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Resistance of the Internal Mammary Artery to Restenosis: A Histomorphologic Study of Various Porcine Arteries

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Key Words
Intervention · Vessel injury · Proliferation · Restenosis · Vascular remodeling · Percutaneous transluminal angioplasty

Abstract
Background/Aims: Restenosis after percutaneous transluminal angioplasty (PTA) of the internal mammary artery (IMA) grafts is much less pronounced than in other arteries and venous grafts. The aim of the study was to test whether various arteries respond differently to dilatation. ***Methods:*** PTA of the IMA, carotid, renal and circumflex coronary (RCx) arteries was performed in 9 pigs (balloon to artery ratio of 1:1.5). After 8 weeks, angiography was repeated and vessels prepared for histological analysis. Immunohistochemical staining was done to examine proliferative activity (Ki67) and to identify the vasa vasorum of the adventitia (F VIII-RA). ***Results:*** The intima-media ratio after PTA was lowest in the IMA (0.06), followed by the carotid (0.27) and renal arteries (0.49) and the RCx (0.69). Proliferation of the intima was seen at 287° of the vessel circumference in the RCx, at 286° in the renal and at 166° in the carotid artery. No proliferative activity was seen in the IMA. The intima-adventitia ratio was lower in the IMA than in the RCx and renal arteries ($p < 0.05$). ***Conclusion:*** Intima proliferation after PTA varies between the different vessels, with best results seen in the IMA. There are differences in remodeling after PTA between muscular, muscular/elastic and elastic arteries.

Coronary-artery disease due to advanced atherosclerosis can be treated by percutaneous vascular interventions. Indeed, percutaneous transluminal coronary angioplasty and stent implantation are the interventions of choice under conditions of stable or unstable cardiac symptoms [1]. However, restenosis after percutaneous transluminal angioplasty (PTA) is a problem, and even stent implantation with or without drug release cannot yet completely abolish this important complication [2, 3]. In addition, the problem of late stent thrombosis is increasing with the use of drug-eluting stents [4]. New technologies such as absorbable material or local antiprolif-

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erative drug administration at the time of PTA are promising novel treatment options [5, 6].

However, coronary-artery bypass grafting is a suitable technique for revascularization, and it is widely accepted that the internal mammary artery (IMA) is ideal for grafting [7]. Several publications have highlighted the superior patency rate of the left and right IMAs in comparison to radial-artery or saphenous-vein grafts (SVG) [8, 9]. Even bilateral IMA grafting plus SVG was shown to have a lower mortality in diabetic patients than single IMA bypass grafting plus SVG [10, 11]. Additionally, long-term follow-up of multiple IMA grafting is associated with a reduction in death and cardiac events and fewer reoperations compared to single IMA grafting [12]. Angiographic studies have shown that the incidence of restenosis of the IMA is lower than that of radial artery or SVG [13].

For treatment of graft restenosis, PTA of the IMA graft represents a safe and feasible option before reoperation, and PTA might even be superior to stent implantation at the site of anastomosis [14, 15]. Despite these impressive clinical observations the reason for this phenomenon remains incompletely understood.

To our knowledge, a systematic comparison of the responses to PTA of various vessel sites within the same organism has not been reported to date. We used a pig model to investigate the hypothesis that the proliferative response to PTA differs between the IMA, the carotid artery, the left circumflex coronary artery (RCx) and the renal artery, by means of histology, immunohistochemistry and angiography.

Materials and Methods

Experimental Protocol

PTA with oversizing of the arteries by 50% of the angiographically measured diameter was performed in the RCx, left renal artery, left common carotid artery and right IMA of 9 German Landrace pigs. The left anterior descending coronary artery (LAD) and the untreated contralateral arteries served as histological controls.

German Landrace pigs of either sex weighing 29.2 ± 1.4 kg were premedicated with an intramuscular injection of azaperone (3 mg/kg), atropine (0.03 mg/kg) and ketamine (2 mg/kg). Anesthesia was induced with a bolus of propofol (20–40 mg) and piritramid (7.5 mg) followed by intubation and ventilation on a positive pressure ventilator (Servo 900, Siemens, Erlangen, Germany). Anesthesia was maintained by infusion of propofol (6–8 mg/kg · h infusion) and additional bolus application of piritramid. The animals were mechanically ventilated (O_2 in room air, FiO_2 0.21, pCO_2 controlled), and venous and arterial lines were introduced via the femoral vessels. A 6-lead ECG and the arterial blood pressure were recorded continuously on a Siemens Sirecust 404-1 (Erlangen, Germany). Heparinized arterial blood (1 ml) was drawn from the femoral artery, and blood gas analysis was performed with a Radiometer Copenhagen Arterial Bloodgas Laboratory 3 (ABL 3, Radiometer A/S, Brønshøj, Denmark).

Coronary angiography was performed with an OEC 9800 General Electric angiographic system (GE Medical Systems, Salt Lake City, Utah, USA). After quantitative vascular angiography, a PTA balloon was chosen with a balloon to artery ratio of 1:1.5 at its nominal pressure (6–8 bar). For each dilatation a balloon with a length of 20 mm was used. The inflation was held for 1 min and repeated twice with recovery intervals of 1 min between passes. After the interventions, a control angiography of the intervened vessel was performed to document the patency of the vessel. No acute vessel occlusion was seen in any of the treated animals or vessel segments.

Anesthesia was then withdrawn and a pressure dressing was applied, and when breathing spontaneously, the animals were extubated and taken back to the animal facility. Eight weeks after the manipulation of the 4 arteries, the animals were reanesthetized, and following angiography they received deep anesthesia and cardioplegia with potassium chloride. The heart was excised under pressure-controlled perfusion fixation (70 mm Hg), and the RCx was prepared at the dilated part, including a small segment proximal and distal to the intervention to clearly identify the site of injury. The LAD was excised as a control vessel. The same procedure was performed on the renal and carotid arteries and the IMA. The contralateral vessels served as controls. Digitized angiographical pictures were examined in a blinded fashion using a computer-based software system (Image tool 3.0 Freeware, University of Texas Health Science Center, San Antonio, Tex., USA).

All investigative procedures and animal facilities conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee.

Histological and Immunohistological Staining Methods

The heart was excised after perfusion fixation with neutral buffered formaldehyde (4%) at a pressure of 70 mm Hg. The same procedure was applied for fixation of the other vessels. Despite perfusion fixation we observed shrinkage of the lumen in pilot experiments after overnight fixation. Therefore, the diameter of the dilated vessels was measured by angiography. The dilated part of the vessel was divided into 5 segments of 4 mm in length. For light microscopy the tissue was fixed overnight in neutral buffered formaldehyde (4%) and embedded in paraffin, and 4-μm-thick sections were prepared from each vessel segment. Histological examination of the intima and media was conducted after staining with hematoxylin and eosin. For each dilated segment the mean value of 5 sections was determined. Representative cross-sections of each artery and a control are depicted in figure 1. To identify collagen and elastic fibers, Elastica-van Gieson (EvG) staining was used. Changes in collagen deposition were analyzed qualitatively by polarization microscopy of Sirius-red-stained sections.

For immunohistochemistry, we used the conventional indirect immunoperoxidase-method with the avidin-biotin complex. After deparaffinization, endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in methanol for 15 min. The

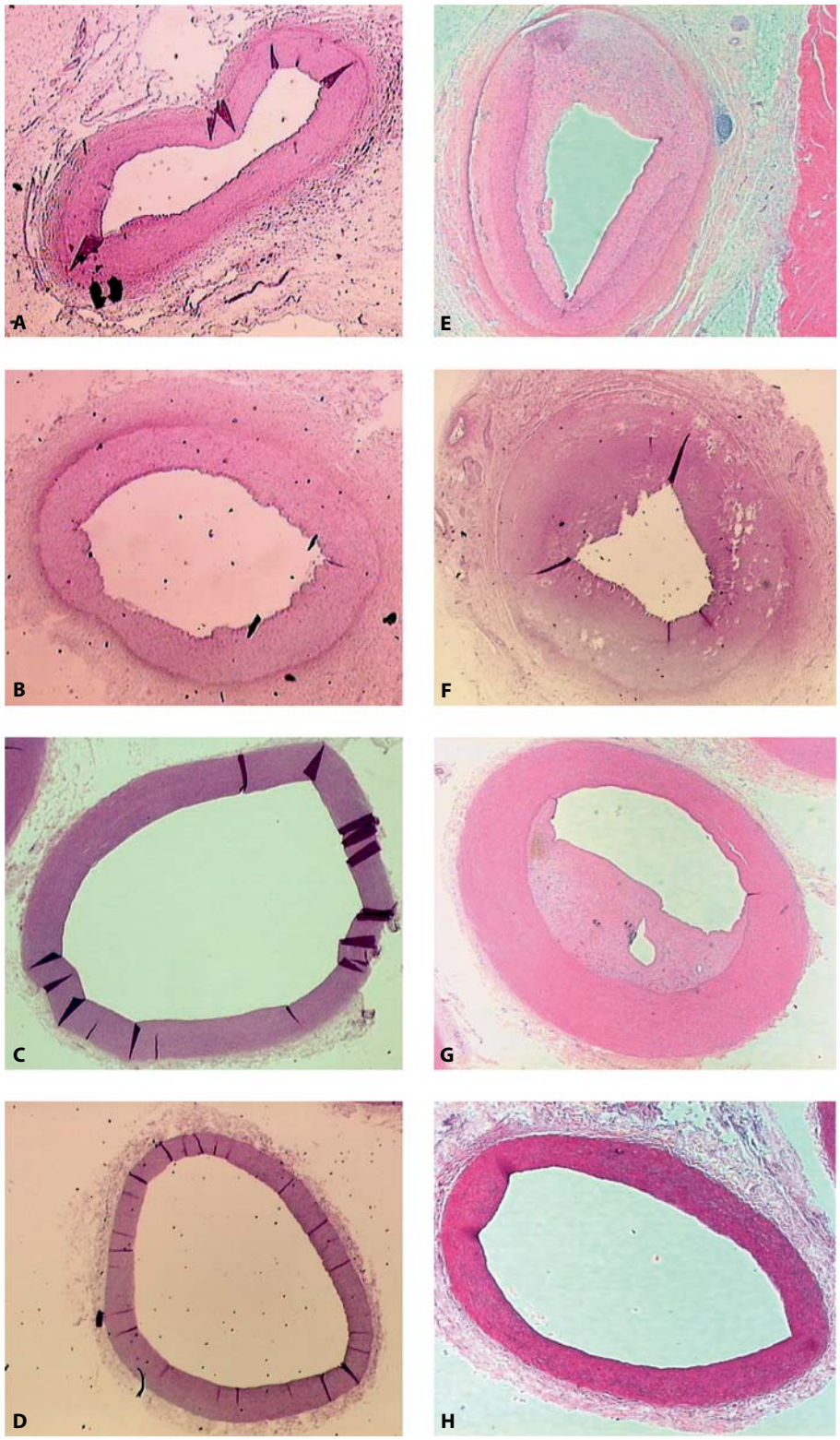

Fig. 1. Representative cross-sections of control (**A–D**) and post-PTA (**E–H**) RCx (**A**, **E**), renal (**B**, **F**) and carotid (**C**, **G**) arteries and IMAs (**D**, **H**). HE. Magnifications 20× (RCx, renal artery and IMA) and 10× (carotid artery).

sections were then incubated with the primary antibody for 1 h at room temperature. To quantify the density of microvessels particularly in the adventitia, a polyclonal rabbit antibody against factor-VIII-related antigen was used (Dako, Hamburg, Germany; dilution 1:500; trypsin 0.1% pretreatment), and for documentation of proliferative activity, a monoclonal antibody against Ki67 (MIB-1; Dako; dilution 1:100, microwave pretreatment). If necessary, the slides were counterstained with hematoxylin. The staining against factor-VIII-related antigen yielded visualization of individual microvessels which were counted on each histological slice.

The microscope slides were digitized using a high-resolution camera (DCM 1200, Nikon) and imported into a personal computer for offline planimetry of the circumference and cross-sectional areas of each vessel and of the thicknesses of intima and media. Examinations under standardized calibrated magnification were performed using a computer-based software system (Image tool 3.0 Freeware, University of Texas Health Science Center). As with angiography, these histological images were examined in a blinded fashion.

Angulation of Intima Proliferation and Eccentricity

To determine the circumference of the examined vessel in the histological short-axis section, we viewed the vessel as a circle of 360° from the middle of the lumen. Then the angle of intima proliferation was determined. Intima proliferation of the total circumference resulted in a complete neointima of 360° and of half of the circumference in a neointima of 180°.

Remodeling of the vessels after PTA resulted in either concentric or eccentric vessel proliferation or a combination of the two. To quantify the type of remodeling of each vessel, the longest diameter of the histological slice was measured and defined as the 'major axis'. The 'minor-axis' length was measured perpendicular to the major axis. Numerical eccentricity was defined as $\varepsilon = e/a$, where 'e' is the linear eccentricity, 'a' the half of the major axis, and 'b' the half of the minor axis.

$$e = \sqrt{a^2 - b^2}$$

Therefore the numerical eccentricity 'ε' is expressed as:

$$\varepsilon = \frac{\sqrt{a^2 - b^2}}{a}$$

'ε' of a circle is zero, and ellipsoids have values between 0 and 1. Values of numerical eccentricity <0.25 are considered as concentric, and values >0.25 as eccentric [16]. Oblique cross-sections were excluded from analysis, and folded or squeezed sections were reinserted from the respective paraffin blocks prior to analysis.

Statistical Analysis

Data are presented as mean values ± SEM. Statistical analysis was performed with Sigma Stat® 3.0 (Jandel Corp., USA). The statistical significance of differences before and after PTA was determined with the paired Student's t test, between the control vessels and the dilated vessels with the t test, and between groups of vessel segments with one-way analysis of variance. If data sets did not show normal distribution, the Kruskal-Wallis one-way analysis of variance by ranks test was used. A 2-sided p value of <0.05 was considered statistically significant.

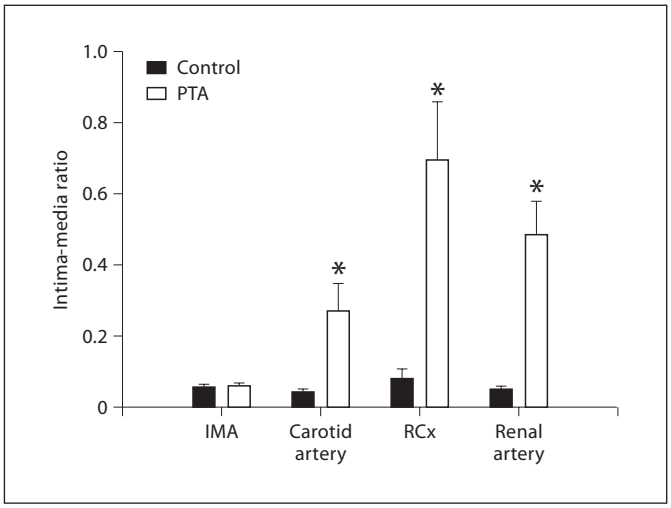

Fig. 2. Intima-media ratios of the IMA, carotid artery, RCx and renal artery and their respective control vessels. The intima-media ratios increased significantly after PTA in the carotid, RCx and renal arteries (* $p < 0.05$).

Results

All animals survived the PTA procedure. Their body weight increased during the observation period from 29.2 ± 1.4 to 64.6 ± 3.8 kg.

Intima-Media Ratio

Eight weeks after PTA, the intima-media ratio of the IMA was significantly different from that of the RCx and the renal arteries (fig. 2). The highest intima-media ratio was measured in the RCx (0.69 ± 0.16) followed by the renal artery (0.48 ± 0.09; $p < 0.05$). The intima-media ratio of the carotid artery (0.27 ± 0.08) was lower than those of the renal artery and the RCx, but the difference was not statistically significant. The lowest values were found in the IMAs (0.06 ± 0.005). Indeed, no statistical differences were seen between the intima-media ratios of the post-PTA IMAs and those of the control vessels (LAD: 0.08 ± 0.03; renal artery: 0.05 ± 0.007; carotid artery: 0.04 ± 0.008; IMA: 0.06 ± 0.005; fig. 2).

After PTA, the increase in the intima-media ratio compared to the control vessels was highest in the renal artery (+871%), followed by the RCx (+744%) and the carotid artery (+651%). The IMA showed virtually no increase in the intima-media ratio (+4.2%). Seven of the 9 histological specimens from the coronary arteries and 8 from the renal arteries showed tears in the internal elastic lamina. No injuries were observed in the IMAs, and only

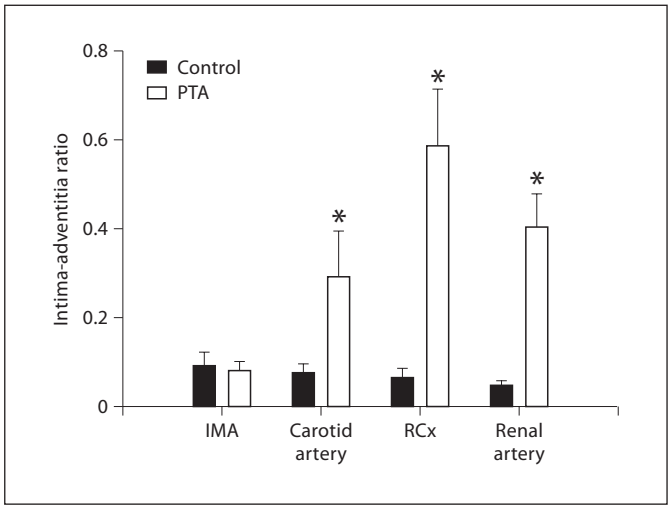

Fig. 3. Intima-adventitia ratio of the IMA, carotid artery, RCx and renal artery and their respective control vessels. The intima-adventitia ratio increased significantly after PTA in the carotid, RCx and renal arteries (* $p < 0.05$).

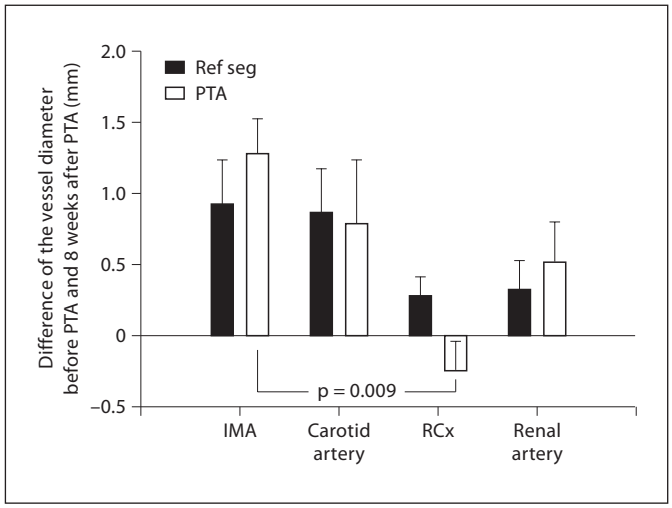

Fig. 4. Differences in angiographically determined vessel diameter before and 8 weeks after PTA in the reference segment (Ref seg) and the dilated segment (PTA). The difference in diameter between the IMA and the RCx was significant ($p = 0.009$).

3 in the carotid arteries. Collagen deposition as seen by Sirius-red staining and polarization was minimal and occurred only in vessels with tears in the internal elastic lamina.

Circumference and Eccentricity of Vessel Proliferation

With regard to the complete circumference of the vessel perimeter, proliferation in the circumflex artery was seen at 287° and in the renal artery at 286°. Proliferation of the carotid artery was observed at 166°. The IMA did not show any significant localized proliferation. A significant difference in proliferation was seen between the RCx and renal artery versus the carotid artery and versus the IMA ($p < 0.05$).

The RCx of all animals showed histomorphologically eccentric proliferation, with a numerical eccentricity index >0.25. The renal artery was eccentric in 10% and the carotid artery in 25% of all examined segments. The IMA remained largely unchanged.

Intima-Adventitia Ratio

Eight weeks after PTA, the intima-adventitia ratio was significantly different between the RCx and the renal artery versus the IMA. The intima-adventitia ratio of the carotid artery was in between the values of the muscular arteries and the IMA, but did not show any statistically significant differences (RCx: 0.59 ± 0.12; renal artery: 0.40 ± 0.07; carotid artery: 0.29 ± 0.10; IMA: 0.08 ± 0.02; $p < 0.05$: RCx vs. IMA and renal artery vs. IMA, fig. 3).

The control vessels did not show any significant differences between vascular segments (RCx: 0.07 ± 0.02; renal artery: 0.05 ± 0.01; carotid artery: 0.08 ± 0.02; IMA: 0.09 ± 0.03). The increase in the intima-adventitia ratio after PTA compared to the control vessels was significant for the RCx ($p = 0.007$) and for the renal artery ($p = 0.001$), and showed a clear trend for the carotid artery ($p = 0.057$).

Number of Mitotic Cells

Mitotic cells were determined by immunohistochemistry with Ki67. The absolute number of positive cells is given in table 1. In the neointima, statistically significant differences were seen neither between the vessel segments nor between the treated and the respective control vessels. Likewise, the number of Ki67-positive cells per cross-section in the media did not show any statistical differences between the vessel segments. Neither did the results of the control and the PTA-treated vessels show any significant differences. Indeed, the numbers of mitotic cells were very low at the time of explantation after PTA in all of the examined segments.

Adventitial Vessels

The numbers of arterial and venous vessels in the adventitia were counted from the sections stained with he-

Table 1. Vessel diameters of the reference and dilated segments, adventitial areas, vasa vasorum and proliferating cells

	IMA		Carotid artery		RCx		Renal artery	
	Control	PTA	Control	PTA	Control	PTA	Control	PTA
Reference segment diameter, mm	2.76 ± 0.17	3.75 ± 0.24*	4.31 ± 0.18	5.18 ± 0.22*	2.90 ± 0.22	3.21 ± 0.21*	3.58 ± 0.28	3.92 ± 0.25
Dilated segment diameter, mm	2.74 ± 0.18	4.02 ± 0.14[a]	4.48 ± 0.30	5.27 ± 0.37	2.92 ± 0.25	2.68 ± 0.21	3.20 ± 0.21	3.72 ± 0.35
Adventitia, mm^2	1.26 ± 0.38	2.09 ± 0.379	3.04 ± 0.48	4.55 ± 0.58	1.18 ± 0.27	2.34 ± 0.32	2.54 ± 0.41	3.69 ± 0.41
Vasa vasorum	0[b]	0.75 ± 0.75	3.43 ± 0.68	9.67 ± 2.39[c]	1.50 ± 1.19	4.56 ± 1.48	3.00 ± 1.21	4.44 ± 1.17
Proliferating cells in the intima	0.25 ± 0.25	0.38 ± 0.26	1.0 ± 0.43	2.44 ± 0.63	0	0.78 ± 0.43	0.14 ± 0.14	0.78 ± 0.32
Proliferating cells in the media	2.20 ± 1.02	2.50 ± 0.89	3.86 ± 0.59	15.44 ± 8.43	4.60 ± 4.12	5.11 ± 1.35	1.15 ± 0.55	10.0 ± 3.33

Data show means ± SEM of the angiographically determined vessel diameters, adventitial areas, the vasa vasorum inside the adventitia and the numbers of proliferating cells in the intima and media.

* The angiographically determined diameters of the reference segments increased significantly in the IMA, carotid artery and RCx ($p < 0.05$)

[a] The diameter increase after PTA was significant for the IMA ($p < 0.05$).

[b] The adventitial tissue of the IMA was feathery, and preparation especially of the control vessel was difficult as seen in previous experiments [17]. Therefore, determination of the vasa vasorum in the adventitia of the untreated IMA from histological sections was difficult.

[c] The number of vasa vasorum in the dilated carotid artery was significantly higher than in the control vessels or the other dilated vessels ($p < 0.05$).

matoxylin-eosin or immunostained with the endothelium-specific factor VIII (table 1). After PTA, the number of adventitial microvessels was significantly higher in the carotid artery than in the RCx and the renal artery ($p < 0.05$). The control vessels of the RCx and carotid and renal arteries did not show any significant differences. Untreated IMAs contained very scant adventitial tissue with only few microvessels, as known from previous experiments [17]. The difference between control and post-PTA vessels was significant only for the carotid artery ($p < 0.05$).

Coronary Angiography and Vessel Diameter

Offline analysis of the angiographies yielded comparable values for the inner luminal diameters before PTA for the IMA, RCx and renal artery, but not for the larger carotid artery. After PTA the inner lumen diameter increased significantly in the IMA versus the control (table 1). The untreated control reference vessel segments proximal to the intervention sites showed comparable values to the treated areas before intervention. Eight weeks later, the diameters had significantly increased in all reference segments except for the renal arteries, which showed only a trend (table 1). With regard to the differences in vessel diameter in the dilated segments before and 8 weeks after PTA, there was a significant difference between the IMA and the RCx ($p = 0.009$). Indeed, the RCx was the only artery in which no enlargement was observed after 8 weeks (fig. 4).

Discussion

In this large-animal study we were able to demonstrate that the PTA of various arteries in the same individual leads to different effects on intima proliferation. Most importantly, we found no noteworthy increase in the intima-media ratio of the IMA. Applying the same degree of relative oversizing by PTA, muscular arteries such as the RCx and the renal artery showed the most striking neointima proliferation and the elastic IMA virtually none, and the mixed muscular and elastic carotid artery was found to be in between the 2 extremes. One histomorphological correlation with the increase in the intima-media ratio in the RCx and renal artery was the increased number of tears in the internal elastic lamina. Virtually no such discontinuities of the internal elastic membrane were found in the IMA, which might be one explanation for the absence of intimal proliferation [18, 19]. The extent of the neointima after PTA of the carotid artery was between the others which may be explained by the increased elastin content [18]. Elastic vessels have the potential to recoil better than predominantly muscular arteries, and hence PTA-induced injury and subsequent neointimal proliferation may be reduced in vessels with an increased elastin component. Similar results have also been published for platelet deposition in pig carotid versus coronary arteries [20].

One limitation of the study could be the fact that young, disease-free and nonatherosclerotic vessels were injured. This does not allow the difficult interpretation of the proliferative effects on the inflammatory back-

ground of diffuse atherosclerosis. The remodeling effect of the arteries in this experimental setup was only due to the PTA and not affected by prior atherosclerotic injury, which could also show differences in local intensity. In any case, the investigation of atherosclerotic remodeling has been hampered by the absence of a good animal model [21].

Additionally, the results clearly demonstrate that PTA of healthy vessel areas is harmful to the renal, coronary and carotid arteries unless the intervention is limited to the atherosclerotic constricted area. However, PTA of focal IMA restenosis, since it is mostly at the anastomosis of the IMA bypass graft, seems to be less harmful to the healthy part if this is also dilated by intervention. The healing process after plaque rupture may result in inward remodeling, but this was not seen by angiography, which could be due to the chosen animal model [19, 21]. Predominantly elastic arteries seem to respond to balloon dilatation with relatively good outward remodeling and little intimal hyperplasia, whereas especially in pigs predominantly the muscular arteries respond with incomplete outward remodeling. Pilot experiments without overexpansion of the vessels showed almost no intima proliferation. The present data are supported by further comparative examinations of overstretching de novo coronary and iliac arteries, in which the iliac arteries showed increased inward remodeling, in contrast to the coronary arteries [22]. Therefore, caution is warranted when extrapolating results from one artery to another when investigating vascular remodeling after angioplasty.

Clinically, vascular interventions are mostly performed in the coronary arteries, but usually not the IMA or radial artery, apart from a small number of patients with restenosis of grafts after coronary-artery bypass grafting. In coronary-artery bypass graft surgery, the patency rate of the IMA is superior to those of radial artery grafts. When fibromuscular hyperplasia of the IMA occurs at all, it is mostly around the anastomosis to the muscular coronary artery [13]. Further observations that the more fibromuscular distal radial artery showed lower patency rates than the more elastic proximal radial artery are in perfect agreement with the present results [23]. However, implantation of the radial graft into the coronary circulation may lead to changes of the radial artery tending towards a vasoregulation of elastic arteries such as the IMA [24].

It is known that the denuded IMA has an abolished reaction to dilatation induced by vascular endothelial growth factor or other vasoregulatory mediators [25, 26]. However, traumatic stimuli did not inhibit nitric oxide-mediated late vasodilatation in vivo [27]. Further publications have reported the concept that arteries respond differently to stimulation depending on their endothelial and smooth-muscle cell functions [24, 28–31]. Also, it has been shown that endothelium-independent contraction and relaxation of the IMA smooth-muscle cells were unaffected by any type of mechanical dilatation [32, 33]. The carotid artery has the capacity to produce nitric oxide despite removal of the endothelium, and similar mechanisms of vasoregulation may prevail in other elastic arteries [34]. Nitric oxide inhibits proliferation after PTA and after stent implantation [35–38] and endogenous nitric oxide production by nonendothelial vascular cells might therefore explain the reduced intima proliferation observed in the IMA in our experiments [34]. The outlined mechanisms may explain – at least in part – the excellent acute and long-term results after PTA of the IMA [39–42]. Furthermore, comparative examinations of various arteries in patients have shown that atherosclerotic changes are diminished in the IMA compared with the other arteries [43, 44].

The experiments raise the question of comparability between the 4 different arteries in respect of the time point of examination. Eight weeks after PTA, the rate of mitosis was very low in the dilated areas, as assessed by Ki67 immunohistochemistry. The results are in accordance with a model of cell proliferation after PTA in various species, in which it was reported that total cell proliferation in pigs is completed at 56 days after PTA [45].

In parallel to the intima proliferation, we also found an increase in the intima-adventitia ratio, with the adventitia per se not exhibiting any relevant proliferation. However, we found a significant increase in the number of vasa vasorum in the adventitia of the carotid arteries, and a corresponding trend in the RCx and renal arteries, as assessed by immunohistochemistry of factor-VIII-related antigen. This supports the concept of induced angiogenesis in the adventitia in response to vessel injury, as previously shown in the coronary-artery system with and without atherosclerotic plaques [46–49]. Recently published data reported the existence of endothelial precursor and stem cells in the area between the smooth-muscle cells and the adventitial layer in the human adult vascular wall. These cells are capable of differentiating into mature endothelial, hematopoietic and local immune cells, such as macrophages [50]. As previously published, cell labeling in pigs is difficult, including endothelial progenitor cells, while the Ki67-positive cells were predominantly seen in the proliferating intima and media [51]. However, the increase in neointima proliferation, especially of

the RCx and renal arteries, did not parallel the increase in the number of vasa vasorum, and hence argues against the notion that the vasa vasorum play the major role in the early stages of neointima formation after PTA. Histological preparation of the vessels proved to be another limitation, especially in the untreated IMA, even though the explantation was performed extensively, including as much as possible of the loose periadventitial tissue.

Previous publications focused on the effect of neointima development after PTA in various species [19, 45, 52]. We now present data which concentrate on the importance of the site of intervention.

This study emphasizes the importance of the type of artery chosen for studies on restenosis after PTA and underlines the excellent suitability of the IMA as an ideal arterial graft in cardiac-bypass surgery.

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