Journal of Biotechnology 210 (2015) 59–65

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Comparing effects of perfusion and hydrostatic pressure on gene profiles of human chondrocyte.

Ge Zhu[a,1], Susanne Mayer-Wagner[a,*,1], Christian Schröder[a], Matthias Woiczinski[a], Helmut Blum[b], Ilaria Lavagi[b], Stefan Krebs[b], Julia I. Redeker[a], Andreas Hölzer[a], Volkmar Jansson[a], Oliver Betz[a], Peter E. Müller[a]

[a] Department of Orthopaedic Surgery, Physical Medicine and Rehabilitation, Campus Großhadern, Ludwig-Maximilians-University, Marchioninistr. 15, 81377 Munich, Germany
[b] LAFUGA Genomics, Gene Center Munich, Ludwig-Maximilians-University, Feodor-Lynen-Str. 25, 81377 Munich, Germany

ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form 17 June 2015
Accepted 22 June 2015
Available online 29 June 2015

Keywords:
Perfusion
Hydrostatic pressure
Bioreactor
Sequencing
Human chondrocytes

ABSTRACT

Hydrostatic pressure and perfusion have been shown to regulate the chondrogenic potential of articular chondrocytes. In order to compare the effects of hydrostatic pressure plus perfusion (HPP) and perfusion (P) we investigated the complete gene expression profiles of human chondrocytes under HPP and P. A simplified bioreactor was constructed to apply loading (0.1 MPa for 2 h) and perfusion (2 ml) through the same piping by pressurizing the medium directly. High-density monolayer cultures of human chondrocytes were exposed to HPP or P for 4 days. Controls (C) were maintained in static cultures. Gene expression was evaluated by sequencing (RNAseq) and quantitative real-time PCR analysis. Both treatments changed gene expression levels of human chondrocytes significantly. Specifically, HPP and P increased COL2A1 expression and decreased COL1A1 and MMP-13 expression. Despite of these similarities, RNAseq revealed a list of cartilage genes including ACAN, ITGA10 and TNC, which were differentially expressed by HPP and P. Of these candidates, adhesion related molecules were found to be upregulated in HPP. Both HPP and P treatment had beneficial effects on chondrocyte differentiation and decreased catabolic enzyme expression. The study provides new insight into how hydrostatic pressure and perfusion enhance cartilage differentiation and inhibit catabolic effects.

1. Introduction

In cell-based therapies for cartilage regeneration, a large number of differentiated chondrocytes are required to repair cartilage defects. However, chondrocytes undergo dedifferentiation and lose their characteristic phenotype when expanded in monolayer culture (von der Mark et al., 1977). Biomechanical stimulation is widely used to stimulate chondrogenic differentiation (Madeira et al., 2015). Various forms of bioreactors have been implemented to apply mechanical stimuli to chondrogenic cells (Darling and Athanasiou, 2003; Responte et al., 2012). Hydrostatic pressure is among the most important forces used in chondrocyte culturing. Hydrostatic pressure on bovine chondrocytes results in an increased collagen and glycosaminoglycan content (Benjamin et al., 2009) and an increased COL2A1 expression in human osteoarthritic (OA) chondrocytes (Smith et al., 2011). Hydrostatic pressure combined with medium perfusion at a very low rate upregulates the expression of COL2 and COL1 and maintained the expression of aggrecan in bovine chondrocytes on collagen gels (Mizuno and Ogawa, 2011). There have also been studies using perfusion without hydrostatic pressure, which showed decreased ACAN and collagen type 2 expression of bovine chondrocytes in a three-dimensional tubular perfusion system after 7 days and re-increased at day 14 (Yu et al., 2014). The question remains whether it is necessary to invest so much effort in applying hydrostatic pressure, or could perfusion by itself, be sufficient enough to improve chondrocyte differentiation? Due to different application methods, cell sources and other parameters it is problematic to compare the outcomes of various bioreactor systems. To obtain an overview of processes regulated by hydrostatic pressure and perfusion in bioreactors, it is important to leave behind restrictions of relative gene expression levels. Microarray analyses of chondrocytes under dynamic expression revealed mechanosensitive genes in mouse chondrocytes (Bougault et al., 2012). To our knowledge there has been no approach to compare perfusion versus perfusion combined with

* Corresponding author. Fax: +49 89 44007 4863.
E-mail address: susanne.mayer@med.uni-muenchen.de (S. Mayer-Wagner).
[1] Both authors contributed equally.

http://dx.doi.org/10.1016/j.jbiotec.2015.06.409
0168-1656/

hydrostatic pressure using gene profiles. Another problem regarding bioreactor studies is the use of animal cells or human OA chondrocytes. Animal cells have been shown to be less sensitive to biomechanical influences and might therefore not reflect clinical findings (Grogan et al., 2012; Tran et al., 2011). Human OA chondrocytes do not reflect clinical conditions, as healthy human chondrocytes are used in the repair of cartilage defects. In order to approach clinical applications, healthy human chondrocytes are required to fully examine the effects of hydrostatic pressure and perfusion.The aim of the present study was to compare effects of HPP and P on the complete gene profile screen of human chondrocytes.

2. Materials and methods

2.1. Cell culture

Human articular cartilage was obtained through triple arthrodesis (16 years, male). The study was approved by the responsible Ludwig-Maximilians-University medical center ethics committee. Chondrocytes were isolated by pronase (Roche Diagnostics GmbH, Mannheim, Germany) and collagenase (Sigma–Aldrich Co., St. Louis, USA). Isolated cells were cultured in a humidified atmosphere at 37 °C, 5% CO_2 until passage 2. Culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1, Biochrom AG, Berlin, Germany), 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany), 1% MEM amino acids (Biochrom AG, Berlin, Germany), 25 μg/ml ascorbic acid (Sigma–Aldrich Co., St. Louis, USA), 50 IU/ml Penicillin/Streptomycin (Biochrom AG, Berlin, Germany) and 0.25 μg/ml Amphotericin B (Biochrom AG, Berlin, Germany). The cells were plated 24 h prior to stimulation at a density of 10^6 cells/cm^2 in 6-well plates.

2.2. Bioreactor and stimulations

A simplified bioreactor (Fig. 1) described earlier (Schröder et al., 2015), was used to culture human chondrocytes in high-density monolayer cultures. In brief, loading and perfusion were applied through the same piping by pressurizing the medium directly. Parallel chambers were used for different stimulations: Perfusion (P) ($n = 6$), hydrostatic pressure plus perfusion (HPP) ($n = 6$) and control (C) ($n = 6$), where cells were kept in static cultures. For P and HPP, perfusion was applied with a medium flow rate of 2 ml/min for 20 h/day for 4 days. The perfusion flow rate of 2 ml/min was chosen in accordance with cell viability tests (data not shown). In HPP, hydrostatic pressure of 0.1 MPa for 2 h (loading), followed by 2 h rest (off-loading), was applied each day. During this 4 h period perfusion was stopped in P simultaneously.

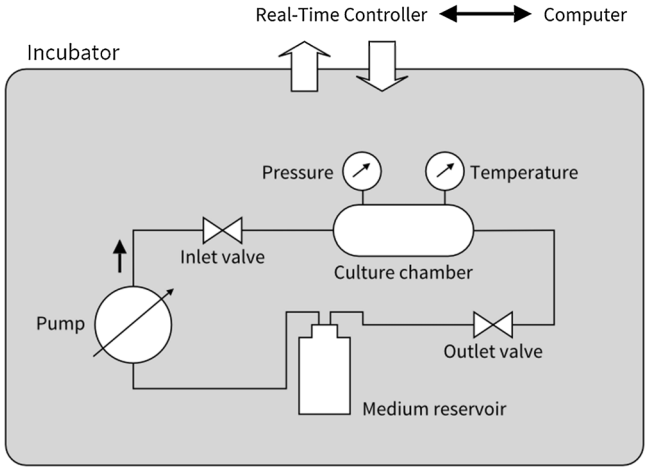

Fig. 1. Schematic setup of the bioreactor system including culture chamber, peristaltic pump and medium reservoir in an incubator.

2.3. Finite element method analysis

To analyze fluid flow of the medium and interaction with cells a computer fluid dynamics (CFD) simulation of the test chamber was developed (Ansys 14.0, Inc., Canonsburg, Pennsylvania, USA). In the numerical simulation, the final mesh for the bioreactor had a maximum element size of 1 mm and the fluid had a maximum mesh size of 0.5 mm. The medium was given the characteristic properties of water. Fluid turbulences were taken into account with the k-epsilon option which is used for small eddies. The inlet and outlet mass flow rates were set to 3.3 e-5 kg/s, matching a 2 ml/min volume flow there. All results were analyzed at end of the transient simulation which occurred after 3 s.

2.4. RNA isolation

After 4 days of culture, the total RNA was isolated using Qiazol™ Lysis Reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, 1 ml of Qiazol™ Lysis Reagent was added in each well of 6-well plates. RNA was extracted with 0.2 ml of chloroform (Sigma–Aldrich Co., St. Louis, USA), precipitated with 0.5 ml isopropanol (Sigma–Aldrich Co., St. Louis, USA) and washed with 75% ethanol. Isolated total RNA was checked for purity (Nanodrop ND-1000, ThermoFisher, Waltham, USA) and integrity (Bioanalyzer 2100, Agilent, Santa Clara, USA). Total RNA with A260/A280 > 1.8 and integrity > 9 was used for analysis.

2.5. Generation of RNAseq libraries

An amount of 100 ng total RNA was first treated with double-strand specific DNAse (Fermentas Inc., Hanover. MD, United States) to remove any traces of genomic DNA. After heat-inactivation of the DNAse, cDNA was synthesized and converted to Illumina-compatible sequencing libraries with the Encore complete RNAseq kit from NuGen (NuGen, San Carlos, USA). Briefly, first strand cDNA was generated by selective priming, second strand cDNA was synthesized using dUTP and the generated double stranded cDNA was fragmented using a Covaris M220 sonicator (50W peak incident power, 20% duty factor, 200 cycles per burst, 160 s treatment time). Then cDNA was end-repaired, ligated to Illumina-Adapters and the second-strand was selectively removed. After 18 cycles of PCR the final library was quantified on an Bioanalyser (Bioanalyzer 2100, Agilent, Santa Clara, USA) and diluted to 10 nM prior to pooling and sequencing. The pooled libraries were sequenced on two lanes of a rapid flowcell in 100 bp single end mode on a HiSeq1500 instrument (Illumina, San Diego USA).

2.6. RNAseq data analysis

Raw sequence reads were demultiplexed and mapped to the human genome (hg19 release) using Tophat 2.0 (Kim et al., 2013). Read counts for each gene were obtained by the python script HTseq count (Anders et al., 2015). Read counts were then normalized by r-log transformation with the program DESeq2 (Love et al., 2014). Differentially expressed genes were identified by pairwise comparison with DESeq2 using an fdr threshold of 5%. A list of cartilage relevant genes was obtained from www.genecards.org using "cartilage" as keyword including all genes with a relevance score of >0.5. The scoring was calculated by a Lucene defined algorithm. From the lists of significant genes that overlapped with the list of relevant cartilage genes was plotted as a heatmap. In order to visualize the difference of the expression between C, P and HPP, the data