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Differential response of porcine osteoblasts and chondrocytes in cell or tissue culture after 5-aminolevulinic acid-based photodynamic therapy

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Summary

Objective: Outcome in osteochondral allografting is limited by the immunological incompatibility of the grafted tissue. Based on a resistance of chondrocytes to photodynamic therapy in cell culture it is proposed that 5-aminolevulinic acid-based photodynamic therapy (5-ALA-PDT) might be used to inactivate bone while maintaining viability of chondrocytes and thus immunomodulate bone selectively.

Methods: Chondrocytes and osteoblasts from porcine humeral heads were either isolated (cell culture) or treated *in situ* (tissue culture). To quantify cytotoxic effects of 5-ALA-PDT (0–20 J/cm^2, 100 mW/cm^2) an (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) (MTT)-assay was used in cell culture and *in situ* hybridization in tissue culture to assess metabolic active cells (functional osteoblasts: colα_1(I) mRNA, functional chondrocytes: colα_1(II) mRNA).

Results: In cell culture, survival after 5-ALA-PDT was significantly higher for chondrocytes (5 J/cm^2: 87 ± 12% compared to untreated cells) than for osteoblasts (5 J/cm^2: 12 ± 11%). In tissue culture, the percentage of functional chondrocytes in cartilage showed a decrease after 5-ALA-PDT (direct fixation: 92 ± 2%, 20 J/cm^2: 35 ± 15%; $P < 0.0001$). A significant decrease in the percentage of bone surfaces covered by functional osteoblasts was observed in freshly harvested (31 ± 3%) compared to untreated tissues maintained in culture (11 ± 4%, $P < 0.0001$), with no further decrease after 5-ALA-PDT.

Conclusion: Chondrocytes were more resistant to 5-ALA-PDT than osteoblasts in cell culture, while in tissue culture a loss of functional chondrocytes was observed after 5-ALA-PDT. Since osteoblasts - but not chondrocytes - were sensitive to the tissue culture conditions, devitalized bone with functional cartilage might already be achieved by applying specific tissue culture conditions even without 5-ALA-PDT.

Key words: Osteoblasts, Chondrocytes, Osteochondral allograft, Aminolevulinic acid, Photodynamic therapy.

Introduction

Osteochondral allografts are used clinically for the treatment of full-thickness articular cartilage lesions[1]. The long-term outcome depends on the incorporation of the osteochondral grafts which is successful when the host-graft interface has united and tolerates physiological weight burdens without fracture or pain[2]. However, failure of allogeneic osteochondral implants due to insufficient incorporation occurs frequently[3]. The immune response of the host targeting the cells within the allogenic bone[3,4], which is associated with inflammation[5], may account for such limited graft integration. It was proposed earlier that pretreatment of osteochondral allografts yielding a devitalized osseous portion but a cartilage with functional chondrocytes could provide an optimized allograft for transplantation[6]. Even though various pretreatment approaches successfully reduced the antigenicity by removal of the cellular components from the osseous portion[7–11], chondrocytes were not resistant to the pretreatments[12]. Therefore, the need to establish a protocol to obtain osteochondral allografts with a devitalized osseous portion but preserved chondrocyte function still remains.

5-aminolevulinic acid-based photodynamic therapy (5-ALA-PDT) follows a two step protocol: (1) after exogenous administration, the prodrug 5-ALA is metabolized within the mitochondria to the photosensitive drug protoporphyrin IX (PpIX), which accumulates in target cells; (2) subsequent light activation of the photosensitizer PpIX initiates photochemical reactions in the presence of oxygen[13] and, depending on the amount of accumulated PpIX[14], leads to mitochondrial damage and cytochrome *c* release triggering apoptosis[15,16].

Previous experiments with bovine cells[17] revealed that chondrocytes were resistant to a treatment protocol with 5-ALA-PDT, whereas osteoblasts proved to be sensitive. The low mitochondrial content of the chondrocytes, which is an adaptation to their hypoxic environment *in vivo*[18–20], may account for a lower level of PpIX accumulation compared to osteoblasts and may therefore render the chondrocytes more resistant. Furthermore, 5-ALA-PDT applied to treat an antigen-induced arthritis in a murine model did not reveal any toxic effect on the cartilage[21].

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Table I
*Experimental groups in **(A)** cell culture and **(B)** tissue culture. The light dose applied in both "light alone" groups was 20 J/cm²*

Group	Untreated	Ala-dark	Light alone	5 J/cm²	10 J/cm²	15 J/cm²	20 J/cm²
(A) Experimental groups in cell culture							
Culture	+	+	+	+	+	+	+
5-ALA	−	+	−	+	+	+	+
Light	−	−	+	+	+	+	+
	Direct fixation	Culture	Ala-dark	Light alone	5 J/cm²	10 J/cm²	20 J/cm²
(B) Experimental groups in tissue culture							
Culture	−	+	+	+	+	+	+
5-ALA	−	−	+	−	+	+	+
Light	−	−	−	+	+	+	+

Therefore, we proposed that 5-ALA-PDT devitalizes the osseous portion of an osteochondral allograft selectively, whereas the chondrocytes are resistant to this treatment. Thus, the aim of this study was to investigate the feasibility of 5-ALA-PDT as a novel pretreatment protocol for osteochondral allografts. In a first step, cell culture experiments using porcine cells were performed to confirm previous experiments with bovine cells revealing the higher resistance of chondrocytes - compared to osteoblasts - to 5-ALA-PDT. In a second step, experiments on porcine osteochondral tissues were performed to extend the investigations from cell- to tissue culture.

Material and methods

EXPERIMENTAL PROTOCOL

Osteoblasts, chondrocytes, and osteochondral tissues were harvested from humeral heads of 6-months-old pigs, obtained from a local slaughterhouse, within three hours after death. Six animals were used in total for cell culture (three animals) and tissue culture (three animals) experiments. The cells and the osteochondral tissues were incubated with 5-ALA and subsequently exposed to light. The effect of 5-ALA-PDT in cell culture was assessed 20 h after light application using a cell viability assay. The tissues were assessed histologically 4 and 20 h after light application by *in situ* hybridization for transcripts encoding collagen type I and II in order to detect functional osteoblasts and chondrocytes, respectively.

CELL CULTURE EXPERIMENTS

Chondrocyte culture

Articular chondrocytes were harvested from porcine humeral heads by enzymatic digestion and cultured in a three dimensional culture system using alginate as described previously[22]. Briefly, the articular cartilage was sequentially digested with 0.2% pronase E (Sigma-Aldrich, Buchs, Switzerland) and 0.03% collagenase P (Roche Diagnostics, Rotkreuz, Switzerland) to release the chondrocytes. Alginate beads were then generated with 4×10^6 chondrocytes per ml of 1.2% alginate (alginic acid sodium salt; Sigma-Aldrich). The beads were cultured for 6 days prior to experiments in 45% Ham's F-12 (Oxoid AG; Basel, Switzerland)/ 45% Dulbecco's Modified Eagle Medium (DMEM) (DMEM; Invitrogen, Basel, Switzerland) (high glucose, Oxoid)/ 10% heat-inactivated fetal bovine serum (FBS H.I.; Oxoid)/ L-glutamine (Sigma-Aldrich)/ penicillin/streptomycin (P/S; Sigma-Aldrich) at 37°C and 5% CO_2. Thereafter, one alginate bead per well was placed in 96-well tissue culture plates for the experiments.

Osteoblast culture

Porcine osteoblasts were prepared using an outgrowth method[23]. Fragments of cancellous bone were harvested from humeral heads, incubated in 300 U/ml collagenase type IV (Sigma-Aldrich) for 2 h and cultured in DMEM (high glucose w/o calcium; Invitrogen, Basel, Switzerland)/ 10% FBS H.I./ L-glutamine/ P/S for 1−2 weeks at 37°C and 5% CO_2. The culture medium was changed twice per week. At confluence, the cells growing from the bone particles were released with trypsin/ Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), replated in tissue culture flasks and subcultured. Released cells were stored in liquid nitrogen and upon thawing expanded in DMEM (high glucose with calcium)/ 10% FBS H.I./ L-glutamine/ P/S at 37°C and 5% CO_2. At confluence, they were released with trypsin/ EDTA, washed, resuspended, and 20,000 cells per well were plated in 96-well tissue culture plates for further experiments.

5-ALA-PDT on chondrocytes and osteoblasts

Based on previous publications[24,25] the cells were incubated for 4 h at 37°C, 5% CO_2 in 1 mM 5-ALA (Sigma-Aldrich) in serum-free medium (DMEM (high glucose); P/S), since serum has been shown to reduce intracellular levels of PpIX[26]. The cells were subjected to increasing light doses up to 20 J/cm² with an intensity of 100 mW/cm² (600−660 nm, Aktilite CL128, Galderma, Düsseldorf, Germany). Thereafter, the medium was replaced by DMEM (high glucose)/ 10% FBS H.I./ L-glutamine/ P/S. 5-ALA-PDT was performed independently three times on chondrocytes and osteoblasts obtained from different animals ($N = 3$). In order to minimize intraexperimental variability in the cell viability assays subsequently to 5-ALA-PDT, the cell cultures of chondrocytes and osteoblasts of one animal were subdivided into three aliquots (triplicates, for each of the experimental groups shown in Table I-A) before 5-ALA-PDT was performed.

Assessment of cell viability

To detect cytotoxic effects of 5-ALA-PDT, the survival of the cells treated with 5-ALA-PDT was determined by performing an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide) based colorimetric assay 20 h after illumination according to the manufacturer's instructions (Cell proliferation kit I; Roche Diagnostics). Prior to the solubilization of the formed crystals during the MTT-assay, chondrocytes were released from the beads by dissolving the alginate with a solubilization buffer (55 mM Na-citrate (Sigma-Aldrich), 20 mM EDTA (VWR international, Dietikon, Switzerland), 150 mM NaCl (VWR international)).

TISSUE CULTURE EXPERIMENTS

Harvest of and 5-ALA-PDT on osteochondral tissues

Cylindrical osteochondral tissues of a size used for mosaic plasty (diameter: 5 mm, length: 10 mm) were harvested from porcine humeral heads with a commercially available device (Osteochondral Autologous Transfer System; Arthrex, Naples, FL, USA) as used in the clinical setting[27]. During this procedure the articular cartilage was irrigated with physiological saline (Invitrogen).

The tissues were incubated for 4 h in 1 mM 5-ALA in serum-free medium (DMEM (high glucose), P/S) in 6-well tissue culture plates. One osteochondral plug was placed into each well of the tissue culture plates. After incubation, the culture plates were placed on a table with the light source above. Thus, the direction of the illumination was perpendicular to the longitudinal axis of the osteochondral cylinders. The tissues were subjected to increasing light doses with a maximum of 20 J/cm² since we reported previously that this light dose yielded a survival rate of less than 12% in osteoblasts, fibroblasts, bone marrow cells and dendritic cells whereas survival of chondrocytes was not affected[25]. After 5-ALA-PDT, the tissues were cultured at 37°C for 4 h in DMEM (high glucose)/ 10% FBS H.I./ L-glutamine/ P/S and subsequently either fixed in paraformaldehyde or stored for a further 16 h at 4°C before fixation to imitate a potential clinical setup. The tissues were thus assessed at two different time points after 5-ALA-PDT (4 h and 20 h). All tissues for one experiment were harvested from the same animal. Tissues assessed after 4 h were harvested from one humeral head whereas the tissues assessed after 20 h were from the opposite side. Additionally, an osteochondral cylinder was harvested from every humeral head and directly fixed in paraformaldehyde. 5-ALA-PDT was performed independently three times on tissues obtained from three different animals ($N = 3$). The tissues for the different experimental groups (as shown in Table I-B) were obtained from the same animal and were treated with 5-ALA-PDT at the same time.