Behaviour of human physeal chondro-progenitor cells in early growth plate injury response in vitro

Karin Pichler · Barbara Schmidt · Eva E. Fischerauer ·
Beate Rinner · Gottfried Dohr · Andreas Leithner ·
Annelie M. Weinberg

Received: 28 January 2012 / Accepted: 9 May 2012 / Published online: 25 May 2012
© Springer-Verlag 2012

Abstract

Purpose The aim of this study was to investigate the proliferation and differentiation behaviour of a defined cell population gained from the human growth plate, namely, chondro-progenitor cells (CPCs), in the initial inflammatory phase of growth plate injury response in vitro.

Methods Growth plate cells were sorted via FACS and differentiated along adipogenic and osteogenic lineage to confirm their progenitor features. To mimic the inflammatory phase of injury response at the growth plate they were treated with IL-1 β and exposed to cyclic mechanical loading. A BrdU assay was used to investigate CPC proliferation. CPC differentiation behaviour was analysed by RT-PCR.

Results CPCs (CD45-, CD34-, CD73+, CD90+, and CD105+) showed a successful differentiation along adipogenic and osteogenic lineage. Under conditions simulating the inflammatory phase of injury response at the growth plate in vitro CPCs differentiated towards hypertrophy while chondrogenesis and

ossification were inhibited. Proliferation was not significantly altered.

Conclusion This study showed that CPCs can be isolated from the human growth plate and expanded in vitro. In the first phase of injury response at the growth plate these cells differentiate towards hypertrophy. As longitudinal growth is obtained by chondrocyte proliferation and volume increase during hypertrophy this maturation might be the first step towards post-traumatic growth disorders such as unwanted premature ossification of the growth plate.

Introduction

The growth plate at the ends of long bones is a unique feature of a child's bone. This structure controls longitudinal bone growth. The growth plate contains chondrocytes at different stages of differentiation, which are organised into several zones: resting, proliferative, pre-hypertrophic and hypertrophic. Within the growth plate, enchondral ossification occurs, which gradually converts cartilage into bone [1]. This well orchestrated process can be easily disrupted by fractures involving the growth plate. If large enough, such fractures impair the growth process, leading to growth disorders due to destruction of the epiphyseal circulation and formation of a bone bridge [2]. A bone bridge evokes limb length discrepancies and predisposes to angular deformity, as the chondrocytes of the remaining parts of the growth plate continue the normal growth process [3].

Following a growth plate injury four different phases of injury response have been identified in various animal models. The first response is the inflammatory phase, followed by the fibrogenic phase, where stromal progenitor cells infiltrate the injury site. During the osteogenic and maturation phases bone cells differentiate and form bony tissue [4]. The molecular and cellular mechanisms regulating these injury healing phases at the growth plate, however, have not been clarified yet.

K. Pichler (✉) · B. Schmidt · E. E. Fischerauer
Department of Pediatric and Adolescent Surgery,
Medical University Graz,
Auenbruggerplatz 34,
8036 Graz, Austria
e-mail: karin.pichler@medunigraz.at

B. Rinner
Center for Medical Research – ZMF, Medical University Graz,
Stiftingtalstraße 24,
8010 Graz, Austria

G. Dohr
Institute of Cell Biology, Histology and Embryology,
Center for Molecular Medicine, Medical University of Graz,
8010 Graz, Austria

A. Leithner · A. M. Weinberg
Department of Orthopaedic Surgery, Medical University of Graz,
8036 Graz, Austria

During the inflammatory phase of injury response the growth plate is influenced mainly by two stimuli: (1) highly up-regulated pro-inflammatory cytokines and (2) differential mechanical loading forces in terms of micro-movements occur at the injury site, even if the fracture is osteosynthetically stabilised. Pro-inflammatory cytokines stimulate the remodeling of the extracellular matrix and cell differentiation towards both osteogenic and chondrogenic lineages [5, 6]. Mechanical stimuli influence growth plate chondrocyte metabolism, dependent on loading type and frequency [7].

The aim of our study was to mimic for the first time the initial inflammatory phase of injury response at the growth plate in vitro, and to investigate under these conditions the proliferation and differentiation behaviour of a defined cell population gained from the human growth plate, namely, chondro-progenitor cells (CPCs). Thus, we isolated cells from the human growth plate and sorted them via FACS in order to obtain CPCs. These CPCs were treated with IL-1 β and exposed to cyclic mechanical loading using the Flexcell Tension Plus System 3000 (Flexcell Int, Hillsborough, NC) to simulate the up-regulation of inflammatory cytokines and the presence of micro-movements at the injury site, respectively. Proliferation behaviour of CPCs was then analysed with a BrdU proliferation assay and mRNA expression of the main chondrogenic and osteogenic marker genes was investigated in a RT-PCR. We expected both mechanical and inflammatory stimuli to greatly influence CPC proliferation and differentiation.

Material and methods

Cell culture

Samples of the infant growth plate were obtained from the supernumerary digit of eight different donors with polydactylism at the time of surgical excision. The study protocol was approved by the local ethics committee (EK-No. 20–344 ex 08/09). Growth plate tissue was digested in 300 units/ml collagenase B (Worthington Biochemical Corp., Lakewood, NJ) overnight, and then 6×10^5 cells were re-suspended in growth medium (DMEM/F-12 1:1 supplemented with 5 % FCS, 1 % penicillin/streptomycin and 0.1 % Amphotericin B; all reagents from Invitrogen, Carlsbad, CA) and seeded into a 75 cm² flask. After one to three passages, cells were harvested and sorted via fluorescence-activated cell sorting (FACS). All experiments were performed under standard cell culture conditions of 37 °C, 5 % CO₂.

Cell sorting

Up to 5×10^6 cells from growth plate cartilage were incubated with primary conjugated antibodies specific for CD45-APC-CyTM7, CD34-APC, CD73-PE (all BD Bioscience, San Diego,

CA), CD90-FITC, or CD105-PerCP/Cy5.5 (both BioLegend, San Diego, CA) for one hour at 4 °C. Labeled cells were re-suspended in PBS and subjected to FACS (BD FACS Aria, BD Bioscience, Heidelberg, Germany). The appropriate IgG controls were run in parallel. Sorted CPCs were expanded in growth medium.

Osteogenic and adipogenic lineage differentiation

In order to confirm their progenitor features, CPCs were subjected to adipogenic and osteogenic lineage differentiation. CPCs were kept in adipogenic or osteogenic differentiation medium (STEMPRO[®] Adipocyte/Osteocyte Differentiation Medium, Invitrogen) for 21 days respectively. To evaluate lipid droplet formation, cells were fixed in 10 % formaldehyde and stained with Oil Red O for one hour (Sigma-Aldrich, Vienna, Austria). To evaluate the mineralised matrix an alkaline phosphatase (ALP) staining was performed. Cells were fixed in 10 % formaldehyde and stained with a solution of naphthol As-MX-PO₄ and Fast red violet LB salt (both Sigma-Aldrich) for 45 min.

Mimicking the inflammatory phase of injury response at the growth plate in vitro

CPCs in the first to third passages were used in this experiment. Cells for the control and experimental conditions were at the same passage number. Four groups were analysed: mechanical loaded CPCs (ML), mechanical loaded CPCs treated with IL-1 β (ML/IL-1 β), CPCs treated with IL-1 β (IL-1 β), and untreated CPCs (control). A total of 1.5×10^5 CPCs/well were seeded in collagen I coated flexible-bottomed culture plates (Flexcell Int) and incubated for 24 hours. One hour prior to mechanical loading 2-ng human recombinant IL-1 β /ml media (Sigma-Aldrich) were added to the wells of the corresponding groups. The Flexcell Tension Plus System 3000 (Flexcell Int) was then used to apply cyclic mechanical stretching with 3 % surface elongation at 0.1 Hz for five days, which was considered being representative for micro-movements [8]. The experiment was run in triplicate.

BrdU proliferation assay

CPC proliferative activity was measured following IL-1 β treatment and/or mechanical loading based on the BrdU incorporation during DNA synthesis using a commercial chemiluminescent BrdU ELISA kit (Roche Molecular Biochemicals, Vienna, Austria) according to the manufacturer's instructions.

Real time RT-PCR

Following IL-1 β treatment and/or mechanical loading total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden,

Germany) and reverse transcribed using the RevertAid cDNA Synthesis Kit (Fermentas) according to the manual. Real-time RT-PCR reactions were performed and monitored using the Light Cycler LC480 instrument (Roche Diagnostics). cDNA samples were analysed in triplicate. Sequences of primers for the reference gene (β -actin) and genes of interest, i.e. Sox9, type II collagen (Col2a1), aggrecan (ACAN), Runx2, type X collagen (Col10a1) and osteocalcin, are listed in Table 1. All primers were provided from MWG, Ebersberg, Germany. Results were normalised to β -actin levels using the formula ΔCt (threshold cycle) = Ct of target gene – Ct of β -actin. The comparative Ct method was used to investigate the amount of target gene relative to the calibrator. All graphs demonstrate the fold change and its range of each experimental group when the control group was normalised as 1.

Statistical analysis

For statistical analysis SPSS® 18.0 software (SPSS Inc., Chicago) was used. Possible statistical significance comparing the groups in terms of proliferation was analysed by Friedman's test. To determine statistically significant gene expression differences analysed by real time RT-PCR, the Wilcoxon signed-rank test was performed. A p -value <0.05 was considered significant.

Results

Samples

Growth plate cells from eight different donors were selected for the study. Ages ranged from 0.25 years to 2.3 years (mean $\pm$ SD, 1.14 ± 0.68 years) with no significant difference

Table 1 Human primer pairs used for real time RT-PCR

Gene	Primer
Sox9	Forward: 5-AGACCTTTGGGCTGCCTTAT-3
	Reverse: 5-TAGCCTCCCTCACTCCAAGA-3
Col2a1	Forward: 5-CTATCTGGACGAAGCAGCTGGCA-3
	Reverse: 5-ATGGGTGCAATGTCAATGATGG-3
ACAN	Forward: 5-CAGCACCAGCATCCAGA-3
	Reverse: 5-CAGCAGTTGATTCTGATTAC-3
Runx2	Forward: 5-AGAAGGCACAGACAGAAGCTTGA-3
	Reverse: 5-AGGAATGCGCCCTAAATCACT-3
Col10a1	Forward: 5-TCCTTGAACCTGGTTCATGGAGT-3
	Reverse: 5-ACTGTGTCTTGGTGTGGGTAGT-3
Osteocalcin	Forward: 5-GGCGCTACCTGTATCAATGG-3
	Reverse: 5-TCAGCCAACCTCGTCACAGTC-3
β -actin	Forward: 5-CTGGAACGGTGAAGGTGACA-3
	Reverse: 5-AAGGGACTTCCTGTAACAATGCA-3

between males and females. CPCs showed the following surface protein expression profile previously described to be characteristic for progenitor cells [9]: CD45–, CD34–, CD73+, CD90+, and CD105+ (Fig. 1).

CPCs from the human growth plate are inducible to osteogenic and adipogenic lineage differentiation

After a period of 21 days CPCs subjected to adipogenic differentiation demonstrated scattered aggregates of positive Oil Red O stained cells in the experimental group (Fig. 2a), which were absent in the control group (Fig. 2b). CPCs subjected to osteogenic differentiation stained clearly positive for ALP (Fig. 2c), whereas no expression of this enzyme could be observed in control groups (Fig. 2d).

Mechanical loading and IL-1 β treatment did not alter CPC proliferation significantly compared to control

The BrdU immunoassay showed that IL-1 β promotes proliferation of CPCs when compared to mechanical loading (Fig. 3). However, proliferation of CPCs that were exposed to both mechanical loading and IL-1 β did not significantly differ from control CPCs.

Under conditions simulating inflammatory injury response at the growth plate in vitro CPCs differentiate towards hypertrophy

To examine the effects of mechanical loading and IL-1 β on the differentiation behavior of CPCs, mRNA expression of the main markers for chondrogenesis, hypertrophy and osteogenesis was investigated by real-time RT-PCR. As markers for chondrogenesis we evaluated expression of Sox9 (Fig. 4a), the main chondrogenic differentiation transcription factor, as well as expression of type II collagen (Fig. 4b) and aggrecan (Fig. 4c), both cartilage-specific matrix molecules. Our results showed a significant down-regulation of Sox9 in the study groups IL-1 and ML/IL-1 compared to control (Fig. 4a). As markers for hypertrophy and osteogenesis type X collagen (Fig. 4e), Runx2 (Fig. 4d) and osteocalcin (Fig. 4f) were analysed. Our results showed a dramatic up-regulation of type X collagen in the study groups IL-1 and ML/IL-1 compared to control (Fig. 4e). In these same study groups expression of osteocalcin was significantly down-regulated (Fig. 4f).

Discussion

This study showed for the first time that CPCs can be isolated from the human growth plate, expanded in vitro and differentiated along osteogenic and adipogenic lineage (Fig. 2a–d). Conditions simulating the inflammatory phase

Fig. 1 Strategy for gating and sorting of CPCs via FACS. In *plot 1* CPCs were analysed on a dot plot of FSC versus SSC. A region of 68 % of cells representing the viable cells (debris and cell doublets excluded) was positioned. *Plot 2* represents CD105 (47.3 % from total) positive cells. CD105+ cells were gated in *plot 3*, where cells expressed CD 73+ and CD90+. *Plot 4* represents only CD105+, CD73+ and CD90+ cells, whereby CD 45- and CD34- were sorted. One of more than three independent experiments is shown here

of injury response at the growth plate in vitro did not affect human CPC proliferation (Fig. 3). Under such conditions, CPCs differentiated towards hypertrophy, while chondrogenesis and ossification were inhibited (Fig. 4a–f).

To our knowledge this is the first in vitro study on primary human growth plate cells. Being of human origin and thereby showing normal biological variability, these cells make results more comparable to the human body than

Fig. 2 Differentiation of growth plate CPCs along adipogenic (a–b) and osteogenic lineage (c–d). **a** Positive Oil Red O stain showing fat accumulation in adipocytes (counterstained with Haematoxylin), absent in the control group (**b**). **c** Alkaline phosphatase staining showing osteocyte differentiation of CPCs (counterstained with Nuclear Fast Red), and undifferentiated control CPCs (**d**). Pictures were taken at 20× magnification

Fig. 3 Mean BrdU up-take was measured in the studied groups as absorbance (A370 nm – A492 nm). IL-1 β treatment significantly enhanced CPC proliferation when compared to mechanical loading ($p < 0.05$, labelled with an asterisk)

results from animal or cell line model studies and therefore are of great value for bone growth studies.

In our experiments we used chondro-progenitor cells, a defined cell population obtained from the human growth plate. These cells have the highest proliferation and differentiation potential within the growth plate [10]. We therefore postulated that they would contribute to a major extent to the healing process of a growth plate injury.

Our data shows that under conditions simulating the inflammatory phase of injury response at the growth plate CPC proliferation is not affected. Interestingly, we discovered that inflammatory and mechanical stimuli have a contrasting effect on CPC proliferation, with IL-1 β promoting it and mechanical loading inhibiting it. The phenomenon of overgrowth due to inflammatory stimuli has clinically been reported in children suffering from pauci-articular juvenile

rheumatoid arthritis. In these children an increased longitudinal bone growth was seen in the affected limbs. However, overgrowth was dependent on the patients' age and the disease' onset, with only young children showing limb overgrowth [11, 12]. In our study the donor's age was not above four years, leaving the possibility that the proliferative effect of IL-1 β and the consequent contrasting effect to mechanical loading were related to donors age. Mechanical loading, i.e. tensile strain, was reported to enhance proliferation within the growth plate [13]. This, however, has been shown to be dependent on the loading frequency [7]. In our experiments we exposed CPCs from the human growth plate to a relatively low loading frequency of 0.1 Hz and a surface elongation of 3 %. This mechanical stimuli showed an inhibitory effect on CPC proliferation. Our loading protocol was chosen because it represented less than physiological locomotion [8] and therefore best mimics the situation after growth plate fracture, when the affected limb is immobilised and only micro-movements remain present at the injury site.

Our study indicated that under conditions simulating the inflammatory phase of injury response at the growth plate in vitro chondrogenesis and ossification are inhibited while differentiation towards hypertrophy of CPCs is dramatically promoted. The inhibition of chondrogenesis and ossification is reflected in the down-regulation of Sox9, the main chondrogenic differentiation transcription factor [14] and the down-regulation of osteocalcin. Osteocalcin is involved in the calcification of hypertrophic cartilage matrix [15]. In this study the inhibition of chondrogenesis and ossification was shown to be almost exclusively the effect of inflammatory stimuli, as CPCs exposed to mechanical loading did not show any significant difference in gene expression compared

Fig. 4 Changes in mRNA expression of genes indicating chondrogenic, hypertrophic and osteogenic differentiation of CPCs from the human growth plate: Sox9 (a), Col2a1 (b), Aggrecan (c), Runx2 (d), Col10a1 (e), Osteocalcin (f). Data are expressed as ratios of expression

levels of target genes over the internal control gene β -actin. Data are presented as average $\pm$ SEM. Significance was assumed when $p < 0.05$ (labelled with an asterisk)

to the control. This result agrees with literature; under inflammatory conditions chondrogenesis is inhibited [11, 14] and longitudinal bone growth in children is delayed [11, 16]. Differentiation of CPCs towards hypertrophy under mechanical loading and IL-1 β treatment in vitro resulted in a significant increase in type X collagen expression. Type X collagen is a specific marker for hypertrophy of growth plate chondrocytes [17]. Hypertrophic maturation of growth plate chondrocytes is responsible for up to 60 % of longitudinal bone growth [18] and represents the last differentiation step before the cells enter the process of apoptosis and allow invading osteoblasts to deposit osteoid and to form bone. An increased expression of type X collagen has already been reported in chondrocytes under the influence of pro-inflammatory cytokines [19]. Interestingly, in our study mechanical loading potentiated the effect of IL-1 β . This was unexpected, as mechanical loading of low magnitudes has been reported to act as an antagonist of IL-1 β , i.e. inhibiting IL-1 β 's catabolic effects [20, 21]. However, in this particular context our results indicate the opposite. They suggest that hypertrophy of growth plate cells might be the first step towards a disturbed longitudinal growth and an unwanted pre-mature ossification of the growth plate, as it may occur after growth plate injuries. Clinically, this finding indicates that reducing the local amount of inflammatory cytokines could be one possible target therapy to prevent the unwanted process of premature growth plate hypertrophy and subsequent ossification. To analyse this, future studies focussing on CPC behaviour under anti-inflammatory and different controlled mechanical loading conditions in this delicate early phase of injury response at the growth plate are planned. We believe that the ability to manipulate CPCs in an early phase of injury response may provide a powerful and adaptable tool to prevent complications of growth plate injuries, as formation of a bone bridge or angular deformity in children.

Conclusion

This study showed for the first time that CPCs can be isolated from the human growth plate, expanded in vitro and differentiated along osteogenic and adipogenic lineage. Furthermore the study showed that in the first phase of injury response at the growth plate CPCs differentiate towards hypertrophy, proliferation remains unaffected and chondrogenesis and ossification are inhibited. This reaction of CPCs might be the first step towards an unwanted premature ossification of the growth plate.

Acknowledgements The authors appreciate support from the Laura Bassi Center of Expertise BRIC (Bioresorbable Implants for Children; FFG -Austria). Furthermore they would like to acknowledge Rudolf Schmied for his valuable technical assistance. They declare no actual or potential conflict of interest.

References

- Olsen BR, Reginato AM, Wang W (2000) Bone development. *Annu Rev Cell Dev Biol* 16:191–220
- Wattenbarger JM, Gruber HE, Phieffer LS (2002) Physeal fractures, part I: histologic features of bone, cartilage, and bar formation in a small animal model. *J Pediatr Orthop* 22(6):703–709
- Shapiro F (1987) Epiphyseal disorders. *N Engl J Med* 317(27):1702–1710
- Chung R, Foster BK, Xian CJ (2011) Injury responses and repair mechanisms of the injured growth plate. *Front Biosci (Schol Ed)* 3:117–125
- Zhou FH, Foster BK, Sander G, Xian CJ (2004) Expression of proinflammatory cytokines and growth factors at the injured growth plate cartilage in young rats. *Bone* 35(6):1307–1315
- Chung R, Cool JC, Scherer MA, Foster BK, Xian CJ (2006) Roles of neutrophil-mediated inflammatory response in the bony repair of injured growth plate cartilage in young rats. *J Leukoc Biol* 80(6):1272–1280
- Ueki M, Tanaka N, Tanimoto K, Nishio C, Honda K, Lin YY et al (2008) The effect of mechanical loading on the metabolism of growth plate chondrocytes. *Ann Biomed Eng* 36(5):793–800
- Winter DA (1983) Biomechanical motor patterns in normal walking. *J Mot Behav* 15(4):302–330
- Kolf CM, Cho E, Tuan RS (2007) Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 9(1):204
- Ballock RT, O'Keefe RJ (2003) The biology of the growth plate. *J Bone Joint Surg Am* 85(4):715–726
- MacRae VE, Farquharson C, Ahmed SF (2006) The pathophysiology of the growth plate in juvenile idiopathic arthritis. *Rheumatology (Oxford)* 45(1):11–19
- Simon S, Whiffen J, Shapiro F (1981) Leg-length discrepancies in monoarticular and pauciarticular juvenile rheumatoid arthritis. *J Bone Joint Surg Am* 63(2):209–215
- Stokes IA, Aronsson DD, Dimock AN, Cortright V, Beck S (2006) Endochondral growth in growth plates of three species at two anatomical locations modulated by mechanical compression and tension. *J Orthop Res* 24(6):1327–1334
- Murakami S, Lefebvre V, de Crombrughe B (2000) Potent inhibition of the master chondrogenic factor sox9 gene by interleukin-1 and tumor necrosis factor- α . *J Biol Chem* 275(5):3687–3692
- Gerstenfeld LC, Shapiro FD (1996) Expression of bone-specific genes by hypertrophic chondrocytes: Implication of the complex functions of the hypertrophic chondrocyte during endochondral bone development. *J Cell Biochem* 62(1):1–9
- Pass C, MacRae VE, Ahmed SF, Farquharson C (2009) Inflammatory cytokines and the GH/IGF-I axis: novel actions on bone growth. *Cell Biochem Funct* 27(3):119–127
- Linsenmayer TF, Eavey RD, Schmid TM (1988) Type X collagen: a hypertrophic cartilage-specific molecule. *Pathol Immunopathol Res* 7(1–2):14–19
- Wilsman NJ, Farnum CE, Leiferman EM, Fry M, Barreto C (1996) Differential growth by growth plates as a function of multiple parameters of chondrocytic kinetics. *J Orthop Res* 14(6):927–936
- Sandell LJ, Aigner T (2001) Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res* 3(2):107–113
- Long P, Gassner R, Agarwal S (2001) Tumor necrosis factor alpha-dependent proinflammatory gene induction is inhibited by cyclic tensile strain in articular chondrocytes in vitro. *Arthritis Rheum* 44(10):2311–2319
- Xu Z, Buckley MJ, Evans CH, Agarwal S (2000) Cyclic tensile strain acts as an antagonist of IL-1 beta actions in chondrocytes. *J Immunol* 165(1):453–460