EXPRESSION PATTERN OF HISTONE H3 SUBTYPES IN ARTICULAR CHONDROCYTES

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Abstract

Three-dimensional cell culture used for tissue engineering has its own rules and directions. There is a deficiency of proliferative markers suitable for tissue engineering research when cells are cross-linked in network of fibers or suspended in hydrogel. It limits cell harvesting or impairs the flow to cell area conventional chemicals used as proliferative markers. According to current published data, the expression of replication-dependent histone H3 genes could be novel proliferative marker of cells. The intensive synthesis of H3 histones is tightly correlated with DNA synthesis and H3 mRNA is rapidly degraded when the S phase is completed or inhibited by cell cycle inhibitors. Based on this relation, non-dividing cells contain no H3 mRNA. The aim of the study was to determine expression pattern of replication-dependent H3 subtypes and tissue-specific H3/t subtype in normal human connective tissue cells. Analyzed cellular model was chondrocytes cell line due to the phenomenon that articular cartilage doesn't have natural ability to heal its injuries, consequently development of cartilage engineering is necessary. Evaluation of expression pattern was performed using Reverse Transcription PCR and reaction products were visualized on the gel electrophoresis. This study demonstrated that RT-PCR technique can be successfully used to study the expression of different histone H3 subtypes. Presented electrophoregram showed differential expression of the analyzed subtypes (no expression of H3/g and H3/t subtypes). Incubation with sodium butyrate and quantitative Real Time PCR enabled quantification of mRNA level of selected H3/d subtype. This part of study showed a significant reduction in the mRNA level of H3/d when the sodium butyrate was added. Obtained results indicated the possibility of using the expression of individual histone H3 subtype as a new proliferative marker.

Keywords: *histone H3 subtypes, proliferative marker, chondrocytes, RT-PCR, sodium butyrate.*

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Introduction

Tissue engineering allows researchers to form three-dimensional living constructs where cells proliferate, gradually settle the biomaterial and restore the desired tissue. It exploits three main components like cells, scaffolds (made of natural or synthetic polymers) and growth factors [1]. Cartilage engineering is useful in treatment of cartilage defects by ACI (autologous chondrocyte implantation), reconstruction of growth plate, facial reconstruction surgery, reconstruction of long segmental tracheal defects or treatment of urinary incontinence and vesicoureteral reflux [2]. Conventional proliferative tests, like flow cytometry, sulforhodamine B test, MTT test, alamarBlue test, immunohistochemical staining, BrdU labelling, aren't sufficient for these types of culture. In spatial cultures, when cells are suspended in hydrogel or cross-linked in a network of woven or non-woven fibers, there are some limitations in cell harvesting or flow of conventional proliferative markers to cell area. Immunostaining of proliferation-associated antigens *e.g.* Ki-67 or PCNA (proliferating cell nuclear antigen) also has some limitations. It was found that PCNA accumulates in cell nuclei, not only in the S phase of the cell cycle, but also during DNA repair. In turn, Ki-67 is characterized by a long half-life and the ability to accumulate throughout the cell cycle. Generally it can be concluded that there is a deficiency of methods for measuring proliferative activity for tissue engineering studies [3-5].

Because of the unique histone H3 gene expression and expression products turnover and due to deficiency of methods for measuring proliferative activity and cell viability suitable for tissue engineering studies, the expression of histone H3 subtypes may be a new marker for this field of biomedical science. H3 subtypes were chosen for two main reasons. Firstly, the intensive synthesis of DNA during S phase is tightly correlated with intensive synthesis of replication-dependent subtypes of H3 [6]. Secondly, phosphorylation of H3 (10Ser) is required in chromosome segregation and it'll be probably a very good marker of cell division [7].

The human genome contains 11 replication-dependent histone H3 genes. Ten of these codes for H3.1 protein (*H3/a*, *H3/b*, *H3/c*, *H3/d*, *H3/e*, *H3/f*, *H3/g*, *H3/h*, *H3/i*, *H3/j*) and one code for H3.2 protein (*H3/n*). Histone H3.1, compared to H3.2, has a single amino acid variation (Ser96 instead of Cys96). The genes encoding histone 3.1 and 3.2 are localized in three clusters on chromosome 6 (6p21.3-22) and chromosome 1 (1q21) [8]. Other genes which encode histone H3 subtypes are replacement histone H3 genes – *H3.3a*, *H3.3b* and tissue-specific histone H3 gene – *H3t*. Histone H3/t probably occurs only in testis and is probably the rat TH3 homologue. The structure of that gene is similar to replication-dependent histone genes but its expression level has not been elucidated more precisely [9]. Genes which code replication-dependent subtypes are intronless and contain a stem-loop structure called 'hairpin' that forms 3'-end of mRNA. This structure is involved into processing, transport and stability of nuclear histone mRNA during replication. Histone mRNA of these subtypes is rapidly degraded both when the S phase is completed and when the DNA synthesis is inhibited by *e.g.* hydoxyurea. In other words, non-dividing cells contain no H3 mRNA [10,11].

The aim of the study was to determine the expression pattern of replication-dependent H3 subtypes and H3/t subtype in human connective tissue cells. Due to correlation between expression of these subtypes and DNA replication they may be new markers of proliferative activity in tissue cultures.

Materials and methods

Cell culture

Chondrocytes were purchased from Lonza. Cells were obtained from articular cartilage and maintained at 37°C and 5% CO_2 in CGM™ Chondrocyte Growth Medium. This growth medium contained supplements and growth factors like fetal bovine serum, R3-IGF-1, bFGF, transferrin, insulin and GA-1000 (aqueous solution of gentamicin sulfate and amphotericin-B) (Lonza). To study the expression of genes, chondrocytes were placed at the initial density of 2-5*10^3 cells/cm^2 in Nunclon™ culture dishes (Nunc). The RNA isolation was performed when cells reached the state of 60% confluation.

For inhibition of DNA synthesis, 1mM, 3mM and 10mM sodium butyrate (Sigma-Aldrich) were administered to exponentially growing cells 48 hours before RNA isolation. Sodium butyrate selectively affects the activation of genes encoding proteins that block cell cycle such as, *eg.* p21WAF1 protein, which blocks kinase CDK2 and inhibits cell transition to the next stage of the cell cycle. The administration of this compound enabled a comparison of expression levels of selected H3 subtype in treated and untreated cells and visualized the potential changes in proliferative activity of cells.

RNA isolation

RNA preparation was carried out using NucleoSpin® kit (Macherey-Nagel). The RNA extracts were digested with DNase I to eliminate DNA contamination during the isolation procedure. RNA quantification was performed by ultrasensitive fluorescent RNA stain using Quant-IT™ RiboGreen® RNA Reagent (Invitrogen).

Reverse transcription PCR analysis and quantitative Real time PCR analysis

Evaluation of expression pattern was performed using RT-PCR (Reverse Transcription PCR). Gene-specific primers were designed using Primer Express™ 1.0 software (Abi Prism). To ensure efficient and accurate amplification of template, conditions for these reactions were optimized. The most important step in pre-standardization was the optimization of annealing temperature. The greatest specificity and efficiency of amplification for each pair of primers was at 60°C and this temperature was used in both types of reactions. The reaction mix at the final volume of 20 µl consisted of: 50 ng of RNA template, 500 nM of each primer, 2.5 units MasterAmp *Tth* DNA Polymerase, 2.5 mM $MgCl_2$, 0.5 mM $MnS0_4$, 400 µM dNTP mix, 1x MasterAmp *Tth* PCR Buffer, 1x MasterAmp PCR Enhancer (Epicentre) and sterile water. The RT-PCR reactions were performed by C-1000 Thermal Cycler (Bio-Rad). Cycling conditions were as follows: one step at 60°C for 30 min, one step at 94°C for 5 min, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. RT-PCT products were detected using 2% agarose gel electrophoresis and ethidium bromide staining. Qualitative analysis of RT-PCR was carried out using LabWorks 4.0 software.

QRT- PCR (quantitative Real Time PCR) enabled detection and quantification of mRNA level of selected H3 subtype for each reaction cycle. The reaction mixture at final volume of 20 µl consisted of: 50 ng of RNA template, 200 nM of each primer, 1x Power SYBR®Green RT-PCR Mix, 1x RT Enzyme mix (Applied Biosystems) and sterile water. Reference gene was GAPDH (glyceraldehydes-3-phospate dehydrogenase). QRT-PCR reaction was performed by DNA Engine Opticon system (Bio-Rad). Thermal conditions were as follows: one step at 48°C for 30 min, one step at 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. At the end of the QRT- PCR reaction, the melting curves were plotted.

Statistical analysis

The relative expression of selected *H3* gene in untreated and treated cells, normalized by *GAPDH* expression, was evaluated by REST© 2009 software (Qiagen).

Results

Each of 12 sequences (H3/a-H3/n, H3/t) for primer designing was obtained from http://www.ncbi.nlm.nih.gov/sites/entrez. The designed primers fulfilled the basic criteria for length, melting temperature, GC content, GC clamp, primer secondary structures or repeats. The length of primers was in the range from 18 bp to 28 bp, the GC content from 43% to 74%, and the melting temperature difference for each pair of primers was 2.6-2.7°C. The expected sizes of amplimers were 124-366 bp (TABLE 1).

TABLE 1. The accession numbers of sequences for primer designing and expected lengths of amplimers [bp].

H3 subtype	Accession No	Amplimer's length [bp]
H3/a	NM_003529.2	220
H3/b	NM_003537.3	224
H3/c	NM_003531.2	324
H3/d	NM_003530.3	190
H3/e	NM_003532.2	366
H3/f	NM_021018.2	226
H3/g	NM_003534.2	196
H3/h	NM_003536.2	268
H3/i	NM_003533.2	174
H3/j	NM_003535.2	303
H3/n	NM_001005464.2	124
H3/t	NM_003493.2	309

RNA was isolated from chondrocyte cell culture and measured using ultrasensitive fluorescent RNA stain. RNA concentrations isolates were between 139 and 195 µg/ml. After the pre-standarization, the proper RT-PCR reactions were carried out and products were visualized in 2% agarose gel electrophoresis. No expression of replication-dependent H3/g subtype and tissue-specific H3/t subtype was detected. All other subtypes were visualized in agarose gel with different efficiencies. The most strongly expressed subtypes were H3/d, H3/e and H3/h. The weakest expressed subtypes were H3/b and H3/n (FIG. 1A, B, TABLE 2).

In the assessment of cell cycle inhibition by sodium butyrate, H3/d subtype was used. It was chosen because of its detected expression in all types of connective tissue cell lines and its optimal length - 190 bp which reduces the risk of degradation by RNase. Results of this part of experiment were presented as levels of relative expression of H3/d. The higher decrease in expression was noticeable in the case of 3 mM sodium butyrate (FIG. 2). Melting curve analysis proved the specificity of the performed amplification reaction (FIG. 3). The results of the QRT-PCR and statistical analysis (REST©2009 software) indicated that there was a significant reduction of H3/d mRNA level when the sodium butyrate was added. The difference in transcriptional activity of the H3/d between treated chondrocytes (and untreated chondrocytes (control) was statistically significant ($p<0,05$) (TABLE 3).

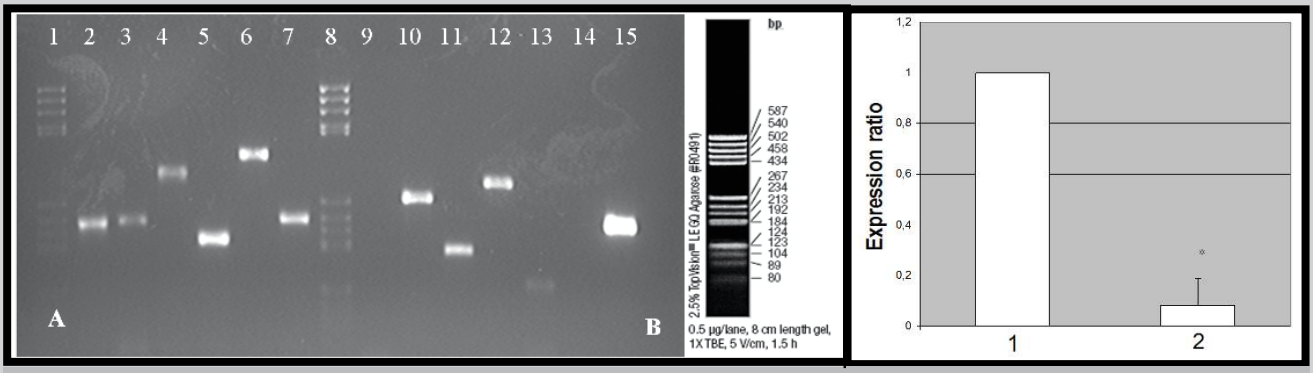

FIG. 1. A) The expression pattern of histone H3 subtypes in chondrocytes: lane 1, 8 – molecular weight marker - pBR322/BsuRI , lane 2-7, 9-14 – histone H3 subtypes, lane 15 – GAPDH. 2% agarose gel stained with ethidium bromide; B) Molecular weight marker pBR322/BsuRI(HaeIII) (Fermentas).

FIG. 2. The level of expression of H3/d in: 1 – untreated cells (control), 2 – cells treated by 3mM sodium butyrate.

TABLE 2. RT-PCR products visualized in agarose gel electrophoresis.

Lane	Product
1	Molecular weight marker pBR322/BsuRI
2	H3/a
3	H3/b
4	H3/c
5	H3/d
6	H3/e
7	H3/f
8	Molecular weight marker pBR322/BsuRI
9	H3/g
10	H3/h
11	H3/i
12	H3/j
13	H3/n
14	H3/t
15	GAPDH

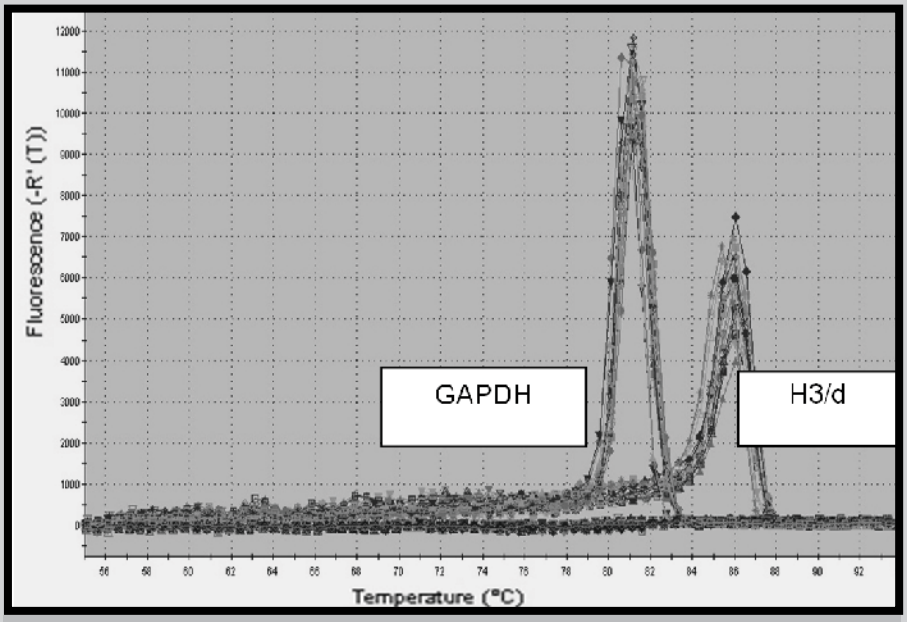

FIG 3. Melting curve analysis. First peak is for GAPDH product and second is for H3/d product.

Discussion

Tissue engineering is rapidly growing field of biomedical science. The aim of this science is to enable an effective treatment of degenerative diseases, burns and traumatic lesion using *eg.* autogenic cells which eliminate the risk of graft rejection in the recipient body. To create these implants, cells, scaffolds and growth factors are used. The presence of scaffolds allows cells to restore interactions between them and ECM (extracellular matrix) and normal cell proliferation [12]. Valid and rapid cell proliferation is needed for gradual settlement of scaffold by cells. The conventional proliferative assays, like counting mitotic figures, counting in hemocytometer or BrdU labeling, aren't sufficient for these types of cultures. It's necessary to create a rapid and effective proliferative test which enables an assessment of accurate proliferative of cells in these constructs.

This new alternative proliferative marker may be expression of replication-dependent subtypes of histone H3. In previous studies, histone mRNA was initially used in the hybridization *in situ* technique (ISH). Results of experiments using this method allow to evaluate the proliferative status at any given time, even in fixed archival samples.

TABLE 3. The values of relative expression levels and standard deviations calculated using REST©2009 software.

Sample	Relative expression level	Standard deviations
Untreated cells	1,000	-
3 mM sodium butyrate	0,081	0,039-0,186

Non-dividing cells have no detectable mRNA levels of histone H3 subtypes due to their rapid degradation after the cell division. The reason is that these subtypes don't have polyA-tail [13,14]. Unfortunately, in the case of three-dimensional scaffolds organic reagents affect the degradation of the scaffold during the preparation of material for analysis so ISH is not recommended.

In recent studies researchers have used quantitative Real time-PCR technique and RNA protection assay to analyze expression of histone H4/i and subtypes of histone H3. Evaluation of the expression of H4/i, using QRT-PCR, was performed by Ignatus *et al* [15]. Osteoblasts used in the experiment were seeded on collagen type I scaffolds. The results indicated that the increase in mechanical load caused an increase in expression of histone H4/i in these cells. This confirmed the assumption that mechanical forces increase the proliferation of osteoblasts compared to untreated control. Koessler *et al* [16] determined the expression of 11 replication-dependent H3 subtypes in three fetal tissues (bladder, lung and liver), a diploid fibroblast line IMR-90 and seven tumor cell lines (HEK-293, Hela-S3, SAOS-2, HL-60, Hep-G2 and Capan-1) by RNase protection assay. The expression pattern of H3 subtypes in all fetal human tissues and IMR-90 line was similar. The most strongly expressed genes were *H3/m* and *H3/n*. On the other hand, the weakest expressed genes were *H3/a*, *H3/d*, *H3/f*, *H3/h* and *H3/j*. In the tumor cell lines – in the case of *H3/m*, *H3/n* and *H3/k* genes – the expression level was low or undetectable. Only in the case of Tera-2 tumor line expression of all histone H3 subtypes occurred, which is likely due to the cell line being pluripotential.

The aim of our study was to determine expression pattern of replication-dependent H3 subtypes in human connective tissue cells. According to current published data the analysis of expression of all 11 replication-dependent H3 subtypes in valid adult human cell line hasn't been performed yet. In this research, the expression pattern of histone H3 subtypes in human chondrocytes – showed no expression of histone H3/g and H3/t subtypes. No expression of histone H3/t subtype confirms probably the specificity of its occurrence in the male testis. However, no detectable level of H3/g expression may indicate either a low promoter activity, its structural changes and what's interesting a high degree of differentiation of these cells. Structural changes within promoter domains affect the regulation of expression. What is more, also alternations in the distance between two domains - CCAAT box and TATA box or CCAAT box and CCAAT box – resulted in significant loss of promoter activity [17]. Incubation with sodium butyrate depicted a decrease in the expression of H3/d gene in treated compared to untreated cells. It confirmed the statement that histone mRNA is rapidly degraded when the DNA synthesis is blocked.

The obtained results of the experiment indicate the need for further studies, using cells growing on three-dimensional scaffolds, individual chosen subtype of H3 and QRT-PCR technique, to confirm the possibility of using changes in mRNA levels of histone H3 subtype as a reliable proliferative marker for tissue engineering research.

Conclusions

(1) The results of the study demonstrated that RT-PCR can be successfully used to analyze the expression of different histone H3 subtypes. It is possible to design sets of primers allowing the specific and efficient amplification of their transcripts. (2) No expression of histone H3/g and H3/t subtypes was detected in analyzed connective human cell line. (3) Incubation with sodium butyrate – cell cycle inhibitor – led to a significant reduction in the expression level of *H3/d* gene. This conclusion indicates the possibility of using the expression of individual histone H3 subtype as a proliferative marker.

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