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Original Article

Improving the angiogenic potential of collagen matrices by covalent incorporation of *Astragalus* polysaccharides

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Abstract: The high degree of degradation and the low angiogenic capabilities of temporary tissue substitutes still represent a major challenge in the field of tissue engineering. In an attempt to meet some of these challenges we covalently incorporated *Astragalus* polysaccharides, a plant extract with angiogenic properties, into collagen matrices. This contribution aims at developing a three-dimensional scaffold for temporarily covering tissue defects in tissue engineering and wound healing e.g. third degree burn wounds. Collagen matrices were modified by incorporating *Astragalus* polysaccharides (Ap) by means of covalent cross-linking with the watersoluble carbodiimide EDC. Matrices with different Ap/EDC ratios were prepared. After intensively washing of collagen matrices, the Ap modified and non-modified collagen matrices were exposed to the *chorioallantoic* membrane or implanted into subcutaneous pockets of rats. The number of capillaries in the *chorioallantoic* membrane in the vicinity of the samples, the hemoglobin contents within the explants and the hydroxyproline contents in the tissues attached to the explants were enhanced. Immunohistochemical evaluation of the explants revealed an increase in the recruitment of CD34+-cells in the modified matrices, indicative of improved angiogenic capabilities. To explore the underlying mechanisms, human umbilical vascular endothelial cells (HUVECs) were exposed to varying concentrations of Ap, collagen I and combinations thereof. The proliferative and chemotactic activities of HUVECs, as well as the protein expression of integrin αV, were strongly enhanced. The modification of collagen matrices with *Astragalus* polysaccharides of Ap with the cross-linking agent EDC leads to matrices with an increased angiogenic potential. The angiogenic capabilities of the modified collagen matrices appeared to depend on the Ap to EDC ratio. The presented results demonstrate that the incorporation of *Astragalus* polysaccharides into collagen matrices is an interesting and promising alternative for making wound dressings more angiogenic and improving their capabilities for covering tissue defects.

Keywords: *Astragalus* polysaccharide, VEGF, collagen matrices, *chorioallantois* membrane and angiogenesis

Introduction

Wound healing is a complex integrated sequence of cellular, physiologic, and biochemical events initiated by the stimulus of injury to tissue which contains mainly 3 stages, i.e. inflammation, proliferation and maturation [1]. Many factors influence the process of wound healing, among these factors nutrient supply is very important because cells cannot survive at distances larger than 1 mm from blood supply. There is thus a need for angiogenic tissue engineered biomaterials. The introduction of selected angiogenic growth factors (e.g. vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)) have been shown to be useful for enhancing angiogenesis [2]. Since simply admixing of these growth factors to the matrices generally leads to a rapid clearance from the defect site, several attempts to immobilize growth factors into three dimensional matrices have been made. Thus, Bentz et al. covalently coupled TGF-β_2 to injectable collagen by means of a homobifunctional cross-linking agent, and they observed a substantial slower release of the immobilized growth factor as compared to the admixed growth factor [3]. In another approach, Wissink et al. [4] made use of the heparin binding affinity of bFGF for physically binding this growth factor to heparin covalently incorporated into collagen films [5].

This procedure also led to a slow release of the growth factor from the collagen matrix [5]. Nevertheless, there are still some disadvantages in the application of angiogenic growth factors,

Incorporated, Shanghai, China), 1 ml of 10% fetal bovine serum (FBS), 20 μg of collagen I or 20 μg of collagen I with 20 μg of Ap dissolve in 1ml of 10% FBS were filled into the lower chamber. Cells were allowed to migrate for 6, 12 and 24 hours. After removal of the insert from the chamber, the unattached cells were washed out, attached cells were fixed with 10% formalin for 10 min. Then the non-migrated cells on the upper side of the filter were gently removed. Afterwards, the migrated cells were counted on a grid under high power field (amplification x 30), 5 high power fields were counted and the average was used for the results.

Western Blot analysis for evaluating the protein expression of angiopoietin 1, VEGF and integrin α_V

The total protein of the harvested cells was extracted by the cell keratoprotein extract solution (1 % Triton X-100, 20 mM Tris, 60 mM KCl, pH 7.0). Protein concentrations were determined by the Bradford method (Quantitate, MDBio, Inc, Qingdao, China). 40 μg of above extracted protein were run on the SDS PAGE and blotted on nitrocellulose. The blot was blocked for 2 hours with bovine serum albumin (BSA) and blocking was followed by incubation overnight with primary antibody (Rabbit anti-human angiopoietin 1; VEGF from Beijing Biosynthesis Biotechnology Co,.LTD, Beijing, China and rabbit anti-human integrin α_V from Cell Signaling Technology Inc. Boston, USA). The second antibody (goat anti-rabbit IgG-HRP, Beijing Biosynthesis Biotechnology Co,.LTD, Beijing, China) was added and incubation for 2 hours after PBS buffer rinsed 3 times. Finally, the exposed blots were quantitatively evaluated for angiopoietin 1, VEGF and integrin α_V with the ECL chromogenic system by using Image-J software analysis. β-actin was used as a loading control.

Chorioallantois membrane assay

Fertilized chicken eggs were obtained from Nanjing Agriculture Science Institute, Nanjing, China. The *chorioallantoic* membrane assay (CAM-assay) was performed essentially as described in Yao et al. [13] and Vargas et al. [15]. Collagen matrices modified with or without Ap immobilization were prepared according to the modification procedures described above and sterilized by immersion in 70% ethanol for 24h. Subsequently they were equilibrated with either PBS or serum free ECM under sterile conditions. Circular specimen of the various collagen matrices (diameter 12 mm, thickness 2 mm) were carefully placed on the *chorioallantoic* membrane and the eggs were put into a static incubator for 7 days at 37^{0}C. At the end of this period, the collagen implant was removed for subsequent analysis and the membrane surrounding the implant were excised and mounted on a slide and fixed with 2% formalin.

Quantitation of the capillaries was carried out by counting them at 50-fold magnification in three non-overlapping areas. The average number of small vessels (diameter smaller than 20 – 40 μm) in the defined areas was taken as an index for the angiogenic potential.

Animal model experiments

Collagen matrices (size: diameter 10 mm; thick 5 mm) modified to parameters described above were implanted in 2 dorsal subcutaneous pockets of Sprague Dawley rats, each one at a distance of 1 cm to the incision of the skin and at 4 cm distance between the implants. The different collagen modifications were implanted in different animals, they were either non-modified or modified with Ap1E1 as described in Materials and Methods at day 3, day 7 and day 14 day post implantation, the animals were sacrificed and the implants were explanted. Explants were weighed and used for determining their hemoglobin contents. Furthermore a sample of tissue surrounding the implant was excised and used for determining the hydroxyproline content.

Evaluation of hemoglobin contents with ELISA

Explants were immediately washed with water for removing attached blood, samples were then put into a reagent tube with 1 ml of water. Slightly coloured solutions were stored at -20^{0}C for determining the hemoglobin concentration by ELISA (CSB-E13036r Kit from Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturers' procedure. The hemoglobin contents were determined in μg hemoglobin/g wet tissue.

Determination of the hydroxyproline content in tissue samples surrounding the implant with ELISA

Tissue samples surrounding and attached to the explants were cut off and weighed after lyophilization. Samples were submersed in 1 ml

such as their short half life time, and the possibility of being tumorogenic.

Many plants have been proposed for the natural therapies by supporting angiogenesis [6]. Among the Chinese traditional herbs, the root of *Radix Astragalis* is widely used to enhance the human resistance to disease and to treat chronic ulcerations [7]. Extracts from *Radix Astragalis* (Astragalus polysaccharides) is one of the main extracts of *Radix Astragalis)* have been shown described to enhance the proliferation of vascular endothelial cells [8,9], to improve the adherence to the ECM [10] and to modulate the immune activities in rats with gastric cancer [11]. We previously observed that loading of *Astragalus* polysaccharides into collagen matrices leads to similar angiogenic effects as those observed earlier for rhVEGF$_{165}$ loading to collagen matrices [12].

Here we report on experiments to covalently incorporate *Astragalus* polysaccharides in collagen matrices for attempting to improve the angiogenic behaviour of these scaffolds for being applied as temporary dressings for treating tissue defects in e.g. burn wounds. The modification effects will be evaluated by exposing the modified matrices to the *chorioallantoic* membrane and by subcutaneous implantation. Furthermore we monitored the proliferative and migratory effects of soluble collagen and *Astragalus* polysaccharides on HUVECs in proliferation assays and chemotaxis experiments. Moreover we report on the effects of collagen I and *Astragalus* polysaccharide on the expression of angiopoietin1, VEGF and integrin α_V.

Materials and Methods

Collagen matrices

The matrices were obtained through lyophilization of collagen suspensions of mainly collagen type I (Beijing Yerkang Bioengineering Development Center, Beijng, China). The collagen matrices were cut into circular specimen (diameter 10 and 12 mm; thickness 2 and 5 mm).

Covalent incorporation of Astragalus polysaccharides

Immobilization of *Astragalus* polysaccharides (Ap) was performed essentially as described in Yao et al. [13] and Wissink et al. [5]. Carboxylic acid groups of Ap were activated with 1-Ethyl-3 (3-dimethylaminopropyl) dicarbodiimide (EDC) and N-hydroxy-succinimide (NHS). Ap was purchased from Beijing Centre Biology Co., Ltd and EDC and NHS were from Sigma-Aldrich. A typical modification experiment is performed as follows: 1 mg Ap were activated with 1 mg EDC/0.6 mg NHS in 500 µl 0.05 M MES-buffer (pH 5.6) for 10 minutes at 37°C, collagen specimen were immersed into the reagent solution and the solutions were evacuated to remove the air from the specimen. After a reaction period of 4 h at 37°C, under gentle shaking, the collagen matrices were washed with 0.1 M Na_2HPO4 (2h), 4M NaCl (4 times in 24 h) and deionized water (5 times in 24 h). Finally, modified sponges were frozen overnight at –80°C, lyophilized and stored at room temperature. Collagen matrices prepared according to these parameters are designated Ap1E1 (1 mg Ap; 1 mg EDC/0.6 mg NHS). The modification parameter Ap1E2 refers to 1 mg Ap and 2 mg EDC/1.2 mg NHS, Ap0E0 refers to collagen matrices which went through all the procedures, except that no Ap and EDC/NHS were added.

Endothelial cells

Human umbilical vein endothelial cells were isolated from the umbilical cord and cultivated in endothelial cell basal growth medium (ECBM, Cell Systems, Shanghai, China) [14]. For the experiments only 2nd passage cells were used.

MTT assay for cell proliferation evaluation

In each well of a 6-well plate 100,000 HUVEC cells were allowed to adhere for 1 day in 2 ml of the ECBM. On day 2 in each well a series of different Ap concentrations (0 to 120 µg/ml) and/or different collagen I concentrations (from 0 to 40 µg/ml) were placed in the wells. For reference, on each plate one well was filled with ECBM only.

After incubation for 24 hours, 20µl of Cell Counting Kit-8 (Beyotime Biology Co. Ltd, Shanghai, China) were transferred to each well and followed by incubating for additional 24 hours. After this period, absorption at 490nm was measured and calculated into proliferation rates and expressed in arbitrary units.

Chemotaxis assay with transwell chamber

100,000 HUVECs were placed in the upper chamber of Transwell chambers (8 µm; Corning

buffer (0.01 mol/L Tris-HCL, 0.0001 mol/L EDTA-2Na, 0.01 mol/L Sucrose, 0.8% NaCl, pH 7.4) and homogenized ultrasonically for about 30 seconds in an ice bath, this procedure was repeated several times until the tissue was disintegrated and almost dissolved. The cell remnants were precipitated by centrifugation at 1000 x g for 15 minutes and the hydroxyproline contents in the supernatants were determined by ELISA (CSB-E08838r Cusabio Biotech Co., Ltd., Wuhan, China) according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis was performed by using the software package SPSS for Windows 13 (SPSS Inc.). For evaluating the significance the Student's T-test was used: p-values <0.05 were considered to be statistically significant.

Results

Initially we prepared a number of collagen matrices, which were modified according to the specifications in Materials and Methods. Modified and non-modified matrices were exposed to the *Chorioallantois* membrane as described earlier [13, 17]. The exposure of unmodified matrices (Ap0E0) to the membrane induced increases of the capillary numbers by 13±4 as compared to the control (no implant). The incorporation of Ap at higher EDC/Ap-ratios (Ap0.5E1; Ap1E1) led to increases of the capillary numbers with 27±14 and 37±9 (n=10) resp., these numbers were significantly different from the control group (p<0.01) (**Figure 1**). The incorporation of higher Ap to EDC ratios e.g. Ap2E1 led to an increase of the number of capillaries with about 26, which is similar to the effects exerted by Ap0.5E1 matrices, i.e. matrices with a lower Ap to EDC ratio. Altogether, the Ap1E1-matrices induced more capillaries than Ap0.5E1- and Ap2E1-matrices, the differences between the Ap0E0 and Ap1E1 matrices were statistically significant (p<0.01).

Next we evaluated the angiogenic capabilities by subcutaneously implanting unmodified (Ap0E0) and modified (Ap1E1) in rats. The implants were explanted after periods of 3, 7 and 14 days, the specimen were taken out carefully and attached surrounding tissues were excised for evaluation of the hydroxyproline contents and CD34+-cell recruitment (see below). The

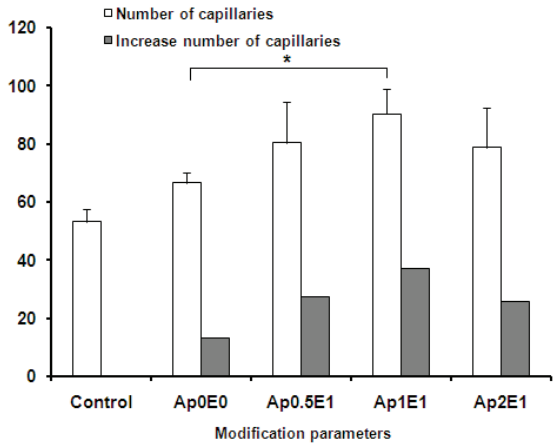

Figure 1. Evaluation of angiogenic potential by exposing the chicken *chorioallantoic* membrane to unmodified (Ap0E0) and modified (Ap0.5E1 – Ap2E1) collagen matrices. The increases in the number of capillaries were obtained by subtracting the mean number of capillaries from the number of capillaries observed in control experiments (no implant). Columns show the mean values, error bars represent the corresponding standard deviations (n=10). * indicates statistically significant differences (p<0.05).

explants were carefully washed and the hemoglobin contents in the washing solutions were determined by ELISA. **Figure 2** demonstrates that Ap1E1 explants after implantation periods of 7 and 14 days were characterized by significantly increased hemoglobin contents as compared with the explants of the unmodified matrices Ap0E0. This effect was also clearly observed by visual evaluation (photographs not shown). At day 7, the hemoglobin contents in the Ap1E1-matrices had increased by 100% as compared with the Ap0E0-matrices. At day 14, the hemoglobin contents of the Ap1E1-matrices were still 82% higher than those of control matrices (Ap0E0).

Simultaneous with the explantation of the implants, tissue samples attached to the matrices were excised carefully. Half of the tissue samples were lyophilized, weighed and used for the determination of the hydroxyproline contents, the other half were used for evaluating the recruitment of CD34+-cells. The contents of both the unmodified (Ap0E0) and modified (Ap1E1) explants increased with the length of the implantation period, after 14 days the hydroxyproline contents had increased by 140%

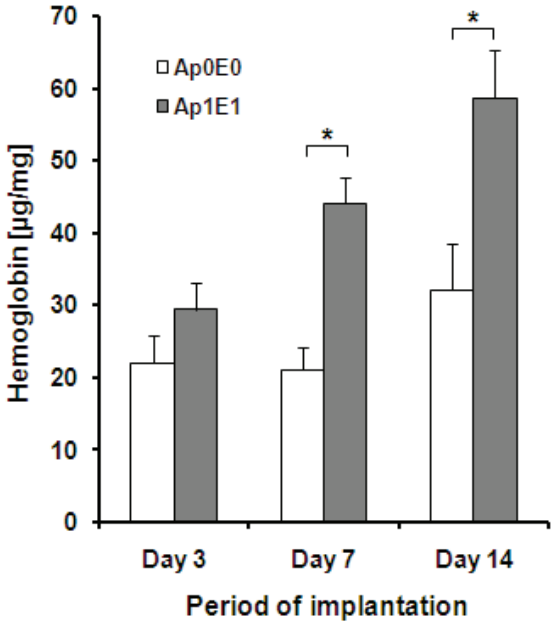

Figure 2. Evaluation of angiogenic potential by animal model experiments. Unmodified (Ap0E0) and modified (Ap1E1) collagen matrices were implanted subcutaneously in rats. Implants were explanted after 3, 7 and 14 days. Hemoglobin contents of explants were determined by ELISA as described in Materials and Methods. Columns show the mean values, error bars represent the corresponding standard deviations (n=5). * indicates statistically significant differences ($p<0.05$).

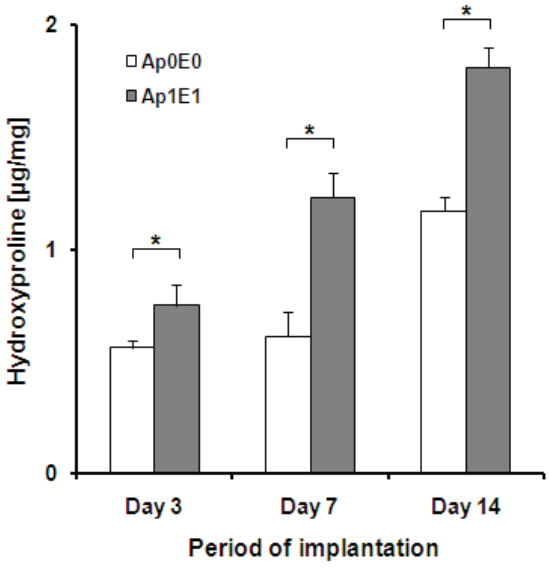

Figure 3. Animal model experiments: the amount of hydroxyproline in the excised tissue samples surrounding the implants during 3, 7 and 14 days. The hydroxyproline contents of the tissue samples were determined by ELISA as described in Materials and Methods. Columns show the mean values, error bars represent the corresponding standard deviations (n=5). * indicates statistically significant differences ($p<0.05$).

as compared to the contents after 3 days of implantation. The hydroxyproline contents of the tissues attached to the Ap1E1 explants, however, were after all implantation periods significantly higher than the tissues attached to the unmodified (Ap0E0) explants (**Figure 3**).

The other half of the tissue samples were used for (evaluating the recruitment) identifying of CD34+-cells by immunohistochemistry. **Figure 4** shows that the surrounding tissues of the modified matrices (Ap1E1) explanted on day 14 were characterized by a much more intense staining of CD34+ cells as compared to the unmodified matrices (Ap0E0). These results were in accordance with the results of hemoglobin contents and the increases in hydroxyproline contents as shown in **Figure 3**.

We then evaluated whether the proliferation rates of HUVECs were dependent on varying concentrations of *Astragalus* polysaccharides (Ap). Ap concentrations varied from 0 to 80 µg/ml. The results show that the optimal concentration of Ap was 20 µg/ml with an almost 100% increase as compared to the control group. Lower and higher concentrations of Ap led to less good results.

In subsequent experiments we investigated the effects of a series of different concentrations of Ap and collagen I on the proliferation of HUVECs. Proliferation was evaluated with the MTT assay. Results obtained with medium were calculated to give a proliferation index of 1. The addition of Ap to the medium had a positive effect on the proliferation of HUVECs (Figure 5A). Effects were maximal after adding 20 µg Ap/ml. **Figure 5B** show that concentrations of 10 and 20 µg/ml of collagen I induced proliferation increases of 100% and 115% resp. The concentration of 40 µg/ml of collagen I was, however, leading to a reduced proliferation (0.66 *vs* 0.84). Next, we tried to obtain more optimal proliferation conditions by mixing 20 µg/ml of Ap and 20 µg/ml of collagen I. Results

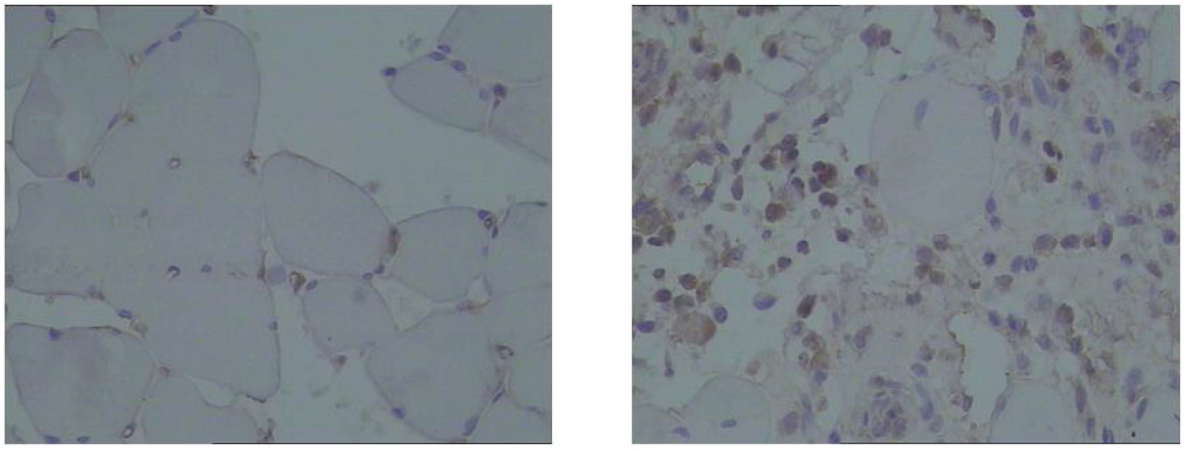

Figure 4. Collagen matrices with incorporated Astralagus polysaccharides attract more CD34+-cells. **A**. Unmodified (Ap0E0) and **B**. modified (Ap1E1) collagen matrices were implanted subcutaneously and explanted after 14days implantation. Sections of the explant were stained for CD34-positive cells by immunohistochemistry.

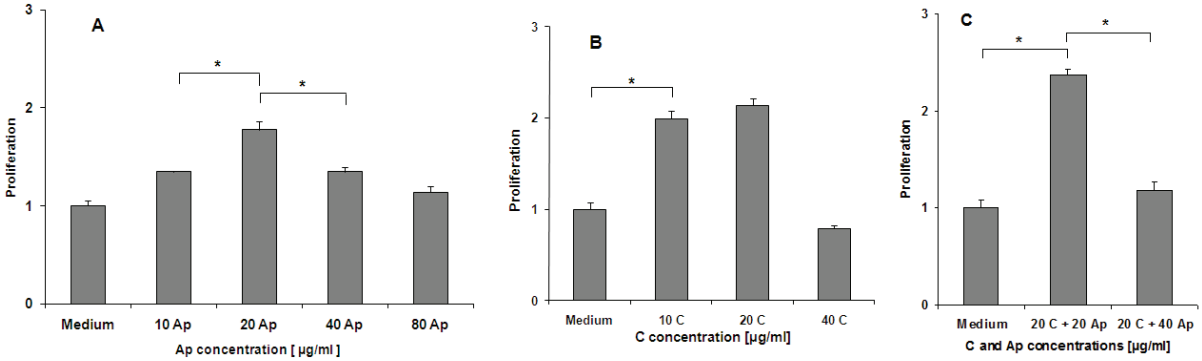

Figure 5. Proliferative exerted effects by *Astralagus* polysaccharides, collagen I and mixtures thereof, on HUVECS. **A**. Effects of Ap concentrations (from 0 µg/ml to 80 µg/ml) on the proliferation of HUVECs. **B**. Proliferative effects of collagen I (C). **C**. Effects of collagen I/Ap mixtures on HUVECs. Results given in arbitrary units normalized to the medium. Columns show mean values, error bars represent the corresponding standard deviations (n = 3). * indicates statistically significant differences ($p<0.05$).

demonstrated that this mixture leads to an even higher proliferation rate than those of 20 µg/ml of Ap (2.00 *vs* 0.85) and 20 µg/ml of collagen I (2.00 *vs* 1.80) (**Figure 5C**). The mixture of 20 µg/ml of Ap and 40 µg/ml of collagen I led to a lower proliferation of HUVECs. From these results, we conclude that a mixture of 20 µg/ml of Ap and 20 µg/ml of collagen I appears to be optimal for stimulating cell proliferation.

Next we used these experimental conditions for further testing the effects on directed cell migration and on the protein expression of angiopoietin 1, VEGF and integrin α_V.

Figure 6 shows the results of chemotaxis experiments. The lower chambers of the chemotaxis setup were filled according to 3 different conditions: 10% FBS (control), 20 µg collagen I/ml, 20 µg collagen I plus 20 µg Ap/ml. Chemotaxis was evaluated at three time points: 6h, 12h and 24h. Collagen I induced in the presence of 20 µg collagen I significant increases of the chemotactic activity with 42% and 41% after 12 and 24 h resp. . Upon loading 20 µg collagen I and 20 µg Ap/ml to the lower chamber increases of the chemotaxis with 73% and 71% after 12 and 24h resp. as compared to the control conditions were observed. At 6 hours, no significant differ-

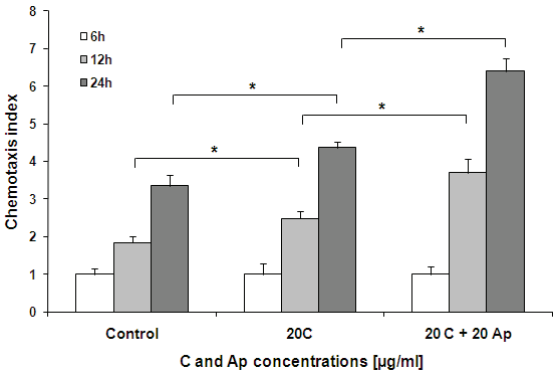

Figure 6. Chemotactic activity of HUVECs as function of variable concentrations of soluble collagen I in combination with Ap in Transwell assays. 20 µg/ml of collagen I (20 C) or 20 µg/ml of collagen I and 20 µg/ml of Ap (20 C + 20 Ap) were dissolved into the lower chamber of the Transwell setup. Medium was used as control. Columns show mean values, error bars represent the corresponding standard deviations (n = 3). * indicates statistically significant differences ($p<0.05$).

ences between the 3 groups could be observed.

In order to elucidate the underlying mechanism for enhancing HUVECs proliferation and migration, we evaluated the effects of the Ap/collagen mixtures on the expression of angiopoietin 1, VEGF and integrin α_V by Western blot analysis. **Figure 7** shows the expression of angiopoietin 1, VEGF and integrin α_V in HUVECs in contact with various concentrations of Ap and collagen I and mixtures thereof. Whereas the protein expression of angiopoietin 1 and VEGF demonstrated only little variation, the expression of integrin α_V was characterized by a large increase: the protein expression being increased 40-fold. The expression of VEGF was maintained at a high level and did not change; the protein expression of angiopoietin 1 appeared to occur at a lower level and did not change.

Discussion

A common approach in tissue engineering is to apply highly porous matrices in which invading cells have the potential to develop into the tissue of interest and then implanting this construct for development *in situ*. Major concerns still exist in relation with implants not being adequately vascularized for long-term survival. Both mass transfer limitations and fibrosis associated with the foreign body response can contribute to failure of the implantation. Therefore, biomaterials with improved angiogenic capabilities might be of great interest for the development of scaffolds for tissue regeneration in wound healing. Up to date, many methods have been used for enhancing the angiogenic functions of biomaterials, among these are the incorporation of angiogenic growth factors such as basic fibroblast growth factor (bFGF) [4,5]

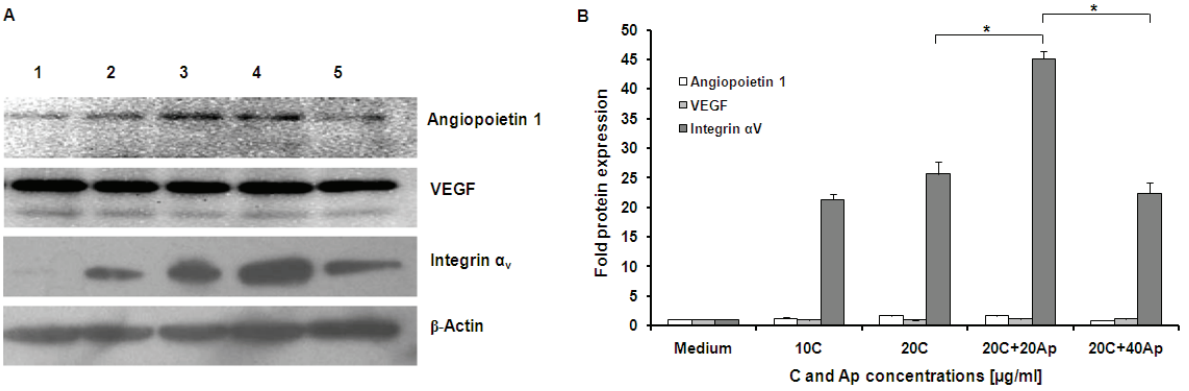

Figure 7. Expression levels of angiopoietin 1, VEGF and integrin α_V observed in HUVECs exposed to variable concentrations of collagen I (C) and *Astragalus* polysaccharides (Ap) as determined by Western blot analysis. **A**. Western blot analysis of angiopoietin 1, VEGF, integrin α_V and β-actin. The different lanes refer to experimental conditions. HUVECs were exposed to: Lane 1: Medium; Lane 2: 10 µg collagen I/ml; Lane 3: 20 µg collagen I/ml; Lane 4: 20µg collagen I + 20µg Ap/ml; Lane 5: 20µg collagen I + 40µg Ap/ml. **B**. Columns show intensities as observed by Western blot analysis. Intensities were normalized to the expressed β-actin. Columns show mean values, error bars represent the corresponding standard deviations (n = 3). * indicates statistically significant differences ($p<0.05$).

and vascular endothelial growth factor (VEGF) [13,17]. Although quite some progress has been achieved in the development of biomaterials by incorporating angiogenic growth factors, there are still shortcomings, such as short half-life time of the growth factor and problems with sterilization as well as shelf storage [18].

Radix Astragalis, known as one of the most widely used tonic drugs, is considered to convey resistance to many human diseases and to heal chronic ulcerations [19]. It can ameliorate the impairment of vascular endothelial cells [7] and enhance their proliferation [8] and adherence to the ECM [9]. The injection of astragal has been used in clinical practice for many years to cure coronary artery disease with good therapeutic results and only little side effects have been reported [20]. The *Astragalus* polysaccharides (Ap) are easily extracted from the *Astragali* root and applications are cost-efficient. They have been shown to improve angiogenesis through increasing proliferation and migration of HUVECs *in vitro* [21]. Therefore, we thought it might be interesting to incorporate Ap into collagen matrices by creating a zero-length amide cross-link using the water soluble carbodiimide EDC in conjunction with NHS. The angiogenic properties of the modification products were evaluated with the *chorioallantoic* membrane assay (CAM), results show that the modified matrices Ap1E1 were more angiogenic than the non-modified matrices Ap0E0 and the modified matrices Ap0.5E1 and Ap2E1.

The next step was to confirm these results by implanting modified and non-modified collagen matrices into subcutaneous pockets of rats and to evaluate the hemoglobin contents within the collagen matrices and hydroxyproline contents in the surrounding tissues. The highest hemoglobin contents were observed with the A1E1 matrices. Since more blood supply means more nutrients and therefore more tissue regeneration, we evaluated the changes of the dry weights of the explanted collagen matrices. These dry weights are assumed to correspond to the amount of invaded cells and deposited ECM components [19]. The obtained results were in accordance with the hemoglobin contents. This time we also tried to monitor the effects of the implanted collagen matrices on the surrounding tissue by determining their hydroxyproline contents. We observed that the hydroxyproline concentrations in the tissue samples in close vicinity of the modified collagen matrices of Ap1E1 were much higher than those of their non-modified counterparts. Furthermore, the immunohistochemical pathological sections demonstrated an increase of CD_{34}-positive cells indicative of an improved vascularization in the neighbourhood of the Ap1E1 matrices as compared with those of Ap0E0. These data confirmed the initial observations of Ap1E1-matrices being more beneficial to the growth and regeneration of the surrounding tissue.

Zhang et al. [22] proposed that 20 μg Ap/ml was an optimal concentration of Ap for vascular endothelial cell proliferation. In line with this observation we performed experiments with HUVECs for evaluating the effects of variable concentrations of Ap and collagen I on their proliferation. In accordance with Zhang et al. [22], we observed that 20 μg Ap/ml is indeed an optimal concentration and in addition, we observed that a concentration of 20 μg collagen I/ml was optimal for proliferation. Higher Ap concentrations provoked, in accordance with the *in vivo* results obtained from the CAM assays, less good results. Furthermore, we investigated whether HUVECs migration assay for the evaluation of the interaction of suitable concentration of Ap and collagen compared with the suitable concentration of collagen. The results revealed that the concentrations which were optimal for proliferation also showed the strongest effects on the chemotaxis of HUVECs.

Furthermore we investigated whether soluble collagen I and Ap, as well as mixtures thereof, had effects on the expression of a number of proteins relevant for endothelial cell behaviour by Western analysis. The protein expression of integrin α_V was observed to be increased to a larger extent as compared to the protein expression levels of angiopoietin1 and VEGF. As we know, endothelial cell growth, proliferation and differentiation also depend on the recruitment of specific integrins on the cell surface. These integrins must bind to its proper ligands in order to initiate distinct signalling pathways that finally lead to the transformation of the endothelial cell into a more angiogenic phenotype [23]. Therefore, we assume that the induction of the up regulation of integrin α_V may be part of the mechanism leading to the improvement of the angiogenic potential of Ap1E1-matrices. It was also found that integrins α_V have strong effects

on the vascular endothelial cells migration, which may explain why we observed more intense effects of the modifications on the cell migration than on proliferation.

Though it has been concluded previously from experimental and clinical research that Ap are beneficial to angiogenesis and ischemic diseases, such as myocardial ischemia, Ap have so far not been used to enhance angiogenesis in collagen matrices. To our knowledge, this manuscript represents the first investigation of incorporating covalently *Astragalus* polysaccharides into collagen matrices and investigating the effects on their angiogenic capabilities. We earlier observed that the cross-linking of collagen itself had a positive angiogenic effect and that VEGF loading only gave rise to relatively small additional effects [24]. From this paper, we conclude that the observed positive effect of cross-linking of collagen scaffolds by EDC/NHS might be due to the up regulation of the expression of integrin α_V, since the expression levels of VEGF and angiopoietin 1 did not change upon contact of endothelial cells with cross-linked collagen. In the future, we will expose endothelial cells and to cross-linked and non-cross-linked collagen matrices and evaluate their effects on relevant signal transduction pathways.

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