Attachment of Flexible Heparin Chains to Gelatin Scaffolds Improves Endothelial Cell Infiltration

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Long-term survival of implanted cells requires oxygen and nutrients, the need for which is met by vascularization of the implant. The use of scaffolds with surface-attached heparin as anchoring points for angiogenic growth factors has been reported to improve this process. We examined the potential role of surface modification of gelatin scaffolds in promoting endothelial cell infiltration by using a unique macromolecular conjugate of heparin as a coating. Compared to other heparin coatings, this surface modification provides flexible heparin chains, representing a new concept in heparin conjugation. *In vitro* cell infiltration of scaffolds was assessed using a three-dimensional model in which the novel heparin surface, without growth factors, showed a 2.5-fold increase in the number of infiltrating endothelial cells when compared to control scaffolds. No additional improvement was achieved by adding growth factors (vascular endothelial growth factor and/or fibroblast growth factor-2) to the scaffold. *In vivo* experiments confirmed these results and also showed that the addition of angiogenic growth factors did not significantly increase the endothelial cell infiltration but increased the number of inflammatory cells in the implanted scaffolds. The endothelial cell-stimulating ability of the heparin surface alone, combined with its growth factor-binding capacity, renders it an interesting candidate surface treatment to create a prevascularized site prepared for implantation of cells and tissues, in particular those sensitive to inflammation but in need of supportive revascularization, such as pancreatic islets of Langerhans.

Introduction

IN ORGAN AND TISSUE ENGINEERING, for example, the generation of bone, cartilage, artificial kidneys, and insulin-producing organs, a key factor for success is providing cells with oxygen and nutrients after implantation. Thus, rapid vascularization at the site of tissue transplantation or regeneration is of importance. Endothelial cell infiltration is a prerequisite for this vascularization. In addition to fulfilling these requirements, biomaterial scaffolds^{1,2} provide three-dimensional support for the cells and often facilitate the engraftment of cells into the implantation tissue.

Inflammation has been suggested as the main driving force for vascularization of implanted scaffolds.³ However, the inflammatory processes may be hazardous for certain cell types. For instance, the delicate beta cells of pancreatic islets can be damaged by the presence of cytokines such as IL-1 β , TNF- α , and IFN- γ , which can all be generated by material-induced inflammation.⁴ Thus, when such cell types are involved, it is important to keep the inflammatory process at a low level while still promoting vascularization.

Vascularization can be promoted by anchoring angiogenic growth factors to supporting scaffolds.² One strategy is to

use immobilized heparin, which, in addition to being anti-inflammatory,⁵ can provide both an endothelial cell-stimulating and migration-promoting ability⁶ and contains binding motifs for regulatory proteins such as angiogenic growth factors. The growth factors vascular endothelial growth factor (VEGF)-A and fibroblast growth factor (FGF)-2 are well known to stimulate endothelial cell infiltration,^{7,8} and binding of these growth factors to a biomaterial surface promotes endothelial cell infiltration in implanted scaffolds.^{2,9,10}

The aim of the present study was primarily to create a prevascularized site as a model, for example, for islet transplantation by using a biomaterial that promotes endothelial cell infiltration while keeping inflammation low. We therefore examined the role of a heparin surface novel to tissue engineering as a material surface modification of a gelatin foam-based biomaterial scaffold^{5,11} and investigated the ability of this surface to support endothelial cell migration and proliferation.

The structure of this heparin surface differs from those produced by more random cross-linking techniques, such as the commonly employed N-hydroxysuccinimide (NHS)-1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) immobilization, in that it consists of dual layers of flexible macromolecular heparin complexes, each ~1000 kDa in size.

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The individual conjugate is made up of ~70 heparin molecules, each end point-attached by selective covalent binding to an extended polyamine core. This structure produces a thick and flexible layer of heparin molecules protruding into the fluid phase and exposing accessible anti-thrombin binding sites^{12,13} (Fig. 1). This heparin surface reduces the activation of both coagulation and complement,^{5,11,14–16} thereby potentially reducing inflammation in implanted scaffolds. Thus, heparin also needs to be flexible, making it necessary to ensure that the immobilized heparin is linked to its substrate through a single point of attachment. It has been shown that if heparin is linked by multiple bonds, its biological activity is lost, and a coating involving such heparin is quite thrombogenic.¹⁷

In previous reports, heparinized scaffolds without growth factors have shown, at best, a moderate effect on the endothelial cell infiltration in implants.² The ability of the present heparin surface to improve endothelial cell infiltration in a biomaterial scaffold, with and without the addition of angiogenic growth factors, was assessed *in vitro* as well as *in vivo*. We set up an *in vitro* model to determine to what extent the heparin surface could promote endothelial cell infiltration in the absence or presence of growth factors. *In vivo* experiments were performed to investigate whether the heparin-coated scaffolds, alone or preloaded with growth factors, would trigger significant endothelial cell infiltration without elevating the levels of infiltrating inflammatory cells.

Our results indicate that the heparinized scaffold works as a climbing frame and promotes the ingrowth of endothelial cells both *in vitro* and *in vivo*, and this effect can potentially be improved by adding growth factors, despite the concomitant generation of increased inflammation.

Materials and Methods

Scanning electron microscopy of the gelatin scaffolds

The structure of the gelatin scaffolds (Spongostan[®] Dental; Ferrosan Medical Devices A/S, Søborg, Denmark) was examined by scanning electron microscopy (SEM). In brief, dry scaffold samples were mounted and coated with gold and palladium using a Polaron SC7640 Sputter Coater. Images were then taken with a LEO 1550 Gemini Field Emission high-resolution scanning electron microscope at different magnifications.

Heparinization of the gelatin scaffolds

Scaffolds (with sizes for designated experiments specified below) were incubated with the polymeric amine polyallylamine (0.25 mg/mL; Corline Systems AB, Uppsala, Sweden) dissolved in sodium borate buffer (250 mM, pH 9) for 15 min at room temperature (RT) and washed three times in milli-Q-quality water (mQ). The pieces were then incubated with a solution of a macromolecular conjugate of heparin (Corline Heparin Conjugate, 0.1 mg/mL; Corline Systems AB) in Na acetate buffer (0.1 M, 0.5 M NaCl, pH 4), for 60 min at RT and rinsed three times in mQ. These two initial steps were repeated once. The pieces were then incubated in Na borate buffer (250 mM, pH 9), for 15 min at RT to remove excess conjugate. Finally, the pieces were incubated in acetic anhydride solution (0.1% in 250 mM Na borate buffer, pH 10.5), for 10 min to block residual amino groups. The pieces were rinsed four times in mQ and stored in phosphate-buffered saline (PBS) at 4°C until use. The amount of heparin bound was determined by the toluidine blue assay as previously described.¹⁸

Heparinization of the scaffolds was visualized by confocal microscopy after incubating scaffolds at RT for 15 min with antithrombin (1 U/mL) labeled with AlexaFluor 488. Image acquisition was performed with a Zeiss LSM 510 Meta (Carl Zeiss, Jena, Germany). Z-stacks were acquired using a 488-nm laser. Blue autofluorescence of the scaffolds was achieved by overexposure of the 405-nm laser. Three-dimensional projections of the acquired z-stacks were analyzed using Imaris software (Bitplane, Zurich, Switzerland). The effect of the heparinization procedure on the scaffolds was also assessed by comparing scaffolds in microscopy pictures taken before and after heparinization of ten scaffolds.

Heparin was detected with avidin (Pierce Biotechnology, Rockford, IL) conjugated with Texas Red (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Scaffolds (control and heparinized) transplanted under the kidney capsule were excised after 1 day *in vivo*, and cryosections were incubated with avidin-Texas Red diluted in TNE blocking buffer (Perkin Elmer, Waltham, MA) for 1 h at 4°C. The sections were analyzed by fluorescence microscopy (Nikon Elipse E600; Nikon, Tokyo, Japan).

FIG. 1. Comparison of heparin-coating techniques. **(A)** With 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) N-hydroxysuccinimide (NHS) immobilization, the heparin chains are covalently bound to each other and to the surface via multiple randomly distributed bonds. **(B)** In the new approach described herein, macromolecular heparin complexes are bound to the surface by charge. The individual heparin chains (red short lines) are end-point-attached to the polyamine backbone (black lines). Color images available online at www.liebertpub.com/tea

Growth factor loading of scaffolds

After heparinization, scaffolds were incubated in 7 µg/mL VEGF-A (rmVEGF-A₁₆₄; PeproTech EC Ltd., London, United Kingdom), 7 µg/mL FGF-2 (rmFGF-2; PeproTech EC Ltd.), or 3.5 µg/mL VEGF-A and 3.5 µg/mL FGF-2, in 0.1% bovine serum albumin in 100 µL of PBS for 60 min at RT. The scaffolds were then rinsed in PBS three times, with repeated compressions to remove all unbound growth factor. This was the standard growth factor loading procedure, used in all experiments in which scaffolds loaded with growth factor were used.

In vitro cell infiltration of scaffolds

The mouse MS1 pancreatic islet endothelial cell line (a kind gift from Dr. Jack L. Arbiser, Children's Hospital, Harvard Medical School, Boston, MA) was grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco) and 100 µg/mL penicillin/streptomycin. MS1 cells were seeded at a concentration of 4000–4500 cells/well in a 24-well plate. In parallel, human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HDMEC) were cultured at 15,000–20,000 cells/well in a 24-well plate in endothelial cell culture medium (ECGM MV; PromoCell, Heidelberg, Germany). After 24 h of culture, scaffolds (control, heparinized, or heparinized and preloaded with growth factors; $n=6$ for each group) of $\sim 5 \times 5 \times 5$ mm in size were added to the wells, and the medium was changed to starvation medium (0.5% FCS). The scaffold density was 13 mg/cm³, which required lightly weighing the scaffolds down to ensure contact with the cells. The scaffolds were lightly compressed when adding them to the cell cultures. The cells were incubated with scaffolds for 3 days, and then the scaffolds were removed, rinsed briefly in PBS, and further processed for cell quantification. The thymidine incorporation was performed as described earlier.⁶ Briefly, 4000 MS1 cells/24-well were cultured in starvation medium during 24–48 h. Then, 30 ng/mL of rmVEGF-A was added to the starvation cultures and 16 h later 1 µCi/mL of ³H-thymidine was added to the cultures during additional 4 h. Triplicate samples were then analyzed by scintillation counting.

Scaffolds with MS1 cells for confocal analysis was prepared as described above, except that after 3 days of incubation, the scaffolds with cells were fixed for 10 min by the addition of 4% paraformaldehyde (PFA) 1:1 to the culture medium at RT and then further fixed in 1% PFA at 4°C for 2 days. Thereafter, the scaffolds were washed in PBS and permeabilized with 0.1% Triton X-100/PBS for 15 min. The cytoskeleton of the cells was then stained with phalloidin–Texas Red (dilution 1:200; Molecular Probes #T7471) overnight at 4°C. Nuclei were stained with DAPI (10 µg/mL), and the scaffolds were then processed for confocal analysis. Z-stack image acquisition was performed with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss). The gelatin scaffolds were visualized by green autofluorescence utilizing the 488-nm laser and the range depth for the confocal analyses was ~ 100 µm.

Cell quantification with the PicoGreen assay

Scaffolds cultured with MS1 cells were immersed in 180 µL ATL buffer (Qiagen, Hilden, Germany); 20 µL of

proteinase K (Qiagen) was then added, and the mixture was incubated at 56°C for 60 min with agitation. The resulting lysate was diluted 1:100 in Tris-EDTA (TE) buffer, and 100 µL was added to a black 96-well plate. PicoGreen (100 µL; Invitrogen, Stockholm, Sweden), diluted 1:200 in TE buffer, was added to each well. The plate was incubated for 3 min in the dark and then analyzed in a fluorometer (485 nm excitation, 520 nm emission). The amount of DNA present in the scaffolds was analyzed against a DNA standard curve (0, 0.25, 2.5, 10, 100, and 1000 µg) according to the manufacturer's instructions. The weight of DNA in a single cell was approximated as follows: The total number of base pairs in a diploid male mouse cell, from which the MS1 cell line was derived, was calculated using data from the sequenced mouse genome, available at NCBI (www.ncbi.nlm.nih.gov/genome/?term=mus%20musculus). The average weight of a base pair was calculated to be 615.88 g/mol. Disregarding the %GC content of the genome, which at a theoretical maximum can only vary by 0.16% in molecular weight, the base pairs of the diploid genome were multiplied by 615.88 and divided by Avogadro's number to give the weight of the genome of a single mouse cell as 5.42 pg.¹⁹

Animals

Female C57BL/6/Bkl mice were obtained from Scanbur BK (Sollentuna, Sweden) and maintained in the animal facilities at the Rudbeck Laboratory, Uppsala University. Animals were matched for age within each experiment. All experiments were approved by the local ethics committee (No. 255/7).

Scaffold implantation and explantation

Gelatin scaffolds, $\sim 3 \times 0.8 \times 0.8$ mm in size, were implanted underneath the kidney capsule of isoflurane-anesthetized mice ($n=5-9$) using a Dialamatic microdispenser (10 µL; Drummond Scientific Co., Broomall, PA). Untreated, heparinized, and heparinized scaffolds preincubated with FGF-2 and VEGF-A were implanted. The mice were sacrificed, and the scaffolds were explanted after 1, 3, or 7 days.

Preparation of tissue for immunohistochemistry

After explantation, mouse kidneys were fixed in 1% PFA at 4°C overnight and cut in half through the center of the graft. One half of the kidney was placed in 70% ethanol until paraffin-embedded and cut into 6-µm sections. The other half was placed in PBS at 4°C overnight, then in 20% sucrose/PBS overnight, and finally stored at -70°C; cryosections (6 or 40 µm) were cut from the kidney biopsies after mounting in optimal cutting temperature (O.C.T.) medium (Tissue-Tech; Sakura Finetek, Zoeterwoude, Netherlands).

Immunostaining

Cryosections of grafts were stained for granulocytes (Gr-1 dilution 1:100, eBioscience #14-5931, San Diego, CA, USA) endothelial cells (CD31 dilution 1:500, Becton Dickinson [BD] Biosciences #550389, Franklin Lakes, NJ) and vascular endothelial-cadherin (VE-cadherin dilution 1:100, Santa Cruz Biotechnology #sc-6458, Santa Cruz, CA), activated phagocytes (CD11b dilution 1:500; Serotec #MCA711G, Oxford, United Kingdom), T cells (CD90.2 dilution 1:100; BD

Biosciences #550543) and FITC-conjugated α -smooth muscle actin (ASMA, dilution 1:1000; Sigma-Aldrich #F3777, St. Louis, MO). Slides were permeabilized for 15 min in 0.3% Triton X-100 in PBS and then stained in a DAKO Autostainer according to the following protocol: 30 min of blocking (TNE blocking buffer; Perkin Elmer), 60 min incubation with primary antibody diluted in TNE blocking buffer, followed by 30 min of Alexa Fluor 568 or Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) diluted 1:500 in TNE blocking buffer, and 10 min in DAPI (10 μ g/mL). Rinsing was carried out between all steps. Stained sections were mounted with Fluoromount G (SouthernBiotech, Birmingham AL) and stored in the dark at 4°C until analyzed. Paraffin sections of kidney biopsies were deparaffinized and stained with hematoxylin and eosin (H&E) and then digitally scanned (ScanScope Aperio, Vista, CA) and visually examined using the ImageScope software from Aperio, freely downloadable at www.aperio.com/download-imagescope-viewer.asp.

Blinded evaluation of endothelial cell infiltration of scaffolds *in vivo*

Blinded visual evaluation of grafts explanted on day 1, 3, or 7 ($n=5$) and stained for endothelial cells with anti-CD31

was performed separately by three investigators to confirm the quantification. The grafts were scored from 0–3 according to the number of DAPI-positive cells (“nuclei” score). The CD31-positive cells in the grafts were scored from 0–3 according to the amount, length, and thickness of the formed vessels (“length & thickness” score). The branching of vessels and the amount and size of the formed lumen (“maturation” score) were scored from 0–3. The category “nuclei” was based on one scoring criterion; “length & thickness” and “maturation” were both based on three scoring criteria. The mean score values of the three investigators for the grafts on day 7 *in vivo* are presented.

Microscopy and cell marker quantification

Microscopy was performed on the paraffin sections and the cryosectioned samples (Nikon Elipse E600, Nikon TS100; Nikon, Zeiss LSM 510 Meta, and Zeiss AxioImager M2; Carl Zeiss). All pictures were modified for clarity (linear corrections). To quantify each specific cell type, the total area of the fluorescence of the respective marker was measured and divided by the total area of the graft, that is, the area above the kidney parenchyma and below the kidney capsule. In Figure 6A, C and D the border of the graft (region of interest [ROI]) is marked by a broken line toward the kidney parenchyma and by arrowheads to mark the

FIG. 2. Scanning electron micrograph images of (A–C) a cross section of the gelatin scaffold at different magnifications, and (D) confocal microscopy image of a heparinized gelatin scaffold, as visualized by binding of antithrombin-labeled with Alexa Fluor 488. In panel (D), scaffold autofluorescence exposed by sectioning appears blue, and anti-thrombin is green. White bars in the top right corner of each panel correspond to 100 μ m. (E) A control nonheparinized scaffold on day 1 *in vivo*, stained with avidin–Texas Red, shows only nonspecific binding to the kidney capsule and parenchyma. The scaffolds are located between the kidney capsule, seen as a thin layer of cells, and the kidney parenchyma. The border between the transplanted scaffold and the kidney parenchyma is marked with white dashed lines. The heparin surface in heparinized scaffolds on day 1 *in vivo* after transplantation is stained with avidin–Texas Red (F). Scale bars = 100 μ m.

kidney capsule. Adobe Photoshop CS3 v10.0.1 was used for all image processing.

To further address the vascular morphology in the 7-day *in vivo* scaffolds ($n=9$), the images were analyzed accordingly: The ROI for all images was done in Fiji image pro-

cessing software (<http://fiji.sc/>) using size-calibrated images ($0.79 \times 0.79 \mu\text{m}$). The ROI measurement gave the total area within the ROI. Next, the images were split from RGB in to red, green, and blue single images. Using the ROI on all the blue images (DAPI-stained nuclei), the amount of nuclei

FIG. 3. *In vitro* infiltration of MS1 mouse endothelial cells into gelatin scaffolds, as measured by total DNA content and confocal image evaluation. Schematic figure of endothelial cells (red) growing into the gelatin scaffold drawn in blue (**A**). Infiltration of endothelial cells (amount of cells based upon DNA content) in untreated scaffolds (S), heparinized scaffolds (SH), heparinized scaffolds with both vascular endothelial growth factor (VEGF)-A and FGF-2 (SHVF), heparinized scaffolds with VEGF-A (SHV), and heparinized scaffolds with FGF-2 (SHF), (**B**). Data are means $\pm$ standard deviation, $n=6$. Significantly increased infiltration in all sample groups when compared to untreated scaffolds ($*p < 0.01$). Confocal images at different magnifications of phalloidin-Texas Red-stained cells growing in untreated scaffolds (**C**), heparinized scaffolds (**D**), and heparinized scaffolds with VEGF-A and FGF-2 (**E**). The third pictures in panels (**C–E**) show high magnification of the boxed regions in the pictures to the left and the last pictures in panels (**C–E**) show additional cellular events in each condition. The scaffolds are visualized by green autofluorescence. Asterisk “*” indicates cytoskeletal formations and arrowhead indicates filopodia. Nuclei stained by DAPI (blue). Scale bars = $100 \mu\text{m}$.

present in each ROI, that is, graft, was obtained. The image was converted from gray to binary and then the "analyze particles" option was used to calculate the amount of DAPI present in each image.

Using the red split image on the same ROI, an extraction of that portion of the image was examined using the CellProfiler image analysis software (www.cellprofiler.org/).²⁰ After converting the images from red to gray and subsequent thresholding, the CD31 objects were identified by using the same two classes "Otsu PerObject thresholding" on all images. CD31 objects <9 pixels were excluded from the analysis. The new CD31 object image was saved and the objects size, shape, and number on all images were measured using the MeasureObjectSizeShape module.

Using the CD31 object image from the CellProfiler image analysis software, we analyzed the branching of the vessels using the skeleton plug-in in Fiji (<http://fiji.sc/AnalyzeSkeleton>). The first step was to convert the gray scale image into a binary image, skeletonize the image, and then analyze the skeletonization. The analysis gave information about the number of junctions and vessel length. The vessel length was analyzed based upon CD31 branches connected through junctions, creating the mean length of CD31 objects. Using the given number of CD31 objects from the CellProfiler analysis combined with the number of junctions from the Fiji skeleton analysis, the junction ratio (number of junctions/number of CD31 objects) was calculated.

Statistical analyses

The effects of the different treatments of the scaffolds on cell infiltration in the scaffolds were compared by using the Kruskal-Wallis one-way analysis of variance by using the GraphPad Prism (La Jolla, CA), with significance set to $p \leq 0.05$. Data are presented as means $\pm$ standard deviation.

Results

Properties of the gelatin scaffolds

When we examined the morphology of the scaffolds by SEM (Fig. 2A–C), we saw a mesh with narrow trabeculae. The space-to-material ratio was high, an observation that was confirmed by a material density of 13 mg/cm^3 . The pores were interconnected, and their sizes ranged from ~ 50 to $400 \mu\text{m}$ (Fig. 2A–C). After the scaffolds had been coated with heparin, the amount of bound heparin was $8.6 \pm 2.2 \mu\text{g}$ per mg scaffold ($n=3$), as evaluated by the toluidine blue assay.¹⁸

Confocal microscopy of the heparinized scaffolds demonstrated excellent penetration and even distribution of heparin, as determined by Alexa Fluor 488-labeled anti-thrombin (Fig. 2D). Sectioning of the heparinized scaffolds exposed the gelatin surface, yielding the blue auto-fluorescence seen in Figure 2D.

Detection of the heparinized scaffolds at day 1 *in vivo* was achieved by avidin–Texas Red staining, revealing distinct

FIG. 4. *In vivo* infiltration of cells in gelatin scaffolds transplanted under the kidney capsule. **(A)** Macroanalysis of explanted mice kidneys on day 7 showing a representative result with a dark blood-containing heparinized scaffold graft (SH), compared to a control (S) or growth factor-loaded scaffold (SHVF). The arrowhead and broken line mark the scaffold, still under the kidney capsule. Inserts show the grafts in close up after removal from the kidneys. **(B)** Hematoxylin and eosin staining of paraffin sections of 7-day grafts of unmodified scaffolds (S), heparinized scaffolds (SH), and heparinized scaffolds incubated with VEGF-A and FGF-2 (SHVF). Scale bars = $100 \mu\text{m}$. The panel below shows a higher magnification ($2.25\times$) of the boxed region in the corresponding picture above. The borders between the transplanted scaffold and the kidney parenchyma are marked with black dashed lines.

binding of avidin to the heparinized gelatin scaffold beneath the kidney capsule (Fig. 2F). The use of avidin for detection of heparin is because avidin harbors heparin-binding domains.²¹ The specificity of avidin–Texas Red binding to the heparin surface was shown by the complete absence of avidin binding in the nonheparinized control scaffolds (Fig. 2E). Unspecific interaction with biotin within the kidney parenchyma, in combination with autofluorescence, created an equal degree of red staining of the kidney in the control and heparinized scaffolds.

In vitro cell infiltration of the scaffolds

Untreated and heparin-coated scaffolds preincubated with the various combinations of growth factors were placed on top of a subconfluent layer of MS1 mouse endothelial cells in

culture to analyze cell infiltration (Fig. 3A, schematic figure), as measured by DNA quantification (Fig. 3B) and confocal analysis (Fig. 3C–E). After 3 days in culture, 3400 ± 200 cells had infiltrated the unmodified scaffolds (S, Fig. 3B). Approximately 2.5 times more cells (8200 ± 1300) were found in the heparinized scaffolds (SH) without growth factors than in the unmodified scaffolds. The heparinized scaffolds preloaded with both VEGF-A and FGF-2 (SHVF) harbored an average of 7400 ± 700 cells; the scaffolds with VEGF-A alone (SHV) had 9400 ± 1400 cells; and the scaffolds with FGF-2 alone (SHF) had 8500 ± 1900 cells. All modified scaffolds had significantly more infiltrating cells than did the untreated control scaffolds ($n=6$; $p<0.01$). No significant differences in cell infiltration were observed between the heparinized scaffolds with and without growth factors. The basal proliferation of MS1 endothelial cells was high, and

FIG. 5. Infiltration of granulocytes and activated phagocytes into scaffolds *in vivo*. **(A)** Infiltration of granulocytes (Gr-1, red) into grafts of unmodified scaffolds (S), heparinized scaffolds (SH), and heparinized scaffolds incubated with VEGF-A and FGF-2 (SHVF) on day 1, 3, or 7 after transplantation. Nuclei are stained with DAPI (blue). Scale bars = 100 μ m. **(B)** CD11b⁺ cells (green) present in the scaffolds after 7 days *in vivo* interacting with vessels stained for VE-cadherin (red). The panel below shows a higher magnification of the boxed regions in the corresponding picture above. Scale bars in upper panel = 100 μ m and in the lower panel = 50 μ m. The borders between the transplanted scaffold and kidney parenchyma are marked with white dashed lines.

measurements of two-dimensional cultures by thymidine incorporation showed an ~ 1.24 -fold increase in proliferation in the presence of VEGF-A (17936 ± 4606 cpm) when compared to control cultures (14443 ± 4087), and an ~ 2 -fold increase in primary HDMEC (374 ± 38 and 163 ± 25.4), and an ~ 1.3 -fold increase in HUVEC (2371 ± 136 and 1816 ± 204) for VEGF-A versus control respectively.

Confocal analysis of MS1 endothelial cells cultured *in vitro* in the presence of S, H, or SHVF gelatin scaffolds revealed phalloidin-stained cells (red) adhering to the surface of the scaffolds (Fig. 3C–E). The cells formed a network of endothelial sprouts, but single cells adhering to the scaffolds were also seen. Experiments with HUVEC showed similar in-growth of cells (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Endothelial cells in the presence of heparinized scaffolds showed an elongated phenotype, and the cells flattened out against the surface, showing clear cytoskeletal formation and filopodia (Fig. 3D, asterisk and arrowhead, respectively).

In vivo cell infiltration of the scaffolds

Scaffolds with the various modifications were implanted under the kidney capsules of C57BL/6/Bkl mice and then explanted after 1, 3, 7, or 21 days ($n=5-9$) for analysis. To focus on a phase of active angiogenesis, while the degradation of the scaffold was minimized to enable possibility of islet deposition within the scaffold, we investigated the first week after transplantation in detail when the structure of the scaffolds appeared to be intact. After 21 days *in vivo*, the scaffolds were equally degraded but still remained to some extent in the three groups (data not shown), verifying that 7 days was the optimal time point for creating a vascular bed, a prevascularized site.

Pools of red blood cells were clearly observed in the heparinized grafts (SH) when compared to the controls (S) and scaffolds with growth factors (SHVF) at the time of kidney removal (Fig. 4A; grafts still in the kidney are marked with an arrowhead and a broken line. Inserts in Figure 4A show grafts after removal from the kidney). Investigation of H&E-stained grafts showed only a moderate increase in cell infiltration by day 7 in the unmodified scaffolds, in contrast to the modified scaffolds, which were completely penetrated

by cells on day 7 (Fig. 4B). Furthermore, no giant-cell body formation or fibrin deposition was found in either type of scaffold at 1, 3, or 7 days. High-resolution scanned images (Supplementary Fig. S2) can be viewed by utilizing the free downloadable ImageScope software from the Aperio web link (www.aperiotech.com/download-imagescope-viewer.asp).

Generally, few cells were found in the scaffolds on day 1, but on day 3 an increased cell infiltration was observed. Staining for granulocyte receptor (Gr-1) revealed the presence of granulocytes at equal levels in all samples on day 1 (Fig. 5A). The Gr-1-expressing granulocytes disappeared from the control scaffolds on day 3, and their presence was slightly decreased in heparinized scaffolds, but the amounts remained unchanged in the scaffolds with growth factors on day 3. On day 7, the number of Gr-1⁺ cells was reduced in all groups (Fig. 5A).

The infiltration of CD11b⁺ cells (activated granulocytes) and their possible interaction with the newly formed vessels was investigated *in vivo* on day 7. The CD11b⁺ cells were spread throughout the scaffold, closely interacting with the VE-cadherin-positive vessels (red). This interaction was quite evident in the SHVF scaffold, in which the CD11b cells were spread around the vessels (Fig. 5B: higher magnification of the boxed regions above).

To visualize the topography of the sectioned *in vivo* material differential interference contrast (DIC) microscopy was performed. DIC in combination with immunofluorescence for CD31 revealed that the endothelial cells at day 7 *in vivo* grow along the scaffold (Supplementary Fig. S3), similar to the findings of MS1 cells growing in the scaffolds *in vitro* (Fig. 3).

The biopsies explanted after 1, 3, or 7 days *in vivo* were also cryosectioned and stained for CD31 (endothelial cells, Fig. 6A, B), CD11b (activated phagocytes, Fig. 6C), and CD90.2 (T cells, Fig. 6D), in order to more accurately define and quantify the infiltrating cells. When cross sections of vessel lumens were assessed, an increase was observed in the treated scaffold groups, SH and SHVF (Fig. 6B, inserted boxes). Also, a blinded evaluation of the CD31- and DAPI-stained scaffolds further supported this observation (Fig. 6E), showing an increased amount of nuclei, length and thickness of the vessels, and maturation of the vessels (the amount and size of the formed lumen and network formation between

FIG. 6. Immunofluorescence staining of sections from grafts explanted on day 7. Images shown are representative of unmodified scaffolds (S), heparinized scaffolds (SH), and heparinized scaffolds incubated with VEGF-A and FGF-2 (SHVF), respectively. Sections were stained in red with fluorescent antibodies against CD31 (endothelial cells, panels A and B), CD11b (activated phagocytes, panel C), and CD90.2 (T-cells, panel D). Nuclei were stained with DAPI (blue). In panel (B) (CD31 staining): The sectioned vessel lumens of a heparinized scaffold and heparinized scaffold with VEGF-A and FGF-2 are compared to the control scaffold (higher magnifications of the boxed regions are shown to the right). The borders between the transplanted scaffold and kidney parenchyma are marked with white dashed lines. Arrowheads indicate the kidney capsule in respective panel. Blinded evaluation (by three investigators) of CD31 and DAPI staining after day 7 *in vivo* (E). The stained grafts were evaluated with regard to the number of nuclei within the graft (Nuclei); the amount, length, and thickness of the vessels formed (Length & thickness); and the network formation between the vessels, together with the appearance and size of the lumen (Maturation). Data are presented as mean values of the summarized scores for each investigator. Image quantification of grafts after 7 days *in vivo* (F–H). Analysis of infiltrating cells (stained for DAPI) into the scaffold (region of interest) showing a significant increase of the amount of DAPI positivity in the SHVF scaffold compared to control (F, $*p=0.0022$). The mean vessel length showed nonsignificant difference between the different groups (G). The junction ratio, the amount of junctions/CD31 objects within the scaffolds showed significant increase in both heparin (SH) scaffold and in heparin scaffold with growth factors (SHVF) compared to the control scaffold (H, $*p=0.0042$). Confocal images of 40- μ m sections of control scaffold (I), heparinized scaffold (J), and heparinized scaffold loaded with growth factors (K) on day 7 *in vivo*, stained for CD31 (red) and α -smooth muscle actin (green). Scale bars in panels (A, C, D) = 100 μ m. Scale bars in panel (B) = 10 μ m. Scale bars in panel (I–K) = 100 μ m.

FIG. 7. Quantification of cell infiltration in explanted unmodified scaffolds (S, $\diamond$), heparinized scaffolds (SH, $\blacksquare$), and heparinized scaffolds incubated with VEGF-A and FGF-2 (SHVF, $\blacktriangle$), explanted after days 1, 3, or 7. Specific infiltration was calculated as the signal area per the total graft area for endothelial cells: (A), CD31⁺; heparin vs. control, $p=0.0094$; (B) activated monocytes/macrophages (CD11b⁺), and (C) T cells (CD90.2⁺). Data are presented as means $\pm$ standard error of the mean. For CD31 analyses, $n=8-9$, and for CD11b and CD90.2 analyses, $n=5-9$.

vessels) in the heparinized scaffolds with and without growth factors, when compared to untreated scaffolds (Fig. 6E). The amount of infiltrating cells was analyzed by quantification of DAPI-stained nuclei within the ROI, that is, the graft (DAPI/ROI), showing significant increase of cells in the SHVF scaffold compared to control (Fig. 6F, $p=0.0022$). Quantification of the vessel length showed a tendency toward increased vessel length in the SH and SHVF grafts in comparison to control although it was not significantly defined (Fig. 6G). To investigate branch points of the vessels formed within the scaffolds, the number of junctions was compared to the amount of CD31 objects. The analysis showed that there was a significant increase of junction ratios in the scaffolds both without and with growth factors in comparison to control (Fig. 6H, $p=0.0042$). To investigate the recruitment of vascular supportive cells, the scaffolds were double-stained after 7 days *in vivo* for vascular ASMA in combination with CD31 (Fig. 6I–K, green and red, respectively). ASMA-positive cells were recruited in all conditions. In the untreated scaffolds, the ASMA-positive cells were elongated and in close contact with the developed vessels (Fig. 6I), whereas the ASMA-positive cells appeared thicker in the heparinized scaffolds (Fig. 6J) and were less organized and covered the vessels to a larger extent in the scaffolds with growth factor (Fig. 6K).

Quantification revealed that the CD31⁺ cell infiltration into the scaffolds tended to increase throughout the 7-day observation period (Fig. 7A). On days 1 and 3, no differences were observed between the unmodified and modified scaffolds in terms of amount of CD31⁺ cells (Fig. 7A). In the case of the unmodified scaffolds, infiltration with CD31⁺ cells had increased slightly by day 7 (Fig. 7A). Heparinization of the scaffolds significantly increased this infiltration ($p<0.05$). Addition of growth factors (VEGF-A and FGF-2) did not significantly increase the endothelial cell infiltration when compared to heparinized scaffolds (Fig. 7A).

The amount of CD11b⁺ cells also increased over time, with similar levels seen for all three types of scaffolds on days 1 and 3. However, pronounced differences were observed on day 7 (Figs. 6C and 7B): The heparinized scaffolds showed significantly higher numbers of infiltrating CD11b⁺ cells than did the control scaffolds ($p<0.05$). Also, the infiltration of CD11b⁺ cells into the heparinized scaffolds preloaded with growth factors was significantly higher than that of the heparinized scaffolds without growth factors ($p<0.05$)

(Fig. 7B). On day 7, the modified scaffolds were infiltrated with a larger number of CD11b⁺ cells than on day 3 ($p<0.05$), while the levels of CD11b⁺ cells in the control scaffolds were unchanged (Fig. 7B).

T-cell infiltration was monitored by staining the grafts with anti-CD90.2 (Fig. 6D). The quantification showed more CD90.2⁺ cells in all scaffolds on day 7 than on days 1 or 3 (Fig. 7C, $p<0.05$). By day 7, more CD90.2⁺ cells had infiltrated the scaffolds preloaded with growth factors (Figs. 6D and 7C) than the control scaffolds or heparinized scaffolds ($p<0.05$). There was no difference in T-cell infiltration between the control and the heparinized scaffolds without growth factors (Fig. 7C).

Discussion

In the present study, we have investigated whether the use of a heparin coating that is novel to tissue engineering applications could improve the vascularization of a scaffold, as part of an attempt to prepare a prevascularized site for tissue transplantation. The scaffold selected for these tests was a commercially available and clinically approved gelatin foam. Initial reports have shown that this denatured collagen sponge has no adverse effect on the surrounding tissue and is degraded within 30–40 days *in vivo*.²² This scaffold has also been reported to be well suited for supporting cells.^{23,24} The scaffold integrity also remained relatively unaffected up to day 7. Our examination of the scaffold showed pore sizes in the vicinity of those previously reported to be optimal for cell infiltration.²⁵ Research on biomaterials used in prosthesis therapy has shown that a pore size $<50\mu\text{m}$ negatively affects the ingrowth capacity of cells.²⁶ We believe that the scaffold used in our study, with a pore size range far exceeding that of the material in the study of Zhang and colleagues,²⁶ can function as a climbing frame for cells. Furthermore, its pore size of 50–400 μm enables the deposition of cells and also of larger cell clusters, such as pancreatic islets (which in humans have an average size range of 50–200 μm).

The heparin coating carried $\sim 13\%$ of the amount of heparin (based on our calculation of heparin per scaffold weight) reported in studies using EDC NHS coupling.^{2,27} However, the actual amount of heparin attached to the scaffold is of lesser importance than the functional integrity of the heparin. The heparin surface used in our study has been shown to bind substantial amounts of antithrombin (up

to 19 pmol per cm^2)¹⁵; although heparin retains its integrity after EDC NHS binding, its use has not been described for surfaces prepared with EDC NHS and should not be expected to be successful considering the random nature of this type of binding (c.f. Fig. 1). The importance of single-point attachment of the heparin conjugate used in this study needs to be stressed; in comparison, multiple bonded heparin shows a loss of biological activity.¹⁷ In earlier publications we have presented the positive effect of coating human islets with the heparin conjugate creating a protection against coagulation *in vivo*¹⁶ and increased adhesion of endothelial cells toward the islet surface *in vitro*⁶ showing biological function.

To evaluate the effects of the heparin coating on endothelial cell infiltration and proliferation, we established a three-dimensional proliferation assay. The main advantage of this new assay is that, as opposed to conventional two-dimensional proliferation assays in which cells disrupted by trypsinization are seeded onto unfamiliar surfaces,²⁸ cells enter log phase before exposure to the new material. In addition, quantification of proliferation is not substrate-dependent during culture; instead, a quantification of genomic DNA, adjusted to account for cell number,¹⁹ is conducted at the end of the experiment. This approach ensures monitoring of proliferation without adverse effects caused by assay components, such as possible metabolic imbalances caused by using labeled substrates. The results of this *in vitro* assay showed that the heparin coating indeed stimulated cell proliferation, producing a 2.5-fold increase in cell number when compared to untreated control scaffolds.

We have recently published data indicating that immobilized heparin (the same unique heparin used herein), in combination with VEGF-A, promotes the adherence and proliferation of primary human endothelial cells.⁶ To determine whether endothelial cell proliferation driven by the heparin coating could be further improved by the addition of a combination of growth factors identical to those used in previous publications,^{2,27} we loaded heparinized scaffolds with VEGF-A and FGF-2. Interestingly, no significant additional increase in endothelial cell proliferation was observed when heparinized scaffolds were preloaded with these growth factors. This result is in contrast to studies using EDC NHS-coupled heparin, which did not find any positive effects of the heparin on *in vitro* proliferation in the absence of growth factors.^{2,29} Our results suggest that the heparin coating in this new application is functional with respect to promoting endothelial cell proliferation, perhaps because of the improved accessibility of the end point-attached heparin.

In vivo, increasing numbers of infiltrating cells were observed over time, with a more pronounced rise seen in the modified scaffolds than in the controls. Scaffolds preloaded with growth factors exhibited the highest numbers of cells, supporting the biological functionality of the loaded growth factors *in vivo* although the effect upon endothelial proliferation *in vitro* was only slightly improved in the presence of growth factors, as shown by amount of DNA and thymidine incorporation. The infiltration of CD11b⁺ cells on days 1 and 3 was more rapid than that of the CD31⁺ cells and was unaffected by scaffold modifications. In the case of the untreated scaffolds, the CD11b⁺ cell infiltration did not increase past day 3, but it did in the case of the heparinized scaffolds. Preloading of scaffolds with VEGF-A and FGF-2

caused an even greater increase, most likely as a result of the inflammatory properties of VEGF-A and FGF-2.^{27,30} *In vivo*, heparan sulfate proteoglycan can protect growth factors, as shown by Saksela *et al.*, who demonstrated decreased proteolytic capacity and reactivation after oxidation.^{31,32} It is not unlikely that the heparin conjugate used in our study provides growth factor protection, thereby creating increased bioavailability. Confocal analyses of biopsies after 7 days *in vivo* showed a close interaction between the infiltrating CD11b⁺ cells and the vasculature within the scaffolds. In the case of the growth factor-loaded scaffolds, the CD11b⁺ cells occasionally covered the vessels almost completely, indicating a possible high trans migratory capacity through the vessels, likely as a result of growth factor stimulation from the scaffolds. This phenomenon also could indicate an increased permeabilization of the vessels growing on the SHVF scaffolds. It should be noted, however, that no fibrin deposits were found when blinded analyses of H&E-stained paraffin sections were performed. Inflammation is a driving force in angiogenesis.³³ In islet transplantation, the inflammatory process that triggers cytokine release may be hazardous for the delicate islets.³⁴ Therefore, we believe that the amount of growth factor used in combination with the heparin conjugate needs to be fine-tuned. It should, however, be noted that there was no giant-cell body formation within the scaffolds, except for the occurrence of one or two cells at the 21-day time point (data not shown).

In vivo endothelial cell infiltration showed no significant change during the first 3 days, by day 7 there were approximately twice as many CD31⁺ cells infiltrating the heparinized scaffolds as in the untreated scaffolds. Preloading of the heparinized scaffolds with growth factors did not significantly increase the total CD31⁺ cell infiltration further on day 7, although blinded examination suggested not only that the lumen formation was more distinct in the SHVF scaffolds but also that there was more branching of the vessels, including increases in their length and thickness. These morphological effects may indicate a maturation of the vessels in the presence of growth factors. Image quantification showed significant infiltration of cells into the scaffolds with growth factors in comparison to control. The infiltration of cells in the heparinized scaffolds was not significantly increased compared to untreated scaffolds. Furthermore, the vascular network, analyzed as the amount of formed junctions based on the amount of CD31 objects within the whole graft, was significantly increased both in heparinized scaffolds and in heparinized scaffolds with growth factors verifying that the vessels growing into the heparin scaffolds formed an increased vascular network indicative of a spreading and maturing vasculature and that the total cell infiltration is increased in the presence of growth factors. Also, the vasculature showed recruitment of vascular smooth muscle cells, indicating maturation of the vessels. As already reported, the increase in CD11b⁺ cells may cause a more rapid release of growth factors as a result of matrix metalloproteinase activity, resulting in increased endothelial cell infiltration.⁵ Also, double-positive CD11b⁺ CD31⁺ cells, that is, CD31⁺ macrophages, may be present among the CD11b⁺ cells. These CD31⁺ macrophages have been reported to have preangioblast-like properties that may support angiogenesis.³⁵ On day 7, T-cell infiltration was markedly increased when the heparin surface was preloaded

with growth factors. Throughout the observed period, heparinization of the scaffolds did not change the T-cell (CD90.2⁺ cell) response when compared to that of the control scaffolds, while preloading with growth factors caused an ~2.5-fold increase in T-cell infiltration.

In conclusion, our results demonstrate that the use of this novel heparin coating provides a promising means of enhancing endothelial cell infiltration, as demonstrated in both *in vitro* and *in vivo* models. The heparinized scaffold works as a climbing frame to stimulate CD31⁺ cell infiltration in combination with a relatively low inflammatory response. When the heparin coating was loaded with growth factors, that is, VEGF-A and FGF-2, we saw no further increase in CD31⁺ cell infiltration, despite the occurrence of a significantly increased inflammatory cell infiltration. However, since heparin-bound VEGF alone stimulates endothelial cell proliferation and the VEGF-A/FGF-2 combination tended to increase CD31⁺ cell infiltration *in vivo*, it is likely that fine-tuning of growth factor concentration and ratio will result in increased vascularization. Heparin coating of the gelatin scaffold creates an inviting surface for endothelial cells, indicating the possibility of the material to be used in the creation of a prevascularized site. The heparin surface treatment in combination with the generous pore size makes the scaffold useful in preparation of a site for islet transplantation. Our results suggest that the heparin coating will be a useful scaffold surface modification to promote basic vascularization *in vivo*, to which titrated amounts of growth factors can be added to improve CD31⁺ cell infiltration, albeit at the expense of slightly increased inflammation.

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Disclosure Statement

No competing financial interests exist.

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