

# Early Host Tissue Response to Different Types of Vascular Prostheses Coated with Silver Acetate or Vaporized Metallic Silver

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## WHAT THIS PAPER ADDS

Two major types of silver-coated vascular prostheses with anti-bacterial properties are currently available: Intergard Silver (IS) and Silver Graft (SG). They differ markedly in terms of their silver release kinetics. In the present study, the angiogenic and inflammatory host tissue response to both types of prostheses was analyzed in a chronic in vivo model during the first 14 days after implantation. This is of particular interest, because insufficient early integration of vascular prostheses is considered to contribute to perigraft seroma formation and subsequent infection. It was found that silver acetate-coated IS exhibits an improved vascularization and reduced perigraft inflammation when compared with vaporized metallic silver-coated SG. Accordingly, the risk for this mechanism of infection may be reduced for IS vascular graft implants.

**Objectives:** In vascular surgery, the infection of prosthetic vascular grafts represents a serious life-threatening complication. Due to the increasing resistance of hospital micro-organisms to standard antibiotic therapies, maximum effort should be put in the primary prevention of such infections. For this purpose, grafts may be coated with different antibacterial silver formulations. In the present study the different effects of silver acetate-coating and vaporized metallic silver-coating on the vascularization and perigraft inflammation during the initial phase after implantation of Intergard Silver (IS) and Silver Graft (SG) were compared.

**Methods:** Silver acetate-coated IS and vaporized metallic silver-coated SG were implanted into the dorsal skinfold chamber of C57BL/6 mice ( $n = 8$  per group) to study angiogenesis and leukocyte inflammation at the implantation site by means of repetitive intravital fluorescence microscopy over a 14-day period. At the end of the in vivo experiments, apoptosis and cell proliferation in the newly developed granulation tissue surrounding the implants was analyzed by immunohistochemistry.

**Results:** IS exhibited an improved vascularization, resulting in a significantly higher functional capillary density when compared to SG. Moreover, the leukocyte inflammatory response to IS was less pronounced, as indicated by a reduced number of adherent leukocytes in perigraft venules. This was associated with a higher proliferative activity of the granulation tissue incorporating the IS when compared to SG. The numbers of apoptotic cells in the perigraft tissue were low and did not differ between the two groups.

**Conclusion:** Silver acetate-coated IS exhibits an improved vascularization and reduced perigraft inflammation during the first 14 days after implantation when compared to vaporized metallic silver-coated SG. This may contribute to reducing the risk of early perigraft seroma formation and subsequent infection.

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## INTRODUCTION

A major problem associated with synthetic vascular prostheses is the risk of bacterial infections, mostly sustained by Gram-positive bacteria.<sup>1</sup> Unfortunately, the prevalence of

such infections has not changed much over the past two decades, with continuing high morbidity and mortality rates.<sup>2,3</sup> Moreover, consensus about the optimal treatment of vascular graft infections is still lacking.<sup>4,5</sup> The complex multimorbidity of vascular disease patients with infections makes it difficult to draw valid conclusions from clinical research studies.<sup>6,7</sup>

In the past, many efforts have been made to identify appropriate coatings for vascular prostheses that are refractory to bacterial adhesion.<sup>8–11</sup> As a consequence, two types of silver-coated vascular prostheses have been

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introduced into clinical practice: Intergard Silver (IS; Maquet, Rastatt, Germany) and Silver Graft (SG; B. Braun, Melsungen, Germany). IS is a silver acetate-coated polyester graft that releases substantial amounts of silver into the surrounding tissue within the first 2–3 weeks. Of interest, we have recently demonstrate that this promotes the early vascularization of the grafts without inducing inflammatory side effects at the implantation site.<sup>12</sup> Early vascularization is, in turn, considered as an essential prerequisite for adequate incorporation of prosthetic vascular grafts into the surrounding tissue, reducing the risk of perigraft seroma formation and subsequent infection.<sup>13–15</sup>

In contrast, SG is a vaporized metallic silver-coated polyester graft that only releases marginal amounts of silver in the perigraft tissue during the first 12 months after implantation.<sup>11</sup> Up to now, it is unknown how the type of silver coating affects the early angiogenic and inflammatory host tissue response to the grafts. Accordingly, SG and IS were implanted into the dorsal skinfold chamber of mice to analyze angiogenesis and inflammation of the perigraft tissue using intravital fluorescence microscopic and immunohistochemical techniques.

## MATERIALS AND METHODS

### Animals

For the experiments a total of 16 C57BL/6 mice (12–16 weeks old, 24–26 g; standard inbred laboratory mouse strain from Charles River Laboratories GmbH, Sulzfeld, Germany) were used. C57BL/6 mice have been used by us and other groups in many former dorsal skinfold chamber studies focusing on the interaction of various biomaterials with the surrounding host tissue.<sup>12,16</sup> They were housed one per cage and had free access to tap water and standard pellet food (Altromin, Lage, Germany) throughout the study period.

All experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985). They were approved by the local governmental animal care committee.

### Implantation of vascular grafts into the dorsal skinfold chamber

The mouse dorsal skinfold chamber as a model for in vivo testing of implanted vascular grafts and its preparation has previously been described in detail.<sup>12,16</sup> In the present study, the two silver-coated, clinically used synthetic vascular prostheses IS and SG, which were kindly provided by Maquet and B. Braun, were implanted. SG is a warp-knitted, double-velour vascular prosthesis made of polyester (polyethylene terephthalate) that is impregnated with modified bovine gelatin (polygelin). IS is a knitted polyester prosthesis that is impregnated with collagen. Both impregnations prevent the passage of blood components into the surrounding tissue. Accordingly, the water permeability of the prostheses is  $\leq 5 \text{ mL} \times \text{cm}^{-2} \times \text{min}^{-1} / 120 \text{ mmHg}$ . IS

contains silver acetate in the collagen layer and in the graft material itself, which leads to substantial silver release during the first 2–3 weeks after implantation followed by a sustained release thereafter. In contrast, SG retains ~80% of its vaporized metallic silver coating during the first 12 months. Thus, effective silver release within the first 14 days after implantation does not occur.<sup>11</sup> The amount of silver coated on the two prostheses is comparable (IS 0.06–0.18 mg/cm<sup>2</sup> and SG 0.07–0.16 mg/cm<sup>2</sup>).

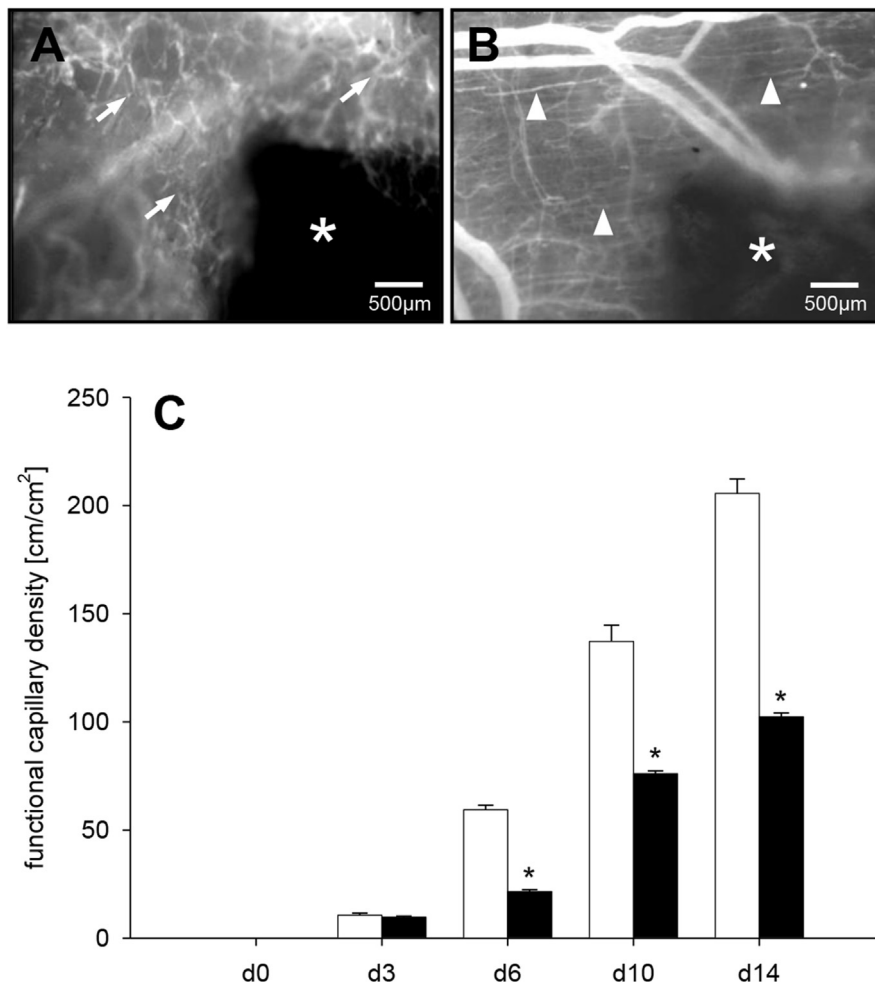
The vascular prostheses were implanted 48 hours after preparation of the dorsal skinfold chamber under laboratory conditions in an operating theatre for small animals at the Institute for Clinical and Experimental Surgery, University of Saarland. All implantations were performed by the same scientist. For this purpose, the mice were anesthetized by an intraperitoneal injection of 75 mg/kg ketamine hydrochloride (Ketavet; Parke Davis, Freiburg, Germany) and 25 mg/kg dihydroxyidinothiazine hydrochloride (Rompun; Bayer, Leverkusen, Germany) and fixed in the right lateral decubital position on a Plexiglas stage. The coverslip of the chamber was temporarily removed and 2 mm<sup>2</sup> piece of either IS (weight:  $1.48 \pm 0.04 \text{ mg}$ ) or SG ( $1.94 \pm 0.05 \text{ mg}$ ) was carefully placed with the exterior side onto the striated muscle tissue within the center of each chamber, taking care to avoid contamination, mechanical irritation, or damage to the chamber preparation.

### Intravital fluorescence microscopy and microcirculatory analysis

For intravital fluorescence microscopy, the anesthetized mice received an intravenous injection of 0.05 mL of 5% fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight: 150,000 Da; Sigma-Aldrich, Taufkirchen, Germany) for the visualization of the intravascular space and 0.05 mL 1% rhodamine 6G (Sigma-Aldrich) for the in vivo staining of leukocytes in postcapillary and collecting venules at the implantation site. Subsequently, the mice were positioned under a Zeiss Axiotech microscope (Zeiss; Oberkochen, Germany) equipped with a camera and DVD recording system, as previously described in detail.<sup>12</sup>

The microscopic images were analyzed quantitatively by means of the computer-assisted off-line analysis system CapImage (Zeintl, Heidelberg, Germany). Angiogenesis was analyzed in eight different microvascular regions of interest (ROIs; size 0.4 mm<sup>2</sup>), which were directly located at the interface between the prosthetic vascular grafts and the surrounding host tissue. The extent of vascular ingrowth into the different ROIs was assessed by the quantitative measurement of the functional capillary density, i.e. the length of blood-perfused capillaries per observation area and is given in cm/cm<sup>2</sup>.<sup>17</sup>

In four different ROIs in the border zone of the prosthetic vascular grafts, that is in close vicinity to the implanted material, we additionally measured leukocyte–endothelial cell interaction and microhemodynamics in postcapillary and collecting venules of the host tissue. For this purpose, rhodamine 6G-stained leukocytes were classified according



**Figure 1.** (A, B) Intravital fluorescent microscopic images at the border of an Intergard Silver (IS) (A, asterisk) and a Silver Graft (SG) implant (B, asterisk) at day 14 after implantation into the dorsal skinfold chamber of C57BL/6 mice. Note the high density of newly formed microvessels at the border of IS (A, arrows), overgrowing the striated muscle capillaries of the host tissue. SG shows only few newly formed microvessels without overgrowth of the host microvasculature (B). Thus, the parallel arranged autochthonous muscle capillaries can still be detected (arrowheads, B). Blue light epi-illumination with contrast enhancement by 5% FITC-labeled dextran 150,000 i.v. (C) Functional capillary density (cm/cm<sup>2</sup>) within the border of IS (white bars;  $n = 8$ ) and SG implants (black bars;  $n = 8$ ), as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means  $\pm$  SEM. \* $p < .05$  versus IS implants.

to their interaction with the vascular endothelium as adherent, rolling, or free-flowing cells.<sup>18</sup> Adherent leukocytes were defined in each vessel segment as cells that did not move or detach from the endothelial lining within a specified observation period of 20 seconds, and are given as number of cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment studied, assuming a cylindrical vessel geometry. Rolling leukocytes were defined as cells moving with a velocity less than two-fifths of the centerline velocity, and are given as number of cells per minute, passing a reference point within the microvessel.<sup>19</sup>

Diameters, centerline red blood cell (RBC) velocity, volumetric blood flow, and wall shear rate were determined in those venules, in which leukocyte–endothelial cell interaction was analyzed. Diameters ( $d$ ) were measured in micrometers perpendicularly to the vessel path. Centerline velocity ( $v$ ) was analyzed by the computer-assisted image

analysis system using the line shift method.<sup>20</sup> Volumetric blood flow was calculated by  $Q = \pi \times (d/2)^2 \times v/1.6$  [pL/s], where 1.6 represents the Baker–Wayland factor to correct for the parabolic velocity profile in microvessels.<sup>21</sup> Moreover, wall shear rate ( $\gamma$ ) was calculated based on the Newtonian definition:  $\gamma = 8 \times v/d$  (given per second).

#### Experimental protocol

Samples of IS ( $n = 8$ ) and SG ( $n = 8$ ) were implanted into the dorsal skinfold chamber of 16 C57BL/6 mice. Intravital fluorescence microscopic analyses of angiogenesis, leukocyte–endothelial cell interaction, and microhemodynamics were performed immediately as well as 3, 6, 10, and 14 days after implantation. At the end of the in vivo experiments, the animals were sacrificed with an overdose of the anesthetics, and the dorsal skinfold chamber preparations were carefully excised for further immunohistochemical analyses.

**Table 1.** Diameter ( $\mu\text{m}$ ), centerline red blood cell velocity ( $\mu\text{m/s}$ ), volumetric blood flow ( $\mu\text{L/s}$ ) and wall shear rate ( $\text{s}^{-1}$ ) of postcapillary and collecting venules within the border zones of IS ( $n = 8$ ) and SG implants ( $n = 8$ ) immediately (d0) as well as 3, 6, 10, and 14 days after implantation into dorsal skinfold chamber of C57BL/6 mice. Means  $\pm$  SEM.

	d0	d3	d6	d10	d14
Diameter ( $\mu\text{m}$ )					
IS	28.1 $\pm$ 1.4	30.1 $\pm$ 2.0	27.2 $\pm$ 1.5	27.8 $\pm$ 1.6	24.5 $\pm$ 1.1
SG	24.4 $\pm$ 0.7	23.1 $\pm$ 0.5	23.3 $\pm$ 1.1	23.2 $\pm$ 1.2	25.4 $\pm$ 1.0
Centerline RBC velocity ( $\mu\text{m/s}$ )					
IS	174.1 $\pm$ 15.4	259.4 $\pm$ 5.2	272.0 $\pm$ 15.9	336.6 $\pm$ 20.1	323.5 $\pm$ 19.9
SG	108.6 $\pm$ 10.5	233.5 $\pm$ 16.8	262.9 $\pm$ 16.1	312.8 $\pm$ 19.6	315.3 $\pm$ 30.3
Volumetric blood flow ( $\mu\text{L/s}$ )					
IS	71.6 $\pm$ 18.7	115.2 $\pm$ 14.0	85.9 $\pm$ 10.9	103.6 $\pm$ 17.0	84.5 $\pm$ 6.8
SG	32.4 $\pm$ 3.1	63.1 $\pm$ 4.8	76.1 $\pm$ 11.8	85.8 $\pm$ 9.8	113.7 $\pm$ 6.1
Shear rate ( $\text{s}^{-1}$ )					
IS	61.3 $\pm$ 6.8	81.0 $\pm$ 7.7	79.8 $\pm$ 7.1	94.0 $\pm$ 7.3	89.0 $\pm$ 4.9
SG	36.4 $\pm$ 4.5	81.9 $\pm$ 6.3	91.2 $\pm$ 4.6	110.8 $\pm$ 10.4	98.1 $\pm$ 11.4

### Immunohistochemistry

At day 14 after the implantation of prosthetic vascular grafts, formalin-fixed specimens of the dorsal skinfold preparations were embedded in paraffin and 3- $\mu\text{m}$ -thick sections were cut. For immunohistochemical detection of apoptotic cells, cleaved caspase-3 (casp-3) staining was performed using a rabbit polyclonal anti-casp-3 antibody (1:100; New England Biolabs, Frankfurt, Germany) followed by a biotin-conjugated goat anti-rabbit antibody (abcam; Cambridge, UK) and streptavidin peroxidase. Proliferating cells were detected by a mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (1:100; Dako GmbH, Hamburg, Germany) as primary antibody followed by a horseradish peroxidase-conjugated goat anti-mouse antibody (1:100; Dianova, Hamburg, Germany). 3,3'-Diaminobenzidine was the chromogen used. The sections were counter-stained with hemalaun and examined by light microscopy (BX60; Olympus, Hamburg, Germany). Casp-3- and PCNA-positive cells were assessed in nine high power fields (HPFs) per section and are given as percentage of the total number of cells per HPF.

### Statistics

After testing the data for normal distribution and equal variance, differences between the two groups were analyzed by an unpaired Student *t* test (SigmaStat; Jandel Corporation, San Rafael, CA, USA). All values are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical significance was accepted for a  $p < 0.05$ .

## RESULTS

### Vascularization of prosthetic vascular grafts

The two types of synthetic vascular grafts induced an angiogenic host tissue response in the dorsal skinfold chamber. Microvascular sprout formation originating from host capillaries and venules in the border zones of the grafts could already be observed 3 days after implantation. During the following days, the sprouts progressively grew into the surrounding of the grafts' surface to finally form new

microvascular networks (Fig. 1A, B). Of interest, angiogenesis and perigraft vascularization were markedly pronounced in IS grafts, which exhibited a significantly higher functional capillary density in the border zones at days 6, 10, and 14 than the SG (Fig. 1C).

### Microhemodynamics at the implantation site

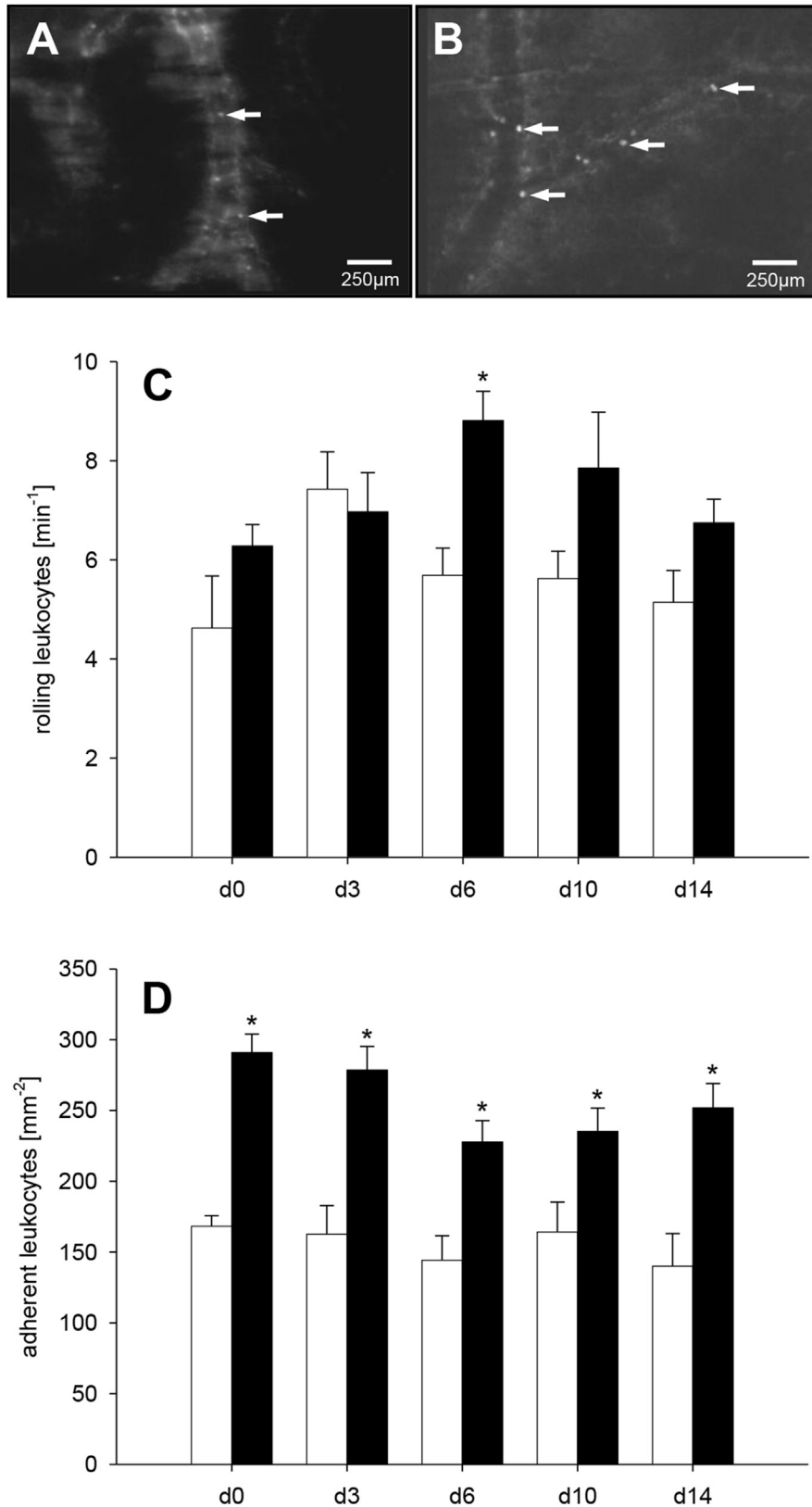
Venules in the direct vicinity of the vascular grafts exhibited a diameter of 23–30  $\mu\text{m}$  (Table 1). The RBC velocity within these vessels was between 109 and 323  $\mu\text{m/s}$  throughout the observation period. Accordingly, calculated values of volumetric blood flow and wall shear rate were 32–115  $\mu\text{L/s}$  and 36–111 per second (Table 1). There were no significant differences between the two experimental groups, indicating comparable microhemodynamic conditions for the analysis of leukocyte–endothelial cell interaction as a parameter for the inflammatory response to the implants.

### Leukocyte–endothelial cell interaction at the implantation site

During the observation period of 14 days, the numbers of rolling leukocytes in postcapillary and collecting venules ranged between 4.8 and 8.9  $\text{min}^{-1}$  without significant differences between the IS and the SG group (Fig. 2A–C). However, the numbers of adherent leukocytes were significantly higher in SG implants than IS grafts, indicating a stronger inflammatory activation of leukocytes in the perigraft host tissue (Fig. 2D).

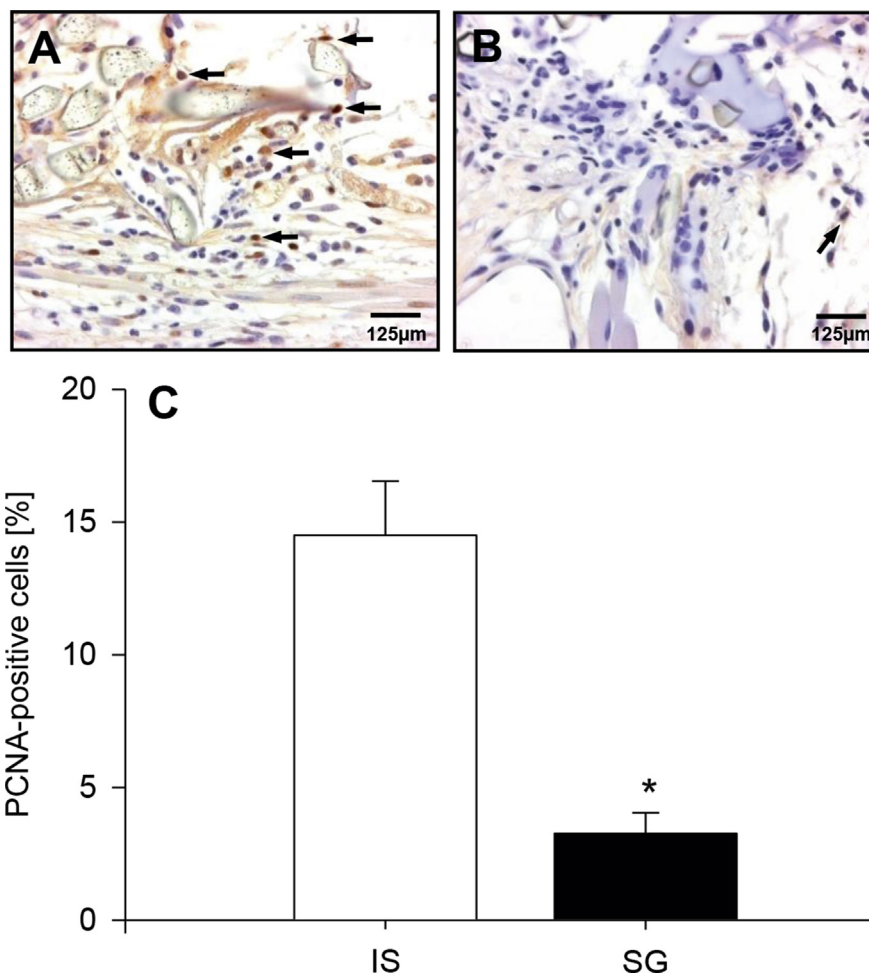
### Cell proliferation and apoptosis in the perigraft tissue

After the observation period of 14 days, immunohistochemical detection of PCNA showed a significantly higher number of proliferating cells in the granulation tissue surrounding the IS grafts compared to that of SG implants (Fig. 3). In contrast, the numbers of apoptotic cells in the perigraft tissue were low without marked differences between the two groups (Fig. 4).



**Figure 2.** (A, B) Intravital fluorescent microscopic images of collecting venules in the direct vicinity of Intergard Silver (IS) (A) and Silver Graft (SG) implants (B) at day 14 after implantation into the dorsal skinfold chamber of C57BL/6 mice. Green light epi-illumination for the detection of adherent rhodamine 6G-stained leukocytes (arrows). (C, D) Rolling leukocytes (C,  $\text{min}^{-1}$ ) and adherent leukocytes (D,  $\text{mm}^{-2}$ ) in postcapillary and collecting venules within the border zones of IS (white bars;  $n = 8$ ) and SG implants (black bars;  $n = 8$ ), as assessed by intravital fluorescence microscopy and computer-assisted image analysis. Means  $\pm$  SEM. \* $p < .05$  versus IS implants.





**Figure 3.** (A, B) Immunohistochemical cross sections of the border zones of an Intergard Silver (IS) (A) and a Silver Graft (SG) implant (B) at day 14 after implantation onto the striated muscle tissue within the dorsal skinfold chamber of C57BL/6 mice. The sections were stained with an antibody against PCNA for the visualization of proliferating cells (arrows). C: PCNA-positive cells (%) in the border zones of IS (white bars;  $n = 8$ ) and SG implants (black bars;  $n = 8$ ) at day 14 after implantation. Means  $\pm$  SEM. \* $p < .05$  versus IS implants.

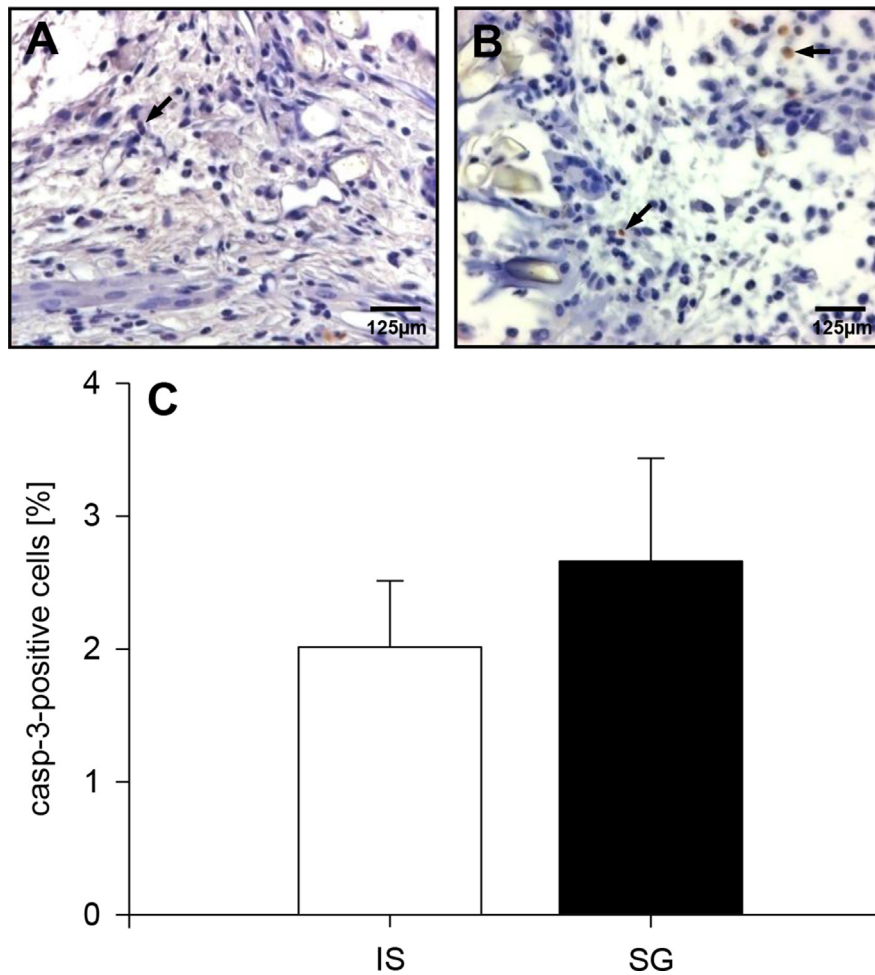
### Discussion

According to the data of Moran et al.<sup>22</sup> and Ali et al.,<sup>23</sup> infection rates of autologous venous and arterial grafts are generally low. In contrast, the implantation of synthetic vascular prostheses is associated with a relative high risk of infections with devastating complications, including anastomotic disruption with massive hemorrhage and pseudoaneurysm formation, graft thrombosis, limb loss, and high perioperative and late mortality.<sup>24,25</sup> For the prevention of such infections, the antibacterial silver-coated vascular grafts IS and SG have been introduced into clinical practice, which markedly differ in terms of their silver-release rates.<sup>26,27</sup> Silver acetate-coated IS rapidly releases silver into the surrounding tissue within the first 2–3 weeks. Thus, these prostheses exhibit a time limited antimicrobial efficacy.<sup>28,29</sup> SG is coated with vaporized metallic silver that is only released to a negligible extent during the first 12 months after implantation.<sup>11</sup> Accordingly, the antibacterial efficacy of its surface remains intact much longer, but the graft is less protective in the surrounding tissue against bacterial infections.<sup>11,30</sup> In the present study,

evidence is provided for the first time that the differences between the two vascular prostheses crucially determine the early angiogenic and inflammatory host tissue response.

The aim of the present study was to compare two silver-coated prostheses with different release kinetics. For this purpose it would have been much better to use two prostheses from the identical manufacturer produced in an identical manner, which solely differ in their silver release kinetics. However, unfortunately this was not possible, because such prostheses are not available on the market. Accordingly, two silver-coated prostheses from different manufacturers, which are currently widely used in clinical practice were compared.

In a previous study it has already been demonstrated that silver acetate-coated IS exhibits improved vascularization compared with uncoated grafts,<sup>12</sup> which may be caused by direct pro-angiogenic silver effects, such as the stimulation of cellular vascular endothelial growth factor production and reactive oxygen species formation.<sup>12,31</sup> Besides the well-known antibacterial properties of silver,<sup>32</sup> this observation may further contribute to the prevention of infections by an improved early graft incorporation into the



**Figure 4.** (A, B) Immunohistochemical cross sections of the border zones of an Intergard Silver (IS) (A) and a Silver Graft (SG) implant (B) at day 14 after implantation onto the striated muscle tissue within the dorsal skinfold chamber of C57BL/6 mice. The sections were stained with an antibody against casp-3 for the visualization of apoptotic cells (arrows). (C) Casp-3-positive cells (%) in the border zones of IS (white bars;  $n = 8$ ) and SG implants (black bars;  $n = 8$ ) at day 14 after implantation. Means  $\pm$  SEM.

host tissue.<sup>13,33</sup> However, these novel data show that this is not the case for vaporized metallic silver-coated SG. In fact, in the dorsal skinfold chamber model, SG exhibited a significantly reduced vascularization during the first 14 days after implantation when compared to IS. Accordingly, a markedly decreased number of PCNA-positive cells in the newly developing granulation tissue surrounding the SG grafts was also observed. This indicates that the extent of vascularization directly correlates with the incorporation of the grafts at the implantation site. Further studies are required to clarify whether this is also the case after longer observation periods.

In addition, it was found that throughout the observation period of 14 days there were an increased number of adherent leukocytes in postcapillary and collecting venules in the direct vicinity of SG when compared to IS. Because the analyzed microvessels of both experimental groups exhibited a comparable diameter, centerline RBC velocity, volumetric blood flow, and wall shear rate, this increased leukocyte–endothelial cell interaction may not be attributed to different microhemodynamic conditions, but is due to a more pronounced leukocyte inflammatory reaction.

Therefore, it may be concluded that despite its very low release rate the vaporized metallic silver of SG activates pro-inflammatory mechanisms at the implantation site. Nonetheless, SG grafts have still to be considered as biocompatible, because the number of adherent leukocytes was constantly  $<300/\text{mm}^2$ , which is far below the typical values of adherent leukocytes in severely inflamed dorsal skinfold chambers ( $>700/\text{mm}^2$ ).<sup>17,34,35</sup> Moreover, both experimental groups presented with low numbers of casp-3-positive apoptotic cells in the perigraft granulation tissue, further indicating the lack of direct cytotoxic effects of the different silver coatings of IS and SG.

## CONCLUSION

Insufficient integration of a vascular prosthesis due to inadequate neovascularization may contribute to perigraft seroma formation and subsequent infection.<sup>13–15</sup> The present study demonstrates that silver acetate-coated IS exhibits an improved vascularization and reduced perigraft inflammation during the first 14 days after implantation when compared to vaporized metallic silver-coated SG.

Accordingly, the risk for this mechanism of infection may be reduced in case of IS vascular graft implants.

## FUNDING

None.

## CONFLICT OF INTEREST

For the present study, the vascular prostheses IS and SG were kindly provided without charge by Maquet and B. Braun. In October 2012 the corresponding author Mohammed R. Moussavian was invited by Maquet to a Maquet symposium of the Congress of the Deutsche Gesellschaft für Gefäßchirurgie in Wiesbaden, Germany. He was compensated by Maquet for his service as a speaker.

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