Expression of Actin Isoforms in Human Auricular Cartilage

(human auricular cartilage / chondrocytes / α-smooth muscle actin / CD34 antigen / myochondrocytes)

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Abstract. The objective of this study was to determine whether human auricular chondrocytes can also express α–-smooth muscle actin. Immunohistochemistry using monoclonal antibodies for α-smooth actin, muscle-specific actin, β-actin, S-100 protein, CD34, and desmin was performed on samples of human ear cartilage obtained from 20 individuals during a partial resection of the ear for different reasons. Moreover, the RT-PCR analysis of actin isoforms in auricular chondrocytes was performed. Approximately 60 % of the chondrocytes of the ear cartilage expressed α-smooth muscle actin as demonstrated by immunohistochemistry in all the examined samples. Actin-positive chondrocytes occurred in both external subperichondrial layers of the auricular cartilage. This finding was confirmed by the RT-PCR technique. The knowledge of this fact could help us to better understand the chondrocyte changes occurring during the healing and transplantation of auricular cartilage. The question of whether it is necessary to refer to these predominating cells in ear cartilage as myochondrocytes is considered. This is the first report of an unusual immunophenotype and contractile potential for human auricular chondrocytes.

Cartilage is further classified as hyaline, elastic cartilage or fibrocartilage based primarily on morphological criteria and secondarily on the collagen and elastin contents. The different cartilage subtypes were characterized by the immunohistochemical localization of the elastin and collagen types in a rabbit (Nauman et al., 2002). Auricular cartilage is distinguished from other subtypes by interterritorial elastin staining and no staining for type VI collagen.

Many phenotypic traits have been reported in respect of chondrocytes from articular cartilage tissue in states of health, disease and response to injury and selected stages of the healing process (Buckwalter and Mankin, 1997; Povýšil et al., 2005). One of the features of auricular chondrocytes that has not yet been investigated is the specific actin composition of the cytoskeleton. The presence of muscle actin isoforms has not been shown previously in human auricular chondrocytes and therefore we are reporting the results of our immunohistochemical and molecular biological study of the occurrence of different actin isoforms in auricular cartilage. The knowledge of this fact could help us to better understand the cytoplasmic changes of the chondrocytes accompanying cartilage healing and transplantation.

Our aim was to determine, by means of an extensive analysis, the distribution of the α-smooth muscle actin in auricular cartilage free of significant pathological changes.

Actins are ubiquitous eukaryotic proteins and are involved in diverse cellular functions which include cell contraction, cell motility, cell adhesion, cell division and the maintenance of cell shape (Ramaekers and Bosman, 2004). Four muscle actin isofoms, namely α-skeletal actin, α-cardiac actin, α-smooth muscle actin and γ-smooth muscle actin, are predominantly seen in muscle tissues – skeletal, cardiac, vascular and enteric. Two other actin isoforms are ubiquitous, i.e. cytoplasmic β- and γ-actins. β-Actin is the most abundant protein constituent of the cytoskeleton of most eukaryotic cells. Many questions still remain to be answered as regards specific actin isoform functions. It would be of interest to investigate the distribution pattern and relative expression and function of α-smooth muscle actin in non-muscle cells such as auricular chondrocytes. Each actin molecule is a polypeptide unit of 375 amino acids with a tightly associated molecule of adenosine-triphosphate (ATP). Actin filaments are organized in linear bundles that form a network, mainly concentrated just beneath the plasma membrane. They also form protrusions and extensions of the plasma membrane and have the dynamic function to form the cell cortex.

Material and Methods

Immunocytochemical staining

Twenty representative bioptic samples of auricular cartilage obtained during the extirpation of basalioma

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Abbreviations: bp – base pairs; α-SKA – α-skeletal muscle actin; α-SMA – α-smooth muscle actin; α-CAA – α-cardiac muscle actin; γ-SMA – γ-smooth muscle actin.

of the ear skin or a plastic operation of the ear were chosen for the purpose of our study. Histological examination and immunohistochemical staining were performed on formalin-fixed paraffin-embedded tissue. For the purpose of our immunohistochemical studies we used the avidin-biotin complex (ABC) technique. Primary monoclonal mouse antibodies were applied against S-100 protein (1 : 200, DAKO, Glostrup Denmark), α-smooth muscle actin (1 : 100, Sigma, St. Louis, MO), muscle-specific actin HHF35 (1: 100, DAKO), CD34 (1 : 400, Immunotech, Prague, CR), vimentin (1 : 100, DAKO) and desmin (1: 200, DAKO). Negative controls were performed by substituting the primary antibody with non-immune mouse serum.

RNA isolation, cDNA preparation and RT-PCR analysis

Isolation of total RNA, synthesis of cDNA and RT-PCR analysis were performed by standard procedures described in our previous work (Tvrdík et al., 2005). The complete list of primer sets including their sequences, annealing temperatures, and PCR product sizes are presented in Table 1.

PCR condition were: 95°C for 3 min, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, followed by 45 cycles with the programmes of denaturation at 95 °C for 1 min, annealing at 47-57°C (see Table 1) for 1 min, and extension at 72 °C for 1 min. The reaction was accomplished with a final extension at 72°C for 10 min.

Results

The histological appearance of the specimens of human auricular cartilage evaluated in this study did not differ from typical normal auricular cartilage.

In all cases we observed distinct immunostaining of the majority of chondrocytes in the peripheral and central part of the auricular cartilage with specific antibody against S-100 protein. α-smooth muscle actin-positive chondrocytes also occurred in all the samples and represented approximately 60 % of the cell population of the auricular cartilage including the cells of the perichondrium. They were concentrated in the peripheral layers of the auricular cartilage (Figs. 1 and 2). In contrast, in the central part of the cartilage samples, actin-negative cells predominated. Identically distributed and concentrated positive cartilage cells were observed in slides stained with antibody against β-actin representing a regular component of the cytoskeleton. Exclusively α-smooth muscle actin-positive cells were also positively stained with antibody against muscle-specific actin. In four samples we also identified chondrocytes containing CD34 antigen. These CD34-positive chondrocytes

Table 1. List of primers used for RT-PCR analysis

Gene	Primer	Sequence (5′ to 3′)	T_a (°C)	PCR product (in bp)
α-SKA	sense	TTCCATTTTCTTCCACAGGG	53	103
α-SKA	antisense	GCTGCCATCGTAAACTGACA	53	103
α-SMA	sense	AGGTAACGAGTCAGAGCTTTGGC	53	199
α-SMA	antisense	CTCTCTGTCCACCTTCCAGCAG	53	199
α-CAA	sense	CCTTCTCTCTCCATCTACCTTCC	47	177
α-CAA	antisense	AGGTTGCAAGTCCTAGTCTGG	47	177
γ-SMA	sense	CCACCTTCCAGCAGATGTG	47	155
γ-SMA	antisense	AGGCTTGTAGGTTTTAATGTTTCA	47	155
β-actin	sense	AGGCCAACCGCGAGAAGATGACC	53	332
β-actin	antisense	GAAGTCCAGGGCGACGTAGCAC	53	332
γ-actin	sense	GTCTGTGGCTTGGTGAGTCT	57	166
γ-actin	antisense	GAAACTGGGTCCTACGGCTT	57	166
Desmin	sense	TCAGCTTCAGGAACAGCAGG	57	224
Desmin	antisense	GGTGTCGGTATTCCATCATC	57	224
CD34	sense	TCAGTCCCCCAACAGATGC	57	203
CD34	antisense	CAACCGTCATTGAAACCAGG	57	203

Each primer was chosen to span introns. Specific annealing temperature (Ta) of each primer and the size of expected PCR products are listed above. The abbreviations used are: bp base pairs; α-SKA α-skeletal muscle actin; α-SMA α-smooth muscle actin; α-CAA α-cardiac muscle actin; γ-SMA γ-smooth muscle actin.

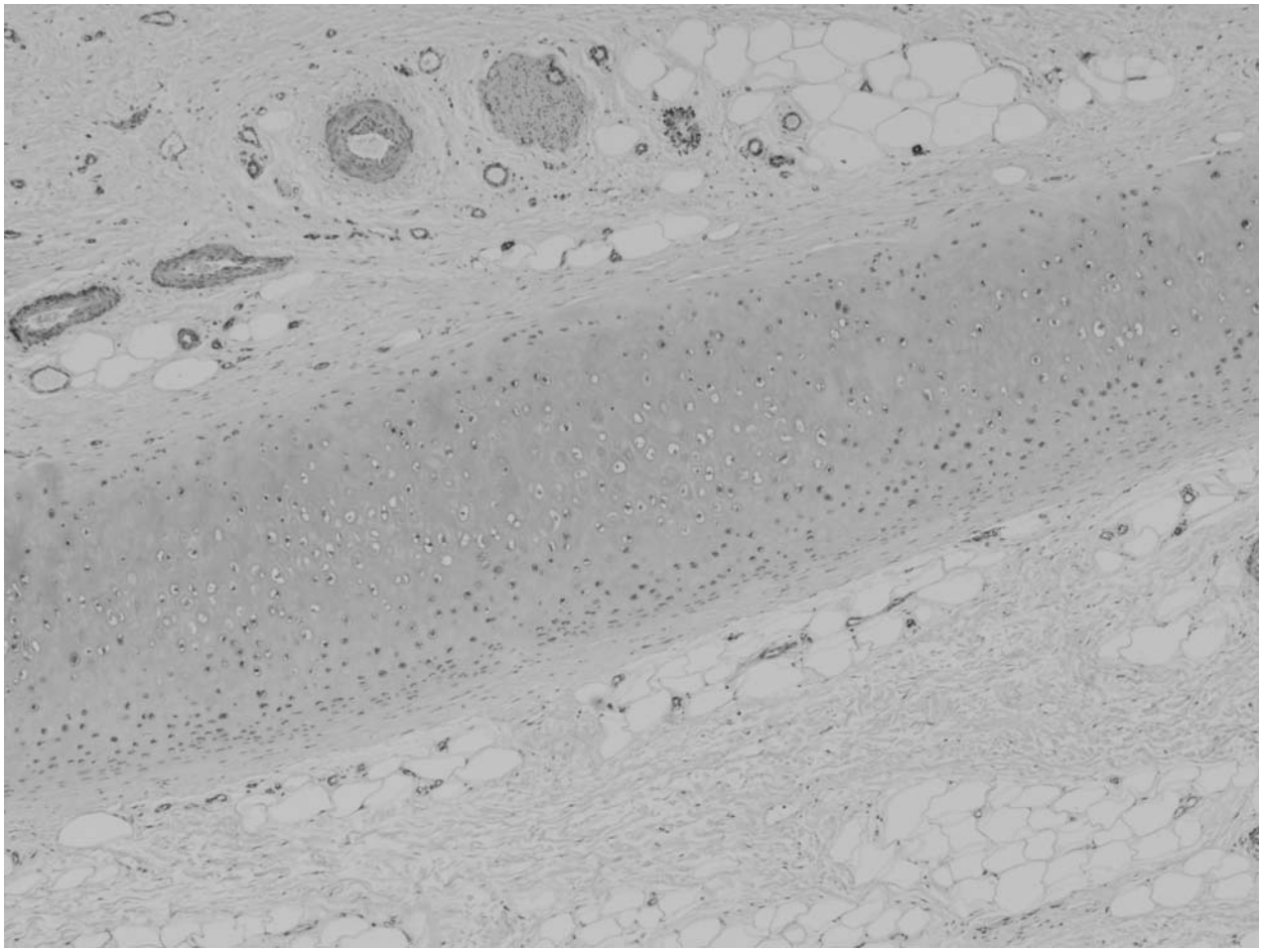

Fig. 1. α-smooth muscle actin-positive chondrocytes in human ear cartilage predominate in the subperichondrial external part. Original magnification × 10.

occurred only rarely in the thin subperichondrial area in three cases. The presence of desmin was not observed in any sample.

The ear perichondrium contained many vessels with α-smooth muscle-positive cells in their wall corresponding to smooth muscles.

Positive vimentin staining was apparent in the dermal fibroblasts and vessels, and in the perichondrial tissue and cytoskeleton of practically all the auricular chondrocytes.

RT-PCR analysis detected putative α-smooth muscle actin and β- and γ-actin in all the examined specimens and confirmed the results of our immunohistochemical examinations (Fig. 3). The presence of other actin isoforms, desmin, and CD34 was not proved (not shown).

Discussion

The results of our study have shown that auricular cartilage contains actin-positive chondrocytes. Approximately 60 % of the auricular chondrocytes contained this contractile actin isoform with the exception of the central part of the auricular cartilage, where the majority of chondrocytes were negative in the immunohistochemical reaction with monoclonal antibody against α-smooth muscle actin. A similar distribution of the positively stained chondrocytes was also observed in the slides stained with antibody against β-actin representing a regular component of chondrocyte cytoskeleton. The present study is the first to report the presence of α-smooth muscle actin in the auricular cartilage by means of immunohistochemistry. The actin-positive cells had a typical chondrocyte appearance and simultaneously showed S-100 protein and sometimes also muscle-specific actin and CD34 expression. These actin-positive cells of the ear were surrounded by a typical intercellular matrix of elastic cartilage. Our immunohistochemical findings were verified by means of the RT-PCR techinque in the same sample and the results of this molecular method confirmed our immunohistochemical findings.

Actins are ubiquitous eukaryotic proteins and are involved in diverse cellular functions which include cell contraction, cell motility, cell adhesion, cell division and the maintenance of cell shape (Ramaekers and Bosman, 2004). Four muscle actin isofoms, namely

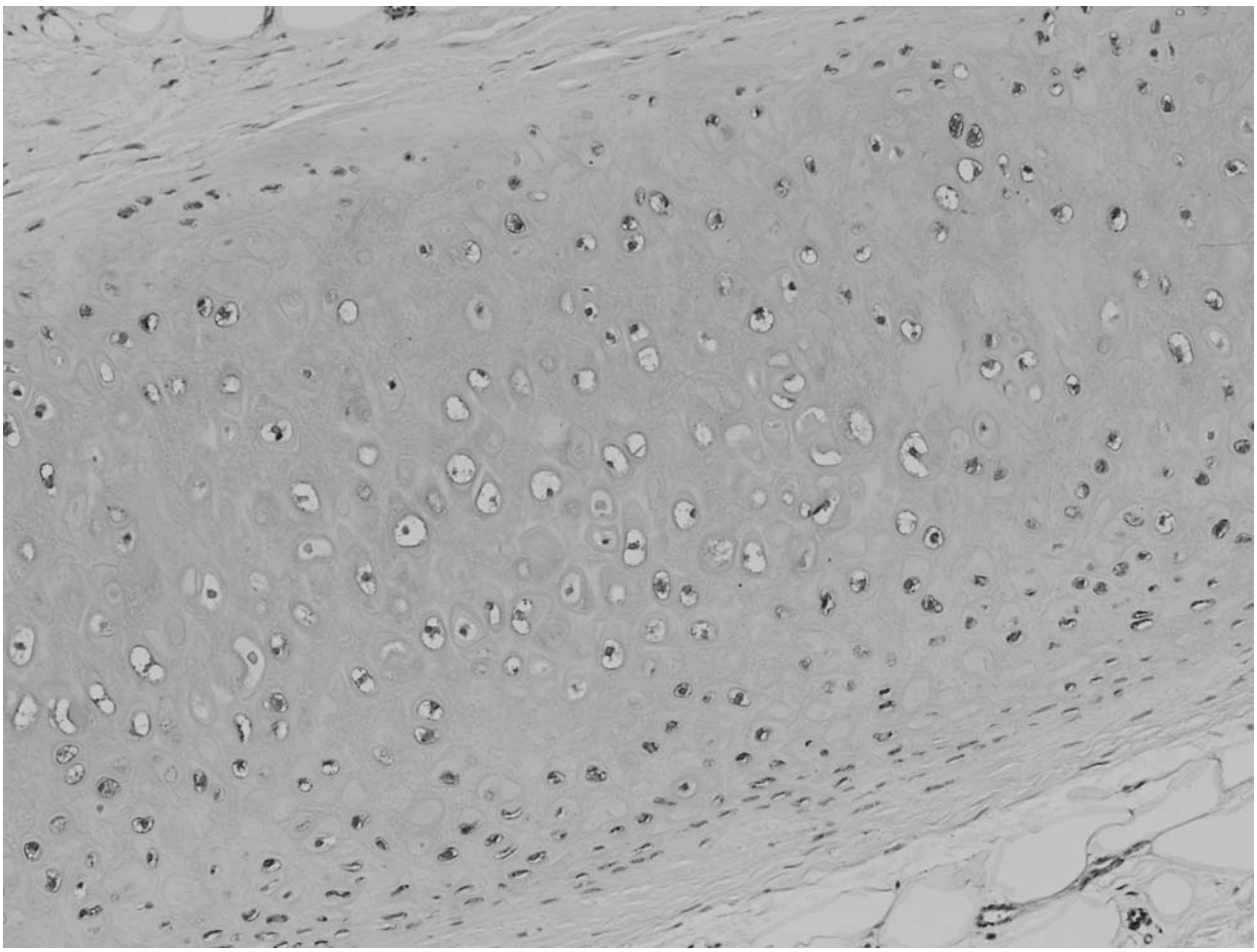

Fig. 2. α-smooth muscle actin-positive chondrocytes in detail. Original magnification × 20.

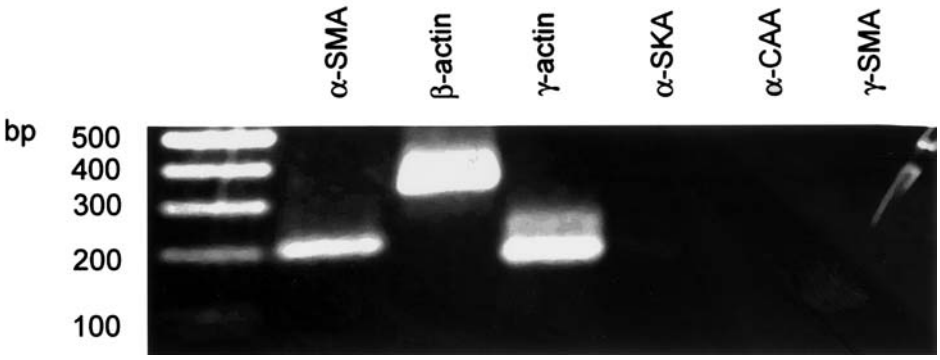

Fig. 3. RT-PCR analysis of actin isoforms in auricular chondrocytes. The presence of actin isoforms was analysed by RT-PCR. The PCR products were then separated by electrophoresis on agarose gel and visualized by using ethidium bromide. Molecular weight of the DNA markers is given in base pairs (left). The abbreviations used are: α-SKA α-skeletal muscle actin; α-SMA α-smooth muscle actin; α-CAA α-cardiac muscle actin; γ-SMA γ-smooth muscle actin.

α-skeletal actin, α-cardiac actin, α-smooth muscle actin and γ-smooth muscle actin, are predominantly seen in muscle tissues – skeletal, cardiac, vascular and enteric. Two other actin isoforms are ubiquitous, i.e. cytoplasmic β- and γ-actins. β-Actin is the most abundant protein constituent of the cytoskeleton of most eukaryotic cells. Many questions still remain to be answered as regards specific actin isoform functions. It would be of interest to investigate the distribution pattern and relative expression and function of α-smooth muscle actin in non-muscle cells such as auricular chondrocytes. Each actin molecule is a polypeptide unit of 375 amino acids with a tightly associated molecule of adenosine-triphosphate (ATP). Actin filaments are organized in linear bundles that form a network, mainly concentrated just beneath the plasma membrane.

They also form protrusions and extensions of the plasma membrane and have the dynamic function to form the cell cortex.

Relatively little is known about the cytoskeleton of the human chondrocytes of the different cartilage tissues. One of the authors of this article (Povýšil et al., 1997) reported on the occurrence of muscle-specific actin and α-smooth muscle actin in some S-100 protein-positive chondrocytes and chondroblasts of the bone chondroblastoma and exceptionally also in chondrosarcomas for the first time several years ago. These findings prompted us to suggest that such cells might be referred to as myochondroblasts and myochondrocytes (Povýšil et al., 1997). Further research confirmed our results and demonstrated the presence of α-smooth muscle actin in cells of a chondrocytic appearance in a canine meniscus (Mueller et al., 1999) and intervertebral discus (Schneider et al., 1999) as well as in human articular cartilage with osteoarthritic changes (Kim and Spector, 2000), and in chondromyxoid fibroma (Nielsen et al., 1999) as well as in defective human articular cartilage (Povýšil et al., 2005).

This finding raises the question of the role of a contractile actin isoform. The role of actin-enabled cell contraction in normal auricular cartilage tissue is as yet unknown. Several immunohistochemical studies have demonstrated that osteoblasts, chondrocytes, fibrochondrocytes and musculoskeletal fibroblasts can express smooth muscle actin in intact tissue (Kinner et al., 2002). Moreover, α-smooth muscle actin is the isoform typical of smooth muscle cells, and pericytes and myofibroblasts. It is believed that myofibroblasts appear to exert some functions that are related to tissue contraction processes (Gabbiani et al., 1971). It has been suggested that these cells with a contractile phenotype play a role in wound healing, being capable of generating an intra-cellular contracting force and possessing a means of transmitting this force to the surrounding tissue via collagen and fibronectin extracellular matrix. These α-smooth muscle actin-containing cells have demonstrated the capacity to contract a collagen-glycosaminoglycan analogue of extracellular matrix *in situ* (Kinner et al., 2002). It has been proposed that contractile forces generated by an α-smooth muscle actin-incorporated cytoplasmic apparatus may facilitate the cell manipulation of its extracellular matrix to impart tissue-specific architecture (Kim and Spector, 2000; Kinner and Spector, 2002).

Auricular cartilage is generated from the perichondrium in a process whereby the perichondrial cells differentiate and mature into the chondrocytes. The findings of our study support the plasticity of chondrocyte population composed of α-smooth muscle actin-negative and actin-positive cells with a predominance of actin-positive chondrocytes. Nobody knows if this composition is stable or not. It is possible that the phenotype of chondrocytes can change during different pathological processes or as a result of mechanical stress, but we have not found any information about this phenomenon in the literature. On the other hand, our finding shows that actin-containing auricular chondrocytes could be histogenetically closely related to myofibroblasts, cells also containing α-smooth muscle actin in their cytoplasm. Generation of contractile force is correlated with the expression of α-smooth muscle actin. Auricular cartilage has a unique property characterized by unusual elasticity and the ability to change the shape under the influence of mechanical stress without any resulting damage. It is well known that mechanical deformation of the ear is restored immediately by its own elasticity after the release of mechanical stress. It is possible that this phenomenon is the result of the presence of numerous cartilage cells containing a large number of smooth muscle actin filaments in their cytoplasm that are able of contractile activity and relaxation. Their contraction helps restore all deformities of the auricular cartilage in cooperation with the network of elastic fibres.

The details of how mechanical loading of the ear influences the function of chondrocytes remain unknown, but deformation of the matrix produces mechanical, electrical and physico-chemical signals that may play a major role in stimulating the chondrocytes. Compression of the ear cartilage deforms the matrix and may directly deform the chondrocytes similarly as supposed in articular cartilage (Buckwalter and Mankin, 1997). The unique biological and mechanical properties of auricular cartilage depend on the design of the tissue and the interactions between the chondrocytes and the matrix that maintain the tissue. Chondrocytes form the macromolecular framework of the tissue matrix from three classes of molecules: collagens, proteoglycan and elastin.

In conclusion, these findings confirm, for the first time, the fact that approximately 60 % of human auricular chondrocytes express α-smooth muscle actin. This expression should be regarded as phenotypically characteristic of auricular chondrocytes, thus seeming that they have a contractile potential. Actin as a component of the cytoskeleton is involved in diverse cellular functions including cell contraction, cell adhesion, cell division and the maintenance of cell shape. It is supposed that auricular chondrocytes could be histogenetically closely related to non-muscle cells also containing α-smooth muscle actin in their cytoplasm. As examples can be mentioned myofibroblasts, osteoblasts and pericytes. The roles of α-smooth muscle actin in these cells warrant further investigation and the knowledge of this fact could help us to better understand the changes that also take place in the chondrocytes during the healing and transplantation of auricular cartilage.

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