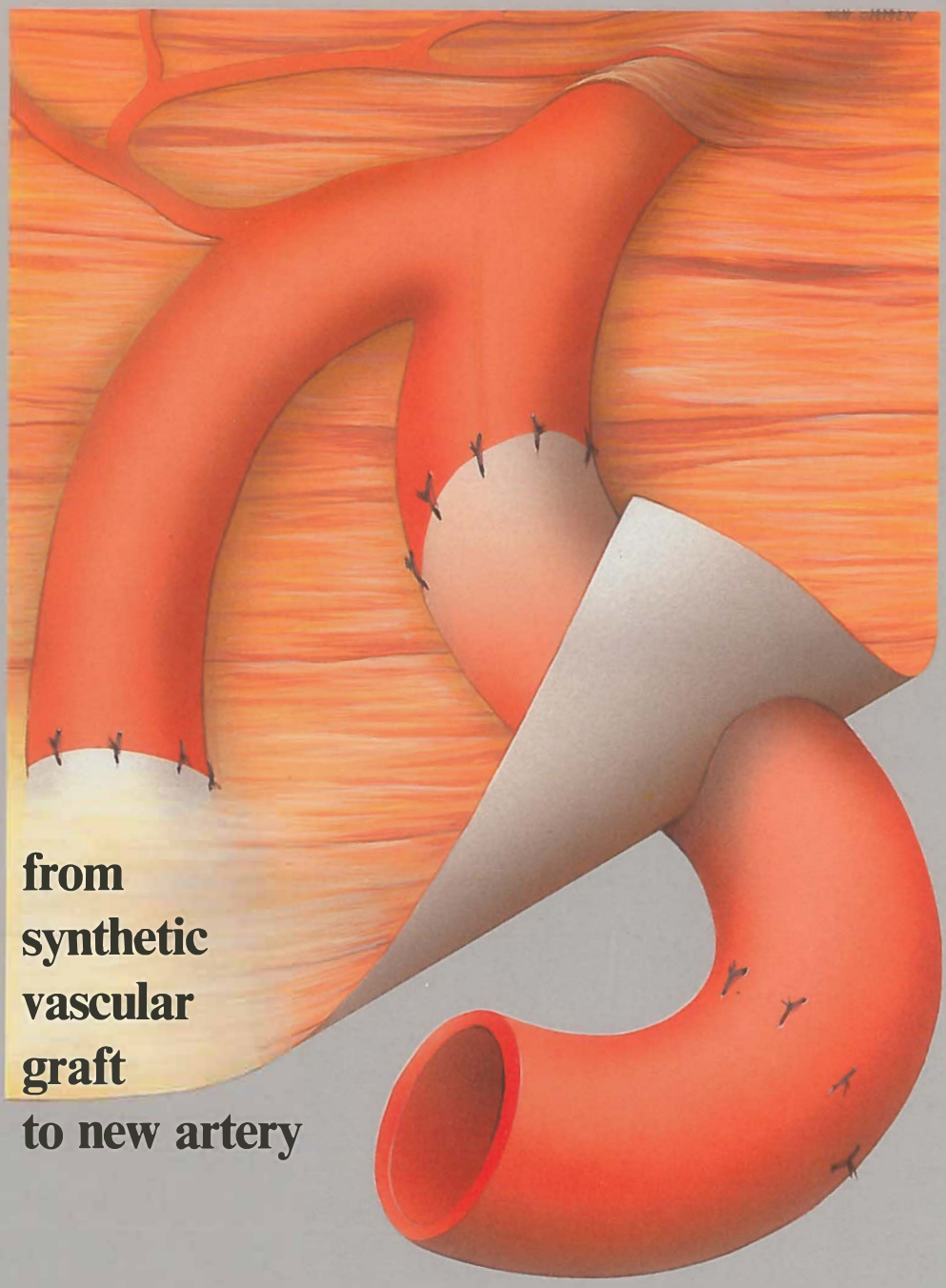
From synthetic vascular graft to new artery

Lei, Berend van der



berend van der lei

FROM SYNTHETIC VASCULAR GRAFT TO NEW ARTERY

Stellingen

1. Klinisch succes van geheel afbreekbare kunstbloedvaten zal niet mogelijk zijn zonder inzaaien van gladde spiercellen.

2. De afbraakprodukten van ideale biomaterialen zouden lichaamseigen stoffen moeten zijn.

3. Celgroei op kunstmateriaal in vitro wordt, ondanks de geabsorbeerde eiwitten aan het polymeer, voornamelijk bepaald door de vrije oppervlakte energie.

   Schakenraad JM et al., J Biomed Mat Res 20: 773-784, 1986.

4. De "leegstrijkproef" ter controle van de doorgankelijkheid van microchirurgisch herstelde bloedvaten dient vermeden te worden.

   Petry JJ et al., Plast Reconstr Surg 77: 960-963, 1986.

5. De chirurg zal steeds meer oog moeten krijgen voor de effecten van zijn handelingen op cellulair en moleculair biologisch niveau.

6. Veel routinematig preoperatief laboratorium onderzoek is overbodig.

   Kaplan EB et al., JAMA 253: 3576-3581, 1985.

7. De epidemiologische gegevens van het buruli ulcer suggereren dat transmissie van mycobacterium ulcerans plaats vindt via grond en/of gewas.

   Graaf Wvd, Tropenscriptie, Groningen 1986.

8. Alveolair surfactant verwijderen door middel van longlavages is geen adequaat model voor het testen van het effekt van surfactant instillaties bij het "adult respiratory distress syndrome (ARDS)".

   Ennema JJ, persoonlijke mededeling.

9. Pauzes, borrels en gezamenlijke diners tijdens een congres bevorderen de wetenschappelijke kennisoverdracht.

10. Goede militairen zijn mensen die zich voortdurend op iets voorbereiden waarvan ze vurig hopen dat het nooit zal voorkomen.

11. Aan de oprichting van het landelijk Rijksmestbureau hangt een vreemd luchtje.

Stellingen behorende bij het proefschrift van
Berend van der Lei
from synthetic vascular graft to new artery
Groningen, 1986

RIJKSUNIVERSITEIT TE GRONINGEN

FROM SYNTHETIC VASCULAR GRAFT TO NEW ARTERY

PROEFSCHRIFT

ter verkrijging van het doctoraat in de Geneeskunde
aan de Rijksuniversiteit te Groningen
op gezag van de Rector Magnificus Dr. E. Bleumink
in het openbaar te verdedigen op woensdag 29 oktober 1986
des namiddags te 4.00 uur

door

BEREND VAN DER LEI

geboren te Wijnjewoude

Promotores: Prof. Dr. Ch.R.H. Wildevuur
Prof. Dr. P. Nieuwenhuis
Prof. Dr. I. Molenaar

Promotiecommissie: Prof. Dr. J.D. Elema
Prof. Dr. J.N. Homan van der Heide
Prof. Dr. A.J. Pennings

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"To restore and maintain mechanical function an implanted segment need only temporarily restore mechanical continuity and serve as a scaffolding or bridge for the laying down of an ingrowth of tissue derived from the host"

C.C. Guthrie, 1919

aan Yvonne,
Charlotte,
Cees Jan
en onze ouders

Voorwoord

Het onderzoek zoals beschreven in dit proefschrift vond plaats in de Afdeling Experimentele Thoraxchirurgie (Prof. Dr. Ch. R.H. Wildevuur) van het Academisch Ziekenhuis te Groningen, in nauwe samenwerking met het Histologisch Laboratorium (Prof. Dr. P. Nieuwenhuis), het Centrum voor Medische Elektronenmicroscopie (Prof. Dr. I. Molenaar) en het Laboratorium voor Polymeerchemie (Prof. Dr. A.J. Pennings) van de Rijksuniversiteit te Groningen. De experimenten vonden plaats in het Centraal Dieren Laboratorium (Drs. H. Dikken), het Histologisch Laboratorium en het Centrum voor Medische Elektronenmicroscopie van de Rijksuniversiteit te Groningen en in het Pharmakologisches Institut (Prof.Dr. K. Schör) van de Universiteit te Keulen.

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Curriculum vitae

Berend vander Lei was born on November 15, 1958, in Wijnjewoude, The Netherlands. After his high school graduation in 1977, he studied Chemistry for one year at the University of Groningen, The Netherlands. In 1978 he started to study Medicine at the University of Groningen. In 1981 he entered as a student research-fellow the Research Division of the Department of Cardiopulmonary Surgery of the University Hospital of Groningen. At this Department, in 1983, he started his Ph.D. research project, which is described in this thesis. In June 1986, he graduated from medical school.

Berend van der Lei, geboren op 15 november 1958 te Wijnjewoude, begon zijn studie Geneeskunde in 1978 aan de Rijksuniversiteit te Groningen, na 1 jaar Scheikunde te hebben gestudeerd wegens uitloting. In 1981 begon hij als studentassistent te werken in de Afdeling Experimentele Thoraxchirurgie van het Academisch Ziekenhuis te Groningen. Vanaf 1983 heeft hij in deze afdeling onderzoek verricht naar een nieuw (kunst)bloedvat, zoals dat beschreven is in dit proefschrift. In juni 1986 is hij afgestudeerd als arts.

Contents

CHAPTER 1

General introduction and aim of the studies

General introduction

In the past four decades the development of a variety of biological and synthetic vascular grafts has enabled many patients to undergo routine surgical repair of their diseased or damaged arteries. Unfortunately, despite enormous experimental and clinical research, none of these vascular grafts appears to be an ideal arterial substitute, especially not for small-caliber arteries. Limited availability, graft deterioration, and complications such as thrombosis, aneurysm formation, and excessive subintimal hyperplasia at the anastomotic sides are still major problems.[1,2]
The demand for a graft that will replicate the role of natural, undiseased arterial tissue is clear, but such a graft is not yet available. However, in this thesis a new type of small-caliber graft will be presented, which in principle may approximate the desired properties of such a graft: a microporous, compliant, biodegradable vascular graft that functions as a temporary scaffold for the regeneration of a new arterial wall.
Prior to the description of the basic concepts and construction of this graft, a survey of the major catagories of biological and synthetic vascular grafts with their advantages and disadvantages is given.

Venous autografts

Venous autografts, in particular autogenous saphenous veins, have become the preferred standard material for the reconstruction of medium-caliber and small-caliber arteries.[3-5] They are flexible, viable, biocompatible, and usually available, although not always in the proper size or length. These facts together with their historical and continued successful performance account for saphenous veins being an attractive arterial substitute.
The first clinical applications of venous autografts as arterial substitute were performed successfully in the beginnings of the 20th century. Goyannes (1906) used the popliteal vein to repair a syphilitic popliteal artery aneurysm[6]. Lexer (1907) used the saphenous vein to repair a traumatic aneurysm of the axillary artery[7]. Pringle (1913) and Bernheim (1915) both replaced a large syphilitic popliteal artery aneurysm with a segment of the saphenous vein[8,9]. Despite these successful clinical applications, there was a hiatus of nearly four decades before the explosive development in clinical application of venous autografts, in particular autologous saphenous veins, for peripheral arterial reconstructive surgery[10-13] and coronary artery bypass surgery.[14-18]
Nevertheless, venous autografts are not considered to be ideal arterial substitutes.[3-5] First, veins may be unsuitable because of inappropriate size, limited length, varicosis or multiple branching. Secondly, progressive late degenerative alterations, including atherosclerosis, subintimal hyperplasia, dilatation, and aneurysm formation occur in vein grafts, as already noted by Carrel and Guthrie at the turn of this century[19] and can eventually lead to graft failure.[3-5,20-29] Factors contributing to these degenerative changes are multiple and not

completely understood. Implicated factors include surgical trauma to the vein wall,[30,31] distention pressure,[32,33] the storage media,[22,34,35] exposure to cardioplegic agents,[36] the higher pressure and pulsatile flow in the arterial system,[37,38] shear stress forces,[39] anastomotic configuration,[39-41] graft compliance,[42,43] platelet involvement,[44,45] factors that prevent fast revascularization of the vasa vasorum of the vein and thus promote graft ischemia,[23,46,47] dietary factors,[30] hyperlipidemia,[40,48,49] and last but not least natural progression of the preexcisting atherosclerotic process.[30] All of these factors more or less lead to vessel wall injury, which is postulated to play a key role in the pathogenesis of vein graft degeneration.

Arterial autografts

Arterial autografts have often been described as the ideal arterial substitute.[50-52] Technically, they are easy to handle. Their clinical function is comparable to that of a normal artery; arterial autografts possess normal compliance, flexibility, and anti-thrombogenicity. After implantation, they remain viable, grow in proportion to the growth rate of the arterial tree when used in growing children, regularly heal in infected areas, and resist late degeneration (stenosis or aneurysmal dilatation). Consequently, for one or more of these reasons, surgeons prefer to use an arterial autograft in certain clinical situations.

The clinical use of arterial autografts was first introduced by Wylie in 1964.[53] In his initial report his primary indications were replacement of arteries adjacent to an active joint and the presence of wound infection.

Nowadays, arterial autografts are mainly used as a renal artery graft or as an aorta-coronary bypass graft. The iliac artery autograft is preferentially used as renal artery graft, especially in growing children and in adults with nonatherosclerotic renovascular hypertension.[54-57] As renal artery graft the iliac artery autograft yields excellent long-term patency rates and extremely low incidence of graft stenosis or aneurysmal dilatation. The donor site is replaced with a synthetic (Dacron) vascular graft when the common and/or external iliac arteries are used. The internal mammary artery autograft is widely used as an aorta-coronary bypass graft.[25-29,58,59]

The internal mammary artery also yields excellent long-term patency rates, does not develop intimal hyperplasia, and seldom develops atherosclerosis. The donor site is not replaced.

General use of arterial autografts, however, is hampered by their limited availability and/or limited length.

Venous allografts

The use of venous allografts has been abandonned clinically, because they show a high incidence of graft deterioration and complications. Modified venous allografts reinforced with a polyester mesh, however, are still used, especially in peripheral arterial reconstructive surgery.[60-62]

The possible use of vein allografts was considered as early as 1912 when Carrel reported that a jugular vein allograft had remained patent in a canine aorta for 2 years[63]. In the early 1970s, Tice et al. and Ochsner et al. reported encouraging early clinical results with venous allografts.[64-66] However, in a follow-up, Ochsner reported a very high failure rate[67]. Immunologic studies have demonstrated that fresh venous allografts are normally antigenic and sensitize the recipient. The immunologic response eventually results in excessive intimal hyperplasia, perigraft fibrosis, medial degeneration and elastic disruption of the venous allografts.[68-70]

Therefore, various techniques, such as proteolytic enzyme digestion, dialdehyde starch-tanning, glutaraldehyde tanning, and freezing have been developed to decrease immunogenicity and to increase strength of venous allografts.[60-62,69,71-77] In general, these techniques result in loss of viability of the graft, leaving a nonviable fibrocollagenous tube. Although viability may not always be required for the complete period during which the graft is expected to function, fibrocollagenous tubes have proved to be an unsatisfactory graft.[69,72,73,77] They eventually lose their tensile strength and become aneurysmal. Therefore, additional use of an external mesh reinforcement, as suggested by Dardik and coworkers in experimental trials with umbilical vein allografts, seems to be necessary to prevent aneurysm formation of these venous allografts.
Nowadays, glutaraldahyde-stabilized umbilical vein grafts with a polyester mesh reinforcement (Biograft) seem to be an acceptable alternative to autologous saphenous veins in peripheral arterial reconstructive surgery.[78-82] Some surgeons even prefer Biograft because its use decreases the operation time, and preserves the saphenous vein for other operative procedures that may be required in the future for the patient. Nevertheless, graft deterioration and complications have also been reported for Biograft.[62,83] Moreover, the patency rates after 5 years and longer are still not superb.[62]

Arterial allografts

The use of arterial allografts has also been abandonned clinically, because of the poor results with these grafts.[2,60] The 5-year and 10-year patency rates that have been reported range from 56-77% and 36-38% respectively for aortic allografts and 26-36% and 3% respectively for femoral artery allografts.[84,85]
Arterial allografts were first introduced for clinical use by Gross and his associates (1948), who used these grafts for the correction of cardiovascular defects.[86] Early reports like those of Dubost et al., Oudot et al., Julian et al., DeBakey et al., and of Szilagyi and Overhulse indicated further encouraging results.[87-91] These reports suggested that arterial allografts function as well as arterial autografts.
Enthusiasm for these grafts, however, was tempered by longer-term studies, which began to appear in the 1960s.[84,85,92,93] The incidence of graft deterioration and complications, including graft occlusion, calcification, aneurysm formation, and graft rupture appeared to increase rapidly with time. Graft deterioration, seen both in fresh and preserved arterial allografts is, just as in venous allografts, mainly due to the immunologic response of the host. Consequently, the graft walls lose much of their structural identity; they become progressively less cellular and thinner and lose the integrity of the collagen and elastin network. The remaining elements do not have sufficient tensile strength to withstand the arterial pressure, eventually leading to further structural breakdown of the graft.

Arterial xenografts

Modified xenografts, as for example xenografts produced from bovine carotid arteries, are nowadays not used very often, mainly as a hemodialysis access shunt.[94]
The bovine xenograft for clinical use was first introduced by Rosenberg et al., after extensive experiments with implantation into the canine thoracic and thoracoabdominal aorta.[94] The graft is prepared by enzymatic digestion of bovine carotid arteries with ficin, followed by dialdehyde starch-tanning. Although the graft was initially widely used for both femoropopliteal and iliac artery bypass operations, its use in peripheral arterial reconstructive surgery

has been discontinued because of a high incidence of aneurysm formation in the femoropopliteal position, the high cost of the graft, and its poor resistance to infection.[94-100] Moreover, there are limitations in the availability of small-caliber grafts of less than 8 mm in diameter.

Synthetic vascular grafts

Vascular grafts prepared from Dacron and Teflon are nowadays widely used for the reconstruction of large-caliber and medium-caliber arteries and perform reasonably satisfactorily.[101]

These grafts have evolved from the Vinyon-N hand-sewn cloth tubes of Voorhees et al., who initially inserted these porous tubes into the abdominal aorta of dogs.[104,105] Their idea for a porous fabric tube as an arterial graft came from their serendipitous observation that a loose silk thread transversing the right ventricle of the heart of a dog had become coated with an endothelial-like thrombus free surface in a few months. They continued their experimental work with a successful clinical series in 1954: 17 abdominal aneurysms and 1 popliteal aneurysm were replaced.[106] However, since Vinyon-N could not be sterilized in an autoclave, it gradually disappeared as a clinical application.[102]

Since the experiments of Voorhees et al. several other materials have been evaluated both experimentally and clinically as arterial substitute during the late 1950s.[107-112] However, many of these materials appeared to be unsuitable. Nylon and Ivalon, for example, were found to lose most of their tensile strength after brief periods of implantation, which subsequently could lead to aneurysmal dilatation and graft rupture.[108] Marlex was found to kink too easily.[112]

Dacron and Teflon, however, appeared to function quite satisfactorily and still are the most common materials for the construction of fabric grafts to the present day.[101-103,109,113-118]

The versatility of Dacron, polyethylene terephthalate, has resulted in the creation of many types of Dacron fabric grafts.[101] Actually, Dacron fabric grafts come in three basic structures: woven, knit, and velour. A woven structure consists of yarns running both longitudinally (warp) and circumferentially (weft). Woven grafts generally yield low porosity and high strength, and are rather difficult to handle because they are rather stiff.

A knit structure consists of yarns running either predominantly longitudinally (warp knit) or predominantly circumferentially (weft knit).[101] Knitted grafts generally yield high porosity and have both better handling and better healing characteristics. However, knitted grafts have to be preclotted before use and have a greater tendency towards dilatation.[119,120]

A velour structure is made in such a manner that loops of yarn extend almost perpendicular from the fabric graft.[101] A velour structure can be formed in a basic woven, weft knit, or warp knit graft. The characteristics of a velour structure depend strongly on the basic approach used. A woven velour will heave strength and handling characteristics closely related to those of woven fabrics, a velour structure formed in a weft or warp knit will have strength and handling characteristics closely related to those of knit fabrics. However, a velour structure of the same porosity as a knit or woven structure is generally more easy to preclot. Inner velour structure in fabric grafts was introduced by DeBakey et al., because such a structure could serve to anchor fibrin deposits and pseudointima and as a consequence prevent embolization or sloughing of the inner cellular lining.[121] Sauvage introduced the outer velour structure in fabric grafts because such a structure would support fibroblast ingrowth.[122]

Teflon, a polymer of tetrafluoroethylene, is more difficult to manufacture into fabric construction and is far less versatile in regard to varying the fabric construction.[101-103] Therefore, Teflon fabric grafts come mainly in the knit structure.

Although both Dacron and Teflon fabric grafts perform reasonably satisfactorily in high-

flow, low-resistance locations conditions, such as aorta, iliac, and proximal femoral arteries, neither of these two materials perform quite satisfactorily for small-caliber arterial reconstructions. This is mainly due to a high incidence of graft complications, including mural thrombus formation, excessive subintimal hyperplasia, stenosis at the anastomotic sides, and anastomotic aneurysm formation. These complications are closely associated with the thrombogenicity,[123-128] the poor healing characteristics,[129-132] and the lack of compliance[133,134] of these materials.

In the early 1970s, nontextile vascular grafts prepared from expanded polytetrafluoroethylene (PTFE) were introduced.[135] Numerous reports have confirmed the usefulness of these PTFE vascular grafts for medium-caliber and small-caliber arterial reconstructions when autologous vein is not available.[135-141] However, their late patency rates have always been inferior to those achieved with venous autografts. Poor healing characteristics,[131,132,142-145] and the lack of compliance[133,134] of these grafts seem to be the major causes for their insufficient performance.

The "Groningen" concept: microporous, compliant, biodegradable vascular grafts

In attempt to develop an "ideal" small-caliber arterial substitute a new type of vascular graft was designed in Groningen. It was postulated that the combination of graft porosity, graft compliance, and the use of a suitable biodegradable material could create a vascular graft that would function as a temporary scaffold for the regeneration of a new arterial wall of small-caliber arteries. Such a graft should allow proper tissue in- and overgrowth and in such a graft the ingrowing tissue should be functionally stressed so that a neoartery with natural functional properties would regenerate.[146] Therefore, this vascular graft should be:

(1) *microporous* to provide a stable anchorage for cells at the luminal side of the graft and to stimulate rapid cell ingrowth from the perigraft region.
(2) *compliant* to stimulate the ingrowing tissue mechanically to form new elastic components of the arterial wall.
(3) *biodegradable* so that the ingrowing tissue would eventually take over the mechanical function of the graft.

It is of interest to note that it was already Guthrie who concluded as early as 1919 that to restore and maintain mechanical function, an implanted segment need only temporarily restore mechanical continuity and serve as a scaffolding or bridge for the laying down of an ingrowth of tissue derived from the host.[147]

In the literature, graft porosity, graft compliance, and the application of biodegradable materials in regard to vascular grafts are described as follows:

Porosity. Porosity, the first factor that was investigated extensively, is regarded as an important factor in graft healing. At present, however, neither the influence of pore volume nor the effect of pore size on graft incorporation and graft healing are thoroughly understood.[101]

In 1957 Edwards demonstrated that perforated polyethylene tubes developed a smooth cellular lining, whereas nonperforated tubes healed only partly at the anastomotic sides.[148] Wesolowsky et al. discovered an inverse relationship between the porosity of synthetic vascular grafts and the degenerative calcification of the inner lining in these grafts: the higher the degree of porosity the lower the degree of calcification and vice versa.[149] Furthermore, Fry et al. reported that the failure of nonporous, woven, Teflon prostheses was caused by dissection of the inner developed cellular lining, due to poor adherence.[150]

Apparently, pores at the luminal side should be of sufficient size to provide a stable

anchorage for the development of a viable cellular lining. However, the luminal pores should not be too large; some investigators demonstrated that large luminal pores are associated with a lower patency rate than small luminal pores.[151,152]
To support rapid capillary and fibrohystiocytic tissue ingrowth into the wall of vascular grafts, pore sizes should be in the range of 25 to 150 μm.[153,154]

Compliance. Poor compliance is one of the most important factors responsible for the poor performance of synthetic vascular grafts for the reconstruction of small-caliber arteries.[133,134] Several studies demonstrated a significant correlation between graft compliance and patency rate. Lyman et al. and Seifert et al. used polyurethane vascular grafts of different elasticities for the reconstruction of canine femoral arteries.[155,156] They found a higher patency rate for grafts with an elasticity which was much like that of the normal femoral artery. Mismatch in compliance between artery and graft results in a mechanical incongruity, high shear stress, and turbulence of the blood flow with local stagnation.[134] These factors can lead to local thrombosis[157] and can damage the arterial wall,[158] subsequently leading to graft thrombosis,[159] anastomotic aneurysm formation,[160] excessive subintimal hyperplasia,[161,162] and impairment of neoendothelial healing.[163,164]

Biodegradability. The first introduction of biodegradable materials for arterial reconstructive surgery was based on the concept to create a graft of low porosity at implantation and of high porosity during graft healing.[165] By using biodegradable material, one could control hemorrhage in more porous materials. Several experimental and clinical applications of biodegradable materials were performed in the 1960s. Teflon and Dacron, impregnated with gelatin,[166-168] or bovin collagen,[169] showed reduced operative bleeding and yielded satisfactory long-term results. Despite the apparent efficacy of the application of biodegradable material, it never achieved widespread application because of the success of the preclotting technique.[170]
Wesolowsky et al. designed a compound vascular graft consisting of biodegradable and nonbiodegradable components.[171] Many of these grafts became aneurysmal when tested. Nevertheless, Wesolowsky et al. showed in following experiments that an appropriate combination of materials could be developed that would function appropriately.[165]
Bowald et al. were the first to report on the use of a totally biodegradable vascular graft and demonstrated the great arterial regenerative potential of these grafts.[172-175] They implanted a polyglactin 910 (Vicryl) mesh graft into the thoracic aorta of growing pigs. This material, which is degraded by hydrolysis and totally absorbed within 70 days,[176] functioned as a temporary scaffold for the regeneration of a neoarterial wall. In grafts not exceeding 15 cm length a neoarterial wall regenerated with a microscopic appearance which was very much like normal arterial tissue. Underneath the endothelial lining, there were layers of smooth muscle cells. However, these neoarterial substitutes did not regenerate an elastic network, which was probably due to the lack of compliance of the polyglactin 910 (Vicryl) mesh graft.

Chemical composition and construction of microporous, compliant, biodegradable vascular grafts. The group led by Pennings (Department of Polymer Chemistry of the University of Groningen) had already for several years great interest in the physical properties, the preparation, and the biomedical application of poly-L-lactide (PLLA).[177-190] PLLA is a biodegradable, biocompatible, non-toxic polymer, which is also reasonably bloodcompatible.[191-197] Moreover PLLA is a low cost material which is available from renewable resources. Although the rate of degradation is slow, as compared to e.g. polyglycolide, special modifications of PLLA can be applied such that PLLA degrades much faster.[184,198,199]
At the onset, PLLA was chosen as a starting material for the preparation of microporous,

compliant, biodegradable vascular grafts. However, vascular grafts prepared from PLLA appeared to be too rigid and too brittle to handle surgically. Therefore, PLLA was combined with an elastomeric polyurethane (PU) to increase compliance and surgical handling characteristics. Polyurethanes are well known synthetic polymers, which have been applied for several medical devices.[200-204] Moreover, some polyurethanes are also biodegradable and biocompatible, as well as bloodcompatible.[205-209] In vitro experiments revealed that was essential to use high amounts (80% to 95% weight) of PU in the PU/PLLA mixtures to produce a biomaterial of sufficient compliance and surgical handling characteristics.
Essentially, the PU/PLLA vascular grafts were prepared by a multi-step dipcoating procedure. Several layers of the solution of the polymer mixture were deposited (precipitated) step by step on a Teflon-coated stainless-steel mandril (1.5 mm diameter), until the required wall thickness was obtained (0.3 mm). A pore size gradient, ranging from 10 μm in the inner region to 100 μm in the outer region of the graft lattice could be produced by gradual dilution of the starting polymer solution. Residual solvents were removed by soaking the grafts for 10 hours in distilled water and subsequently for 2 hours in ethanol.
The microstructure of the graft lattice actually can be described as a network of interpenetrating fibrils composed of the PU/PLLA mixture.

In vivo experiments. The first in vivo experiments with PU/PLLA vascular grafts in rats were carried out by E.Lommen et al.[146] They reported that these grafts could indeed function as a temporary scaffold for the regeneration of a new arterial wall of small-caliber arteries. They observed a newly formed arterial wall, which was already established as early as six weeks after implantation. This wall consisted of an inner endothelial lining (neointima), with underneath several layers of smooth muscle cells (neomedia) in which elastin and collagen regenerated, and a disintegrated graft wall organized by fibrohistiocytic tissue (neoadventitia).

Aim of the studies

In this thesis, the following issues were investigated to determine the basic mechanisms of arterial wall regeneration in PU/PLLA vascular grafts, and to determine the scope and the limitations of the use of these vascular grafts (*chapter 8*):

(1) the neoendothelial healing characteristics of PU/PLLA vascular grafts in comparison with polytetrafluoroethylene grafts (*chapter 2*).
(2) the most suitable composition for PU/PLLA vascular grafts to ensure an optimal regeneration of neoarterial tissue (*chapter 3*).
(3) the ultrastructure of neoarterial tissue regenerated in PU/PLLA vascular grafts (*chapter 4*).
(4) the specific influence of both compliance and biodegradation of microporous vascular grafts on the regeneration of neoarterial tissue, especially on the regeneration of elastic laminae (*chapter 5*).
(5) the sequential events leading to the arterial wall regeneration in PU/PLLA vascular grafts (*chapter 6*).
(6) the ultimate biological fate of neoarterial tissue regenerated in PU/PLLA vascular grafts (*chapter 7*).

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CHAPTER 2

Arterial wall regeneration in small-caliber vascular grafts in rats

Neoendothelial healing and prostacyclin production

Berend van der Lei,[1] Harald Darius,[2] Karsten Schrör,[2] Paul Nieuwenhuis,[3] Izaäk Molenaar,[4] and Charles R.H. Wildevuur[1]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Pharmacological Institute, University of Köln, West Germany
[3] Department of Histology, University of Groningen, The Netherlands
[4] Centre for Medical Electron Microscopy, University of Groningen, The Netherlands

Summary

Clinically available synthetic graft materials frequently fail when used as a small-caliber arterial substitute. Therefore, we developed a new type of graft material, prepared from a mixture of polyurethane (PU) and poly-L-lactic acid (PLLA), to be used as a scaffold for the regeneration of the arterial wall. In this study microporous, compliant, biodegradable PU/PLLA grafts (n = 16) and polytetrafluoroethylene (PTFE) grafts (n = 16) were implanted into the rat abdominal aorta and evaluated 3, 6, and 12 weeks after implantation. First, we evaluated the extent of neoendothelial healing (n = 8) by means of light microscopy and scanning electron microscopy. Next, we studied the ability of the neoendothelial cells to produce prostacyclin (n = 8) by means of bioassay for prostacyclin and radioimmunoassay for its stable hydrolysis product, 6-oxo-prostaglandin $F_{1\alpha}$.
There were no significant differences between the two graft types in the amount of prostacyclin production per unit graft area covered with neoendothelium, and this amount was the same as for normal endothelium. However, the PTFE grafts showed incomplete neoendothelial healing, even after 12 weeks of implantation, in contrast to the PU/PLLA grafts. The better healing characteristics of the PU/PLLA grafts ensured the fast development of a complete neoarterial wall, possessing strength, compliance, and thromboresistance equivalent to normal arterial wall tissue.
These results demonstrate that arterial wall tissue regeneration in PU/PLLA grafts may open new perspectives in the field of arterial reconstructive surgery.

Introduction

The thrombogenic nature of prosthetic material used in vascular reconstructive operations limits the patency of grafts, particularly of small-caliber vascular grafts.[1-4] This thrombogenicity is mainly determined by the reactivity of the prosthetic material with platelets.[1, 5-7] Fast and complete neoendothelial healing may reduce this thrombogenicity[8-10] by producing

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prostacyclin, the most potent inhibitor of platelet aggregation.[11] However, since most prosthetic grafts have poor healing characteristics, as reported for various species including man,[12-16] the risks of thromboembolic complications and graft occlusion remain.
Therefore, an autologous vein graft is still the first choice as a small-caliber arterial substitute for clinical use.[4, 17, 18] However, because harvesting of a vein of appropriate length and size extends the operation time and because such a vein sometimes is not even available, it would be most advantageous to have readily available synthetic vascular grafts in various sizes.
In attempt to improve the healing characteristics, we have developed a new type of small-caliber vascular graft, prepared from a mixture of polyurethane (PU) and poly-L-lactic acid (PLLA),[19, 20] which is biodegradable. Bowald, Busch, and Eriksson[21, 22] already have demonstrated that biodegradable vascular graft material can function as a temporary scaffold for the regeneration of arterial tissue. Moreover, PU/PLLA grafts are microporous, which enables rapid perigraft tissue ingrowth,[23] and compliant, which prevents high shear forces at the anastomosis.[24] Preliminary results of studies in which this biodegradable, microporous, compliant PU/PLLA graft material was used as a substitute for rat abdominal aorta showed development of a neoartery.[25, 26]
The aim of this study in rats was to quantify and qualify the neoendothelial healing characteristics of these PU/PLLA grafts by determining the extent of neoendothelial healing as well as the prostacyclin production, in comparison with polytetrafluoroethylene (PTFE) grafts, which are clinically used.

Materials and methods

Graft implantation procedure. Male Wistar rats (TNO, Zeist, The Netherlands, n = 35) weighing 250 to 350 gm were premedicated with atropin (0.25 $mg.kg^{-1}$ body weight, administered intramuscularly) and anesthetized with 1% halothane (Fluothane®). Under sterile conditions, the abdominal aorta was exposed by a midline abdominal incision and dissected free from the adjacent caval vein and surrounding tissue. All grafts were gas sterilized (ethylene oxide) and evacuated under high vacuum (10^{-5} torr) for 24 hours. A 1 cm segment of the abdominal aorta was resected and replaced by a PTFE (Impra, Inc., Tempe, Ariz.) (n = 16) or a PU/PLLA vascular graft[19] (n = 16) by means of microsurgical techniques, using an operation microscope (Zeiss OPMI 7-D, Carl Zeiss, Inc., Thornwood, N.Y.) (Table I). Interrupted sutures, Ethilon 9-0 (BV-4 needle, Ethicon, Inc., Somerville, N.J.), were used. The grafts were not preclotted. Neither heparin nor spasmolytics were administered. After hemostasis had been secured by gentle pressure, patency was determined by direct inspection. The wound was then irrigated with saline solution and closed with Dexon 4-0 sutures.
The rats had free access to standard rat food and water. Eight rats of each group, selected at random, were used for neoendothelial healing determination; the other eight rats of each group for prostacyclin measurements (Table II).

Graft harvesting. The rats were anesthetized with urethane (1.5 $gm.kg^{-1}$ body weight, administered intraperitoneally). Patency was evaluated and heparin was administered (1,000 IU, intravenously) 2 to 3 minutes before graft harvesting to prevent clotting.

Determination of neoendothelial healing. The abdominal aorta was cannulated distal and proximal to the graft. Immediately after cannulation, pressure-controlled perfusion was started at a pressure of 180 mm Hg with, successively, 0.9% sodium chloride (for 30 seconds), 2% polyvinylpyrrolidon K30, 0.4% sodium nitrite in 0.1 M phosphate buffer, pH 7.4 (for 60

Table I. *Material characteristics of the vascular grafts*

	PTFE grafts	*PU/PLLA grafts*
Chemical composition	100% PTFE	95% PU/5% PLLA
Internal diameter	1.5 mm	1.5 mm
Wall thickness	0.5 mm	1.5 mm
Pore size	30 μm	40-50 μm

Table II. *Evaluation scheme of the PTFE and PU/PLLA vascular grafts*

	Weeks after implantation		
	3	*6*	*12*
Neoendothelial healing determination (No. of rats)	2	4	2
Prostacyclin measurements (No. of rats)	2	2	4

seconds), and finally for fixation with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (for 15 minutes). Next the cannulated aorta with implanted graft was removed and postfixed with 2% glutaraldehyde at 4 °C at 180 mm Hg for 24 hours.
To determine the extent of endothelial cell coverage, almost the entire graft was prepared for scanning electron microscopy (SEM). For light microscopy (LM) and transmission electron microscopy (TEM) a 3 mm distal or proximal segment was cut off. LM was applied to determine the subendothelial graft healing and the perigraft tissue ingrowth into the prosthetic graft walls. TEM was used for positive identification of the cells observed with SEM and LM.

Preparation for SEM. The specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide and sputter coated with gold palladium. The specimens were examined in a JSM-35 C scanning electron microscope operated at 15 to 25 kV.

Preparation for LM and TEM. The 3 mm graft segments were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 4 hours in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol, the segments were embedded in Epon. Semithin sections (1 μm) for LM were cut and stained with toluidine blue and basic fuchsin. Areas of interest were selected; this material was routinely prepared and stained for TEM.

Prostacyclin measurements. The grafts were excised and rinsed with Krebs-Henseleit buffer solution to remove the intraluminal blood. Two mm of the distal and proximal ends of the grafts, containing the sutures, were removed. The remaining segments were incubated in oxygenated (95% oxygen, 5% carbon dioxide) Krebs-Henseleit buffer solution at 37 °C for a stabilization period of 60 minutes. Afterwards, the segments were incubated in a reaction vial containing 400 μl Krebs-Henseleit buffer solution at 37 °C. After 1, 5, 10, and 20 minutes, aliquots of the supernatant (2 × 20 μl, duplicate determinations) were taken for the bioassay of prostacyclin. The bioassay for prostacyclin was performed by measuring the inhibition of primary adenosine diphosphate (0.1 to 1 μM)-induced aggregation of human platelets in platelet-rich plasma.[27] The inhibitory effect of the supernatant was matched with that of synthetic prostacyclin-Na (Schering AG, Berlin/Bergkamen, West Germany).
Platelet aggregometry was measured turbidometrically in a two-channel aggregometer

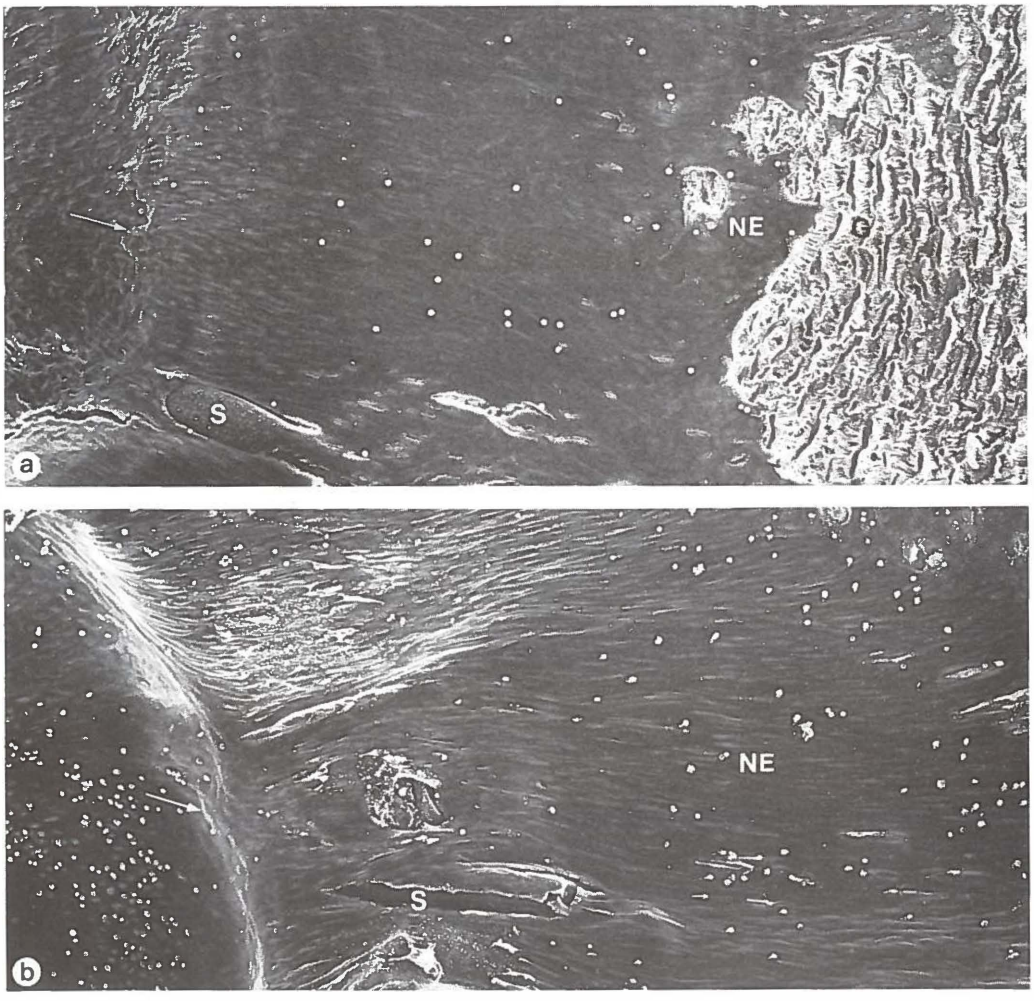

Fig. 1a, b Scanning electron micrographs of the luminal surface of a PTFE graft (**a**) and of a PU/PLLA graft (**b**) near the proximal anastomosis, 3 weeks after implantation. The layer of neoendothelium (*NE*) has hardly grown over the PTFE graft, in contrast to the PU/PLLA graft. Note the smooth anastomotic conjunctions (*arrows*) between aorta and both graft types. *S* suture; *G* inner surface of the PTFE graft. (magnification × 105).

(Labor, Hamburg, West Germany). After 20 minutes of incubation, 200 μl of the supernatant was taken and stored at room temperature for 2 hours to complete the hydrolysis of prostacyclin into 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), the stable hydrolysis product of prostacyclin. The amount of 6-oxo-$PGF_{1\alpha}$ was measured by radioimmunoassay (RIA) with a specific antibody.[28] The RIA values of the grafts of the two groups were divided by the graft area covered with endothelium, as determined by means of SEM, and compared with each other as well as with the RIA values of abdominal aorta of unoperated control rats (n = 6). For SEM, the segments were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, after 20 minutes' incubation.

Statistics. All values are presented as mean ± standard deviation. The Mann Whitney U test was used to compare the prostacyclin results of the groups.[29]

Results

Surgical results. The rigid PTFE grafts required 10 to 12 stitches to achieve anastomotic seal, whereas the compliant PU/PLLA grafts required 8 stitches. Consequently, the average time to accomplish both anastomoses of the PTFE grafts was almost twice (40 minutes) the time needed for the PU/PLLA grafts (25 minutes). A total of 35 rats were operated on to obtain two groups of 16 rats each. Three rats died postoperatively within 24 hours; two rats because of leakage at the anastomosis (PTFE grafts) and one rat because of an unkown reason (PU/PLLA graft). The PU/PLLA grafts showed arterial pulsations immediately after recirculation, as well as at the moment of graft harvest, in contrast to the PTFE grafts. None of the grafts showed aneurysm formation or graft occlusion after the observation periods of 3, 6, and 12 weeks.

Determination of neoendothelial healing. SEM and LM of the PTFE and PU/PLLA grafts showed that there was a smooth transition from artery to graft in all 16 rats. Continuous sheets of flat, elongated cells, resembling endothelium, grew from the anastomotic sides over the prosthetic surface in both graft types (Fig. 1). Endothelial islands were not observed. TEM confirmed that the lining cells bore the morphological characteristics of endothelium: tight junctions, numerous pinocytotic vesicles and occasional Weibel-Palade bodies (not shown).

Neither platelets nor fibrin were found adhering to the neoendothelial cells, not even to the front-line cells. On the nonendothelialized areas a thin platelet-fibrin layer was deposited. Statistical analysis with regard to the endothelial cell coverage was not performed, because the differences in the extent of the neoendothelial graft healing between the PTFE and PU/PLLA grafts were obvious (Fig. 2). The neoendothelial healing of the PTFE grafts was incomplete, even after 12 weeks of implantation (Figs. 2, 3a, 4a). In contrast, the PU/PLLA grafts were progressively endothelialized (Figs. 2, 3b, 4b). Six weeks after implantation the PU/PLLA grafts were already almost completely covered with a neoendothelial lining.

There were also striking differences between the two graft types in the subendothelial graft healing and perigraft tissue ingrowth into the micropores of the graft walls (Fig. 3). When semithin sections of the PTFE grafts were made for LM, the endothelial lining was frequently detached from the graft, which indicates poor attachment to the prosthetic surface. Sometimes some smooth muscle cells could be observed near the anastomosis underneath the poorly developed neoendothelial lining. There was only limited fibroblast and histiocyte penetration into the micropores of the PTFE graft walls, and no capillary ingrowth. Some multinucleated giant cell activity was observed along the outer surface of the PTFE grafts.

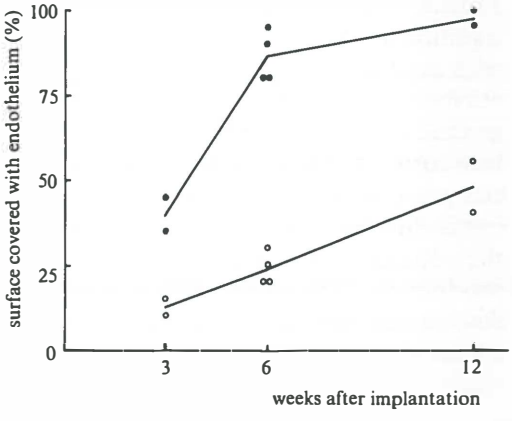

Fig. 2 Endothelial cell coverage of the PTFE grafts (o) and the PU/PLLA grafts (•) in relation to time after implantation.

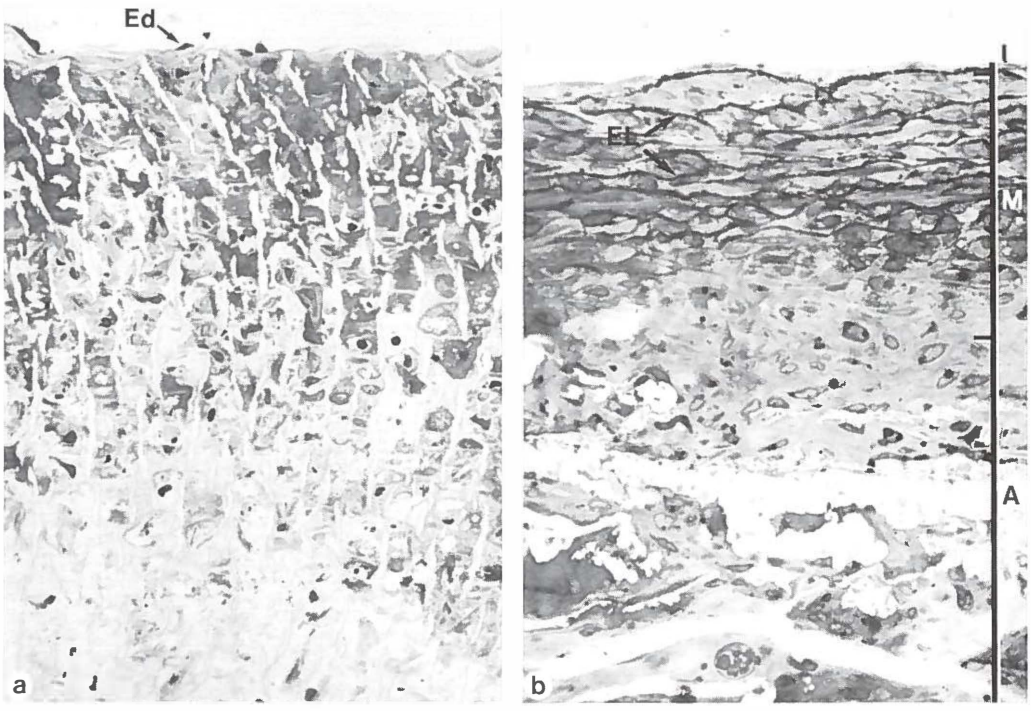

Fig. 3a, b Light micrographs of a PTFE graft (**a**) and of a PU/PLLA graft (**b**), 6 weeks after implantation. The PTFE graft surface is scarcely covered with endothelium (*Ed*). There is limited perigraft tissue ingrowth. In the PU/PLLA graft a neoartery has developed, composed of a neointima (*I*), a neomedia (*M*), and a neoadventitia (*A*). Note the elastic laminae (*EL*) in the neomedia and the extensive perigraft tissue ingrowth into the PU/PLLA lattice. (magnification × 300).

In contrast, the PU/PLLA grafts had the microscopic appearance of a neoartery (Fig. 3). Multiple layers of smooth muscle cells (neomedia) had developed underneath the endothelial lining, containing collagen and elastic laminae. These layers did not narrow the lumen. There was an extensive ingrowth of capillaries, fibroblasts, and histiocytes into the micropores of the PU/PLLA lattices (neoadventitia). The PU/PLLA graft material clearly showed fragmentation and disintegration. Multinucleated giant cells frequently surrounded remnants of the prosthetic material.

Prostacyclin measurements. All 16 graft aliquots added to the platelet-rich plasma showed significant inhibition of adenosine diphosphate-induced platelet aggregation, as compared with control aggregation. The antiaggregatory activity was time-dependent, reaching a maximum after 10 minutes of incubation and hardly increasing after 20 minutes (Fig. 5). The prostacyclin-like activity disappeared when the supernatant was inactivated either by heating for 20 minutes at 60 °C, or after 1 hour incubation at pH 4, and was not present when the graft segments had previously been incubated in Krebs-Henseleit buffer solution containing indomethacin (cyclo-oxygenase inhibitor) (data not shown). This indicates that the antiaggregatory activity, measured in the graft supernatants, was due to prostacyclin production.[11] The results of this bioassay, by means of which the prostacyclin production was determined, were consistent with the results obtained with the RIA, by means of which the prostacyclin production was determined by its stable hydrolysis product, 6-oxo-$PGF_{1\alpha}$ ($r = 0.852$, $n = 16$).

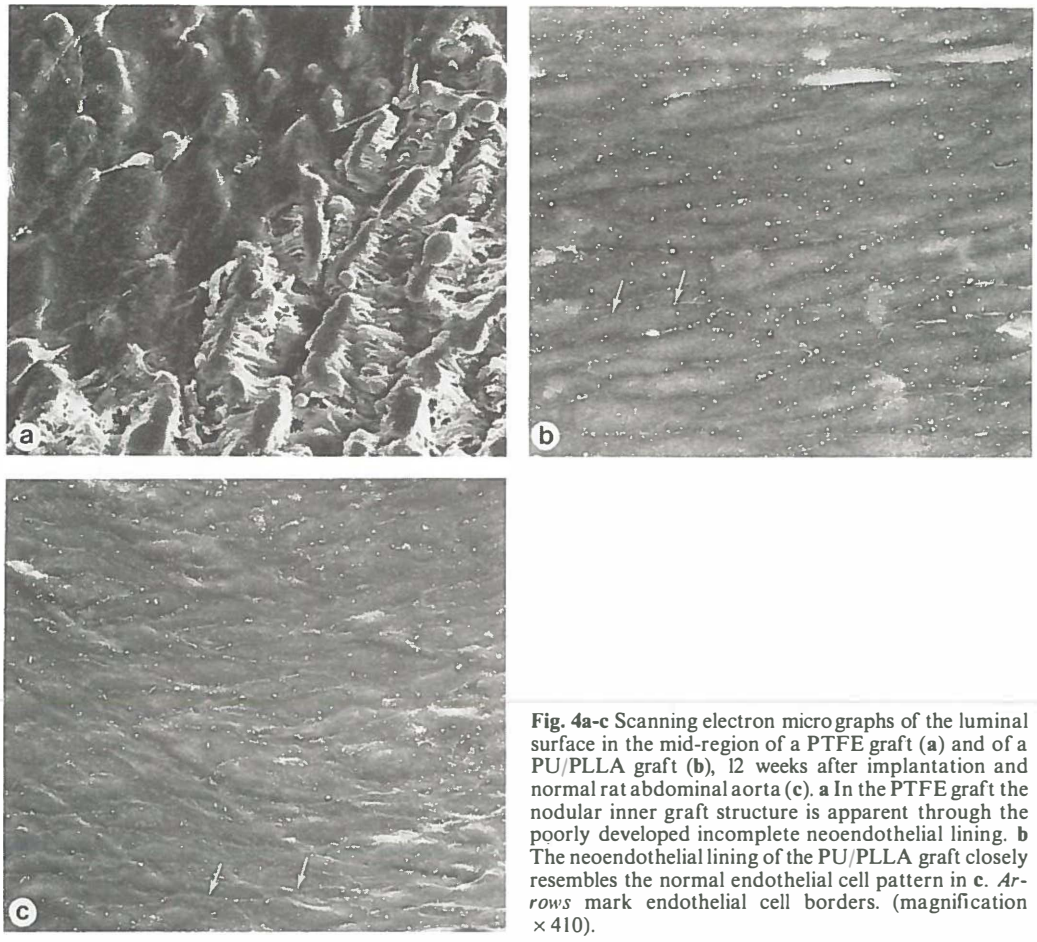

Fig. 4a-c Scanning electron micrographs of the luminal surface in the mid-region of a PTFE graft (**a**) and of a PU/PLLA graft (**b**), 12 weeks after implantation and normal rat abdominal aorta (**c**). **a** In the PTFE graft the nodular inner graft structure is apparent through the poorly developed incomplete neoendothelial lining. **b** The neoendothelial lining of the PU/PLLA graft closely resembles the normal endothelial cell pattern in **c**. *Arrows* mark endothelial cell borders. (magnification ×410).

For further statistical analysis, the RIA values for 6-oxo-$PGF_{1\alpha}$ were used (Fig. 6); these values present the total prostacyclin production within the incubation time, being independent of any inactivation that may occur with prostacyclin itself. It was concluded that there were no significant differences between both graft types in prostacyclin production, as expressed by the RIA value for 6-oxo-$PGF_{1\alpha}$ per unit graft area covered with neoendothelium ($p < 0.05$, two-sided, Mann Whitney U test). On the average, the endothelium of all graft segments used for prostacyclin measurements ($n = 16$) had a mean RIA value for 6-oxo-$PGF_{1\alpha}$ of 156 ± 96.4 pmol.cm^{-2} (without one extreme value, 134.6 ± 42.9 pmol.cm^{-2}, $n = 15$). This value did not differ significantly from the RIA values in abdominal aortic segments of unoperated control rats (154 ± 40 pmol.cm^{-2}, $n = 6$, $p < 0.05$, two-sided, Mann Whitney U test).

Discussion

Fast and complete neoendothelial healing of synthetic small-caliber vascular grafts will reduce the risks of thromboembolic complications and graft occlusion,[8-10] because of the production of prostacyclin[11] by neoendothelial cells. In this study we compared the neoendo-

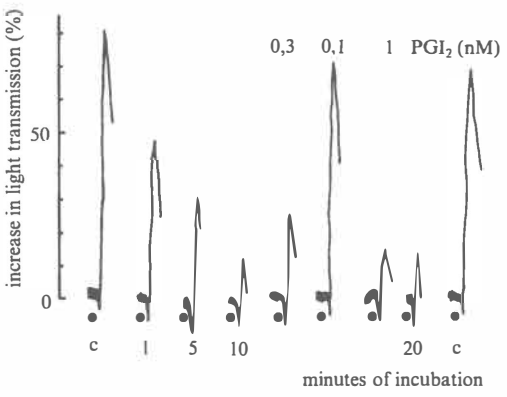

Fig. 5 Typical experiment on the inhibition of primary adenosine diphosphate-induced platelet aggregation by the supernatant of a PU/PLLA graft, 12 weeks after implantation. There is an increasing inhibition of platelet aggregation after 1, 5, 10, and 20 minutes of incubation compared with control agggregations (*c*). The amount of antiaggregatory activity is matched with synthetic prostacyclin-Na (*PGI_2; 0.1, 0.3, and 1 nM*).

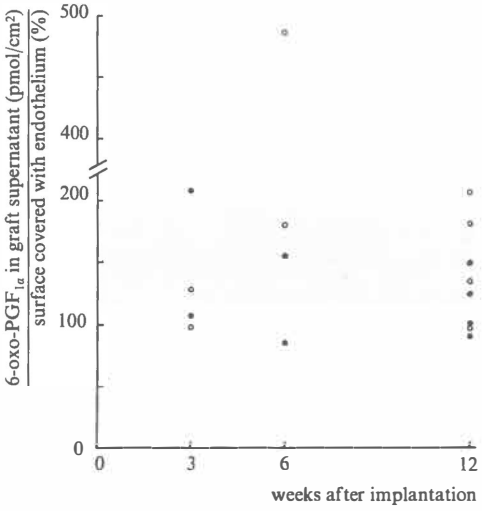

Fig. 6 Amount of 6-oxo-$PGF_{1\alpha}$ in graft supernatant per unit graft area covered with neoendothelium of the PTFE grafts (○) and the PU/PLLA grafts (●). The *stippled area* represents mean ± standard deviation for 6-oxo-$PGF_{1\alpha}$ of normal rat abdominal aorta (n = 6).

thelial healing of PU/PLLA grafts with PTFE grafts qualitatively and quantitatively. We chose the rat abdominal aorta implantation as the test model because in this model highly reproducible results can be achieved and the prostacyclin can be measured easily. Both graft types showed excellent patency rates. However, the PU/PLLA grafts were much easier to handle and to suture than the PTFE grafts.

The results of this study clearly demonstrated that, although in both graft types the neoendothelial cells were qualitatively the same as normal endothelial cells concerning their prostacyclin production (Fig. 6), there were striking quantitative differences concerning the process of neoendothelial graft healing (Figs. 1-4). The PTFE grafts showed incomplete neoendothelial healing, even after 12 weeks of implantation, in contrast to the PU/PLLA grafts. In the PU/PLLA grafts the process of neoendothelialization was fast and almost complete six weeks after implantation, a time frame which corresponds with observations of the healing process of artificially denuded arterial wall tissue.[30-32]

The striking quantitative differences in the process of neoendothelial graft healing noted between the two graft types has to be ascribed to the differences in chemical and material characteristics of the grafts. The PTFE grafts are rigid, nonbiodegradable, and have an inner luminal surface structure of circumferentially oriented nodes, interconnected by smaller fibrils oriented longitudinally. By contrast, the PU/PLLA grafts are compliant, biodegradable, and have a fibrous lattice as inner luminal surface structure. The process of luminal graft healing and neoendothelialization is determined by the graft fabrication, the inner structure of a graft wall, the speed and amount of perigraft tissue ingrowth.[33-36] The perigraft tissue ingrowth into the PTFE graft walls was minimal and smooth muscle cells did not grow over the prosthetic surface (Fig. 3a). In addition, the neoendothelium was poorly attached to the PTFE material. These observations of poor healing of PTFE grafts with a largely incomplete neoendothelial lining are consistent with our findings in a previous study[37] and with those of other investigators,[12-15, 34] although some studies reported better neoendothelial healing of PTFE grafts.[38, 39]

In contrast, in the PU/PLLA grafts the perigraft tissue ingrowth was rapid and extensive (Fig. 3b), which was made possible by virtue of fragmentation and degradation of the graft wall. Moreover, smooth muscle cells had grown over the PU/PLLA lattices. Apparently, these smooth muscle cell layers (neomedia) provided a good structural base and anchorage to the proliferating endothelial cells. The neoendothelial lining was part of the regenerated neoartery that had taken over the strength and compliance from the disintegrating PU/PLLA lattice, as demonstrated by discernible arterial pulsations, the presence of elastic laminae and collagen in the neomedia, and the absence of aneurysm formation at the moments of graft harvest. However, basically the process of neoendothelial healing in both graft types was the same. Endothelial cells grew in as a continuous sheet across the anastomosis from the host artery, as has also been described by other investigators studying the healing of vascular grafts.[40, 41] The absence of isolated patches of endothelial cells in both graft types in our study does not support the theory that circulating cells are additionally seeded on the grafts and contribute to the process of neoendothelial healing.[42]

In both graft types, the amount of prostacyclin production per unit graft area covered with endothelium was the same as for the normal rat abdominal aorta. Apparently, neither graft material was detrimental to the functional development of the neoendothelial lining, concerning the production of prostacyclin. Prostacyclin, the most potent inhibitor of platelet aggregation yet discovered,[11] mainly determines the antithrombogenic nature of the luminal surface of blood vessels. The enzyme that metabolizes prostaglandin endoperoxides to prostacyclin (prostacyclin synthetase) is most highly concentrated in the intimal layer of the arterial wall,[43] and endothelial cells are the most active producers of prostacyclin, which was demonstrated in studies using cultured cells from vessel walls.[44, 45]

In addition, our results emphasize the dominant contribution of endothelial cells to the prostacyclin production and also support our findings in a previous study[37] and the observations of other investigators, who were able to correlate normalization of platelet survival with the extent of neoendothelial healing.[46, 47] The scanning electron microscopic observations demonstrated that the neoendothelial cells in both graft types possessed thromboresistance even in an early stage of their development: no platelets or fibrin were found adhering to the front-line cells of the endothelial sheets running from the anastomotic sides. However, our results also demonstrate that subendothelial tissue, which was present as a neomedia in the disintegrating PU/PLLA grafts and was absent in the PTFE grafts, virtually does not contribute to prostacyclin production, as has been suggested by some investigators.[10] In conclusion, our results demonstrate that, although PTFE graft material is not detrimental to the functional development of neoendothelium, it is detrimental to the process of neoendothelial healing. Therefore, no normal antithrombogenic neoendothelial flow surface is achieved quantitatively. This may explain the reports of the failure of PTFE grafts, when used as alternative for autologous vein grafts.[4, 48-50] In contrast, the better healing characteristics of the PU/PLLA grafts ensured the fast development of a complete neoarterial wall with the same thromboresistance, strength, and compliance as normal arterial tissue. These results justify further investigation of the application of PU/PLLA graft material as a scaffold for the regeneration of arterial wall tissue. We believe that this new type of vascular graft material may open new perspectives in the field of arterial reconstructive surgery.

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CHAPTER 3

Microporous, compliant, biodegradable vascular grafts for the regeneration of the arterial wall in rat abdominal aorta

Berend van der Lei,[1] Hans L. Bartels,[2] Paul Nieuwenhuis,[3] and Charles R.H. Wildevuur[1]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Central Animal Laboratory, University of Groningen, The Netherlands
[3] Department of Histology, University of Groningen, The Netherlands

Summary

Microporous, compliant, biodegradable vascular grafts prepared from mixtures of polyurethane (PU) and poly-L-lactic acid (PLLA) can function as temporary scaffolds for the regeneration of the arterial wall in small-caliber arteries. This study was undertaken to determine the most suitable composition for PU/PLLA vascular grafts to ensure an optimal regeneration. Four types of PU/PLLA vascular grafts, differing in percent weight of the PU/PLLA mixture, viscosity-average molecular weight of PLLA, and pore size were implanted into the abdominal aorta of rats (n = 32). Six weeks after implantation two implants of each graft type were evaluated by means of scanning electron microscopy and six implants were evaluated by means of light microscopy.

In two types of the PU/PLLA vascular grafts, both of which were prepared from a 95%/5% weight PU/PLLA mixture with PLLA of viscosity-average molecular weight 500,000, but which had a different pore size, there was (I) absence of aneurysm formation and maintenance of arterial implant pulsations, (II) regeneration of a complete antithrombogenic neointima, (III) regeneration of a neomedia of comparable thickness to the media of normal rat abdominal aorta with the regeneration of elastic laminae almost throughout its thickness, and (IV) regeneration of a sufficiently supporting neoadventitia.

These results demonstrate that a 95%/5% weight PU/PLLA mixture with PLLA of viscosity-average molecular weight 500,000 is the most suitable composition for PU/PLLA vascular grafts to ensure an optimal regeneration of a neoarterial wall that is of sufficient strength, compliance, and thromboresistance to function as small-caliber arterial substitute. Pore size of these PU/PLLA grafts does not affect regeneration.

Introduction

Synthetic vascular grafts that are currently used clinically are not ideal substitutes for the reconstruction of small-caliber arteries. They frequently fail as a result of their thrombogenicity, poor healing characteristics, and insufficient elasticity.[1-6] Even autologous vein grafts, still regarded as the best replacement for small-caliber arteries, appear not to be ideal arterial substitutes. Clinical and experimental studies demonstrated that in vein grafts chronic

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structural changes, such as excessive subintimal hyperplasia, atherosclerosis, and aneurysm formation will eventually develop.[7-12]

However, small-caliber vascular grafts prepared from mixtures of polyurethane (PU) and poly-L-lactic acid (PLLA)[13, 14] have some ideal arterial substitute characteristics: they are easy to handle, they are compliant, and they have excellent healing characteristics.[15-17] When implanted into the rat abdominal aorta, these microporous, biodegradable vascular grafts can function as a temporary scaffold for the regeneration of a neoarterial wall, which is as thromboresistant as normal rat abdominal aorta.[15, 16]

This study was undertaken to determine the most suitable composition for PU/PLLA vascular grafts to ensure an optimal regeneration. Four types of PU/PLLA vascular grafts, differing in percent weight of the PU/PLLA mixture, viscosity-average molecular weight ($\bar{M}_v$) of PLLA, and pore size were implanted into the abdominal aorta of rats. Six weeks after implantation two implants of each graft type were evaluated by means of scanning electron microscopy (SEM) and six implants by means of light microscopy (LM).

Materials and methods

PU/PLLA vascular grafts. Each of the four types of PU/PLLA vascular grafts (Table I; Fig. 1) had an internal diameter of 1.5 mm and a wall thickness of 0.3 mm. The grafts were sterilized with ethylene oxide and evacuated for 24 hours under high vacuum (10^{-5} torr). The grafts were not preclotted. Each type of PU/PLLA vascular graft was implanted in eight rats.

Animals. Male Wistar rats (TNO, Zeist, The Netherlands) (n = 32), weighing 250 to 350 gm were used. The rats had free access to standard rat food and water.

Surgery. The rats were premedicated with atropin (0.25 $mg.kg^{-1}$ body weight, administered intramuscularly) and anesthetized with 1% halothane (Fluothane®). Under sterile conditions a midline abdominal incision was made and the abdominal aorta was exposed and carefully dissected free from the adjacent caval vein and surrounding tissue. One cm of the abdominal aorta was resected and replaced by a PU/PLLA vascular graft by means of microsurgical techniques. The grafts were easy to suture and conformed easily to the aorta. The end-to-end anastomoses were made with eight interrupted sutures, Ethilon 9-0 (BV-4 needle, Ethicon, Inc., Somerville, N.J.). The mean operation time was 40 minutes, the mean aortic cross-clamping time 25 minutes. Neither heparin nor spasmolytics were administered. After hemostasis had been secured by gentle pressure, patency of the graft was determined by direct inspection.[18] Arterial pulsations were observed in all implanted grafts. The abdominal wound was then irrigated with saline solution and closed with Dexon 4-0 sutures. A total of 34 rats were operated on to obtain the four groups of 8 rats: one rat died 4 hours after surgery because of blood leakage as a result of an improperly placed suture; the other rat died 3 hours after surgery because of blood leakage from a ruptured small intestinal blood vessel.

Table I. *Characteristics of the PU/PLLA vascular grafts*

Group	*PU/PLLA (% weight)*	*$\bar{M}_v$ of PLLA*	*Pore size (μm)*
A	80/20	100,000	10-100*
B	80/20	500,000	10-100*
C	95/ 5	500,000	10-100*
D	95/ 5	500,000	40

$\bar{M}_v$ viscosity-average molecular weight.

* Pore size gradient ranging from 10 μm in the inner region of the graft lattice to 100 μm in the outer region.

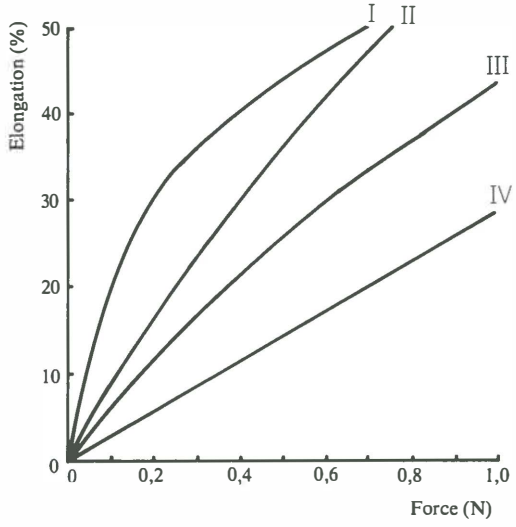

Fig. 1 Stress-strain dependence of PU/PLLA vascular grafts with PLLA concentration compared with normal rat abdominal aorta. *I* Abdominal aorta; *II* 95%/5% weight PU/PLLA material composition; *III* 80%/20% weight PU/PLLA material composition; *IV* 50%/50% weight PU/PLLA material composition.

Graft Harvesting. The implants were harvested 6 weeks after implantation. Before the implants were removed, heparin (1,000 IU, intravenously) was administered to prevent clotting. Two implants were prepared for SEM and six implants for LM. SEM and LM were used for morphologic evaluation of the endothelial lining (neointima); LM was used for morphologic evaluation of the subendothelial smooth muscle cell layers (neomedia) as well as the fibrohistiocytic tissue in the graft lattice (neoadventitia).

Preparation for SEM. The implants were fixed by pressure-controlled perfusion[16] at a pressure of 180 mm Hg with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 minutes. Then the implants were dissected and left in the same fixative for 24 hours at 4 °C. Next, the specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide. Then the specimens were cut in three longitudinal segments and sputter coated with gold paladium. These segments were examined in a JSM-35C scanning electron microscope operated at 15 to 25 kV.

Preparation for LM. The implants were dissected and rinsed in Ringer's salt solution to remove excess intraluminal blood. Subsequently the implants were fixed in 4% paraformaldehyde, cut into halves, and embedded in 2-hydroxyethyl methacrylate (Technovit 7100).[19] Longitudinal and transverse sections (2 μm) of either the distal half of the implant or the proximal half were stained with haematoxylin-eosin and orcein (elastin stain).

Morphologic Evaluation. The neointima was inspected for its completeness.
The neomedia was inspected in the longitudinal sections for its thickness and elastin content by means of a light microscope (magnification × 320) with a square grid placed in its ocular.[20] The thickness of the neomedia was measured in both sides of the implants in the longitudinal sections, both in the middle part of the implants as well as near the anastomotic sides. These results were compared with the thickness of the media of normal rat abdominal aorta. The elastin content was determined by measurement of the ratio of the thickness of that part of the neomedia, which contained elastic laminae, to the total thickness of the neomedia. These results were compared among the four groups.

The neoadventitia was inspected for the extent of fibrohistiocytic tissue ingrowth into the graft lattices by means of a light microscope with a square grid placed in its ocular. The extent of tissue ingrowth was determined in both sides of the implants in the longitudinal sections by measurement of the ratio of the number of grid points laying over tissue to the total number of grid points used. These measurements were performed in two equally large regions of the PU/PLLA lattices: in the inner third region and the outer third region of the PU/PLLA lattices. The amount of fibrohistiocytic tissue in these regions was compared among the groups.

Statistics. To analyse the morphometric measurements of the neomedia we used the Kruskal-Wallis one-way analysis of variance; to analyse the morphometric measurements of the neoadventitia we used the parametric one-way analysis of variance.[21] All data are presented as mean ± standard deviation.

Results

Macroscopic examination. At the moment of graft harvesting all implants were patent except one from group B. Examination of this occluded implant showed an organized thrombus, revealing early thrombosis. In group A all implants had developed into aneurysms; two rats had even died because of rupture of the aneurysmal implant. A turbulent blood flow was discernable through the wall of the aneurysmal implants. In addition, no arterial pulsations of the aneurysmal implants were observed.
In contrast, in groups B, C, and D the implants had not developed into aneurysms: they were not dilated al all. In group B arterial pulsations of the implants were hardly discernable, whereas in groups C and D pulsations were clearly discernable.

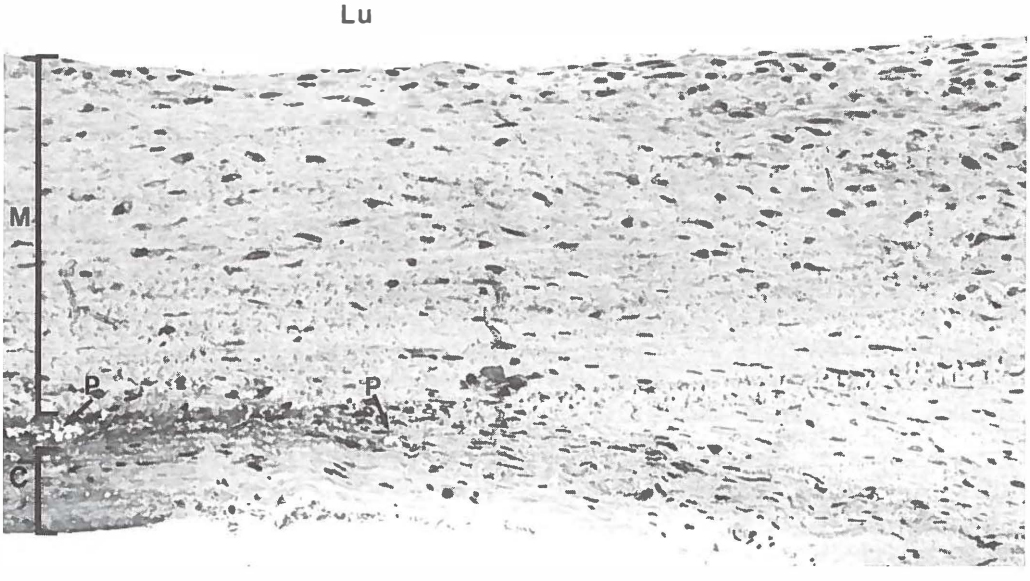

Fig. 2 Light micrograph of a transverse section of an aneurysmal implant of group A. The endothelial lining is virtually absent. The wall of the implant consists of a thick layer of smooth muscle cells (*M* neomedia), and a thin layer of dense collagenous neoadventitial tissue (*C*). Note the remnants of prosthetic material (*P*). *Lu* lumen. (Haematoxylin-eosin, magnification × 165).

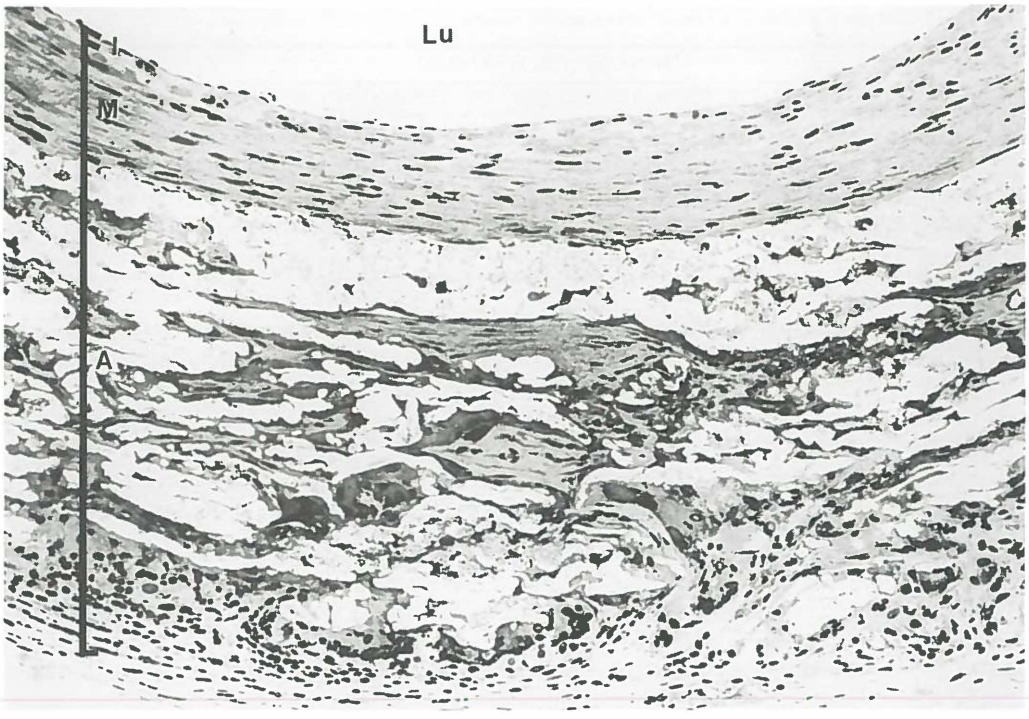

Fig. 3 Light micrograph of a transverse section of an implant of group B. Note inner endothelial lining (*I* neointima), several subintimal layers of smooth muscle cells (*M* neomedia), and fibrohistiocytic tissue in the disintegrating graft lattice (*A* neoadventitia). *Lu* lumen. (Haematoxylin-eosin, magnification × 165).

Microscopic examination. The microscopic appearance of the wall of the aneurysmal implants of group A (Fig. 2) differed strikingly from that of the wall of the implants of groups B, C, and D (Fig. 3), as will be described in detail.

Neointima. The SEM observations supported the LM observations. In the aneurysmal implants of group A the endothelial lining had not advanced more than 1 mm from both anastomotic sides. This lining continued with a pseudoendothelial lining of polygonal, flat-shaped cells that had no recognizable orientation in relation to the blood flow (Fig. 4a). Apparently smooth muscle cells of the neomedia had formed this pseudoendothelial lining (Fig. 2). No fibrin or platelets were found to adhere to the pseudoendothelial lining.
In contrast, in the implants of groups B, C, and D there was a complete endothelial lining: a monolayer of flat elongated cells was aligned parallel to the blood flow (Figs. 3, 4b). Here also no fibrin or platelets adhered.

Neomedia. The smooth muscle cells were predominantly longitudinally arranged. In groups C and D the thickness of the neomedia, both in the middle part of the implants and near the anastomotic sides, was of comparable thickness to the media of normal rat abdominal aorta, but this was not true in groups A and B (Table II).
In addition, the neomedia in groups C and D contained significantly more elastic laminae than did the neomedia in groups A and B (Table II). In group A there were hardly no or no elastic laminae (Fig. 5a), in group B the elastic laminae were restricted to the luminal layers (Fig. 5b), and in groups C and D the elastic laminae were present almost throughout the neomedia (Fig. 5c).

Table II. *Thickness and elastin content of the neomedia (mean ± standard deviation)*

Group	n*	Thickness of the neomedia at		Elastin content (%)
		Anastomoses (μm)	Mid-region (μm)	
A	8	202 ± 61†	182 ± 40‡	8 ± 12§
B	10	291 ± 175†	132 ± 84	57 ± 15§
C	12	96 ± 59	88 ± 31	95 ± 7
D	12	91 ± 52	81 ± 48	95 ± 7

* Number of measurements.
† Significantly different from groups C, D, and the media of normal rat abdominal aorta (137 ± 27 μm, n = 8) ($p < 0.005$).
‡ Significantly different from groups B, C, D, and the media of normal rat abdominal aorta ($p < 0.005$).
§ Significantly different from groups C and D ($p < 0.001$).

Neoadventitia. In group A an insufficiently supporting neoadventitia had developed, composed of a thin layer of collagenous tissue and of some remnants of prosthetic material (Fig. 2). In groups B, C, and D, however, a sufficiently supporting neoadventitia had developed, composed of fibrohistiocytic tissue, which had organized the disintegrating PU/PLLA lattices (Figs. 3, 6). Capillaries were present as well as multinucleated giant cells, which surrounded prosthetic particles (Fig. 6). There was no evidence of necrosis or calcification. The relative amount of fibrohistiocytic tissue ingrowth was significantly higher in the inner

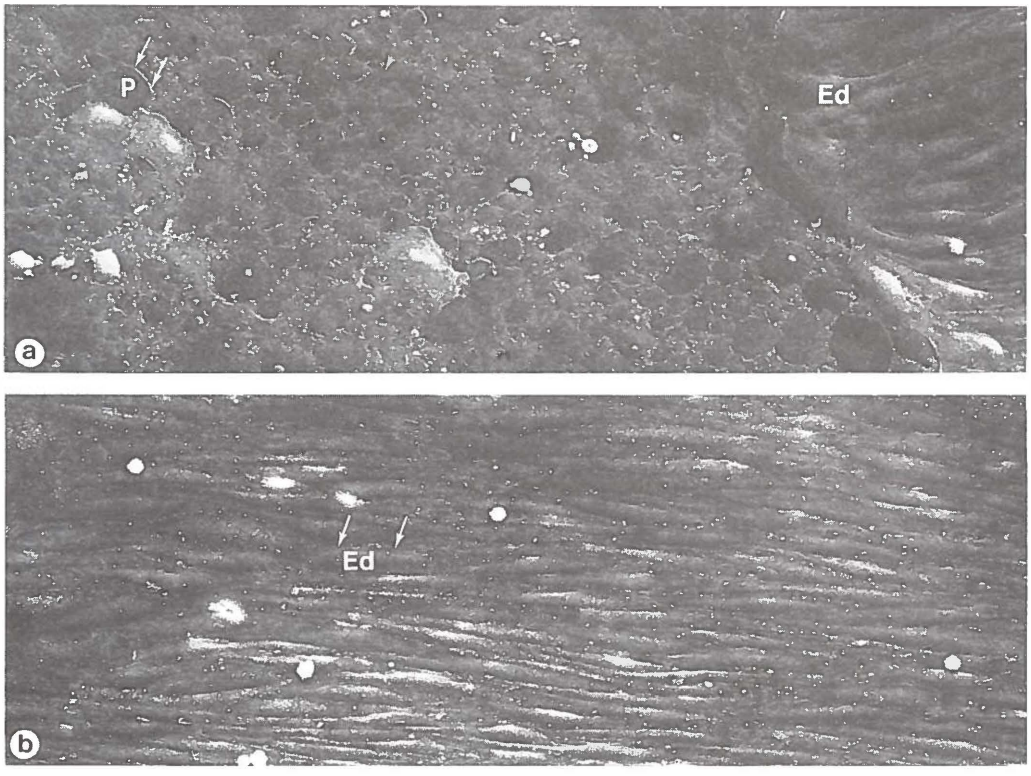

Fig. 4a, b Scanning electron micrographs of the lining cells. **a** The distal part of an aneurysmal implant of group A. The endothelial lining (*Ed*), which is hardly present, continues with a pseudoendothelial lining (*P*), which is composed of polygonal, flat-shaped cells. **b** The middle part of an implant of group D. The endothelial lining resembles that of normal arterial tissue: flat, elongated cells are aligned parallel to the blood flow. (*Arrows* mark cell borders, magnification × 375).

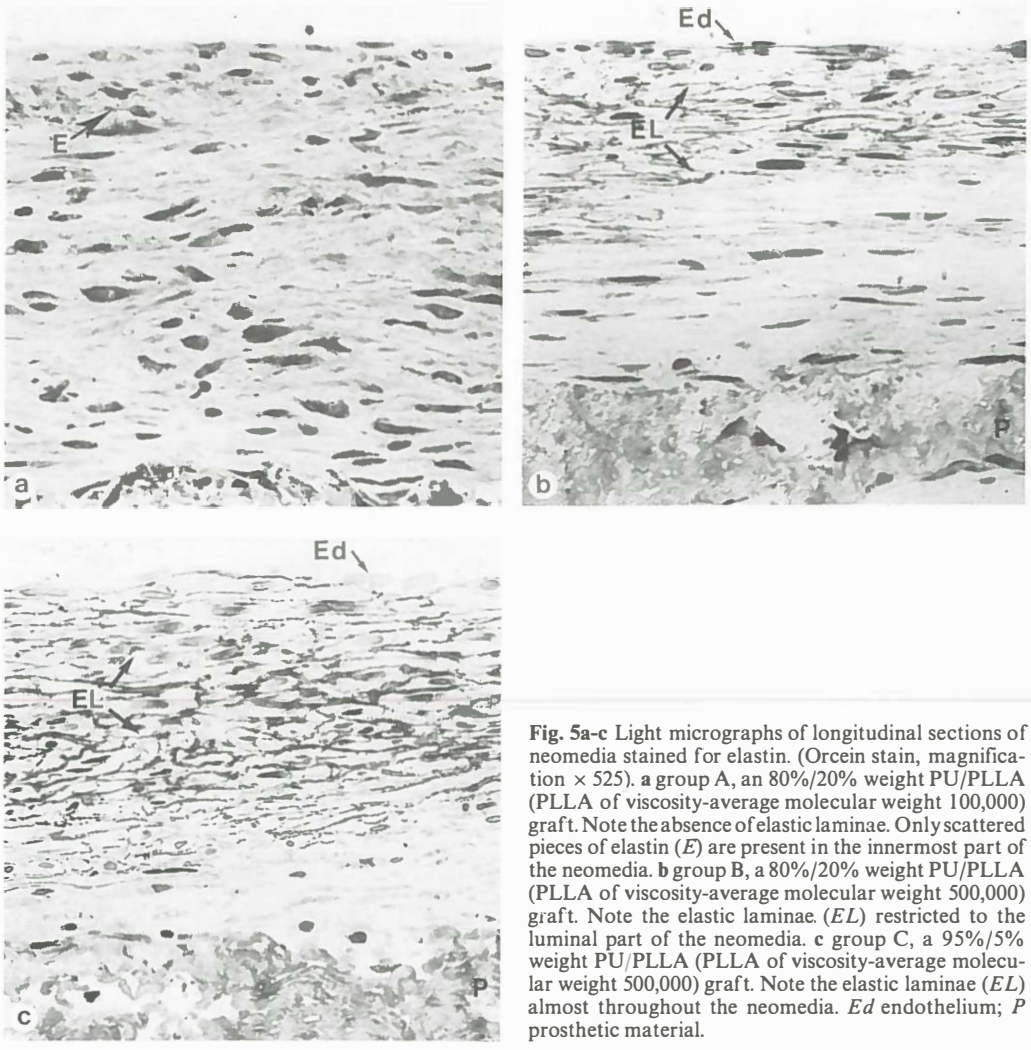

Fig. 5a-c Light micrographs of longitudinal sections of neomedia stained for elastin. (Orcein stain, magnification × 525). **a** group A, an 80%/20% weight PU/PLLA (PLLA of viscosity-average molecular weight 100,000) graft. Note the absence of elastic laminae. Only scattered pieces of elastin (*E*) are present in the innermost part of the neomedia. **b** group B, a 80%/20% weight PU/PLLA (PLLA of viscosity-average molecular weight 500,000) graft. Note the elastic laminae (*EL*) restricted to the luminal part of the neomedia. **c** group C, a 95%/5% weight PU/PLLA (PLLA of viscosity-average molecular weight 500,000) graft. Note the elastic laminae (*EL*) almost throughout the neomedia. *Ed* endothelium; *P* prosthetic material.

third region of the PU/PLLA lattices of groups C and D than of group B (Table III). In the outer third region no significant differences between groups B, C, and D could be measured.

Discussion

Our results clearly demonstrate that the 95%/5% weight PU/PLLA mixture with the PLLA of $\bar{M}_v$ 500,000 is the most suitable composition for PU/PLLA vascular grafts to ensure optimal regeneration of a neoarterial wall, because in the grafts prepared from this material (groups C and D) there was (I) absence of aneurysm formation and maintenance of arterial graft pulsations, which means compliance, (II) regeneration of a complete antithrombogenic neointima, (III) regeneration of a neomedia of comparable thickness to the media of normal rat abdominal aorta, with the regeneration of elastic laminae almost throughout its thickness, and (IV) regeneration of a sufficiently supporting neoadventitia. Pore size of these PU/PLLA vascular grafts did not influence regeneration.

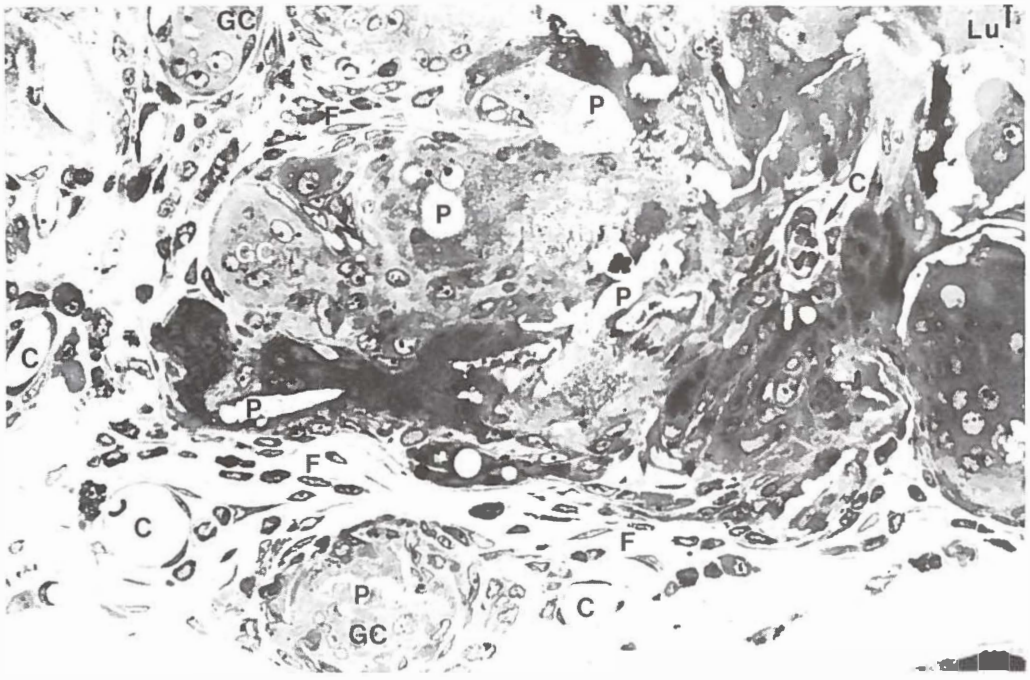

Fig. 6 Light micrograph of a longitudinal section of the neoadventitia in the outer boundery region of the lattice of a 95%/5% weight PU/PLLA graft (group D). Fibroblasts (*F*), capillaries (*C*), and multinucleated giant cells (*GC*) can be observed. The latter frequently surround remnants of prosthetic material. *Lu* lumen. (Heamatoxylin-eosin stain, magnification × 660).

The percent weight of the PU/PLLA mixture and the $\bar{M}_v$ of the PLLA determine the biodegradation and the compliance (Fig. 1) of PU/PLLA vascular grafts and thus the quality of the regenerated arterial wall. The PU/PLLA vascular grafts prepared from the 95%/5% weight PU/PLLA mixture with the PLLA of $\bar{M}_v$ 500,000 (groups C and D), which had a well matched compliance in combination with a well balanced degradation, induced the regeneration of neoarterial tissue of sufficient strength and compliance. The PU/PLLA vascular grafts prepared from the 80%/20% weight PU/PLLA mixture with the PLLA of $\bar{M}_v$ 100,000, which degraded too fast (group A), induced aneurysm formation despite the fact that smooth muscle cells were stimulated to form a thick neomedia. The formation of a thick neomedia most likely is a compensatory mechanism to withstand the high tensile forces. The PU/PLLA vascular grafts prepared from the 80%/20% weight PU/PLLA mixture with the PLLA of $\bar{M}_v$ 500,000 (group B), which had a less matched compliance in combination with a less balanced

Table III. *Relative amount of fibrohistiocytic tissue in the neoadventitia (mean ± standard deviation)*

		Relative amount of tissue ingrowth into	
Group	*n**	*Inner region (%)*	*Outer region (%)*
A	8	—†	—†
B	10	45 ± 7‡	82 ± 10
C	12	65 ± 7	80 ± 7
D	12	68 ± 5	82 ± 6

* Number of measurements.
† Not evaluated because of the presence of a thin layer of dense, collagenous, insufficiently supporting neoadventitia.
‡ Significantly different from groups C and D ($p < 0.001$).

degradation, induced the regeneration of neoarterial tissue of sufficient strength, but of insufficient compliance.

Pore size of the PU/PLLA vascular grafts neither affected the regeneration of neoarterial tissue (Tables II, III) nor the initial graft thrombogenicity. Nevertheless, we suggest the use of pore size gradients in PU/PLLA vascular grafts, because this pore structure can reduce the initial graft thrombogenicity by its smaller luminal pores[22] in more thrombogenic species, such as man, without impairing the regeneration of neoarterial tissue.

The results of this study confirm our previous findings[15, 16] that PU/PLLA vascular grafts have excellent neoendothelial healing characteristics, which resemble those of normal arterial tissue that is denuded from endothelium.[23, 24] This is most likely due to the underlaying smooth muscle cells of the neomedia, which provide a natural, structural layer to regenerating endothelial cells that grow from the anastomotic sides. Despite the presence of a neomedia, the absence of an endothelial lining in the aneurysmal implants of group A is most likely due to the turbulent blood flow in these implants. It is known from other studies that the direction and magnitude of shear stress can significantly affect the ingrowth of endothelial cells.[25, 26] The regeneration of the neomedia on the luminal side of the disintegrating PU/PLLA lattices resembles the process as described in studies of arterial wall damage.[27, 28] Most likely the smooth muscle cells have grown from the anastomotic sides. The growth of these smooth muscle cells may have been stimulated by several factors, including a growth factor released by platelets.[29] Additionally, mechanical stimulation by the arterial pulsations may have been an important stimulus for the smooth muscle cells to produce elastic laminae and collagen.[30]

The regeneration of a neoadventitia (i.e., the fibrohistiocytic tissue organization of the disintegrating PU/PLLA lattices/), which has an important role with regard to the regeneration of neoarterial tissue of sufficient strength, resembles the reaction of tissue against foreign body implants:[31, 32] macrophages, originating from monocytes, fuse to multinucleated giant cells,[33] which can contribute to the degradation of prosthetic material,[34] and macrophages stimulate perigraft tissue ingrowth[35] and capillary ingrowth.[36, 37] The capillaries of the neoadventitia in the implants ensured a sufficiently nutritial supply for the fibrohistiocytic tissue, as demonstrated by the absence of necrosis.

In conclusion, our results demonstrate that the 95%/5% weight PU/PLLA (PLLA of $\bar{M}_v$ 500,000) mixture is the most suitable composition for PU/PLLA vascular grafts to ensure the regeneration of a neoarterial wall of sufficient strength, compliance, and thromboresistance. Pore size of the grafts does not affect this regeneration. The potential of neoarterial tissue to function as small-caliber arterial substitute merits further investigation towards the basic mechanisms of its regeneration, especially of the elastic laminae, and towards its long-term behaviour.

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CHAPTER 4

Regeneration of the arterial wall in microporous, compliant, biodegradable vascular grafts after implantation into the rat abdominal aorta

Ultrastructural observations

Berend van der Lei,[1] Charles R.H. Wildevuur,[1] Paul Nieuwenhuis,[2] Engbert H. Blaauw,[3] Freark Dijk,[3] Caesar E. Hulstaert,[3] and Izaäk Molenaar[3]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Department of Histology, University of Groningen, The Netherlands
[3] Centre for Medical Electron Microscopy, University of Groningen, The Netherlands

Summary

The ultrastructure of a new type of vascular graft, prepared from a mixture of polyurethane (95% weight) and poly-L-lactic acid (5% weight), was examined six weeks after implantation into the abdominal aorta of rats. These microporous, compliant, biodegradable vascular grafts function as temporary scaffolds for the regeneration of the arterial wall. Smooth muscle cells, covering the grafts, regenerated a neomedia underneath an almost completely regenerated endothelial layer (neointima). These smooth muscle cells varied in morphology from normal smooth muscle cells to myofibroblasts. They were surrounded by elastic laminae and collagen fibrils. Macrophages, epithelioid cells, multinucleated giant cells, fibroblasts, and capillaries were present in the disintegrating graft lattices. The epithelioid cells and multinucleated giant cells engulfed polymer particles of the disintegrating grafts. The regeneration of the endothelial cells and smooth muscle cells is similar to the natural response of arterial tissue upon injury. The presence of macrophages, epithelioid cells, multinucleated giant cells, fibroblasts, and capillaries in the graft lattices resembles the natural response of tissue against foreign body implants. Both of these responses result in the formation of a neoartery that possesses sufficient strength, compliance, and thromboresistance to function as a small-caliber arterial substitute.

Introduction

The application of synthetic vascular grafts for the reconstruction of small-caliber arteries is limited. This is mainly a result of their thrombogenicity, insufficient elasticity, and poor healing characteristics.[1-3] Thus the necessity for a continuous search for better grafts or alternative principles in arterial reconstructive surgery remains. We have developed microporous, compliant, biodegradable grafts to function as temporary scaffolds for the regeneration of the arterial wall.[4-7] These grafts prepared from mixtures of polyurethane (PU) and

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poly-L-lactic acid (PLLA)[8,9] were implanted into the abdominal aorta of rats. After implantation of PU/PLLA grafts prepared from a 95%/5% weight PU/PLLA mixture arterial tissue regenerated optimally within six weeks: a sufficiently strong, compliant, antithrombogenic neoarterial substitute was formed as was revealed by macroscopic inspection, by microscopic inspection, and by bioassay and radioimmunoassay for prostacyclin.[5-7] Electron microscopic examination of these neoarterial substitutes should provide more detailed information concerning the nature of the processes that transform these grafts into neoarterial substitutes. Therefore, we have examined the ultrastructure of PU/PLLA vascular grafts six weeks after implantation into the abdominal aorta of rats.

Materials and methods

Animals and surgery. Six male Wistar rats (TNO, Zeist, The Netherlands) weighing 250 to 350 gm were used. The rats were premedicated with atropin (0.25 $mg.kg^{-1}$ body weight, administered intramuscularly) and anesthetized with 1% halothane (Fluothane®). Under sterile conditions, a 1 cm segment of the abdominal aorta was resected and replaced by a PU/PLLA vascular graft (Table I) by means of microsurgical techniques using an operation microscope (Zeiss OPMI 7-D, Carl Zeiss, Inc., Thornwood, N.Y.).[5-7] After implantation, arterial pulsations of the grafts could be observed. Six weeks after implantation of the grafts the implants were fixed by means of a pressure-controlled perfusion fixation. The rats had free access to standard rat food and water.

Perfusion fixation procedure. Under general pentobarbital (Nembutal®) anesthesia (50 $mg.kg^{-1}$ body weight, administered intramuscularly), the abdominal aorta with implant was carefully exposed. Heparin (1,000 IU, intravenously) was administered 2 to 3 minutes before cannulation to prevent blood clotting. Immediately after cannulation, pressure-controlled perfusion was started at a pressure of 180 mm Hg with, successively, (a) 0.9% sodium chloride for 30 seconds, (b) 2% polyvinylpyrrolidon K30, 0.4% sodium nitrite in 0.1 M phosphate buffer, pH 7.4, for 60 seconds, and (c) finally for fixation with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 minutes. The segment of cannulated abdominal aorta with implant was then removed and left in the same fixative for 24 hours at 4 °C at a pressure of 180 mm Hg. Either the distal or the proximal end of the implant was prepared for transmission electron microscopy (TEM), whereas the other end was prepared for scanning electron microscopy (SEM).

Preparation for TEM. Small rings of approximately 1 to 2 mm width were cut from the implants and rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH

Table I. *Material characteristics of the PU/PLLA vascular grafts*

Chemical composition	95% weight polyurethane 5% weight poly-L-lactic acid
Internal diameter	1.5 mm
Length	1 cm
Wall thickness	0.3 mm
Pore size	pore size gradient, ranging from 10 μm in the inner region of the graft lattice to 100 μm in the outer region.

7.4, and postfixed for 4 hours at 4 °C in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4.[10] After dehydration in alcohol, the samples were embedded in Epon. Semithin sections were stained with toluidine blue and basic fuchsin. After screening these semithin sections with the light microscope, areas of interest were selected for ultrathin sectioning. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope operated at 60 kV.

Preparation for SEM. After fixation in glutaraldehyde (see *Perfusion fixation procedure*), the

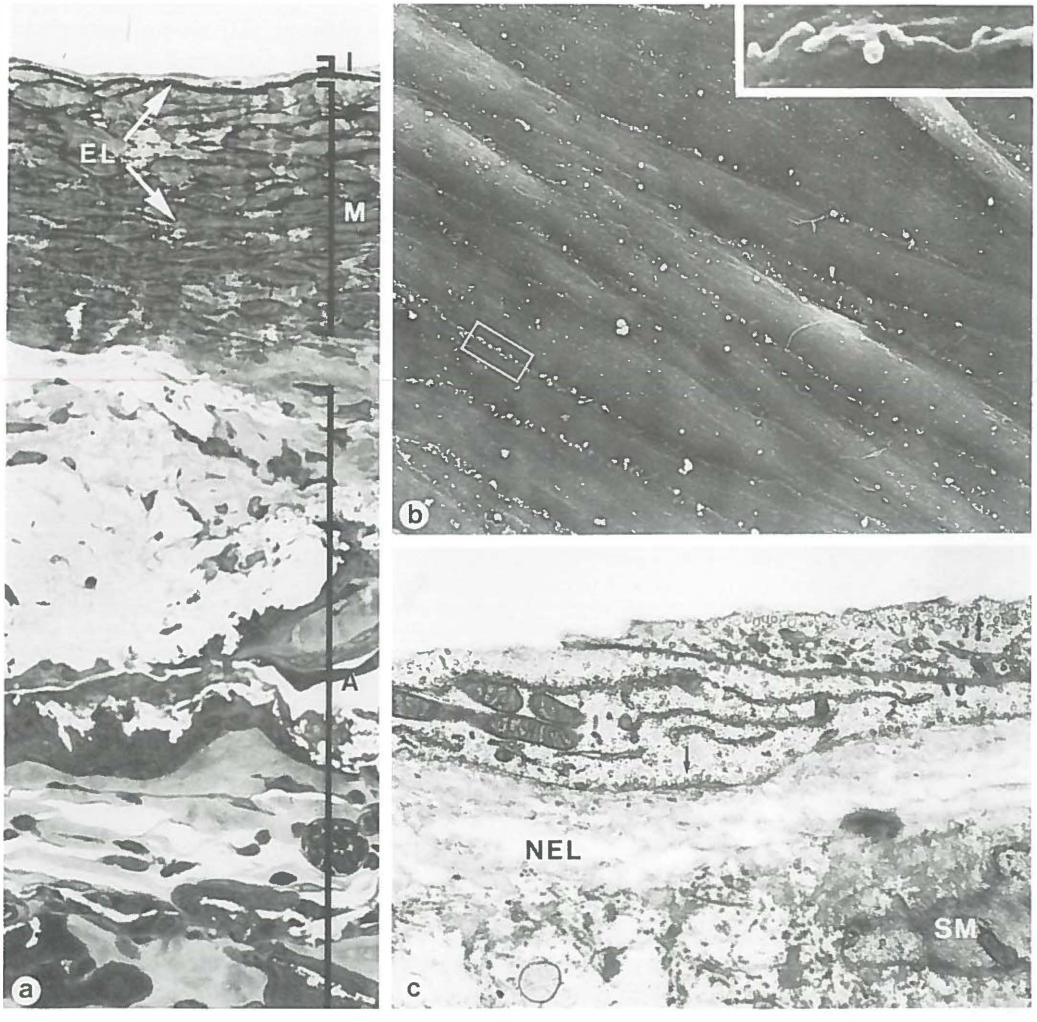

Fig. 1a Light micrograph of a longitudinal section of a PU/PLLA vascular graft, six weeks after implantation into the rat abdominal aorta. The implant takes the form of a neoarterial substitute in which three separate layers can be recognized: (I) a neointima (*I*), composed of endothelial cells, (II) a neomedia (*M*) composed of smooth muscle cells and (III) a neoadventitia (*A*), composed of fibrohistiocytic tissue and capillaries in the disintegrating graft lattice. Note the elastic laminae (*EL*) in the neomedia. (magnification × 375). **b** Scanning electron micrograph of the neointima. The endothelial cells are elongated in the direction of the blood flow. *Inset:* Endothelial cell border with a row of small knobs and villi. (magnification × 1,350; inset × 16,500). **c** Transmission electron micrograph of a portion of an endothelial cell. Note the numerous pinocytotic vesicles (*arrows*) and the new internal elastic lamina (*NEL*). *SM* smooth muscle cell. (magnification × 13,500).

specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide. The implants were cut in three longitudinal segments and sputter coated with gold palladium. These segments were examined in a JSM-35 C scanning electron microscope operated at 15 to 25 kV.

Results

At the moment of graft harvesting the implants were still pulsating. No aneurysm formation was observed. Microscopically three separate layers could be recognized in these implants, as described previously:[4-7] (I) an inner endothelial layer (neointima), (II) several subintimal layers of smooth muscle cells (neomedia), and (III) fibrohistiocytic tissue in a disintegrating graft lattice (neoadventitia) (Fig. 1a). The ultrastructure of these layers is described as follows:

(I) Neointima. The luminal surface of the implants was almost completely covered with flattened elongated endothelial cells (Fig. 1b, c). The cells were uniform in size and shape and were arranged with their long cellular axis parallel to the blood flow. The cell margins were

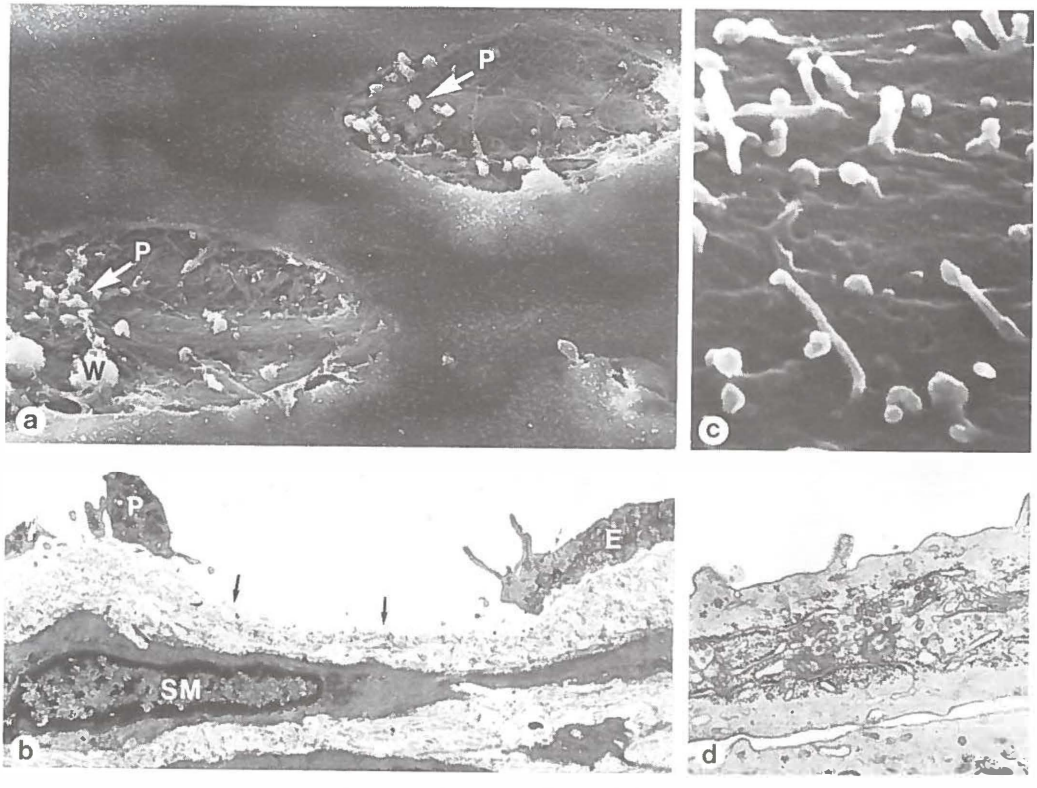

Fig. 2a Scanning electron micrograph of two small neointimal lining defects where platelets (*P*) and white blood cells (*W*) adhere. (magnification × 825). **b** Transmission electron micrograph of part of a small neointimal lining defect, where smooth muscle cells (*SM*) with their surrounding extracellular material (*arrows*) are exposed to the lumen. *E* endothelial cell; *P* platelet. (magnification × 4,500). **c** Scanning electron micrograph of part of an endothelial cell near a neointimal lining defect. Numerous microvilli are present. (magnification × 16,500). **d** Transmission electron micrograph of part of an endothelial cell near a neointimal lining defect. The endothelial cell contains a large number of organelles. (magnification × 16,500).

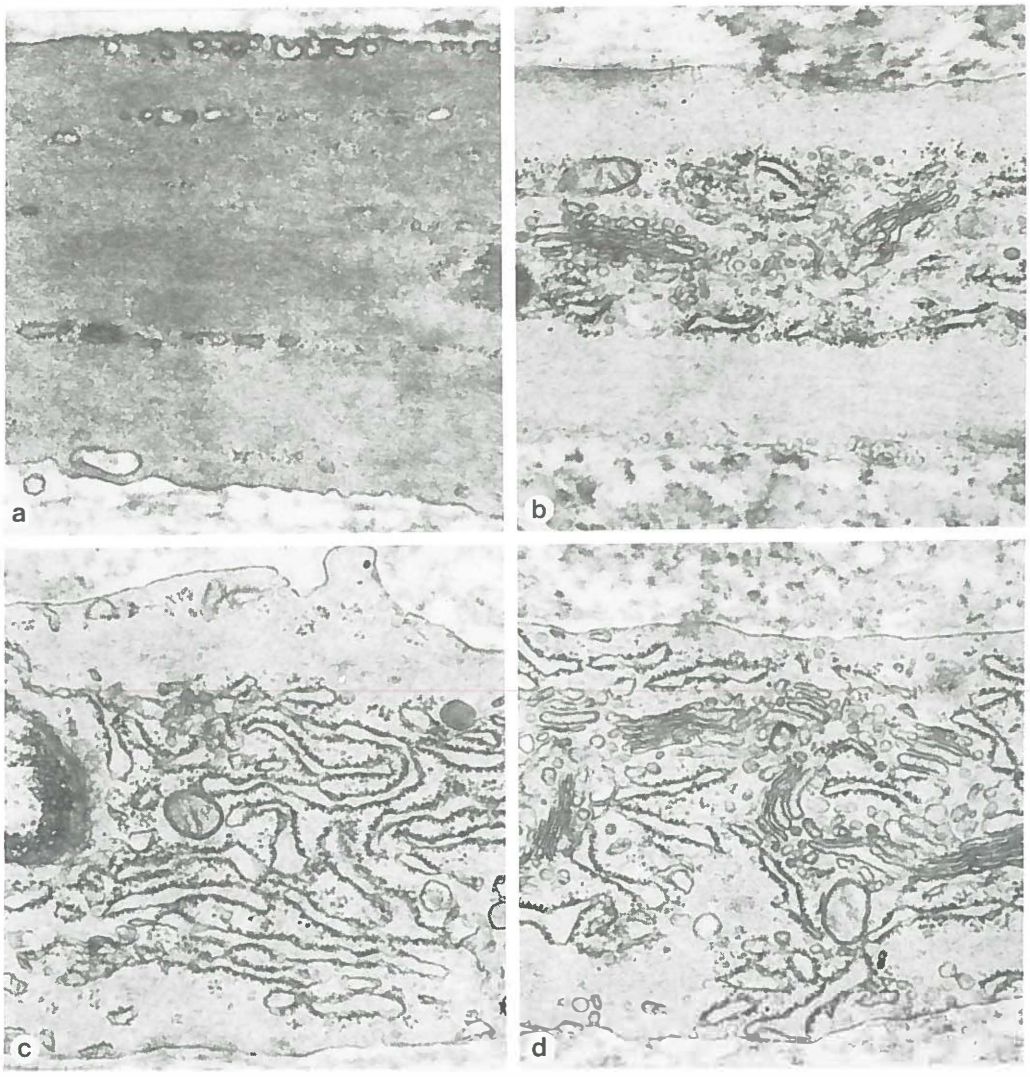

Fig. 3a-d Transmission electron micrographs of different types of smooth muscle cells. **a** Smooth muscle cell, containing packed myofilaments and peripheral vesicles. **b** Smooth muscle cell, showing myofilaments restricted to the cell periphery. **c** Dedifferentiated smooth muscle cell (myofibroblast), containing few myofilaments but abundant rough endoplasmic reticulum. **d** Myofibroblast, containing large numbers of rough endoplasmic reticulum and Golgi complexes. (magnification × 28,500).

smooth, often with some small knobs or villi (Fig. 1b, inset). No platelets or fibrin adhered to the endothelial cells.

In a few places the layer of endothelial cells was interrupted (Fig. 2a). Here smooth muscle cells of the neomedia were exposed to the lumen (Fig. 2b). The endothelial cells in the vicinity of these lining defects had numerous short microvilli of different size and shape over their entire surface (Fig. 2c). In addition, these cells had a large number of organelles (Fig. 2d).

(II) Neomedia. This layer contained a mixed population of smooth muscle cell types (Fig. 3). These cells varied in morphology from normal smooth muscle cells to myofibroblasts. Normal smooth muscle cells were characterized by a modest number of organelles, packed

myofilaments parallel to the long cellular axis, peripheral dense bodies, and peripheral vesicles (Fig. 3a). Intermediate cell types (Fig. 3b, c) were characterized by a larger number of organelles (Golgi complexes, mitochondria, free ribosomes, and rough endoplasmatic reticulum) and a smaller number of myofilaments, which were restricted to the periphery of the cell. Myofibroblasts, which had the appearance of fibroblast-like cells, were characteri-

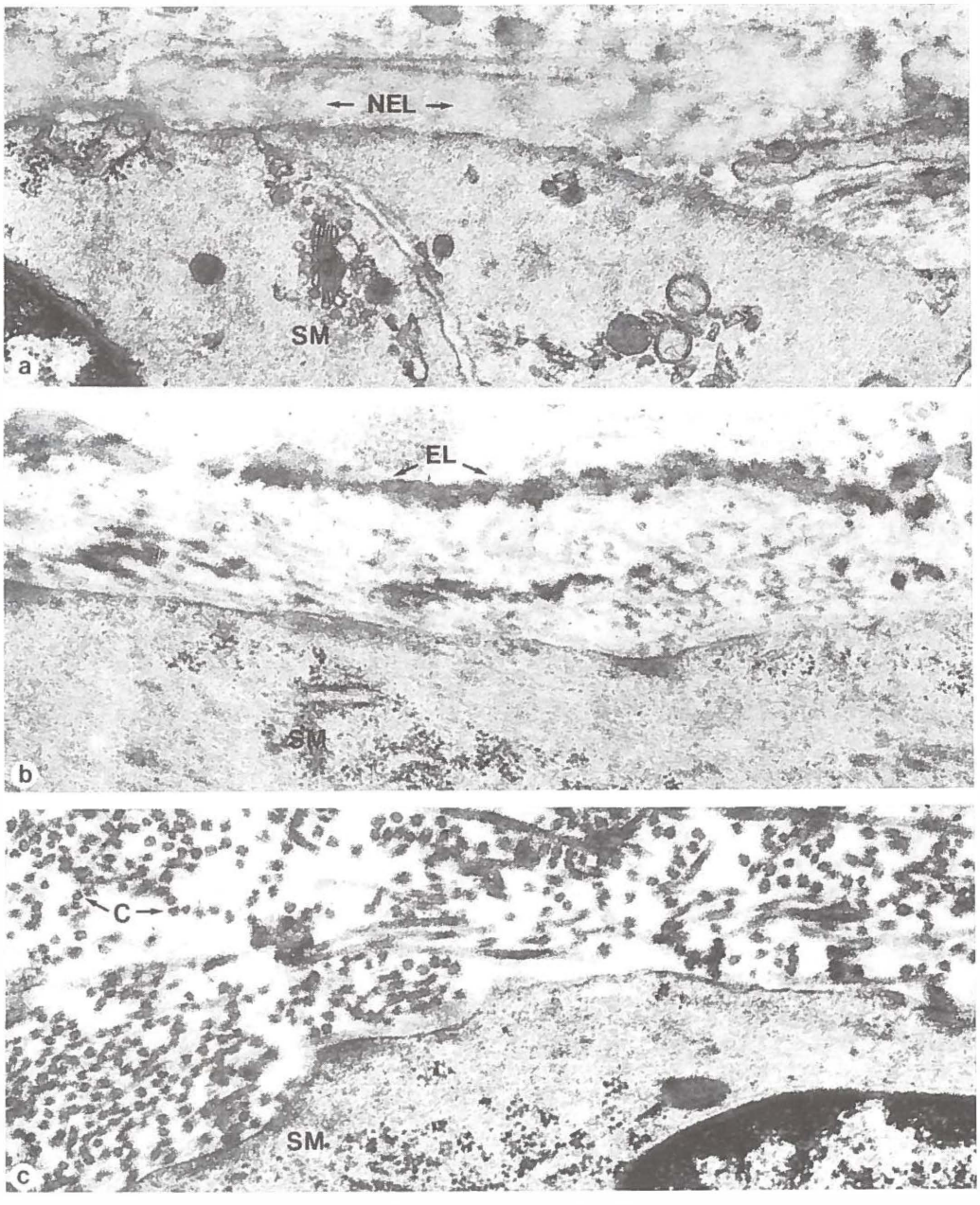

Fig. 4a-c Transmission electron micrographs of extracellular material at different levels of the neomedia. **a** New elastic laminae (*NEL*) as found in the luminal region of the neomedia. **b** Maturing elastic laminae (*EL*) found in a deeper region of the neomedia. **c** Numerous collagen fibrils (*C*) found in the deep regions of the neomedia. *SM* smooth muscle cell. (magnification × 35,300).

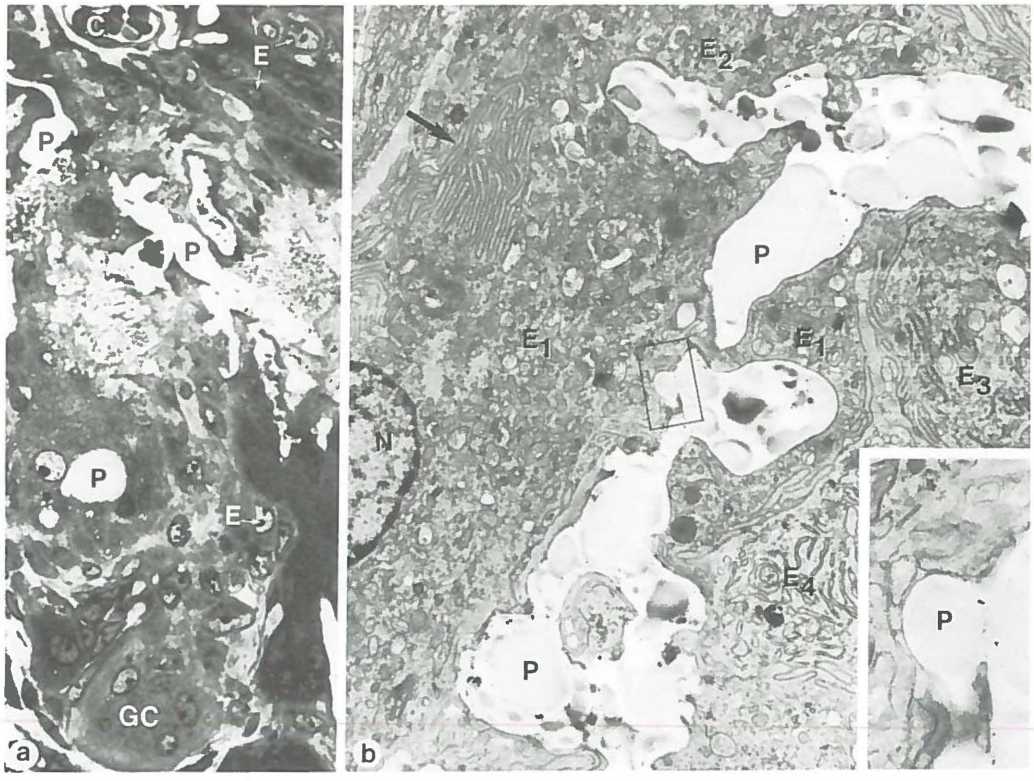

Fig. 5a Light micrograph of part of a disintegrating PU/PLLA lattice. Note the epithelioid cells (*E*) and the multinucleated giant cell (*GC*); their nuclei contain abundant euchromantin and serveral nucleoli. *P* prosthetic material; *C* capillary. (magnification × 825). **b** Transmission electron micrograph of several epithelioid cells (*E1-E4*) in a disintegrating PU/PLLA lattice. They surround and engulf polymer particles (*P*) (see *inset*). Their cytoplasm contains many mitochondria and lysosomes, abundant endoplasmic reticulum, and several Golgi complexes. Note the interdigitating cytoplasmic extensions between *E1* and *E2* (*arrow*). *N* nucleus. (magnification × 6,000; *inset* × 21,800).

zed by an almost complete absence of myofilaments and the presence of large numbers of rough endoplasmatic reticulum and Golgi complexes (Fig. 3d). All these cell types appeared to be randomly distributed.

The intercellular matrix mainly consisted of collagen fibrils and elastin material. Although elastin material was found throughout the entire neomedia, there were differences in elastin maturation, dependent upon the location in the neomedia. Complete elastic laminae (Figs. 1a, c, 4a) were present in the luminal part of the neomedia, whereas maturing elastic laminae and conglomerates of elastin material were present in the deep regions of the neomedia (Fig. 4b). In contrast to the elastin, collagen fibrils were more numerous in the deep regions of the neomedia (Fig. 4c).

(III) Neoadventitia. In the interstices of the disintegrating graft lattice there were large numbers of epithelioid cells and multinucleated giant cells (Figs. 1a, 5a). Macrophages were present at the periphery of these cells together with fibroblasts, collagen fibrils, and capillaries.

The epithelioid cells and the multinucleated giant cells contained a large number of lamellapodial extensions, which increased the plasma membrane considerably. These lamellapodial extensions were either free (Fig. 6) or in contact with lamellapodia of other cells (Fig. 5b). In the latter case they interdigitated with each other, forming an intricate lamellar

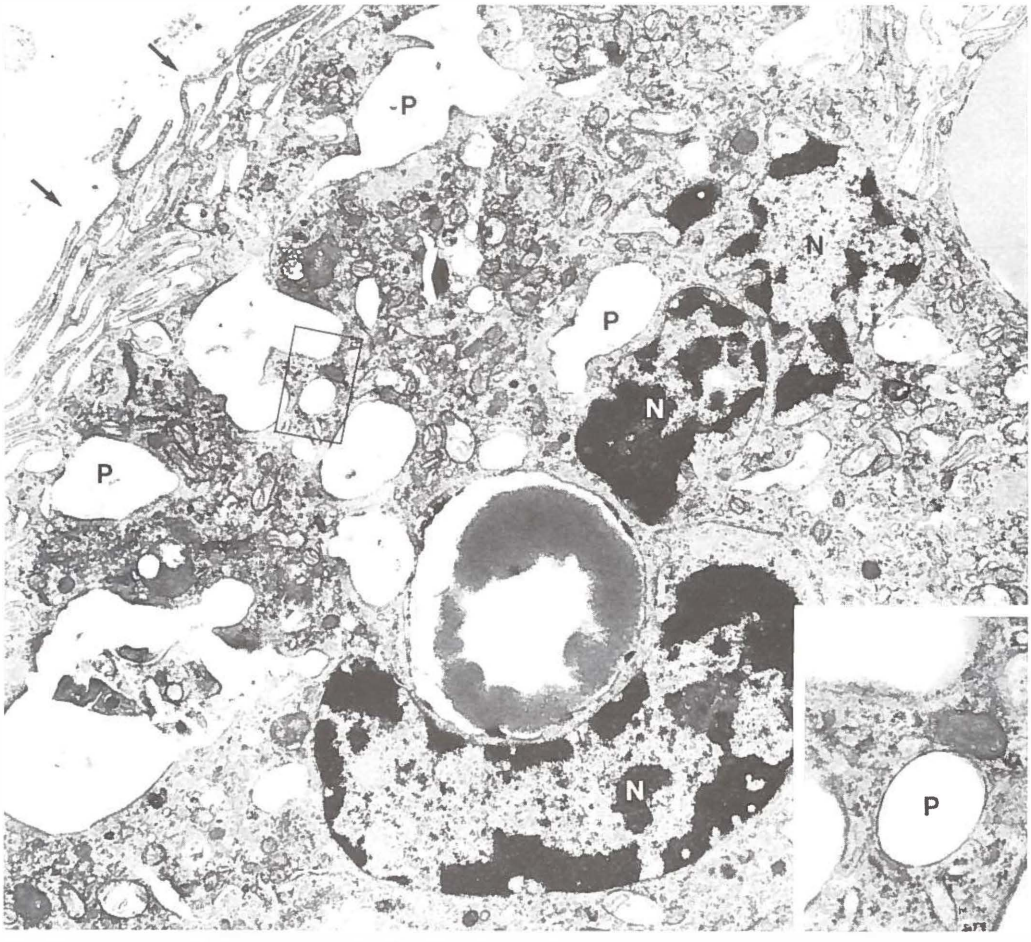

Fig. 6 Transmission electron micrograph of multinucleated giant cell. The cell is filled with prosthetic material (*P*). Note the elongated lamellapodial extensions that are present at the free border of the giant cell (arrows). Inset: Vacuole containing prosthetic material. *N* nucleus. (magnification × 8,250; *inset* × 26,300).

pattern between the the main bodies of the cells. The plasma membrane near the prosthetic material was smooth, giving no indication of micropinocytosis. However, there were clear signs of both polymer encapsulation and macropinocytosis (Figs. 5a, b, 6).

The cytoplasm of the epithelioid cells and multinucleated giant cells contained many organelles such as rough endoplasmic reticulum, free ribosomes, numerous mitochondria, extensive Golgi complexes, lysosomes, and vacuoles of varying sizes (Figs. 5b, 6). The nuclei of the epithelioid cells and multinucleated giant cells contained considerable amounts of euchromatin and several nucleoli, suggesting an active metabolic state (Fig. 5a).

Discussion

In this study we have examined the ultrastructural appearance of microporous, compliant, biodegradable PU/PLLA vascular grafts six weeks after implantion into the abdominal aorta of rats. The present results demonstrate that the regeneration of arterial wall tissue in these grafts resembles the natural response of arterial tissue upon injury. The reaction against

the PU/PLLA material itself resembles the natural response of tissue against foreign body material. Both of these responses result in the formation of a neoartery, which is of sufficient strength, compliance, and thromboresistance to function as arterial substitute.

Although some studies have suggested that endothelial cells in vascular grafts are derived from blood-born cells seeded on the graft surface,[11-13] it has been established that these cells only grow as a continuous sheet from undamaged endothelial cells at the anastomotic sides.[6, 14, 15] Most probably, their stimulus to proliferate is the interruption of the continuity of the endothelial cell layer.[16-18] The regeneration of an almost complete endothelial lining within the six-week period corresponds to that of arterial tissue that is denuded from endothelium.[16, 19-23] This regeneration in our PU/PLLA vascular grafts may be positively influenced by the underlaying smooth muscle cells with their surrounding extracellular matrix; they provide a natural structural layer for the proliferating endothelial cells.[5, 6, 24, 25] The small defects in the endothelial lining observed after 6 weeks (Fig. 2a, b) may be caused by (1) the faster growth of regenerating endothelial cells in the axial direction of the blood flow than in its circumferential direction[16, 22] and (2) the variable growth rate of these cells along the cut edge.[15] The numerous microvilli on endothelial cells in the vicinity of the defects in the lining (Fig. 2c, d; also observed by Schaper et al.[26] on endothelial cells during their early stage of growth in newly developing coronary collateral arteries) could be an indication of increased plasma membrane activity of growing cells. This is supported by the observation of large numbers of organelles in these cells (Fig. 2d).

Our observations do not provide evidence concerning the origin of the smooth muscle cells of the neomedia, but their most probably source is the media of the adjacent host artery.[27-29] Factors released by platelets,[30] by regenerating endothelial cells,[31-34] and by monocytes,[35] low density lipoproteins,[36, 37] and fibrin[38] can stimulate proliferation of smooth muscle cells at the anastomotic sides and may regulate their growth over the prosthetic segment. The observation of different types of smooth muscle cells (Fig. 3), including myofibroblasts, indicates their synthetic activity. It has been demonstrated that modified smooth muscle cells, i.e., myofibroblasts, are responsible for the formation of extracellular material in the arterial wall.[39-43]

Our findings of the increased maturation of elastic laminae towards the luminal layers of the neomedia and of the increase of collagen fibrils towards the deeper layers of the neomedia (Fig. 4) correspond to the results described in reports concerning (1) the response of arterial tissue to injury,[19, 44-48] (2) developing elastin,[49, 50] and (3) developing arterial tissue.[39, 42, 43] The implantation of a microporous, compliant, biodegradable graft seems to resemble ontogeny, in which smooth muscle cells are stimulated to produce new elastic laminae and collagen. Mechanical stimulation of these cells by the arterial pulsation is an important stimulus for the formation of elastic laminae and collagen. In vitro and in vivo studies have demonstrated that repeated stretching of smooth muscle cells stimulates their cell metabolism[51, 52] and the formation of elastic laminae.[53, 54]

The appearance of macrophages, epithelioid cells, multinucleated giant cells, fibroblasts, and capillaries in the lattices of the PU/PLLA grafts (Figs. 1a, 5, 6) resembles a chronic inflammatory response: an attempt to phagocytose or to encapsulate prosthetic material to facilitate healing.[55] Macrophages play a central role in this response.[56, 57] They differentiate into epithelioid cells.[58, 59] Both macrophages and epithelioid cells merge by fusion into multinucleated giant cells.[55, 60, 61] This, together with the contact between cells with interdigitating lamellapodia, could be an attempt to isolate the foreign material by forming a barrier between the material and the surrounding environment.[62] Macrophages stimulate capillary ingrowth,[63-65] fibroblast proliferation,[66] and thus, indirectly, collagen formation.

Although the compliance of the implants is well maintained for as long as 3 months, as demonstrated by visible pulsation of the implants and the presence of elastic laminae,[6] this

may ultimately be hampered by fibrous tissue substitution of the degrading PU/PLLA grafts. Only long-term follow-up in vivo will clarify this point. However, substitution of the degrading PU/PLLA grafts by fibrous tissue is to a certain extent necessary to contribute to the integrity of the implants.[7] The observation of polymer particles engulfed by epithelioid cells and multinucleated giant cells (Figs. 5b, 6) indicates that the PU/PLLA lattice is degradable. However, the exact mechanism of degradation remains to be elucidated.

In conclusion, our observations indicate that the regeneration of arterial wall tissue in PU/PLLA grafts is similar to the natural response of arterial tissue upon injury. The reaction against the PU/PLLA material resembles a chronic inflammatory response. Both of these responses result in the formation of a neoarterial substitute that possesses sufficient strength, compliance, and thromboresistance to function. Therefore, the use of vascular grafts as temporary scaffolds for the regeneration of the arterial wall may open new perspectives in the field of arterial reconstructive surgery.

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CHAPTER 5

Compliance and biodegradation of vascular grafts stimulate the regeneration of elastic laminae in neoarterial tissue

An experimental study in rats

Berend van der Lei,[1] Charles R.H. Wildevuur,[1] and Paul Nieuwenhuis[2]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Department of Histology, University of Groningen, The Netherlands

Summary

Microporous vascular grafts that are compliant and biodegradable can function as scaffolds for the regeneration of the arterial wall in small-caliber arteries. The purpose of this study was to determine the specific influence of both compliance and biodegradation of microporous vascular grafts on this regeneration, especially on the regeneration of elastic laminae. Therefore we implanted three different types of microporous vascular grafts into the abdominal aorta of rats. These grafts were (I) compliant, biodegradable (group I; n = 6), (II) compliant, biostable (group II; n = 8), and (III) noncompliant, biodegradable (group III; n = 8). Six weeks after implantation the implants were evaluated by means of light microscopy and electron microscopy.

The compliance of the implants, as indicated by arterial pulsations, was well maintained in group I but not in group II. In all groups a neomedia had regenerated, composed of smooth muscle cells that were predominantly longitudinally arranged. Elastic laminae were present almost throughout the neomedia in group I, restricted to the luminal layers of the neomedia in group II, and totally absent in the neomedia of group III.

These results demonstrate that both compliance and biodegradation stimulate the regeneration of elastic laminae in neoarterial tissue. Because of the compliance of microporous vascular grafts, smooth muscle cells are mechanically stimulated by the arterial pulsations to produce elastin arranged in laminae. Because of the biodegradation of these grafts, compliance is maintained, which therefore favours the regeneration of elastic laminae.

Introduction

An ideal graft for the reconstruction of small-caliber arteries has not yet been developed.[1] However, microporous vascular grafts, prepared from a mixture of 95% weight polyurethane (PU) and 5% weight poly-L-lactic acid (PLLA) of viscosity-average molecular weight 500,000, combine some ideal graft characteristics: these compliant, biodegradable grafts can function as a temporary scaffold for the regeneration of a neoarterial wall that possesses strength, compliance, and thromboresistance equivalent to those of normal arterial tissue.[2-7]

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We consider that this regeneration is most likely due to an appropriate combination of both compliance and biodegradation of these microporous vascular grafts.
The aim of this study is therefore to determine the specific influence of both compliance and biodegradation of microporous vascular grafts on the regeneration of arterial tissue, especially on the regeneration of elastic laminae: this could contribute to the understanding of the basic mechanism and development of the still-experimental way of vascular grafting, using vascular grafts as a temporary scaffold for the regeneration of the arterial wall. Therefore we implanted three types of microporous vascular grafts into the abdominal aorta of rats. These grafts were (I) compliant and biodegradable, (II) compliant and biostable, and (III) noncompliant and biodegradable. Six weeks after implantation we evaluated the implants by means of light microscopy (LM) and electron microscopy.

Materials and methods

Vascular Grafts. We implanted the following types of microporous vascular grafts into the abdominal aorta of rats (Table I): (I) compliant, biodegradable 95%/5% weight PU/PLLA (PLLA of viscosity-average molecular weight 500,000) vascular grafts (group I; n = 6); (II) compliant, biostable PU vascular grafts (group II; n = 8; these PU grafts had the same pore structure as the grafts of group I); (III) noncompliant, biodegradable grafts (group III; n = 8). The group III grafts were constructed from group I grafts and rigid polytetrafluoroethylene (PTFE) grafts (Impra, Inc., Tempe, Ariz.); the PTFE grafts were circumferentially stretched and fitted around the PU/PLLA grafts to prevent these grafts from pulsating.

Animals. Male Wistar rats (TNO, Zeist, The Netherlands) (n = 22) weighing 250 to 350 gm were used. The rats had free access to standard rat food and water.

Surgery. The rats were premedicated with atropin (0.25 $mg.kg^{-1}$ body weight, administered intramuscularly) and anesthetized with 1% halothane (Fluothane®). One cm of the abdominal aorta was resected and replaced by a vascular graft by means of sterile microsurgical techniques.[5,6] The grafts were not preclotted. Neither heparin nor spasmolytics were administered. Patency was determined by direct inspection.[8] Immediately after reconstruction, arterial pulsations were clearly present in the grafts of groups I and II and absent in the grafts of group III. A total of 23 rats were operated on to obtain the three groups of rats (n = 22): one rat died 2 hours after surgery because of blood leakage at the proximal anastomosis.

Graft Harvesting. The implants were harvested 6 weeks after implantation. Heparin (1,000 IU, intravenously) was administered before graft harvesting to prevent clotting. The implants were fixed by pressure-controlled perfusion[5,7] at a pressure of 180 mm Hg with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 minutes, removed, and left in the same fixative for 24 hours at 4 °C. Alternately, the distal or proximal end of an implant was

Table I. *Characteristics of the vascular graft materials*

Vascular grafts	*Wall thickness (mm)*	*Pore size (μm)*
PU/PLLA	0.3	10-100*
PU	0.3	10-100*
PTFE	0.5	30

* Pore size gradient ranging from 10 μm in the inner region of the graft lattice to 100 μm in the outer region.

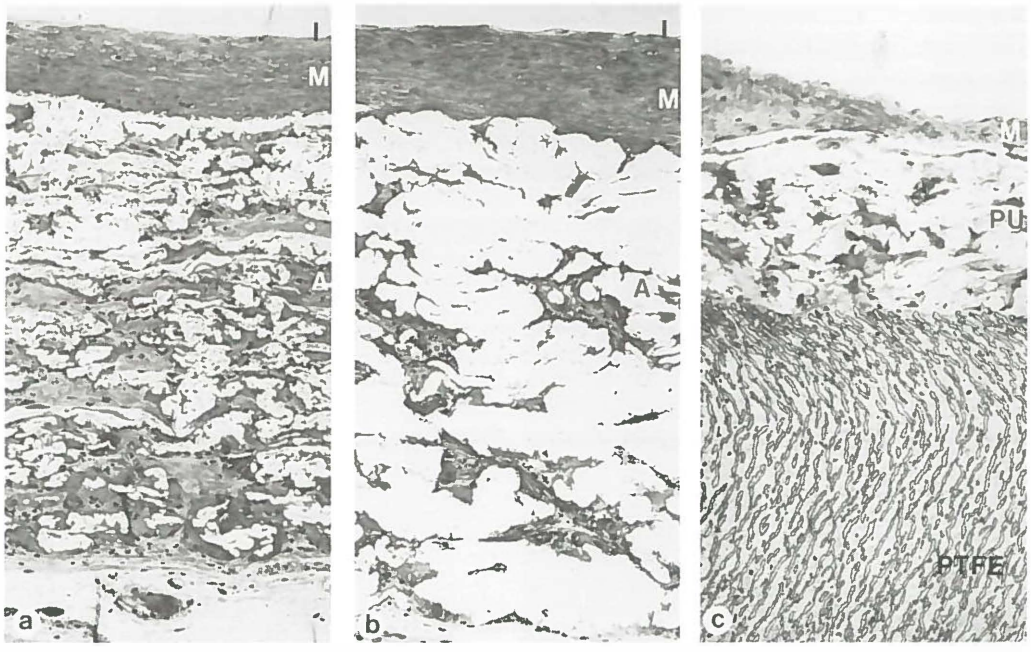

Fig. 1a-c Light micrographs of implants. **a** Group I: note the three separate layers that can be recognized: an inner endothelial lining (*I* neointima), several layers of subintimal smooth muscle cells (*M* neomedia) and fibrohistiocytic tissue in the disintegrating graft lattice (*A* neoadventitia). **b** Group II: note the significantly smaller extent of fibrohistiocytic tissue ingrowth compared with group I. **c** Group III: note the significantly thinner neomedia. The PU/PLLA graft (*PU*), which is surrounded by a PTFE graft (*PTFE*), is crushed to about one third of its original thickness. (magnification × 165).

prepared for LM and transmission electron microscopy (TEM), the other end for scanning electron microscopy (SEM). SEM was used for morphologic evaluation of the endothelial lining (neointima), LM for morphologic evaluation of the subendothelial smooth muscle cell layers (neomedia) and the fibrohistiocytic tissue in the graft lattice (neoadventitia). TEM was used for positive identification of the cells observed with LM and SEM.

Preparation for LM and TEM. The specimens were cut in small rings, rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 4 hours in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol, the rings were embedded in Epon. Longitudinal semithin sections (1 μm) were cut and stained with toluidine blue and basic fuchsin. The semithin sections were used for morphologic evaluation with a light microscope. Areas of interest were selected for ultrathin sectioning. The ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope operated at 60 kV.

Preparation for SEM. The specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide and cut in three longitudinal segments. These segments were sputter coated with gold palladium and examined in a JSM-35 C scanning electron microscope operated at 15 to 25 kV.

Morphologic evaluation. The neointima was inspected for its completeness. The neomedia was inspected for its thickness, both in the middle part of the implants and near the anastomotic sides, and for its elastin content and evaluated as described earlier.[6, 9] The neoadventitia was inspected for the extent of fibrohistiocytic tissue ingrowth into the graft lattices and evaluated as described earlier.[6, 9] Only the grafts of groups I and II were evaluated, because these grafts had the same pore structure.

Statistics. To analyse the morphometric measurements of the neomedia we used the Kruskal-

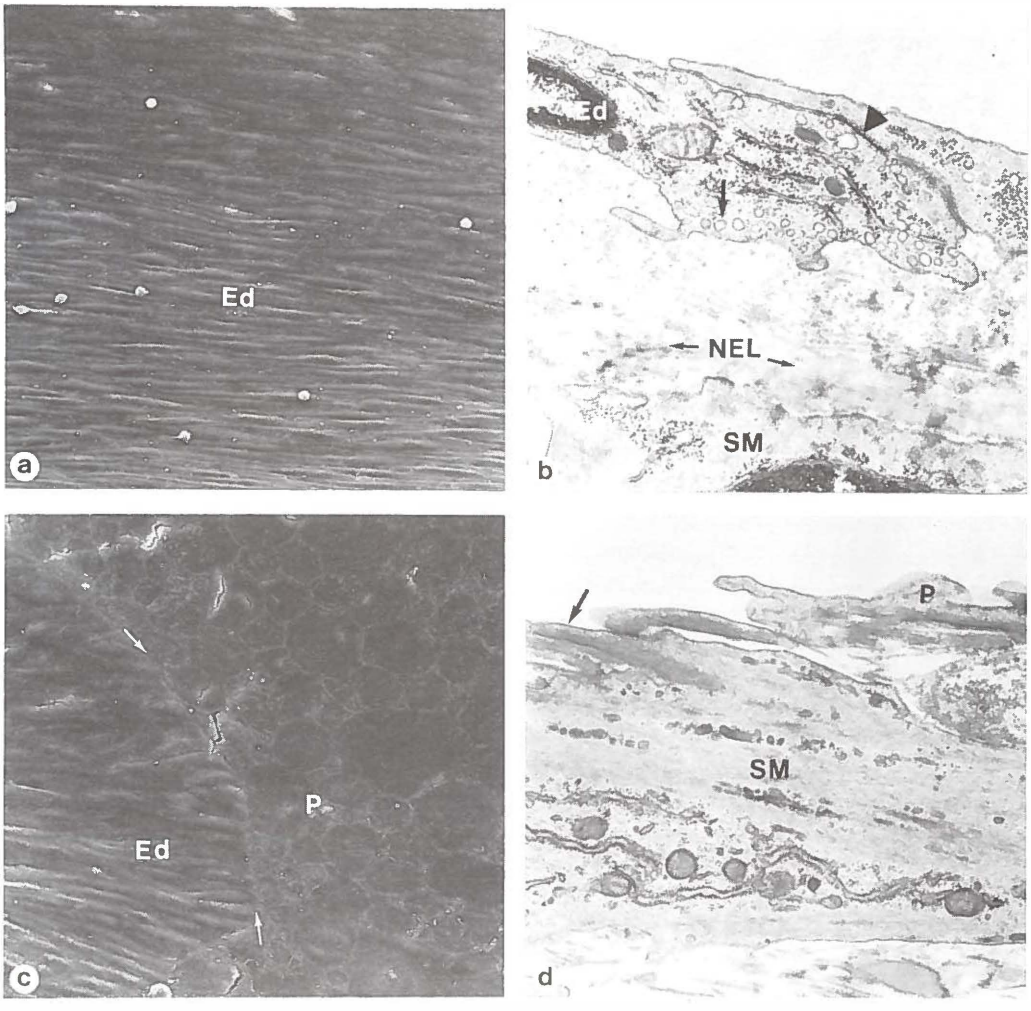

Fig. 2a Scanning electron micrograph of the lining cells in the middle part of an implant of group I. A confluent layer of flat, elongated endothelial cells (*Ed*) is well established. (magnification × 375). **b** Transmission electron micrograph of the endothelial lining cells of an implant of group I. Note the tight intercellular junction (*triangle*), the pinocytotic vescicles (*vertical arrow*), and the Weibel Palade body in cross-section (*horizontal arrow*) of the endothelial cell and the new internal elastic laminae (*NEL*). *SM* smooth muscle cell. (magnification × 21,800). **c** Scanning electron micrograph of the lining cells in the proximal part of an implant of group III. Polygonal flat-shaped cells (*P*) form a pseudoendothelial lining. *Arrows* mark the border between the endothelial lining and the pseudoendothelial lining. (magnification × 375). **d** Transmission electron micrograph of the pseudoendothelial lining cells of an implant of group III. These cells (*P*) appear to be smooth muscle cells (*SM*) of the neomedia, which are more or less exposed to the lumen (*arrow*). Note the myofilament bundles in the smooth muscle cells. (magnification × 21,800).

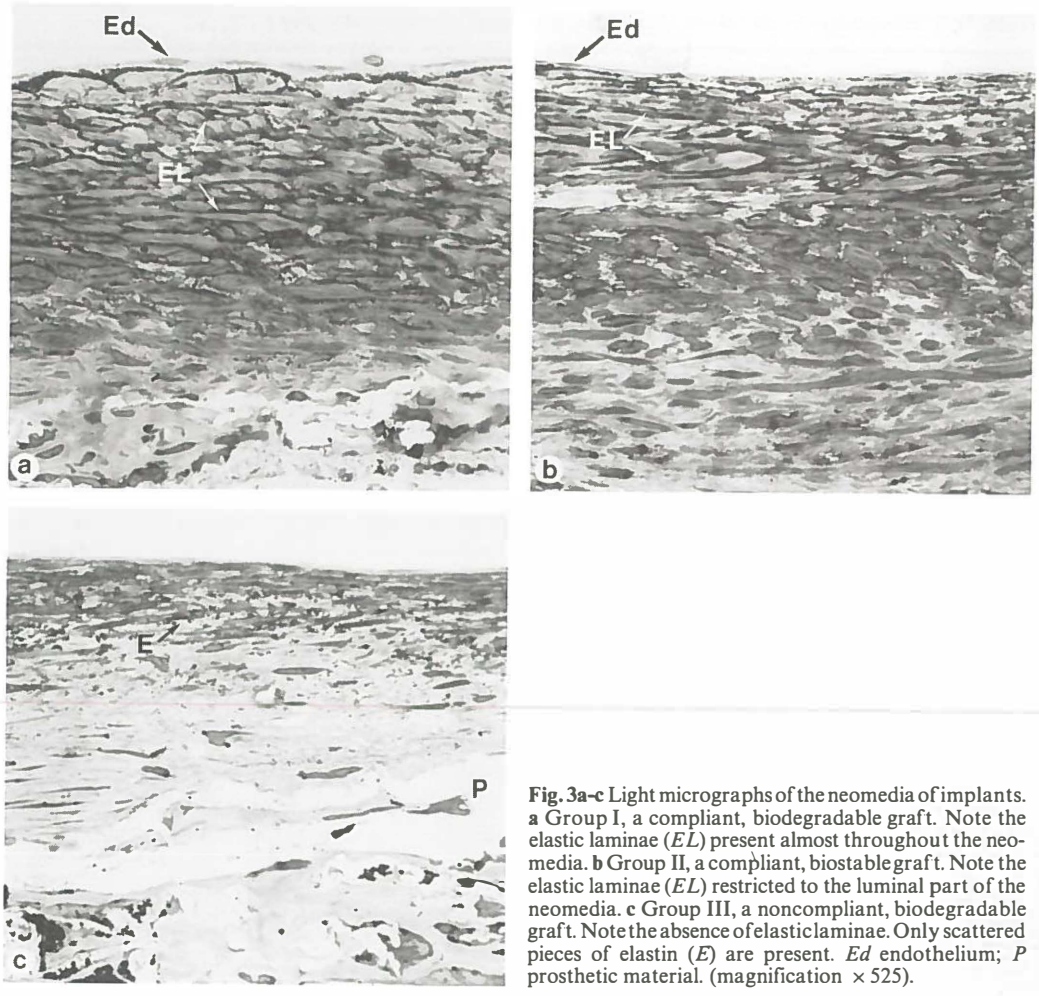

Fig. 3a-c Light micrographs of the neomedia of implants. **a** Group I, a compliant, biodegradable graft. Note the elastic laminae (*EL*) present almost throughout the neomedia. **b** Group II, a compliant, biostable graft. Note the elastic laminae (*EL*) restricted to the luminal part of the neomedia. **c** Group III, a noncompliant, biodegradable graft. Note the absence of elastic laminae. Only scattered pieces of elastin (*E*) are present. *Ed* endothelium; *P* prosthetic material. (magnification × 525).

Wallis one-way analysis of variance; to analyse the morphometric measurements of the neoadventitia we used the Student t test.[10] All data are presented as mean ± standard deviation.

Results

Macroscopic examination. At the moment of graft harvesting, in group I all six implants were patent, in group II seven out of the eight implants were patent, and in group III six out of eight implants were patent. Examination of the occluded implants (n = 3) showed an organized thrombus revealing early thrombosis.

Arterial pulsations were still clearly present in the implants of group I, hardly present in the implants of group II, and absent in the implants of group III. In five implants (of which two were occluded) of group III, the inner PU/PLLA grafts were folded along the longitudinal axis, causing a slight narrowing of the lumen. This was due to shrinking of the externally applied PTFE grafts, which had to be circumferentially stretched to fit around the PU/PLLA grafts.

Table II. *Thickness and elastin content of the neomedia (mean ± standard deviation)*

Group	n*	Thickness of the neomedia at		Elastin content (%)
		Anastomoses (μm)	Mid-region (μm)	
I	12	92.7 ± 27.9	87.5 ± 28.7	90.7 ± 8.8
II	14	103.6 ± 27.5	105.4 ± 58.8	29.8 ± 18.1†
III	12	45.8 ± 27.4‡	57.3 ± 40.8§	—#

* Number of measurements.
† Significantly different from group I ($p < 0.01$).
‡ Significantly different from groups I, II, and the media of normal rat abdominal aorta (83 ± 9 μm; n = 8; $p < 0.005$). This value is lower as the value reported previously,[6] because of a different fixation procedure.
§ Significantly different from groups I, II, and the media of normal rat abdominal aorta ($0.1 < p < 0.05$).
# No elastic laminae were present.

Microscopic examination.

Group I. The microscopic appearance of all implants in group I was as described previously[5, 6] (Fig. 1a). A complete neointima had regenerated, composed of a confluent layer of endothelial cells (Fig. 2a, b). Under this neointima, a neomedia had regenerated, which was composed of smooth muscle cells, which were predominantly longitudinally arranged (Figs. 1a, 3a). In both the middle part of the implants and near the anastomotic sides, the neomedia was of comparable thickness to that of the media of normal rat aorta (Table II). Elastic laminae were found almost throughout the entire neomedia (Figs. 3a, 4a).
In the disintegrating PU/PLLA lattices a neoadventitia had regenerated, which was composed of capillarized fibrohistiocytic tissue (Figs. 1a, 5a; Table III).

Group II. Microscopically the implants of group II resembled the implants in group I (Fig. 1b). However, there were two striking differences between these two groups. First, the amount of elastic laminae in the neomedia of the implants of group II was significantly smaller than in the implants of group I (Table II): in group II the elastic laminae were

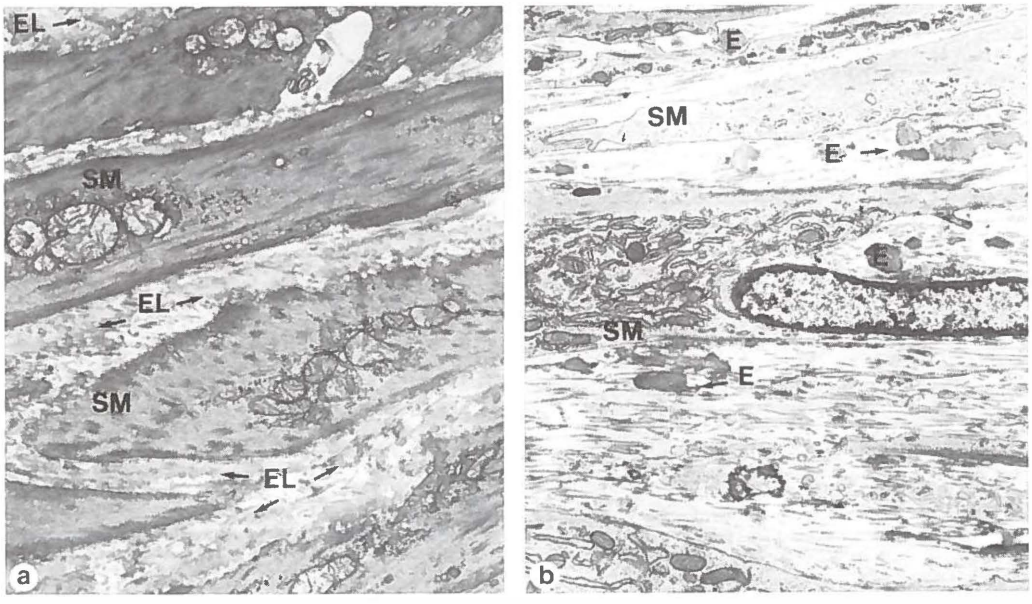

Fig. 4a Transmission electron micrograph of elastin arranged in laminae (*EL*) between the smooth muscle cells (*SM*) of the neomedia of an implant of group I. **b** Transmission electron micrograph of irregular scattered pieces of elastin (*E*) between the smooth muscle cells (*SM*) of the neomedia of an implant of group III. (magnification ×8,250).

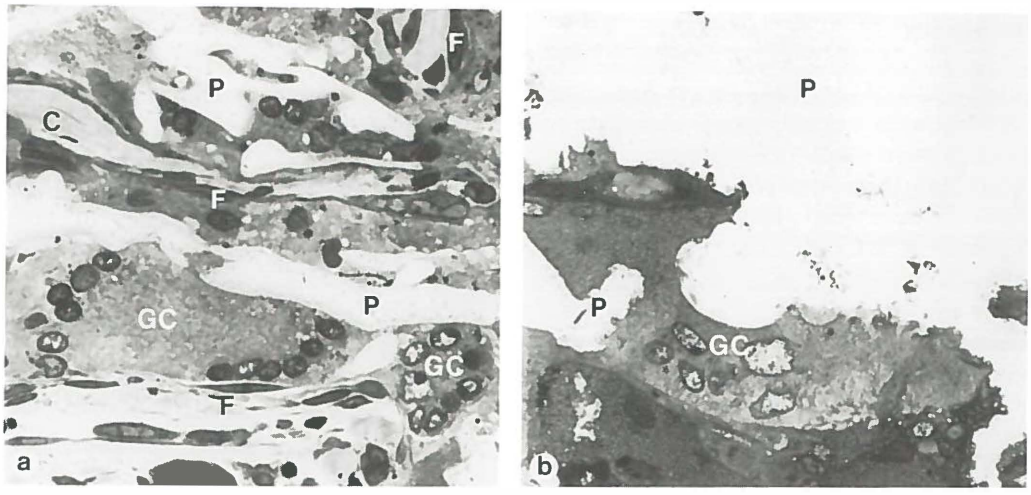

Fig. 5a, b Light micrographs of part of the graft lattice of implants. **a** Group I, a biodegradable graft. Note the extensive fibrohistiocytic tissue ingrowth into the disintegrating graft lattice. **b** Group II, a biostable graft. Note the significantly less fibrohistiocytic tissue ingrowth into the graft lattice. *P* prosthetic material; *GC* multinucleated giant cell; *F* fibroblast; *C* collagen. (magnification × 600).

restricted to the luminal layers of the neomedia (Fig. 3b). Second, the extent of fibrohistiocytic tissue ingrowth into the hardly disintegrating graft lattices of group II was significantly less than into the disintegrating graft lattices of group I (Figs. 1b, 5b; Table III).

Group III. Microscopically, group III differed significantly from group I (Fig. 1c). Only about 30% of the surface area of the implants of group III was lined with endothelial cells. The remaining surface area was lined with the same polygonal, flat-shaped pseudoendothelial cells as that of aneurysmal implants, which have been described in a previous report.[6] These pseudoendothelial cells appeared to be luminal smooth muscle cells of the neomedia (Fig. 2c, d). No thrombus adhered to these cells. The thickness of the neomedia was significantly less in this group than in groups I and II (Fig. 1c; Table II). Most strikingly, there were no elastic laminae present in the neomedia; only small scattered pieces of irregular elastin were seen (Figs. 3c, 4b).

In both the externally applied PTFE grafts and in the inner PU/PLLA grafts there were limited amounts of fibroblasts and histiocytes and no capillaries. The inner PU/PLLA grafts were crushed to about a third of their original thickness (Fig. 1c).

Table III. *Relative amount of fibrohistiocytic tissue in the neoadventitia (mean ± standard deviation)*

Group	*n**	*Relative amount of tissue ingrowth (%)*
I	12	68.5 ± 4.9
II	14	28.9 ± 5.9†
III	12	—‡

* Number of measurements.
† Significantly different from group I ($p < 0.001$).
‡ Not evaluated because these are compound grafts.

Discussion

The results of this study clearly demonstrate that both compliance and biodegradation of microporous vascular grafts stimulate the regeneration of arterial tissue, especially the regeneration of elastic laminae.

Until now there were only some indications that mechanical stimulation of smooth muscle cells by the arterial pulsations might be an important stimulus for the formation of elastic laminae in arterial tissue. Bunting[11] already discerned the possible effect of mechanical stimulation on elastin formation, based on the observations of dense elastic tissue in scars after myocardial infarction and in pleural adhesions. Both of these scars are subjected to alterations in tension and relaxation. Other investigators who observed the formation of elastic laminae in developing arterial tissue,[12, 13] subintimal hyperplasia,[14] and autologous veins that were transplanted in the arterial circulation[15] also discerned the possibility that mechanical stimulation caused by the arterial pulsations might stimulate elastin synthesis. In addition, in vitro experiments demonstrated that repeated stretching of arterial smooth muscle cells stimulates their cell metabolism.[16]

However, our results give direct evidence that mechanical stimulation of smooth muscle cells by arterial pulsations is an important stimulus for the formation of elastic laminae in arterial tissue.[17] This was demonstrated by the presence of elastic laminae between the smooth muscle cells that had developed in the compliant grafts of groups I and II (Figs. 3a, b, 4a) and by the absence of elastic laminae between the smooth muscle cells that had developed in the noncompliant grafts of group III (Figs. 3c, 4b).

Mechanical stimulation of smooth muscle cells not only stimulates their synthesis of elastin but it also appears to stimulates their growth. The smooth muscle cell layers were significantly thicker in the compliant grafts of group I and II than in the noncompliant grafts of group III (Table II). The tensile forces in the smooth muscle cell layers during expansion of the grafts might be of major importance for the growth, because when vascular graft material degrades too fast, smooth muscle cells are also stimulated to grow.[6]

Biodegradation of compliant vascular grafts, which serve as a scaffold for the regeneration of the arterial wall, is a prerequisite to maintain compliance of the implants, which is again a prerequisite to stimulate the regeneration of elastic laminae. At the moment of graft harvest in our study, the arterial pulsations were still clearly present in the biodegradable, compliant grafts of group I, but not in the biostable grafts of group II. Moreover, the smooth muscle cells had produced elastic laminae almost throughout their layer thickness in the biodegradable, compliant grafts of group I (Fig. 3a), whereas the elastic laminae were restricted to the luminal layers in the compliant, biostable grafts of group II (Fig. 3b; Table II).

This effect of biodegradation might be explained as follows: tissue growth into and over vascular grafts reduces compliance, whereas degradation of vascular grafts increases compliance despite the fact that it also enhances tissue ingrowth (Table III). Bowald, Busch, and Eriksson[18, 19] who used biodegradable, noncompliant vascular grafts for the regeneration of the arterial wall in large-caliber arteries, also observed some "compliance" effect of the biodegradation of these vascular grafts: they also observed regeneration of some elastic laminae but not to the same extent that we did in our compliant vascular grafts.

In addition, our results demonstrate that the regeneration of the neointima is affected by the compliance of vascular grafts. The neoendothelial healing of the noncompliant grafts of group III was greatly impaired despite the presence of a neomedia, which provides a natural structural layer to regenerating endothelial cells.[5] The mismatch in compliance between graft and connected artery leads to turbulance and thus to high shear stress,[20-22] which impairs endothelial cell ingrowth.[6, 23] In addition, the folding of the inner PU/PLLA grafts in three implants might have attributed to turbulence.

However, this cannot explain the poor neoendothelial healing in all noncompliant grafts. In conclusion, our results demonstrate that both compliance and biodegradation of microporous vascular grafts stimulate the regeneration of arterial tissue, especially the regeneration of elastic laminae. Because of the compliance, smooth muscle cells are mechanically stimulated by the arterial pulsations to produce elastin arranged in laminae. Because of the biodegradation, compliance of the implants is maintained, which therefore favours the regeneration of elastic laminae. Therefore microporous vascular grafts, which are used as a scaffold for the regeneration of the arterial wall, should be both compliant and biodegradable to ensure an optimal regeneration.

Acknowledgements. We thank S. Gogolewski and A.J. Pennings for supplying the PU/PLLA vascular grafts, H.L. Bartels for his microsurgical assistance, E.H. Blaauw for the LM and TEM handling procedures, F. Dijk for the SEM handling procedures, J. Koudstaal for his suggestions concerning the morphometric measurements, H.G. Seijen for helping with the statistical analysis, D. Huizinga for the photography, J.J.P. Bruyns for typing the manuscript, and C.E. Fontijne-de Bruyn for correcting the manuscript.

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CHAPTER 6

Sequential studies of arterial wall regeneration in microporous, compliant, biodegradable small-caliber vascular grafts in rats

Berend van der Lei,[1] Charles R.H. Wildevuur,[1] Freark Dijk,[2] Engbert H. Blaauw,[2] Izaäk Molenaar,[2] and Paul Nieuwenhuis[3]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Centre of Medical Electron Microscopy, University of Groningen, The Netherlands
[3] Department of Histology, University of Groningen, The Netherlands

Summary

Microporous, compliant, biodegradable vascular grafts prepared from a mixture of polyurethane (PU; 95% weight) and poly-L-lactic acid (PLLA; 5% weight) can function as a temporary scaffold for the regeneration of the arterial wall in small-caliber arteries. This study was undertaken to document the sequential events leading to this regeneration. Therefore, PU/PLLA vascular grafts were implanted into the abdominal aorta of rats (n = 28), and were harvested at regular intervals from 1 hour up to 12 weeks after implantation. The implants were evaluated by means of light microscopy and electron microscopy.
At each time of harvesting the implants were patent and showed arterial pulsations. No stenosis or dilatation was observed. Endothelial cells grew from the adjacent aortic intima across the anastomoses from Day 6 onwards, to form already an almost complete neointima after 6 weeks of implantation. Smooth muscle cells grew from the adjacent aortic media over the graft lattice through the platelet-fibrin coagulum also from Day 6 onwards. The smooth muscle cells, predominantly longitudinally arranged on Week 6, but also circularly arranged in some areas on Week 12, formed a neomedia in which collagen and elastic laminae were newly deposited. Polymorphonuclear leucocytes and monocytes initially invaded the graft lattices. Fibroblasts, histiocytes, and capillaries grew from the perigraft tissue into the PU/PLLA lattices from Day 6 onwards, resulting in the formation of a neoadventitia. The PU/PLLA lattices started to disintegrate from Day 12 onwards.
These sequential regenerative processes in the disintegrating PU/PLLA grafts resulted in the formation of neoarteries, which were of sufficient strength, compliance, and thromboresistance to function as small-caliber arterial substitutes.

Introduction

At present small-caliber arterial reconstructions with autologous saphenous vein grafts are used widely in patients with coronary artery disease and peripheral vascular disease. These reconstructions, however, are frequently compromised by late graft stenosis, atherosclerosis,

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and aneurysm formation.[1-6] Therefore, there is still a demand for a readily available ideal substitute for these reconstructions.
Autologous arterial tissue is indisputably the best material available. Its use, however, is hampered by its limited availability. Synthetic vascular grafts that are currently used clinically are not reliable for small-caliber arterial reconstructions. They frequently fail as a result of their thrombogenicity, poor healing characteristics, and insufficient elasticity.[7-12]
Small-caliber vascular grafts prepared from a mixture of polyurethane (PU) and poly-L-lactic acid (PLLA),[13, 14] however, combine some ideal arterial substitute characteristics.[15-20] When implanted into the rat abdominal aorta, these grafts can function as a temporary scaffold for the regeneration of a neoarterial wall that possesses strength, compliance, and thromboresistance equivalent to normal arterial tissue.
Evaluation of the dynamics of this regeneration could contribute to the development of this new way of vascular grafting. Therefore, this study was undertaken to document the sequential events leading to the arterial wall regeneration in PU/PLLA vascular grafts. PU/PLLA vascular grafts prepared from a 95%/5% weight PU/PLLA mixture were implanted into the abdominal aorta of rats and were evaluated by means of light microscopy and electron microscopy at regular intervals from 1 hour up to 12 weeks after implantation.

Materials and Methods

Graft implantation procedure. Male Wistar rats (TNO, Zeist, The Netherlands) (n = 28) weighing 250 to 350 gm were premedicated with atropin (0.25 $mg.kg^{-1}$ body weight, administered intramuscularly) and anesthetized with 1% halothane (Fluothane®). By means of sterile microsurgical techniques, one cm of the abdominal aorta was resected and replaced by a PU/PLLA vascular graft (Fig. 1a, b) prepared from a 95%/5% weight PU/PLLA mixture.[13, 14] The PU/PLLA vascular grafts had a pore size gradient in their 0.3 mm thick graft wall, ranging from 10 μm in the inner region of the graft lattice to 100 μm in the outer region. All grafts were sterilized with ethylene oxide and for 24 hours evacuated under high vacuum (10^{-5} torr). Twenty-nine rats had to be operated on to obtain a group of 28 rats; one rat died within 24 hours (reason unknown).
At 1 hour and 1 day after implantation 2 implants, and at 3 days, 6 days, 12 days, 24 days, 6 weeks, and 12 weeks after implantation 4 implants were harvested and prepared for scanning

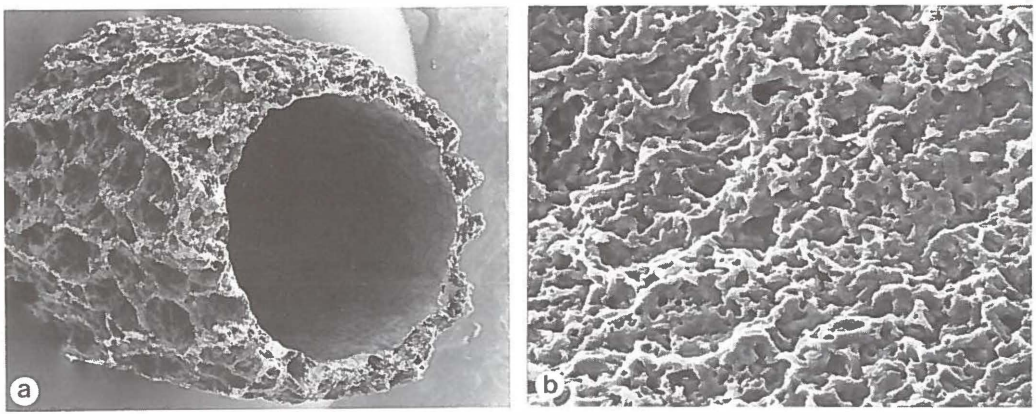

Fig. 1a, b Scanning electron micrographs of a microporous, compliant, biodegradable PU/PLLA vascular graft. Note the large pores at the outer surface (**a**; magnification × 23) and the small pores at the luminal surface (**b**; magnification × 150).

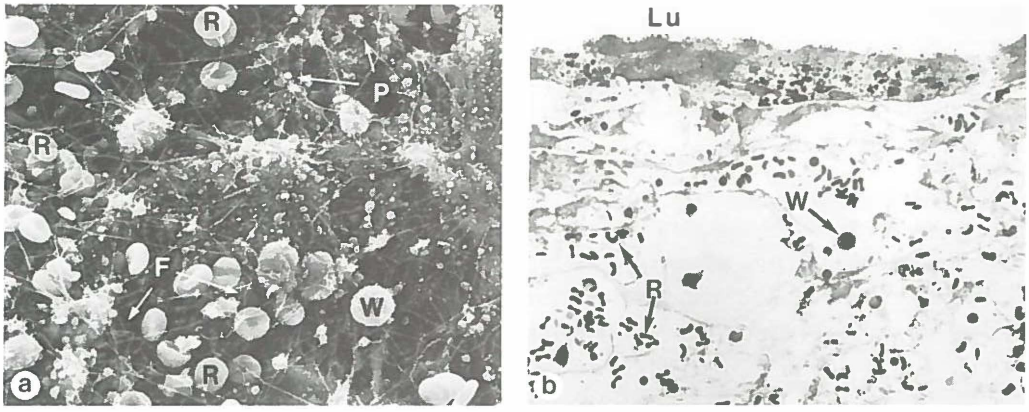

Fig. 2a, b Scanning electron micrograph (**a**) and light micrograph (**b**) of a 1-hour implant. **a** On the luminal surface platelets (*P*), red blood cells (*R*) and some white blood cells (*W*) are entrapped in a fibrin meshwork (*F*). (magnification × 825). **b** In addition, the graft lattice is filled with platelets, red blood cells (*R*), and a few white blood cells (*W*) enmeshed in a fibrin matrix. *Lu* lumen. (magnification × 225).

electron microscopy (SEM), light microscopy (LM), and transmission electron microscopy (TEM). SEM was used to inspect the endothelial healing process of the implants, LM was used to determine the subendothelial healing and the tissue ingrowth into the implants. TEM was used for ultrastructural detail and identification of the cells observed with SEM and LM. The rats had free access to standard rat food and water.

Graft Fixation procedure. The rats were anesthetized with pentobarbital (Nembutal®; 50 $mg.kg^{-1}$ body weight, administered intraperitoneally). The patency and compliance of the implants was checked by direct inspection looking for arterial pulsations.[21] Heparin (1,000 IU, intraveneously) was administered to prevent clotting during cannulation of the aorta. Immediately after cannulation the implants were fixed by pressure-controlled perfusion[22] at a pressure of 180 mm Hg for 15 minutes with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After dissection the implant was left in the same fixative for 24 hours at 4 °C at a pressure of 180 mm Hg. Alternately, either the distal or the proximal end of the implant was prepared for SEM, the other end was prepared for LM and TEM.

Preparation for SEM. The specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide. Next the specimens were cut in three longitudinal segments and sputter coated with gold palladium. These segments were examined in a JSM-35 C scanning electron microscope operated at 15 to 25 kV.

Preparation for LM and TEM. The specimens, cut into rings of approximately 1 to 2 mm width, were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phophate buffer, pH 7.4, and postfixed for 4 hours at 4 °C in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4. After dehydration in alcohol, the rings were embedded in Epon. Semithin sections (0.5 to 1.0 μm) were cut and stained with toluidine blue and basic fuchsin. Areas of interest were selected for ultrathin sectioning. The ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and were examined in a Philips EM 300 electron microscope operated at 60 kV.

Results

Macroscopic examination. At each time of harvesting the implants were patent and showed arterial pulsations. No stenosis or dilatation was observed.

Microscopic examination.

One-hour implants. The graft surface (Fig. 1b) was covered with platelets, red blood cells, and a few white blood cells entrapped in a fibrin meshwork (Fig. 2a). In addition, in the graft lattice red blood cells and a few white blood cells were enmeshed in a fibrin matrix (Fig. 2b).

One-day implants. The graft surface was covered similar to that of the implants of one Hour. The aorta, laying just in the slightly oversized graft, was well adjusted to the graft (Fig. 3a); no tissue components were found to protrude into the lumen. At the anastomoses, at the cut ends, the endothelial cells seemed to flatten, most likely due to cell damage as a result of the operation (Fig. 3b). In the graft lattice, there were no differences observed compared to the implants of one Hour.

Three-day implants. Nearly all of the red blood cells in the fibrin clot had lysed (Fig. 4a-c). Therefore, the coagulum on the graft surface now appeared more homogenous. At the anastomoses, at the cut edges, a distinct flat area of damaged endothelium, with platelets adhering to it, could be clearly distinghuised from the intact aortic endothelial cells (Fig. 4b). In the graft lattice there were large numbers of polymorphonuclear leucocytes (Fig. 4d). In addition, there were some monocytes and macrophages. The macrophages contained large numbers of lysosomes, phagolysosomes, and vacuoles as a result of incorporation of (cellular) debris (Fig. 4e). Prosthetic material was not yet found to be incorporated.

Six-day implants. The graft surface remained similarly covered to that of the implants of Day 3, being mainly composed of platelet-fibrin coagulum. At the cut ends, flat, elongated cells, resembling endothelial cells, started to grow over the area of damaged and denuded endothelium. At some places, these cells were already present on the graft area (Fig. 5a, b).

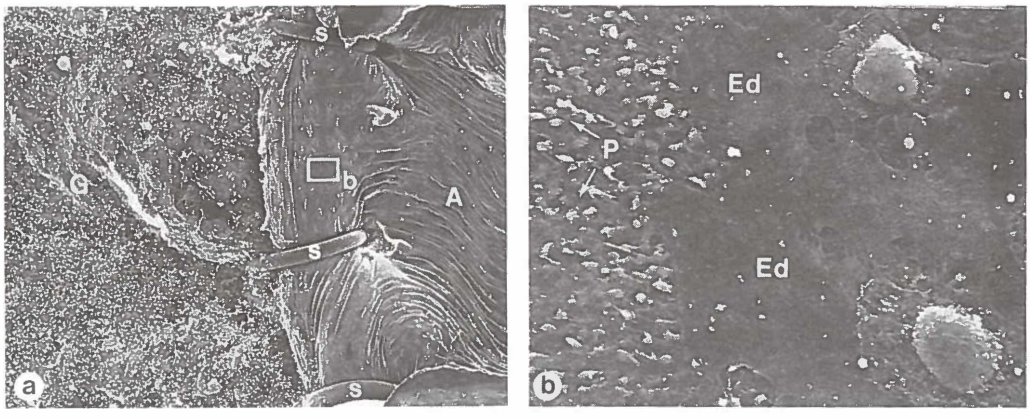

Fig. 3a, b Scanning electron micrographs of the distal anastomosis of a 1-day implant. **a** Note the good approximation of the graft (*G*) and aorta (*A*), and the absence of protruding tissue components. *S* suture. (magnification × 53). **b** At the cut end endothelial cells (*Ed*) flatten, most likely due to cell damage as a result of the implantation procedure. *P* platelets. (magnification × 825).

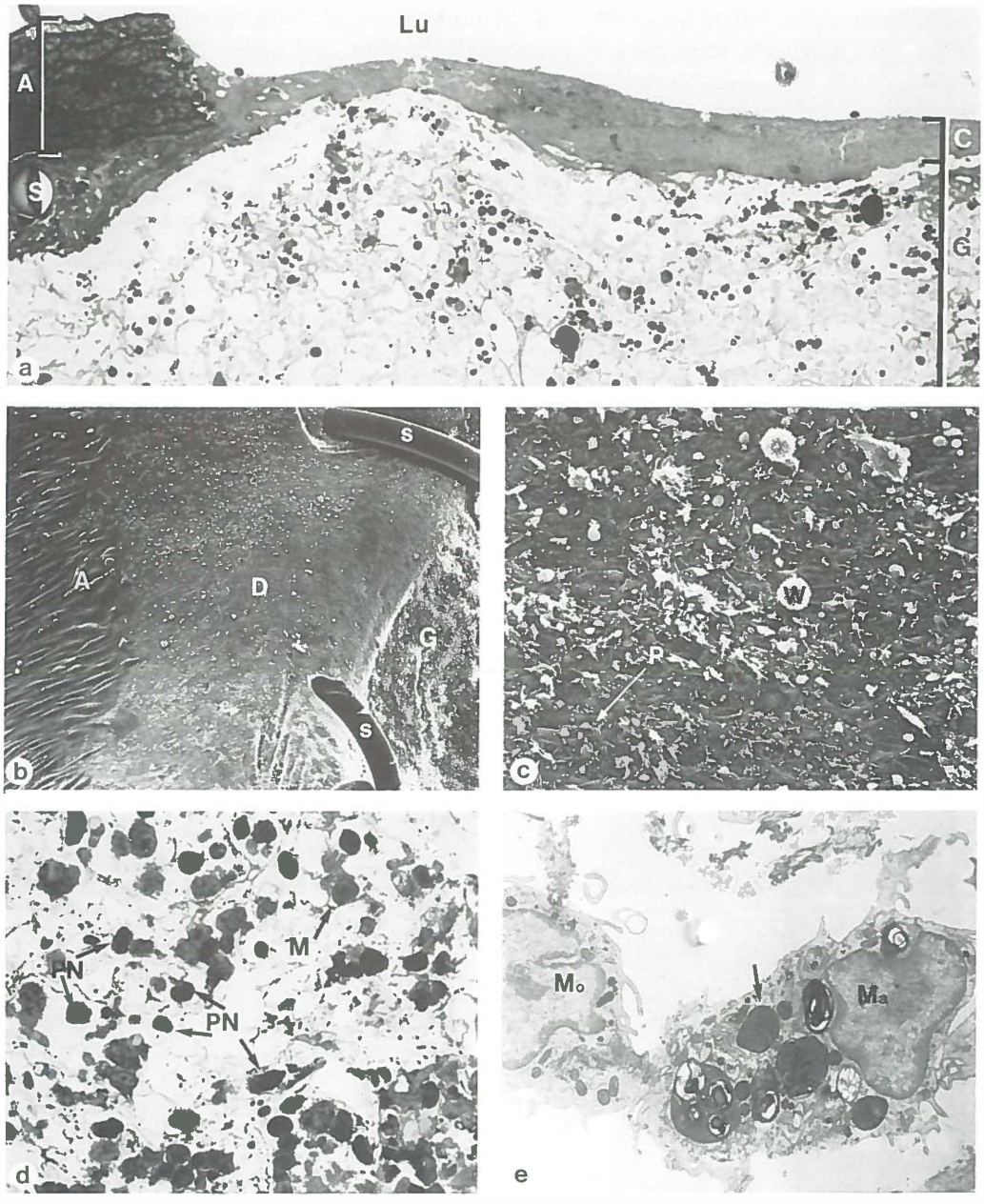

Fig. 4a-e Micrographs of 3-day implants. **a** Light micrograph of a longitudinal section of the proximal anastomosis. A layer of homogenous platelet-fibrin coagulum (*C*) is present on the graft surface (for detail, see **c**); in the graft lattice (*G*) there are large amounts of polymorphonuclear leucocytes and some monocytes and macrophages (for detail, see **d, e**). *Lu* lumen. (magnification × 190). **b** Scanning electron micrograph of the proximal anastomosis. Three separate areas can be distinguished: the intact aortic endothelial cells (*A*), a flat area of damaged and denuded endothelium (*D*), and the graft surface (*G*). *S* suture. (magnification × 120). **c** Scanning electron micrograph of the luminal surface. Note the smooth lining formed by the platelet-fibrin coagulum; some white blood cells (*W*), but no red blood cells, adhere to this lining. *P* platelet. (magnification × 825). **d** Light micrograph of a longitudinal section of the middle part of the graft lattice. Large amounts of polymorphonuclear leucocytes (*PN*) and some monocytes (*M*) and macrophages are present. (magnification × 375). **e** Transmission electron micrograph of a monocyte (*Mo*) and a macrophage (*Ma*) in the graft lattice. Note the large numbers of phagolysosomes in the macrophage (*arrows*). (magnification × 4,000).

TEM demonstrated that these cells had the ultrastructural characteristics of endothelial cells: tight junctions, numerous of pinocytotic vesicles, and occasional Weibel-Palade bodies. In addition, cells originating from the media of the aorta seemed to migrate towards the graft through the platelet-fibrin coagulum (Fig. 5a). TEM confirmed that these cells bore the ultrastructural characteristics of smooth muscle cells: myofilaments parallel to the long cellular axis, peripheral dense bodies, and peripheral vesicles. In the graft lattice polymorphonuclear leucocytes now were scarce. In contrast, a large number of macrophages and epithelioid cells were now present, which seemed to migrate mainly from the perigraft tissue (Fig. 5c). The macrophages were still found to phagocytose (cellular) debris, and started to form, together with epithelioid cells, cell clusters around the polymer. The epithelioid cells contained a large number of lamellapodial extensions, which were either free or in contact with neighbouring cells (Fig. 5d). In addition, fibroblasts penetrated from the perigraft tissue into the graft lattice. Some multinucleated giant cells were also present in the outer region of the graft lattice (Fig. 5c).

Twelve-day implants. No accumulation of fresh thrombus was observed; the platelet-fibrin

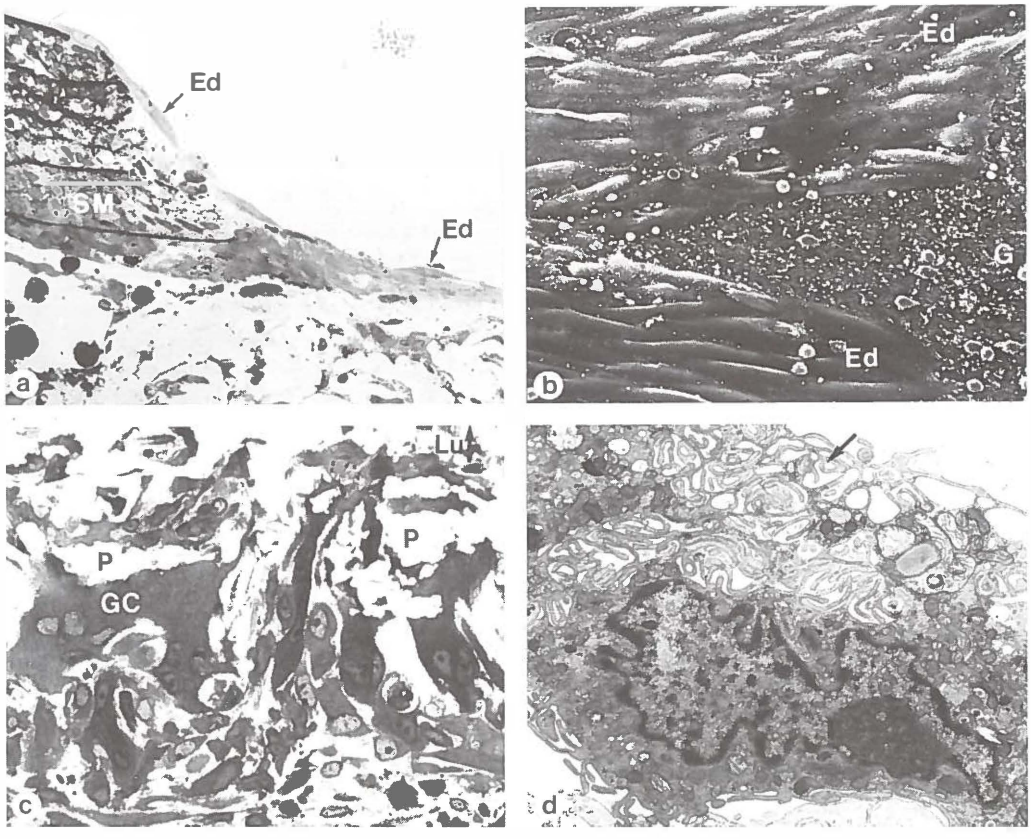

Fig. 5a-d Micrographs of 6-day implants. **a** Light micrograph of a longitudinal section of the proximal anastomosis. Endothelial cells (*Ed*) start to grow from the aortic intima over the graft area, smooth muscle cells (*SM*) start to grow from the aortic media through the platelet-fibrin coagulum. (magnification × 300). **b** Scanning electron micrograph of the proximal growth zone. The endothelial (*Ed*) cell ingrowth is not equal at all front places. *G* graft surface. (magnification × 375). **c** Light micrograph of a longitudinal section of the outer boundary region of the graft lattice. Fibrohistiocytic tissue seems to migrate from the perigraft tissue into the graft lattice. *GC* multinucleated giant cell; *P* prosthetic material; *Lu* lumen. (magnification × 450). **d** Transmission electron micrograph of an epithelioid cell in the graft lattice. Note the elongated lamellapodia at the free border of the epithelioid cell (*arrow;* magnification × 4,000).

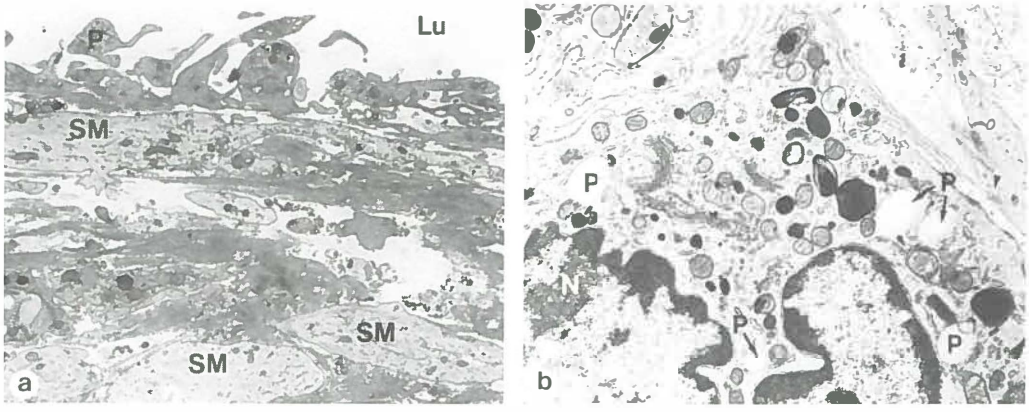

Fig. 6a Transmission electron micrograph of the platelet-fibrin coagulum, lining the non-endothelialized graft areas of a 12-day implant. Note smooth muscle cells (*SM*) growing through and organizing the layer of platelet-fibrin coagulum. *P* platelet; *Lu* lumen. (magnification ×4,700). **b** Transmission electron micrograph of a portion of a multinucleated giant cell in the graft lattice of a 12-day implant. The presence of numerous of organelles as well as the presence of much euchromantin and a nucleolus (*N*) points to the active metabolic stage. Note the presence of small incorporated polymer particles (*P*). (magnification ×4,000).

coagulum formed a smooth layer. The front of endothelial cells had advanced for 1 to 3 mm. The smooth muscle cells, which varied in morphology from normal smooth muscle cells to myofibroblasts, had advanced further (Fig. 6a). In the graft lattices macrophages, epithelioid cells, and multinucleated giant cells were abundantly present. They all were found both to encapsulate polymer and to engulf small polymer particles (Fig. 6b). Fibroblasts had already produced small areas of collagen. Some capillaries were found to penetrate into the graft lattice.

Twenty-four-day implants. The endothelial cell ingrowth had further advanced (Fig. 7a-c); half of the inner lumen was now covered. The endothelial cells formed a continuous monolayer. Only near the front small intercellular gaps were noted (Fig. 7c). In the middle of the non-endothelialized graft area, isolated patches of polygonal shaped cells of different size could be found (Fig. 7a, b). No platelets or fibrin adhered to these cells. TEM demonstrated that these cells, which have also been described in a previous report,[20] are luminal smooth muscle cells, which have penetrated and organized the platelet-fibrin coagulum. Elastic laminae could be found between the smooth muscle cells, which had formed several layers (Fig. 7d, e). Fibrohistiocytic tissue organization of the graft lattice, which by now showed more signs of disintegration, had continued. More capillaries were present. There were no clear indications that fibrohistiocytic tissue or capillaries penetrated through the disintegrating graft lattice to contribute to the luminal healing of the PU/PLLA grafts.

Six-week implants. An almost complete endothelial layer, forming a neointima, was present (Fig. 8a). Some white blood cells were found to adhere to this layer. Underneath this layer there was a neomedia, containing a mixed population of predominantly longitudinally arranged smooth muscle cell types (Fig. 8a), as described in detail previously.[19] Elastic laminae were present almost throughout the entire thickness of the neomedia. The graft lattices were futher disintegrated and organized by fibrohistiocytic tissue, and further penetrated by capillaries, forming thus a neoadventitia (Fig. 8a).

Twelve-week implants. A complete endothelial layer was well established (Fig. 8b, c). In the

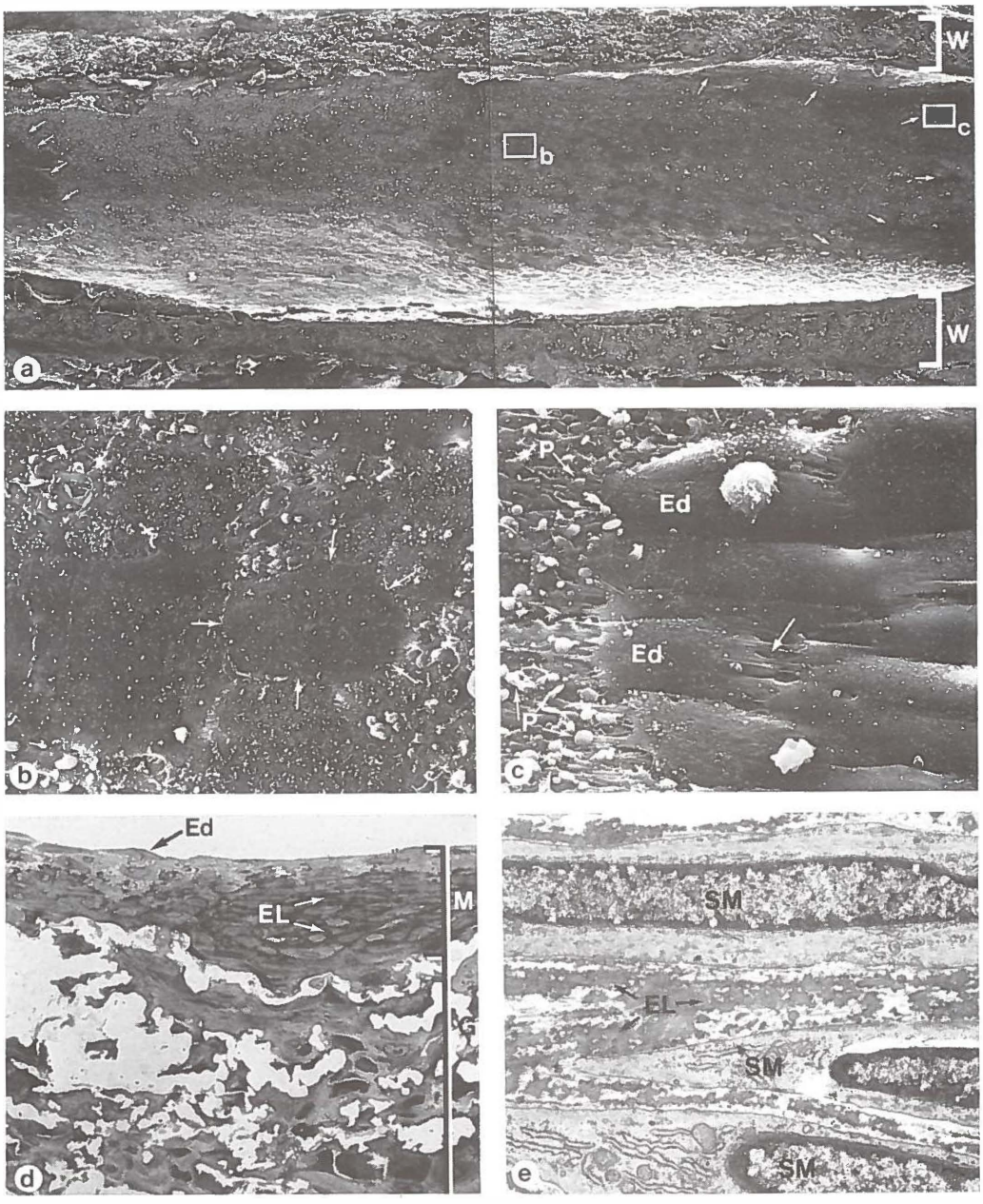

Fig. 7a-c Scanning electron micrographs of the middle part of a 24-days implant. **a** Note both growth zones of endothelial cells (*arrows*). (magnification × 30). **b** At some places of the non-endothelialized mid-area, there are isolated patches of polygonal flat shaped cells, which appeared to be luminal smooth muscle cells (*arrows* mark cell border). (magnification × 1,000). **c** Large number of microvilli were present on the front cells. Note the small intercellular gaps (*arrow*). *P* platelet. (magnification × 1,000). **d** Light micrograph of a longitudinal section of the proximal mid-region of a 24-days implant. Note the several layers of smooth muscle cells (*M*) surrounded by elastic laminae (*EL*) and the ongoing fibrohistiocytic tissue organization of the disintegrating graft lattice (*G*). The front of the sheet of regenerating endothelial cells (*Ed*) is just visible. (magnification × 340). **e** Transmission electron micrograph of smooth muscle cells (*SM*), which have formed several layers and have produced new elastic laminae (*EL*). (magnification × 10,700).

neomedia there were by now areas in which the smooth muscle cells were circularly arranged (Fig. 8b, d). The fibrohistiocytic tissue organization of the disintegrating PU/PLLA lattices was rather similar to that of the implants of Week 6. Some larger capillaries could be found.

Discussion

This study was undertaken to document the sequential events leading to the arterial wall regeneration in PU/PLLA vascular grafts. Our results demonstrate that both endothelial cells and smooth muscle cells grow from the anastomotic sides over the luminal side of the grafts from Day 6 onwards; the endothelial cells from the adjacent aortic intima to form a neointima, the smooth muscle cells from the adjacent aortic media to form a neomedia. Fibroblasts, histiocytes, and capillaries grow from the perigraft tissue into the disintegrating PU/PLLA lattices also from Day 6 onwards to form a neoadventitia.

The origin of the endothelial lining in vascular grafts has been the subject of investigation for many years and has led to several hypotheses. Some investigators have suggested that endothelial cells or multipotential cells, conveyed by the bloodstream and seeded on the graft surface, may contribute to the process of neoendothelialization.[23-28] Other investigators have suggested that the endothelial lining cells may be derived from the perigraft tissue, either from multipotential cells or from endothelial cells of capillaries that penetrate the graft lattice.[29-33] In 1968, Ts'ao[34] suggested that endothelial cells originate from smooth muscle cells. Schwartz et al.,[35] however, presented evidence that smooth muscle cells laying under denuded natural arterial tissue are able to form a prelimanary lining. This lining is subsequently covered by endothelium, originating from the wound edges. Our results in healing PU/PLLA vascular grafts support this notion: three weeks after implantation, there were already patches of smooth muscle cells, which formed a preliminary lining (Fig. 7a, b). These smooth muscle cells that penetrated the platelet-fibrin coagulum were subsequently overgrown with regenerating endothelial cells originating from the adjacent aortic intima (Figs. 5a, b, 7a-c).

The smooth muscle cells seemed to originate from the adjacent media of the aorta, forming a neomedia (Fig. 5a). Our results gave no direct evidence that multipotential cells or fibroblasts, penetrating through the PU/PLLA lattice, contributed to the formation of the neomedia. However, this process can not be excluded since modified smooth muscle cells i.e. myofibroblasts look very much like fibroblasts.[36, 37]

Smooth muscle cells apparently have the capacity to restore a normal arterial wall architecture, as can be concluded from the observation of circularly arranged smooth muscle cells in the 12 Week implants (Fig. 8b, d). However, since the smooth muscle cells have to grow from the anastomotic sides and thus in the direction of the blood flow, they will first arrange predominantly in this direction. Later on, they can become circularly arranged, possibly stimulated by the pulsatile graft distention.[38] Theoretically, this circularly arrangement of the smooth muscle cells is more efficient to withstand the arterial pulsations.

For the initial development of the neomedia the following mechanisms can be considered. Immediately after reestablishing the circulation in the abdominal aorta, the intrinsic and extrinsic coagulation cascade and the complement cascade will be activated: platelets will adhere to the prosthetic segment, aggregate, and release the content of their granules, which also includes a growth factor for smooth muscle cells.[39] Additionally, smooth muscle cells at the cut edges are exposed to fibrin,[40] to factors released by the regenerating endothelial cells[41, 42] and monocytes,[43] to low density lipoproteins,[44] and to other blood components. Therefore, smooth muscle cells at the cut edges will start to proliferate and grow over the prosthetic segment, penetrating the platelet-fibrin coagulum in an attempt to heal the wound, i.e. the graft segment. Additionally, the arterial pulsations stimulate the smooth muscle cells to produce elastic laminae and collagen.[20, 45, 46]

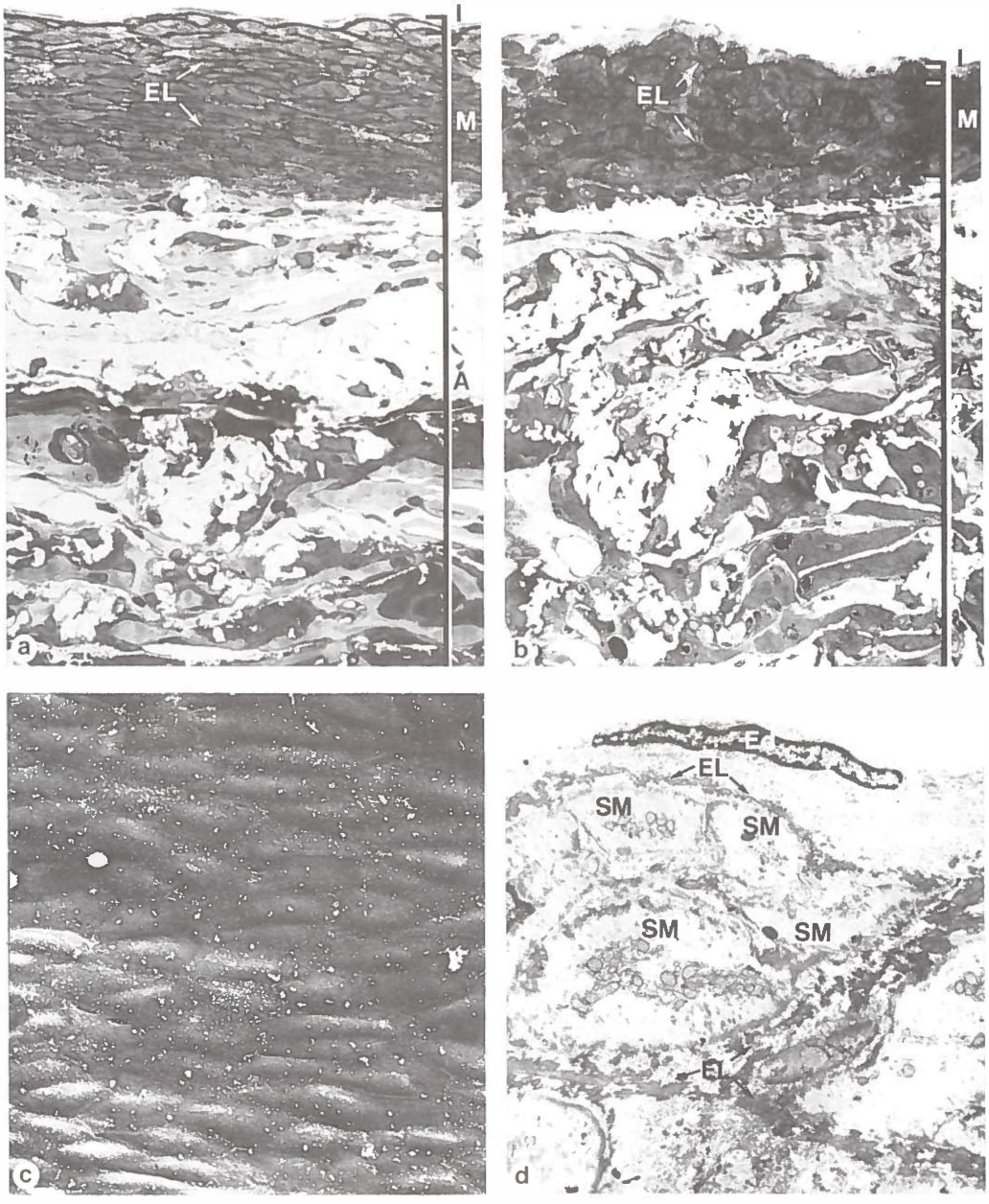

Fig. 8a, b Light micrographs of longitudinal sections of a 6-week implant (**a**) and of a 12-week implant (**b**). Three seperate layers can clearly be recognized: a neointima (*I*), composed of endothelial cells, a neomedia (*M*), composed of several layers of smooth muscle cells surrounded by collagen and new elastic laminae (*EL*), and a neoadventitia (*A*), composed of fibrohistiocytic tissue which organizes the disintegrating graft lattice. Note the circularly arranged smooth muscle cells in the neomedia of the 12-week implant (**b**; magnification × 225). **c** Scanning electron micrograph of the neointima of the middle part of a 12-weeks implant. A complete layer of endothelial cells is well established. (magnification × 450). **d** Transmission electron micrograph of the neomedia of a part of a 12-weeks implant. Note the circularly arranged smooth muscle cells (*SM*), surrounded by elastic laminae (*EL*). *Ed* endothelial cell. (magnification × 5,900).

The reactions against the PU/PLLA material, as we observed, is like an inflammatory response; a natural response to heal a wound.[19, 47, 48] Initially, complex interactions of the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin- and prostaglandin-generating system, and the platelets presumably all are involved.[49] Migration of polymorphonuclear leucocytes and monocytes into the graft lattice, which is filled with fibrin-platelet coagulum, is then mediated through chemotactic stimuli.[49, 50] The polymorphonuclear leucocytes, the initial predominant cells that are very short-lived compared to monocytes, will phagocytize and/or die and release their cytoplasmic and granular components, subsequently mediating the inflammatory response. The monocytes differentiate into macrophages, which play a central role in the inflammatory response.[48, 51] They form epithelioid cells and multinucleated giant cells.[52] Additionally, macrophages can stimulate capillary[53-55] and fibroblast ingrowth.[42, 56] The epithelioid cells and multinucleated giant cells can contribute to the degradation of the PU/PLLA material.[19, 57] The fibroblasts produce collagen, replacing the degradating PU/PLLA lattice. The eventual effect of this fibrous tissue organization at the site of the graft on its functionality, i.e., its compliance, should be evaluated in long-term studies.

The observation of polymer particles engulfed by macrophages, epithelioid cells, and multinucleated giant cells (Fig. 6b) clearly indicates that the PU/PLLA graft lattice is being degraded. Most likely, the process of biodegradation initially is merely a matter of biofragmentation. Since the PU/PLLA grafts are microporous, the polymer fibrils are highly accessible to diffusion of body fluids, and therefore PLLA readily degrades.[13, 14, 58] Upon biodegradation of PLLA, in which lactate dehydrogenase also may play an important role, lactic acid is produced (lactate dehydrogenase has been localized in the cytoplasm of giant cells adjacent to absorbing polyglactin, a lactide-glycolide copolymer[59]). This will effect the hydrolytic stability of the PU matrix and enhance chain scission of hydrolysable groups of the PU, thus leading to fragmentation of the graft lattice. What eventually happens with the fragments of PU remains to be elucidated. Marinesco et al.,[60] however, demonstrated that polyurethane eventually can vanish after several years of implantation.

The healing process of PU/PLLA vascular grafts, as described in detail in this report, differs significantly from polytetrafluoroethylene (PTFE) vascular grafts.[15-17, 61-63] Upon implantation into the rat abdominal aorta, PTFE vascular grafts show limited platelet and fibrin deposition, poor neoendothelial healing, absence of neomedia formation, and limited perigraft tissue ingrowth. All of these differences can be explained by the chemical and material characteristics of PTFE grafts, as well as the graft construction and texture. The physical inertness, the negative surface charge, and the hydrophobic properties of the PTFE grafts all inhibit the formation of an initial thin platelet-fibrin coagulum on the luminal surface, as occurs in PU/PLLA vascular grafts, and therefore limit the process of graft healing.[64-66] Moreover, the construction and texture of PTFE grafts provide poor anchorage to regenerating arterial tissue,[15-17, 67] limit perigraft tissue ingrowth, and thus also limit graft healing.[68, 69] All of these poor healing characteristics of PTFE vascular grafts may have contributed to their poor clinical performance.[9, 10, 70, 71]

The present results may suggest that arterial tissue regeneration in PU/PLLA vascular grafts is limited to short segments, because endothelial cells and smooth muscle cells seem to grow only from the anastomotic sides. In long vascular grafts, which will be needed clinically, one cannot expect endothelial cell and smooth muscle cell ingrowth from the anastomotic sides along the entire graft length within the period of graft disintegration.[72] However, when penetrating perigraft tissue[29-33] or cell seeding[73-75] can contribute to the regeneration of a neoarterial wall, this point may be solved in long vascular grafts.

In conclusion, our results demonstrate that both endothelial cells and smooth muscle cells that regenerate in PU/PLLA vascular grafts grow from the anastomotic sides with the

connected vessel over the luminal side of the grafts. Fibrohistiocytic tissue that organizes the disintegrating graft lattices grows from the perigraft tissue. These sequential regenerative processes result in the formation of a neoarteries, which are of sufficient strength, compliance, and thromboresistance to function as small-caliber arterial substitutes. Therefore, the use of microporous, compliant, biodegradable vascular grafts might open new perspectives for the reconstruction of diseased small-caliber arteries.

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CHAPTER 7

Long-term biological fate of neoarteries regenerated in microporous, compliant, biodegradable, small-caliber vascular grafts in rats

Berend van der Lei,[1] Paul Nieuwenhuis,[2] Izaäk Molenaar,[3] and Charles R.H. Wildevuur[1]

[1] Department of Cardiopulmonary Surgery, Research Division, University Hospital of Groningen, The Netherlands
[2] Department of Histology, University of Groningen, The Netherlands
[3] Centre for Medical Electron Microscopy, University of Groningen, The Netherlands

Summary

Microporous, compliant, biodegradable vascular grafts prepared from a mixture of polyurethane (PU; 95% weight) and poly-L-lactide (PLLA; 5% weight) can function as temporary scaffolds for the regeneration of the arterial wall in small-caliber arteries. The purpose of this study was to determine the long-term biological fate of these neoarteries regenerated in PU/PLLA vascular grafts. Therefore, PU/PLLA vascular grafts (internal diameter 1.5 mm, length 1 cm) were implanted into the abdominal aorta of rats (n = 8) and were evaluated one year after implantation by means of macroscopic inspection, light microscopy, and electron microscopy.

All implants were patent; 3 implants were normally shaped, 2 implants were slightly dilated,($\pm$ 10% of the original internal diameter) and 3 implants were aneurysmal. Arterial pulsations were reduced but still visible in the normally shaped implants but absent in the other implants. In all implants the neointima was complete. The neomedia varied among the implants; in the normally shaped implants smooth muscle cells were predominantly circularly arranged as in normal arterial tissue, in the other implants smooth muscle cells were predominantly longitudinally arranged. The neoadventitia showed a completely fragmentated graft lattice, organized by fibrohistiocytic tissue.

These results suggest that the pattern of arrangement of smooth muscle cells in the neomedia determines the ultimate biological fate of neoarteries regenerated in microporous, compliant, biodegradable vascular grafts. Only those neoarteries with predominantly circularly arranged smooth muscle cells in the neomedia were able to function normally as an arterial substitute for a one year period after implantation into the rat abdominal aorta.

Introduction

During the past three decades, many types of synthetic vascular grafts have been evaluated experimentally and clinically in attempt to find a useful substitute for the reconstruction of diseased arterial segments. Although vascular grafts constructed of either Dacron or Teflon appeared to be clinical useful for the reconstruction of large- and medium-caliber arteries,[1-3] none of them appeared to perform quite satisfactory for the reconstruction of small-caliber

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arteries.[4-9] Therefore, the autologous vein graft is still the first choice as a small-caliber arterial substitute for clinical use.[7, 10-13] However, because its use is frequently compromised by late graft deterioration,[14-16] there is still a demand for a better small-caliber arterial substitute.

Since autologous arterial tissue is indisputably the best material available for the reconstruction of diseased arterial segments, we started to develop a new type of vascular graft which should function as a temporary scaffold for the regeneration of a new arterial wall of small-caliber arteries.[17-19] Microporous, compliant, biodegradable vascular grafts were prepared from mixtures of polyurethane (PU) and poly-L-lactide (PLLA)[20, 21] and were implanted into the abdominal aorta of rats. In vascular grafts prepared from a 95%/5% weight PU/PLLA mixture there was an optimal regeneration of neoarterial tissue, which was of satisfactory strength, compliance, and thromboresistance to function as small-caliber arterial substitute up to 3 months after implantation.[19, 22-25] No stenosis, no aneurysm formation, and no degenerative changes were observed in these neoarterial substitutes. This study was undertaken to determine the long-term biological fate of neoarteries regenerated in PU/PLLA vascular grafts. Therefore, PU/PLLA vascular grafts were implanted into the abdominal aorta of rats and were evaluated one year after implantation by means of macroscopic inspection, light microscopy, and electron microscopy.

Materials and methods

Surgery. Male Wistar rats (TNO, Zeist, The Netherlands) (n = 8), 2 to 3 months old and weighing 250 to 350 gm, were premedicated with atropin (0.25 mg.kg^{-1} body weight, administered intramuscularly) and were anesthetized with 1% halothane (Fluothane®). By means of sterile microsurgical techniques, using an operation microscope (Zeiss OPMI 7-D, Carl Zeiss, Inc., Thornwood, N.Y.), one cm of the abdominal aorta was resected and replaced by a PU/PLLA vascular graft prepared from a 95%/5% weight PU/PLLA mixture.[20, 21] These PU/PLLA vascular grafts had a pore size gradient in their 0.3 mm thick graft wall, ranging from 10 μm in the inner region of the graft lattice to 100 μm in the outer region, and had an internal diameter of 1.5 mm. All grafts were gas sterilized (ethylene oxide) and evacuated for 24 hours under high vacuum (10^{-5} torr). The rats had free access to standard rat food and water. One year after implantation the implants were harvested.

Graft Harvesting. The rats were anesthetized with penthobarbital (Nembutal®, 50 mg.kg^{-1} body weight, administered intraperitoneally). Patency and compliance of the implants were checked by direct inspection looking for arterial pulsations.[26] Heparin (1000 IU, intravenously) was administered 2 to 3 minutes before the fixation procedure was started to prevent clotting. The implants were fixed by pressure-controlled perfusion at a pressure of 180 mm Hg with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 15 minutes, as has been described in detail previously.[19, 27] After dissection, the implant was left in the same fixative for 24 hours at 4 °C. Alternately the distal or the proximal end of the implant was prepared for scanning electron microscopy (SEM), the other end for light microscopy (LM) and transmission electron microscopy (TEM). SEM was used for morphologic evaluation of the endothelial lining (neointima) of the implants, LM was used for morphologic evaluation of the subendothelial smooth muscle cell layers (neomedia) and the fibrohystiocytic tissue organization of the completely disintegrated and fragmentated graft lattices (neoadventitia). TEM was used for ultrastructural analyses and positive identification of the cells observed with SEM and LM.

Preparation for LM and TEM. The specimens, cut into rings of approximately 1 to 2 mm width, were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, p H 7.4, and postfixed for 4 hours at 4 °C in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M phosphate buffer, pH 7.4. After dehydration in alcohol, the rings were embedded in Epon. Semithin sections (0.5 to 1.0 μm) were cut and stained with toluidine blue and basic fuchsin. Areas of interest were selected for ultrathin sectioning. The ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate. Finally, the ultrathin sections were examined with a Philips EM 300 electron microscope operated at 60 kV.

Preparation for SEM. The specimens were rinsed for 30 minutes in 6.8% sucrose solution in 0.1 M phosphate buffer, pH 7.4, and postfixed for 3 hours in 1% osmium tetroxide in 0.1 M phosphate buffer, p H 7.4, at 4 °C. After dehydration in alcohol and substitution with amyl acetate, the specimens were critical-point dried with carbon dioxide. Next the specimens were cut in three longitudinal segments and sputter coated with gold palladium. These segments were examined in a JSM-35 C scanning electron microscope operated at 15 to 25 kV.

Morphologic evaluation. The neointima was inspected for its completeness and cellular appearance.

The neomedia was inspected for its thickness and elastin content by means of a light microscope (magnification × 320) with a square grid placed in its ocular.[28] The thickness of the neomedia was measured in both sides of the implants in the longitudinal sections, both in the middle part of the implants as well as near the anastomotic sides, as reported earlier.[22] The elastin content was determined by measurement of the ratio of the thickness of that part of the neomedia, which contained elastic laminae, to the total thickness of the neomedia.

The neoadventitia was inspected for the extent of fibrohistiocytic tissue ingrowth into the completely fragmentated graft lattices by means of a light microscope with a square grid placed in its ocular. The extent of tissue ingrowth was determined in both sides of the implants in the longitudinal sections by measurement of the ratio of the number of grid points laying over tissue to the total number of grid points used.

Table I. *Macroscopic and microscopic evaluation of PU/PLLA vascular grafts, one year after implantation into the abdominal aorta of eight rats*

Rat no.	*Macroscopic appearance*	*Arterial pulsations*	*Thickness of the neomedia at the*		*Elastin content (%)*	*Orientation smooth muscle cells*	*Relative amount of tissue ingrowth into neoadventitia (%)*
			Anastomoses (μm)	*Mid-region (μm)*			
1	normal	±	100	113	94	C	88
2	normal	±	88	94	93	C	79
3	normal	±	100	100	100	C	85
4	dilated	–	100	110	98	C/L	89
5	dilated	–	115	125	70	C/L	85
6	aneurysmal	–	113	188	47	L	86
7	aneurysmal	–	163	275	32	L	86
8	aneurysmal	–	38	25	0	L	84
normal rat abdominal aorta		+	91 ± 9 (n = 8)			C	

+ : normal arterial pulsations
± : just visible arterial pulsations
– : absence of arterial pulsations
C : circular orientation
L : longitudinal orientation

Results

Macroscopic examination. All implants (n = 8) were patent at the moment of harvesting. Remnants of prosthetic material were present as fragmented, isolated pieces of white/yellowish material within regenerated tissue (Fig. 1a, b). Three implants were normally shaped, two implants were slightly dilated (± 10 % of the original internal diameter) and three implants were aneurysmal (one aneurysm dissecans and two uniformely dilated implants) (Fig. 1a, b; Table I). Arterial pulsations were just visible in the normally shaped implants but were absent in the other implants. Neither microscopic nor macroscopic pathologic changes at the site of the implants or in the organs of the rats were observed.

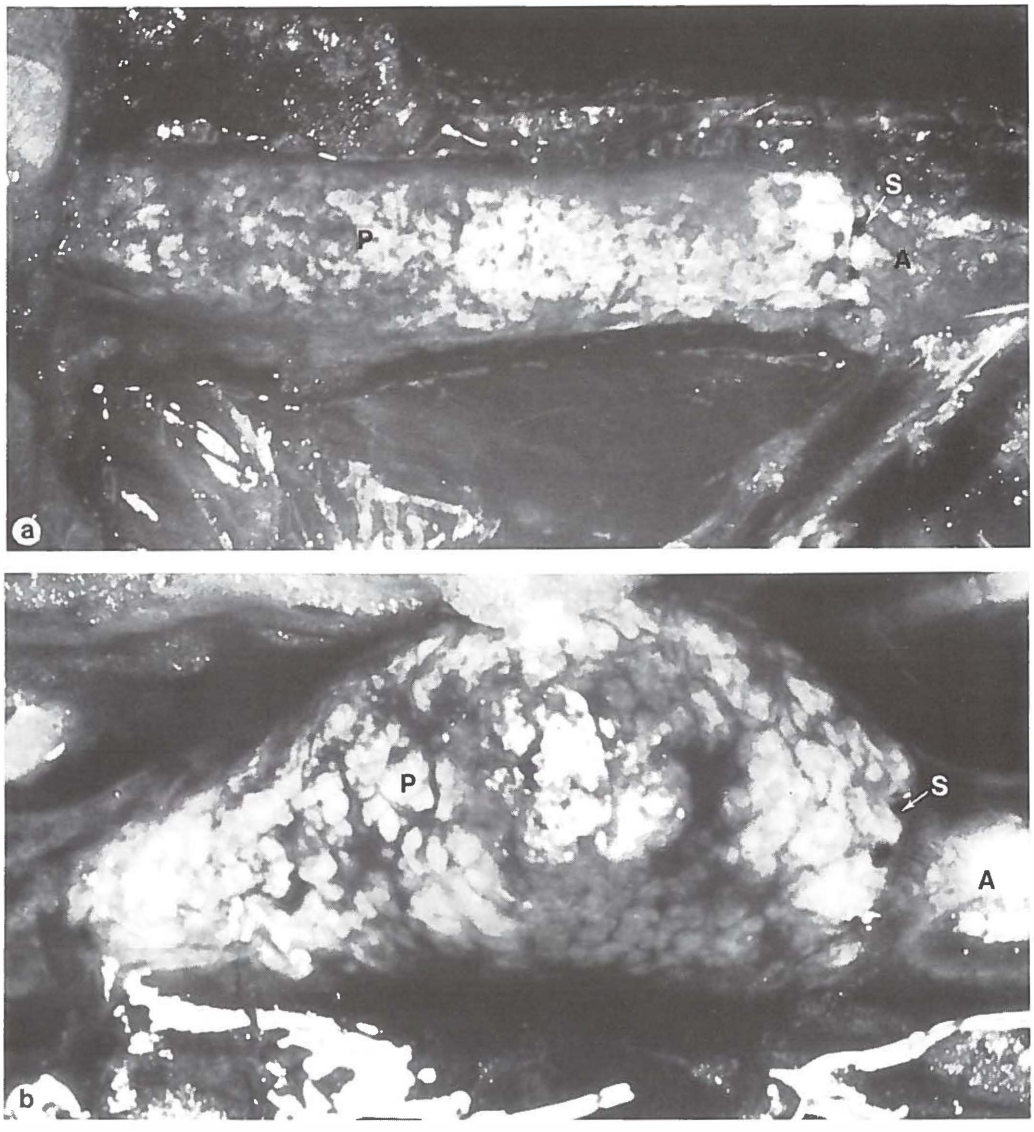

Fig. 1a, b A normally shaped implant (**a**) and an aneurysmal implant (**b**) one year after implantation of a PU/PLLA vascular graft into the rat abdominal aorta. Note the isolated fragments of prosthetic material (*P*) within regenerated tissue. *A* rat abdominal aorta; *S* suture. (magnification 11x).

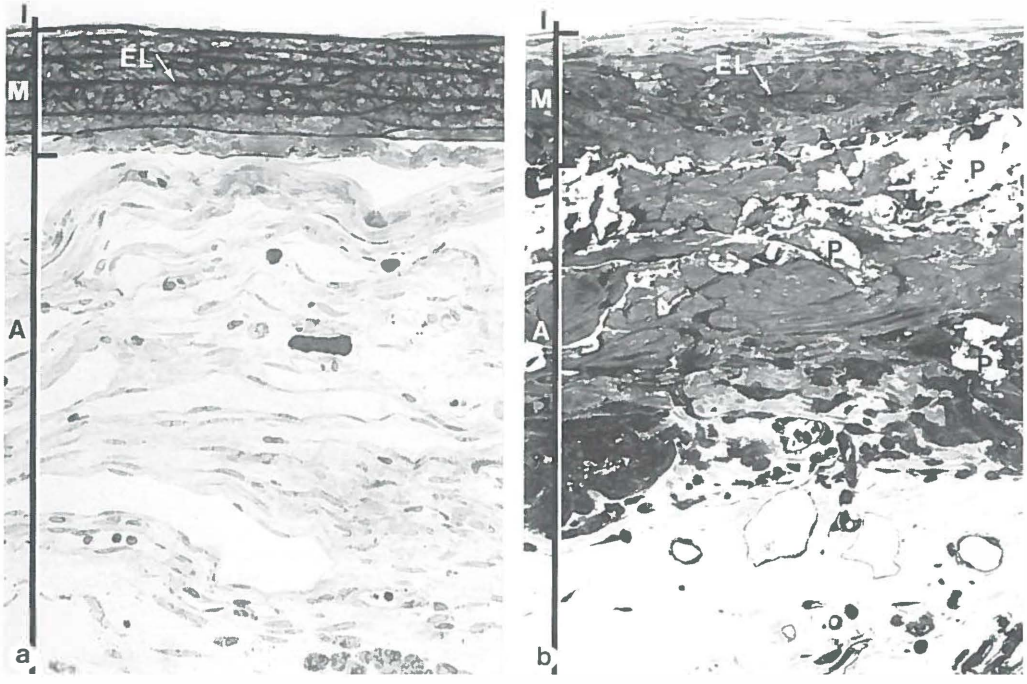

Fig. 2a, b Light micrographs of longitudinal sections of normal rat abdominal aorta (**a**) and of a normally shaped implant one year after implantation of a PU/PLLA vascular graft (**b**). In both sections, three separate layers can be recognized: (1) an inner intima (*I*), composed of endothelial cells, (2) a media (*M*), composed of smooth muscle cells, and (3) an adventitia (*A*), composed of either loose connective tissue (**a**) or composed of fibrohistiocytic tissue that has organized the completely fragmentated graft lattice (**b**). Note the circularly arranged smooth muscle cells in the media of the implant (**b**). *EL* elastic laminae; *P* prosthetic material. (magnification × 190).

Microscopic examination. The microscopic appearance of the wall of the normally shaped implants resembled that of the wall of rat abdominal aorta (Fig. 2a, b). In contrast, the other implants differed strikingly, especially with regard to the smooth muscle cell orientation in the neomedia. The microscopic appearance of the implants is described as follows:

Neointima. A complete layer of endothelial cells, as confirmed by TEM, lined the lumen of all implants, even of the aneurysmal implants. Mostly, the endothelial cells were rhomboidally shaped, parallel aligned to the blood flow (Fig. 3a). Only in the aneurysmal implants at the extremely dilated areas, the endothelial cells were more or less polygonally shaped (Fig. 3b). No fibrin or platelets were found to adhere to the neointima.

Neomedia. In the normally shaped and slightly dilated implants the neomedia was of comparable thickness to the media of normal rat abdominal aorta (Table I). In the aneurysmal implants the neomedia was either thick, especially in the mid-region, or thin (Table I). At some places in the neomedia of the aneurysmal implants there were degenerative changes: lipid accumulation in foam cells (Fig. 4). In the normally shaped and slightly dilated implants, elastic laminae were present almost throughout the entire neomedia. In the aneurysmal implants elastic laminae were only present either in the deep layers of the neomedia, or were hardly present at all (Table I). TEM demonstrated that the cells in the neomedia had the morphology of normal smooth muscle cells (Fig. 5a, b). These smooth muscle cells were predominantly circularly arranged in the normally shaped implants (Fig. 5a), whereas they were predominantly longitudinally arranged in the other implants (Fig. 5b).

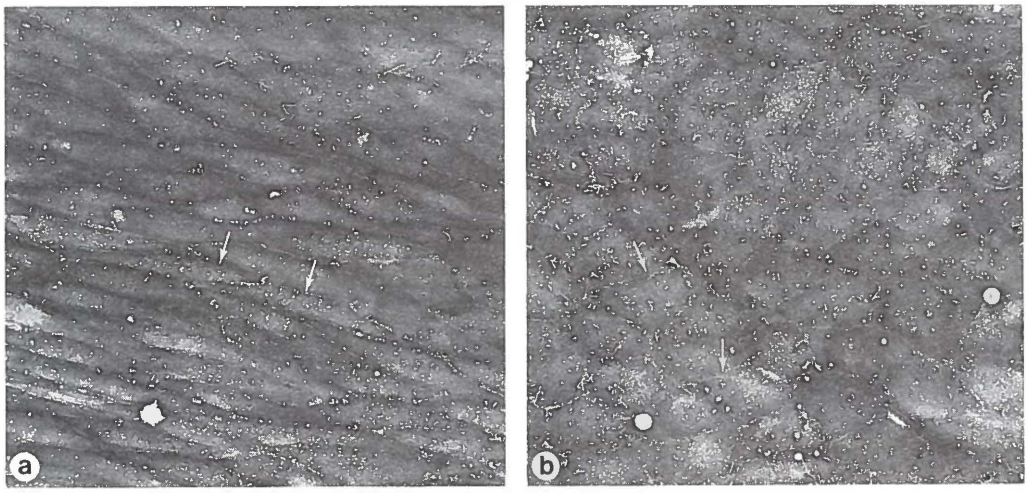

Fig. 3a, b Scanning electron micrographs of the luminal surface in the mid-region of a normally shaped implant (**a**) and of an aneurysmal implant (**b**) one year after implantation of a PU/PLLA vascular graft. In the normally shaped implant (**a**) the endothelial cells are rhomboidally shaped, elongated in the direction of the blood flow. In the aneurysmal implant the endothelial cells are more or less polygonally shaped. *Arrows* mark cell borders. (magnification ×410).

Neoadventitia. In all the implants, a more or less similar neoadventitia was present composed of an extensive amount of fibrohistiocytic tissue, which had organized the completely fragmentated PU/PLLA lattices (Fig. 6a, b). The relative amount of tissue in the neoadventitia, especially the amount of collagen, had increased (Table I; Figs. 2b, 6a) as compared with previously described implants at 6 or 12 weeks after implantation.[22-25]

Discussion

The present results suggest that the pattern of arrangement of smooth muscle cells in the neomedia determines the ultimate biological fate of neoarteries regenerated in microporous, compliant, biodegradable vascular grafts. In the three normally shaped implants, the smooth muscle cells were predominantly circularly arranged as in normal arterial tissue, whereas in the other slightly dilated or aneurysmal implants the smooth muscle cells were predominantly longitudinally arranged. Although neoarteries with predominantly longitudinally arranged smooth muscle cells in the neomedia can function for certain periods of time as arterial substitute (we never observed aneurysm formation up till 3 months after implantation of 95%/5% weight PU/PLLA vascular grafts[19, 25]), apparently over long-term they eventually will fatigue from the continuous arterial pulsations.

Aneurysmal dilatation of neoarteries regenerated in biodegradable vascular grafts has also been reported by Marinescu et al.[29] and Greisler et al.[30, 31] Marinescu et al.[29] believed that abnormal healing of biodegradable vascular grafts and their subsequent failure mainly results from technical imperfections in preparing the grafts. Greisler et al.,[30, 31] however, pointed to the importance of degenerative changes associated with atherosclerosis.

Both factors indeed may play a role in the failure of biodegradable vascular grafts. Because our PU/PPLA vascular grafts were hand made,[20, 21] one could expect minor production imperfections, which subsequently could have attributed to the observed graft failures. In addition, lipid-laden foam cells were observed in the neomedia of the aneurysmal implants, suggesting a tendency towards atherosclerosis of neoarteries. Nevertheless, we feel that the

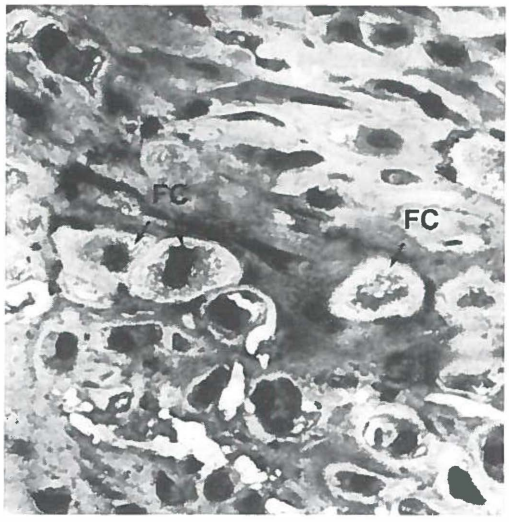

Fig. 4 Light micrograph of the neomedia of an aneurysmal implant one year after implantation of a PU/PLLA vascular graft. Several foam cells (*FC*) containing lipid vacuoles can be observed. (magnification × 660).

arrangement of the smooth muscle cells in the neomedia of the neoarteries is of major importance for their ultimate fate.

The initial primarily longitudinal arrangement of the smooth muscle cells in the neomedia, as observed at six weeks after implantation of PU/PLLA vascular grafts,[22-24] is most likely due to the fact that these smooth muscle cells originate from the anastomotic sides[25] and thus grow in the direction of the blood flow. Synthesis of elastin[24, 32] and collagen[23] then leads to the formation of an elastin- and collagen-network with a predominantly longitudinal orienta-

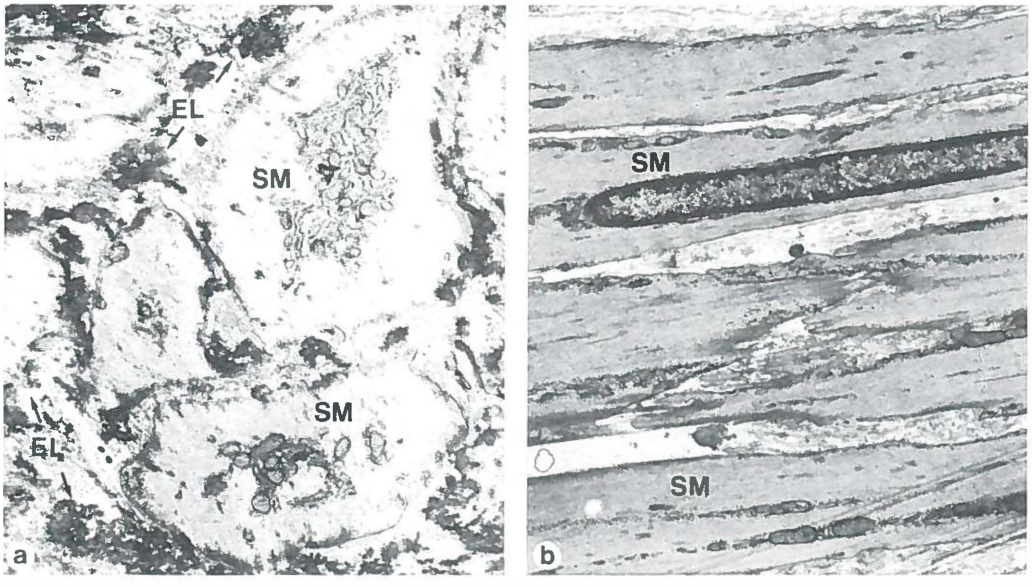

Fig. 5a, b Transmission electron micrographs of longitudinal sections of the neomedia of a normally shaped implant (**a**) and of an aneurysmal implant (**b**) one year after implantation of a PU/PLLA vascular graft. In the normally shaped implant (**a**) the smooth muscle cells (*SM*) are circularly arranged, whereas in the aneurysmal implant (**b**) longitudinally arranged. The smooth muscle cells are clearly characterized by the presence of packed myofilaments parallel to their long cellular axis, peripheral dense bodies, peripheral vesicles, and a modest number of organelles. Note the elastic laminae (*EL*) in the normally shaped implant (**a**). (magnification × 8,500).

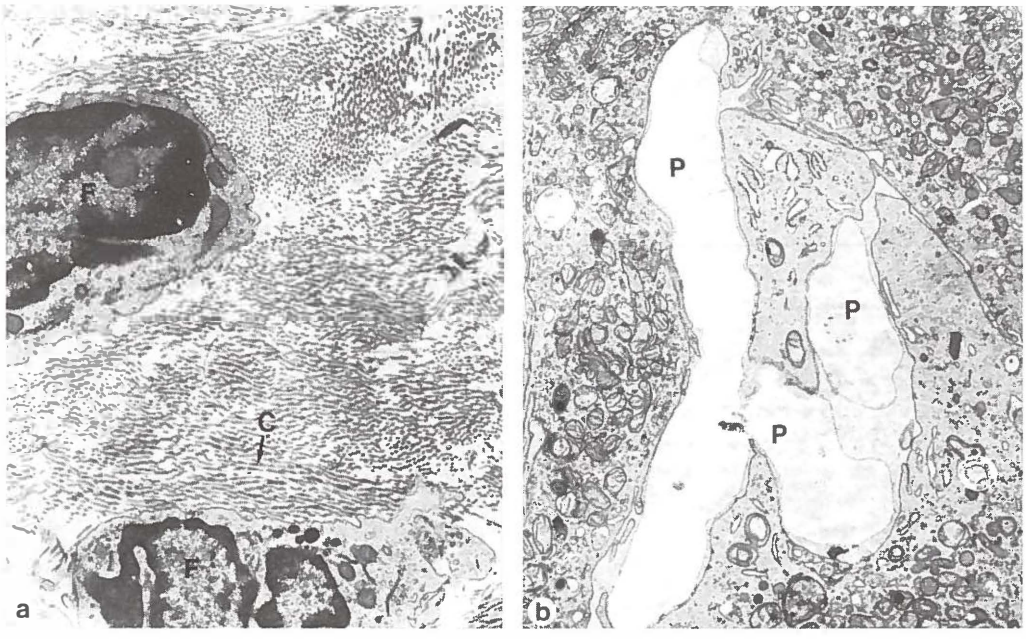

Fig. 6a, b Transmission electron micrographs of the neoadventitia of a normally shaped implant one year after implantation of a PU/PLLA vascular graft. Large amounts of collagen fibrils (*C*) can be found, surrounding fibroblasts (*F*) (**a**), and isolated fragments of prosthetic material (*P*) encapsulated and/or incorporated by histiocytes (**b**). The prosthetic fragments seemed to be infiltrated by intercellular components. Note the large amounts of organelles, indicating a high metabolic activity. (magnification × 7,100).

tion, which in addition may lead to further cell orientation in this direction through contact guidance.

With time, however, smooth muscle cells apparently can become circularly arranged (Figs. 2b, 5a). This way of (re)orientation can be considered as a biologic mechanism of adaptation to the continuous mechanical stress caused by the arterial pulsations. Noishiki[33] demonstrated that the arrangement of smooth muscle cells in vascular grafts is largely determined by the direction of tension to which they are subjected. He implanted stretchable prostheses, which stretched only longitudinally, and expansile prostheses, which expanded only circumferentially, into the thoracic aorta of dogs. In the stretchable prostheses smooth muscle cells were found to orient longitudinally, whereas in the expansile prostheses circumferentially. Dartsch et al.,[34] who subjected cultured arterial smooth muscle cells grown on silicone membranes to directional cyclic stretching, confirmed that smooth muscle cell orientation is determined by the direction of tension, and additionally demonstrated that smooth muscle cell orientation is reversible, depending upon alterations of mechanical stimuli.

Therefore, the observed predominantly longitudinal arrangement of the smooth muscle cells in the slightly dilated and aneurysmal implants may represent a point in time at which the mechanical stimulus for the smooth muscle cells to reorientate has vanished because of loss of compliance of the implants. This loss of compliance, which is to a lesser extent also present in the normally shaped implants, most likely results from the fibrocollagenous neoadventitial tissue organization of the degrading graft lattices, which had increased in comparison to implants at 6 or 12 weeks after implantation (Table I; Fig. 2b).[17-19, 22-25] Initially, this fibrocollagenous tissue organization is to a certain extent neccessary to contribute to the integrity of the neoarterial substitutes.[22, 35] However, since the fibrocollagenous tissue organization continues, stimulated by the chronic inflammatory response as a result of the

continuous presence of prosthetic material,[23, 36-38] the compliance apparently eventually will be impaired. Although only isolated fragments of prosthetic material were present at one year, complete disappearance of these fragments, which is most likely pure PU as based upon in vitro experiments,[20, 21, 39] probably will take some years, as demonstrated by Marinescu et al.[29]

The long-term presence of prosthetic material in the living body may also have carcinogenic effects. Therefore (although we did not observe microscopic or macroscopic pathologic changes at the site of the implants or in the organs of the rats) the ultimate effect of the PU/PLLA material and its degradation products in the living body, especially of the PU components, remains to be evaluated in more specific studies.

To maintain an optimal long-term compliance of the implants and consequently an optimal long-term stimulus for the smooth muscle cells to reorientate eventually, the fibrocollagenous tissue organization as a result of the chronic inflammatory response should be reduced. Graft modifications, as for example (1) grafts prepared from less amounts of polymer (i.e. increasing the graft porosity and/or reducing the graft wall thickness), (2) grafts prepared from other material compositions that degrade fast after graft disintegration, and (3) grafts impregnated with fibroblast-inhibiting factors, might reduce this fibrocollagenous tissue organization.

In conlusion, our results suggest that the pattern of arrangement of the smooth muscle cells in the neomedia determines the ultimate biological fate of neoarteries. Therefore, further studies evaluating the various factors that may enhance the circularly (re)orientation of smooth muscle cells, as for example special graft modifications or smooth muscle cell seeding,[40, 41] are of utmost importance for the continuing development of microporous, compliant, biodegradable vascular grafts, which can function as temporary scaffolds to restore the normal arterial wall architecture of small-caliber arteries.

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CHAPTER 8

General discussion

The studies reported in this thesis demonstrate that the process of arterial wall regeneration in microporous, compliant, biodegradable vascular grafts, prepared from a PU/PLLA mixture, is similar to the natural reaction of arterial tissue upon injury. This regeneration, which is a natural attempt of arterial tissue to restore its normal architecture, is determined by the functional characteristics of the graft: *microporosity, compliance*, and *biodegradability*.

Injury and repair of the normal arterial wall

Normally, the endothelial cells of the arterial wall form an antithrombogenic lining with the circulating blood. Although several factors contribute to the antithrombogenicity of endothelial cells, such as their binding sites for thrombin,[1] the presence of antithrombin III,[2] and glycosaminoglycans[3] on their surface, their capicity to synthesize and release prostacyclin[4-7] is of major importance. Prostacyclin is the most powerful, natural occurring inhibitor of platelet aggregation yet discovered.[4]
When a part of the arterial wall has been damaged, i.e. a part of the endothelial lining and possibly a part of the media (smooth muscle cells) have been damaged and denuded, the natural antithrombogenicity of the arterial wall is much reduced. Platelets then rapidly adhere to the exposed connective tissue, aggregate and release their components, including those from the alpha granules (platelet-derived growth factor, platelet factor 4, b-thromboglobulin, fibrinogen and other coagulation factors) and those from the amine storage granules or dense bodies (serotonin, adenine nucleotides, pyrophosphate, and calcium).[8,9] Consequently, fibrin forms and entwines the platelets, thus forming a stable platelet-fibrin coagulum over the denuded area.[10]
Endothelial cells surrounding the damaged and denuded area start to proliferate and to migrate in an attempt to cover the defect.[11-16] Growth of these endothelial cells may be stimulated and regulated by interruption of cell-cell contact,[14,17,18] underlying smooth muscle cells,[19,20] polymorphonuclear leucocytes,[21] macrophages,[22] fibrin,[23] and serum mitogens.[24] The endothelial cells secrete plasminogen activator,[23,25] which stimulates plasminogen to form plasmin. Plasmin in its turn lyses the platelet-fibrin coagulum.
Smooth muscle cells surrounding and beneath the damaged and denuded area also start to proliferate and to migrate in an attempt to restore the defect.[26-28] They penetrate the platelet-fibrin coagulum, form several layers (which is called subintimal hyperplasia), and produce collagen and elastin.[29-33] In this way, even damaged elastic laminae of the arterial wall can be repaired.[34,35] Factors released by platelets,[36] by regenerating endothelial cells,[37-41] and by monocytes,[42] low density lipoproteins,[43,44] and fibrin[45] may stimulate and regulate the growth of the smooth muscle cells.
Possible defects of the adventitial layer of the arterial wall heal fast by granulation tissue.[29,46] Regeneration of the arterial tissue components, which is closely related to the extent of the defect in the arterial wall and the cell types involved,[47-50] continues until the vascular integrity and thus the balance in the microenvironment of the arterial wall has been restored.
Nonetheless, the exact growth regulating mechanisms and the interrelations of growth of the cell types involved in the process of arterial wall regeneration remain obscure.

Healing of PU/PLLA vascular grafts

Implantation of PU/PLLA vascular grafts into the rat abdominal aorta evoke similar responses to those which have just been described (*chapters 2-7*).
Initially, a thin platelet-fibrin layer is deposited on the graft surface (*chapter 6*). Then, from day six onwards, both endothelial cells originating from the adjacent host intima and smooth muscle cells originating from the adjacent host media grow in across the anastomoses, in an attempt to heal the grafted segment. The ingrowing endothelial cells establish a new intima (neointima), which is as thromboresistant as a normal intima (*chapter 2*). Underneath this neointima, the ingrowing smooth muscle cells that penetrate the platelet-fibrin coagulum establish a new media (neomedia), in which elastic laminae and collagen are newly deposited. From day 6 onwards, granulation tissue originating from the perigraft tissue and histiocytes also start to grow into the pores of the graft and organize the disintegrating PU/PLLA lattices, resulting in the formation of a new adventitia (neoadventitia), which additionally contributes to the integrity of the regenerated arterial wall.

Cell polymer interactions

The reaction of tissue against the biodegradable PU/PLLA material itself (*chapters 3, 4, and 6*) is similar to the natural response of tissue to synthetic material: a chronic inflammatory response.[51-53]
Initially complex interactions of the extrinsic and intrinsic coagulation systems, the fibrinolytic system, the complement system, the kinin- and prostaglandin-generating systems and platelets are presumably all involved.[54] Polymorphonuclear leucocytes and monocytes migrate into the graft lattice, mediated through chemotactic stimuli.[55] The polymorphonuclear leucocytes are initially the predominant cells, which start to phagocytose cellular debris (*chapter 6*). After 1 to 2 days these cells die and then release their cytoplasmic and granular components, consequently mediating the inflammatory response. The monocytes differentiate into macrophages, which play a central role in the inflammatory response and in the process of biodegradation of the PU/PLLA material.[56] They start to phagocytose cellular debris as well as polymer particles, differentiate into epithelioid cells,[57] and can stimulate ingrowth of granulation tissue, i.e. capillary ingrowth[58-60] and fibroblast ingrowth.[42,61] Both macrophages and epithelioid cells merge by fusion into multinucleated giant cells,[62,63] which also contribute to the degradation of the graft material.[64] The ongoing process of biodegradation coincides with an ungoing fibrocollagenous tissue organization of the degrading PU/PLLA graft lattices, which ultimately impairs the compliance of the implants (*chapter 7*).
Although the exact mechanisms of biodegradation of PU/PLLA grafts has not yet been studied in full detail, initially this process is merely a matter of biofragmentation (*chapter 6*). PLLA readily degrades after implantation of a PU/PLLA vascular graft, in which diffusion of body fluids and lactate dehydrogenase of histiocytes may play an important role.[65,66] Lactic acid is produced after degradation of PLLA, which will effect the hydrolytic stability of the PU matrix and will enhance chain scission of hydrolysable groups of PU. Consequently, the graft lattices will fragmentate and disintegrate. Graft disintegration, observed from day 12 onwards, is completed probably between 6 weeks and 3 months after implantation. Complete biodegradation of the PU/PLLA graft is a time-consuming process that probably will take several years.[67]
We never observed pathological changes at the site of implantion or in the organs of the rats after harvesting of an implant. Still, the ultimate effect of PU/PLLA material and its

degradation products in the living body, especially of the PU components, remains to be elucidated in more specific studies to exclude the possibility of long-term carcinogenic effects of PU/PLLA material.

Essential functional characteristics of PU/PLLA vascular grafts

The *microporous structure* of the PU/PLLA vascular grafts, which to a certain extent also determines their compliance and rate of biodegradation, creates a stable anchorage for both the initially deposited platelet-fibrin coagulum and the ultimately established neointima and neomedia, and determines the speed and amount of perivascular fibrohystiocytic tissue ingrowth.[68-70] The two pore structures of PU/PLLA vascular grafts which were tested, were a constant pore size of 40 μm throughout the graft lattice and a pore size gradient ranging from 10 μm in the inner region to 100 μm in the outer region of the graft lattice (*chapter 3*). These two pore structures did not significantly differ in their effect on regeneration. Nevertheless, we suggest the use of the pore size gradient in these vascular grafts, because such a pore structure can reduce initial graft thrombogenicity its smaller luminal pores in more thrombogenic species, such as man, without impairing the regeneration of neoarterial tissue.[71]
The *compliance* and *rate of biodegradation* of the PU/PLLA vascular grafts are determined by the material composition of the PU/PLLA, and determine the regeneration of both the arterial cellular components and the extracellular components (*chapters 3 and 5*). Since PU/PLLA vascular grafts prepared from a 95%/5% weight PU/PLLA mixture with the PLLA of viscosity-average molecular weight 500,000 have a well-matched compliance in combination with a well-balanced degradation, there is an optimal regeneration of neoarterial tissue in these grafts. Because of the well-matched compliance smooth muscle cells are mechanically stimulated by the arterial pulsations to produce elastin arranged in laminae. Because of the well-balanced degradation of these grafts both the integrity and the compliance of the implants is well maintained, despite the fact that degradation also enhances tissue ingrowth into the graft lattices. Therefore, elastic laminae are produced almost throughout the neomedia.

Smooth muscle cell orientation

At six weeks after implantation the neomedia in the regenerated implants is already of comparable thickness to the media of normal rat abdominal aorta (*chapters 3 and 5*). However, in contrast to normal arterial tissue, the smooth muscle cells are predominantly longitudinally arranged. This arrangement is most likely due to the fact that these smooth muscle cells originate from the anastomotic sides and thus grow in this direction. In time, however, the smooth muscle cells can reorient towards a circular arrangement: at three months after implantation, there are already areas in the neomedia with circularly arranged smooth muscle cells, and one year after implantation, all smooth muscle cells can be circularly arranged (*chapters 6 and 7*). This circular reorientation, which can be considered as a biological mechanism of adaptation to the continuous stress caused by the arterial pulsations,[72,73] seems to be of crucial importance for the ultimate fate of neoarteries. After one year only those neoarteries were normally shaped, in which the smooth muscle cells in the neomedia were predominantly circularly arranged, whereas the neoarteries with predominantly longitudinally arranged smooth muscle cells in the neomedia were more or less aneurysmal (*chapter 7*).

A prospective view on further development of microporous, compliant, biodegradable vascular grafts

So far, experiments have only been performed with PU/PLLA vascular grafts of 1 cm length, implanted in young healthy rats. These grafts could ensure regeneration of neoarteries of sufficient strength, compliance, and thromboresistance within six weeks after implantation. However, long-term function of these neoarteries, which seemed to depend on whether or not the smooth muscle cells eventually reoriented towards a circular arrangement, could not always be ensured. Although the exact factors that determine whether or not the smooth muscle cells eventually reorient towards a circular arrangement are not fully understood, too early loss of compliance of the implants by fibrocollagenous neoadventitial tissue formation as a result of the chronic inflammatory response to the PU/PLLA material seems to be of major importance (*chapter 7*). Therefore, various factors that may reduce this fibrocollagenous tissue formation, such as special graft constructions and/or application of other material compositions that degrade fast after graft disintegration, should be evaluated.
Clinically, long vascular grafts usually will be needed for patients whose blood vessels are usually diseased as a result of general atherosclerosis. This implies that arterial wall regeneration may require more time and may be impaired by unfavourable conditions of the patient. Therefore, various factors that may enhance fast and uniform regeneration in long vascular grafts, such as cell seeding (endothelial cells and smooth muscle cells) should also be evaluated. Recent studies revealed that smooth muscle cell seeding in microporous, compliant, biodegradable vascular grafts is feasable.[74,75] By means of a preclotting technique, high amounts of smooth muscle cells were seeded on grafts of 1 cm length, which subsequently were implanted into the rat abdominal aorta. This cell seeding could result in the formation of a neomedia of normal thickness with predominantly circularly arranged smooth muscle cells within 1 week. Inasmuch as a normal arterial architecture appears to be the most important element permitting proper long-term function of neoarteries, it is not unreasonable to speculate that with the application of this cell seeding technique we might be on the threshold of a new phase in the continuing development of microporous, compliant, biodegradable grafts. The major issue therefore seems to be extending smooth muscle cell seeding in long vascular grafts.
Finally, microporous, compliant, biodegradable vascular grafts implanted in adult animals of various species should be tested, including in animals fed to atherogenic diets, in order to mimic the human situation as closely as possible.
Thus, tremendous tasks remain for both polymer chemists and biomedical researchers: only careful synthesis of polymer science and cell biology of the vascular wall will further develop vascular grafting with microporous, compliant, biodegradable vascular grafts so that eventually safe clinical applications can be considered.

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CHAPTER 9

Summary

An ideal arterial substitute, in particular for small-caliber arteries, is not yet available. Vascular grafts, prepared from a mixture of polyurethane (PU) and poly-L-lactic acid (PLLA), however, may approximate the desired properties of an ideal arterial substitute; these microporous, compliant, biodegradable vascular grafts can function as a temporary scaffold for the regeneration of a new arterial wall in small-caliber arteries. This thesis describes a research project which was focussed on the elucidation of the basic mechanisms of the arterial wall regeneration in these vascular grafts and the scope and limitations of the use of these vascular grafts.

Microporous, compliant, biodegradable PU/PLLA vascular grafts (length 1 cm, internal diameter 1.5 mm) were implanted into the abdominal aorta of rats by means of sterile microsurgical techniques. The grafts were evaluated until one year after implantation in the several studies which are reported in this thesis.

In the general introduction, *chapter 1*, a survey of the major categories of biological and synthetic vascular grafts is presented, together with the basic concepts and construction of PU/PLLA vascular grafts. Finally, the aim of the studies reported in this thesis is outlined.

In *chapter 2* is shown that microporous, compliant, biodegradable vascular grafts prepared from a PU/PLLA mixture have better healing characteristics than rigid, non-degradable polytetrafluoroethylene grafts, which are clinically used. Although in both graft types the neoendothelial cells were qualitatively the same as normal endothelial cells concerning their prostacyclin production, there were striking quantitative differences concerning the process of neoendothelial healing. The polytetrafluoroethylene grafts showed incomplete healing, even after 12 weeks of implantation, whereas the PU/PLLA vascular grafts showed almost complete healing after 6 weeks of implantation.

In *chapter 3* is shown that a 95%/5% weight PU/PLLA mixture with the PLLA of viscosity-average molecular weight 500,000 is the most suitable composition for PU/PLLA vascular grafts to ensure an optimal regeneration of a new arterial wall. There was no difference in effect on the processes of regeneration between PU/PLLA vascular grafts with a constant pore size of 40 μm throughout the graft lattice and grafts with a pore size gradient ranging from 10 μm in the inner region to 100 μm in the outer region in the graft lattice.

In *chapter 4* a description of the ultrastructure of neoarterial tissue regenerated in PU/PLLA vascular grafts, six weeks after implantation, is given. The ultrastructural observations demonstrated that the processes which transform PU/PLLA vascular grafts into neoarterial substitutes, resemble the natural healing response of arterial tissue upon injury and the natural response of tissue against synthetic material.

In *chapter 5* is shown that both compliance and biodegradation of microporous vascular grafts stimulate the regeneration of elastic laminae in neoarterial tissue. Because of the compliance of these vascular grafts, smooth muscle cells are continuously mechanically stimulated by the arterial pulsations, resulting in the production of elastin arranged in laminae. Because of the balanced biodegradation of these vascular grafts, compliance is well maintained despite tissue ingrowth and therefore favours the regeneration of elastic laminae.

In *chapter 6* the dynamics of the arterial wall regeneration in PU/PLLA vascular grafts were evaluated. It is demonstrated that both endothelial cells and smooth muscle cells grow from the anastomotic sides with the connected vessel over the luminal side of the grafts. Fibrohis-

tiocytic tissue that organizes the disintegrating graft lattice grows from the perigraft tissue. In *chapter 7* it is suggested that the pattern of arrangement of smooth muscle cells in the neomedia determines the ultimate biologic fate of the neoarteries. Only the neoarteries with predominantly circularly arranged smooth muscle cells were able to function as a small-caliber arterial substitute for a one year period without dilatation or aneurysm formation. In the general discussion, *chapter 8*, it is stated that the processes of arterial wall regeneration in microporous, compliant, biodegradable vascular grafts, prepared from PU/PLLA mixtures, is similar to the natural healing response of arterial tissue upon injury and the natural response of tissue against synthetic material. These processes are determined by the functional characteristics of the graft: *microporosity*, *compliance*, and *biodegradibility*. The basic mechanisms of regeneration, and the scope and the limitations of the new way of vascular grafting are summarized and discussed. In addition, some implications of our studies for further research are given.

CHAPTER 10

Samenvatting

Nog steeds blijkt er geen ideaal vervangingsmateriaal te bestaan voor dichtgeslibte kleine bloedvaten, zoals bijvoorbeeld voor de kransslagaderen van het hart. Microporeuze, elastische, afbreekbare kunstbloedvaten echter, kunnen mogelijkerwijs een doorbraak betekenen als ideale vervanging voor kleine arteriën door als tijdelijk geraamte te fungeren voor het ontstaan van een nieuw bloedvat.
In dit proefschrift zijn dergelijke kunstbloedvaten, gemaakt van een mengsel van polyurethane en poly-L-melkzuur, op systematische wijze onderzocht. De kunstbloedvaten, met een inwendige diameter van 1,5 mm en een lengte van 1 cm, werden met behulp van microchirurgische technieken geïmplanteerd in de buikslagader van ratten. Op verschillende tijdstippen tot 1 jaar na implantatie werden deze geïmplanteerde (kunst)bloedvaten onderzocht.
De nieuwe kunstbloedvaten blijken veel betere genezingseigenschappen te hebben dan de huidig toegepaste niet-afbreekbare polytetrafluoroethyleen (PTFE) kunstbloedvaten (*hoofdstuk 2*). De genezing in de nieuwe kunstbloedvaten verloopt op een zelfde wijze als die na beschadiging van een normaal bloedvat (*hoofdstuk 4 en 6*). Vanaf de aanhechtingsplaatsen van het kunstbloedvat met het normale bloedvat groeien endotheelcellen en gladde spiercellen over en door het laagje bloedstolsel, dat zich direct na implantatie op de binnenkant van het kunstbloedvat heeft gevormd. Het kunstmateriaal roept een ontstekingsreaktie op, waardoor bindweefselcellen (fibroblasten) en haarvaatjes vanuit het omliggende weefsel in de wand van het kunstbloedvat gaan groeien. Het kunstmateriaal zelf wordt geleidelijk afgebroken door ontstekingscellen (macrofagen en reuscellen).
Door deze processen wordt het kunstbloedvat binnen zes weken na implantatie omgebouwd tot een nieuw bloedvat, dat dezelfde gelaagde opbouw heeft als een normaal bloedvat (*hoofdstuk 2, 3, en 5*).
De laag aan de binnenkant, de intima, bestaat uit een enkele laag endotheelcellen. Deze nieuwe intima produceert evenveel prostacycline (PGI_2) als de intima van een normaal bloedvat, waardoor bloedstolling voorkomen wordt (*hoofdstuk 2*).
Onder de intima vormt zich het spiergedeelte van de nieuwe vaatwand, de media. Deze bestaat uit meer lagen van gladde spiercellen omgeven door collageen en zelfs, wat vooral bijzonder is, elastine. Interessant is dat alleen kunstbloedvaten gemaakt van een 95% polyurethaan / 5% poly-L-melkzuur mengsel tot elastine vorming door de gehele media heen leiden (*hoofdstuk 3*).
De vorming van elastine wordt bepaald door de combinatie van de elasticiteit en de afbreekbaarheid van de kunstbloedvaten (*hoofdstuk 5*). Doordat de kunstbloedvaten elastisch zijn worden de gladde spiercellen door de pulsaties van de bloedstroom steeds even uitgerekt, en daardoor gestimuleerd tot de vorming van elastine. Doordat de kunstbloedvaten tegelijkertijd ook worden afgebroken, worden ze door de in- en overgroei van vaatwandweefsel niet stugger, maar behouden ze beter hun oorspronkelijke elasticiteit; dit bevordert de elastine vorming.
In de wand van het kunstbloedvat, die geleidelijk door ontstekingscellen wordt afgebroken, vormt zich een omgevende laag, de adventitia. Deze bestaat dus feitelijk uit ontstekingscellen (macrofagen en reuscellen), bindweefsel en capillairen (*hoofdstuk 4*).
Zes weken na implantatie is de wand van het nieuw gevormde bloedvat ongeveer even dik als de wand van de buikslagader van de rat (*hoofdstuk 3 en 5*). In tegenstelling tot het normale

bloedvat, zijn de gladde spiercellen in de nieuwe media nog voornamelijk in de lengterichting van het bloedvat gerangschikt. Deze rangschikking blijkt in de loop van de tijd te kunnen veranderen. Drie maanden na implantatie zijn er al gebieden in de nieuwe media met circulair gerangschikte gladde spiercellen (*hoofdstuk 6*). Een jaar na implantatie bij een optimaal verlopend genezingsproces zijn bijna alle gladde spiercellen circulair gerangschikt (*hoofdstuk 7*). De nieuw gevormde bloedvaten zien er dan op het blote oog normaal uit.
Indien het genezingsproces echter niet optimaal verloopt en de gladde spiercellen niet circulair maar nog steeds in de lengterichting zijn gerangschikt, zijn de bloedvaten na 1 jaar implantatie in meer of mindere mate uitgezet (aneurysmatisch). Dit komt waarschijnlijk doordat de wand van dergelijke bloedvaten gedurende langere tijd minder bestand is om weerstand te kunnen bieden aan de bloeddruk.
Geconcludeerd wordt (*hoofdstuk 8*) dat verder onderzoek met microporeuze, elastische, afbreekbare kunstbloedvaten nodig zal zijn om de circulaire rangschikking van gladde spiercellen te bevorderen opdat alle nieuwgevormde bloedvaten op de lange duur geen afwijkingen vertonen en normaal blijven functioneren.

Colophon

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