The effect of cross-linking of collagen matrices on their angiogenic capability

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Abstract

The poor vascularization rate of matrices following cell invasion is considered to be one of the main shortcomings of scaffolds used in tissue engineering. In the past decade much effort has been directed towards enhancing the angiogenic potential of biomaterials. A great many studies have appeared reporting about enhancement of vascularization by immobilizing angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-2 (FGF-2). We have also tried to achieve this goal by modifying collagen matrices by covalent incorporation of heparin into the matrices and loading them with VEGF. We and others have observed that loading angiogenic factors to heparinized materials markedly increases angiogenic capacity. In the present paper we also investigated the angiogenic properties of collagen matrices which were only cross-linked, i.e. in the absence of heparin. The angiogenic capacity of the modified matrices was evaluated using the chorioallantoic membrane assay. Differences in angiogenic potential were deduced from macroscopic and microscopic analyses of the chorioallantoic membrane, as well as from dry weight changes. Cross-linked only matrices and matrices both cross-linked and heparinized appeared to show a significantly larger angiogenic potential than unmodified matrices. As previously observed, loading VEGF to these matrices further stepped up angiogenic potential. Quite surprisingly, cross-linking had a substantial impact on angiogenic potential. In terms of magnitude, this effect was similar to the effect of loading VEGF to heparinized matrices. Both modification procedures resulted in an increase of average pore size within the collagen matrices, and this observation may explain the more rapid invasion of mouse fibroblasts into cross-linked and heparinized matrices. Form changes of the implants were also monitored during the \textit{in vivo} contacts: cross-linked and heparinized matrices showed far better resistance against contraction, as compared to unmodified matrices.

Results from the chorioallantoic membrane assay experiments were compared with data obtained from rat model experiments, which confirmed the results from the chorioallantoic membrane assay. This relatively simple assay was again shown to be extremely helpful in evaluating and predicting the angiogenic capabilities of biomaterials for use in tissue engineering and wound healing.

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1. Introduction

Although tissue engineering has been recognized in recent years as a promising technique for repairing tissue defects, many problems still remain to be solved before large volume tissue defects can be adequately treated. One of the major problems with engineered matrices is their poor ability to become vascularized within a reasonable time \cite{1}. There appears to be a need for biomaterials with which angiogenesis—essential for oxygen and nutrient supply—correlates with cell invasion. The application of selected angiogenic growth factors (e.g. vascular endothelial growth factor (VEGF) and basic fibroblast growth
factor (bFGF or FGF-2)) may be useful for enhancing angiogenesis [2]. Simply admixing these growth factors to the matrices generally leads to a rapid clearance from the implant site. However, the achievement of a matrix combining high loadability with controlled release of growth factors still represents a major challenge in the field of tissue engineering. The poor resistance of many matrices against contraction is a further challenge. This is likely to be of importance not only for the reduction of scarring, but also in terms of restoring the functionality of the regenerated tissue.

The immobilization of growth factors in three-dimen-
sional matrices has already been introduced by several investigators [3–6] and a range of approaches has been investigated. One approach, introduced by Bentz et al. [7], focuses on covalent coupling of transforming growth factor (TGF-β) to injectable collagen by means of a homobifunc-
tional cross-linking agent. It has been demonstrated that under these conditions, release of the immobilized growth factor is substantially reduced in comparison to release from collagen matrices in which the growth factor is simply admixed. In an alternative approach, Wissink et al. [8] made use of the heparin-binding affinity of bFGF for physically binding this growth factor to heparin covalently incorporated into collagen films. In this approach the heparinization of the cross-linked collagen was shown to have a stimulative effect on the proliferation of endothelial cells, both in in vitro and in vivo experiments [9]. Alternatively, Pieper et al. [10] inserted heparansulfate into collagen matrices and observed that the incorporation of this glycosaminoglycan with EDC/NHS had positive effects on the angiogenic potential, and that these effects could be further enhanced by loading bFGF to these matrices [4]. VEGF has also been used for enhancing angiogenesis in tissue engineering [11–14].

The lack of suitable methods for quantifying angiogenic responses remains a major problem in angiogenesis research. Angiogenic responses of graft materials can generally be evaluated by in vitro or in vivo methods. In vitro assays are based on the principle that endothelial cells form tubule-like structures [15]. The in vitro assays are considered to be the most informative, but they are expensive and time-consuming [16]. Among the in vitro methods, the chorioallantoic assay represents a rather simple and cost-effective procedure that makes use of the easy access of the chorioallantoic membrane in fertilized chicken eggs [17].

A previous paper we have published deals with characterization of the angiogenic properties of collagen matrices, modified by covalent incorporation of heparin and loading with VEGF [18]. In the present paper, we report in more detail on the evaluation of the angiogenic effects arising from cross-linking of collagen, both in the presence and absence of heparin. Also, we evaluate the effects of loading VEGF to cross-linked and heparinized matrices. In the heparinized matrices, VEGF is assumed to be physically immobilized via its high-affinity binding site for heparin and released at a rate which is controlled by the degradation of the collagenous scaffold [19]. This growth factor has been shown to enhance angiogenesis by positively stimulating migration, proliferation and morphogenesis of endothelial cells [20]. The angiogenic potential of the collagen matrices is evaluated by exposure to the chorioallantoic membrane, both with macroscopic and microscopic investigation of the membrane. Also, conclusions are drawn from the dry weight and size changes of the explants. The results are compared with data obtained from rat model experiments.

2. Materials and methods

2.1. Collagen matrices

Collagen matrices were produced by Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany. The matrices were obtained through lyophili-
lation of collagen (from bovine skin) suspensions containing primarily bovine collagen type I. The porous structure is non-directed and pore size varies in the range 15–30 μm, while overall porosity is approx. 98%. The collagen matrices were cut into circular specimens (12 mm diameter, 2 or 5 mm thick, weights varying according to diameter in the range 6.5 ± 0.2 mg to 16.2 ± 0.5 mg, respectively.).

2.2. Modifications of collagen matrices

Modification procedures were performed using a procedure adopted from Wissink et al. [9]. Briefly, carboxylic acid groups of heparin were activated with EDC/NHS. EDC and NHS were added at a fixed ratio of 1:0.6, and at varying EDC/NHS-to-heparin ratios (w/w). For example, 1 mg of heparin was activated with 1 mg EDC/0.6 mg NHS in 500 μl of 0.05 M MES buffer pH 5.6 for 10 min. After 10 min the collagen specimens were immersed in this solution and, to remove the air from the collagen matrices, the solution was evacuated at 20 mmHg for about 2 min. The reaction was allowed to proceed for 4 h at 37 °C, after which the collagen matrices were extensively washed with 0.1 m Na2HPO4 (2h), 4 m NaCl (four times in 24 h) and distilled water (five times in 24 h). Collagen matrices obtained under these conditions are designated H1E1, H1E0.5 and H0E2 refer to matrices modified with 1 mg heparin and 0.5 mg EDC/0.3 mg NHS and 0 mg heparin and 2 mg EDC/1.2 mg NHS per 500 μl, respectively. Modified collagen matrices were frozen at −80 °C overnight and then lyophilized. After lyophilization, the collagen matrices were stored at room temperature.

2.3. Chorioallantoic membrane assay

Circular specimens of collagen matrices (12 mm diameter, 2 mm thick) were disinfected by incubation in ethanol (70%) for 24 h, and then thoroughly washed with sterile PBS for 24 h. Fertilized chicken eggs (Brüterei West, Hamminkeln, Germany) were incubated in rotating incubators for 6 days. Procedures were performed essentially as described by Zwadlo-Klarwasser et al. [17]. Ahead of implantation, the moisture contents of the collagen specimen were reduced under sterile conditions by contact with sterile gauze. Specimens were then loaded with 10 μl of solutions containing varying amounts of rhVEGF165 (0–300 ng; R&D Systems) in 0.1% (w/v) bovine serum albumin (Sigma). Following implantation, the eggs were incubated for an additional 7 days in static incubators. On day 14, the hole on the upper side was enlarged to enable better macroscopic evaluation of the chorioallantoic capillaries and size changes of the collagen specimen. Each parameter condition was assayed with at least five eggs, and one batch of five eggs with no implants was used as a control group.
After in situ fixation of the membranes in 2% formalin, the membranes were carefully excised and mounted on a slide. The degree of angiogenesis was evaluated by counting the microvessels in three randomly selected non-overlapping areas of 125 × 125 μm² at 100-fold magnification. The results are presented both as absolute numbers and as changes in capillary density. Changes to explants were determined immediately after explantation using graph paper and expressed as mm².

2.4 Migration of mouse L929 fibroblasts in collagen matrices

L929 fibroblasts (ATCC no. CCL 1) were cultivated in RPMI 1640 (5% FCS, penicillin/streptomycin 0.8%, l-glutamine 1.6%). A total of 20 μL containing 80,000 cells were pipetted onto unmodified (H0E0), cross-linked (H1E1) and heparinized (H1E1) collagen matrices (cylinders of 13 mm diameter and 1 mm thick) and placed in a 24-well plate. Matrices were disinfected with 70% ethanol overnight and equilibrated with medium. Cells were pipetted onto the top of the matrices and allowed to adhere for 2 h, after which the wells were filled with medium. Cells were cultivated at 37 °C and 5% CO₂ for periods of 3, 6, 12 and 21 days. The cell culture media were changed every 2-3 days. After these periods, matrices were taken out of the wells and fixed with a 4% formalin solution for 24 h at room temperature and embedded in paraffin. Cellular invasion and migration were evaluated from hematoxylin–eosin-stained sections of the matrices. These experiments were performed as triple experiments (n = 3).

2.5 Determination of dry weights

The matrices were explanted and adhering tissue was removed. The matrices were then washed in 1 mL water for 72 h (with three water changes). After freezing at −20 °C, the matrices were lyophilized. To evaluate tissue ingrowth, the dry weights were determined and compared with the original weights.

2.6 Animal model experiments

Lewis rats (200–250 g) under deep pentobarbital sedation were used for the experiments. Collagen matrices (12 mm diameter, 5 mm thick), modified according to the parameters described above, were implanted in four mid-line dorsal subcutaneous pockets of Lewis rats, each at a distance of 1 cm to the incision of the skin and with 4 cm between the implants. Sterile specimens of the variously modified collagen matrices were implanted in animals either non-loaded or loaded with 300 ng of recombinant rat VEGF. After 2, 8 or 14 days the animals were sacrificed and the matrices were explanted. Explants were either directly used for histological evaluation or washed with 1 mL water for about 24 h and the hemeprotein contents were determined spectrophotometrically at the Soret band (absorbance 410 nm). Calibration curves were obtained by determining the absorbance of solutions prepared with lyophilized human hemoglobin [6,21]. Alternatively, after the washing procedure, explants were lyophilized and used in determining changes in dry weight. The dry weights of the explants were compared with the weights of the specimens prior to implantation. The explants to be used for histology were treated with a buffered formaldehyde solution (4% w/v). The formalin-fixed fragments were dehydrated in an ascending series of alcohol and embedded in paraffin. Tissue sections of 6 μm were prepared and stained with hematoxylin–eosin. The animal experiments were performed as triple experiments (n = 3).

2.7 Statistical analysis

In evaluating significance, the Student’s t-test was used. In relevant cases, the significance of differences between data sets is highlighted by one star (p < 0.05), two stars (p < 0.01) or three stars (p < 0.005). In other comparisons, in which data sets are not significant, this fact is shown by the entry NS.

3. Results

In this study we investigated matrices cross-linked in the presence (H1Ex) and absence of heparin (H0Ex). Matrices going through all the procedures in the absence of both the cross-linking agents and heparin were used as controls (H0E0). Matrices were produced as described in Section 2, and their characteristics have been published previously [18]. To evaluate their angiogenic capacity, unmodified (H0E0), cross-linked (H0E0.2–2) and heparinized (H1E0.2–2) collagen matrices were exposed to the choriovallantoic membrane of the chicken embryo (see Section 2 and Zwadlo-Klarwasser et al. [17] for a detailed description of the applied methodology).

Table 1 summarizes a selection of macroscopic observations. Special attention was given to direction of vessel growth and structural stability of implants. Whereas in the control group (no implant) vessel growth was always undirected, at increasing concentrations of EDC/NHS, the orientation of the vessels progressively shifted towards higher percentages of directed vessel growth: e.g. 50% undirected, 50% spoke-like for H0E0 and 0% undirected, 100% spoke-like for H0E2. The implantation of the various matrices had no impact on mortality rates, indicating that matrices and modification procedures were not cytotoxic. We also macroscopically evaluated size changes in the implants, initially observing that the matrices modified with higher concentrations of the cross-linking agents showed better resistance against size reduction. Following macroscopic examination, the choriovallantoic membrane (about 1 cm²) was excised in the vicinity of the implant, mounted on a slide and evaluated microscopically. We first compared the number of capillaries induced by matrices cross-linked in the absence of heparin (H0Ex) and matrices cross-linked in the presence of heparin (H1Ex). The data show that the number of capillaries in both sets of conditions increased with the concentration of cross-linking agents, while EDC concentrations varied from 0 to 2 mg EDC per 500 μL (Fig. 1). The data obtained for the matrices cross-linked in the absence of heparin were similar to data published earlier for H1Ex matrices [18]. The capillary densities induced by the heparinized matrices (H1Ex) were in most cases somewhat higher than by the matrices which were cross-linked only (H0Ex). However, the differences between the two groups are either not or only marginally significant, indicating that the incorporation of heparin has only limited impact on angiogenic properties.

To evaluate the effects of loading VEGF to heparinized matrices, we determined the dose dependency of VEGF loaded to collagen specimens. Varying amounts (0, 30, 100 and 300 ng) of rhVEGF165 were loaded to H0E0 and H1E1 matrices ahead of implantation on the choriovallantoic membrane. The VEGF effects from loading 100 and 300 ng were only slightly greater than that from loading 30 ng (data not shown). Apparently, 100 ng was sufficient for inducing the full VEGF effect. This observation led us to...
the decision to restrict further experiments to the comparison of matrices modified according to the parameters H0E0, cross-linked (H0E1) and heparinized (H1E1) collagen matrices.

The angiogenic effects induced by cross-linking and by loading VEGF in and to these matrices are shown in Fig. 2. Compared to the control (no implant), the implantation of H0E0 matrices induced an increase of the number of capillaries from $51 \pm 4$ to $58 \pm 4$ (mean $\pm$ SD ($n = 5$)). Implantation of H0E1 and H1E1 matrices induced larger number of capillaries in the chorioallantoic membrane with $88 \pm 5$ and $92 \pm 5$ capillaries, respectively. The modifications, i.e. cross-linking in the absence (H0E1) and presence of heparin (H1E1) thus resulted in higher numbers of capillaries. Loading of the unmodified and modified matrices with VEGF induced further increases in the number of capillaries. These VEGF effects, expressed as differences in numbers of capillaries between non-loaded and loaded matrices, were relatively small for the unmodified (H0E0) matrices and the matrices cross-linked only (H0E1): $5 \pm 2$ and $11 \pm 5$ capillaries, respectively. In the case of the heparinized (H1E1) matrices, more substantial VEGF effects were observed: $24 \pm 7$ capillaries.

Rather surprisingly, we observed that the angiogenic effects due to cross-linking (H0E1 vs. H0E0 (30 $\pm$ 2)) and (H1E1 vs. H0E0 (34 $\pm$ 5)) were superior to the angiogenic effects induced by VEGF loading (H0E1 + V100 vs. H0E1 (11 $\pm$ 5) and H1E1 + V100 vs. H1E1 (24 $\pm$ 7)).

Since the structural stability of the matrices is also an important parameter in the development of templates for tissue regeneration, we also investigated changes in the dimensions of the implants under these conditions. After an exposure period of 1 week, collagen matrices were carefully excised and the surface areas were determined. Fig. 3 shows both surfaces of the explanted specimens of unmodified (H0Ex) and heparinized (H1Ex) collagen matrices. After 1 week, the unmodified (H0E0) matrices showed quite substantial contraction: the absolute areas diminished from $124.0 \pm 1.6 \text{mm}^2$ (mean $\pm$ SD ($n = 5$)) to $57.4 \pm 4.6 \text{mm}^2$, corresponding to changes in size of approx. 54%. The area reductions for the cross-linked (H0E1) and heparinized (H1E1) matrices were much greater.

### Table 1

<table>
<thead>
<tr>
<th>Modification and loading parameters</th>
<th>Death incidence (%)</th>
<th>Area reduction (%)</th>
<th>Vessel structure (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>–</td>
<td>100</td>
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<tr>
<td>H0E0</td>
<td>20</td>
<td>50</td>
<td>60</td>
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<tr>
<td>H0E0.2</td>
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<td>30</td>
<td>70</td>
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<tr>
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<td>10</td>
<td>20</td>
<td>75</td>
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<tr>
<td>H0E1</td>
<td>10</td>
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<td>H0E2</td>
<td>25</td>
<td>5</td>
<td>100</td>
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<tr>
<td>H1E0</td>
<td>20</td>
<td>30</td>
<td>60</td>
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<tr>
<td>H1E0.2</td>
<td>20</td>
<td>40</td>
<td>50</td>
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<tr>
<td>H1E0.5</td>
<td>15–20</td>
<td>30</td>
<td>75</td>
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<tr>
<td>H1E1</td>
<td>20</td>
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<td>H1E2</td>
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<td>10</td>
<td>75</td>
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<tr>
<td>Control + VEGF</td>
<td>20</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>H1E0 + VEGF</td>
<td>20</td>
<td>50</td>
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<td>H1E0.2 + VEGF</td>
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<td>H1E0.5 + VEGF</td>
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<td>H1E1 + VEGF</td>
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<td>H1E2 + VEGF</td>
<td>20</td>
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</table>

Heparinized collagen matrices were either non-loaded (H1Ex) or loaded with 100 ng rhVEGF165 (H1Ex + VEGF).
smaller, at approx. 16% and 23%, respectively. This data again demonstrates that chemical cross-linking has a large impact on structural stability. The small differences in area reduction between matrices cross-linked in the absence (H0E1) and presence of heparin (H1E1) is most likely due to the consumption of EDC by carboxyl groups on the heparin, leaving fewer EDC molecules available for the cross-linking reaction.

After determination of their dimensions, the explants were washed carefully and lyophilized to determine their dry weights. The differences in the dry weights of the collagen specimens before and after exposure to the chorioallantoic membrane are considered to correlate to the extent of tissue ingrowth. Fig. 4 shows the dry weights of the explanted unmodified (H0E0), cross-linked (H0E1) and heparinized (H1E1) collagen matrices. The control refers to the mean weights of the matrices ahead of implantation. The VEGF effects refer to the differences in dry weight between non-loaded matrices and matrices loaded with VEGF. Columns show mean values, while error bars refer to the corresponding standard deviations (n = 5).

towards a decrease in dry weights, indicating that with these matrices, weight losses by degradation exceed weight increases by tissue ingrowth. Loading of these matrices with 100 ng VEGF resulted only in a slight VEGF effect. The explanted H0E1 matrices were characterized by dry weight increases of 7.04 ± 0.32 mg in the absence of VEGF (mean ± SD (n = 5)), and of 7.4 ± 0.64 mg when loaded with 100 ng VEGF. The heparinized matrices (H1E1) showed dry weight increases of 7.92 ± 0.23 mg for the non-loaded matrices and 8.94 ± 0.37 mg for matrices loaded with 100 ng VEGF. Thus, also when using this method, the impact of VEGF loading is greater with heparinized matrices: 1.02 mg for the H1E1 matrices vs. 0.35 mg for the H0E1 matrices. The differences in dry weight were smaller, at approx. 16% and 23%, respectively. This data again demonstrates that chemical cross-linking has a large impact on structural stability. The small differences in area reduction between matrices cross-linked in the absence (H0E1) and presence of heparin (H1E1) is most likely due to the consumption of EDC by carboxyl groups on the heparin, leaving fewer EDC molecules available for the cross-linking reaction.

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significant when comparing the group of non-loaded H0E0 and H0E1 matrices ($p < 0.005$) and the group of VEGF loaded H0E1 and H1E1 matrices ($p < 0.01$). The difference between the effects of loading VEGF to H1E1 matrices was significant ($p < 0.005$), while the effects of loading VEGF to H0E1 matrices were not ($p = 0.173$).

Based on the rather surprising observations of the effects induced by cross-linking, we decided to look into the impact these modifications had on the structure of the collagen matrices. We therefore evaluated the pore size of the collagen matrices by microscopy of hematoxylin–eosin-stained sections. Both the heparinized matrices (H1E1) and the matrices, which were cross-linked only (H0E1), were characterized by an increase in average pore size from approx. 20–30 μm to approx. 30–50 μm (data not shown, unpublished data of M. Markowicz).

The observation of an increase in pore size after cross-linking the collagen matrices prompted us to investigate whether this increase would impact the migration rates for cells within these matrices. We therefore loaded unmodified (H0E0), cross-linked (H0E1) and heparinized (H1E1) collagen matrices with mouse fibroblasts from the cell line L929 and histologically evaluated the migration rates within these matrices. Fibroblasts migrated significantly faster ($p < 0.005$) in H0E1 and H1E1 matrices than in unmodified matrices (H0E0) (Fig. 5). The differences in migration between H0E1 and H1E1 matrices were not significant, and this last observation may be due to the similarity in pore size in heparinized (H1E1) and cross-linked (H0E1) matrices (unpublished results of M. Markowicz).

To be able to compare the results obtained using the chorioallantoic membrane model to an alternative in vivo system, we evaluated the angiogenic potential of the unmodified, cross-linked and heparinized collagen matrices with an animal model. Collagen specimens were implanted subcutaneously in the back of Lewis rats and explanted after 2, 8 and 14 days. One set of explants was used for the determination of the hemeprotein contents, while the other set (8 and 14 days only) of explanted specimens was washed with water and lyophilized for determination of the dry weights.

The hemeprotein contents of the collagen specimen explanted after 2, 8 and 14 days are shown in Fig. 6. In the case of the unmodified matrices (H0E0), only minor amounts of hemeproteins could be traced. However, the cross-linked (H0E1) and heparinized (H1E1) matrices show a completely different picture. Increases in hemeprotein content are clearly detectable and correlate to implantation time. Also, we observe that the additional impact on the heparinized matrices with VEGF loading are substantial during the first 8 days, but decrease to some extent over longer implantation times.

We also evaluated the dry weight changes in collagen specimens explanted after 8 and 14 days (Fig. 7). Again, clear differences were observed between unmodified, cross-linked and heparinized collagen matrices. The dry weights
of the unmodified matrices decreased with increasing implantation time, while VEGF loading only produced a negligible VEGF impact. The dry weights of the cross-linked and heparinized matrices clearly increased with implantation time, the impact of VEGF loading being substantial in the case of the heparinized matrices. However, this effect decreases with time of implantation.

The degree of neovascularization was evaluated by immunohistochemical investigation of the explants. Fig. 8 shows photographs of sections of the H0E0, H0E1 and H1E1 explants stained with hematoxylin. Sections of H0E1 and H1E1 specimens explanted after 2 weeks showed enhanced neovascularization and cell invasion. Mature vessels filled with erythrocytes were observed throughout the whole matrix. H0E0 explants were characterized by a lower cell density and a lower number of capillaries. The newly formed blood vessels in both specimens showed mature characteristics, i.e. the capillaries consisted of endothelial cells layered on a basal membrane surrounded by pericytes. The arterioles and venules consist of endothelial cells, smooth muscle cells and connective tissue. In addition, the lumen of the vessels is filled with erythrocytes, which proves connection to the general circulation of the rat.

4. Discussion

This investigation deals with the evaluation of the angiogenic capability of cross-linked and heparinized collagen matrices, either non-loaded or loaded with VEGF, using the chorioallantoic membrane assay and animal model experiments. In previous publications we have reported about the characteristics of heparinized collagen matrices and we gave a preliminary report about their angiogenic properties [18,22]. Heparinized collagen matrices have appeared to be characterized by increased angiogenic potential, which could be increased by VEGF loading. The heparin was incorporated by covalent cross-linking to the collagen using the cross-linking agent 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) in conjunction with N-hydroxysuccinimide (NHS). This procedure not only produces zero-length amide cross-links between the heparin and collagen, but also leads to cross-links between collagen fibers, which in turn increase their resistance towards degradation by collagenases [18,22,23]. Since these heparinized matrices, including in the absence of VEGF, show clear increases in angiogenic potential, we...
became interested in the angiogenic capability of matrices which were cross-linked only, i.e. cross-linked in the absence of heparin. To analyze the angiogenic properties of these matrices, we exposed the chorioallantoic membrane of the chicken embryo to unmodified, cross-linked and heparanized collagen matrices, either non-loaded or loaded with VEGF. After an exposure period of 7 days, the chorioallantoic membrane was macroscopically investigated and vessel structure and size changes were observed and documented. The presence of an unmodified collagen implant already produced effects on the structural orientation of the vessels. Undirected vessel structure changed into a more spoke-like vessel structure, in which the vessels were oriented towards the implants. These effects increased when matrices modified with increasing EDC/NHS concentrations were implanted, both in the absence (H0Ex) and presence of heparin (H1Ex). The effects peaked at EDC/NHS concentrations of about 1 mg ECD/0.6 mg NHS per 500 μL, and cross-linked (H0Ex) and heparinized (H1Ex) collagen matrices behaved in a rather similar way. None of the investigated collagen matrices produced any increase in the mortality rates of the chicken embryos, demonstrating that the collagen matrices were well accepted and did not induce any cytotoxic effects.

Angiogenic potential was further evaluated by determining the increase of capillaries in proximity to the implanted collagen matrices. We observed that in the case of the matrices which were cross-linked only (H0Ex), the number of capillaries in the vicinity of the implants increased with the concentrations of EDC/NHS applied in the modification procedure. These matrices therefore behaved very much like the matrices which were cross-linked with identical EDC/NHS concentrations in the presence of heparin [18]. We also investigated the angiogenic effects of loading VEGF to unmodified, cross-linked and heparinized collagen matrices. Loading of VEGF to these matrices provoked various responses. H0E0 and H0E1 matrices were characteristic of relatively small VEGF effects, while loading VEGF to heparanized matrices (H1E1) provoked substantially greater VEGF effects. These results correlate to earlier results of our group [18], as well as with results of experiments in which bFGF was loaded to collagen matrices with incorporated heparan-sulfate [4]. However, the VEGF effects observed were relatively slight in comparison to the effects due to chemical cross-linking. Although increases in the number of capillaries with the heparinized collagen matrices (H1Ex) were generally somewhat greater than with the cross-linked matrices (H0Ex), these differences were only marginally significant. Based on these results, we deduce that incorporation of heparin has only a small angiogenic effect.

We also investigated the changes in size and in dry weight of collagen specimens after in vivo exposure to the chorioallantoic membrane. The first parameter is important in evaluating structural stability and may therefore be relevant to the development of temporary templates for repair of dermal tissue with reduced wound contraction and improved functionality of the replaced tissue. Both the cross-linked (H0E1) and the heparinized (H1E1) matrices appeared to be far better in maintaining their original size than the unmodified (H0E0) matrices. Also, with respect to the differences in dry weights, the cross-linked and the heparinized matrices scored considerably better than the unmodified matrices. Actually, dry weight changes result from two processes which may have an impact on dry weight changes in two opposed ways. Whereas cell invasion leads to higher dry weights, degradation results in a reduction in dry weights. The impact of VEGF loading in this procedure was relatively slight in comparison to the dry weight changes due to cross-linking.

We observed that cross-linking produced larger pore sizes, with average pore sizes changing from 20–30 μm to 30–50 μm. These observations have been recently confirmed by others [24,25]. The question arises about whether these pore size changes may have an impact on cell migration. We therefore loaded the differently modified collagen matrices with mouse fibroblasts and investigated the migration rates of these cells within these matrices. The migration rates within the heparinized (H1E1) and cross-linked (H0E1) matrices were significantly greater than in the unmodified (H0E0) matrices.

To both evaluate and substantiate the data obtained with the chorioallantoic membrane assay, we performed animal model experiments with rats. These experiments, in which we evaluated the hemeprotein contents and dry weights of the explants after various periods of subcutaneous implantation, provide confirmation of the data obtained with the CAM assay. Thus, in the absence of VEGF loading, cross-linked (H0E1) and heparinized (H1E1) matrices appeared to be far better vascularized than the unmodified (H0E0) matrices. At the same time, under these conditions, loading of VEGF to the heparinized (H1E1) matrices induced greater angiogenic effects than loading the same amount of VEGF to cross-linked (H0E1) matrices. Also, determination of the dry weights confirmed the results obtained from the CAM assay. Both cross-linking and VEGF loading positively impacted angiogenic potential. In these experiments, the unmodified matrices decreased in weight due to degradation, which apparently exceeded the increase in weight due to cellular invasion. The improved angiogenic capability of the H1E1 and H0E1 matrices were further substantiated by histological investigation of the explants. These matrices demonstrated a clearly enhanced cell and vessel density as compared to the matrices which were not cross-linked (H0E0).

5. Conclusions

The results presented here demonstrate that chemical cross-linking contributes quite substantially to the angiogenic properties of collagen matrices. In comparison, the angiogenic effects due to heparinization in combination with VEGF loading were relatively slight. Nevertheless, we
observed that the greatest angiogenic effects were induced by a combination of the two, i.e. heparinization with concomitant cross-linking and VEGF binding. The increases observed in the angiogenic capacity of heparinized collagen matrices thus may be the result of the cumulative effects of controlled release of VEGF [19], higher cellular migration rates due to larger pore size (this publication and [26,27]), lower degradation rates [18] and a more rigid structure of the collagen scaffold [25]. The heparinized matrices therefore offer opportunities for producing low-cost matrices of enhanced angiogenic capacity for tissue engineering and wound-healing purposes, the heparin then perhaps functioning as a sink for exogenous and/or endogenous heparin-binding growth factors.

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