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Lipidic implants for controlled release of bioactive insulin: Effects on cartilage engineered in vitro

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Abstract

Controlled release systems for growth factors and morphogens are potentially powerful tools for the engineering or the treatment of living tissues. However, due to possible instabilities of the protein during manufacture, storage, and release, in the development of new release systems it is paramount to investigate into the maintenance of bioactivity of the protein. Within this study, recently developed protein releasing lipid matrix cylinders of 2 mm diameter and 2 mm height made from glycerol tripalmitate were manufactured in a compression process without further additives. Insulin in different concentrations (0.2%, 1%, and 2%) served as model protein. The bioactivity of the protein released from the matrices was investigated in a long-term cartilage engineering culture for up to four weeks; additionally, the release profiles were determined using ELISA. Insulin released from the matrices increased the wet weights of the cartilaginous cell-polymer constructs (up to 3.2-fold), the amount of GAG and collagen in the constructs (up to 2.4-fold and 3.2-fold, respectively) and the GAG and collagen content per cell (1.8-fold and 2.5-fold, respectively), compared to the control. The dose-dependent effects on tissue development correlated well with release profiles from the matrices with different insulin loading. In conclusion, the lipid matrices, preserving the bioactivity of incorporated and released protein, are suggested as a suitable carrier system for use in tissue engineering or for the localized treatment of tissues with highly potent protein drugs such as used in the therapy of brain cancer or neurodegenerative CNS diseases.

Keywords: Protein; Insulin; Controlled release; Lipid; Bioactivity; Cartilage; Tissue engineering

1. Introduction

The field of tissue engineering (Langer and Vacanti, 1993) aims at the regeneration of mechanical and functional body tissue or organ defects that cannot be sufficiently cured by self-healing processes. One strategy in tissue engineering is to seed and culture cells on 3-D scaffold structures in vitro in order to generate tissue constructs for implantation. Cell proliferation and differentiation as well as the formation of an adequate extracellular matrix (ECM) in an in vitro culture largely depend on the supplementation of growth factors and other morphogens (Babensee et al., 2000). In addition growth factors can strongly improve the integration of the engineered tissue after implantation. These effects render growth factors an important tool for tissue engineering purposes, however, their efficacy is limited by their short half-lives and their potential toxicity at systemic levels (Babensee et al., 2000). To overcome these problems the use of protein carriers that ensure a sustained release and at the same time retain the biological activity of the growth factors is desirable (Tabata, 2003). Unfortunately, protein stability is easily compromised during the manufacture, storage, and drug release (Schwendeman et al., 1996). For example, for the well established biocopolymer poly(lactic-co-glycolic acid) (PLGA) it has been demonstrated that degradation products from the release matrix can influence protein stability due to changes in the microclimate of the microspheres during degradation, e.g., higher osmotic pressure or acidic environment (Lucke et al., 2002; Lucke and Goepferich, 2003). In order to overcome such problems, stabilizing additives were introduced such as $Mg(OH)_2$ (Zhu et al., 2000), $Ca(OH)_2$ (Zhu and Schwendeman,

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2000), and, especially for insulin, zinc salts (Kim et al., 2001; Choi and Kim, 2003; Surendrakumar et al., 2003). The latter was additionally used to prolong the release of insulin (Brange and Langkjaer, 1992, 1993; Cai et al., 2002). As an alternative approach, controlled release systems based on lipids as a matrix material have recently attracted increasing attention, as they avoid detrimental effects of breakdown products of the biomaterial (Thomas et al., 2004; Dellamary et al., 2004; Prego et al., 2005). However, the processes used for the production of a lipid matrix often include organic solvents likely resulting in organic–water interfaces, which in turn are known as destabilizing factor for proteins (Fu et al., 2000). We recently developed cylindrical matrices based on solid triglycerides, especially designed for the purpose of a long-term release (Vogelhuber et al., 2003). For the production of these protein-loaded matrices neither emulsions with organic solvents, surfactants nor ultrasonification are needed, which in other systems may lead to a loss of bioactivity of the incorporated proteins (Maschke et al., 2004). These matrices may not only be of major interest in the field of tissue engineering, but also can be loaded with proteins and other types of drugs for the local treatment of tissues such as needed in the treatment of brain cancer (Vogelhuber et al., 2003) or neurodegenerative CNS diseases.

Previously, we established a 3-D cartilage engineering culture that can be utilized as a test system for sustained-release carriers (Kellner et al., 2001). Readily available insulin is used as a model protein; insulin was demonstrated to have strong anabolic effects on engineered cartilaginous constructs similar to those of insulin-like growth factor-I (IGF-I). The model provides quantifiable data and responds sensitively to supplemented insulin in a dose-dependent manner over a cultivation period of several weeks (Kellner et al., 2001). Even if sustained-release carriers are typically applied in an in vivo situation, this 3-D culture offers the opportunity to evaluate newly developed release systems with regard to their effects within a defined tissue engineering setting.

In this study, insulin-loaded triglyceride matrices were manufactured in order to investigate the biological effects of released insulin in the 3-D cartilage engineering culture. The first specific aim was the determination of the release kinetics of matrices with varying amounts of incorporated insulin. Further specific aims were the analysis of the effects of released insulin on the tissue construct weight, cell number, and amounts of ECM components, namely glycosaminoglycans and collagen, within the engineered tissue.

2. Materials and methods

2.1. Materials

Glycerol tripalmitate (Dynasan 116®) was a kind gift from Sasol (Witten, Germany). Human insulin was provided by Sanofi-Aventis (Frankfurt/Main, Germany). The human insulin immunoassay (ELISA) was purchased from Mercodia (Uppsala, Sweden).

Knee joints from three-months-old bovine calves were obtained from a local slaughterhouse within 6–12 h of slaughter. Collagenase II and papain were obtained from Worthington (CellSystems, St.Katharinen, Germany). Phosphate buffer solution (PBS), Dulbecco's Modified Eagle Medium with 4.5 g/l glucose (DMEM), fetal bovine serum (FBS), MEM non-essential amino acids solution, penicillin, and streptomycin were purchased from Gibco (Karlsruhe, Germany). One hundred and forty-nine micrometer pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA).

Spinner flasks were self-made (250 ml volume, 6 cm bottom diameter, side arms for gas exchange); silicone stoppers were obtained from Schuber & Weiss (München, Germany). Silicon tubing was from Cole Palmer (Niles, IL, USA), needles from Unimed (Lausanne, Switzerland). Polyglycolic acid (PGA) non-woven meshes (12–14 μm fiber diameter; 96% porosity; 62 mg/cm^3 bulk density; initial molecular weight of PGA approximately 70 kDa) were obtained from Albany International (Mansfield, MA, USA) and die-punched into discs 5 mm in diameter and 2 mm thick (scaffolds). Pipettes, petri dishes, falcon tubes, and well plates were purchased from Corning (Schiphol-Rijk, Netherlands).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA). Ascorbic acid, deoxyribonucleic acid, dimethylmethylene blue, eosin, glutaraldehyde, hematoxylin, HEPES buffer, proline, safranin-O, tween 80, and pepsin were purchased from Sigma–Aldrich (Taufkirchen, Germany). Formalin 37%, glutaraldehyd, hydrochloric acid (HCl), *p*-dimethylaminobenzaldehyde (*p*-DAB), and chloramine-T were from Merck (Darmstadt, Germany). Chondroitin sulfate A was from ICN (Aurora, OH, USA).

2.2. Lipid matrices

For the aseptic production of insulin-loaded matrices, Dynasan 116 (glycerol tripalmitate) was sterilized for 2 h at 160 °C. Subsequently crystallization of the lipid in the stable beta-orientation was achieved by tempering the molten lipid for three days at 55 °C. Lipid modification was investigated by scanning calorimetry using a 2920 differential scanning calorimeter (TA Instruments, Alzenau, Germany) at a heating rate of 5 K/min. The lipid was powdered in a mortar and sieved through a sieve with a pore size of 106 μm under aseptic conditions. For the preparation of the desired insulin/lipid powder mixtures, 58.84 mg human insulin were dissolved in 300 μl 0.01N HCl and 11 ml double-distilled water were added. The solution was filtered through a 0.22 μm filter (Corning, Schiphol-Rijk, Netherlands) and the concentration of insulin was determined by HPLC (see Section 2.3). Calculated amounts of the insulin solutions required for the desired insulin/lipid ratios were added to mortars filled with sterile Dynasan 116® (glycerol tripalmitate). The mixtures were freeze-dried in a desiccator, cooled with dry ice, and evacuated using a RV5 two-stage pump (Edwards, Crawley, Sussex, UK) for 24 h under aseptic conditions. The dried powder mixtures were manually homogenized in a mortar. For the manufacture of lipid matrix cylinders, a set of 2 mm diameter cylindrical punches and a die were machined from hardened steel and V4A steel, respectively. Cylindrical matrix discs (2 mm height, 2 mm diameter) were obtained by manual compression

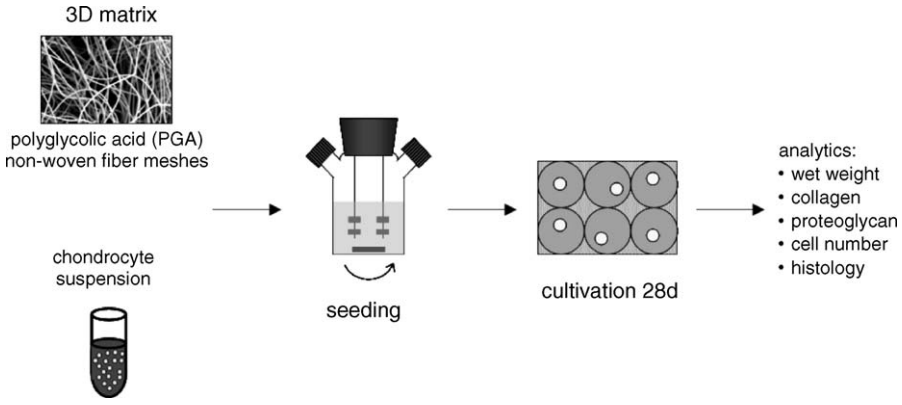

Fig. 1. Experimental tissue engineering set-up including seeding in spinner flask bioreactors and cultivation in well-plates.

of accurately weighed amounts of the insulin-loaded lipid powder in the die, applying a force of approximately 250 N for 10 s. Cylinders with an average weight of 6.3 ± 0.60 mg were used for the experiments. The matrices were visualized by scanning electron microscopy (SEM). For this procedure, samples were mounted on aluminum stubs with conductive carbon tape and coated with gold–palladium. All micrographs were obtained at 10 kV on a DSM 950 (Zeiss, Oberkochen, Germany).

2.3. Insulin measurements

The drug loading of the matrices was determined by HPLC. Matrices were dissolved in 600 μl chloroform each and insulin was then extracted with 600 μl 0.01N HCl. After 10 s of mixing on a vortex mixer, phase separation was achieved by letting the mixture settle for 10 min. This procedure was repeated two times and followed by centrifugation at $1600 \times g$.

The insulin content of the aqueous phase was analyzed by HPLC analysis, using an HPLC system with a degasser (Knauer, Berlin, Germany), LC-10AT pump, FCV-10AT$_{vp}$ gradient mixer, SIL-10Ad$_{vp}$ autosampler, CTO-6a oven, SPD-10AV UV-Detector, RF-551 fluorescence detector and SCL-10A$_{vp}$ controller (all from Shimadzu, Duisburg, Germany).

Hundred microlitres of the insulin solutions were analyzed at 37 °C using a C18-reversed phase precolumn (LC318, 4.6 mm × 20 mm, Supelco, Bellefonte, USA) combined with an analytical C-18 reversed phase column (Supelcosil, LC318, 4.6 mm × 250 mm; Supelco, Bellefonte, USA) and a linear gradient method (mobile phase A: 90% H_2O, 10% acetonitrile, 0.1% TFA; mobile phase B: 90% acetonitrile, 10% H_2O, and 0.1% TFA) with a flow rate of 1 ml/min. A linear gradient from 20% to 36% B over 24 min was applied. The chromatograms were recorded at wavelengths of 210 nm and 274 nm (UV-detection) and fluorescence detection was carried out at 274 nm excitation and 308 nm emission.

Cell culture media in the four-weeks trial (see below) were collected at the times of media change every two to three days and frozen at −80 °C. The insulin content was determined by enzyme-linked immunoassay (ELISA) in appropriate dilutions. The absorption was measured at 450 nm on a plate reader (Shimadzu, Duisburg, Germany). Possible adsorption of insulin on the surfaces of the well-plates was minimized by the use of FBS in the releasing cell culture media (Brange and Langkjaer, 1993). Previous studies employing two similar culture systems indicated that when exogenous insulin was applied at the time of media change and remaining insulin was assessed at the next medium change after two to three days, approximately 75–100% of the originally applied insulin were measured by ELISA (Weiser, 2002).

2.4. Chondrocyte isolation

Fresh articular cartilage was gained from the surface of the femoral patellar groove. The cartilage was cut into small pieces and primary chondrocytes were isolated by enzymatic digestion with collagenase type II. The digest was filtered through a 149 μm filter, centrifuged at 1200 rpm for 5 min, and washed three times with PBS (Freed et al., 1993). Isolated cells were resuspended in culture medium (DMEM) containing 4.5 g/l glucose, 584 mg/l glutamine, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 0.1 mM non-essential amino acids, 0.4 mM proline, and 50 μg/ml ascorbic acid. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope.

2.5. In vitro cell culture

Cell seeding was performed in spinner flasks (Fig. 1). Scaffolds were threaded onto needles (10 cm long, 0.5 mm diameter) and held in place with small pieces of silicone tubing (1 mm long). Four needles with two scaffolds apiece were inserted into a silicone stopper, which was placed into the mouth of a spinner flask containing a magnetic stir bar. The cell suspension with 5×10^6 isolated chondrocytes per scaffold (i.e., 40×10^6 cells per flask) in 110 ml of culture medium was filled into the flask. Flasks were placed in an incubator at 37 °C, 5% CO_2 and 95% humidity; stirring with 50 rpm on a magnetic stirrer for two days allowed for cell attachment.

Each scaffold was then placed in a 6-well plate filled with 6 ml of culture medium (same medium as above except for 1% FBS instead of 10%); plates were placed on an orbital

shaker at 50 rpm (Stuart Scientific, Surrey, UK). After one day, insulin-containing or unloaded matrices were added to the wells. Alternatively, in one group, exogenous insulin (see below) was added also after one day and from then on with each medium change. In all groups, medium was replaced three times per week.

Two independent studies were conducted: in the first study, which was conducted over two weeks and which in the following is designated "two-weeks trial", cell-polymer constructs were cultured in the presence of matrices loaded with 2% (m/m) insulin; groups with unloaded matrices (0%) or no matrices served as controls. For further comparison, a fourth group was included receiving exogenous insulin at 2.5 μg/ml with each medium change (concentration eliciting maximum response based on previous studies (Kellner et al., 2001)). Four individual constructs were cultured per matrices group, two constructs in each of the no-matrices groups.

In the second study, conducted over four weeks and designated "four-weeks trial", cell-polymer constructs were cultured in the presence of matrices with three different loading concentrations of insulin (0.2%, 1%, 2% (w/w)) or unloaded matrices (0%). Four individual constructs were cultured per group.

2.6. Biochemical analysis of the engineered tissues

Analytical assays were performed as previously described (Kellner et al., 2001). In brief, cell-polymer constructs were weighed (= wet weight, ww) and cut in half. One part was freeze-dried and digested by papain solution (3.2 U/ml in buffer) for 18 h at 60 °C. The cell number was determined measuring the DNA content using Hoechst 33258 dye in a fluorometrical assay (Kim et al., 1988). The sulfated glycosaminoglycan (GAG) content was determined spectrophotometrically at 525 nm as chondroitin sulfate after reaction with dimethylmethylene blue (Taylor and Jeffree, 1969; Farndale et al., 1986). Hydroxyproline content was measured spectrophotometrically at 550 nm after acid hydrolysis and reaction with chloramine-T and *p*-dimethylaminobenzaldehyde (Woessner, 1961). The total collagen amount could be calculated using a hydroxyproline to collagen ratio of 1:10 (Hollander et al., 1994).

2.7. Histology of the engineered tissues

The constructs were fixed in 2% glutaraldehyde for 30 min and stored in 5% formalin. After fixation, the tissues were embedded in paraffin and sliced into 5 μm sections. The deparafinized sections were subjected to a hematoxylin, fast green and safranin-O staining (Martin et al., 1999).

2.8. Statistical analysis

Statistical significance was assessed by one-way analysis of variance ANOVA in conjunction with Tukey's studentized range test using SPSS 12 for Windows from SPSS Software (Munich, Germany).

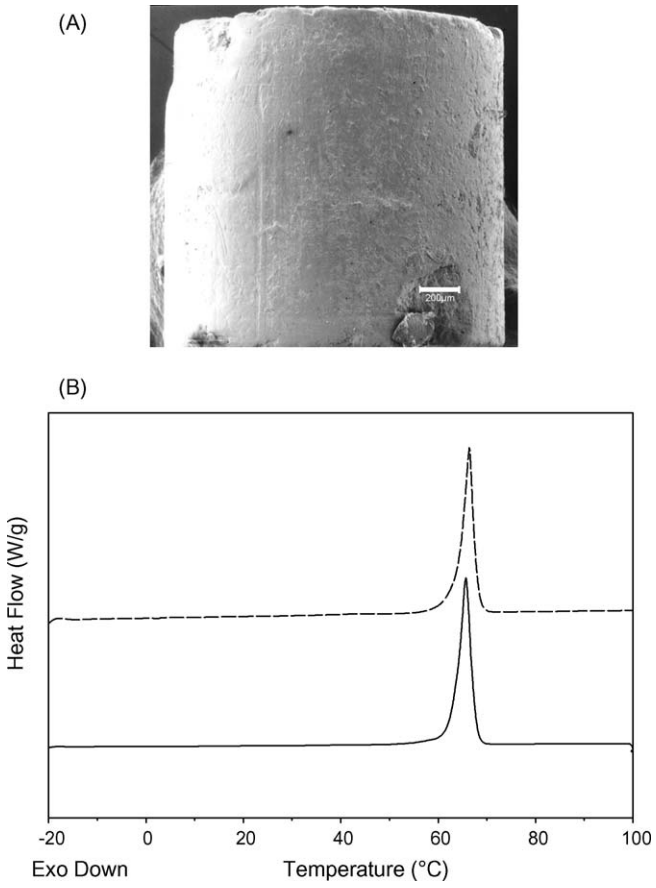

Fig. 2. (A) Scanning electron microscopy image of the manufactured cylindrical matrix, loaded with human insulin. (B) Determination of crystallinity of untreated glycerol tripalmitate (—) and sterilized and tempered glycerol tripalmitate (- -) by differential scanning calorimetry. The thermograms were recorded at a heating rate of 5 K/min. Only the endothermic peak for the melting of the stable β-modification of glycerol tripalmitate was detected before and after treatment, indicating that complete crystallization in the stable modification was achieved also after treatment.

3. Results

3.1. Lipid matrices

The manufactured insulin-loaded matrices had a well-defined cylindrical geometry. Examination by scanning electron microscopy revealed a generally smooth surface (Fig. 2A). After sterilization and tempering, differential scanning calorimetry analysis showed only the endothermic peak caused by the melting of the stable β-modification of glycerol tripalmitate (same peak as before treatment) indicating that complete crystallization of the lipid in the stable orientation was achieved (Fig. 2B).

Insulin loadings of the matrices were 0.20% (w/w) ± 0.003, 1.08% ± 0.043, and 2.08% ± 0.114, as determined by HPLC, and correlated well with the intended and designated insulin contents of 0.2%, 1%, and 2%, respectively.

3.2. Two-weeks trial

In the two-weeks trial, a significant 1.9-fold increase in wet weight was detected for the cell-polymer constructs cultured

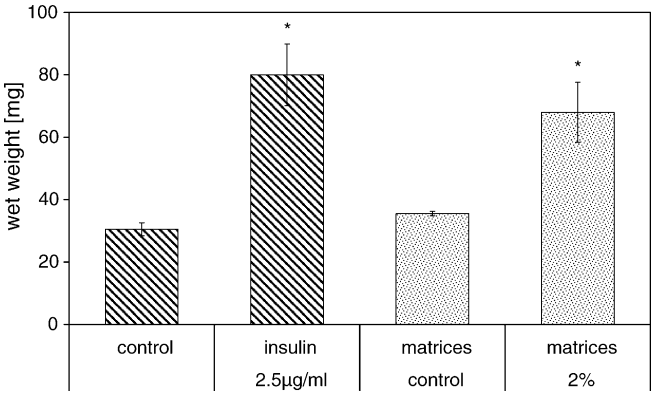

Fig. 3. Effects of unloaded and insulin-loaded lipid matrices on the wet weight of tissue-engineered cartilage in the two-weeks trial. Statistically significant differences to the control and matrices control (unloaded matrices) are denoted by asterisk (*) ($p < 0.05$). Data represents the average ± S.D. of four independent measurements for the matrices groups and two independent measurements for the control and insulin 2.5 μg/ml groups.

for two weeks in the presence of the insulin-loaded matrices (2%) (68 mg), as compared to the control group with unloaded matrices (35.5 mg) (Fig. 3). The wet weight of the unloaded matrices group was not significantly different from the control group with no matrices. The constructs receiving exogenous insulin (2.5 μg/ml) showed similar wet weights as the constructs cultured in the presence of the insulin-loaded matrices (Fig. 3).

The insulin-loaded matrices also significantly increased the absolute amounts of the ECM components GAG and collagen in the cell-polymer constructs (2.4-fold and 3.2-fold, respectively) and the GAG and collagen content per cell (1.8-fold and 2.5-fold, respectively), as compared to the control group with unloaded matrices (Table 1). The cell number per wet weight was decreased to 67% (Table 1); a lower cell density is commonly observed in more mature engineered cartilaginous constructs (Kellner et al., 2001). All values obtained for the constructs grown in the presence of the insulin-loaded matrices were on the same order of magnitude as the values obtained for the constructs receiving exogenous insulin (Table 1).

For all parameters investigated, no significant differences were detected between the two control groups cultured in the absence of matrices or in the presence of unloaded matrices (Table 1).

The histological analysis of the constructs correlated well with the obtained quantitative biochemical data. All cross-sections of the constructs appeared to be cartilaginous with round chondrocytes in lacunae surrounded by large areas of extracellular matrix deposition. However, the control group constructs cultured without insulin had a smaller, more fractured appearance compared to those of the insulin groups. Constructs from the groups cultured either in the presence of insulin-loaded matrices or exogenous insulin showed a more regular and coherent GAG distribution when stained red with safranin-O (data not shown).

3.3. Four-weeks trial

3.3.1. Insulin release

Released insulin was sampled at the times of media change directly from the well plates of the cell culture of the four-weeks trial. Thus, the collected insulin had been released over a period of two to three days. Over the first three days, a small burst release was observed for matrices 2% (10.3%), matrices 1% (23.8%), and matrices 0.2% (21.6%). Within the first 12 days, the matrices continuously liberated up to 67% (matrices 2%), 89% (matrices 1%) and 99% (matrices 0.2%), respectively, of the total loaded protein (Fig. 4A). Insulin concentrations at the times of media change within the first 12 days were between 0.4 μg/ml and 5.7 μg/ml for matrices 2% (day 12, day 8), between 0.9 μg/ml and 3.4 μg/ml for matrices 1% (day 12, day 8), and between 0.01 μg/ml and 1.0 μg/ml for matrices 0.2% (day12, day 8) (Fig. 4B).

Generally, after day 12, lower amounts of insulin were released. However, at the times of media change the insulin concentrations were still above 0.005 μg/ml in the groups of the matrices 1% (except for day 19, 0.004 μg/ml) and matrices 2% (except for day 24, 0.004 μg/ml) (Fig. 4B). Even on day 29, the last day of this study, insulin concentrations of 0.019 μg/ml and 0.025 μg/ml were observed for matrices 1% and matrices 2%. In contrast, insulin concentrations in the group of the matrices 0.2% were always below 0.002 μg/ml after day 17 (Fig. 4B).

3.3.2. Wet weights and cell number

All cell-polymer constructs cultured in the presence of insulin-loaded matrices exhibited significantly increased wet weights after four weeks of culture (54.5–100 mg), as compared to control constructs cultured in the presence of unloaded matrices (31 mg). The dose-dependent increases of the matrices groups were 1.7-fold, 2.7-fold, and 3.2-fold, respectively. Values for the matrices 1% and 2% were significantly higher than those for matrices 0.2% (Fig. 5A).

Table 1
Effects of unloaded and insulin-loaded lipid matrices on tissue-engineered cartilage in the two-weeks trial

	Control	Insulin 2.5 μg/ml	Matrices control	Matrices 2%
GAG [mg]	1.2 ± 0.47	2.7 ± 0.20*	1.2 ± 0.27	2.9 ± 0.63*
GAG per cell [pg]	181 ± 35.9	347 ± 54.2*	200 ± 10.1	368 ± 47.7*
Collagen [mg]	0.39 ± 0.113	1.3 ± 0.24*	0.36 ± 0.047	1.2 ± 0.34*
Collagen per cell [pg]	63.1 ± 4.09	171 ± 8.1*	58.0 ± 1.93	144 ± 29.0*
Cell number per wet weight [1/μg]	202 ± 31.4	96.8 ± 10.27*	174 ± 25.3	117 ± 11.3*

Data represents the average ± S.D. of four independent measurements for the matrices groups and two independent measurements for the control and insulin 2.5 μg/ml groups. Statistically significant differences to the control and matrices control (unloaded matrices) are denoted by asterisk (*) ($p < 0.05$).

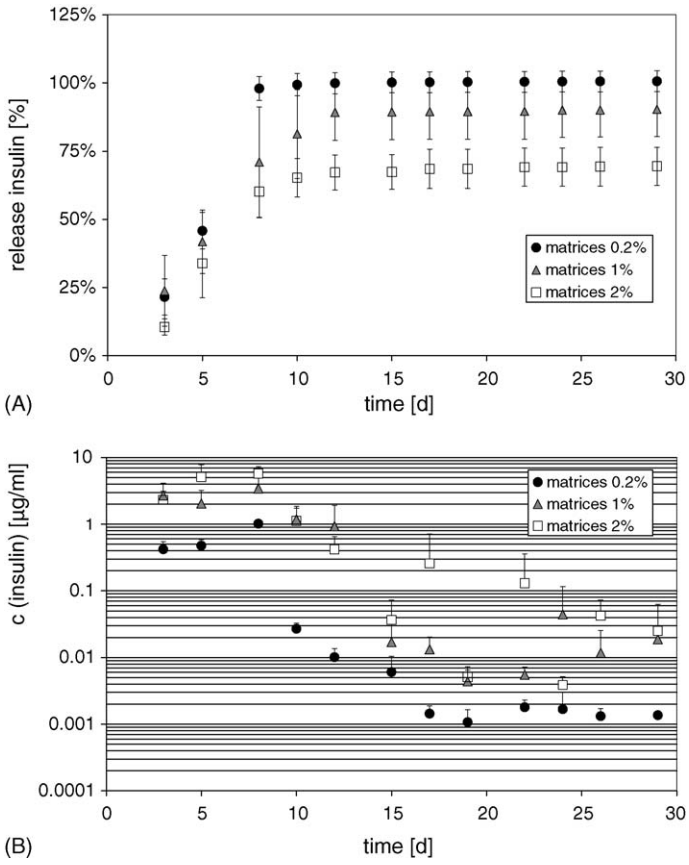

Fig. 4. (A) Cumulative release of insulin from lipid matrices in the four-weeks trial under cell culture conditions determined by ELISA. (B) Insulin concentrations measured in the cell culture medium at the time of media change every second or third day determined by ELISA. Data represents the average ± S.D. of four independent measurements.

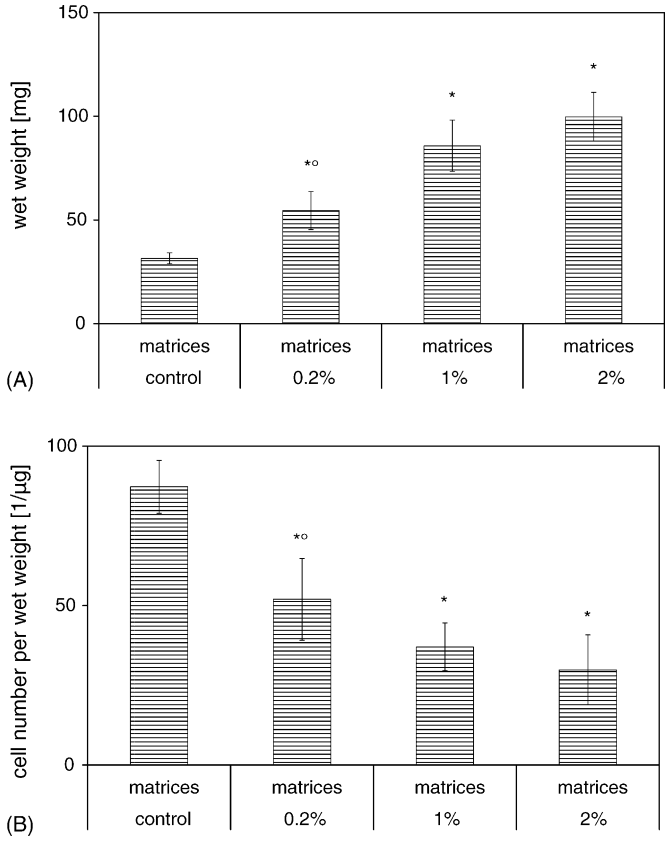

Fig. 5. Effects of insulin-loaded matrices on the wet weight (A), and the cell number per wet weight (B) of engineered cartilage after 28 days in the four-weeks trial. Data represents the average ± S.D. of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by asterisk (*), to the matrices 2% group by symbol (°) ($p < 0.05$).

The cell number per wet weight was reduced in all constructs receiving released insulin, as compared to the constructs receiving no insulin. A dose-dependent trend was observed with lowest cell densities for the highest insulin loading (Fig. 5B).

3.3.3. Collagen and glycosaminoglycans

Insulin released from the lipid matrices dose-dependently increased the absolute amount of collagen within the cell-polymer constructs. Increases were between 4-fold (4.4 mg for matrices 2%) and 2-fold (2.2 mg for matrices 0.2%), as compared to constructs receiving no insulin (1.1 mg) (Fig. 6A). The collagen content per cell was also increased by released insulin; an almost 3-fold increase was observed for matrices 2% (Fig. 6B).

Similar observations were made for the GAG content: absolute amounts of GAG were increased in the matrices 1% and 2% groups (up to 3.5 mg), as compared to the unloaded matrices group (1.2 mg). The matrices 0.2% only led to a slight, but not significant increase (Fig. 7A). The same applied to GAG content per cell, which was increased 2.2-fold in the matrices 1% and 2% groups (Fig. 7B). For all parameters, no significant differences could be detected between the matrix 1% and matrix 2% group (Figs. 6 and 7).

3.3.4. Histology

Histological cross-sections showed an increasing construct size with increasing insulin content of the matrices correlating with the construct weights (Figs. 5A and 8). In all cross-sections, round chondrocytes in lacunae surrounded by large areas stained red with safranin-O for GAG were detected. However, the constructs grown in the presence of matrices 1% and 2% exhibited a more even distribution of GAG, as compared to the constructs of groups with matrices 0.2% and unloaded matrices, which showed an irregular GAG distribution with distinct areas containing no safranin-O stain (Fig. 8).

4. Discussion

Growth factors, cytokines and morphogens are powerful protein molecules that, if adequately released from carrier systems, potentially play a key role in many tissue engineering applications. Unfortunately, in controlled release studies in general, processing during the manufacture of the carriers, storage, and the release itself often strongly compromise protein stability (Schwendeman et al., 1996). Therefore, in the development of new release systems it is paramount to determine not only the release kinetics by standard assays such as ELISA, but also to determine the bioactivity of the released protein in a relevant environment. In this study, recently developed

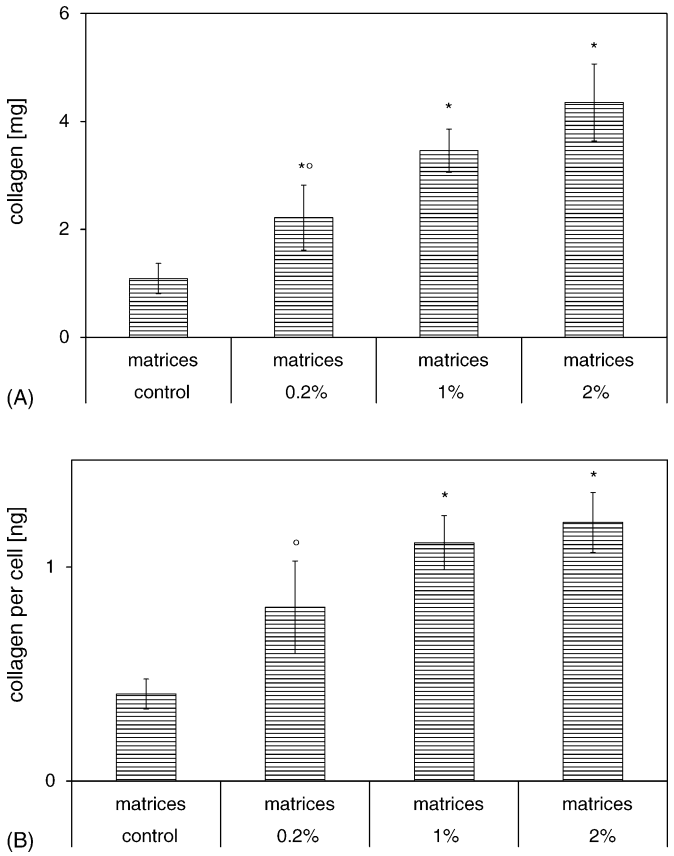

Fig. 6. Effects of insulin on the absolute amount of collagen per construct (A), and on the amount of collagen per cell in engineered constructs (B) in the four-weeks trial. Data represents the average ± S.D. of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by asterisk (*), to the matrices 2% group by symbol (°) ($p < 0.05$).

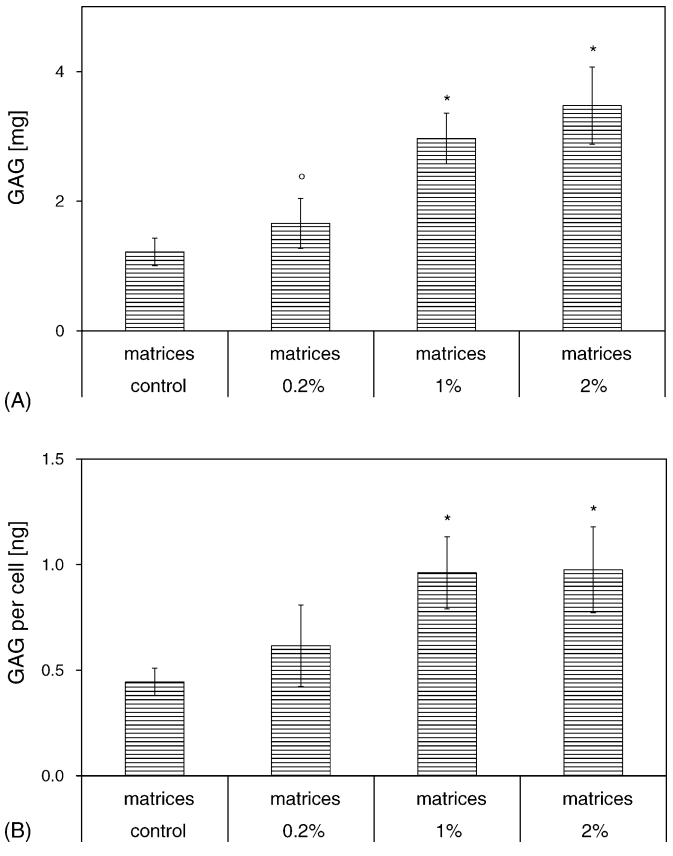

Fig. 7. Effects of insulin on the absolute amount of glycosaminoglycans (GAG) per construct (A), and on the amount of GAG per cell in engineered constructs (B) in the four-weeks trial. Data represents the average ± S.D. of four independent measurements. Statistically significant differences to the matrices control (unloaded matrices) are denoted by asterisk (*), to the matrices 2% by symbol (°) ($p < 0.05$).

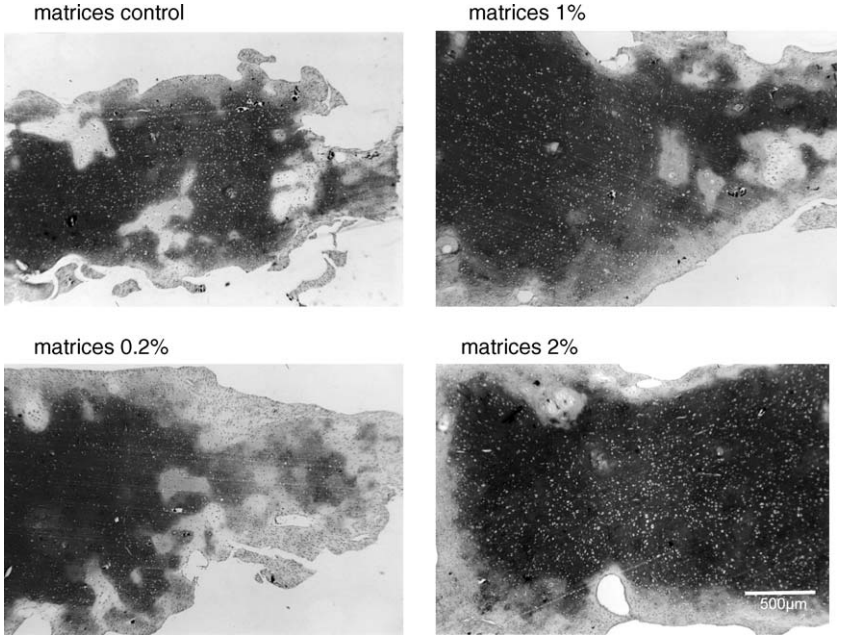

Fig. 8. Glycosaminoglycan (GAG) distributions in cross-sections of tissue-engineered constructs. GAG was stained red with safranin-O (appears dark gray in the black and white print).

cylindrical matrices were investigated with regard to the biological effects of released protein. The lipid devices require neither stabilizing additives within the matrix nor possibly detrimental organic–water interfaces during the production process. Insulin served as model protein and its effects were determined in a recently established cartilage engineering test culture (Kellner et al., 2001).

Cylindrical lipid matrices were produced from glycerol tripalmitate by manual compression with an insulin load of 0.2%, 1%, or 2%. As a possible change in lipid modification could have an impact on release characteristics, the lipid matrices were subjected to differential scanning calorimetry analysis. It was demonstrated that no detrimental effects occurred during processing; the glycerol tripalmitate showed crystallization exclusively in the stable β-modification before and after treatment.

In the two-weeks trial, distinct beneficial effects on tissue construct development were observed elicited by released insulin. Increases in construct wet weight and amounts of ECM components, as compared to controls receiving no insulin, were on the same order of magnitude as for constructs in the group receiving exogenous insulin at a concentration of 2.5 μg/ml (Fig. 3; Table 1); this concentration was previously shown to elicit the maximum response in the same culture system (Kellner et al., 2001). Furthermore, the constructs grown in the presence of the empty control matrices were not significantly different from the control constructs cultured in the absence of any matrices only in basal medium, indicating that the lipid matrices themselves had no effects on tissue construct development (Fig. 3; Table 1). Therefore, in the four-weeks trial the control group cultured only in basal medium was omitted; the insulin-loaded matrices were compared to unloaded controls.

For all three different insulin loadings, an insulin burst release of only 10–24% was detected within the first three days (Fig. 4A). In preliminary experiments, we could show that high concentrations of exogenously applied insulin (up to 50 μg/ml) only during the first days of culture, i.e., simulating a burst release at concentrations by far exceeding the concentrations reached in the presented study, resulted in distinctly inferior constructs compared to a continuous supply of insulin at concentrations below 0.01 μg/ml over 21 days (data not shown). Within the presented study, in general, higher amounts (>0.4 μg/ml) of insulin were released during the first 12 days from the matrices with higher loadings and during the first eight days from the matrices with the lowest loading. Although the released amounts dropped afterwards, for the matrices 1% and 2% still concentrations above 0.005 μg/ml of insulin were achieved (Fig. 4B). In preliminary experiments, 0.005 μg/ml was the minimum insulin concentration eliciting a biological response in the 3-D cartilage engineering culture (data not shown). In the group cultured in the presence of the matrix with the lowest loading, insulin concentrations were always distinctly below this concentration after day 17 (Fig. 4B).

The measured concentrations of the released insulin were well reflected by the effects on the quality of the engineered cartilage constructs. The culture in the presence of the lipid matrices 0.2% resulted in significant increases of the wet weight and collagen content of the constructs, furthermore in a beneficial decrease of the cell number per wet weight, as compared to the unloaded matrices controls (Figs. 5 and 6). This indicated that even the comparably low concentrations of insulin released from these matrices were sufficient to elicit relevant biological effects.

Even stronger effects were observed for the matrices 1% and 2%: large improvements in wet weight, cell number per weight, collagen and GAG amounts and also differences in the histological appearance were detected. Even the amounts of the ECM components per cell were distinctly increased (Figs. 5–7). The effects on wet weight and amounts of ECM components were significantly larger than those of the matrices 0.2%, which was in agreement with the release data. Only a small trend in favor of the matrices with the highest loading (2%) was observed, but no significant differences could be detected between constructs grown in the presence of matrices 1% and 2%, indicating that the minor differences in release data were also of minor relevance for the development of the constructs.

In the presented study, the cartilage engineering culture was successfully employed to prove the bioactivity of the released insulin. Up to now, the only methods to directly investigate the efficacy of an insulin releasing device over a period of weeks involved in vivo assays such as the determination of the blood glucose level in rats over a period of 14 days (Choi and Kim, 2003). Here, it was demonstrated that the cartilage engineering in vitro assay facilitates the testing of controlled release devices with regard to their biological efficacy in a complex system without the need for the expense of laboratory animals.

In conclusion, in this study we demonstrated the sustained release of bioactive insulin from cylindrical lipid matrices. The released insulin elicited strong dose-dependent effects on tissue-engineered cartilage. The lipid matrices, preserving bioactivity of incorporated and released proteins, are suggested as a suitable carrier system for growth factors and morphogens in regenerative medicine.

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