Biotechnol Lett (2006) 28:1641–1648
DOI 10.1007/s10529-006-9144-1

ORIGINAL PAPER

Redifferentiation of chondrocytes and cartilage formation under intermittent hydrostatic pressure

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Received: 9 June 2006 / Accepted: 12 June 2006 / Published online: 11 August 2006

Abstract Since articular cartilage is subjected to varying loads in vivo and undergoes cyclic hydrostatic pressure during periods of loading, it is hypothesized that mimicking these in vivo conditions can enhance synthesis of important matrix components during cultivation in vitro. Thus, the influence of intermittent loading during redifferentiation of chondrocytes in alginate beads, and during cartilage formation was investigated. A statistically significant increased synthesis of glycosaminoglycan and collagen type II during redifferentiation of chondrocytes embedded in alginate beads, as well as an increase in glycosaminoglycan content of tissue-engineered cartilage, was found compared to control without load. Immunohistological staining indicated qualitatively a high expression of collagen type II for both cases.

Keywords Chondrocytes · Cartilage · Hydrodynamic pressure · Bioreactor

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Introduction

Hyaline cartilage has only a limited potential of self-healing and present repair strategies do not yield satisfactory results (Hunter 1995; Petersen et al. 2003). Thus, research in tissue engineering has attempted to generate cartilage substitute, with properties similar to native tissue. However, the existing procedures are not yet satisfactory (Petersen et al. 2003; Darling and Athanasiou 2003). Previous investigations evaluated factors mimicking an in vivo environment as stimulation for the redifferentiation of dedifferentiated chondrocytes (Darling and Athanasiou 2003). Chondrocytes located in the articular cartilage undergo hydrostatic pressure during periods of loading in vivo, thus, it is assumed that mimicking hydrostatic pressure might increase matrix synthesis of in vitro engineered cartilage (Hansen et al. 2001). However, the response of the cartilage matrix to loading is complex and involves many factors, including tissue and cell deformation,

changes in hydrostatic pressure, and fluid flow (Weightman et al. 1979).

There are different techniques to impose hydrostatic pressure: (i) the first uses mechanical forces to compress the gas and liquid phase within a chamber (Darling and Athanasiou 2003), (ii) the second uses a semicontinuous perfusion system that feeds the cells and applies hydrostatic pressure in the same device (Heath and Magari 1996), and (iii) the third, which was applied in these studies as illustrated in Fig. 1, is to exert hydrostatic pressure with compression by overpressure (Pörtner et al. 2005).

Materials and methods

Cells and cultivation procedure for generation of cartilage-carrier constructs

Porcine chondrocytes were isolated from an approximately 18-month-old domestic pig. The cells were isolated from the knee-joint, which was kindly provided by a local slaughter. Cartilage-carrier constructs were generated according to a 4-step-protocoll suggested by Nagel-Heyer et al. (2005). Differing from that protocol redifferentiation and cartilage formation was performed under an atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2.

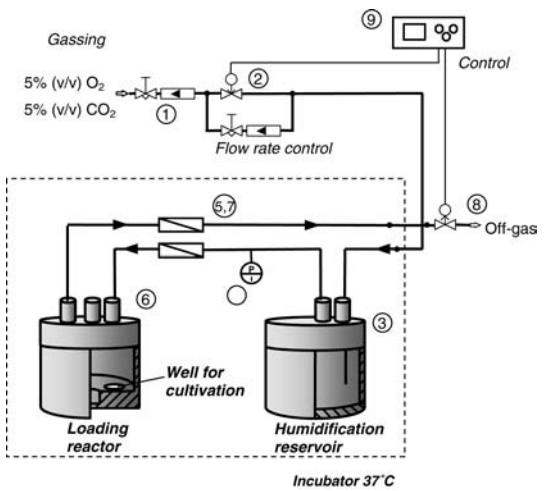

Fig. 1 Scheme of the loading reactor; achieving hydrostatic pressure via gassing with overpressure and cyclic closing of the off-gas valve, controlled by a time-timer. 1 = rotameter, 2 = additional circuit, 3 = humidification reservoir, 4 = manometer, 5,7 = sterile filters, 6 = loading reactor, 8 = off-gas valve

Loading reactor

A special-constructed cylindrical loading reactor, as shown in Fig. 1 containing 7 wells, was used to cultivate either alginate beads or cartilage-carrier constructs. The primary pressure of the gas supply from a gas one bottle containing a premixed gas was set to 0.4 and 0.5 MPa, respectively. The flow rate was controlled by a rotameter. Before the gas mixture reached the loading reactor it was passed through a humidification reservoir containing water to moisten the gas mixture so as to prevent losses of medium in the loading reactor. To ensure the reactor remained sterile, the incoming gas and the off-gas were led through sterile filters (Bioengineering).

Hydrostatic loading inside the reactor was achieved by closing the valve at the off-gas position. A time-timer was used to control the on/off regime, e.g. 1 min on/off means a closed valve in the 'on' position, resulting in the build up of hydrostatic pressure in the loading reactor by the in-streaming gas mixture within 1 min, whereas the 'off' status opens the valve, resulting in a reduction to atmospheric pressure for 1 min. Thereby, the build up of the pressure was similar to a saw tooth curve where the desired pressure was achieved after 1 min. The on/off status was controlled by a second time-timer resulting in a 6 h loading phase and an 18 h recuperation phase per day. The flow rate was set by rotameters in such a way that a hydrostatic pressure of 0.3 MPa was obtained within the time-interval with closed valve. The pressure inside the reactor could be observed by a manometer. When the pressure was being build up, a reduction in the flow rates could be observed due to backpressure.

Since a high volume of gas was needed to build up the pressure, an additional circuit was installed. This led to a lower consumption of the premixed gas during the phases where no pressure was being built up. During normal aeration and the off-phase, the upper valve, which was controlled by the same time-timer as the off-gas valve but switched opposite, was closed and the gas passed through the lower part. A rotameter

positioned downstream was used to reduce the flow rate during that time and this minimized the consumption of gas. To build up the hydrostatic pressure, the upper valve was opened by the time-timer and because the lower part had a higher resistance, the essential flow rate for the creation of the pressure flowed without any resistance through the upper part. The approximate flow rate required to obtain a pressure of 0.3 MPa in the reactor after closing the valve for 1 min was 50 l/h. A flow rate of 4 l/h was realized during relaxation. Prior to use, both the reactor and the humidification reservoir were autoclaved.

Alginate culture procedure

In order to investigate the influence of intermittent loading during redifferentiation, 8 samples of chondrocytes embedded in alginate beads were generated. Four samples were placed in 4 wells of the loading reactor cultivated at the above described conditions and another 4 samples were cultivated in 12-well plates at static conditions. After cultivation the redifferentiated chondrocytes were recovered from the alginate and analyzed. For characterization of matrix synthesis during this phase the collagen type II to I and the ratio of glycosaminoglycane (GAG) to DNA were determined (see below).

Cartilage formation procedure

Intermittent loading was also applied, after showing positive effects during redifferentiation, on cartilage-carrier constructs. Therefore, a total of 9 cartilage-carrier constructs were generated. For 3 constructs loading stress was applied during the alginate culture and during cartilage formation within the last 7 days of cultivation. Another 3 constructs were generated at static conditions during the alginate culture and loading was applied only within the last 7 days of cartilage formation. The remaining 3 constructs were generated at static conditions during the entire time. For all constructs physical parameters were determined. For 2 constructs of each condition, the GAG/DNA ratio was determined and the remaining one was used for immunohistological staining.

Biochemical analysis

In order to determine the glycosaminoglycan (GAG) content and the amount of DNA as an indicator for the number of cells (Nagel-Heyer et al. 2005), the glycosaminoglycan was determined with 1,9-dimethymethylene blue chloride staining after enzymatic digestion with papain. The DNA content was determined using the fluorescence marker H33258 (Buschmann et al. 1992).

Immunohistology analysis

Collagen type I and II determination after redifferentiation of chondrocytes embedded in alginate beads was carried out according to Goepfert et al. (2006) on cytospins. Determination of collagen type II and I was performed separately via specific binding of monoclonal antibodies of mice (Acris Antibodies GmbH) against collagen type I (Ig G2a/k) and collagen type II (IgG1/k). A fluorescent antibody [IgG(H + L), FITC] Goat anti-mouse (Southern Biotech) was used for staining. For staining of the nucleus the DNA-binding fluorochrome, 4-6-diamidino-2-phenyl-dihydrochloride, was used. For qualitative and quantitative analysis purposes, the immunostained samples were analyzed using a fluorescence microscope and the corresponding Lucia G software (Nikon). For quantitative analysis purposes a further step was carried out with the Lucia G software: to this end, the previously created image was analyzed with a macro to determine the total cell numbers (blue DAPI dots) and the amount of cells generating collagen (green FITC areas).

Collagen type I and II determination after cartilage formation was performed analogous to Nagel-Heyer et al. (2005). Determination of collagen type II and I was performed separately via specific binding of monoclonal antibodies of mice against collagen type I (Ig G2a/k) and collagen type II (IgG1/k) and binding of the biotinylated secondary antibody (IgG (H + L)-biotin) Goat anti-mouse (Southern Biotech). Color development was carried out using streptavidin/alkaline phosphates complex and the New Fuchsin chromagen. The immunohistological images were

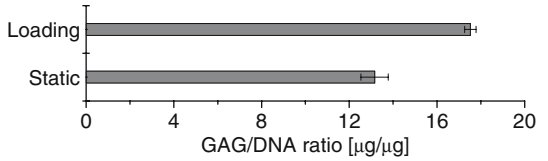

Fig. 2 Relationship between glycosamino-(GAG)- and DNA-content after cultivation of chondrocytes immobilized in alginate beads for redifferentiation at intermittent loading and static conditions, respectively

judged qualitatively. Therefore, the distribution of the chondrocytes within the tissue as well as the intensity of the stainings for collagen type II and I were evaluated.

Physical parameters

At the end of the cultivation the weight and the height of the generated cartilage were determined. Color, shape and homogeneity were judged visually. The stability was characterized via the determination of the Young's modulus following the unconfined compression method suggested by Korhonen et al. (2002). A custom-made high-precision material testing device (Zwicki 1120; Zwick) was used for the mechanical test. After equilibration under an offset of 0.05 N, stepwise stress–relaxation tests (each step 3% of uncompressed cartilage thickness) were carried out up to a strain of 21%. For each step, the criterion for complete relaxation was a relaxation-rate < 0.002 N/min. The Young's modulus was determined from the linear range of the stress–strain curve.

Statistics

Statistics software SigmaStat was applied to evaluate the data obtained from the experiments. Statistical significance was assessed by analysis of variance with $p < 0.05$ (ANOVA).

Results

Alginate culture

Figure 2 clearly shows that the reactor cultivation with intermittent loading gave a higher GAG/DNA ratio compared to the control, which was about 24% higher than the static control. For the collagen type II to I ratio best results were also obtained during cultivation in the loading reactor (Figs. 3, 4). The evaluation in this case showed an increase in the ratio of approximately 65% for the reactor sample compared to the static control.

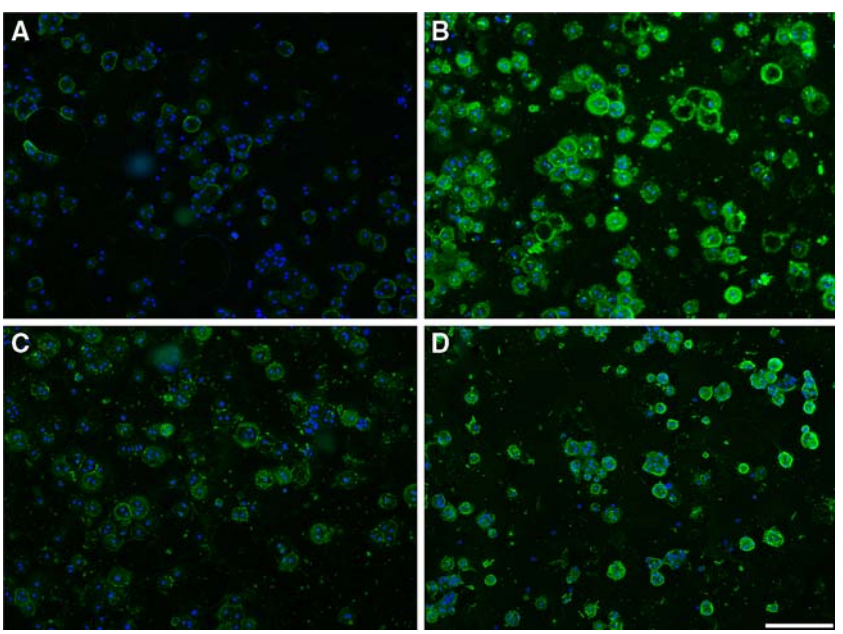

Fig. 3 Comparison of immunostaining for collagen type I and II after alginate culture under intermittent hydrostatic pressure (1 min on/off) to the static control. Stainings for collagen type I and II appear green, whereas blue areas determine nucleus DNA. (A) Collagen type I staining and (B) collagen type II staining for reactor sample. (C) Collagen type I staining and (D) collagen type II staining for static sample. Scale Bar = 100 µm

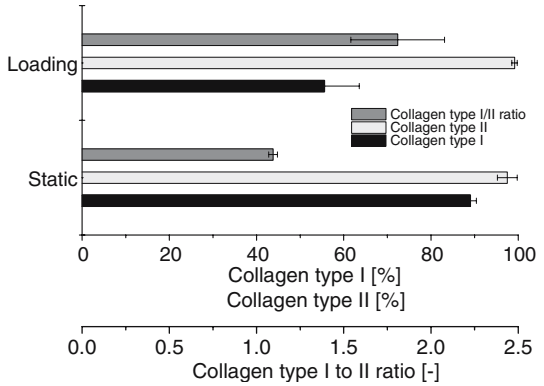

Fig. 4 Relationship between collagen type II to I of samples cultivated at intermittent loading and static conditions in alginate beads

Comparing the images indicates that the intensity of collagen type I of the reactor sample seems to be less significant than it is for the static control (Fig. 3). Furthermore, the images suggest a superior intensity of collagen type II staining for the reactor sample. A comparison of the reactor experiment with the static one shows a better ratio of collagen type II to I, mainly through a decrease in the negative collagen type I expression and with almost 100% collagen type II-producing cells in each sample (Fig. 4).

Cartilage-carrier constructs

The obtained values for weight and height (Fig. 5) indicated almost no differences between the generated cartilages, whereas a significant increase in the Young's modulus was achieved for the cartilage when intermittent loading was applied in the alginate as well as in the cartilage culture. Figure 6 shows the highest GAG/DNA ratios for cultivation performed in the reactor during the alginate as well as during the cartilage culture. While having a small increase when applying loading stress only in the cartilage culture compared to the static control, a further increase was obtained when loading stress was applied in the alginate culture as well as in the cartilage culture.

The immunohistological images for collagen type I and II (Fig. 7) showed homogenous tissue in all cases with evenly-distributed chondrocytes within the tissue. For collagen type I, a sketchy staining could be observed for all cartilages. Also for collagen type II a similar intensive staining could be observed, whereas for the static constructs areas of little more intensively stained tissue were observed, an indication of a higher collagen type II synthesis.

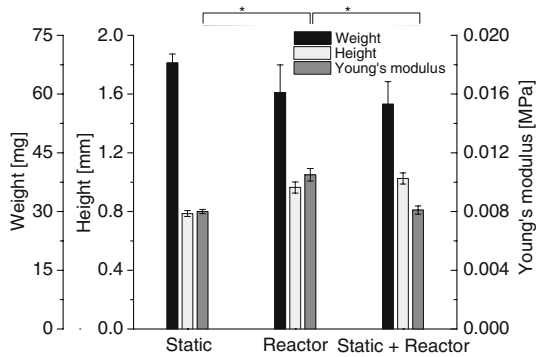

Fig. 5 Physical parameters weight, height and Young's modulus of cartilage-carrier constructs cultivated at intermittent loading and static conditions. static = control cultivated without any intermittent hydrostatic pressure during entire time, reactor = intermittend hydrostatic pressure applied during alginate culture as well as within the last 7 days of cartilage culture; static + reactor = intermittent hydrostatic pressure applied only within the last 7 days of cartilage culture

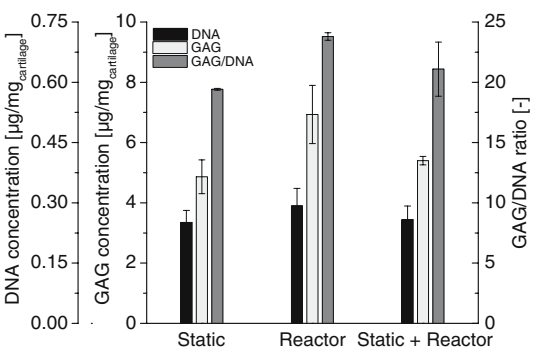

Fig. 6 Relationship between GAG-content and DNA-content of cartilage-carrier constructs cultivated at intermittent loading and static conditions. static = control cultivated without any intermittent hydrostatic pressure during entire time, reactor = intermittend hydrostatic pressure applied during alginate culture as well as within the last 7 days of cartilage culture; static + reactor = intermittent hydrostatic pressure applied only within the last 7 days of cartilage culture

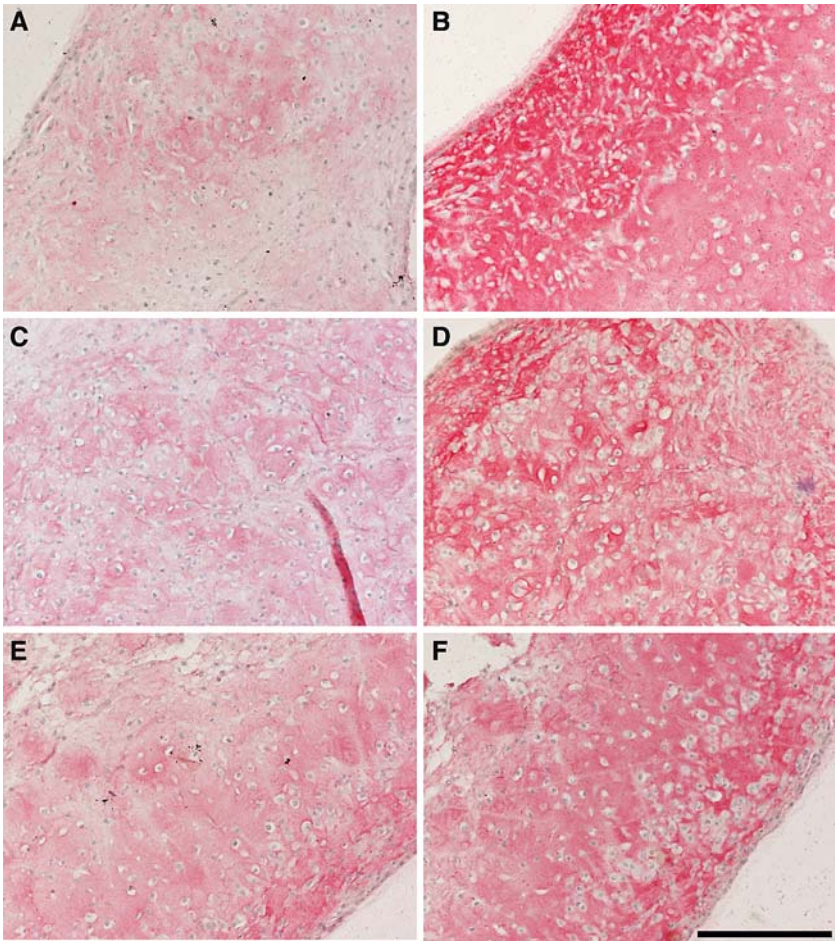

Fig. 7 Immunohistological staining for collagen type I and II of cartilage-carrier constructs cultivated at intermittent loading and static conditions. (A) Collagen type II staining and (B) collagen type II staining for static construct. (C) Collagen type I staining and (D) collagen type II staining for construct with loading stress within the alginate culture as well as in the cartilage culture. (E) Collagen type I staining and (F) collagen type II staining for construct with loading stress within the alginate culture. Scale bar = 100 μm

Discussion

In the present study the response of porcine chondrocytes to intermittent hydrostatic pressure realized by gassing with overpressure at low oxygen concentrations of 5% (v/v) O_2 was investigated. The load was transduced via the uncompressible medium to the cells. The pressure amplitude of 0.3 MPa used here was within the range of amplitudes found to have a stimulating effect in other systems (Darling and Athanasiou 2003; Veldhuijzen et al. 1979). The results of this study show that intermittent loading can influence matrix synthesis during redifferentiation of chondrocytes in alginate beads and during cartilage formation.

Data from experiments with alginate beads using a prepared gas-mixture with a defined atmosphere of 5% (v/v) O_2 and 5% (v/v) CO_2, and a loading regime of 1 min on/off underline that intermittent loading might be a useful tool for inducing the synthesis of extracellular matrix components. A significant increase in the ratio of glycosaminoglycan (GAG) to DNA was found, 25% higher compared to the corresponding static control. A 65% higher ratio of collagen type II to I compared to the static control was observed. Furthermore, the immuno-staining indicated a

superior intensity of collagen type II and a lower intensity of collagen type I compared to the static control. It does, however, seem obvious that intermittent loading can introduce matrix synthesis, although it should be pointed out that little is known about the effects of loading stress. The results indicate that applying intermittent loading during redifferentiation on chondrocytes embeded in three-dimensional alginate beads can induce higher rates of synthesis in the matrix components glycosaminoglycan and collagen type II at low oxygen concentrations.

For chondrocytes cultivated in alginate beads at intermittent hydrostatic loading enhanced chondrogenesis during cartilage formation was observed. The results indicate that loading stress does not affect metabolic parameters (data not shown) or physical parameters significantly. One interesting result is that a better Young's modulus was achieved for loaded cartilage-carrier constructs, indicating a stiffer matrix when applying intermittent loading during both cultivation steps. Obviously, a more stable collagen network with a higher stability is formed when cartilage is cultivated under hydrostatic load. Promising results were found for the ratio of GAG/DNA, where an increase of more than 8% in cartilages with intermittent hydrostatic pressure only during cartilage formation and an increase of more than 22% in cartilage, applying intermittent hydrostatic loading during both cultivation steps, compared to the static controls was determined.

The stimulation observed when intermittent hydrostatic pressure was exerted underlines the positive effects of mimicking in vivo conditions. Although also previous reports describe the stimulating effects of intermittent loading on glycosaminoglycan synthesis of bovine chondrocytes (Darling and Athanasiou 2003; Hansen et al. 2001), no advice in the literature has been found reporting this stimulation for porcine chondrocytes. However, the results demonstrated that intermittent loading applied during redifferentiation in the alginate culture and later on the cartilage-carrier constructs had positive effects on the characteristics of the matrix. Although no increase in collagen type II expression was observed, a more stable matrix was achieved, indicated by the higher Young's modulus. Moreover, glycosaminoglycan production was enhanced throughout loading, although it was also shown that a combination of applied loading during alginate culture and again on cartilage culture yielded the highest values.

In summary, it can be assumed that mimicking in vivo conditions such as loading stress during cultivation, might be a useful tool in cartilage tissue engineering and might lead to optimized culture conditions.

Acknowledgements We would like to thank Ditte Siemesgelüss for here excellent technical support, Prof. Dr. Michael Morlock for is help in statistical analysis and Zellwerk GmbH, Eichstädt for donation of the ceramic carrier. The project was kindly supported by ''Qualitätsoffensive Tissue Engineering'', Hamburg.

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