constant localization at the edges of the vastus intermedius of the quadriceps muscle.

The immunoperoxidase signal for $\alpha_v\beta_3$ integrin was just above the level of detection (Fig. 4) and quantification of the intensity of immunostaining did not reveal any significant difference to arteries from sham operated legs (Fig. 5).

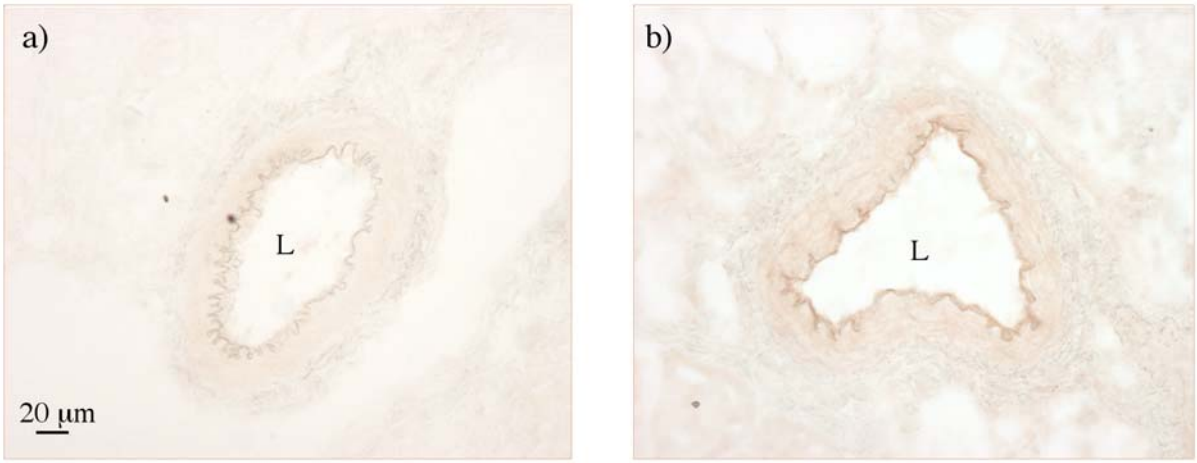


Fig. 4: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in:*
a) Artery after sham operation,
b) Artery 1 day after femoral occlusion.
Both show $\alpha_v\beta_3$ integrin staining intensity just above the level of detection. L=lumen

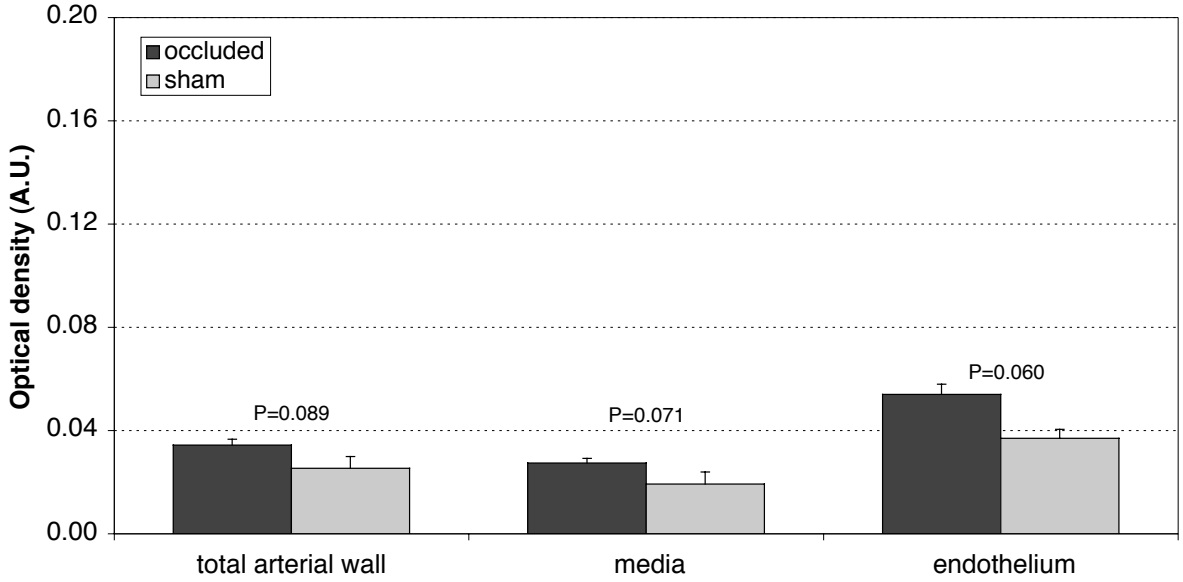


Fig. 5: *Immunoperoxidase signal for $a_v\beta_3$ integrin in arteries 1 day after femoral occlusion and after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated, P≤0.05 was regarded significant.*

1.2 Three days after femoral occlusion

Evaluated were 18 arteries from hind limbs after 3 days of femoral occlusion and 5 arteries after sham operation. Fourteen arteries in tissue after femoral occlusion were clearly positive for Ki-67 and thus identified as growing collaterals. Five of these arteries only showed proliferation, identified by Ki-67, in the endothelium, 3 predominantly in the endothelium but also in the media, and 6 arteries showed similar amounts of proliferating cells in the endothelium and in the media.

A qualitative evaluation of the $\alpha_v\beta_3$ immunostaining allowed the distinction of three different kinds of growing collateral arteries with regard to localization and intensity of integrin staining. Examples of each kind are shown in Figure 6. Figure 7 shows the same arteries in the same sections stained with hematoxylin and eosin after immunoperoxidase labeling. Two proliferating, Ki-67-positive, arteries did not show significant signals for $\alpha_v\beta_3$ integrin in any layer of the arterial wall (endo-/media-), resembling quiescent arteries. The second kind (7 arteries) showed pronounced and clearly stronger staining for $\alpha_v\beta_3$ integrin on the abluminal side of the endothelium but not in the media (endo+/media-), and the third kind, which occurred in 5 arteries, showed staining for $\alpha_v\beta_3$ integrin in both, media and endothelium (endo+/media+), which significantly ($P \leq 0.005$) differed from controls.

The mean optical densities of immunoperoxidase signal in growing collaterals three days after occlusion compared to control arteries are shown in Figure 8. The difference between growing collaterals and controls in the media is not significant probably because most of the growing collaterals did not show staining in the media. In the endothelium significance ($P=0.005$) was achieved.

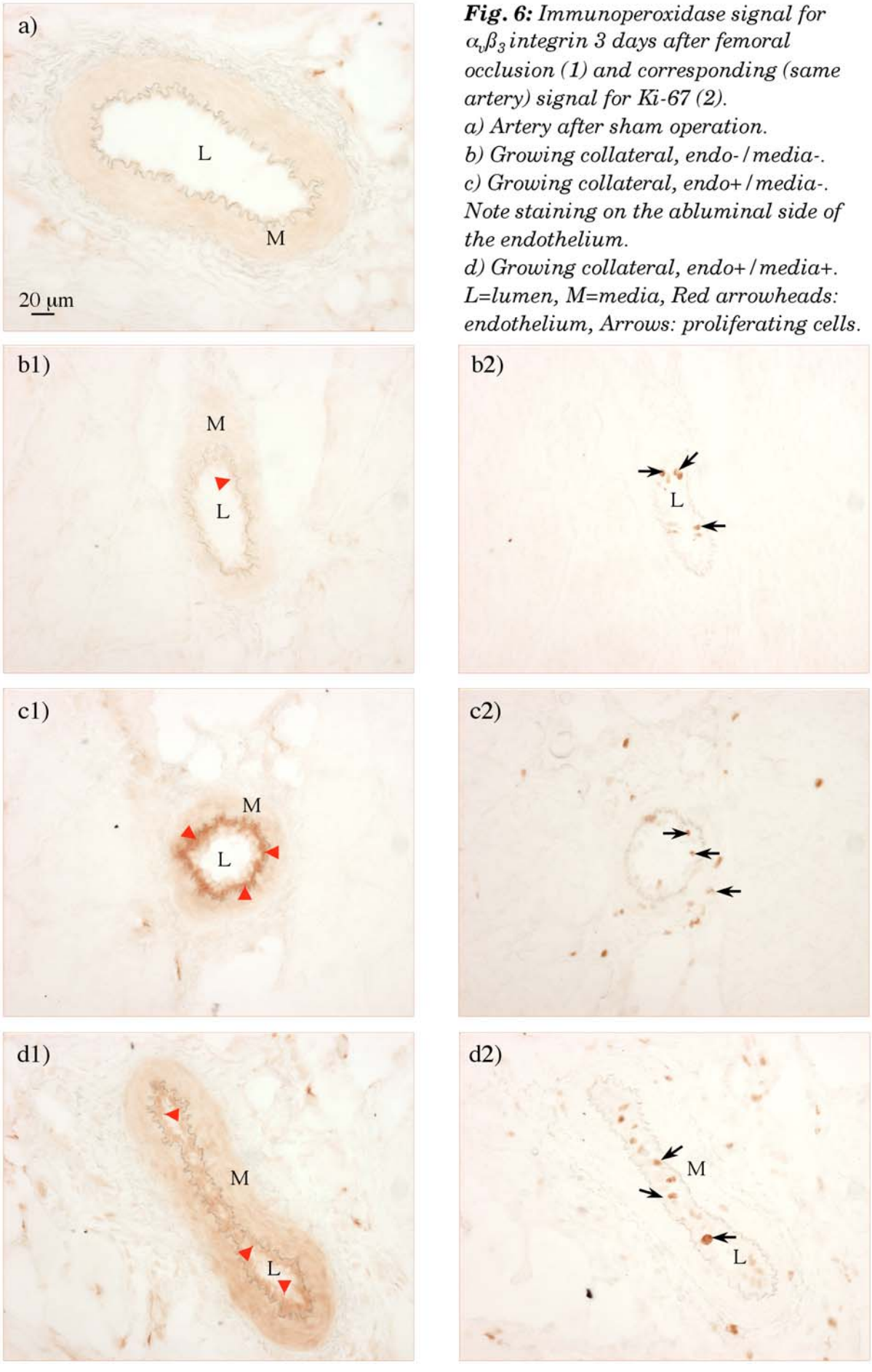


Fig. 6: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin 3 days after femoral occlusion (1) and corresponding (same artery) signal for Ki-67 (2).*
a) Artery after sham operation.
b) Growing collateral, endo- / media-.
c) Growing collateral, endo+ / media-. Note staining on the abluminal side of the endothelium.
d) Growing collateral, endo+ / media+.
L=lumen, M=media, Red arrowheads: endothelium, Arrows: proliferating cells.

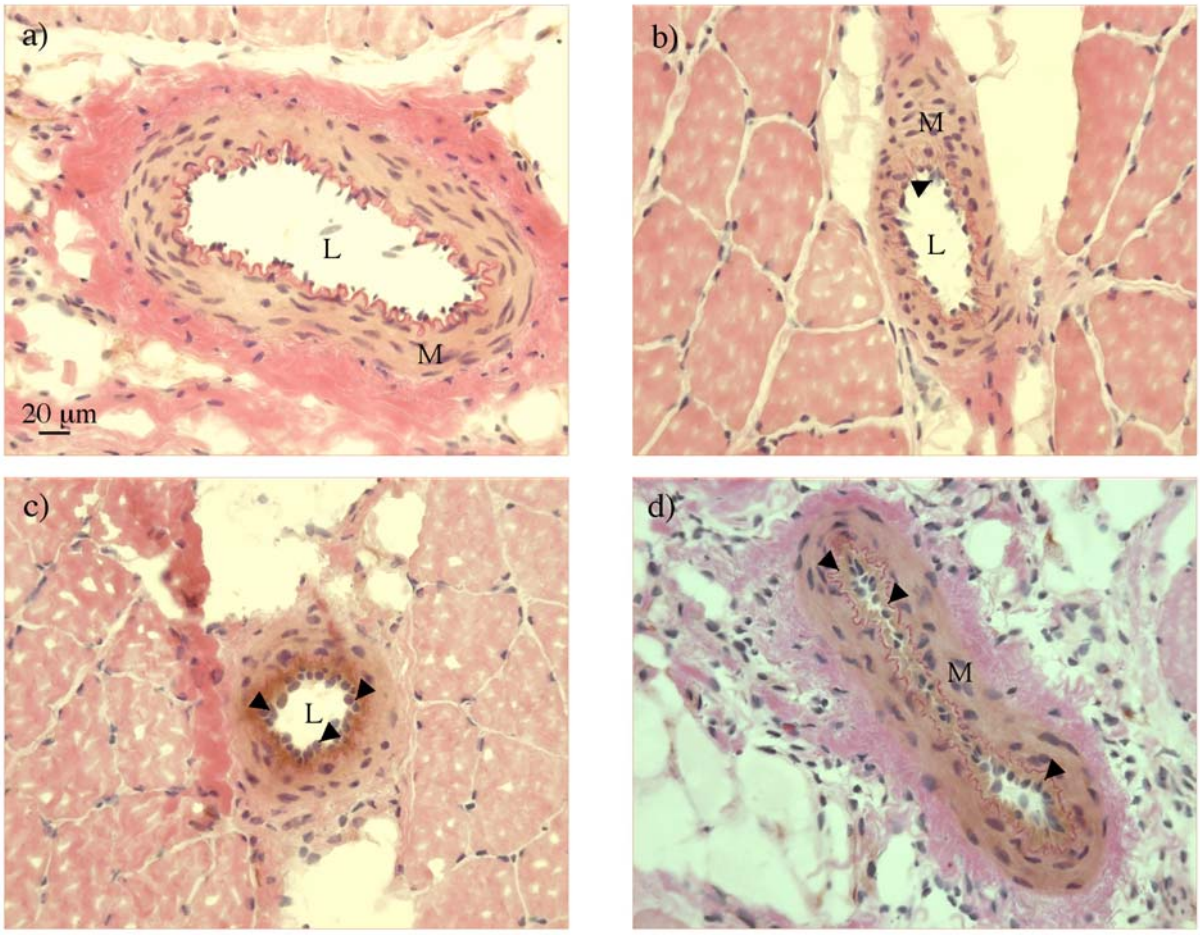


Fig. 7: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin 3 days after femoral occlusion. Same sections as in Fig. 6, counterstained with hematoxilin and eosin.*
a) Artery after sham operation.
b) Growing collateral, endo-/media-.
c) Growing collateral, endo+/media-. Note staining on the abluminal side of the endothelium.
d) Growing collateral, endo+/media+.
L=lumen, M=media, Arrowheads: endothelium.

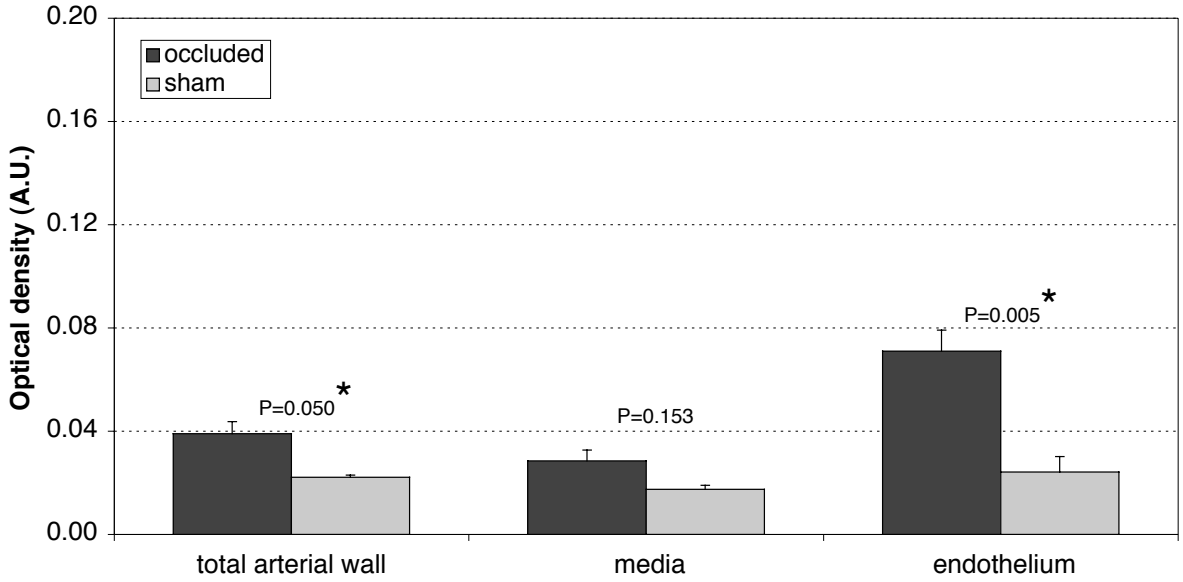


Fig. 8: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in growing collateral arteries 3 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. * indicates statistical significance.*

An additional comparison was made between quiescent, Ki-67 negative arteries from hind limbs after femoral occlusion and quiescent arteries from sham operated samples. The immunoperoxidase signal for $\alpha_v\beta_3$ integrin was just above the level of detection in both groups. No differences were found (Fig. 9).

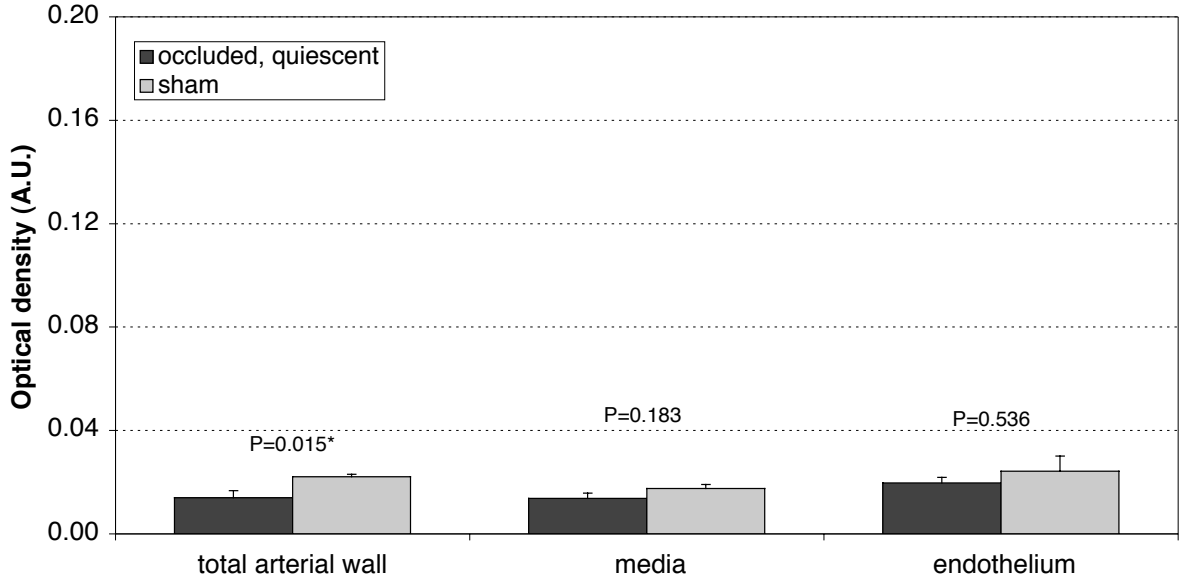


Fig. 9: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in quiescent arteries 3 days after femoral occlusion compared to arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. * indicates statistical significance.*

1.3 Seven days after femoral occlusion

At this time point 16 arteries after femoral occlusion were evaluated, 11 of which were Ki-67-positive collaterals. The other 5 were quiescent arteries. They were compared to 8 arteries from hind limbs after sham operation.

All growing collaterals 7 days after occlusion showed few proliferating cells in both, media and endothelium (Fig. 10, b3 and c3). Three of them showed early neointima formation, which could also be observed in the corresponding hematoxylin / eosin stained sections (Fig. 10, c1 and c2).

The $\alpha_v\beta_3$ integrin immunoperoxidase signal in all growing collaterals reached values that differed significantly from control arteries in endothelium ($P<0.001$) and media ($P<0.001$) as well as neointima (measured together with the media), if present (Fig. 11). The staining pattern was inhomogeneous.

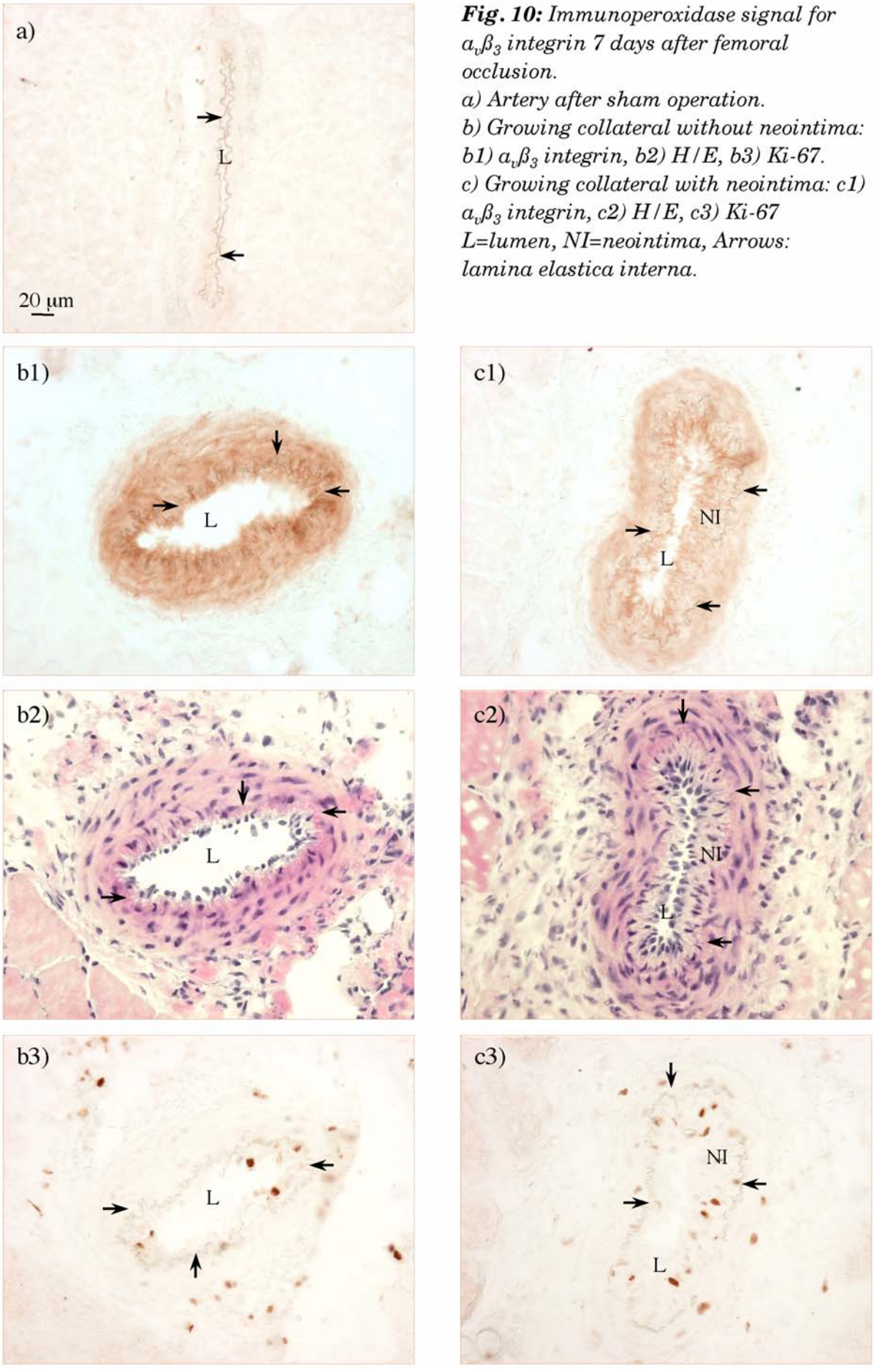


Fig. 10: *Immunoperoxidase signal for $a_v\beta_3$ integrin 7 days after femoral occlusion.*
a) Artery after sham operation.
b) Growing collateral without neointima: b1) $a_v\beta_3$ integrin, b2) H/E, b3) Ki-67.
c) Growing collateral with neointima: c1) $a_v\beta_3$ integrin, c2) H/E, c3) Ki-67
L=lumen, NI=neointima, Arrows: lamina elastica interna.

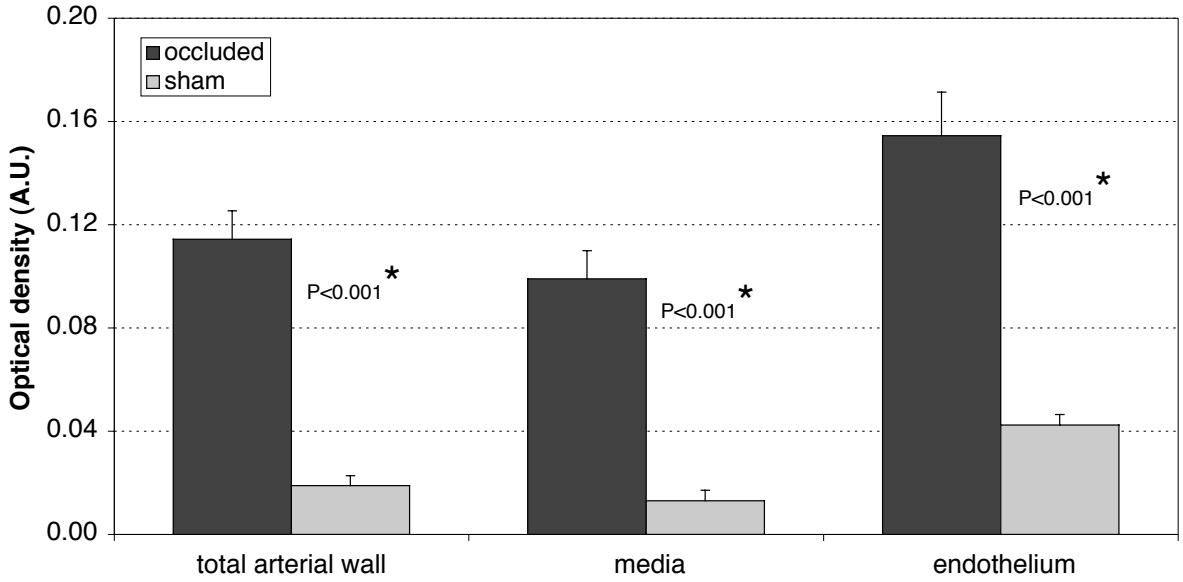


Fig. 11: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in growing collateral arteries 7 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM indicated, P≤0.05 was regarded significant.*
* *indicates significance.*

Again, at this time point, the comparison between arteries after sham operation and quiescent arteries after femoral artery occlusion did not reveal any differences (Fig. 12).

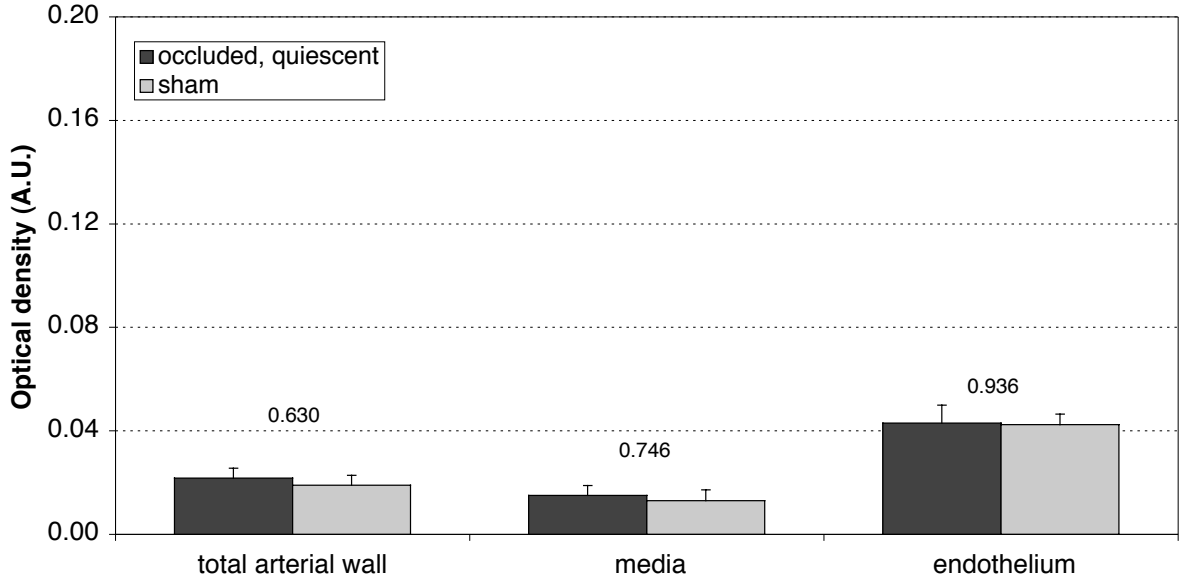


Fig. 12: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in quiescent arteries 7 days after femoral occlusion compared to arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. P≤0.05 was regarded significant.*

1.4 Fourteen days after femoral occlusion

Evaluated were 13 growing collaterals, 6 quiescent arteries from hind limbs after femoral occlusion and 10 arteries from tissue after sham operation. Growing collaterals were easy to identify by the proliferation marker Ki-67, which was expressed in high amounts in the endothelium and in the media (Fig. 13, b2 and c2). Almost 50% of the growing collaterals (6) showed neointima formation and all of them were very large compared to arteries found at earlier time points.

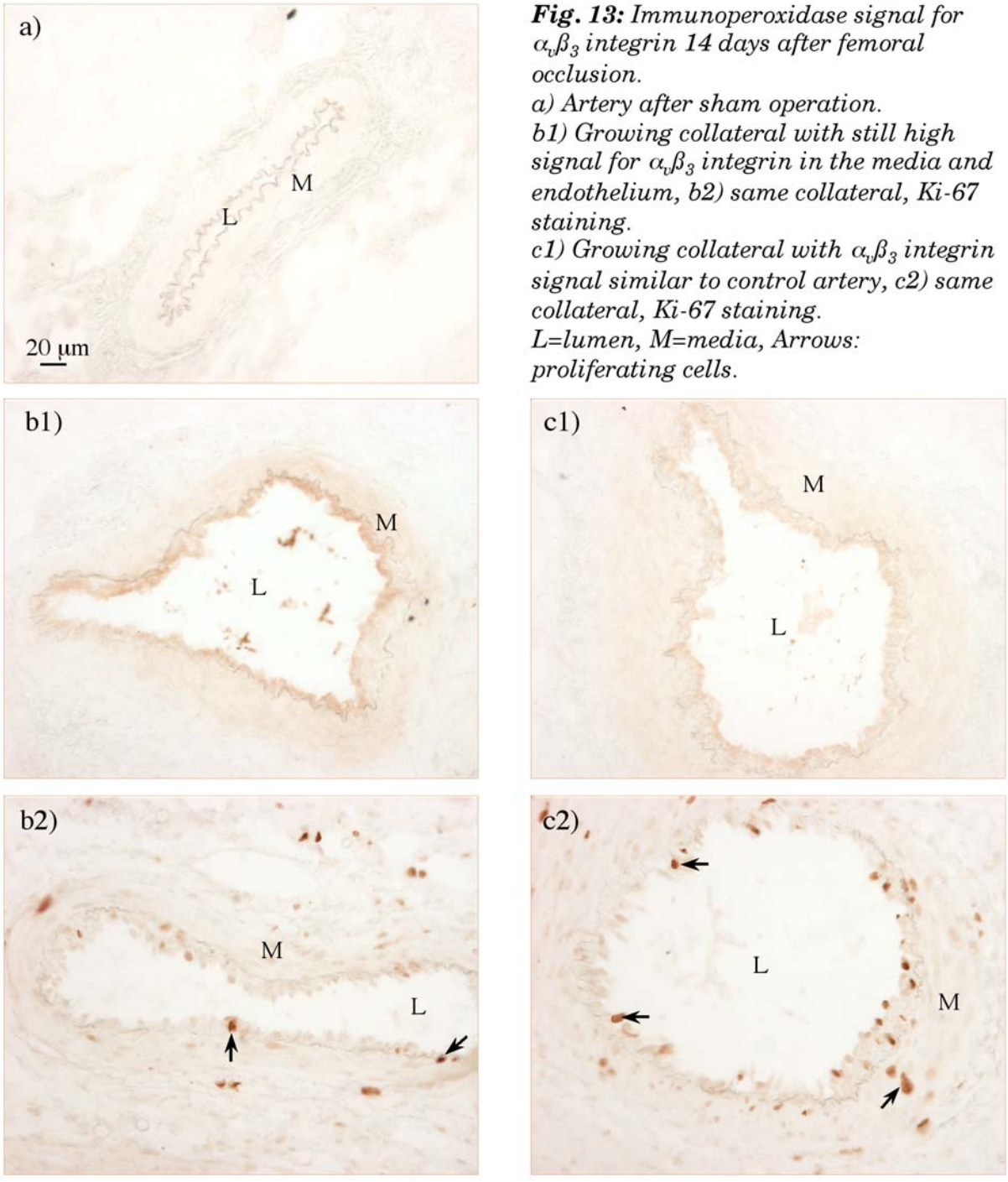


Fig. 13: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin 14 days after femoral occlusion.*
a) Artery after sham operation.
b1) Growing collateral with still high signal for $\alpha_v\beta_3$ integrin in the media and endothelium, b2) same collateral, Ki-67 staining.
c1) Growing collateral with $\alpha_v\beta_3$ integrin signal similar to control artery, c2) same collateral, Ki-67 staining.
L=lumen, M=media, Arrows: proliferating cells.

Some growing collaterals still displayed high levels of $\alpha_v\beta_3$ integrin (Fig. 13, b1) but in most of them, integrin levels were similar to those in control arteries (Fig. 13, a and c1 and Fig. 14).

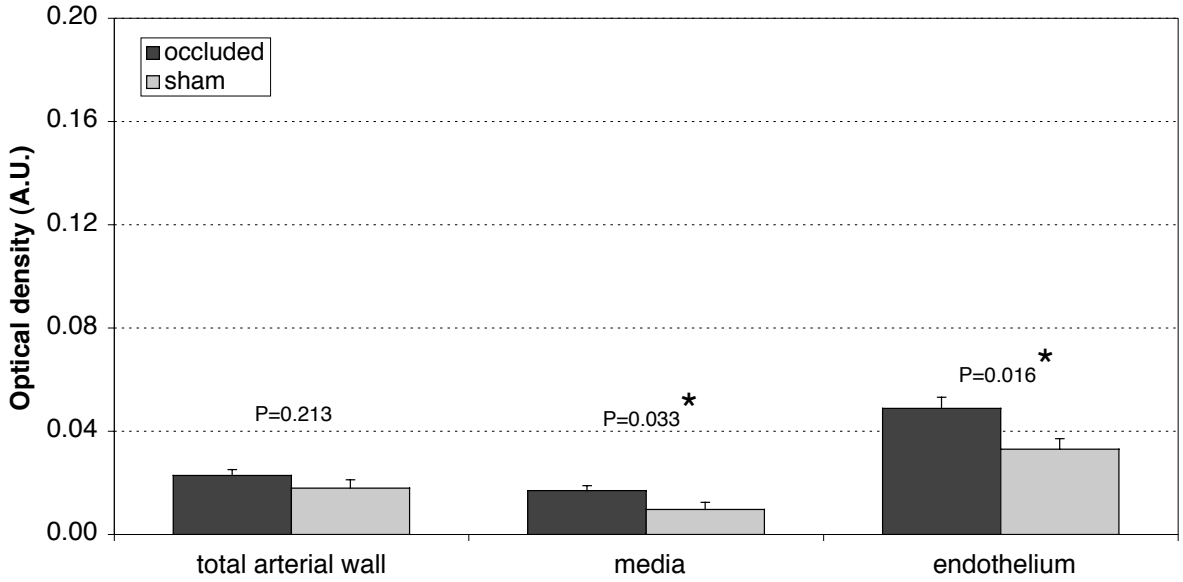


Fig. 14: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in growing collateral arteries 14 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. * indicates statistical significance.*

1.5 Fifty-six days after femoral occlusion

The evaluated arteries were 19 from tissue after femoral occlusion and 11 arteries after sham operation.

None of the arteries were positive for the proliferation marker Ki-67. Thus, growing collaterals were selected by their location in the quadriceps muscle and their histological appearance, especially their unusually large size compared to controls, the existence of a neointima and the appearance of a fragmented lamina elastica interna (Fig. 15, b). In this way 15 of the arteries were identified as growing collaterals. Nine of those showed neointima formation. Compared to earlier time points this neointima was reduced to a thin band (Fig. 15, b) in most of the growing collaterals (6).

In all growing collaterals at this time point the intensity of immunoperoxidase staining for $\alpha_v\beta_3$ integrin was similar to that in arteries from tissue after sham operation and statistical evaluation did not show differences (Fig. 16).

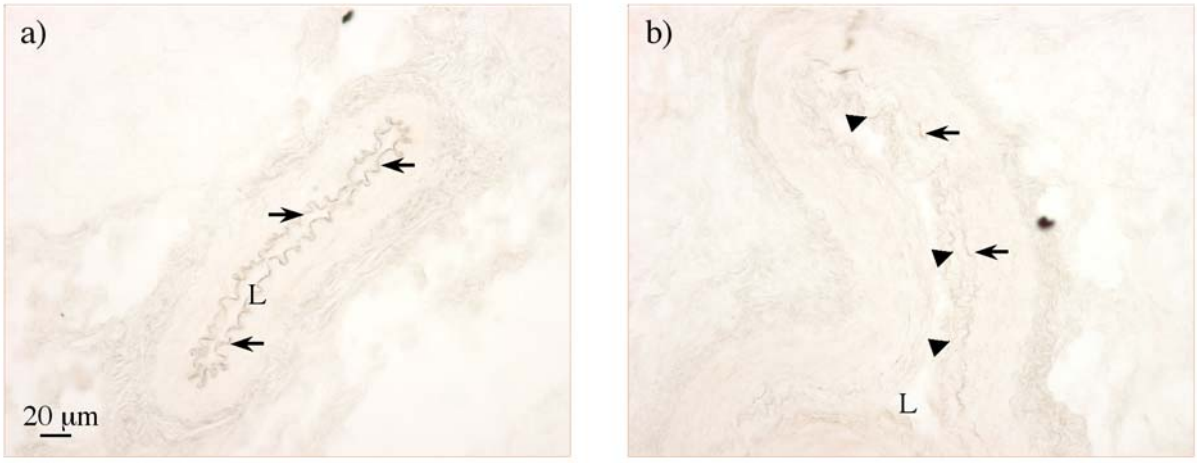


Fig. 15: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin 56 days after femoral occlusion.*
a) Artery after sham operation.
b) Growing collateral 56 days after femoral occlusion.
Both show a similar staining pattern and intensity, the collateral has remarkably grown, and the old and the newly built internal elastic lamina are visible. The neointima is a thin layer. L= lumen, Arrows: lamina elastica interna, Arrowheads: new lamina elastica interna.

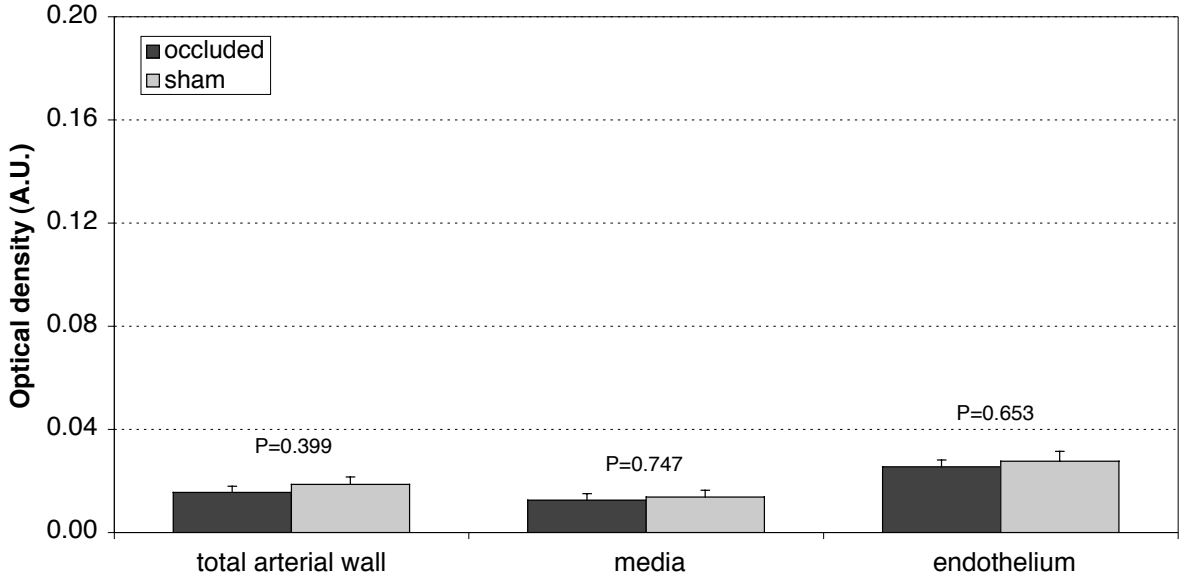


Fig. 16: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in growing collateral arteries 56 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. P≤0.05 was regarded significant.*

1.6 Comparison of findings

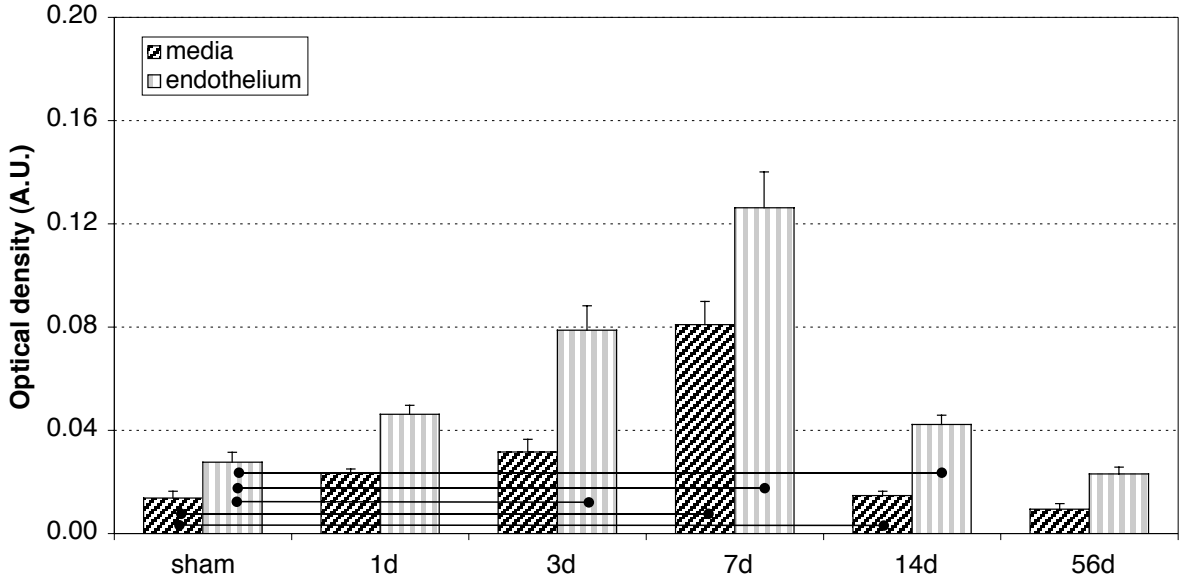


Fig. 17: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin in the media and endothelium of growing collateral arteries at different time points after femoral occlusion and of arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. Connecting lines indicate significance.*

A comparison of the results of $\alpha_v\beta_3$ integrin staining and quantification of the staining intensity for $\alpha_v\beta_3$ integrin at different time points is shown in Figures 17 and 18. Quiescent arteries showed staining for $\alpha_v\beta_3$ integrin in the endothelium and in the media just above the level of detection. The intensity of staining was clearly enhanced after femoral occlusion. After 3 days of occlusion, an increase in $\alpha_v\beta_3$ integrin peroxidase signal was evident in most of the growing collaterals, reaching statistical significance in the endothelium. The increase then spread to the media, reaching maximum staining intensity 7 days after femoral occlusion. After 14 days of occlusion most growing collaterals already showed a decreased level of integrin staining similar to control arteries. Finally, after 56 days of occlusion, $\alpha_v\beta_3$ integrin levels were back to normal in all growing collaterals.

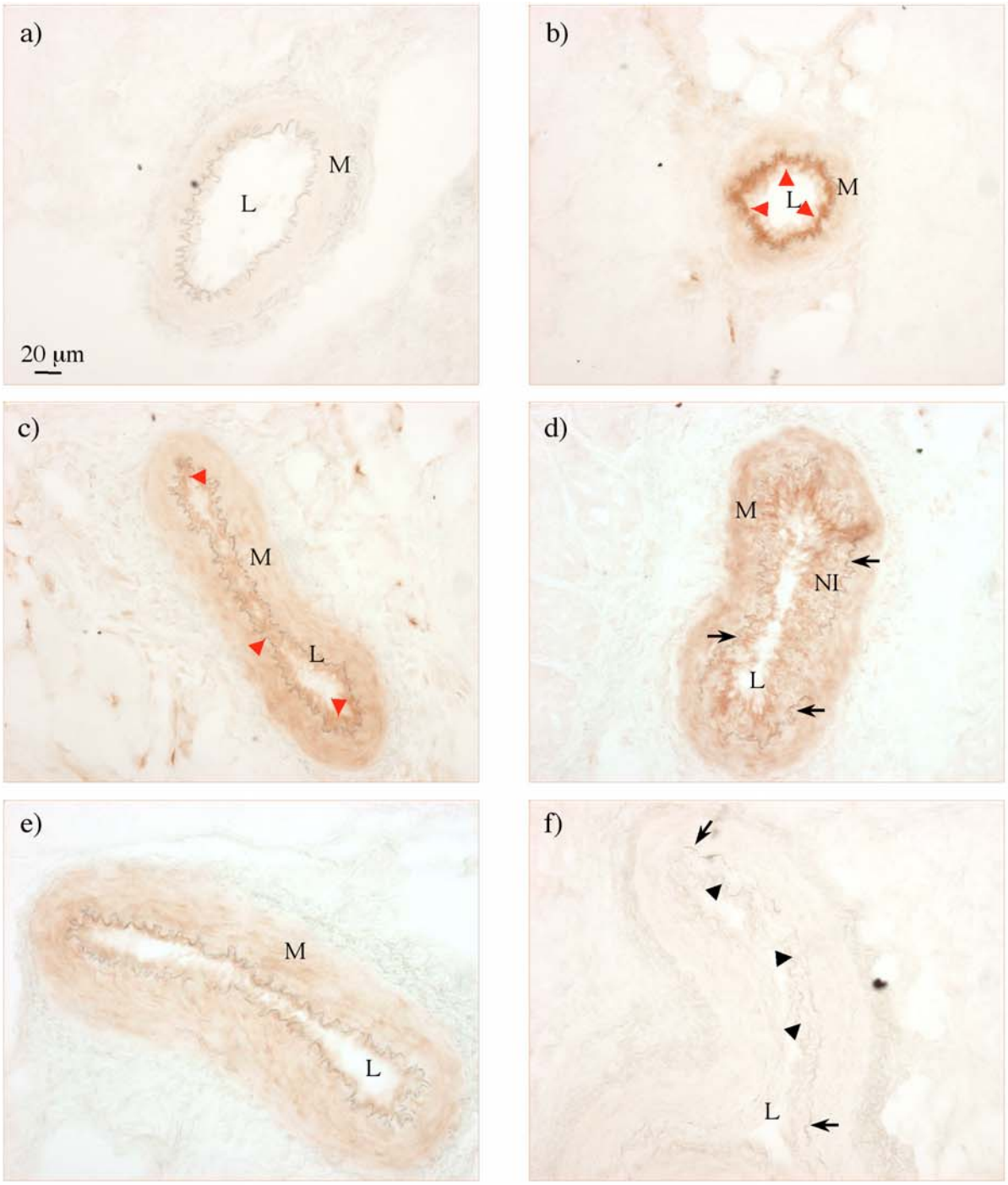


Fig. 18: *Immunoperoxidase signal for $\alpha_v\beta_3$ integrin at different time points after femoral occlusion.*
a) Artery after sham operation: integrin signal is just above the level of detection.
b) Artery 3 days after femoral occlusion: immunoperoxidase signal for $\alpha_v\beta_3$ integrin is strong in the endothelium.
c) Artery 3 days after femoral occlusion: immunoperoxidase signal for $\alpha_v\beta_3$ integrin is evident in the endothelium and media.
d) Artery 7 days after femoral occlusion: integrin signal is strong in endothelium and media.
e) Artery 14 days after femoral occlusion: $\alpha_v\beta_3$ integrin returns to basal levels.
f) Artery 56 days after femoral occlusion: integrin levels are back to basal levels, and the artery has strongly increased in size. L=lumen, M=media, NI=neointima, Arrows: old internal elastic lamina, Black arrowheads: new internal elastic lamina, Red arrowheads: endothelium.

2. Vitronectin

Since the immunoperoxidase signal for $\alpha_v\beta_3$ integrin reached maximum values 7 days after femoral occlusion and was returning to normal levels 14 days after occlusion, these two important time points were further investigated for the expression and localization of the main $\alpha_v\beta_3$ integrin ligand: vitronectin.

2.1 Western blot

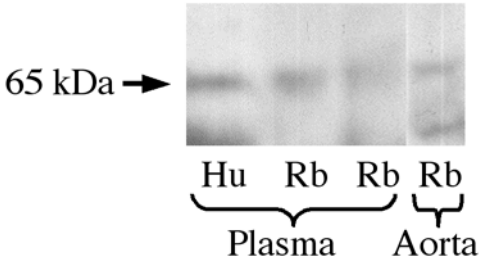


Fig. 19: *Western blot of Vitronectin 65 on samples of human (Hu) and different rabbit (Rb) plasma and rabbit aorta. The blot shows similar bands for different rabbit and human samples at 65 kDa.*

The test for specificity of two vitronectin antibodies by Western blot showed similar bands at 65 kDa for different human and rabbit plasma samples and a rabbit aorta sample only with Vitronectin 65 from Sigma.

2.2 Expression of vitronectin seven days after femoral occlusion

Evaluated were 10 growing collaterals, identified by the presence of proliferation detected with Ki-67 antibody, 7 quiescent arteries from hind limbs after femoral occlusion and 16 arteries after sham operation. The immunoperoxidase signal for vitronectin in quiescent arteries showed a homogeneous pattern within the endothelium or media but the intensity was higher in the endothelium (Fig. 20a). In growing collaterals vitronectin staining in the endothelium was more irregular (Fig. 20b) and significantly ($P<0.001$) lower (Fig. 21) than in the endothelium of arteries after sham operation.

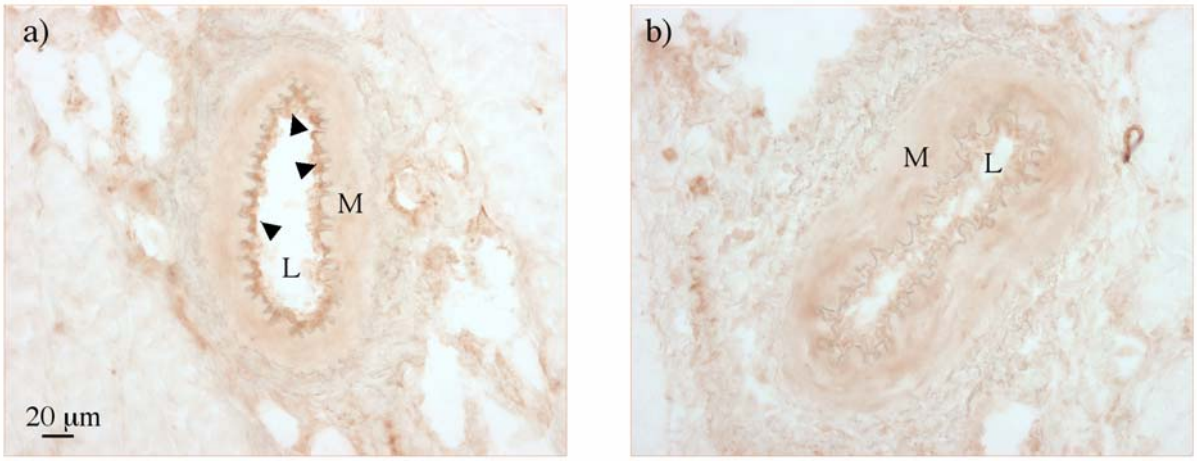


Fig. 20: *Immunoperoxidase signal for vitronectin in:*
a) Artery after sham operation showing strong vitronectin staining in the endothelium (arrowheads).
b) Growing collateral 7 days after femoral occlusion: Immunoperoxidase signal for vitronectin is clearly weaker.
L=lumen, M=media, Arrowheads: endothelium.

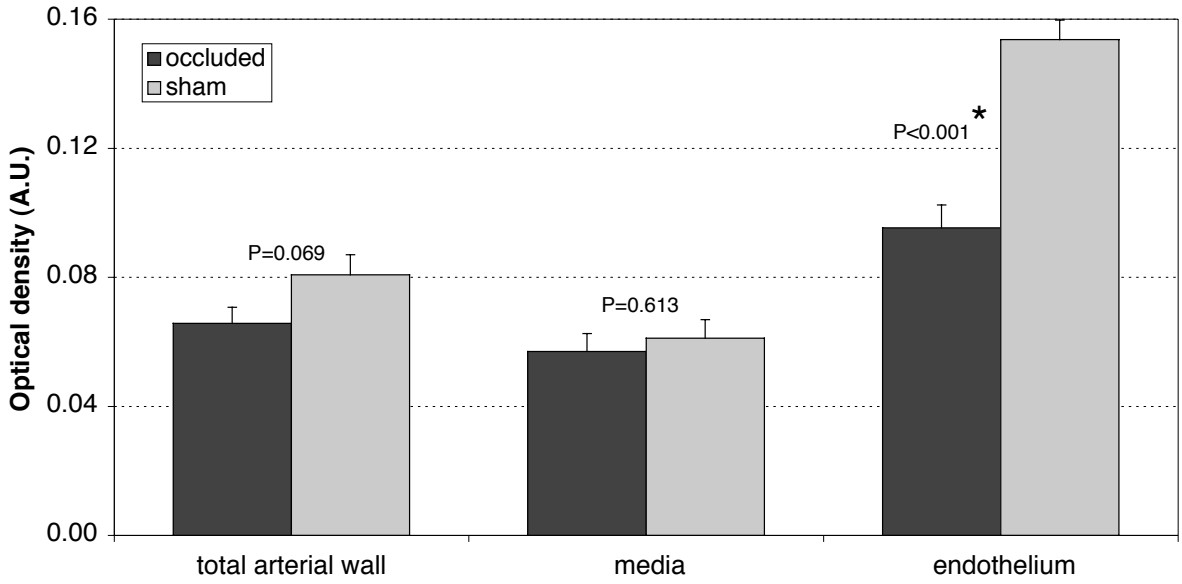
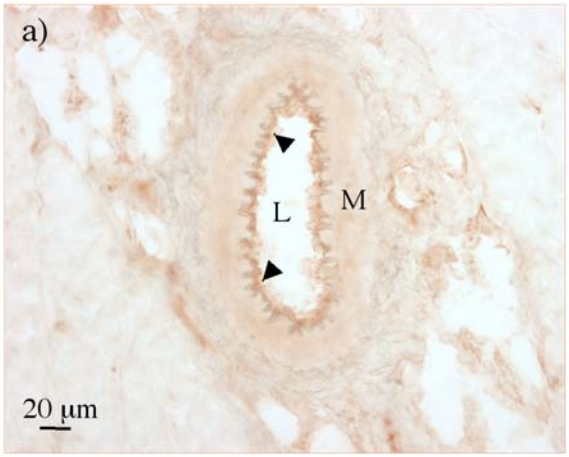


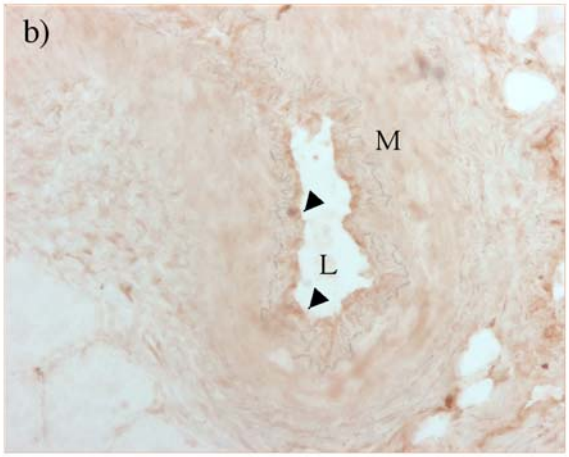
Fig. 21: *Immunoperoxidase signal for vitronectin in growing collateral arteries 7 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. * indicates statistical significance.*

2.3 Expression of vitronectin fourteen days after femoral occlusion

Evaluated were 7 growing collaterals, 8 quiescent arteries after femoral occlusion and 17 arteries from sham operated animals.

Some growing collaterals still showed significantly lower levels of immunoperoxidase signal for vitronectin in the endothelium than arteries after sham operation (Fig. 22b), while in other growing collaterals the vitronectin amount in the endothelium returned to initial high levels (Fig. 22c). The differences found in the quantitative analysis were smaller than at 7 days after occlusion (P=0.032, Fig. 23). Quiescent arteries after occlusion resembled arteries after sham operation.





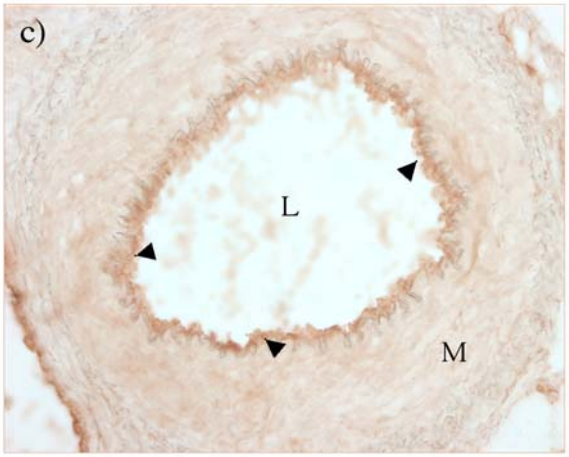


Fig. 22: *Immunoperoxidase signal for vitronectin 14 days after femoral occlusion in:*
a) Artery after sham operation with high vitronectin signal in the endothelium,
b) Artery 14 days after femoral occlusion: vitronectin signal low in endothelium and media,
c) Artery 14 days after femoral occlusion: vitronectin signal back to basal high levels in the endothelium.
L=lumen, M=media, Arrowheads: endothelium.

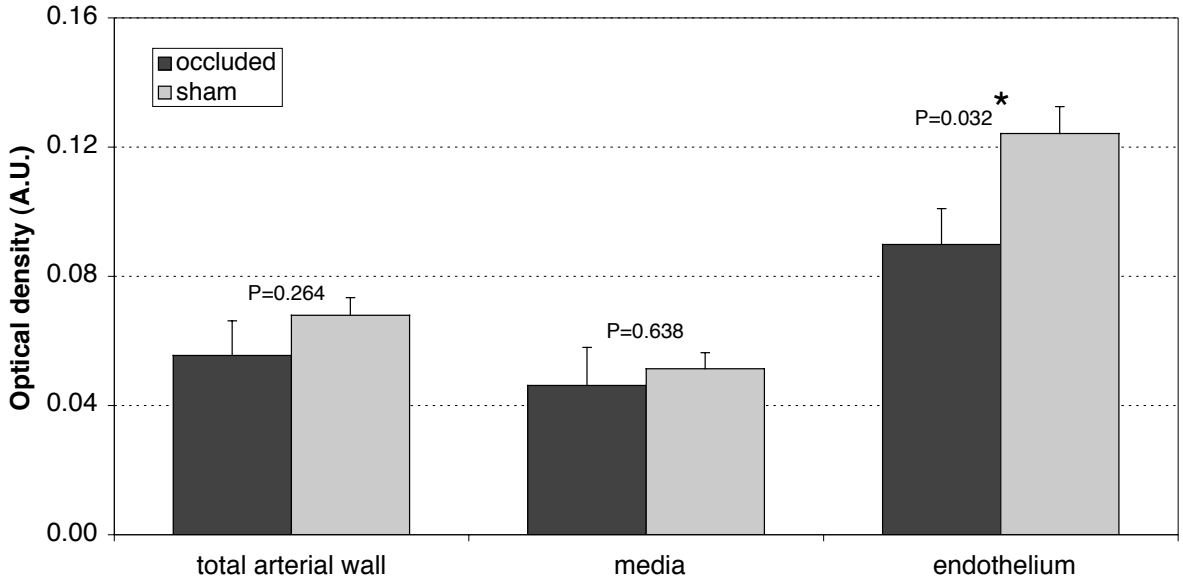


Fig. 23: *Immunoperoxidase signal for vitronectin in growing collateral arteries 14 days after femoral occlusion and in arteries after sham operation. The mean optical density is expressed in arbitrary units (A.U.). SEM is indicated. * indicates statistical significance.*

V. DISCUSSION

The role of $\alpha_v\beta_3$ integrin is well investigated, especially in the vascular system. It has been shown to play an important role in restenosis after vascular injury, in neointima formation and angiogenesis. However, the specialized functions and the exact mechanisms of action are not known and the degree of importance of $\alpha_v\beta_3$ integrin remains unclear, not at last due to the differences between gene ablation and blocking experiments. Moreover, the already known facts about $\alpha_v\beta_3$ integrin in angiogenesis give rise to the question about its role in arteriogenesis. This topic is of special interest since on one hand, $\alpha_v\beta_3$ integrin might reveal possibilities of enhancing arteriogenesis and, on the other hand, using integrin blockers in restenosis prevention might interfere with arteriogenesis, which is an endogenous mechanism of fighting against vascular obstructive diseases.

Thus, the aim of the present study was to investigate the role of $\alpha_v\beta_3$ integrin and its main ligand vitronectin in arteriogenesis.

The results show a time course dependent upregulation of $\alpha_v\beta_3$ integrin together with a downregulation of vitronectin and a later return of both to basal levels. These data clearly indicate that $\alpha_v\beta_3$ integrin plays a role in arteriogenesis and that in this case vitronectin is not the involved $\alpha_v\beta_3$ integrin ligand.

1. The rabbit hind limb model

In this study, the rabbit hind limb model of ischemia was used to investigate arteriogenesis. It is based on the experimental occlusion of the main feeding artery of the limb, the arteria femoralis. It is occluded by two single ligations proximally to the orifices of the arteria poplitea but distal to the branching-point of the arteria profunda femoris leaving the arteria profunda femoris, the arteria circumflexa femoris lateralis and circumflexa abdominis patent. Preexisting collateral arteries are spanning from the arteria profunda femoris and arteria circumflexa femoris lateralis to the arteriae genualis and saphena parva [118].

It is assumed that no unidirectional net forward flow occurs in these collaterals “at rest” [108] but after femoral occlusion the blood takes the path of lowest resistance along these vessels, directed blood flow is established, and blood flow in the receiving artery can reverse [9,108]. The increased shear stress in the collaterals initiates the process of arteriogenesis and the vessels become more easily visible by different methods because they grow [12,81]. Figure 24 shows a schematic view of the arteries in the rabbit hind limb before and after femoral occlusion.

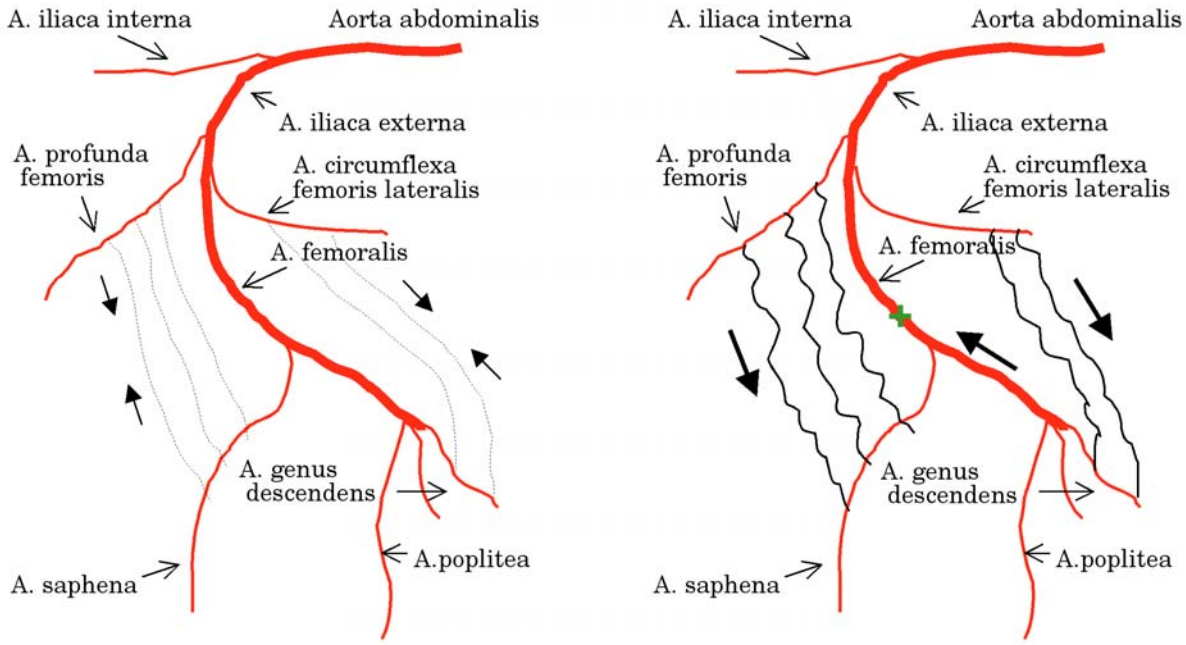


Fig. 24: *Schematic view of arteriogenesis in the rabbit hind limb after femoral occlusion. left: pre-, right: post-occlusion,* ✚ *indicates the occlusion site, arrows indicate blood flow.*

The process of collateral growth in the rabbit hind limb very much resembles the process previously investigated in canine coronary collaterals including neointima formation. Furthermore, arteriogenesis has been studied in other models and it has been shown that the similarities between species and organs are strong enough to arrive at general conclusions [10,15,81]. Compared to other species like mice, which are used for research, rabbits show constant distribution and development of collaterals, which are large enough to be analyzed immunohistochemically, within the quadriceps muscle. Evidently, femoral occlusion of the rabbit hind limb is a good model for research in arteriogenesis.

Another advantage of this model is the fact that both, arteriogenesis and angiogenesis, occur but spatially separated. Part of the lower leg becomes ischemic and angiogenic while arteriogenesis proceeds in the upper thigh muscles where oxygen levels remain unchanged [114]. Thus, both processes can occur in the same tissue at the same time and arteriogenesis can be studied independently and without interference of angiogenic processes.

Nevertheless, both mechanisms are functionally linked. In case of development of large collaterals, which supply blood to the lower limb, the hypoxic stimulus for angiogenesis is eliminated and thus arteriogenesis can indirectly inhibit angiogenesis [291]. The growth of preexisting collaterals is the most efficient mechanism to maintain blood supply to tissue and organs in case of a gradual stenosis or occlusion of a major conducting artery. Collaterals can be recruited as natural bypasses to the occlusion site immediately and their protective role

largely depends on the ability to increase their diameter within short periods of time [15,49]. Since flow is related to the diameter in the fourth potency (Hagen-Poisseuille's law), an enormous amount of capillaries would be required to replace a large conductance artery. Capillary networks are created to locally deliver nutrients and oxygen, rather than to conduct large volumes of blood. Consequentially, angiogenesis is ineffective in case of stenosis or occlusion, whereas collaterals after adaptive growth can transport large amounts of blood and guaranty perfusion. Furthermore, due to the muscular media, blood supply can be adjusted to changing needs. Unfortunately, in spite of remarkable growth, the arteriogenic process stops prematurely and only one third of the conductance of the replaced artery is reached [12,15,109]. Thus, it is important to thoroughly understand arteriogenesis and find therapies to improve, prolong or accelerate the process.

The rabbit hind limb model of ischemia is a suitable model for investigation of the important mechanism of arteriogenesis, which is initiated after arterial occlusion.

Some scientists prefer a model of total excision of the femoral artery. In that model all major branches of the femoral artery as well as the external iliac artery are ligated, and the femoral artery is completely excised from its origin at the external iliac artery to the distal point of bifurcation into the saphenous and popliteal arteries. This results in retrograde thrombus propagation and blood flow depends upon flow through the internal iliac artery [292,293]. In contrast to our model of femoral artery occlusion, this can cause severe gangrene, problems of wound healing, or gross impairment of function. The surgical procedure of mere femoral occlusion is only slightly invasive and none of the mentioned problems can be observed. Thus, influence of wound healing or organization of muscle necrosis on experimental results can be excluded [114] and the model used in the present study is also preferential for animal protection reasons.

2. Evaluation of methods

In the present study we performed numerous trial protocols for immunostaining of $\alpha_v\beta_3$ integrin, such as the direct and indirect immunoperoxidase method as well as direct and indirect immunofluorescence protocols. Finally, we have found that the method of direct immunoperoxidase staining was more sensitive in labeling $\alpha_v\beta_3$ integrin than others. It was sensitive enough to detect even small signals and thus made quantification possible. With this method every staining batch turns out slightly different even under standardized conditions. For this reason,

all tissue samples of one time point and corresponding tissue from sham operations were stained in one batch. Conclusions concerning the absolute amount of protein cannot be made but by this means the relation between the staining intensities of different groups can be determined. Quantification with NIH software was useful in expressing the differences or similarities in numbers and to verify the qualitatively suggested results. Furthermore, it enabled us to evaluate the results statistically. Similar methods for quantification have been previously used by Corjay et al. [23] and by Dugar et al. [290].

Since we wanted to compare the intensity of immunoperoxidase signals for $\alpha_v\beta_3$ integrin at different time points, a method had to be found to obtain comparable values. An additional comparison between arteries from tissue after sham operation from different time points stained together in one batch did not reveal significant differences. Furthermore, no significant differences in $\alpha_v\beta_3$ integrin staining could be found between quiescent arteries of occlusion samples and quiescent arteries after sham operation at any investigated time point. Thus, the process of integrin upregulation is restricted to growing collaterals. Consequentially, any quiescent artery after sham operation or after femoral occlusion could be used as a control for comparison to growing collaterals, indifferent, at which time point it was obtained.

Moreover, it was legitimate to calculate a factor that equalized the control values of the different staining procedures (time points) and to convert the experimental values with this factor to make measurements from different time points comparable.

3. Proliferation in growing collaterals

It has been shown previously that proliferation is confined to growing collaterals [98]. Thus, we used a Ki-67 antibody to identify those. Ki-67 antibody is a marker known to detect proliferation in mammalian cells, even though the exact role of the protein that it binds to is not known [294].

One day after femoral occlusion no proliferation could be detected with this method. For this reason, growing collaterals were selected by their typical morphology (hypertrophy of activated endothelium) and the constant distribution at the edges of the vastus intermedius of the quadriceps muscle. Hypertrophy is a characteristic feature of the activated endothelium in the first stage of arteriogenesis due to loss of volume control. Together with increased endoplasmic reticulum and free ribosomes, it is typical for the proliferative phenotype of endothelial cells. Chloride channels that are responsible for volume control open,

and the ECs appear swollen in scanning electron microscopic images [10,12,97,98]. As a result of the endothelial activation and upregulation of certain cytokines like MCP-1 and ICAM and VCAM, circulating monocytes are attracted, and adhere to and invade the collateral arteries. In turn, they themselves become activated, transform into macrophages [86], and produce more cytokines and growth factors [12,62,99,100], which further enhances proliferation and thus the growth process.

The results for Ki-67 clearly show that proliferation starts in the endothelium around day three after femoral occlusion and then spreads to the media, continuing in these two parts of the arterial wall up to 14 days and ending somewhere between 14 and 56 days after femoral occlusion. Proliferation in the present model is strongest between 7 and 14 days, which is also the time of neointima formation. This corresponds to a study of neointima formation in the rabbit femoral artery by Corjay et al. [23] that showed development of a concentric neointima by 2 weeks after placing a non-occlusive cuff around the vessel. In their study, proliferation determined by bromodeoxyuridine incorporation peaked between day 3 and 5, which could be on one hand due to the different method of detection but on the other hand is due to the different process that is investigated. Since only about half of the growing collaterals showed neointima formation by 14 days, this process seems to often occur during arteriogenesis but it is not a required element. The large size of the growing collateral arteries at 14 days after femoral occlusion compared to arteries from earlier time points additionally shows that the phase of active growth ends after 14 days and is then followed by maturation of the grown vessel [81].

Finally, at 56 days after femoral artery occlusion growing collaterals again had to be selected by their localization at the edges of the vastus intermedius of the quadriceps muscle, their size, and their histological appearance, which is at this later time point a still fragmented old lamina elastica interna together with a new one. The lamina elastica interna is degraded by MMPs during the synthetic phase of arteriogenesis in order to facilitate SMC migration and a new one is built by the new SMCs [12,104,105]. This is histologically visible at 56 days after femoral occlusion because the process of remodeling of the artery is not finished yet.

4. $\alpha_v\beta_3$ integrin

One day after femoral occlusion, quantification of the staining intensity for $\alpha_v\beta_3$ integrin did not reveal significant differences between growing collaterals and quiescent arteries. However, the probability values for the differences were at the limit of significance. Therefore, upregulation of $\alpha_v\beta_3$ integrin may already start one day after femoral occlusion.

The presence of three kinds of growing collateral arteries showing different staining intensity and location regarding $\alpha_v\beta_3$ integrin staining at three days after femoral occlusion together with the absence of growing collaterals showing $\alpha_v\beta_3$ integrin staining only in the media demonstrates that integrin upregulation (as well as proliferation) starts in the endothelium, becomes evident at 3 days after occlusion, and later spreads to the media. The heterogeneity in integrin staining indicates variability in the starting time of arteriogenesis in different collaterals within the same muscle or area. This is probably due to differences in blood flow and shear stress, which is the initiating event, between collaterals. Proliferation detected with Ki-67 does not strictly correlate with $\alpha_v\beta_3$ integrin upregulation, as 15% of the already proliferating collaterals 3 days after femoral occlusion did not show increased levels of $\alpha_v\beta_3$ integrin. This proves that the function of $\alpha_v\beta_3$ integrin in arteriogenesis is different from stimulation of mitosis.

Since the staining intensity for $\alpha_v\beta_3$ integrin in the media 7 days after femoral occlusion was equal to that in the beginning neointima, if present, and since both consist of smooth muscle cells, media and neointima were measured together as one layer. At this time point $\alpha_v\beta_3$ integrin levels in endothelium, media, and neointima reached maximum values with an average 6-fold increase in $\alpha_v\beta_3$ integrin in comparison to quiescent vessels. Apparently, at 7 days post occlusion, the growth process had spread to endothelium and media of all growing collaterals. At 14 days after femoral occlusion, the stage of proliferation reaches a maximum, while $\alpha_v\beta_3$ integrin is already downregulated. Thus, the functions fulfilled by $\alpha_v\beta_3$ integrin in arteriogenesis must be important in the earlier stages of growth.

Another important finding of this study is the localization of $\alpha_v\beta_3$ integrin signals especially on the abluminal side of the endothelium. *In vitro* studies of $\alpha_v\beta_3$ integrin expression in endothelial cell layers subjected to elevated shear stress led to the same characteristic localization [295]. Since shear stress is regarded to be the initiating event of arteriogenesis, it can be presumed that the abluminal localization of $\alpha_v\beta_3$ integrin is a result of elevated shear stress. However it is not known, if this pattern is a characteristic general feature of endothelial cell

reaction or if it is a specific response to shear stress. The first would be more reasonable because active $\alpha_v\beta_3$ integrin located on the luminal side of the endothelium might be highly thrombogenic due to its apparent functions in atherosclerosis and restenosis [154,274].

In summary, the present results demonstrate a time-dependent upregulation of $\alpha_v\beta_3$ integrin levels selectively in the wall of growing collaterals during arteriogenesis. The upregulation is detectable in the endothelium 3 days after femoral occlusion and spreads to the SMCs of the media reaching maximum concentration 7 days after femoral occlusion. By 14 days after occlusion integrin levels are returning to control values. This clearly indicates that $\alpha_v\beta_3$ integrin plays a role in the early events of arteriogenesis.

5. Vitronectin

An important function of $\alpha_v\beta_3$ integrin in vascular biology is the promotion of SMC migration, especially from the media to the neointima, as it occurs after vascular injury or in atherosclerotic and restenotic vessels [27,160]. Vitronectin, the main ligand of $\alpha_v\beta_3$ integrin is known to promote cell adhesion, spreading, and migration by interacting with integrins [25,26]. Vitronectin-binding to $\alpha_v\beta_3$ integrin has been shown to mediate SMC migration during neointima formation in atherosclerotic arteries and restenosis [154,274]. Thus, we additionally analyzed the localization and amount of vitronectin in growing collateral arteries to further elucidate the function of $\alpha_v\beta_3$ integrin in arteriogenesis. Seven days and fourteen days after femoral occlusion were the selected time points for the study apart from sham operation because they coincided with the important events of maximum $\alpha_v\beta_3$ integrin expression (7 days) and its return to the initial level (14 days).

The results of the Western blot confirmed the specificity of the anti-human vitronectin antibody Vitronectin 65 for the rabbit protein.

The substantial downregulation of vitronectin in the endothelium of growing collateral arteries and the return to basal levels 14 days after femoral occlusion, while $\alpha_v\beta_3$ integrin is upregulated, clearly proves that vitronectin is not the main ligand for $\alpha_v\beta_3$ integrin in arteriogenesis.

6. Role of $\alpha_v\beta_3$ integrin-ligand binding in arteriogenesis

Since the pathway, which is activated by integrin binding, depends on the ligand, it seems that the specific functions of $\alpha_v\beta_3$ integrin-vitronectin binding are not needed in arteriogenesis or even are inhibited. These results are opposing arterial injury studies, where $\alpha_v\beta_3$ integrin and vitronectin have been shown to be coordinately upregulated for 7 or even 14 days, respectively [35,154]. This is reasonable considering that $\alpha_v\beta_3$ integrin-vitronectin binding in arterial injury is functioning in a repair process, whereas arteriogenesis is a process of growth. The downregulation of vitronectin at the same time as $\alpha_v\beta_3$ integrin reaches its maximum level suggests that a specific mechanism might even prevent $\alpha_v\beta_3$ integrin interaction with vitronectin.

The transitory upregulation of $\alpha_v\beta_3$ integrin in the first week after femoral occlusion and the return to basal levels after the second week while vitronectin is downregulated suggest that the function of $\alpha_v\beta_3$ integrin-vitronectin binding is important in cell-to-cell and / or cell-to-ECM interactions probably different from those required for neointima formation, which takes place later in the process.

The three processes of atherosclerosis, restenosis, and arteriogenesis all include neointima formation. However, only in atherosclerotic and restenotic vessels $\alpha_v\beta_3$ integrin and vitronectin mediate SMC migration. In atherosclerosis and restenosis the neointima develops in response to an aggressive stimulus, e.g. vascular injury in consequence of balloon angioplasty, in order to limit the damage to the vessel wall. In this case, neointima formation is a protective mechanism rather than a process of growth. On the other hand, in arteriogenesis the formation of a neointima increases the number of cell layers of the vessel wall and the amount of smooth muscle cells, which is part of the elaborate process of growth. Probably, the accumulated smooth muscle cells slip along each other to again decrease the number of cell layers but increase the diameter of the vessel. In this way, they develop to additional layers of the media, which is then thinner than media and neointima together have been but of a larger diameter. Thus, it is probable that in this case the downregulation of vitronectin and the potential binding of $\alpha_v\beta_3$ integrin to a different ligand are required for the development of a neointima that is subsequently remodeled to finally increase the size of the media. In this regard, therapeutic agents that block $\alpha_v\beta_3$ integrin-vitronectin binding may be able to change the pathological result of neointima formation in atherosclerosis and restenosis.

Since $\alpha_v\beta_3$ integrin can bind to other proteins of the ECM apart from vitronectin (osteopontin [158], fibrinogen [159], thrombin, fibronectin, laminin, and proteolysed forms of collagen [147,160]), further studies should be conducted to elucidate which ligand binds to $\alpha_v\beta_3$ integrin in arteriogenesis and which exact mechanism is controlled by their interaction.

One more $\alpha_v\beta_3$ integrin ligand that is known to act in the vascular system is osteopontin, a phosphorylated glycoprotein, which specifically induces β_3-mediated SMC migration but not proliferation [296]. A study by Corjay et al. [23] in a rabbit model of neointima formation using a non-occlusive cuff around the femoral artery revealed coordinated upregulation of $\alpha_v\beta_3$ and $\alpha_5\beta_3$ integrin and osteopontin in the medial and luminal area of the arterial wall from 6 hours to 5 days after cuffing. A concentric neointima had formed by two weeks. The levels of all three proteins as well as proliferation determined by bromodeoxyuridine incorporation peaked between 3 and 5 days post-cuffing, clearly preceding neointima formation [23]. Even though this time course of $\alpha_v\beta_3$ integrin upregulation does not conform to the results of the present study, the fact that $\alpha_v\beta_3$ integrin upregulation precedes neointima formation, which is evident at 2 weeks after injury or occlusion, is the same. Taken together, these studies indicate that osteopontin is the main ligand for $\alpha_v\beta_3$ integrin in arteriogenesis. Unfortunately, an osteopontin antibody that was tested in several different immunohistochemical protocols during this study, did not bind to rabbit osteopontin.

A further ligand for $\alpha_v\beta_3$ integrin that could be important in arteriogenesis is thrombin. It has been shown to support monocyte adhesion to vascular endothelium via $\alpha_v\beta_3$ integrin [297]. The adhesion and activation of monocytes is an important element early in arteriogenesis, which can be used to stimulate collateral growth. It has been shown in different animal models that local infusion of MCP-1, which attracts and activates monocytes, significantly accelerates collateral growth and increases collateral conductance but does not change the final extent of growth [90,116,118-121]. Apart from MCP-1, the infusion of granulocyte monocyte-colony stimulating factor (GM-CSF), a protein, which prolongs the life span of monocytes by inhibiting their apoptosis [122,123] also stimulated arteriogenesis and increased collateral conductance [119].

Moreover, in our study, $\alpha_v\beta_3$ integrin was not only upregulated in SMCs, but also in the endothelium. As mentioned before, the upregulation was strongest prior to neointima formation. Thus, apart from neointima formation, $\alpha_v\beta_3$ integrin probably performs other functions in the arteriogenic process.

Macrophage adhesion and transmigration is an important part of arteriogenesis that in other models has been demonstrated to be mediated by $\alpha_v\beta_3$ integrin interaction with adhesion molecules like ICAM-1 [176] and PECAM-1 [161]. Bishop et al. [35] found significantly reduced macrophage content in vessels after balloon angioplasty and blocking of $\alpha_v\beta_3$ integrin, together with reduced expression of the adhesion molecules ICAM-1 and VCAM-1. Whether this was a direct effect of $\alpha_v\beta_3$ integrin blocking or simply a marker of reduced inflammation was not clear. ICAM upregulation and subsequent adherence of circulating monocytes to the endothelium early in arteriogenesis have previously been shown [81]. Thus, an important function of $\alpha_v\beta_3$ integrin during arteriogenesis could be the regulation of leukocyte recruitment by interaction with ICAM.

Since it has been shown *in vitro* and in rabbit models of balloon angioplasty that $\alpha_v\beta_3$ integrin serves as an anti-apoptotic signal and that antagonizing $\alpha_v\beta_3$ integrin enhances apoptosis [32,33,177,178], $\alpha_v\beta_3$ integrin in the wall of growing collaterals could support growth by serving as an anti-apoptotic signal. Furthermore, it has been shown that β_3 integrins mediate shear stress induced vasodilatation of coronary arterioles [30]. Thus, $\alpha_v\beta_3$ integrin could mediate the early vasodilation of the collateral artery. It can also serve as a mechanosensor that in this case transduces shear stress into chemical signals [298], and it can associate with growth factors in focal adhesion points in order to regulate the receptors' capacity to forward downstream signaling [299].

Considering all the potential functions of $\alpha_v\beta_3$ integrin in arteriogenesis and the different known and possibly unknown ligands of this receptor, it is quite evident that further studies are necessary to elucidate the exact role of $\alpha_v\beta_3$ integrin and to determine its ligand or ligands, if any, in the process of arteriogenesis. This is particularly interesting in regard to anti-restenotic therapies, which are prescribed, for example after balloon angioplasty, and include non-specific integrin blockers [199-201]. This may well interfere with arteriogenesis, which can be regarded as the physiological mechanism for limitation of the consequences of arterial stenosis or occlusion.

VI. SUMMARY

The objective of the present study was to investigate the possible role of $\alpha_v\beta_3$ integrin and its main ligand vitronectin in the growth of collateral arteries (arteriogenesis).

Integrins are membrane receptor proteins that mediate cell-ECM and cell-to-cell interactions. Among other cells, endothelial cells and smooth muscle cells express $\alpha_v\beta_3$ integrin, and it is implicated in biological processes such as angiogenesis, neointima formation, platelet aggregation and macrophage transmigration.

Seventeen male, specific-pathogen-free New Zealand White rabbits with an average weight of 2.5 kg were subjected to experimental occlusion of the femoral artery or sham operated. After 1, 3, 7, 14 or 56 days of femoral occlusion, the vastus intermedius of the quadriceps muscle was excised and cryopreserved. Transversal sections were immunostained with antibodies specific either for $\alpha_v\beta_3$ integrin or vitronectin, and the intensity of immunoperoxidase staining signal was quantified by computer software imaging. The proliferation marker Ki-67 was used to identify growing collateral arteries.

Quiescent arteries in sham operated and experimental animals showed $\alpha_v\beta_3$ integrin staining just above the level of detection. A significant increase in integrin content was found in the endothelium of growing collateral arteries 3 days after occlusion (3.2 fold, $p<0.005$), and in the endothelium and media 7 days after occlusion (3.7 fold, $p<0.001$; 7 fold, $p<0.001$; respectively). Fourteen days after occlusion, $\alpha_v\beta_3$ integrin protein already returned to initial levels. The increase was restricted to growing collateral vessels. Contrarily, vitronectin content in the endothelium of growing collaterals 7 days after occlusion decreased compared to controls and returned to initial, higher levels after 14 days.

These results demonstrate that $\alpha_v\beta_3$ integrin plays a role in the arteriogenic process but that vitronectin is not the main ligand for $\alpha_v\beta_3$ integrin in arteriogenesis. The functions of this integrin in this context are probably related to macrophage recruitment and/or growth factor receptor clustering, both required for proper collateral development during the proliferative phase. It could also play a role in early vasodilation, in formation of a subsequently remodeled neointima, as a mechanosensor in signal transduction, and as an anti-apoptotic signal. The most probable ligand for $\alpha_v\beta_3$ integrin in the process of arteriogenesis as indicated by previous studies is osteopontin, which is also acting in the vascular system.

Considering all the potential functions of $\alpha_v\beta_3$ integrin in arteriogenesis and the different known and possibly unknown ligands of this receptor, it is quite evident that further studies are necessary to elucidate the exact role of $\alpha_v\beta_3$ integrin and to determine its ligand or ligands, if any in the process of arteriogenesis. This is particularly interesting in regard to anti-restenotic therapies, which are prescribed, for example after balloon angioplasty, and include non-specific integrin blockers [199-201]. This may well interfere with arteriogenesis, which can be regarded as the physiological mechanism for limitation of the consequences of arterial stenosis or occlusion.

VII. ZUSAMMENFASSUNG

Das Ziel dieser Dissertation war es, die mögliche Bedeutung von $\alpha_v\beta_3$ Integrin und seines Hauptliganden Vitronektin für das Wachstum von Kollateralarterien (Arteriogenese) zu untersuchen.

Die sogenannten Integrine sind Glykoprotein-Oberflächenrezeptoren, die Zell-Zell Wechselwirkungen und Wechselwirkungen zwischen Zellen und der extrazellulären Matrix vermitteln. Das Integrin $\alpha_v\beta_3$ kommt im Gefäßsystem unter anderem auf Endothelzellen und glatten Muskelzellen vor und ist an so wichtigen biologischen Prozessen wie der Angiogenese (Neubildung von Kapillaren), der Neointima-Bildung, der Plättchenaggregation und der Monozytenwanderung beteiligt.

Bei 17 männlichen, spezifisch pathogen freien Weißen Neuseeländer Kaninchen wurde durch zweifache Ligatur der Arteria femoralis eine chronische, regionale Ischämie des Hinterbeins induziert, beziehungsweise eine Sham-Operation ohne Anziehen der Ligaturen durchgeführt. Nach 1, 3, 7, 14 oder 56 Tagen wurde der Vastus intermedius des Quadrizeps Muskels entnommen und zur immunhistochemischen Untersuchung kryokonserviert. Transversale Serien-Gefrierschnitte der Proben wurden entweder mit spezifischen Antikörpern für $\alpha_v\beta_3$ Integrin oder für Vitronektin gefärbt. Die Intensität des resultierenden Immunoperoxidase-Signals wurde mit Hilfe von Computer Image Software quantifiziert. Eine immunhistologische Färbung mit dem Proliferationsmarker Ki-67 wurde verwendet, um wachsende Kollateralarterien zu identifizieren.

Kollateralen aus Kontrollgewebe (nach Sham-Operation) und nicht-proliferierende Kollateralen aus Gewebe nach Femoralokklusion zeigten ein Signal für $\alpha_v\beta_3$ Integrin nah der Nachweisgrenze. Eine signifikante Steigerung des $\alpha_v\beta_3$ Integrin Gehalts war in wachsenden Kollateralarterien 3 Tage nach Femoralokklusion im Endothel (3,2-fach, $p \leq 0{,}005$) und 7 Tage nach Okklusion im Endothel und der Media (3,7-fach, $p \leq 0{,}001$ bzw. 7-fach, $p \leq 0{,}001$) nachweisbar. Vierzehn Tage nach Femoralokklusion sank der $\alpha_v\beta_3$ Integrin Gehalt in vielen wachsenden Kollateralen bereits auf ursprüngliche Werte zurück. Der beschriebene Anstieg trat nur in wachsenden Kollateralarterien auf. Im Gegensatz dazu sank 7 Tage nach Okklusion die Färbeintensität für Vitronektin im Endothel von wachsenden Kollateralen signifikant ab und ging nach 14 Tagen auf die ursprünglichen, hohen Werte zurück.

Die Ergebnisse dieser Studie zeigen deutlich, daß $\alpha_v\beta_3$ Integrin eine Rolle in der Arteriogenese spielt, wobei in diesem Prozeß Vitronektin nicht der Hauptligand dieses Integrins ist.

Die Funktionen, die $\alpha_v\beta_3$ Integrin in diesem Fall ausübt, stehen wahrscheinlich im Zusammenhang mit der Anziehung von Makrophagen und/oder dem „Clustering“ von Wachstumsfaktoren. Beides sind wichtige Faktoren in der proliferativen Phase der Arteriogenese. Weiterhin könnte $\alpha_v\beta_3$ Integrin bei der frühen Vasodilatation, bei der Bildung der später umgebauten Neointima, als Mechanosensor bei der Übertragung von Signalen und als anti-apoptotisches Signal eine Rolle spielen. Mehrere Studien geben Hinweise darauf, daß der wahrscheinlichste Ligand für $\alpha_v\beta_3$ Integrin in der Arteriogenese Osteopontin ist, welches diverse Funktionen im Gefäßsystem unterstützt.

In Anbetracht der vielen möglichen Funktionen von $\alpha_v\beta_3$ Integrin in der Arteriogenese und der Vielzahl von bekannten und möglicherweise noch unbekannten Liganden dieses Integrins wird klar, daß weitere Studien nötig sind, um die offen gebliebenen Fragen zu klären. Dies ist besonders wichtig im Hinblick auf Anti-Restenose Therapien, die nach Operationen, wie z.B. Ballonkatheter-Gefäßerweiterungen, mit unspezifischen Integrin-Blockern bereits durchgeführt werden. Unter Umständen reduzieren diese Therapieansätze nicht nur die Restenosierung, sondern beeinflussen auch die Arteriogenese, die als natürlicher Mechanismus zur Schadensbegrenzung im Falle von arteriellen Stenosen oder Verschlüssen betrachtet werden kann.

VIII. TABLE OF ABBREVIATIONS

%	percent
µg/kg	micrograms per kilogram
µl	microliter
µm	micrometer
A.U.	arbitrary units
Ab	antibody
Aqua dest.	Aqua destillata
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bFGF	basic fibroblast growth factor (FGF-2)
BSA	bovine serum albumine
bzw.	beziehungsweise
cAMP	cyclo adenosine monophosphate
cDNA	complementary desoxiribunucleic acid
CHD	coronary heart disease
CVD	cardiovascular disease
d	day
DAB	diaminobenzidine
DNA	desoxyribonucleic acid
ECM	extracellular matrix
ECs	endothelial cells
Endo	endothelium
FGF-1	fibroblast growth factor-1
FGF-2	fibroblast growth factor-2 (bFGF)
FGFs	fibroblast growth factors
Fig.	figure

g	gram
GM-CSF	granulocyte monocyte-colony stimulating factor
H/E	hematoxilin/eosin
H_2O_2	hydrogen peroxide
HIF-1	hypoxia inducible factor-1
HIV	human immunodeficiency virus
I.U.	international units
ICAM-1	intra cellular adhesion molecule 1
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IgG	immunoglobulin G
IL-1	interleukin-1
IL-4	interleukin-4
kb	kilobases
kDa	kiloDalton
l	liter
mA	milliAmpere
Max.	maximum/maximal
MCP-1	monocyte chemoattractant proteine-1
mg/kg	milligrams per kilogram
min	minutes
ml	milliliter
MMP	matrix metalloproteinase
MMP-2	matrix metalloproteinase-2
mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase

P	probability value
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCr	phosphocreatinine
PDGF	platelet derived growth factor
PDGF-BB	platelet derived growth factor-BB
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PlGF	placenta growth factor
PTCA	percutaneous transluminal angioplasty
PVD	peripheral vascular disease
RGD	Arg-Gly-Asp
rpm	rounds per minute
RT	room temperature
SDS	sodiumdodecylsulfate
sec	seconds
SEM	standard error of the mean
SMCs	smooth muscle cells
SSRE	stress responsive element
TAT	thrombin-antithrombin (III)
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
Txβ (2)	thromboxane β (2)
uPAR	urokinase receptor
VCAM	vascular cellular adhesion molecule
VEGF	vascular endothelial growth factor

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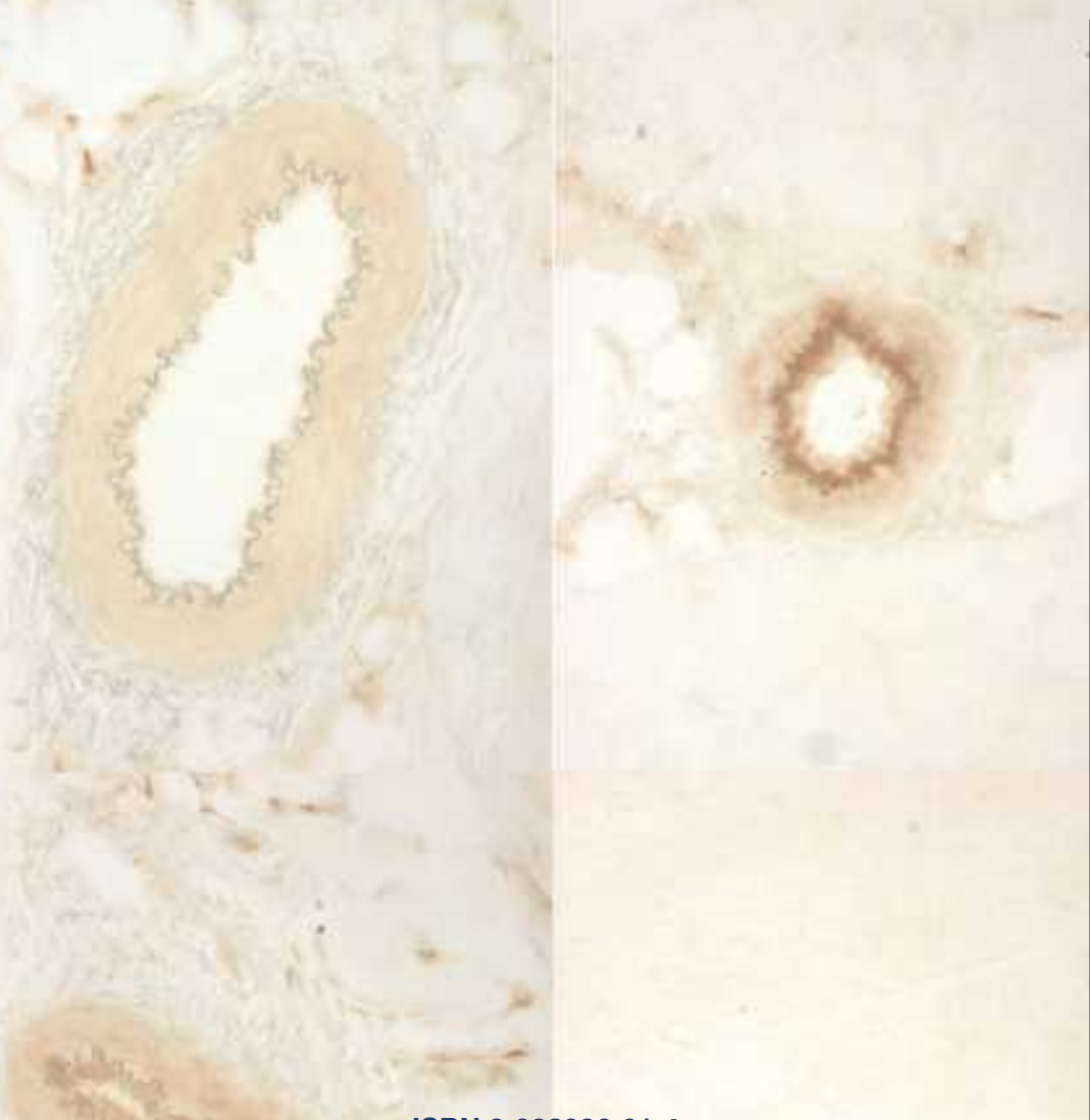

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