Accelerated angiogenesis by continuous medium flow with vascular endothelial growth factor inside tissue-engineered trachea ☆

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Abstract

Objective: To test the effects of a continuous medium flow inside DegraPol® scaffolds on the reepithelialization and revascularization processes of a tissue-engineered trachea prosthesis. **Methods:** In this proof-of-principle study a continuous medium flow was maintained within a tubular DegraPol® scaffold by an inserted porous catheter connected to a pump system. The impact of the intra-scaffold medium flow on the survival of a tracheal epithelial sheet wrapped around and on chondrocyte delivery to the DegraPol® scaffold was studied. In the chick embryo, chorioallantoic membrane (CAM) model angiogenesis within the biomaterial was investigated. **Results:** Scanning electronic microscopy (SEM) images showed an intact epithelial layer after a 2-week support by continuous medium flow underneath. On histology, three-dimensional cell growth was detected in the continuous delivery group. The CAM assay showed that angiogenesis was enhanced within the DegraPol® scaffolds when vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) was added to the perfusate. **Conclusions:** Taken together, these results demonstrated that the built-in perfusion system within DegraPol® scaffolds was able to maintain an intact tracheal epithelial layer, allowed a continuous delivery of cells, and kept an efficient VEGF/VPF expression level which accelerated angiogenic response in the CAM assay. This design combines the in vitro and in vivo parts of tissue engineering and offers the possibility to be used as an in vivo bioreactor implanted for the tissue-engineered reconstruction of trachea and of other organs.
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1. Introduction

Tissue engineering, aimed at alleviating donor organ shortage by providing an alternative treatment for tissue and organ failure, has emerged as a promising new field in medical science [1]. Led by the illusionary simplicity of developing a tubular cartilage tissue to replace the native trachea, many scientists focused their research on tissue-engineered trachea assuming it to be a straightforward procedure. Unfortunately, all animals died within 1 week when a circumferential replacement of the trachea was performed with a tubular cartilage tissue only. Autopsies showed lethal airway obstructions caused by granulation tissue ingrowth or by sputum retention [2]. These results

have highlighted the importance of the reepithelialization process to achieve a functional trachea substitute [3–5]. The experience gained from tissue-engineered skin demonstrated that epithelial cells have better survival chances on a well-vascularized wound surface [6,7], a clear proof that revascularization is the essence of reepithelialization.

In contrast to other parenchymal organs the trachea is supplied by a network of small vessels that makes direct revascularization difficult. Microanastomoses were attempted, but that is not the way to follow [8]. Even immediate orthotopic tracheal replacements were doomed to fail as the capillary networks only penetrate into the implant for no more than 2 cm (i.e., 4 cm bilaterally) across the two anastomoses. This revascularization process normally requires several months [9]. In an implant longer than 3 cm the centrally located epithelial cells die soon after implantation and fail to maintain an intact basement membrane causing tracheal stenosis by granulation tissue hyperplasia.

To improve the survival of a continuous epithelial layer and to accelerate the revascularization process of a tissue-engineered trachea substitute, a perfusion system was designed inside a tubular DegraPol® polymer [10,11], allowing medium to flow and to permeate throughout the scaffold. As it has been proved difficult to successfully simulate an in vivo regenerative environment in vitro [12], the tissue-engineered trachea with its integrated perfusion system is suggested to be implanted into the recipient as soon as possible. After implantation the intra-scaffold perfusion system will be connected to an extra-corporeal pump system which works as nutrition assist device for the avascular tissue-engineered trachea to maintain an intact tracheal epithelial cell layer throughout the whole regenerative period until the slow process of revascularization has been achieved. During this period the recipient will function as an in vivo bioreactor for the implanted tissue-engineered trachea. Further advantages of this in vivo bioreactor design include the possibility of continuous or intermittent cell seeding and the delivery of growth factors to accelerate the revascularization process of newly implanted tissue-engineered prostheses.

In this study we describe a simple proof-of-principle model to demonstrate the feasibility of such an approach.

2. Materials and methods

2.1. Description of the perfusion model

A catheter (6F angiographic catheter, 1 mm inner diameter, Cordis Corporation, Spreitenbach, Switzerland) was inserted into the lumen of a DegraPol® tube (Institute of Polymer Research ETHZ, Zurich, Switzerland) of 2 cm in length, 2 mm in inner diameter, and 6 mm outer diameter. Half of the catheter wall located inside the DegraPol® lumen was cut to allow the medium leak out of the catheter and permeate into the scaffold. The ends of the catheter were connected to two peristaltic pumps (IPC high-precision multi-channel dispenser, ISMATEC, Zurich, Switzerland) through 60-cm long Tygon LFL tubes (1.6 mm inner diameter, ISMATEC, Zurich, Switzerland). One pump continuously supplied perfusate into the scaffold while the other removing the waste products both at a steady volumetric flow of 5 ml/h (Fig. 1A).

2.2. Reepithelialization test

The human tracheal epithelial cell line 16HBE14o (generously provided by Dr Jordi Ehrenfeld, Faculté des Sciences de Nice, France) was cultured as published previously [13]. In short, cells were cultured in Dubecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 584 mg/l L-glutamine, 50 μg/ml gentamycin, 100 μg/ml streptomycin, and 100 U/ml penicillin G, incubated at 37 °C with 5% CO_2. Medium was changed every other day. Cells were trypsinized using trypsin/EDTA solution (0.05%/0.02%, PAN Biotech, Aidenbach, Germany) and 1×10^7 cells were harvested and seeded onto each xenogenic (porcine) acellular dermal matrix (ADM) (Qidong Biotechnologies Ltd., Shanghai, China), 2 cm × 2 cm in size,
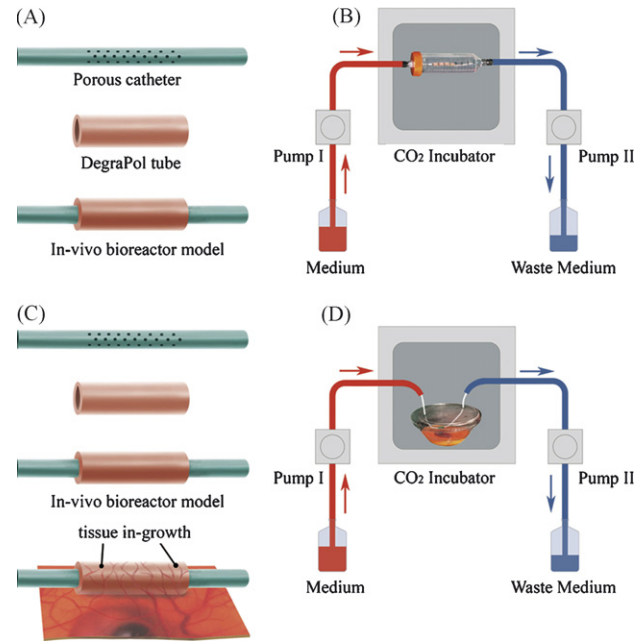

Fig. 1. Scheme for the assembly of an in vivo bioreactor model (A); cell delivery test model, 10^7 rat chondrocytes were added into the medium daily. A centrifuge tube was used to protect the DegraPol® scaffold (B); CAM angiogenesis test model (C,D). The perfusate was changed daily and the flow rate remained at a steady volumetric flow of 5 ml/h in all the tests. CAM: chick embryo chorioallantoic membrane.

and 0.02 cm in thickness [14,15]. Stored in an incubator overnight for cell attachment, five ADM scaffolds seeded with epithelial cells were then wrapped around a DegraPol® tube connected to the perfusion pump system and cultured in an incubator for 2 weeks, at 37 °C with 5% CO_2 (Fig. 1B). Scanning electronic microscopy (SEM) was performed to monitor the continuity of the epithelial layer on the surface of the ADM after 2 weeks.

2.3. Cell delivery test

The same perfusion model was used for delivering chondrocytes to DegraPol® scaffolds. Rat chondrocytes were harvested from Lewis rat xiphoids as previously described by our group [16]. In brief, minced cartilage particles were digested with 0.3% collagenase II (PAN Biotech, Aidenbach, Germany) solution in fresh Ham's F12 medium at 37 °C for 16–18 h; the resulting suspension was centrifuged at $400 \times g$ for 5 min and the chondrocyte pellets were resuspended in Ham's F12 medium with 248.54 mg/l stabilized L-glutamine, 50 μg/ml of gentamycin, and 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) and cultured in 75-cm^2 vented polystyrene cell culture flasks (Costar 3376, Corning, Inc., Corning, NY, USA) in an incubator at 37 °C with 5% CO_2. The medium was changed twice a week. Cells were trypsinized by trypsin/EDTA solution for use. Rat chondrocytes 1×10^7 were harvested daily and resuspended in 250 cc Ham's F12 medium in one 75-cm^2 vented polystyrene cell culture flask which was connected to the inlet pump through a Tygon LFL tube (1.6 mm inner diameter, 60 cm in length, ISMATEC, Zurich, Switzerland). This cell suspension was administered continuously into the DegraPol® scaffold while the outlet pump

removed the fluid with waste products at a steady volumetric flow of 5 ml/h. Topically seeded and static culture methods were used as controls with 1×10^7 rat chondrocytes directly seeded onto a DegraPol® tube of equal size as described above. The samples were then placed overnight for cell attachment and subsequently immersed in 20 cc Ham's F12 medium in a culture dish. The cell-scaffold construction was subsequently cultured in a CO_2 incubator for 2 weeks with 1×10^7 rat chondrocytes reseeded daily. Each group contains five DegraPol® tubes; 2 weeks later the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and histological assessments were performed to detect viable cells within all the scaffolds.

2.4. Angiogenesis assay

We used the ex ovo chorioallantoic membrane (CAM) model of chick embryos [17] to test the impact of the in vivo bioreactor system on angiogenesis. On incubation day 12, sixteen CAMs were randomly allocated into four groups with four CAMs in each group. Part of the surface of the CAM membrane was wounded by a tiny drop of alcohol at the contact site of the DegraPol® tube (1 cm long, 2 mm inner diameter). Four different groups were studied: (I) static control: 1 cm DegraPol® tube only; (II) VEGF pre-coated: DegraPol® tubes were immersed in 5 cc F12 medium containing 4 μg/ml of canine VEGF164 [18] (gift from PD Dr Rolf Jaussi, Paul Scherrer Institute, Villigen, Switzerland) for 1 h before the experiment; (III) in vivo bioreactor: a porous catheter was inserted through the DegraPol® tube and connected to the perfusion pump system as described above. DMEM was used as perfusate at a steady volumetric flow of 5 ml/h (Fig. 1C,D); (IV) in vivo bioreactor plus VEGF: same setting as that in group III, except VEGF164 (40 ng/ml) added to the perfusion medium. After 5 days all samples were collected for histological examinations to assess angiogenesis within the DegraPol® scaffolds.

2.5. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT assay determines viable cell numbers and is based on the mitochondrial conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Briefly, DegraPol® scaffolds seeded with chondrocytes were harvested and placed in a 6-well culture plate. To each well 500 μl medium and 40 μl MTT solution were added and incubated at 37 °C for 1 h. The supernatant was discarded and replaced by 800 μl isopropanol with 10% formic acid. Samples were incubated at 37 °C for an additional 5 min, vortexed for 10 min, and the MTT absorbency values of the resulting solution were measured using an ELISA reader (Dynatech 5000, Dynatech, Billinghurst, UK) at a wavelength of 570 nm.

2.6. Histological and scanning electron microscopy (SEM) analysis

Samples for the SEM examination were fixed in 2% phosphate-buffered glutaraldehyde solution, then dehydrated with a graded isopropanol series, and air-dried. Before analysis, the dried samples were mounted on aluminum supports and sputter-coated with gold.

DegraPol® samples from the cell delivery tests were fixed for histological analysis with 4% phosphate-buffered formalin, embedded in paraffin, and sectioned using standard histochemical technique. Slides were stained with hematoxylin and eosin (H&E).

One hour prior to the sample harvest, the CAMs were injected i.v. with bisbenzimide H 33342 [19] (100 μl, 1 μg/ml, Fluka, Buchs, Switzerland). Frozen sections of DegraPol® samples were imaged for bisbenzimide H 33342 fluorescence using a 360 nm excitation filter and a 450 nm long-pass emission filter. The imaging system consisted of a fluorescence microscope (Leica DM RE, Leica Microsystems, Wetzlar, Germany), a digital camera (Color-CCD Camera 'RETIGA', QImaging, Burnaby, Canada), and an image software (Leica lite, Leica Microsystem, Wetzlar, Germany).

2.7. Statistics

The MTT test data were expressed as mean ± standard deviation. We used the SPSS 8.0 software for statistical analysis. An unpaired t-test was performed, at a given p-value of less than 0.05, which was considered as statistically significant.

3. Results

3.1. Reepithelialization test

The SEM results demonstrated an intact monolayer of tracheal epithelial cells on the surface of acellular dermal matrix after a 2-week support by the in vivo bioreactor device in all the samples (Fig. 2). These cells were physiologically exposed to ambient air instead of having been unphysiologically immersed in the medium during the whole culture period.

3.2. Cell delivery test

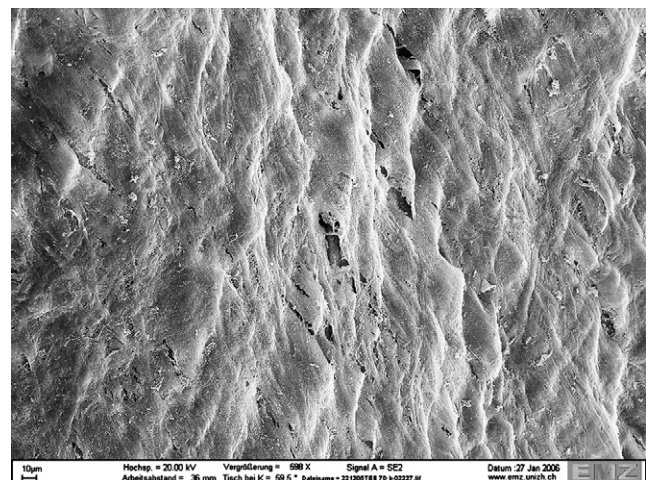The MTT test results showed no significant difference ($p > 0.05$; Fig. 3) between the perfusion delivery group

Fig. 2. SEM demonstrating an intact monolayer of epithelial cells on the surface of xenogenic (porcine) ADM wrapped outside DegraPol® scaffold with a continuous medium flow inside after 2 weeks. ADM: acellular dermal matrix.
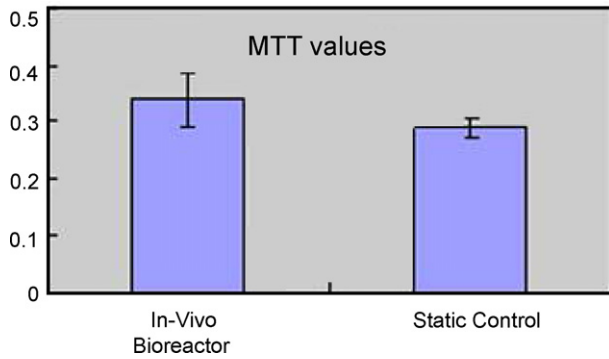
Fig. 3. MTT values at 570 nm showing no significant differences between the in vivo bioreactor group and the conventional topically seeded and static culture group.

Fig. 5. (A) Frozen section showing vessel formation in the ingrowth CAM tissue of the DegraPol® scaffold in group IV ($\times 200$). (B) Bisbenzimide H 33342 staining marked the inside erythrocyte nuclei red showing normal blood vessel functions.

(0.312 ± 0.177) and the topically seeded static culture group (0.288 ± 0.053). The histological results demonstrated a better three-dimensional clone formation inside the Degra-Pol® scaffolds in the perfusion group while there was only a monolayer of chondrocytes formed on the surface of the samples in the static culture group (Fig. 4). Chondrocytes could be efficiently delivered to the scaffolds via the perfusion system and the continuous medium flow improved the extent of proliferation and even distribution of seeded cells inside the scaffolds. It proved to be a promising approach to combine cell seeding and cell culture systems in tissue engineering research.

3.3. Angiogenesis assay

Histological slices from groups I and II showed almost no sign of tissue ingrowth inside the DegraPol® scaffolds, while samples from groups III and IV displayed more than two-third tissue invasion. No blood vessels were found inside the DegraPol® scaffolds in the first three groups, while bisbenzimide H 33342 injections revealed normal functional capillary formation inside the ingrowing tissue in group IV (Fig. 5). Erythrocytes were detected throughout the scaffolds due to the increased vascular permeability which was specifically induced by the circulating VEGF, formerly called vascular permeability factor (VPF) (Fig. 6). In one out of four experiments in group IV we found in histology that the ingrowing CAM tissue completely biodegraded the lower parts of the DegraPol® tube within 5 days (Fig. 7). The

Fig. 6. (A) In all the samples from angiogenesis test group IV erythrocytes migrating throughout the whole DegraPol® scaffolds, even at the upper part without any ingrowth of connective tissue, due to an increased vessel permeability caused by VEGF/VPF (H&E, $\times 200$). (B) Red bisbenzimide H 33342 staining of erythrocyte nuclei proving their migration from normal functional vessels. VEGF/VPF: vascular endothelial growth factor/vascular permeability factor.

continuous medium flow benefits the biocompatibility characteristics of the biomaterial scaffold to facilitate tissue ingrowth and also provide a straightforward approach for the delivery of growth factors.

4. Discussion

In this study we advanced a novel concept of in vivo bioreactor defined as the design of a perfusion system inside the scaffold for tissue-engineered trachea reconstruction. In contrast to traditional bioreactor designs where the cell-
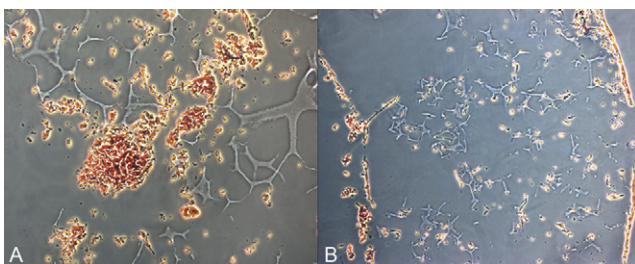

Fig. 4. (A) Histological section of the in vivo bioreactor group showing a three-dimensional chondrocyte cluster formation inside the DegraPol® scaffold. (B) In comparison, only a monolayer of chondrocytes covered the surface of the DegraPol® tube in the topically seeded and static culture group. (H&E staining, $\times 100$).
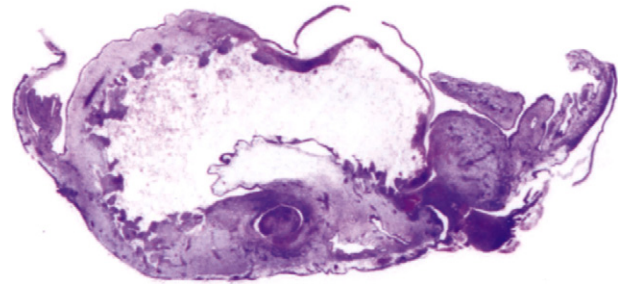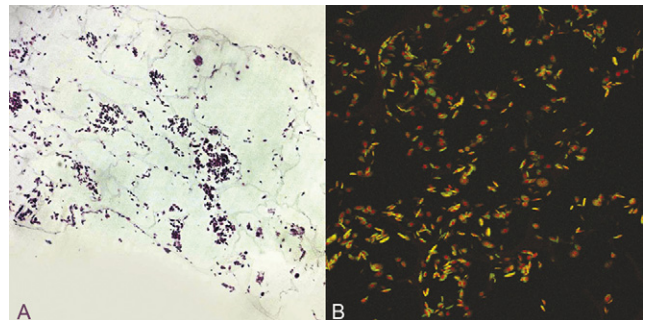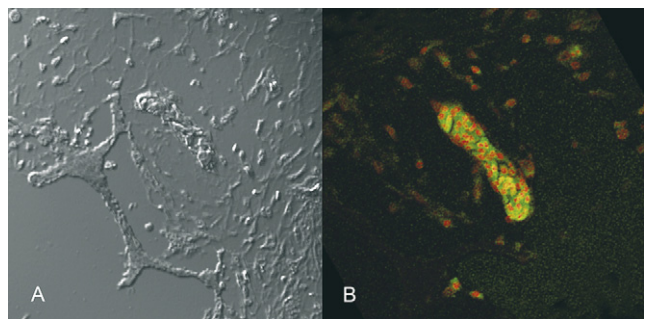

Fig. 7. One histological section from the angiogenesis test group IV showing that the CAM tissue biodegraded the lower part of the DegraPol® tube and reepithelialized the upper part within 5 days (H&E staining, $\times 1$).

scaffold constructions are immersed in the perfusate [12], the in vivo bioreactor contains an inter-connective pipe system with the aim to allow the medium to flow and to permeate throughout the entire biodegradable scaffold. In reepithelialization experiments epithelial cells formed a monolayer on the surface of an acellular dermal matrix with continuous medium perfusion inside a tubular DegraPol® scaffold underneath. The medium infiltrated through two different biomaterials and supported the survival of epithelial cells on the top surface for 2 weeks. Much larger size of scaffolds will be needed in later clinic application, while the structure of the novel tissue-engineered trachea prosthesis will be similar to the one we presented in this paper. The construct we tested can be considered as part of a large size tubular DegraPol® scaffold embedded with several porous feeding tubes inside and covered with tissue-engineered skin over the inner lumen surface. These feeding tubes will be connected to an extra-corporeal pump system to guarantee continuous medium perfusion. The power of the outlet pump should be adjustable to prevent medium overflow leaking out of the tracheal prosthesis causing inappropriate space between scaffolds and local infection. In further studies, the maximal perfusion area by each feeding tube should be calculated and the formation of an intact basement membrane by these tracheal epithelial cells needs to be addressed as it proved to be critical for preventing the unwanted ingrowth of granulation tissues into the tracheal lumen after implantation and for guiding normal epithelial cells ingrowing from the two anastomoses.

The MTT assay results in the cell delivery test showed no significant differences between the perfusion delivery and the conventional topically seeded static culture method. This result may offer advantages for future clinical applications especially in cases of emergency patients who are not able to wait for the time-consuming reconstruction of a tissue-engineered trachea in vitro. In such critical clinical situations an in vivo bioreactor could be first implanted during an emergency operation with appropriate tissues harvested at the same time for later isolation and expansion of cells in the laboratory. These cells could then be seeded into the embedded tissue-engineered trachea scaffold continuously by daily transfusions. The histological sections in our experiments showed three-dimensional cell growth inside the scaffold in the perfusion delivery group while there was only a monolayer on the scaffold surface in the control group. This demonstrates that the dynamic perfusion within the scaffolds efficiently improved the nutrient supply and the metabolic waste removal. The relatively weak internal flow shear stress seemed not to disturb the formation of cellular interconnections within the polymeric scaffolds while providing sufficient internal nutrient flux. The in vivo bioreactor acted as an assist device not only to delivery cells but also to support the metabolic needs of seeded cells deep inside the scaffolds where no efficient diffusion from normal surrounding tissues is available during the initial pre-vascular period after implantation. In previous studies Vacanti and co-workers engineered pre-formed vascular networks on silicon and Pyrex chips with the help of the micro fabrication technique. These microchips were sandwiched between tissue layers to function as an artificial capillary network [20]. In order to reduce thrombogenicity and to

avoid coagulation-related problems the channel surface was coated with endothelial cells. As an alternative for future preclinical and clinical developments we connected the perfusion system inside the biomaterial scaffold to an extra-corporeal pump system separated from the circulation of the recipient. As shown in the CAM model such an in vivo bioreactor is thought to offer a simple and practical approach to solve the problem associated with delayed revascularization of tissue-engineered prostheses.

Despite the time limitation of the in vivo CAM model we observed in the samples from group IV the presence of erythrocytes throughout the scaffold due to the increased vascular permeability induced by the continuous delivery of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) together with a much earlier neo-vessel formation within the scaffolds as compared to the control groups. The intravenously injected bisbenzimide H 33342 demonstrated that the newly formed CAM capillaries within the scaffolds were functionally connected to the circulation system of the chick embryo. These in vivo phenomena showed that the in vivo bioreactor is able to serve as an efficient growth factor delivery system that indeed accelerates the angiogenesis process. Angiogenesis requires various growth factors that might be best applied together or in a timed sequence [21]. In the in vivo bioreactor the expression levels of different kinds of growth factors can be readily controlled through the adjustment of dose concentrations in the perfusate. In future studies a detailed spatial and temporal application schedule could, therefore, be realized in long-term angiogenesis animal models in comparison to other delivery approaches such as gene transfer and growth factor-scaffold binding methods [22,23].

Recently, an in vivo bioreactor for tissue-engineered bone was described by introducing an artificial space between the recipient's normal bone and the periosteum [24]. In contrast, our design emphasizes the possible advantages of continuous cell delivery and growth factor administration together with the direct implantation of selected customized scaffolds into tissue defects. To further improve the initial poor oxygenation within an avascular tissue-engineered prosthesis artificial oxygen carriers such as perfluorocarbone (PFC) emulsion [25] could be added to the perfusate to bridge the gap until revascularization is achieved.

In conclusion, this pilot study demonstrated that continuously perfused biodegradable, tubular DegraPol® scaffolds supported the tracheal epithelial cell survival, allowed growth factor and chondrocyte delivery. By the addition of VEGF to the perfusion medium angiogenesis was enhanced in the CAM model. The in vivo bioreactor described here combines the formerly separated in vitro and in vivo parts in tissue engineering research into an all-in-one concept.

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