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Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF

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Abstract Since the survival of ingrowing cells in biomaterials for regenerative processes largely depends on the supply of nutrients and oxygen, angiogenesis plays an important role in the development of new materials for tissue engineering. In this study we investigated the possibility of enhancing the angiogenic properties of collagen matrices by covalent incorporation of the vascular endothelial growth factor (VEGF). In a previous paper we already reported the use of homo- and heterobifunctional cross-linking agents for modifying collagen matrices [1].

In the present work the angiogenic growth factor was linked to the collagen with the homobifunctional cross-linker disuccinimidyldisuccinatepolyethyleneglycol (SS-PEG-SS) in a two step procedure. The efficiency of the first reaction step–the reaction of SS-PEG-SS with VEGF—was evaluated by western blot analysis. After 10 minutes virtually all of the dimeric molecules VEGF were on average modified by conjugation with 1 cross-linking molecule. The biological activity of the conjugate was investigated by exposing endothelial cells to non-modified VEGF and to VEGF conjugated to the cross-linker. The conjugation only had a limited effect on the mitogenic activity of VEGF. We therefore applied the cross-linking reaction to the VEGF-collagen system. In a first approach the changes were evaluated by the *in vitro* exposure of HUVECs to non-modified matrices, to matrices in which the VEGF was simply admixed and to matrices in which the VEGF was covalently incorporated. The angiogenic properties were evaluated *in vivo* with the chorioallantois membrane model. In this assay the chorioallantois membrane of the chicken embryo was exposed to the same set of matrices. The covalent incorporation of VEGF has a small but significant effect both on the formation of microvessels in the chorioallantois membrane and the tissue ingrowth into the implant. The covalent incorporation of angiogenic growth factors may thus be considered as a promising approach for enhancing the angiogenic capabilities of collagen matrices. Also the cross-linking with the homobifunctional cross-linking agent has a positive effect on the angiogenic potential of the collagen matrices.

Abbreviations: CM , Collagen matrix · PBS, Phosphate buffered saline · VEGF, Vascular Endothelial Growth Factor · $rhVEGF_{165}$, Recombinant human Vascular Endothelial Growth Factor (Splice variant with 165 amino acids) · SS-PEG-SS, Disuccinimidyldisuccinatepolyethyleneglycol · PEG, Polyethyleneglycol · VEGF-PEG, VEGF conjugated to the PEG-derivative · SDS, Sodiumdocedylsulfate · PAGE, Polyacrylamidegelelectrophoresis· ELISA, Enzyme Linked Immuno Sorbent Assay· HUVEC, Human Umbilical Vascular Endothelial Cell· CAM, Chorioallantoic membrane

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1. Introduction

The outcome after surgical application of biomaterials as tissue replacement depends on adequate incorporation by wound healing. Wound healing is a highly complex process composed of overlapping phases like inflammation, angiogenesis, matrix formation and epithelialization. These processes may be limited by an appropriate oxygen and substrate transport throughout the implanted scaffold. Therefore, angiogenesis, the formation of new capillaries by sprouting from pre-existing micro vessels, is essential [2]. Local, controlled induction of angiogenesis remains a challenge that limits tissue-engineering approaches to restore, maintain and enhance tissue and organs [3]. Different developments have been undertaken to enhance the angiogenic potential by either changing the structural parameters or by administration of growth factors. A large number of cytokines, of which basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are extensively characterized [4,5], regulate endothelial cell growth, proliferation and differentiation into tubulous structures. In addition, extracellular matrix (ECM) components, like collagen, laminin, other glycoproteins and the recruitment of specific integrins (avβ3) have been shown to play important roles in angiogenic processes [6,7].

It is generally accepted that fibrillar collagen is weakly antigenic [8] and that the antigenicity can be further reduced by crosslinking [9,10].

Various attempts to immobilize growth factors into collagen and alginate have already been performed. Thus Bentz et al. [11] were able to covalently incorporate TGF-β into injectable collagen by means of a homobifunctional cross-linking agent. This modification leads to an improved local delivery and a prolongation of the *in vivo* TGF-β responses. Wissink et al. [12,13] demonstrated, that physical binding of the growth factor bFGF to heparinized collagen matrices leads to a sustained bFGF release and a positive effect on the proliferation of endothelial cells. In an alternative approach Chinen et al. [14] were able to stabilize bFGF by physical binding to heparin cross-linked to alginate gel and to induce angiogenesis by controlling its release. Pieper et al. [15] physically immobilized bFGF within collagen matrices with covalently bound heparan sulfate.

In the present paper we describe a series of experiments to covalently immobilise the angiogenic growth factor VEGF within three dimensional collagen matrices. The covalent incorporation was performed according to a protocol published previously [1]. The experiments were carried out with the homobifunctional cross-linking agent disuccinimidyldisuccinatepolyethyleneglycol (SS-PEG_{3400}-SS). The angiogenic outcome of these modifications was evaluated by exposure of the modified matrices to endothelial cells and to the chorioallantois membrane of the chicken embryo [16,17]. The results clearly show, that the covalent incorporation has a beneficial effect on the *in vitro* proliferation of endothelial cells, on the *in vivo* induction of capillary growth in the chicken membrane and on the *in vivo* tissue ingrowth as deduced from the dry weight changes of the explanted collagen specimens.

2. Materials and methods

2.1. Collagen matrices

Collagen matrices were obtained from Dr. Suwelack Skin & Health Care AG, Billerbeck, Germany. They are made by freeze drying collagen suspensions derived from bovine skin, and mainly consist of collagen l. The pore structure is non-directed and pore sizes vary from 15–30 μm.

2.2. Immobilisation of VEGF to collagen

The covalent attachment was performed with the homobifunctional crosslinker SS-PEG-SS (Shearwater Polymers, Birmingham, Alabama, USA). A typical crosslinking experiment was carried out as follows: 10 ng $rhVEGF_{165}$ (TEBU-Bio, Offenbach, Germany) were allowed to react with 28 μg SS-PEG-SS in PBS for 10 minutes (room temperature), the reaction mixture (50 μl) was subsequently administrated to collagen matrices (cubes with a size of 5 $\times$ 5 $\times$ 5 mm or disks with a diameter of 12 mm and a thickness of 2 mm) and allowed to react for 30 minutes. Cubes and disks were then used for determining the proliferative effects on HUVECs (2.5), for the evaluation of the angiogenic potential (2.6) and for release experiments (3.0).

2.3. Polyacrylamide gel electrophoresis and Western blot analysis

20 μl aliquots comprising 200 ng protein were mixed with 20 μl sample buffer (mercaptoethanol, 4% SDS; 15% sucrose in Tris/HCI pH 6.8) and heated for 5 minutes at 95°C. 20 μl of the sample solutions were then loaded into pockets of the polyacrylamide gel. The conditions of the polyacrylamide gel electrophoresis procedure were as described in Chen et al. [1]. Marker proteins (BOA Protein Marker Biomol, Hamburg, Germany) were loaded for protein mass identification. After electrophoresis the proteins were blotted on a PVDF-membrane (Bio Trace^{TM} PVDF, 0,45 μm) and identification of VEGF and its derivatives was performed immunologically with rabbit anti-hVEGF (Upstate Biotechnology, Richmond, USA) as the primary antibody and goat anti-rabbit antibodies (Dako, Carpinteria, CA, USA) as the second antibody. The

latter was linked to horse radish peroxidase for identification of the chemiluminescence (Fuji LAS 3000).

2.4. Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to van Wachem et al. [18]. Cells were cultured to the 2nd passage in tissue culture flasks (Cellstar®, Greiner Bio-One GmbH, Germany). Endothelial Cell Medium-2 (EGM™, Clonetics®, Cambrex, Verviers, Belgium) was complemented with SingleQuot®. Cell culturing was carried out at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. HUVECs used were detached (from flasks) for further experiments by incubation with trypsin (Trypsin-EDTA, PAA Laboratories GmbH, Austria). Residual trypsin was inactivated by addition of culture medium.

2.5. Proliferation of endothelial cells

In each well of a 6-well plate (Falcon® Multiwell™ 6 Well, Becton Dickinson Labware, USA) 100,000 HUVECs were allowed to adhere for 24 h in 2 ml of the Endothelial Cell Medium supplemented with SingleQuot®. At day 2, in each well one collagen specimen (cubes of the size 10*10*10 mm), modified according to the specified parameters, was placed in the wells and the wells were filled with 2 ml of medium. As indicated, in some cases matrices were loaded with a sterile solution containing 100 ng $rhVEGF_{165}$ (TEBU-Bio, Offenbach, Germany) in 20 μl PBS. For control, on each plate one well was left without a matrix and filled with medium only. The cells were then allowed to proliferate for 5 days. Medium was changed every 2nd day. At day 7, the medium and the matrices were removed and the HUVECs were trypsinized. Cell proliferation was quantified microscopically by counting the cells in a Neubauer chamber. In order to highlight the changes, the number of cells present at day 1 (100,000), was subtracted from the result at day 7.

2.6. Evaluation of the angiogenic potential of collagen matrices

The evaluation of the angiogenic properties was performed by exposing the chorioallantoic membrane of the chicken embryo to the differently modified and loaded collagen matrices. The procedure was carried out essentially as described in [17]. Fertilized chicken eggs were incubated for 7 days in a rotating, humidified incubator at 37°C. On day 7 a small hole was driven in the egg shell at the wider end and a triangular hole was created on the upper side for allowing the implantation of a collagen specimen on the chorioallantoic membrane. Specimens (diameter 12 mm; thickness 2 mm) were implanted after disinfection in 70% ethanol and equilibration in sterile PBS, including loading of $rhVEGF_{165}$ (R&D Systems) if indicated. The eggs were again incubated for 7 days at 37°C without rotation. On day 14 the eggs and the chorioallantoic membranes were first evaluated macroscopically, after treatment of the embryonated eggs with formalin, the implants were carefully explanted. Part of the explant was used for histologic evaluation, while the other part of the explant was lyophilized after an extensive washing procedure with water for determining the dry weight changes. After the macroscopic evaluation, the chorioallantoic membranes were excised and mounted on a glass plate. The numbers of the microvessels were counted in three independent areas in the vicinity of the implant by microscopy (magnification 22.5). Each set of modification parameters was investigated with 3 groups of 5 eggs each.

2.7. Enzyme linked immuno sorbent assay

ELISA was performed with the human VEGF Elisa Development System (R&D Systems, Minneapolis, USA). Experiments were performed as described by the manufacturer and 96-well plates (NUNC immunoplate max sorb, Wiesbaden, Germany) were used.

2.8. Stability of VEGF in solution

10 μg $rhVEGF_{165}$ (TEBU-Bio, Offenbach, Germany) were dissolved in 100 μl diluent (1% BSA in PBS, pH 7.2–7.4, sterile). This solution was further diluted to 3 ng/ml with diluent. To one of two 10 ml samples of this solution—one of which comprised 20 units collagenase/ml - were used for determining the stability of VEGF. The collagenase (Collagenase Type 1) was purchased from Worthington, Lakewood, NJ, USA. At the specified time intervals 1 ml aliquots were taken and frozen at –20°C. These aliquots were used for VEGF determination by ELISA.

2.9. Cumulative release of VEGF from collagen matrices

10 ng $rhVEGF_{165}$ (TEBU-Bio, Offenbach, Germany) were cross-linked to collagen matrices (cubes 5*5*5 mm) as described above. In the experiments in which the growth factor was simply admixed; the cross-linking agent was omitted. Collagen specimens were then immersed into 1 ml diluent (1% BSA in PBS, pH 7.2–7.4, filtered sterile) or 1 ml diluent comprising 10 units collagenase/ml (Worthington). 1 ml aliquots were collected and replaced by 1 ml fresh diluent after 0, 3, 24 and 48 h. After collection of the 48 h-aliquots 1 ml diluent comprising 200 units collagenase/ml was added to all samples, the release experiment was stopped at 72 h. Aliquots were frozen at –20°C and used for VEGF determination by ELISA. Cumulative release curves were obtained by totalizing the

individual results from the aliquots collected at the various time intervals.

2.10. Statistical analysis

Statistical analysis was performed by using the software package SPSS for Windows 13 (SPSS Inc.). For significance tests the Student's t-Test was used at the 95% confidence level ($p \leq 0.05$).

3. Results and discussion

In a first approach we tested the two step cross-linking procedure as published in [1] on VEGF. Since VEGF is only available at μg amounts we applied the western blot analysis for identifying modified and non-modified VEGF-molecules after the modification reaction.

Figure 1 shows the result of the Western blot analysis of the conjugation reaction of VEGF with SS-PEG-SS. In analogy to the reaction conditions published in [1] a reaction time of 10 minutes was applied. After this period the reaction was stopped by addition of sample buffer, after dissociation in a SDS/mercaptoethanol containing buffer, the reaction mixtures were run on PAGE gels and identified immunologically after blotting.

Clearly two bands are observed, the lower band corresponds to the non-modified VEGF (Mr 19 kDa) and the upper band to a species with a molecular mass between 20 and 30 kDa. The native VEGF molecule under normal conditions occurs as a dimeric molecule with Mr 38 kDa [5], we therefore interprete this result in the following manner. After about 10 minutes on average all the dimeric molecules have reacted with one cross-linking molecule, dissociation with mercaptoethanol leads to dissociation into modified and non-modified VEGF-monomers. Since the two species appear in a 1:1 ratio, we assume that after 10 minutes most of the VEGF-dimer molecules have reacted with one cross-linking molecule and should be able to react with amino groups on the collagen in the second step.

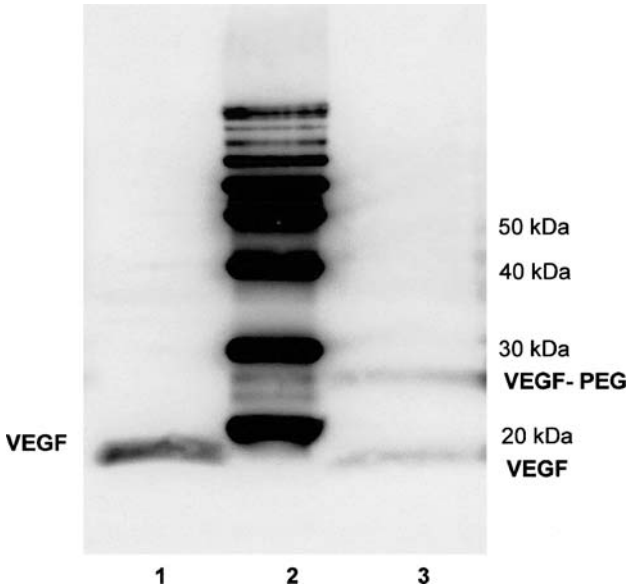

Fig. 1 Western blot analysis of 10 pg rhVEGF$_{165}$ (lane 1) and 10 pg rhVEGF$_{165}$ modified by reaction SS-PEG-SS for 10 minutes (lane 3). In lane 2 7 μl marker proteins were loaded. The immunodetection was performed as described in the paragraph Materials and Methods.

Since the conjugation of the PEG-derivative may have an influence on the biological activity of VEGF, we investigated this issue by exposing human umbilical endothelial cells (HUVECs) to VEGF and VEGF conjugated to the PEG-derivative. Figure 2 demonstrates that the reaction of VEGF with the homobifunctional cross-linker leads to a small, but significant change ($p = 0.005$) in the mitogenic activity i.e. the ability to enhance the proliferation of HUVECs.

As a next step we investigated the response of HUVECs to the exposure to collagen matrices with covalently incorporated VEGF. Figure 3 shows the increase in proliferation of HUVECs when exposed to non-modified collagen matrices (CM), to matrices cross-linked in the absence of VEGF (CM-PEG), to non-modified matrices loaded with VEGF (CM + VEGF), and to matrices cross-linked in the presence of VEGF (CM-PEG-VEGF). A clear cut increase in the proliferation of the HUVECs is observed. The presence of the non-modified matrix (CM) leads to only a small increase, which is further increased by simply admixing VEGF to these non-modified matrices (CM + VEGF). The cross-linking of the matrix in the absence of VEGF also leads to a substantial increase(CM-PEG), the covalent incorporation of VEGF, however, leads to the largest increase. Certainly part of the effect may be due to the cross-linking of the collagen exerted by SS-PEG-SS. The increase due to the covalent incorporation is significant ($p < 0.001$). The observed effects are most likely the result from a paracrine signalling mechanism initiated by HUVECs in direct contact with the collagen and/or VEGF.

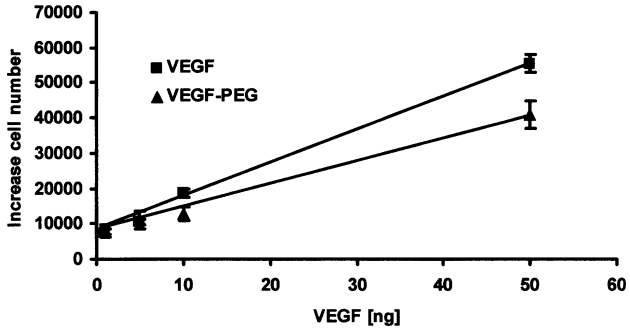

Fig. 2 Comparison of proliferative effects on HUVECs of VEGF and VEGF conjugated to SS-PEG-SS.

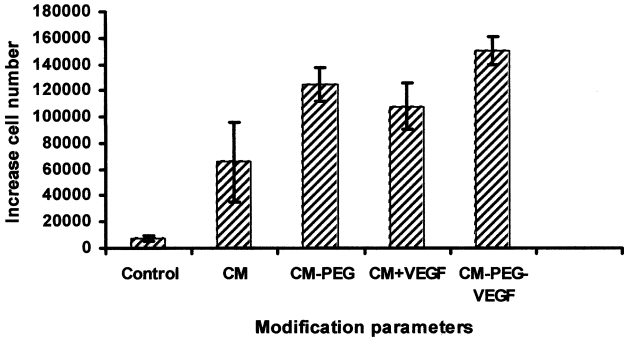

Fig. 3 Proliferative effects of collagen matrices evaluated by exposure to HUVECs. Matrices were either non-modified (CM), loaded with 100 ng rhVEGF$_{165}$ (CM + VEGF), cross-linked with SS-PEG-SS (CM-PEG) or cross-linked with SS-PEG-SS in the presence of 100 ng rhVEGF$_{165}$ (CM-PEG-VEGF). Columns show the increase in cell number, error bars represent the corresponding standard deviations (n = 4).

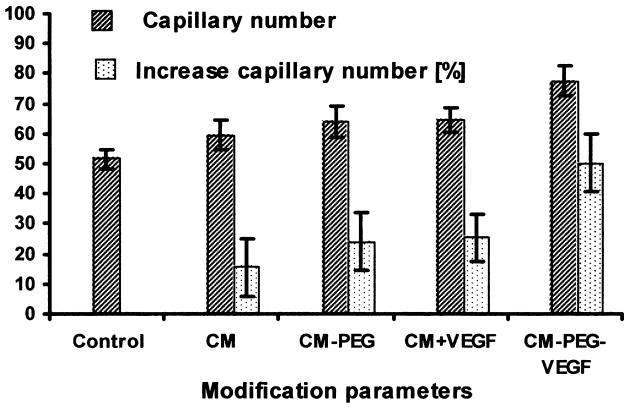

Fig. 4 Evaluation of angiogenic potential of collagen matrices with the chorioallantois membrane assay. Matrices were either non-modified (CM), loaded with 50 ng rhVEGF$_{165}$ (CM + VEGF), cross-linked with SS-PEG-SS (CM-PEG) and cross-linked with SS-PEG-SS in the presence of 50 ng rhVEGF$_{165}$ (CM-PEG-VEGF). Columns show number of capillaries and increase of number of capillaries in percent, error bars represent the corresponding standard deviations (n = 7).

For evaluating the change in the angiogenic properties we tested the various combinations with the chorioallantois membrane assay (CAM-assay) [17]. Figure 4 nicely shows, that the results are very similar to the results observed by exposing HUVECs to the same combinations of modification and loading. The the cross-linking with SS-PEG-SS (CM − PEG) as well as the simple admixing of the VEGF (CM + VEGF) leads to the induction of a larger number of microvessels in the chorioallantois membrane. The largest increases are again seen upon exposure of the CAM to the collagen matrix in which the VEGF is covalently linked to the collagen (CM-PEG-VEGF). This increase is relatively small but significant with $p < 0.001$.

The angiogenic potential was also evaluated by estimating the cell invasion from the CAM into the collagen matrix by

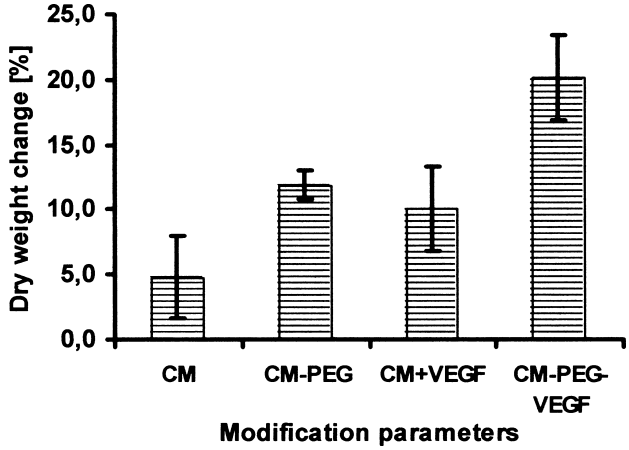

Fig. 5 Dry weight changes of collagen matrices after a 7 day exposure to the chorioallantois membrane of the chicken embryo. Matrices were either non-modified (CM); loaded with 50 ng rhVEGF$_{165}$ (CM + VEGF); cross-linked with SS-PEG-SS (CM-PEG) or cross-linked with SS-PEG-SS in the presence of 50 ng rhVEGF$_{165}$ (CM-PEG-VEGF). Columns show the dry weight changes in %, error bars represent the corresponding standard deviations (n = 7).

determining the dry weights of the explants after having been in contact with the CAM for 7 days. Figure 5 shows the dry weight changes as percentage of the initial weights, the collagen matrices in which the VEGF is covalently incorporated are characterized by the largest increases in tissue ingrowth. Here, the increases of the dry weights of the matrices with the covalently incorporated VEGF (CM-PEG-VEGF) are significantly larger than the dry weight changes of the matrices in which VEGF was simply admixed (CM + VEGF; p = 0.005). The positive effects of cross-linking the VEGF to the collagen are more outspoken with the determination of the dry weights.

In order to further investigate the angiogenic properties of the matrices and to support the *in vitro* and *in vivo* observations we investigated how far the matrices with the covalently incorporated growth factor function as a sustained release system. Being mainly interested in the release behaviour in the first 3 days after implantation we determined the retrieval of VEGF after having been in solution by means of ELISA. Since we intend to follow the release from the collagen matrices under conditions which are to a certain extent similar to *in vivo* conditions, we also added relative low amounts of collagenase to the release medium. For this reason we also investigated the retrieval rate of VEGF after having been exposed to low level collagenase concentrations.

Figure 6 shows that there is only a small decline of the retrieval rate over time, after 48 h still more than 90% of the VEGF-molecules were being retrieved by the ELISA technology. In the presence of collagenase the retrieval rate is somewhat lower and decreases with time from 96 to 84%.

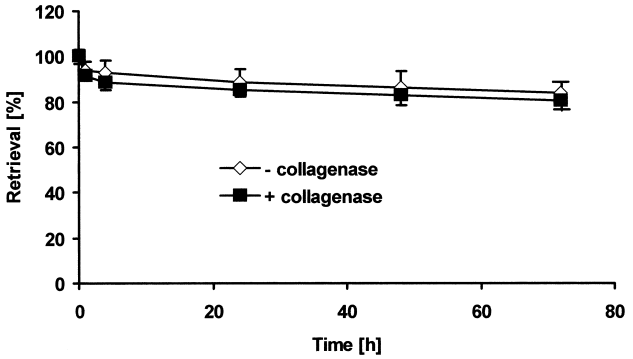

Fig. 6 Stability of rhVEGF$_{165}$ in solution. Retrieval rates were determined in the absence and presence of 10 units collagenase/ml diluent at room temperature. Error bars represent standard deviations (n = 3)

In order to better understand the observations of Figures 4 and 5 we determined the cumulative release curves from collagen matrices in which VEGF was simply admixed and from matrices in which VEGF is assumed to be covalently bound. Release curves were determined in the absence and presence of 10 units collagenase/ml. This amount of collagen in general leads to the complete degradation of non-cross-linked collagen matrices in 48 h, the PEG-treated matrices are degraded to less than 50% (non-published results). The data of Figure 7 show, that i) the releases are strongly dominated by the degradation of the collagen and ii) the cross-linking of VEGF with SS-PEG-SS leads to an additional and significant slow down effect on the releases. After 48 h a relatively large amount of collagenase (200 units/ml) was added to all

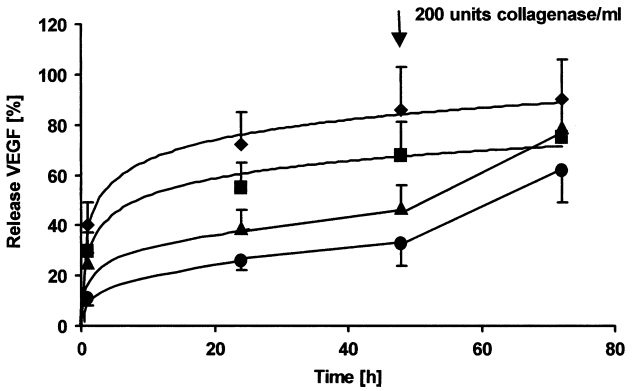

Fig. 7 Cumulative release of VEGF from collagen matrices in which VEGF was simply admixed (CM + VEGF ▲,◆) and collagen matrices in which VEGF was covalently incorporated (CM-PEG-VEGF ●,■). 10000 pg of rhVEGF$_{165}$ were either admixed (CM + VEGF) or covalently linked with SS-PEG-SS (CM-PEG-VEGF) to cubic collagen specimens (cube size 5 mm). Cubes were after admixing or cross-linking immersed in 1 ml diluent in the presence (◆,■) and absence (▲,●) of 10 units collagenase/ml. Solutions were completely removed after 1, 24 and 48 h and frozen at –20°C and replaced by 1 ml diluent. After 48 h (arrow) solutions were replaced by 1 ml aliquots comprising 200 units collagenase/ml diluent. At t = 72 h solutions were taken and frozen at –20°C. VEGF concentrations were determined by ELISA. Error bars represent standard deviations (n = 3).

matrices, in those cases in which collagenase was present from the start and the matrices are completely degraded, no increases in the release rates were observed. In those cases, however, in which no collagenase was present in the first 48 h, clear cut increases in the release rates have been observed.

The results altogether demonstrate, that the covalent incorporation of the angiogenic growth factor VEGF leads to matrices from which VEGF is released in a way, which is apparently governed by the collagen degradation and which has a beneficial effect on the proliferation of endothelial cells and more importantly on the induction of microvessels in the chorioallantoic membrane and the extent of tissue ingrowth as deduced from the dry weights of the explants.

4. Conclusions

In this publication we describe the covalent attachment of the angiogenic growth factor VEGF to collagen in order to develop a matrix with enhanced angiogenic capabilities for applications in the field of tissue engineering. The covalent immobilisation was performed with the homobifunctional cross-linking agent SS-PEG-SS. We initially investigated the reaction between the cross-linker and VEGF. Already after a reaction time of 10 minutes—as deduced from Western blot analysis—all the dimeric VEGF-molecules are characterized by the presence of—on average—one cross-linker molecule. The proliferative activity of the conjugated VEGF-molecules was only reduced to a minor extent. The conjugated VEGF-molecules were then allowed to react with the collagen for the immobilisation reaction. Release experiments demonstrate that in the presence of a low collagenase concentration the VEGF release from the matrices, in which the VEGF is assumed to be covalently linked, is substantially slower than from the matrices in which the VEGF was simply admixed. This difference in release rates was also observed in the absence of the collagenase, the difference between the admixed and cross-linked VEGF is, however, considerably smaller.

The biological effects were tested by exposing HUVECs and chorioallantoic membranes to the various modifications. Both the *in vitro* exposure of the endothelial cells and the *in vivo* approach with the chorioallantoic membrane show that the increase of the angiogenic properties is largest in those collagen matrices, in which VEGF has been covalently immobilized.

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