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A novel method to examine the phenotype of chondrocytes

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Tissue engineering of articular cartilage in order to restore the function of degenerated, diarthrodial joints is currently widely under investigation. The results obtained thus far indicate that proper control of the differentiation of the cells used for this purpose is essential to produce and maintain a hyaline-like matrix. In this study, a procedure is described by which differentiation of chondrocytes *in vitro* and *ex vivo* can be studied. The method involves quantitative assessment of mRNA for different collagens, which are markers for differentiation of chondrocytes, by competitive PCR. In a culture system employing human osteoarthritic chondrocytes, mRNAs for the α_1-chains of collagen types I, II and X are quantified. The procedure is fast, specific and sensitive. However, several controls should be included to ascertain the reliability of the assessment.

1. Introduction

The capacity of articular cartilage defects to repair is very limited. Superficial defects do not heal at all, full depth defects may heal with fibrocartilaginous tissue because of recruitment of mesenchymal stem cells from subchondral bone marrow [1–6]. In order to repair an articular cartilage defect to restore the function of a joint, methods are being developed using tissue engineering of cartilage [7–18].

Autogenous cells capable of producing and maintaining a hyaline cartilage matrix are amplified in a laboratory and then within a suitable scaffold implanted into the defect. Both chondrocytes and chondro-progenitor cells are used for these purposes [7, 10, 14]. Mature chondrocytes do not proliferate. When these cells are cultured in a monolayer, they start proliferating but lose their chondrogenic phenotype (dedifferentiation) [19]. Once the cell number is high enough, the cells have to regain their chondrogenic phenotype. This can be achieved *in vitro* within a scaffold [7], or *in vivo* after implantation within the cartilage defect [10].

Chondro-progenitor cells can be harvested from bone marrow, perichondrium or periosteum [14, 20–23]. Chondro-progenitor cells have to gain the chondrogenic phenotype before or after implantation.

Careful control of these differentiation processes of either chondrocytes regaining their mature phenotype or chondro-progenitor cells gaining a chrondrogenic phenotype is essential for the success of the method. Chondro-progenitor cells not only are able to differentiate along chrondrogenic lineage's, but are also capable of transforming into osteoblast-like cells and adipocytes [23]. Mature chondrocytes may enter the lineage of terminal differentiation. Such cells become hypertrophic, start to express type X collagen and to calcify their surrounding matrix, which will ultimately be replaced by bone. Normally terminal differentiation is only seen in the growth plates of endochondral bones. However, subpopulations of articular chondrocytes in osteoarthritic cartilage also exhibit this hypertrophic phenotype [24–26].

In this study, a method is described to study chrondrocyte differentiation, dedifferentiation and re-differentiation. Total RNA is isolated from cultures of cells in various differentiation stages, or from cartilaginous tissue. The absolute amounts of mRNA of the collagen α_1(II) chain, the collagen α_1(I) chain and the collagen α_1(X) are assessed using competitive PCR [27–30]. The controls, necessary to establish absolute quantification are described [31].

2. Materials and methods

The outline of the method is given in Fig. 1.

2.1. Cell cultures

Articular cartilage was obtained from the operating theater after total hip or knee replacement. The

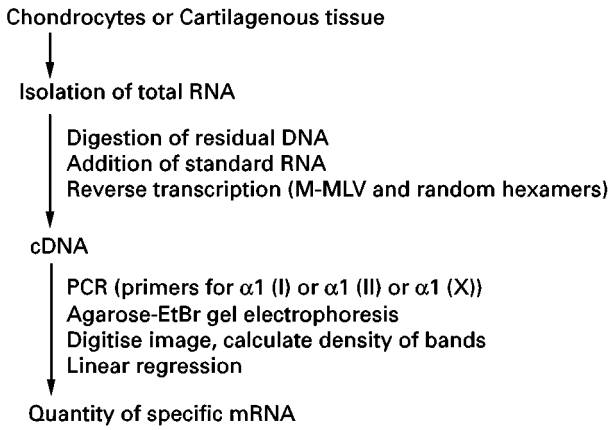

Figure 1 Schematic representation of the method. Total RNA was isolated from cultures of human osteoarthritic chondrocytes or from articular cartilage. Residual genomic DNA was digested using RNase-free DNase. Standard RNA was added. RNA was reverse transcribed and the resulting cDNA was amplified with PCR with specific primers for collagen α_1(I), α_1(II) or α_1(X) cDNA. The PCR products were subjected to ethidium bromide agarose gel electrophoresis. Images of the gel on UV-trays were digitized and the density of the bands was assessed. The amount of respective mRNA was calculated using linear regression analysis.

isolation and culture of cells was performed as described previously with minor modifications [32].

The cartilage was dissected from subchondral bone, diced finely and digested with 300 units/ml of a selected batch of collagenase type II (Life Technologies, Breda, The Netherlands) in Dulbecco's modified Eagle's medium (DMEM) containing HEPES (Life Technologies) and antibiotics for 24 h at 37 °C. The digest was filtered through a 70 μm nylon filter (cell strainer, Falcon, Micronic, Lelystad, The Netherlands), cells were pelleted at 1200 r.p.m. for 8 min and washed three times with phosphate buffered saline (PBS).Cells were counted and 300 000 cells in 1 ml culture medium were pelleted in conical centrifuge tubes at 800 r.p.m. for 4 min. Culture medium was DMEM containing 10% foetal bovine serum (FBS). L-glutamin (2 mM), ascorbic acid (25 $\mu g\,ml^{-1}$), penicillin (500 $U\,ml^{-1}$) and streptomycin (500 $\mu g\,ml^{-1}$). Cells were left undisturbed for 4–5 d, then the medium was refreshed and from that moment on the medium was refreshed each Monday, Wednesday, and Friday. Within the first 4–5 d, the cells aggregate to form a micromass culture. Cultures of cells that did not aggregate, were excluded.

SW1353, a human chondrosarcoma cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in monolayer in 162 cm^2 culture flasks until 80% confluent. Then cells were detached from the flask using trypsin and pelleted as micromass cultures as described above and cultured for 1–18 d.

2.2. RNA isolation

Cultures were maintained for 10–24 d, after which 10–12 micromass cultures were collected in one tube and dissolved in TRIZOL™ (Life Technologies) for isolation of total RNA according to the instructions of the manufacturer.

Articular cartilage was frozen in liquid nitrogen; approximately 100 mg cartilage (wet weight) was powdered in a Spex freezer/mill (Spex industries Inc., Metuchen, N.J.) and allowed to thaw in TRIZOL™. Total RNA was isolated according to a protocol described by Reno *et al.* [33] using the SV total RNA isolation kit (Promega, Leiden, The Netherlands).

2.3. Competitive PCR

Individual polymerase chain reactions (PCR) with primers for α_1(X) [34], for α_1(I) and for α_1(II) [35] were set up and optimized. Primer sets for these collagen chains were obtained from the literature for the α_1(II) and the α_1(X) chain or was selected from the sequence of the cDNA obtained from Genbank (α_1(I)).

Competitive PCR is based on competition of the primers in the amplification reaction for the target sequence and a known amount of an internal standard sequence [27–30]. One internal standard sequence was prepared, that could be used with primer sets for all three α_1-chains. For this purpose, two oligonucleotides were synthesized. The first contained the consecutive forward primers of α_1(X), α_1(I), α_1(II), and ovalbumin; the second contained the reverse primers for the same sequences in the same order (Table I, Fig. 2). Using these oligonucleotides, a PCR was performed on chick DNA. The resulting product was amplified once more with the primers for α_1(X). This PCR product was cloned into vector pCRII using the TA-cloning kit (Invitrogen, Leek, The Netherlands). *E. coli K12* (*1046*) bacteria were transformed and plasmids were isolated using the High pure plasmid isolation kit (Boehringer Mannheim, Germany). Standard RNA was generated using T7 RNA polymerase and quantified by measuring the OD at 260 nm. Different dilutions of standard RNA were stored frozen at −80 °C until further use.

Total RNA was isolated using TRIZOL™ (Life Technologies) according to the instructions of the manufacturer. To remove genomic DNA, the samples were treated with DNase I (Boehringer Mannheim). Absence of genomic DNA was established with a PCR using specific primers for α_1(X) DNA.

Six different dilution of standard RNA were added to six equal samples of total RNA. RNA was reverse transcribed using Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega corporation, Leiden, The Netherlands) and random hexamer primers. The resulting cDNA molecules were amplified in a PCR with primers for either the α_1(X), α_1(II) or α_1(I) message (Table I). PCR products were subjected to agarose gel electrophoresis and DNA bands were visualized using ethidium bromide fluorescence. Images of the gels were digitized using conditions in which bands were not saturated, and the bands were quantified using GELPRO™ software (Media Cybernetics, Silver Spring, MD, USA). The logarithm of the ratio of target DNA over standard DNA was plotted versus the logarithm of the amount of standard RNA added. Using linear regression, the amount of target RNA was assessed. Controls were a negative control

TABLE I

Primers for PCR of different collagen chains

Primers for cDNA of α_1(X) modified after [34]		product cDNA
XP1	5′ACAGGAATGCCTGTGTCTGCTTTT-3′ (1735–1758)	
XP2	5′-TTGGGAAGCTGGAGCCACACCTGGTC-3′ (2063–2038)	329 bp
Primers for cDNA of α_1(I)		
IC41	5′-CCAGCGCTGGTTTCGACTTC-3′ (3694–3713)	
IC566	5′-GGCCACGCTGTTCTTGCAGT-3′ (4244–4225)	551 bp
Primers for cDNA of α_1(I) [35]		
II-9	5′-GAAAAGATGGTCCCAAAGGTGC-3′ (2564–2585)	
II-10	5′-TGTCTCCTTGCTTGCCAGTTGG-3′ (3055–3034)	492 bp
Primers for chick DNA of ovalbumin		
ov1	5′-CCTGCAAAGTGCAGCTGCTG-3′ (1779–1798)	
ov2	5′-GCACAGCTTTTGGAGCCAGC-3′ (2084–2065)	301 bp

Forward oligo for standard

5′-ACAGGAATGCCTGTGTCTGCTTTTCCAGCGCTGGTTTCGACTTCGAAAAGATGGTCCCAAAGGTGCCCTGCAAAGTGCAGCTGCTG-3′

Reverse oligo for standard

5′-TTGGGAAGCTGGAGCCACACCTGGTCGGCCACGCTGTTCTTGCAGTTGTCTCCTTGCTTGCCAGTTGGGCACAGCTTTTGGAGCCAGC-3′

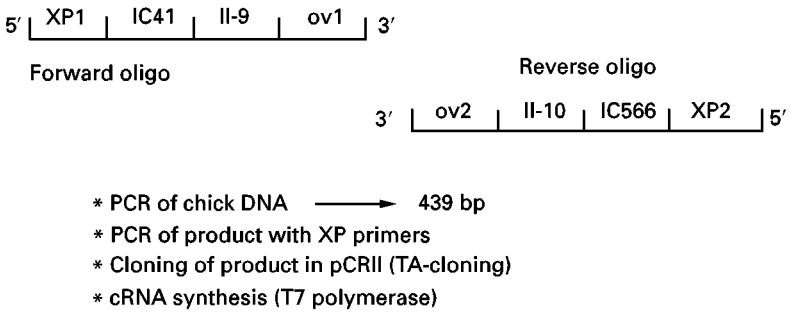

Figure 2 Preparation of standard RNA for quantification of mRNA of collagen α_1(I), α_1(II) and α_1(X) chains. Two oligonucleotides consisting of the consecutive primer sequences for α_1(X), α_1(I), and α_1(II) and ovalbumin were synthesized. These oligonucleotides were used as primers for PCR of chick DNA. The resulting PCR product was amplified with primers XP1 and XP2. The latter PCR product was inserted into vector pCRII using TA-cloning. Standard RNA was synthesized using T7 RNA polymerase.

throughout the entire procedure, a negative control for the PCR only, the slope of the obtained curve (should be -1. At 10% or more deviation from -1, the PCR was repeated with less template [31, 36]). Occasionally, RNA in a ten times diluted sample was quantified.

3. Results

Individual RT-PCR reactions for the α_1(I), the α_1(II) and the α_1(X) chain of collagen type I, type II and type X were set up and optimized using the primers described in Table I (Fig. 3). All PCR reactions proved to work well using an annealing temperature of 66 °C and a magnesium concentration in the PCR buffer of 0.7 mM.

Production of the internal standard required polymerase chain reactions with hot starts, probably because of secondary structures in the DNA to be amplified. The product of the PCR of chicken DNA

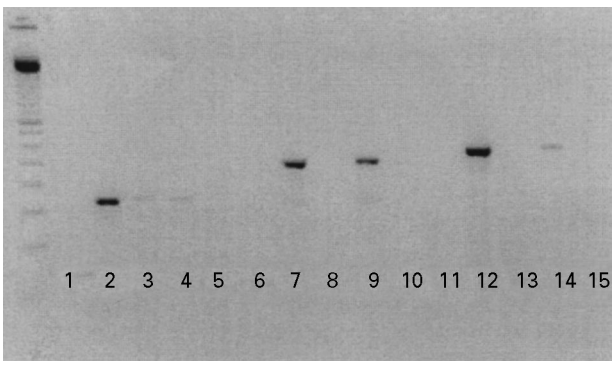

Figure 3 Digitized image of ethidium bromide stained agarose gel after electrophoresis of RT-PCR products of one RNA sample with primers for α_1(I), α_1(II), and α_1(X). Lane 1, negative control for PCR α_1(X) (no template); lane 2, cell culture – PCR α_1(X); lane 3, negative control of RT reaction of sample lane 2 (see text); lane 4, Cartilage sample – PCR α_1(X); lane 5, Cartilage sample (α_1(X)) negative control of RT reaction. Lanes 6–10 are for the same samples as lanes 1–5, but with primers for α_1(II). Lanes 11–15 are for the same samples as lanes 1–5, but with primers for α_1(I).

was cloned into pCRII and after transformation, colonies of *E. coli K12* bacteria were picked and put in a PCR vial. When three PCRs with primers for α_1(I), α_1(II) and α_1(X), respectively, of one colony of bacteria resulted in products of the expected sizes, the bacteria were cultured for plasmid isolation.

T7 Polymerase generated standard RNA was dissolved in double distilled water, quantified and diluted in appropriate amounts. Standard RNA was titrated to six equal portions of total RNA (in duplicate) and all samples were reverse transcribed and amplified. The image of the agarose gel was digitized and the bands were quantified. Then the logarithm of the ratio of the band of total DNA over standard DNA was plotted versus the logarithm of standard RNA (Fig. 4). The slope of the resulting line was calculated using linear regression and had to be -1 (± 0.1). The amount of mRNA was calculated using linear regression analysis. Whenever the slope deviated from -1, the PCR was repeated using 1 µl instead of 4 µl template (RT reaction sample). This usually resulted in a curve with a correct slope. Occasionally a ten-fold dilution of a total RNA sample was tested to assure correct absolute quantification of the assay. Negative controls were always included. In case the negative controls showed positive signals, the data and samples were discarded and the experiment was repeated using fresh buffers throughout the entire procedure.

Using this method, α_1(X) mRNA was detected in human osteoarthritic chondrocyte samples from cultures of approximately 10 d old and beyond. The amounts varied between individual samples from 5 fg up to 5 pg/400 ng total RNA. These cells also expressed mRNA of type I collagen and type II collagen (from 10–10000 pg/200 ng total RNA. SW1353 cells in micromass cultures expressed α_1(I) mRNA (10–45 pg/200 ng total RNA) and after approximately 5 d also α_1(X) mRNA (7–15 pg/400 ng total RNA) (Fig. 5). Analysis of total RNA from human osteoarthritic cartilage revealed expression of types X, II and I collagen (Fig. 3).

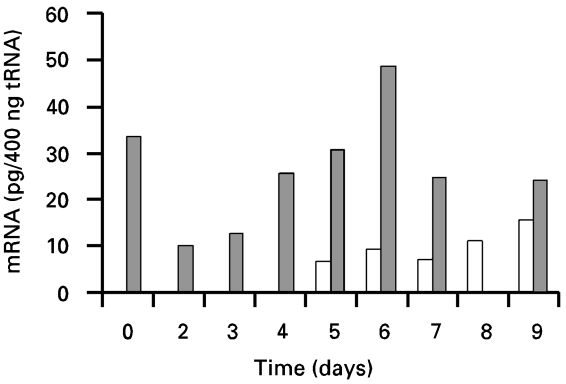

Figure 5 Expression of collagen α_1(I) and α_1(X) mRNA in cell cultures of SW1353 chondrosarcoma cells versus time in culture. Collagen α_1(X) mRNA, (□), was first detected after 5 d culture. Collagen α_1(X) mRNA, (■), was expressed by these cells at all time points indicated.

4. Discussion

Tissue engineering to produce articular cartilage for reconstruction of joints is currently under investigation in a large number of laboratories [37–47]. Independent of the source of the cells, ultimately mature chondrocytes will have to be established within a hyaline cartilaginous matrix. After transplantation, this matrix with its cells will have to survive in the joint and the phenotype of the cells will have to be maintained in order to be able to restore the function of the joint. However, during this process, chondrocytes may easily deviate from the mature phenotype and exhibit different other phenotypes.

Methods used to evaluate the quality of the matrix of the graft involve histology [12, 18, 48–50], histology combined with histomorphometry [18, 51] or with immunohistochemistry using antibodies to type II and type I or III collagen [37, 52]. Special

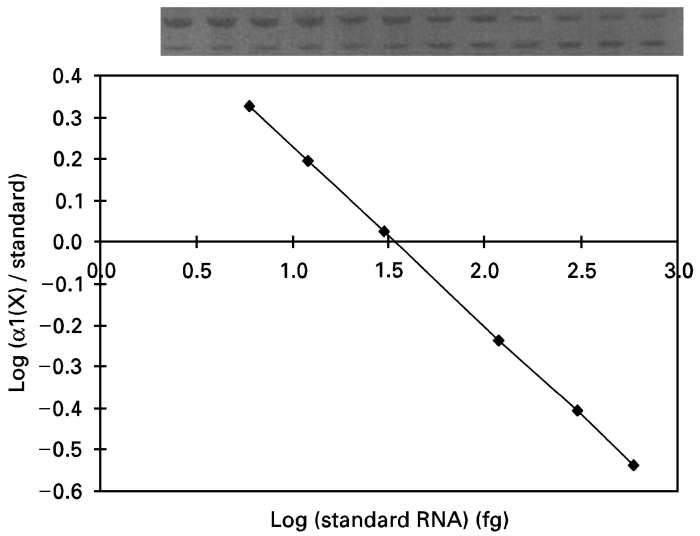

Figure 4 Competitive PCR of a cell culture sample using primers for α_1(X) collagen. The densities of the bands in the upper panel were assessed. A plot of the logarithm of the ratio of α_1(X) over standard RNA versus the logarithm of the amount of standard RNA was made. Linear regression was used to calculate the amount of mRNA of α_1(X) (34.4 fg/400 ng total RNA).

scoring systems were developed for histological grading of the quality of the grafted material [51, 53]. The biochemical methods used involve analysis of cyanogen bromide derived peptides of the collagens in the matrix [54–61], analysis of metabolically labeled collagen (*ex vivo*) [49] and [^{35}S] sulphate incorporation into *ex vivo* synthesized proteoglycans [47]. Additionally, the mechanical characteristics of the newly formed cartilaginous tissue are tested in a number of laboratories [18, 62–64]. With the exception of the *ex vivo* radiolabeling techniques, all these procedures assess the quality of the matrix which has formed over a period of time. *In vivo* the grafted material will adapt to the situation and may, in first instance, slightly deteriorate. After adaptation, restoration may occur. The gathered information with the currently used methods is the result of the history of the matrix from its onset. Considering the low turnover of especially the collagen components of the cartilage matrix it is not surprising that high amounts of type I collagen are retrieved from grafted tissues even 1 y after implantation [65]. Therefore, these data give insufficient information of the present situation of the grafted material, especially of the cells responsible for producing and maintaining the matrix components. Such information will be important to give some kind of prediction of the prognosis of the graft. The method described here enables determination of the phenotype of the cells within the cell cultures, and also after implantation. Unlike the *ex vivo* labeling procedures, this method does not involve radioactive materials.

The described procedure is relatively fast (results within 2 d), is highly specific and requires only small amounts of cells or tissue. A disadvantage of this method is the high sensitivity of RNA for degradation; i.e. working under strictly RNase-free conditions is an absolute requirement. Furthermore, the method is prone to errors. PCR samples can easily become contaminated with foreign material leading to unexplainable results. Therefore, proper controls should be included. These controls for competitive PCR are especially emphasized in this paper. The limitation of the procedure, as described here, is the detection system for the PCR products: agarose gel electrophoresis and ethidium bromide fluorescence detection. Other detection systems may increase the sensitivity of the method considerably.

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