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The impact of proteinase-induced matrix degradation on the release of VEGF from heparinized collagen matrices

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Abstract

The in vivo application of engineered matrices in human wound healing processes is often hampered by the slow rate of vascularization. Therefore much research is directed towards enhancing the angiogenic properties of such matrices. One approach for enhancing the vascularization is the incorporation of angiogenic growth factors. Recently, we and others have reported on immobilizing such factors into collagen matrices either by covalent attachment or by physical binding to covalently incorporated heparin. Especially the latter procedure has been shown to lead to substantial increase rates in vascularization in in vivo experiments. The increases have been proposed to depend on the sustained release of the incorporated angiogenic growth factors from the heparinized collagen matrices.

In this paper, we report on investigations to study the release of vascular endothelial growth factor (VEGF) from collagen matrices under conditions which mimic potential in vivo situations. Relevant proteinase concentrations were deduced from in vitro experiments in which we evaluated the secretion of selected matrix metalloproteinases from fibroblasts in contact with collagen. The release of VEGF from non-modified, cross-linked and heparinized collagen matrices in the absence and in the presence of varying concentrations of proteinases was then determined by ELISA and liquid scintillation counting. The release behaviour appears to be controlled by both the presence of heparin and the levels of proteinases applied. Experiments with matrices containing radioactively labelled heparin suggest that VEGF release results from the consecutive and simultaneous release of three species of VEGF molecules that differ in their binding affinities to the differently modified collagen matrices. The species binding specifically to heparin most likely accounts for the previously observed increases in angiogenic potential between loading VEGF to non-heparinized and heparinized collagen matrices.

Keywords: Collagen; Controlled release; Growth factors; Heparin; Angiogenesis

1. Introduction

Although tissue engineering has in recent years been recognized as a promising technique for repair of tissue defects there are still many problems to be solved before large volume tissue defects may be adequately treated. One of the major problems with most engineered matrices is their poor ability to be vascularized within a reasonable

Abbreviations: VEGF; vascular endothelial growth factor; $rhVEGF_{165}$; recombinant human vascular endothelial growth factor (alternative splicing variant with 165 amino acids); bFGF; basic fibroblast growth factor; TGF-β; transforming growth factor-β; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; H, heparin; BPh, burst phase; Collagenase, crude enzyme preparation containing a collagenase and a gelatinase; C0.1, 0.1 units collagenase/mL; ELISA, enzyme linked immunosorbent assay; PBS, phosphate-buffered saline; MMP, matrix metalloproteinase; H1E1, collagen matrix modified with a reaction mixture comprising 1 mg heparin (H), 1 mg EDC and 0.6 mg NHS per 500 μL reaction volume; ^{35}S-H1E1, collagen matrix modified by incorporating ^{35}S-heparinsulfonate under the same conditions as in H1E1.

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time frame [1]. Thus, there appears to be a need for biomaterials in which angiogenesis—essential for oxygen and nutrient supply—better correlates with cell invasion. The application of selected angiogenic growth factors (e.g. vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF)) may be useful for enhancing angiogenesis [2]. Since the simple admixing of these growth factors to the matrices generally leads to a rapid clearance from the implant site, the development of a matrix, which combines a high loading capacity with a controlled release of growth factors, represents a major challenge in the field of tissue engineering. Several approaches for immobilizing growth factors within collagen matrices have already been investigated. Bentz et al. [3] incorporated transforming growth factor-β (TGF-β) into injectable collagen by use of homobifunctional cross-linking agents. Kanematsu et al. [4] investigated the possibility of using collageneous matrices as release carriers of exogenous growth factors. Among the tested growth factors in that study were VEGF and bFGF. Wissink et al. [5,6] modified collagen matrices by covalently incorporating heparin for physical binding of bFGF. Heparinized collagen matrices were also investigated by Pieper et al. [7]. Alternatively VEGF was encapsulated in alginate beads: a sustained release was observed [8]. The potential advantage of administering several growth factors simultaneously has also been investigated: Richardson et al. observed that the combined delivery of VEGF and platelet derived growth factor (PDGF) synergistically leads to the rapid formation of a mature vascular network [9]. Exogenous VEGF administration can—as has been shown during embryonic vasculogenesis—lead to malformed leaky vessels with unusually large irregular lumens [10]. In a recent article, Ozawa et al. [11] have demonstrated, that the micro-environmental VEGF concentration rather than the overall dose determines the threshold between normal and aberrant angiogenesis.

In two previous papers [12,13], we have reported on the in vitro and in vivo angiogenic properties of heparinized collagen matrices loaded with VEGF. The alternative splice variant of VEGF with 165 amino acids ($VEGF_{165}$) possesses, as many other growth factors acting in the extracellular matrix [14,15], a heparin binding domain. This particular splice variant is further characterized by a high mitogenic and angiogenic activity. We were able to show that—as deduced from in vitro and in vivo experiments [12,13]—loading of VEGF to heparinized collagen matrices leads to an increase in the angiogenic potential when compared to loading the same amount of VEGF to non-heparinized collagen matrices. Similar results were also observed by Pieper et al. [16] with bFGF.

The process of vascularization of collagen matrices is accompanied by a remodelling of the matrix, which includes degradation of the collagen and deposition of newly synthesized collagen. Fibroblasts play an important role in the physiologic processes of remodelling and wound healing. They do this by secretion of matrix metalloproteinases (MMPs), specific tissue inhibitors of metalloproteinases (TIMPs) and by collagen deposition [17–20]. Since the angiogenic process is intimately related to the invasion of fibroblasts and endothelial cells and remodelling of the collagen matrix, we specifically studied the release behaviour of collagen matrices in the presence of those concentrations of MMPs that are expected to prevail in potential in vivo situations. We initially determined the secretion levels of selected proteinases (MMPs) in in vitro experiments with fibroblasts. Based on these findings we then studied the VEGF release behaviour from non-modified, cross-linked and heparinized collagen matrices in the presence of varying relevant concentrations of proteinases by means of enzyme linked immunosorbent assay (ELISA) and liquid scintillation counting of radioiodinated VEGF.

2. Materials and methods

2.1. Collagen matrices

Collagen matrices were produced by Dr. Suwelack Skin and Health Care AG, Billerbeck, Germany. The matrices were obtained through lyophilization of collagen suspensions containing primarily bovine collagen type I. The porous structure is non-directed and pore sizes vary from 15 to 30 μm. The overall porosity amounts to approx. 98%.

2.2. Modification of collagen matrices

Incorporation of heparin into collagen matrices was performed with a procedure that was adopted from Wissink et al. [21] and previously published in [13]. Briefly, carboxylic acid groups of heparin were activated with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS). 1 mg of heparin (H-4784; Sigma, Steinheim, Germany) 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 specimen were immersed into this solution and the solution was evacuated at 20 mmHg for about 2 min to remove the air from the collagen matrices. The reaction was allowed to proceed for 4 h at 37 °C, after which the collagen matrices were extensively washed with 0.1 M Na_2HPO_4 (2 h), 4 M NaCl (4 times in 24 h) and distilled water (five times in 24 h). Matrices obtained under these conditions are designated H1E1; in such matrices about 30 μg heparin/mg collagen were incorporated, matrices treated with heparin in the absence of cross-linking agents did not contain any heparin [13]. Matrices obtained in the absence of the cross-linking reagents are designated H0E0. After the modification procedure, matrices were frozen at −80 °C overnight and lyophilized.

2.3. Cell culture of fibroblasts

CCD18 fibroblasts derived from human colon (American Type Culture Collection [ATCC], Rockville, MD: CRL-1459, passages 14–15) were cultivated in DMEM medium (DMEM, 31966-021, Gibco-Invitrogen, Auckland, New Zeeland) containing 10% fetal calf serum (FCS). Cells were cultured at 37 °C in a humidified atmosphere containing 5% carbon dioxide and subcultured using 500 mg/L trypsin-200 mg/L ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS, Bio Whittaker, BE17-161E, Cambrex Bio Science, Verviers, Belgium) for cell detachment. For experiments in the postconfluent growth state, the adherent growing cells were seeded at defined cell densities and grown for the time intervals indicated.