Tuning the differentiation of periosteum-derived cartilage using biochemical and mechanical stimulations

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Objective: In this study, we aim at tuning the differentiation of periosteum in an organ culture model towards cartilage, rich in collagen type II, using combinations of biochemical and mechanical stimuli. We hypothesize that addition of TGF-β will stimulate chondrogenesis, whereas sliding indentation will enhance collagen synthesis.

Design: Periosteum was dissected from the tibiotarsus of 15-day-old chick embryos. Explants were embedded in between two agarose layers, and cultured without stimulation (control), with biochemical stimulation (10 ng/ml TGF-β1), with mechanical stimulation (sliding indentation), or both biochemical and mechanical stimulations. Sliding indentation was introduced as a method to induce tensile tissue strain. Analysis included quantification of DNA, collagen and GAG content, conventional histology, and immunohistochemistry for collagen type I and II at 1 or 2 weeks of culture.

Results: Embedding the periosteal explants in between agarose layers induced cartilage formation, confirmed by synthesis of sGAG and collagen type II. Addition of TGF-β1 to the culture medium did not further enhance this chondrogenic response. Applying sliding indentation only to the periosteum in between agarose layers enhanced the production of collagen type I, leading to the formation of fibrous tissue without any evidence of cartilage formation. However, when stimulated by both TGF-β1 and sliding indentation, collagen production was still enhanced, but now collagen type II, while sGAG was found to be similar to TGF-β1 or unloaded samples.

Conclusions: The type of tissue produced by periosteal explants can be tuned by combining mechanical stimulation and soluble factors. TGF-β1 stimulated a chondrocyte phenotype and sliding indentation stimulated collagen synthesis. Such a combination may be valuable for improvement of the quality of tissue-engineered cartilage.

Introduction

The limited ability of damaged articular cartilage to repair itself and the morbidity that accompanies this injury pose a major clinical problem. A potential solution for this problem is to use tissue engineering to repair articular cartilage defects. However, to be able to accomplish this, a comprehensive understanding of the chondrogenic process itself is needed. Periosteal chondrogenesis is a well-known model to study chondrogenesis and provides insights in (periosteal) cartilage repair by progenitor cells. The chondrogenic potential of periosteum has been shown in both in vivo and in vitro studies.

O’Driscoll et al. developed an in vitro organ culture model in which rabbit periosteal explants form cartilage when suspended in agarose gels. Using this organ culture model, it was shown that addition of transforming growth factor β1 (TGF-β1) and application of dynamic fluid pressure enhanced periosteal chondrogenesis, observed by increased production of proteoglycans and collagen type II. These studies have shown that the periosteum organ cultures respond to their biochemical and mechanical environments. The major advantage of this approach over more standard approaches is that we can study chondrogenesis without the presence of scaffold material, while we are able to apply a well-defined mechanical loading protocol. This particular combination of possibilities is not available in commonly used cartilage tissue engineering setups.
However, similar to other cartilage tissue engineering systems, only low amounts of collagen type II are synthesized in the formed cartilage in periosteal explants in vitro. The small quantity of collagen type II is a general shortcoming of cartilage tissue engineering and is believed to be the most prominent reason why engineered cartilage does not reach sufficient load-bearing properties. In healthy cartilage, the function of the collagen fiber network is to resist tension. We therefore postulate that in order to stimulate collagen formation, we need to apply tension to the periosteal explants.

In order to apply predefined tensile loads, a numerical-experimental approach was adopted. Periosteal explants were embedded between agarose layers and subjected to sliding indentation, which involves an indenter compressed into the construct and slid over the construct to ensure dynamic straining. Numerical evaluation showed that sliding indentation results in tensile strain in the tissue, perpendicular to the indentation direction. Furthermore, the model allowed us to determine the required indentation depth and thickness of the different layers to obtain the optimal loading regime.

The main goal of the present investigation was to tune the differentiation of chick periosteum in the organ culture model towards cartilage formation, rich in collagen type II, using combinations of biochemical and mechanical stimuli. We hypothesize that addition of TGF-β will stimulate chondrogenesis, whereas sliding indentation will enhance collagen synthesis.

Method

Sliding indentation bioreactor system

A bioreactor was designed to apply sliding indentation to periosteal explants suspended in agarose (Fig. 1). The core part of the bioreactor was constructed of a single piece of aluminum, which was machined such that an inner and an outer frame remained, which were connected through four vertical and two horizontal leaf springs. This construction stabilized motion of the inner frame in the exact horizontal plane. Continuous motion was applied by a DC motor (Maxon, Switzerland). The bioreactor was placed inside an incubator at 37°C and 5% CO2. The two horizontal leaf springs allowed vertical positioning of the inner part by a micrometer (Mitutoyo, Japan) located on top of the bioreactor. Two aluminum subunits were attached to either side of the inner frame, each containing another vertical leaf spring system and a micrometer (Mitutoyo, Japan; resolution 10 μm). This allowed independent vertical positioning of the two subunits, each with six glass rods that served as indenters (diameter 4.55 mm, length 8.5 mm) through a stainless steel l-shaped connector. Through this system, the indenters could move back and forth in an exact horizontal plane at a preset height. The indenters compressed and slid over rectangular agarose constructs (length: 12 mm; width: 9 mm; height: 6 mm) inside stainless steel moulds. The moulds were locked inside a Teflon container that was attached to the bottom plate of the bioreactor. The container was closed with a lid that had a small opening for the aforementioned l-shaped connector unit (Fig. 1).

The strains induced by sliding indentation are not homogenous in depth into the agarose, and depend on the properties of the construct and the loading conditions, including indenter size and indentation depth. To ensure that the periosteal explants in the agarose constructs received high amounts of strain, we determined numerically the depth at which maximum tensile strains would occur in our bioreactor system. Using the finite element method (ABAQUS 6.7, Simulia, USA), the stress field for a 12 mm long and 6 mm high 3% (w/v) agarose construct was computed, which was indented 10% with a 4.5 mm diameter indenter, sliding at 0.2 mm/s. Contact between indenter and sample was assumed frictionless. A validated nonlinear biphasic material model with compressible Neo-Hookean solid matrix behavior was used to describe the behavior of 3% agarose. The simulation was performed in two dimensions, and free fluid outflow was allowed to the top-plane area of the agarose that was not in contact with the indenter. The bulk and shear moduli for agarose were fitted from experimental data (8.5 kPa and 12.2 kPa, respectively). The highest tensile principal strains reached 16% and were found in the region directly under the indenter, in the upper 1/3 of height of the construct (Fig. 2). The periosteal explants were placed in this region of highest strains, at 2 mm depth.

Periosteum isolation and agarose-suspension cultures

Periosteal explants (17 × 8 mm; approximate number: 144) were aseptically dissected from the tibiotarsus of 15-day-old chick embryos (t Anker, Ochten, The Netherlands). Tibirotarsi were carefully dissected, without damaging the periosteum. All remaining surgical procedures were performed in phosphate buffer saline (PBS). A single longitudinal incision through the periosteum along the entire diaphysis was made with a scalpel next to the fibula. Both the proximal and distal cartilages were removed via a scalpel-cut at the epiphysial base. The fibula was removed without additional tissue. Hereby, the periosteum came away from the underlying bone to which it is only loosely attached. Subsequently, the periosteum was placed in between two agarose layers, in the exact plane in which, according to the finite element simulation, high tensile strains occurred. The procedure was as follows: the bottom of each well was filled with a 4 mm thick layer of 3% low-melting temperature agarose type VII (Sigma, Zwijndrecht, The Netherlands) and the agarose was allowed to solidify at 4°C for 10 min. One periosteal explant was placed on the agarose base in each well and was fixed at the corners with thin pins in order to keep the periosteum planar and flat. The periosteum was covered by a 2 mm thick layer of the same agarose. After the agarose had solidified for 15 min, pins were removed and the moulds with explants were placed into medium containers (26 ml of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) medium containing...
10% fetal calf serum, 1% ITS + Premix (human recombinant insulin, human transferrin (162.5 µg each) selenous acid (162.5 ng), bovine serum albumin (BSA) (32.5 mg), and linoleic acid (139 µg)) (BD Biosciences, San Jose, USA), 50 µg/ml ascorbic acid and 1% penicillin–streptomycin.

A pilot experiment was performed in which either TGF-β1 or TGF-β3 was added to chick periosteal explants in the organ culture model, to find the most effective isomer for our culture model.

Four groups were defined. The control group contained explants in standard medium, cultured under free swelling conditions (CTR). In the other groups either the medium was supplemented with 10 ng/ml TGF-β1 (TGF), the samples were loaded by sliding indentation (IND), or 10 ng/ml TGF-β1 was added in addition to sliding indentation (TGF-IND). Sliding indentation was 10% of depth at 20 mm/s and the indenter moved back and forth over the agarose once every 2 s for 4 h/day. Medium was changed 3 times per week.

Analyses

After 0 (n = 16), 1 (n = 16 per experimental group) or 2 (n = 16 per experimental group) weeks of culture, periosteal explants were removed from the agarose layers and analyzed.

DNA, HYP and sGAG assay

Per experimental group and per time point, eight periosteal explants were lyophilized, weighed dry, and digested in papain solution (100 mM phosphate buffer, 5 mM l-cystein, 5 mM ethylenediaminetetraacetic acid (EDTA) and 125–140 µg/ml papain) at 60 °C for 16 h. The amount of DNA was determined using the Hoescht dye method with a reference curve of calf thymus DNA (Sigma). The sulfated-glycosaminoglycan (sGAG) content was determined using a modification of the assay described by Farndale et al., and a shark cartilage chondroitin sulfate reference (Sigma). Subsequently, digested tissue samples were hydrolyzed in 6 M hydrochloric acid (Merck, Whitehouse Station, NJ, USA) and hydroxyproline (HYP) quantity was assessed using an assay modified from Huszar et al. and a trans-4-hydroxyproline (Sigma) reference. The amounts of DNA, HYP and sGAG were expressed per mg dry weight of tissue.

Histology and immunohistochemistry

Per experimental group and per time point, eight periosteal explants were fixed in formalin and paraffin-embedded and 5 µm slides were taken through the middle of the explants and stained with Safranin-O/Fast Green dyes to evaluate the distribution of sGAG and collagen.

Other samples were de-waxed, rehydrated and incubated in TRIS–EDTA buffer at 100 °C for 20 min. Non-specific binding was avoided by incubation in 1% BSA for 30 min. The samples were labeled with PBS containing monoclonal primary antibodies for type I collagen (1:100; Sigma, Zwijndrecht, The Netherlands) and type II collagen (1:100; Acris GmbH, Germany), after testing for specificity. Alexa 555- and 488-conjugated goat anti-mouse secondary antibody labeling (Molecular Probes, Leiden, The Netherlands) for collagen type I and II, respectively, and
4,6-diamidino-2-phenylindole (DAPI) nuclear counterstaining were performed to visualize collagen and cells.

Statistical analysis

Statistics were performed with Statgraphics (Statgraphics, Manugistics, Inc., Rockville, MD, USA). The effects of treatment and time as well as their interaction were examined for significant differences by two-way analysis of variance (ANOVA), with sGAG, DNA and HYP per dry weight as the dependent variables. If no significant interaction was found, a one-way ANOVA with Bonferroni post-hoc testing was used to search for significant differences in either time or treatment within the other factor level. In the case of a significant interaction effect, an independent t-test with Bonferroni correction for number of applied comparisons was used to test for significant differences between each specific time and treatment sub-group. Statistical significance was assumed for \( P < 0.05 \).

Results

Biochemical content

The amount of DNA per dry weight in the periosteal explants in between agarose layers was significantly lower after 1 week of culture than freshly harvested periosteum in all experimental groups (all \( P < 0.0001 \)). Between 1 and 2 weeks of culture, no significant differences were found [Fig. 3(A)].

sGAG content per dry weight increased with culture time in CTR and TGF groups (\( P < 0.0001 \)), and no significant differences between both groups were observed. In contrast, sGAG content in the IND group did not significantly change in time, but was significantly lower compared to the CTR and TGF groups at week 1 and 2 (all \( P < 0.001 \)). Interestingly, in the TGF-IND group, sGAG content per dry weight significantly increased in time (\( P < 0.0001 \)) and was significantly higher than in the IND group at both 1 and 2 weeks of culture (all \( P < 0.0001 \)) and similar to sGAG levels in CTR and TGF groups at both time points [Fig. 3(B)].

HYP content per dry weight did not significantly change with time of culture in CTR and TGF groups. In contrast, in both IND (\( P = 0.007 \)) and TGF-IND (\( P = 0.003 \)) groups the HYP content significantly increased in time, with no significant differences between both groups. The HYP content in the IND group was significantly higher than in the CTR group at week 1 (\( P = 0.002 \)) and week 2 (\( P = 0.001 \)), and also higher than in the TGF group at week 1 and 2 (both \( P < 0.0001 \)). The HYP content in the TGF-IND groups was significantly higher than in the CTR group at week 1 (\( P = 0.002 \)) and week 2 (\( P < 0.0001 \)) and also higher than in the TGF group at week 1 and 2 (both \( P < 0.0001 \)) [Fig. 3(C)].

Histology and immunohistochemistry

In freshly harvested periosteum explants, two layers could be detected: the fibrous layer and the cambium layer [Fig. 4(A)]. Directly after harvesting, collagen type I, but not type II was detected [Fig. 4(B)].

In the CTR and TGF groups, similar cartilage formation was evident after 1 or 2 weeks [Fig. 5(A–B)], with most cartilage formed after 2 weeks, consistent with the absolute amount of sGAG per dry weight. The tissues increased in apparent size, and became smooth, rounded and solid. Immunohistochemical staining showed collagen type II in the chondrogenic area, and collagen type I in the fibrous outer layer [Fig. 5(E–F)].

The periosteal explants in the IND group showed no cartilage formation after 1 or 2 weeks [Fig. 5(C)]. These explants contained mainly collagen, with sparse islands of proteoglycans in three out of 16 explants (data not shown), in agreement with the biochemical data. Immunohistochemical staining showed collagen type I deposition throughout the entire explants and absence of collagen type II [Fig. 5(G)]. Additionally, chondrogenesis was not apparent and no change in size or shape of the periosteal tissue was observed.

In the TGF-IND group, cartilage formation was seen in all explants after 1 or 2 weeks of culture [Fig. 5(D)]. However, the area in the tissue in which cartilage formation was evident, was less rounded, smooth and firmly shaped compared with the CTR and TGF groups. Immunohistochemical staining showed deposition of collagen type II in the chondrogenic area of the explants, similar to CTR and TGF groups, and collagen type I in the fibrous layer [Fig. 5(H)].

Discussion

We have demonstrated that tissue differentiation in chick periosteal explants in organ culture can be tuned by biochemical and mechanical stimulations. Embedding the periosteal explants in between agarose layers induced cartilage formation. Addition of TGF-β1 to the culture medium did not further enhance this chondrogenic response. Applying sliding indentation to the periosteum in between agarose layers enhanced the production of collagen type I, leading to the formation of fibrous tissue without any evidence of cartilage formation. When sliding indentation was

**Fig. 4.** Freshly harvested periosteum. (A) Photomicrograph showing the two layers of a chick periosteal explant, demonstrating the outer fibrous layer and the inner cambium layer (Safranin-O/Fast Green staining; 40× magnification). (B) Section stained with antibodies for collagen type I (red) and II (green), demonstrating the absence of collagen type II in freshly harvested periosteum (40× magnification).
Fig. 5. Sections of periosteal explants after 2 weeks of culture, stained with Safranin-O/Fast Green (A–D, magnification 40×) and with antibodies for collagen type I and II (E–H, magnification 40×). Cartilage was produced by the explants between agarose layers, with and without addition of TGF-β1 (A, B) and collagen type II was synthesized in this cartilage (E–F). Only collagen type I was visible in the explants that were cultured under tension by sliding indentation and no cartilage was formed (C, G). When sliding indentation was combined with TGF-β1 supplementation cartilage formation was visible (D) and deposition of collagen type II can be seen in the chondrogenic area (H).
applied in presence of TGF-b1, enhanced collagen production was still present, but now collagen type II, while similar sGAG was found to unloaded and TGF-b1 treated samples.

The observation that cartilage formed in the embryonic chick periosteal explants is in agreement with previous in vitro studies, which showed that embryonic chick periosteum has chondrogenic potential in vitro\(^\text{26-22}\). Using the organ culture model, O'Driscoll et al. showed a clear chondrogenic response of rabbit periosteum when TGF-b1 was present in the culture medium, whereas chondrogenesis was observed only in few cases in cultures without TGF-b1\(^\text{22}\). This is in contrast with our study, in which cartilage was produced in explants cultured both with and without TGF-b1. This may be explained by the different species or developmental state of the used periosteal tissues. O'Driscoll et al. used periosteum of 2 weeks, 2 months and 6 months old rabbits, whereas embryonic chicks were used in our study. Embryonic tissue is known to be more adaptable to the culture conditions than similar tissue from mature animals. Furthermore, it is known that embryonic perios-tem naturally expresses relatively high amounts of TGF-b, which could affect the chondrogenic potential of our tissue in an autocrine manner, so that additional TGF-b1 was redundant. However, in general the effects of TGF-b on chondrogenic response are irreso- lute, with different studies that show stimulation, inhibition or no effect at all\(^\text{22-25}\). Further, the choice of the TGF-b isomer for enhancing periosteal chondrogenesis has been contradictory. TGF-b1 has been shown to enhance chondrogenic differentiation in chick periosteal cells\(^\text{12,22}\) and rabbit grafts in agarose suspension\(^\text{18,23}\). In other studies using human periosteal cells, TGF-b3 was more effective than TGF-b1 in inducing chondrogenesis\(^\text{26,27}\). Both isomers induced chondrogenesis similarly in our pilot experiments, but explants cultured with TGF-b3 contained considerably more hypertrophic cells (Fig. 6). For that reason TGF-b1 was used.

The periosteum was placed at a height in the agarose at which we computed that sliding indentation would exert the highest tensile strains on the tissue, given the externally applied indentation depth. This particular height does change with indentation depth, and is rather insensitive to variations in tissue stiffness or boundary conditions between agarose and periosteum. However, the absolute magnitude of the strain that is applied to the periosteum does depend on these conditions and may deviate from the values provided in Fig. 2. In our simulation, agarose properties fitted from experimental data\(^\text{19}\) and we measured periosteum properties in our lab in another study\(^\text{28}\). Because the periosteum could easily be separated from the agarose sandwich after the experiment, we assumed frictionless conditions between agarose and periosteum.

Mechanical stimulation through sliding indentation is a novel loading regime, which aims at stimulating collagen formation by inducing tensile strains. Our results on HVP content clearly showed that sliding indentation increases collagen formation in periosteal explants. However, even though we set up our study such that the periosteum was receiving the highest possible tensile strains in our system, it remains to be determined whether tensile strain alone triggered increased collagen synthesis. In addition, sliding indentation also induces fluid flow, compressive and shear forces. Fluid flow alone\(^6\), or fluid flow causing better transport of nutrients and waste products, could theoretically influence the response of the cells to mechanical loading\(^3\). However, because tensile strain has been shown to result in increased collagen production\(^1\), we propose that tensile strain is a likely candidate.

The loading regime was optimized for high tensile strains. However, it is possible that loading with other parameters will have a more favorable effect on cartilage formation in the explants. From literature it is known that chondrocytes and chondrocyte precursor cells react differently to different loading frequencies, magnitudes and periods\(^36-39\). Therefore, future studies aim at investigating the effect of different sliding indentation protocols on cartilage formation in the periosteal explants.

Sliding indentation in presence of TGF-b1 resulted in cartilage formation with high amounts of collagen type II and in absence of TGF-b1 no chondrogenic response was observed and only collagen type I was produced, resulting in a fibrous tissue. It is interesting to speculate what happens in the tissue in response to sliding indentation. Possibly, we stimulated the cells in the fibrous layer or the cambium layer or both. Also, the cells subsequently may have stimulated each other. Further, it is unclear whether the expression of new tissue occurred in the fibrous or the cambium layer. Pluripotent mesenchymal stem cells with the potential to form either cartilage or bone are found in the inner cambium layer of the periosteum. In vitro and in vivo experiments showed cartilage formation in this cambium layer\(^1\). Hence, we suggest the cartilage formation in our study also originates from these cambium cells. However, this study does not allow concluding whether the precursor cells respond directly to sliding indentation or whether an interaction between cell populations was essential. The mechanism by which these responses occur is a separate issue and requires further investigation of the signaling pathways.

In summary, this study confirms our hypothesis that application of sliding indentation stimulates collagen synthesis. We postulate this is caused by tensile strains induced by this loading regime. The type of produced tissue can be tuned, ranging from collagen type I rich fibrous tissue by only sliding indentation to cartilage with high amounts of collagen type II by sliding indentation in presence of TGF-b1. We speculate that this stimulatory effect of sliding indentation on collagen synthesis is generally applicable to cartilage tissue engineering systems and that therefore this technique may be valuable for improvement of the quality of tissue-engineered cartilage.

Contributions

Linda M. Kock: design of the study, acquisition of the data, analysis and interpretation of the data, drafting and revision of the article and final approval.
Agnese Ravetto: design of the study, acquisition of the data, analysis and interpretation of the data, revision of the article and final approval.
Corrinus C. van Donkelaar: design of the study, analysis and interpretation of the data, revision of the article and final approval. Responsible for the integrity of the work.
Jasper Foolen: analysis and interpretation of the data, revision of the article, technical support, and final approval.
Pieter J. Emans: design of the study, analysis and interpretation of the data, technical support, revision of the article and final approval.
Keita Ito: design of the study, analysis and interpretation of the data, revision of the article, and final approval. Responsible for the integrity of the work.

Conflict of interest
The authors state that they have no conflicts of interest.

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