

1–34 PTH Effect on the Chondroprogenitor Cells Differentiation, As Well As on the Microstructure of the Subchondral Bone Tissue, and the Regeneration of Articular Cartilage in Rats¹

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Abstract—The purpose of the present study was to investigate the impact of intermittent and constant administration of 1–34 PTH fragment on bovine chondroprogenitor cells differentiation contained in chondrogenic and osteogenic media. In addition, regeneration of the cartilage tissue of non-native rats and the dependence of this process on the level of parathyroid hormone, as well as changes in the subchondral bone, were studied. Monolayer cultures of bovine chondroprogenitor cells contained in osteogenic and chondrogenic medium with periodic and constant addition of 1–34 PTH, were examined by immunofluorescence analysis to reveal appropriate markers. Rats' knee joints have been operated with the full thickness defect formation, and further investigation of the regeneration processes depending on the introduction of the PTH in vivo by histochemical analysis of the operated knee joints. The findings suggest that the modulating effect of PTH on chondrogenic differentiation of stem cells and the therapeutic potential of this hormone for cartilage regeneration.

Keywords: parathyroid hormone, chondrocytes, stem cells, differentiation

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INTRODUCTION

By previous studies it was established that intermittent 1–34 fragment of parathyroid hormone (PTH) administration increases mass, strength and mineral density of bone, and improves bone microarchitecture and fracture healing [1, 2]. Furthermore, previous in vitro studies have indicated that intermittent delivery of 1–34 PTH enhances the proliferation and differentiation of osteoprogenitor cells in bone marrow, increases osteoblast activity and inhibits osteoblast apoptosis [3]. However, the precise molecular mechanism underlying the effect of 1–34 PTH during the osteogenic differentiation of stem cells remains elusive.

Several studies indicated that 1–34 PTH also affects chondrocyte as well. PTH 1–34 inhibits the terminal differentiation of articular chondrocytes and the progression of osteoarthritis (OA) [4]. In parallel with the suppression of chondrocyte hypertrophy, PTH (1–34) stimulates chondrocyte proliferation and differentiation in the early stage [5–7]. However, the effects of PTH on chondrogenic differentiation of stem cells remain to be elucidated. If it is hypothesized that PTH promotes early chondrogenic differentiation of chondroprogenitor cells, so there is need of investigation of the dosage dependent modulatory effect of PTH.

Thus, it was found that PTH has opposite effects on chondrogenesis, depending on the concentration and the administration of hormone in culture media.

However, despite the number of studies, the effect of different doses of 1–34 PTH on differentiation of bovine chondroprogenitor cells is still poorly understood. Accordingly, the aim of the present study was to investigate the impact of intermittent (I-PTH) and continuous (C-PTH) PTH 1–34 fragment administration on bovine chondroprogenitor cells differentiation in chondrogenic and osteogenic media.

MATERIALS AND METHODS

Chondroprogenitor Cells Isolation and Expansion

Chondroprogenitor cells were isolated on the basis of differential adhesion to fibronectin. Chondrocytes were isolated by surgical dissection from the metacarpal-phalangeal joints of 7-day-old juvenile bovine steers and subjected to sequential pronase (70 U mL⁻¹, 1 h at 37°C) (PRON-RO, “Sigma”, UK), collagenase (300 U mL⁻¹, 3 h at 37°C) (COLLD-RO, “Sigma”, UK) enzymatic digestion. Isolated chondrocytes were subjected to differential adhesion on fibronectin-coated (FN; Sigma, UK). After 20 min, media and non-adherent cells were removed and replaced with standard growth media: Dulbecco's modified eagles

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medium (DMEM Sigma, UK) penicillin 10000 mg mL⁻¹/streptomycin 10000 U mL⁻¹ (Penicillin/streptomycin, "Sigma", UK), 0.1 mM L-ascorbic acid ("Sigma", UK), 100 mM HEPES ("Sigma", UK), and 10% fetal bovine serum (FBS, "Sigma", UK). Cultured cells were maintained in a humidified 37°C, 5% CO₂ incubator for 14 days. Bovine cartilage chondroprogenitor cells were cultured in chondrogenic media containing Insulin-Transferrin-Selenium (ITS, "GIBCO", UK) and TGF-β2 ("PeproTech", UK), and in osteogenic medium DMEM+ plus β-Glycerophosphate ("Sigma", UK). Media were changed every other day.

Chondroprogenitor Transwell Culture

Chondroprogenitor cells were pelleted at a concentration of 1×10^6 cells per 1.5 mL in transwell membranes and grown in chondrogenic medium; DMEM/F12, 1% insulin-transferrin-selenium supplement (Sigma, UK), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.1% gentamicin, 0.1 mM dexamethasone and 10 ng transforming growth factor b2 (TGFβ2) ("PeproTech", UK). According to the PTH 1-34 fragment ("Sigma", UK) administration methods, cells were divided into several groups: intermittent PTH administration (I-PTH) and continuous PTH administration (C-PTH). Media were changed every next day. In the I-PTH administration group PTH was added every 48 h (100 ng/mL). The media were replaced every other day and transwell cultures maintained for a total of 21 days.

Histochemical Studying

Cells prior to confluency were analyzed by histochemical staining. Toluidine blue was applied to reveal acidic glycosaminoglycans, markers of chondrogenesis. Alizarin red was used to detect calcified regions in matrix, which is marker of osteogenesis. Results evaluation was done by phase-contrast microscopy (Eclipse E800; "Nikon", Japan), pictures were taken by built in camera (2000R Fast1394; "Retiga", Japan).

Immunofluorescent Labelling of Chondroprogenitor Monolayer Cultures

Monolayer cultures of 4% paraformaldehyde fixed chondroprogenitor cells were processed for immunodetection of proteins. Primary antibodies, monoclonal anti-rat Col-1, Col-2, osteocrin (bTan20, Developmental Studies Hybridoma Bank, "Iowa", USA), were diluted in tris-buffered salts (TBS) at a concentration of 5 mg ml⁻¹ and sections probed for 12 h at 4°C. Monolayer cultures were probed with either anti-goat peroxidase-conjugated or fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and anti-mouse secondary antibodies ("Sigma", UK).

Fluorescently labelled sections were mounted in Vectashield (VectorLabs) containing 40,6-diamidino-

2-phenylindole (DAPI) as a counter stain for cell nuclei and viewed using an Olympus BX61 fluorescence microscope.

The Formation of Articular Cartilage Full Thickness Defect

Rats aged 8 weeks were operated on to form a defect of the cartilage covering the joint surface of the femur. By means of microsurgery, bilateral defects were formed in the full thickness of the articular cartilage in the knee joints of both hind limbs. At the same time, the rats were anesthetized with intraperitoneal injection (0.5-1.0-mL/kg body weight) of ketamine (100-mg/mL), xylazine (20-mg/mL) and acepromazine (10-mg/mL). Access to the articular bag was obtained by a small (0.5-1.0 cm), parapatellar, a cut of the skin from the medial side, the joint capsule was opened and the knee cap was laterally displaced to expose the articular surface of the tibia. A defect in the total thickness of the cartilage was formed by inserting a sterile 27Gx 1/2 needle into the cartilage in a circular motion. The penetration into the medullary cavity was confirmed by the appearance of a drop of blood on the surface of the cartilage after the needle was removed. The joint capsule was closed with a resorbable polypropylene filament, a continuous seam. The subcutaneous tissue was sewn with a mattress seam and Vet-close skin glue.

In the study of continuous exposure to PTH (C-PTH), 1-34 the fragment was injected every day by intramuscular injection, in the study of intermittent exposure (I-PTH), every other day, with a calculated dose of PTH: 100 ng PTH per 1 g.

Histological Examination

The rats were euthanised 8 weeks after surgery. Both knee joints were fixed in 10% neutral formalin buffered solution and decalcified using 10% formic acid in 5% formaldehyde solution for 48 h at 4°C. The sagittal sections after dewaxing were stained with toluidine blue and studied using the (Eclipse E800; Nikon, Japan) microscope and captured with a built-in camera (2000R Fast1394; Retiga, Japan) for histological examination. There were studied 10 rats in each experimental group.

RESULTS AND THEIR DISCUSSION

Chondroprogenitor cells were isolated from cell suspensions due to their ability to adhere to fibronectin. Cells acquired fibroblast-like stellate structure, already on the 1st day of seeding, in this case bovine cells differ from human by larger cell bodies and higher growth kinetics. So clearly defined colonies were formed on the 7-th day of seeding, and the 14-th day, cells reached a 90% confluent state.

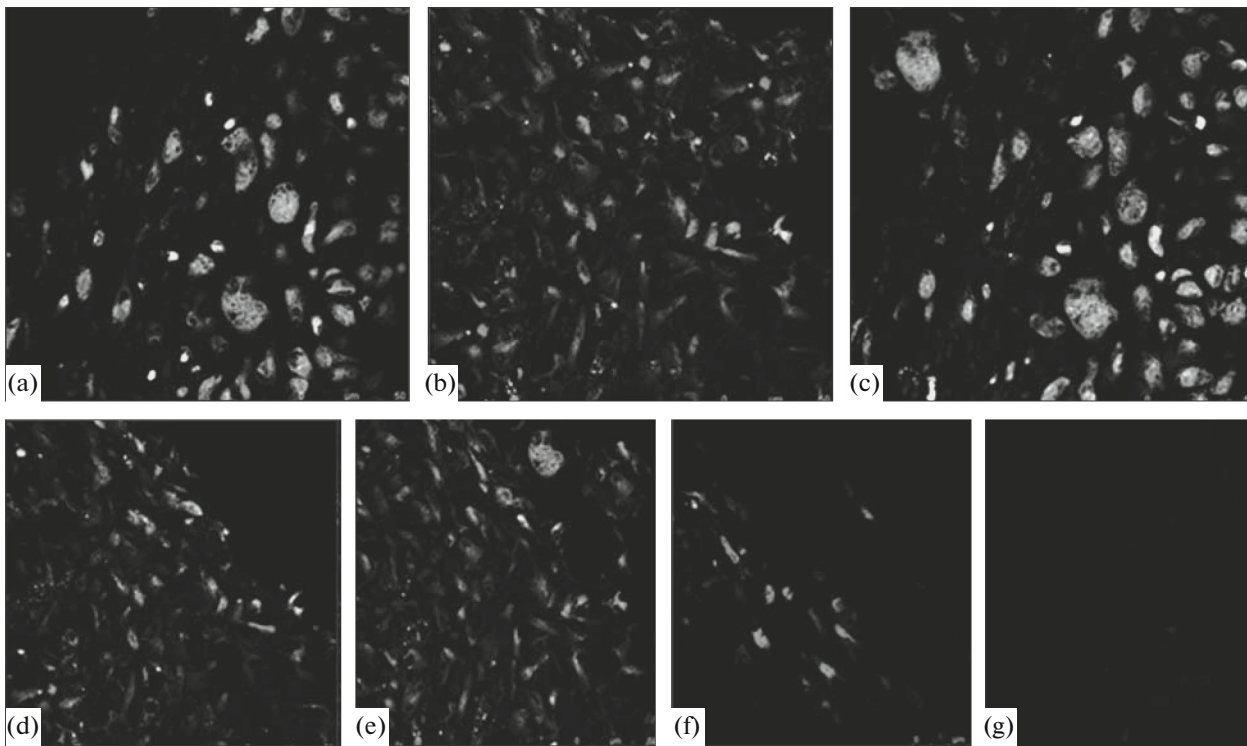


Fig. 1. Immunofluorescent labeling Collagen 1 (a, b); Collagen 2 (c, d); Osteocrin (e, f); negative control (g).

Monolayer cultures were immunofluorescently labeled to detect the activity of collagen types 1 and 2 and osteocrin. In monolayer cultures of chondroprogenitor cells grown in osteogenic medium with periodic addition of parathyroid hormone fragment 1–34 (I-PTH), an intense fluorescent labeling was detected for collagen type 1 and osteocrin. It is known that these proteins are characteristic for bone matrix, i.e. may be considered as markers of bone formation (Figs. 1a, 1e). At the same time in monolayer cultures grown in osteogenic medium with the constant administration of PTH 1–34 fragment (C-PTH) fluorescent labeling was slightly reduced (Figs. 1b, 1f). In contrast to this fact, expressed fluorescence was observed for type 2 collagen contained in a medium with constant PTH addition (Figs. 1c, 1d), which indicates the intensity of chondrogenesis, accompanied by the accumulation of collagen type 2, which is characteristic for cartilage matrix.

Chondroprogenitor cells were also seeded on Transwell membranes in special systems that allow the formation of three-dimensional cell cultures and with their possible subsequent transfer in skeletal tissue defect *in vivo* (Fig. 2).

Transwell cultures were fixed and stained on day 21 after seeding. Thus, toluidine intense staining was revealed in cultures kept in chondrogenic medium with the constant addition of PTH (Fig. 2a), unlike cultures receiving only periodic dose of the hormone (Fig. 2b).

Conversely, the deposition of calcium compounds is noted in the cell cultures contained in a medium with periodic PTH administration (Fig. 2c), unlike tissue obtained from medium with a high constant level of hormone (Fig. 2d). Thus, the present study confirms the fact that the periodic raising of parathyroid hormone concentration is activating bone formation, while the constant high levels of PTH lead to an intensification of chondrogenesis, i.e. can contribute to the joints cartilage regeneration.

We also investigated the effect of a permanent (C-PTH) or periodic (I-PTH) increase in the level 1–34 of PTH on the restoration of cartilage tissue *in vivo* in non-native white rats. It was found that a constant increase in the level of PTH promotes the restoration of articular cartilage. In this case, the area of the articular cartilage defect was filled with a hyaline tissue with a smooth surface and an intensely colored matrix rich in proteoglycans. In structure, the restored site did not differ from the neighboring intact cartilage, i.e. full integration of the regenerated area was noted (Fig. 3). At the same time, intensive resorption of the subchondral bone was observed, which was manifested by the expansion of lacunas of the spongy epiphyseal bone.

At the same time, the periodic administration of PTH to mice promotes thickening of the subchondral bone, however, there is no restoration of the cartilaginous tissue. The area of the articular cartilage defect was filled with a poorly colored fibrous cartilaginous tissue, i.e. there was no cartilage matrix typical of hya-

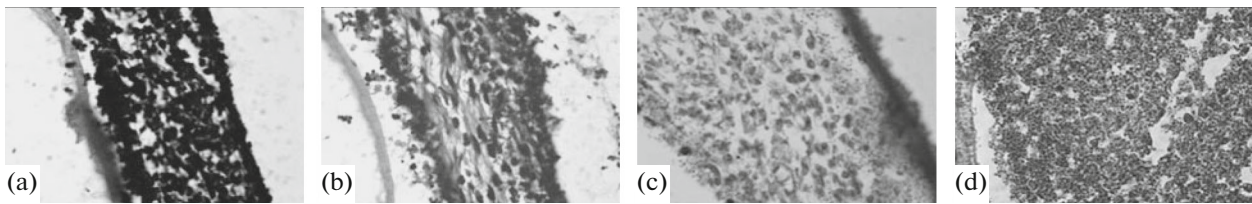


Fig. 2. Chondrogenesis, toluidine blue C-PTH (a); Chondrogenesis, toluidine blue, I-PTH (b); Osteogenesis, alizarin red C-PTH (c); Osteogenesis, alizarin red, I-PTH (d), 10 × 40.

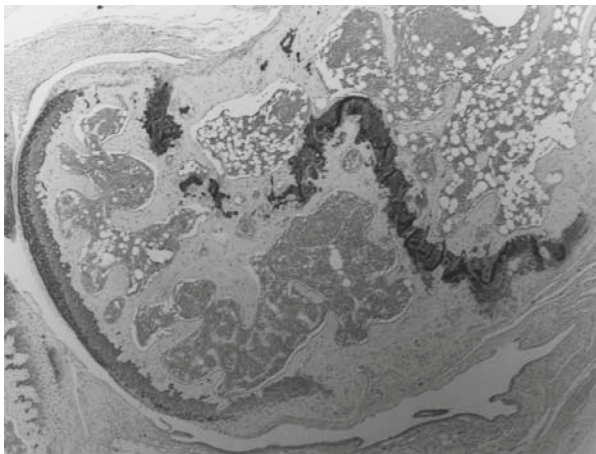


Fig. 3. Continuous PTH administration, 10 × 40.

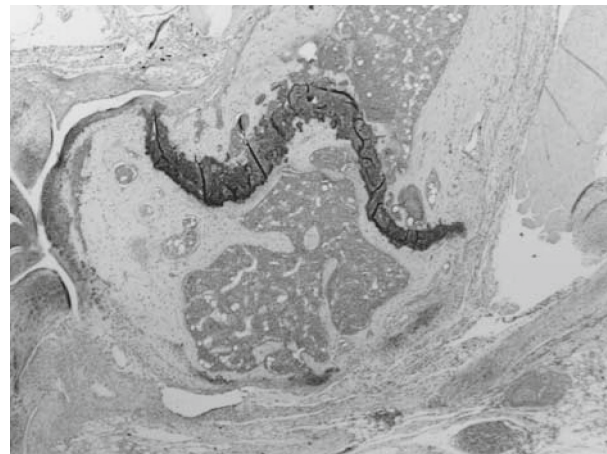


Fig. 4. Periodic PTH administration, 10 × 40.

line cartilage (Fig. 4). Reduced regeneration activity is manifested in histological sections. Thus, in the cartilaginous defect area in rats, a fibrous cartilaginous tissue with a characteristic light coloration of the matrix is revealed, while with constant administration of PTH the defect area is filled with hyaline tissue, with a matrix rich in proteoglycans and a smooth surface characteristic of healthy hyaline cartilage. The regeneration tissue is similar to the surrounding hyaline cartilage in structure and thickness, with full integration along the edges of the defect with the surrounding intact cartilage. Accordingly, the continuous administration of PTH promotes regeneration of the articular cartilage, while the periodic reduces the regenerative activity.

Our study is confirmed by a number of scientific papers. Kao et al. [8] reported that direct activation of cAMP signaling by forskolin in bone mesenchymal stem cells (BMSCs) elevated the expression of osteoblast marker genes, such as RUNX2, osterix, collagen I and osteocalcin, and that treatment of BMSCs with PTH enhanced the ability of the subsequent differentiated osteoblasts to mineralize. As with PTH, exposing BMSCs to forskolin also increased mineralization. In the present study, and consistent with these previous studies, the results of the alkaline phosphatase (ALP) activity assay and RT-qPCR revealed that ALP activity and RUNX2, osterix, collagen I, osteocalcin

and osteopontin expression were all upregulated by intermittent 1–34 PTH application, and that the addition of forskolin enhanced these effects to a greater degree than was achieved by 1–34 PTH alone. Furthermore, the effects of 1–34 PTH were inhibited by H-89. Alizarin Red S staining revealed that intermittent 1–34 PTH treatment significantly increased mineralization via the cAMP/PKA pathway. Together, these data imply that intermittent 1–34 PTH administration can affect osteogenesis by increasing the commitment of BMSCs to the osteogenic lineage, via activation of the cAMP/PKA pathway [9, 10].

The findings suggest that the modulating effect of PTH on chondrogenic differentiation of stem cells and the therapeutic potential of this hormone cartilage regeneration. Based on clinical experience with the efficacy and safety of the hormone for bone metabolism, PTH may also be used in the clinic for cartilage repair.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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