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REIJO SIRONEN

Gene Expression Profiles of Chondrocytic Cells Exposed to Hydrostatic Pressure

Doctoral dissertation

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ABSTRACT

Human articular cartilage is specialized to transmit forces between articulating bones and provide almost frictionless movement of bone ends against each other. Chondrocytes, the only type of cell in this tissue, have to cope with these mechanical forces during movement. One of the components of the overall mechanical force in articular cartilage is hydrostatic pressure. This study was undertaken to analyze gene expression profile of chondrocytic human cells under high and low hydrostatic pressure (HP). Earlier studies concerning the effects of hydrostatic pressure on chondrocyte metabolism have mainly focused on measuring the synthetic capacity of extracellular matrix components under various loading regimes rather than on gene expression on a wide scale.

In this study, a human chondrosarcoma cell line was cultured as a monolayer culture and pressurized in a custom-made pressurization chamber. High static pressure (30 MPa) was used to study the stressful effects of pressure on the cells and the activity of possible mechanosensitive genes under these circumstances. Another part of the study focused on the effects of low static and cyclic pressures (5 MPa) on the gene expression profile of the cells. For comparison, one set of cells was also mechanically stretched. Differential display RT-PCR and cDNA array techniques were used to analyse the expression profiles. Possible differential expression was further confirmed by RNA hybridization analysis, ribonuclease protection assay and immunoblotting analysis. In addition, potential changes in mRNA stability during pressurization were assessed by the cDNA array technique.

Expression profiling revealed induction of heat shock genes, genes involved in growth arrest and control of the cell cycle under high static pressure (30 MPa), indicating adaptation to stressful conditions. In addition, a set of immediate-early genes, for example transcription factors, were up- and down-regulated on the mRNA level, also indicating the beginning of adaptive processes on genetic level. Interestingly, the Id1, Id2 and Id3 proteins, inhibitors of differentiation, were markedly down-regulated under high pressure. Thus, high pressure may activate differentiation processes in the cells under study. In contrast, low static and cyclic (5 MPa) pressure affected only a few genes, ones which are involved in cell adhesion and transcriptional regulation. The stability of mRNA molecules was not increased during pressurization. Furthermore, stretching of the cells affected expression of different set of genes compared to genes involved in pressure response.

In differential display RT-PCR analysis, reticulon 4-B, a member of the reticulon gene family, was found to be up-regulated under high static pressure. This previously uncharacterized gene was not responsive to other kinds of stressful treatments, indicating a possible mechanosensitive element in its transcriptional regulation. Furthermore, the intracellular localization and potential role of a putative di-lysine motif in intracellular transport was characterized.

National Library of Medicine Classification: WE 300

Medical Subject Headings: cartilage, articular/physiology; chondrocytes; hydrostatic pressure; gene expression/physiology; gene expression profiling; stress, mechanical; RNA, messenger; heat-shock proteins; transcription factors; cell differentiation; membrane proteins; reverse transcriptase polymerase chain reaction

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Kuopio, October 2004

Reijo Sironen

ABBREVIATIONS

ATF	Activating transcription factor
Atm	Atmosphere
BMP4-R	Bone morphogenetic protein 4 receptor
Btg	B cell translocation gene
Cdc37	Cell division cycle 37
CDK	Cyclin-dependent kinase
CS	Chondroitin sulphate
CTGF	Connective tissue growth factor
DDLC-1	Drosophila dynein light chain 1
DDRT-PCR	Differential display reverse transcription polymerase chain reaction
DS	Dermatan sulphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGR-1	Early growth response gene 1
Elf-1	E74-like factor 1
ER	Endoplasmic reticulum
EYFP	Enhanced yellow fluorescent protein
Fra	Fos-related antigen
GADD	Growth arrest and DNA damage-inducible gene
GAG	Glycosaminoglycan
GF	Growth factor
GAPDH	Glyseraldehyde phosphate dehydrogenase
HA	Hyaluronan
HCS	Human chondrosarcoma
HDLC-1	Human homologue of the ddlc1 gene
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
hHR23	Human homologue of Rad23
HMG17	High mobility group 17 protein
HP	Hydrostatic pressure
HS	Heparan sulphate
Hsp	Heat shock protein
Id	Inhibitor of differentiation
IGFBP	Insulin-like growth factor binding protein
IGF1R	Insulin-like growth factor I receptor
IL	Interleukin
JAK	Janus kinase
KS	Keratan sulphate
MCL-1	Myeloid cell leukaemia-1 protein
MMP	Matrix metalloproteinase
N-CAML1	Neural cell adhesion molecule L1
NIP3	Nineteen kD interacting protein-3
OA	Osteoarthritis
PBS	Phosphate buffered saline

PDGF-B	Platelet derived growth factor B
PG	Proteoglycan
PML	Promyelocytic leukemia gene
PMSF	Phenylmethylsulfonyl fluoride
PRELP	Proline arginine-rich end leucine-rich repeat protein
PSI	Pounds per square inch
$p21^{(WAF1/CIP1)}$	Cell cycle inhibitor - wild-type activated fragment 1 / CDK-interacting protein 1
RBP-J	Recombination signal binding protein-Jkappa
RHD	Reticulon homology domain
RIPA	Rapid immunoprecipitation assay
RPA	Ribonuclease protection assay
RTN	Reticulon
SOX9	Sry-type HMG box / transcriptional regulator
SSC	Saline sodium citrate
STAT	Signal transducer of transcription
TGF-α	Transforming growth factor α
Tiam1	T-lymphoma and metastasis gene 1
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
Tob	Transducer of ErbB-2
TXBP151	TAX1-binding protein 151
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following reports, referred to in the text by Roman numerals:

I Sironen RK, Karjalainen HM, Elo MA, Kaarniranta K, Törrönen K, Takigawa M, Helminen HJ, Lammi MJ. cDNA array reveals mechanosensitive genes in chondrocytic cells under hydrostatic pressure. Biochim Biophys Acta - Mol Cell Res 2002; 1591: 45-54.

II Sironen RK, Karjalainen HM, Törrönen K, Elo MA, Kaarniranta K, Takigawa M, Helminen HJ, Lammi MJ. High pressure effects on cellular expression profile and mRNA stability. A cDNA array analysis. Biorheology 2002; 39: 111-117.

III Karjalainen HM, Sironen RK, Elo MA, Kaarniranta K, Takigawa M, Helminen HJ, Lammi MJ. Gene expression profiles in chondrosarcoma cells subjected to cyclic stretching and hydrostatic pressure. A cDNA array study. Biorheology 2003; 40: 93-100.

IV Sironen RK, Karjalainen HM, Törrönen K, Elo MA, Hyttinen M, Helminen HJ, Lammi MJ. Reticulon 4 in Human Chondrocytic Cells: Barosensitivity and Intracellular Localization. Int J Biochem Cell Biol 2004; 36: 1521-1531.

CONTENTS

1. INTRODUCTION

The well-being of the musculoskeletal system is a precondition for normal physical activity in all mammals. Articular cartilage is located in a "key" position of this system providing smooth movement of articulating bones in synovial joints. Chondrocytes in the weight-bearing areas (e.g. between femur and tibia in a knee joint) are prone to a variety of physical forces which provide cells with various biomechanical stimuli. These physical stimuli are converted into chemical ones and probably have a marked effect on cellular metabolism. Knowledge of the regulation of chondrocyte metabolism in articular cartilage during joint loading helps us to understand cellular behaviour under normal and stressful conditions.

Several pathological conditions affect the structure and function of articular cartilage. Osteoarthritis (OA), which is characterized by loss of joint cartilage leading to pain and loss of function primarily in the weight-bearing joints, affects 9.6% of men and 18% of women aged over 60 years (Woolf et al. 2003). Among the risk factors of this degenerative disease are obesity, trauma, physically demanding activities and age. Thus, physical factors are closely involved in the disease process.

Articular cartilage, like bone, reacts to changes in the prevailing activity. Immobilization leads to a transient or permanent atrophy and softening of the tissue (Palmoski et al. 1979, Jurvelin et al. 1986, Arokoski et al. 1994) but moderate exercise increases cartilage thickness and, especially, proteoglycan (PG) content (Helminen et al. 2000). The overall mechanical load directed towards articular cartilage is a complex phenomenon that has several components, such as tensile, shear and compressive forces,

water flow, hydrostatic pressure, and osmotic and electrostatic changes (Urban 1994). Due to the relatively small permeability of the cartilage, the interstitial water is pressurized and supports most of the loading during dynamic loading (Helminen et al. 2000). The studies concerning the effects of hydrostatic pressure on the metabolism of chondrocytes have mainly focused on the quantitative changes in matrix production. Few experimental studies have been conducted to determine the influence of hydrostatic pressure on the gene expression profile on a large scale. The present experiments were conducted to obtain more information about the influence of hydrostatic pressure on the genes not previously associated with mechanosensitive qualities.

2. REVIEW OF THE LITERATURE

2.1. Biomechanical environment of chondrocytes in articular cartilage

Chondrocytes are specialised cells which produce and maintain the extracellular matrix of articular cartilage. They are rounded or polygonal cells of mesenchymal origin and, *in vivo*, are long-lived and do not significantly divide after skeletal maturity except in pathological conditions. During the human growth period, chondrocytes are major contributors to body growth (Archer et al. 2003). Articular cartilage is avascular tissue whose nutrition depends on diffusion through matrix from synovial fluid or vessels of subchondral bone. Other forms of cartilaginous tissue are surrounded by the perichondrium, a fibrocellular layer containing blood vessels. Chondrocytes are able to withstand low oxygen tension (ranging from 10% at the surface of cartilage to under 1% in the deep layers) and metabolise glucose primarily by glycolysis. Anaerobic metabolism is maintained under aerobic conditions (Marcus et al. 1973). Partly due to these properties, the tissue has a low reparative potential that predisposes it to degenerative conditions such as osteoarthritis (OA). Intracellular structures of chondrocytes show typical features of metabolically active cells that synthesise and turnover a large matrix volume consisting of collagens, glycoproteins, proteoglycans and hyaluronan (Archer et al. 2003). There is a relatively high matrix to cell volume ratio. Thus, in mammalian articular cartilage chondrocytes occupy about 10% of the total tissue volume (Stockwell 1979) and the matrix makes up 90% of the dry weight of the tissue (Hardingham et al. 1992).

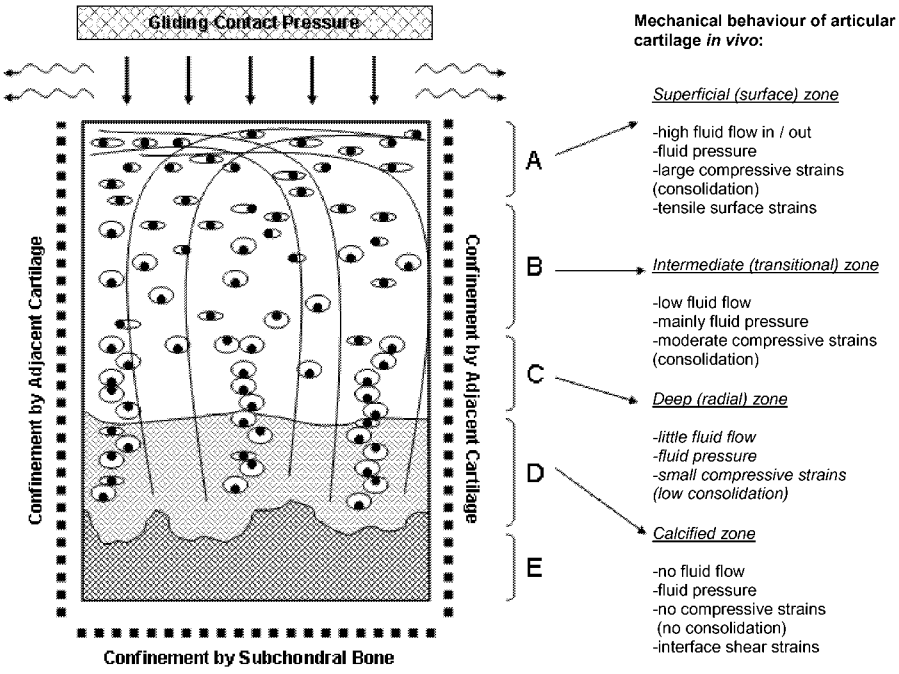

Figure 1. Schematic presentation of the structure of articular cartilage illustrating the zones and *in vivo* mechanical changes of the tissue under joint loading. In the superficial zone (A), the chondrocytes are discoidal, with their long axes parallel to the surface. The collagen fibres are closely packed and the orientation of the fibrils is predominantly tangential to the surface. In the intermediate zone (B), the cells are more spheroidal and the collagen fibres are more randomly oriented. The deep (radial) zone (C) has the highest proteoglycan content, the spheroidal cells forming columns aligned perpendicularly to the surface of cartilage. The collagen fibres are perpendicular (radial) to the surface. The calcified part of the cartilage is adjacent to the subchondral bone and forms the calcified zone (D). In calcified cartilage, the proteoglycan content is low and the matrix is mineralized with crystals of calcium salts. There are few chondrocytes among the radially oriented collagen fibres. The tidemark forms the border line between the deep and calcified zones. Under the cartilage is subchondral bone (E), providing mechanical support and nutrients for the avascular cartilage in young individuals. The biomechanical changes during joint loading and motion taking place in the different zones are presented on the right side of the figure (modified from Helminen et al., 1992 and Wong et al., 2003).

Chondrocytes have to withstand various amounts of compressive load especially in the articular surfaces of weight-bearing areas of the joints and in the intervertebral discs. The tissue has a set of unique mechanical and physicochemical properties which are responsible for its load-carrying capabilities and near-frictionless qualities. Mechanical

load is an important regulator of cellular metabolic activity. The forces transmitted to the cartilage tissue and cells act in several ways. Cellular deformation, hydrostatic pressure (HP), strain, streaming potentials caused by fluid flow, and change in tissue osmolality are among the regulators of the function of cartilage (Figs. 1 and 2). Chondrocytes in adult articular cartilage are isolated from each other by extracellular matrix (ECM). The matrix has a high concentration of polyanionic proteoglycans that form the fixed negative charge in the tissue. These anions are immobilized into tissue by type II collagen fibrils and bind high amount of cations (Na^+, K^+, and Ca^{2+}). The overall concentration of ions and hence tissue osmolality is higher in cartilage than in synovial fluid or serum. As a result of this osmotic pressure difference, fluid is absorbed into tissue and the swelling is resisted by the collagen network. This system tends to reach pressure equilibrium, the level of which depends on the amount and duration of the load. If the load is maintained for a long time, fluid is expressed out of tissue. Even at rest, the osmotic force generates hydrostatic pressure of 0.2 MPa (= 2 atm = 29.39 PSI = 2.03 bar) in human articular cartilage (Maroudas et al. 1981). The pressure may be even higher in the close vicinity of chondrocytes, where the concentration of proteoglycans is the highest. In the articular cartilage, the fluid loss is 1-5% during normal walking (Weightman et al. 1979) and 20% in the intervertebral discs in the upright position (Boos et al. 1993).

Cartilage and chondrocytes are subjected to high stresses, and during normal activity pressures rise to 10-20 MPa (100-200 atm) within milliseconds (Hodge et al. 1986). The cells are directly attached to ECM and are part of the fluid continuum of the tissue. Cellular shape, pressure and chemical environments are altered by deformations created in the ECM and by local fluid pressure and flow (Wong et al. 2003). In the joints, the

cells of the load-bearing area are large and have a great volume of intracellular organelles (Eggli et al. 1988) for the production of more resilient ECM compared with the cells of other areas of articular cartilage. The cartilage is also capable of altering its thickness and proteoglycan content according to the changing loading pattern (Kiviranta et al. 1988). Immobilization leads to loss of cartilage proteoglycans within a few weeks, while the proteoglycan content is mostly restored after remobilization (Säämänen et al. 1987, Haapala et al. 1999). Regular application of a mechanical load appears necessary for the cartilage matrix to maintain its composition (Urban 1994).

Studies *in vitro* have shown that mechanical loads can affect both the synthesis rate and pattern of macromolecules produced by cells. In addition, loading of articular cartilage plugs *in vitro* has revealed that the cellular responses are sensitive to loading patterns (Parkkinen et al. 1992). The magnitude of the load is dependent on body weight, muscle tension, posture and activity. Static loading seems to lead to a fall in the synthesis rate of matrix components proportionally to the applied stress (Gray et al. 1989). Cyclic loading, in contrast, can stimulate synthesis depending on loading frequency. Thus, frequencies resembling a walking cycle induced synthesis rates of proteoglycans, and lower frequencies were inhibitory in this respect (Sah et al. 1989, Korver et al. 1992, Parkkinen et al. 1992).

2.2. Hydrostatic pressure as a component of the loading force in articular cartilage

During daily activities various types of forces are continuously transmitted through the tissues, especially in the weight-bearing parts of the body. In the articular cartilage these

forces consist of direct mechanical stresses, strains, pressure and fluid flow inside the tissue over the contact area of the two opposing cartilage surfaces in the synovial joint (e.g., between the femur and tibia). The nature of the overall stresses that are concentrated on the tissue depends on the magnitude, direction, duration and mode (e.g. continuous vs. cyclic) of the force.

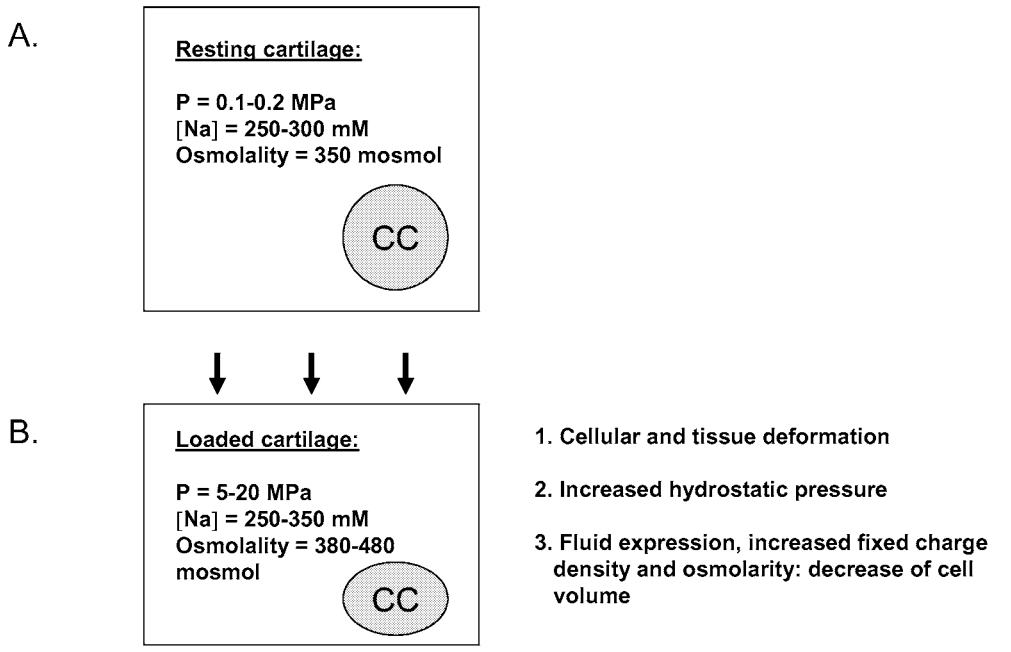

Figure 2. A schematic representation of the effect of load on the chondrocyte's physical and chemical environment. (A) Resting cartilage. (B) Compressive load in a weight-bearing area of articular cartilage deforms the matrix and chondrocytes. Hydrostatic pressure rises in milliseconds and interstitial fluid is squeezed out of the tissue increasing the extracellular concentration of fixed charged ions in seconds to hours (modified from Urban 1994, CC = chondocyte).

There is a very thin fluid film over the solid tissue between the contact areas of two opposing cartilage surfaces. This is called unbound fluid, while the fluid in the intercellular space of cartilage is called bound or interstitial fluid. The contact force immediately causes pressurization of this interstitial fluid and some of it squeezes out of the solid matrix onto the surface of cartilage. This unbound fluid is a lubricating agent that diminishes the friction and transmits forces into the underlying solid matrix. The interstitial fluid is also immediately pressurized and transmits the contact force to the underlying bone (Wong et al. 2003). Thus, the fluid phase is mainly responsible for transmission of the forces through the cartilage, producing variable amount of hydrostatic pressure in the tissue. The excess exudation of fluid out of the cartilage is inhibited by the relatively low fluid permeability of the matrix (Mow et al. 1984). The subchondral bone, impermeable to fluid flow (Maroudas et al. 1968), and adjacent cartilage tissue confines the pressurized part of the cartilage (Fig. 1) and restricts mechanical deformation of tissue under the contact surface. Fluid flow, especially into the superficial zone and to a lesser extent into the transitional zone, is restricted during normal activities. During fluid exudation, the solid matrix of the superficial zone consolidates and cells in this region experience compressive deformations. The porosity, and thereby the permeability of superficial matrix, is also decreased during exudation. This forms a barrier preventing the further exudation of fluid out of the underlying radial and calcified zones. Thus, the fluid flow is negligible in these zones and the pressurization of the interstitial fluid forms the main load carrying capacity of cartilage *in vivo*. The solid collagen network, especially in superficial zones, resists tensile and shear stresses and the fluid component resists the high hydrostatic stresses generated during compressive loads (Wong et al. 2003).

The magnitude of total cartilage thinning as a result of fluid exudation and tissue consolidation during daily activities is relatively small. No significant diurnal changes have been found in magnetic resonance imaging studies in the volume of the articular cartilage of the knee: the thinning has been found to be only 0.6 mm in highly loaded areas during one day (Waterton et al. 2000). During resting periods, the joint is unloaded and increased osmotic activity in the consolidated superficial zones leads to absorption of water back into the tissue, thereby restoring the original amount of interstitial fluid (Suh et al. 1995).

The highest measured contact pressure in humans is approximately 20 MPa during the normal daily activity of 73-year old woman (68 kg) determined by instrumented endoprosthesis of the hip joint. In the study, the pressure was found to be 4-5 MPa during normal walking (Hodge et al. 1986). Contact pressures may be higher in obese persons, or during heavy work or sports activities. During normal daily activities, the periods of loading are usually cyclic, rarely lasting more than one hour, and are interspersed with periods of partial recovery. However, the loading regimes in articular cartilage during standing work or other static loading situations have not been determined.

2.3. Hydrostatic pressure and chondrocyte metabolism

2.3.1. General aspects

Experiments have been performed to determine the effects of HP on cartilage *in vivo*, *ex vivo* (cartilage explants) and on cultured cells. Primary chondrocytes isolated from the

cartilage have been widely used in the studies. In addition, immortalized chondrocytes (Goldring et al. 1994) and continuously growing chondrocytic cell lines of cancerous origin (Takigawa et al. 1989) have also been studied. Tests *in vivo* have shown that load-bearing areas of articular cartilage are exposed to contact pressures between 10 and 20 MPa (100-200 atm) within milliseconds (Hodge et al. 1986) at frequencies ranging from 0.1 to 10 Hz (Wong et al. 2003). The "physiologic" pressure in cartilage has been reported to be in the range 5-15 MPa (Wong et al. 2003).

2.3.2. The proteoglycans and their synthesis

Proteoglycans are a major component of the extracellular matrix of cartilage. These macromolecules consist of a central core protein to which one or more glycosaminoglycan (GAG) side chains are attached (Hardingham et al. 1992, Muir 1995, Knudson et al. 2001). GAGs are unbranched carbohydrates made of repeating disaccharide units of which one residue is N-acetylglucosamine or N-acetylgalactosamine and the other is hexuronic acid or galactose. The cartilage GAGs are chondroitin sulphate (CS), keratan sulphate (KS), dermatan sulphate (DS), heparan sulphate (HS) and hyaluronan (HA). The predominant glycosaminoglycan present is chondroitin sulphate.

All GAGs except HA are sulphated to varying degrees and attached to the core protein by covalent bonds. Because of these sulphate or carboxyl groups on most of their sugar residues, GAGs are highly negatively charged. This negative charge attracts cations, such as Na^+ and K^+, and causes large amounts of water to be absorbed into the matrix.

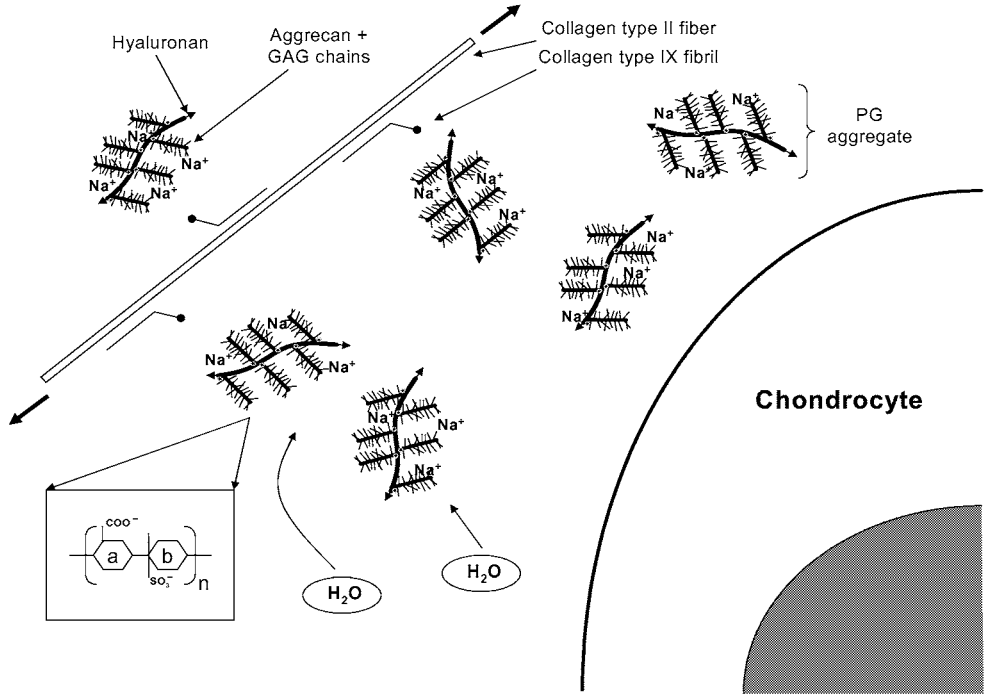

Figure 3. Schematic view of the extracellular matrix of cartilage. Type II collagen fibrils anchor chondrocytes and proteoglycan (PG) aggregates in place. Aggrecan core protein is usually connected with approximately 130 chondroitin sulphate and keratan sulphate side chains and is linked to hyaluronan chain by two link proteins. The GAG side chains are negatively charged due to the carboxyl and sulphate groups in repeating disaccharide sequences, presented in the box in the lower left corner. The fixed negative charges bind mobile cations (Na^+, K^+ and H^+), increasing the osmotic pressure and lowering the extracellular pH of tissue. This causes water influx into tissue and enables the rise of hydrostatic pressure. Tissue swelling is resisted by collagen fibres. (Modified from Urban 1994 and Alberts et al., 2002)

Thus, GAGs tend to adopt highly extended conformations occupying a large volume relative to their mass. This creates a swelling pressure that enables the matrix to withstand compressive forces (Alberts et al. 2002).

The proteoglycan core proteins in cartilage are aggrecan, decorin, biglycan, fibromodulin, lumican, epiphycan, perlecan and PRELP (proline arginine-rich end leucine-rich repeat protein) (Knudson et al. 2001). The predominant proteoglycan in cartilage is aggrecan, and the deposition of aggrecan PG in tissue is considered a hallmark of chondrogenesis (Schwartz et al. 1999). Aggrecan typically forms large proteoglycan aggregates with HA by non-covalent binding. This attachment is stabilised by a link protein. An aggregate consists of approximately 100 aggrecan monomers bound to a single hyaluronan chain. The molecular weight of such a complex can be 10^8 Da or more, and it occupies a volume equivalent to that of a bacterium (2×10^{-12} cm^3). Such giant molecules are an essential part of the system that provides the tissue swelling pressure by absorbing water (Alberts et al. 2002).

The effect of pressure on chondrocyte synthetic activity has been extensively studied during the last two decades (Tables 1 and 2). The results are somewhat controversial, since many different kinds of experimental setups, specimens and loading protocols have been used in these studies. The importance of hydrostatic pressure in cartilage proteoglycan production has been demonstrated in a number of studies (Lippiello et al. 1985, Hall et al. 1991, Parkkinen et al. 1993, Lammi et al. 1994, Smith et al. 1996, Ikenoue et al. 2003). Proteoglycan synthesis has usually been assessed by ^{35}S-sulphate incorporation assay and amino acid uptake / total protein synthesis by ^{3}H-proline or ^{14}C-leucine incorporation assays. These reports show that cyclic (intermittent) pressure, as well as low static pressure, usually stimulate proteoglycan production in tissue and cell cultures, while high continuous hydrostatic pressure inhibits the synthesis (Hall et al. 1991, Lammi et al. 1994). However, proteoglycan synthesis seems to be dependent on the

pressure mode and loading regime (Tables 1 and 2). Thus, different frequencies of cyclic loading can cause different effect to sulphate incorporation rates, even if the pressure magnitude is unaltered (Parkkinen et al. 1993). Hydrostatic load can also modulate the structure of the synthesized proteoglycans. Thus, high static pressure causes a slight increase in the average GAG chain length, which does not take place under milder conditions (Lammi et al. 1994).

Expression of PG core proteins is also pressure dependent. Pressures of 5-10 MPa static or 5 MPa cyclic (0.017 Hz) do not alter the aggrecan mRNA synthesis (Lammi et al. 1994, Smith et al. 1996), while 5 MPa cyclic (1 Hz), 10 MPa cyclic (Smith et al. 1996, Smith et al. 2000, Ikenoue et al. 2003) and 30 MPa static pressure (Lammi et al. 1994) induce expression (Tables 1 and 2). Osteoarthritic human chondrocytes or primary bovine chondrocytes in agarose culture up-regulated their aggrecan synthesis under low (5 MPa) cyclic and static pressure (Lee et al. 2003, Toyoda et al. 2003).

2.3.3. Cartilage collagens and their synthesis

The collagen network plays a vital role in maintaining the structural integrity of the ECM in articular cartilage. After the breakage of this network PGs may diffuse out of the tissue, and the osmotic balance is disturbed. Collagen types II, IX (also a PG), X and XI are often called as "cartilage-specific" collagens. Types VI and XII are present in cartilage as well as in non-cartilaginous tissues. Each collagen molecule, regardless of their type, is composed of three α chains. The amino acid sequence in the triple-helical part of an α chain is typically Gly-X-Y, where X is frequently proline and Y

hydroxyproline. Three α chains form a coiled-coil superhelix which is highly resistant to proteolytic degradation, except to specialised matrix metalloproteinases (MMPs). Collagen types II, IX and XI are organized into matrix fibrils: type II constitutes the bulk of the fibril. The fibrils then form larger collagen fibres in the tissue. Type IX facilitates fibril interaction with PG macromolecules and type XI seems to regulate fibril size. Type X is found only in small amounts in normal human adult cartilage. It is most abundant in areas where hypertrophic chondrocytes are found (i.e. in the growth plate and at healing fracture sites) (Cremer et al. 1998). Canine articular cartilage shows a site-dependent expression of type X at a depth of 50–100 μm from the surface. This may involve a modification of the collagen fibril arrangement at the site of collagen fibril arcades, possibly providing extra support to the collagen network (Lammi et al. 2002). Type X collagen has also been found in mouse articular cartilage, especially at the tidemark (Eerola et al. 1998).

The cartilage collagen fibrils are non-homogeneous structures and vary in size and length. Type II forms the backbone of the cartilage heteropolymeric fibrils and type I plays a similar role in non-cartilaginous fibrils. Type XI is located mainly within the type II fibrils and is covalently linked with these. Type IX is located on the exterior of type II fibrils in an antiparallel orientation. It has been proposed that it functions as a "spacer" between individual fibrils, a "glue" that binds the type II lattice work, and as a device for collagen fibres to interact with PG macromolecules (Cremer et al. 1998).

The effect of hydrostatic pressure on the expression of various collagen molecules has been reported in a few articles. Moderate cyclic pressure (10 MPa) increased the expression of type II collagen mRNA in primary bovine chondrocytes (Smith et al. 1996,

Smith et al. 2000), and in isolated human articular chondrocytes (Ikenoue et al. 2003). In contrast, moderate static pressure (10 MPa) decreased the expression of this collagen type (Smith et al. 1996). Bovine chondrocytes seeded in alginate constructs has been documented to increase the expression of collagen type X expression under 5 MPa cyclic pressure (Wong et al. 2003). Cyclic pressure also contributes to the enhancement of chondrogenic phenotype by increasing the total collagen synthesis in bone marrow derived mesenchymal progenitor cell under 5 MPa cyclic hydrostatic load (Angele et al. 2003). The effects of hydrostatic pressure on fibril formation or on the expression of other types of collagens have not been reported. However, it seems evident that collagen expression is at least somewhat reactive to the physical changes induced by HP (Tables 1 and 2).

Table 1. Summary of published data on the effects of fluid phase *in vitro* loading with static hydrostatic pressure on the cellular metabolism of chondrocytes (Pressure units other than Pascals presented in the original articles have been converted to MPa units according to the SI system). Data is arranged according to the magnitude of pressure used in the experiments (↓= inhibition, ↑= stimulation, ↔ = no effect, cc = chondrocytes, OA = osteoarthritis).

Authors	Material	Pressure (MPa) - loading time		Effect on cellular metabolism
Hall et al., 1991	Steer cartilage slices	30-50	2 h	Proline and sulphate incorporation ↓
		5-10	2 h	Proline and sulphate incorporation ↑
		20-50	20 s	Proline or sulphate incorporation ↔
		2.5-20	20 s	Proline and sulphate incorporation ↑
Hall 1999	Isolated Primary bovine cc	0.1-50	20 s-10 min	Na^+/K^+ pump and $Na^+/K^+/2Cl^-$ cotransporter activity ↓
Takahashi et al., 1997	Human chondrosarcoma cell line HCS-2/8	10 and 50	2 h	Sulphate incorporation ↓, TGF-β1 and Hsp70 mRNA ↑
		1 and 5	2 h	Sulphate incorporation ↑
Takahashi et al., 1998	Human chondrosarcoma cell line HCS-2/8	1, 5, 10 and 50	2 h	Cell viability ↔ , pressure dependent increase in expression of TNF and IL-6 mRNA, TIMP-1 or stromelysin mRNA ↔
Parkkinen et al., 1993	Isolated primary bovine cc	15 and 30	2 h	Reversible compaction of Golgi apparatus, PG synthesis ↓
Parkkinen et al., 1995	Isolated primary bovine cc	30	2 h	Reversible disappearance of stress fibres
Lammi et al., 1994	Isolated primary bovine cc	30	20 h	Sulphate incorporation ↓, mRNA expression of biglycan and decorin ↑ , aggrecan mRNA↔, slight increase in average GAG chain length
		5	20 h	Sulphate incorporation ↔
Kaarniranta et al., 1998	Immortalized human cc	30	12 h	Heat shock response, stabilization of Hsp70 mRNA
Browning et al., 1999	Isolated primary bovine cc	0.1-30	0-300 s	Na^+ x H^+ exchanger activity ↑
Elo et al., 2000	Immortalized human cc (T/C28a4)	30	12 h	Protein synthesis ↓, Hsp70 and Grp78 proteins ↑, Hsp90α and Hsp90β proteins ↔
		4	12 h	Protein synthesis and stress proteins ↔
Jortikka et al., 2000	Isolated primary bovine cc	30	20 h	Sulphate incorporation ↓
		5 and 15	20 h	Sulphate incorporation ↔
Kaarniranta et al., 2001	Isolated primary bovine cc	30	0 - 12 h	Heat shock response ↔
Westacott et al., 2002	chondrocyte cell line (T/AC62), isolated human normal and OA cc	5 and 20	24 h	TNF-receptor type I mRNA ↑ in OA chondrocytes, in other cell types ↓. TNF-receptor II mRNA ↔
Smith et al., 1996	Isolated primary bovine cc	10	4 h	Collagen type II mRNA ↓, aggrecan mRNA in serum free medium ↔. No changes in either mRNA levels with 1% FBS. PG synthesis ↑
Toyoda et al., 2003	Primary bovine cc in agarose gel	5	4 h	Aggrecan and collagen II mRNA ↑, TIMP-1 mRNA ↔
Handa et al., 1997	Intervertebral discs	0.1-3	2 h	Sulphate incorporation ↑, MMP-3 protein ↑
Lippiello et al., 1985	Human and bovine articular cartilage segments	2.6	24 h	Sulphate incorporation ↑
		0.52-2.1	24 h	Sulphate incorporation ↓
Wright et al., 1992	Isolated sheep articular cc	0.016	20 min.	Depolarization of transmembrane potential

Table 2. Summary of published data on the effects of fluid phase *in vitro* loading with cyclic hydrostatic pressure on the cellular metabolism of chondrocytes (Pressure units other than Pascals presented in the original articles have been converted to MPa units according to the SI system). Data is arranged according to the magnitude of pressure used in the experiments (↓= inhibition, ↑= stimulation, ↔ = no effect, cc = chondrocyte, OA = osteoarthritis)

Authors	Material	Pressure (MPa) / frequency (Hz) - loading time		Effect on cellular metabolism
Parkkinen et al., 1993	Isolated primary bovine cc	5, 15 and 30 / 0.125 and 0.05	2 h	No morphological effect on Golgi apparatus
Parkkinen et al., 1995	Isolated primary bovine cc	5, 15 and 30 / 0.125 and 0.05	2 h	Moderate decrease of stress fibers (15 and 30 MPa), no effect (5 MPa)
Kaarniranta et al., 1998	Immortalized human cc (T/C28a4)	30 / 0.5	12 h	Hsp-response ↔
Elo et al., 2000	Immortalized human cc (T/C28a4)	30 / 0.5	12 h	Protein synthesis ↓, Hsp70 protein ↑, other stress proteins ↔
Smith et al., 1996	Isolated primary bovine cc	10 / 1	4 h	Aggrecan mRNA ↑, collagen II mRNA ↔. Both mRNAs ↑ with 1% FBS, PG synthesis ↑
		4 / 0.5	12 h	Protein synthesis and stress proteins ↔
Smith et al., 2000	Isolated primary bovine cc	10 / 1	2-24 h	Aggrecan mRNA ↑, biphasic expression pattern of collagen II mRNA
		10 / 1	4 hrs / day x 4 days	Collagen II and aggrecan mRNA ↑
Lee et al., 2003	Human OA cc	10 / 1	4 h / day x 1-4 days	Nitric oxide release ↓, collagen II and aggrecan mRNA↑
Ikenoue et al., 2003	Isolated human articular cc	1, 5 and 10 / 1	4 h	Aggrecan mRNA ↑ at 5 and 10 MPa, collagen II mRNA ↔
		1, 5 and 10 / 1	4 h / day x 4 days	Aggrecan mRNA ↑ at all loading levels, collagen II mRNA ↑ at 5 and 10 MPa
Parkkinen et al., 1993	Bovine cartilage explants	5 / 0.5	1.5 h	Sulphate incorporation ↑
		5 / 0.0167-0.25	1.5 h	Sulphate incorporation ↔
	Isolated primary bovine cc	5 / 0.05 – 0.5	1.5 h	Sulphate incorporation ↓
		5 / 0.25-0.5	20 h	Sulphate incorporation ↑
		5 / 0.0167	20 h	Sulphate incorporation ↓
Lammi et al., 1994	Isolated primary bovine cc	5 / 0.5	20 h	Sulphate incorporation ↑, aggrecan, decorin and biglycan mRNA expression ↔
		5 / 0.25	20 h	Sulphate incorporation ↔, aggrecan, decorin and biglycan mRNA expression ↔
		5 / 0.017	20 h	Sulphate incorporation ↓, aggrecan, decorin and biglycan mRNA expression ↔
Jortikka et al., 2000	Isolated primary bovine cc	5 / 0.5	20 h	Sulphate incorporation ↑
Islam et al., 2002	Isolated human cc from OA cartilage	5 / 1	2-4 hrs	Induction of apoptosis through up-regulation of TNF-α, iNOS, p53, c-myc and bax-α, and suppression of bcl-2 mRNA
Toyoda et al., 2003	Primary bovine chondrocytes in agarose gel	5 / 1 (and static)	4 h	GAG synthesis and aggrecan mRNA ↑
Wong et al., 2003	Bovine chondrocyte-seeded alginate constructs	5 / 0.5	3 h / day x 3 days	TIMP-1, VEGF and collagen X mRNA ↑, MMP-13 ↓, runx2/cbfa1, TIMP-2 and CTFG mRNA ↔
Mizuno et al., 2002	Isolated bovine primary chondrocytes grown in collagen sponges	2.8 / 0.015 (and static)	15 days	Sulphate incorporation ↑, accumulation of CS and KS proteoglycans
Angele et al., 2003	Bone marrow derived mesenchymal progenitor cells	0.55 – 5.03 / 1	4 h	PG synthesis and collagen protein ↔
		0.55 – 5.03 / 1	4 h / day x 7 days	PG and collagen synthesis ↑ (enhancement of chondrogenic phenotype)
Hansen et al., 2001	Isolated primary bovine chondrocytes	0.2 / 5+5 s (on + off)	4-6 days	Thymidine incorporation / proliferation ↓
		0.2 / 30+2 min (on + off)	4-6 days	Thymidine incorporation / proliferation ↓
		0.2 / 2+30 min (on + off)	4-6 days	Thymidine incorporation / proliferation ↑
Wright et al., 1992	Isolated sheep articular cc	0.016 / 0.33	20 min.	Hyperpolarization of transmembrane potential

2.3.4. Other pressure-induced changes in gene expression and cellular metabolism

Several studies describe pressure-induced changes in cellular metabolism unrelated to PG or collagen synthesis. The results indicate changes in cell membrane potentials, cell organelles, stress response and regulation of specific gene expression on the posttranscriptional level. For example, short-term 0.016 MPa static pressure caused membrane depolarization and a similar cyclic (0.33 Hz) pressure-induced hyperpolarisation in isolated sheep articular chondrocytes (Wright et al. 1992). In that study, specific blockers were used and involvement of Ca^{2+} -dependent K^+ channels and Na^+ channels was documented. Further, cytochalasin B was shown to abolish pressure-induced hyperpolarization in chondrocytes, but caused hyperpolarisation in pressure-treated fibroblasts indicating changes in cytoskeletal organization. The Na^+/K^+ pump and $Na^+/K^+/2Cl^-$ cotransporter of primary chondrocyte plasma membrane are inhibited by all levels of static hydrostatic pressure. Alterations of matrix synthesis by application of load may thus result partly from variations in the intracellular ionic or osmotic composition of chondrocytes arising from changes in the activity of membrane transport pathways (Hall 1999). The results indicate the involvement of membrane channels and/or carriers in the response to hydrostatic loading. Hydrostatic pressure has a strong influence on the interface of liquid and membranes, where it can influence the membrane channels (Stockwell 1987).

Proteoglycan glycosylation and sulphation take place in the Golgi apparatus of chondrocytes. Hydrostatic pressure treatment has been shown to compact the Golgi apparatus of primary bovine articular chondrocytes under a pressure of 15-30 MPa static

pressure. In contrast, cyclic 5-30 MPa pressure had no effect on the packing of this organelle. Such packing may contribute to the well-known inhibition of PG synthesis observed in chondrocytes subjected to high static hydrostatic pressure (Parkkinen et al. 1993). Again, this indicates that the mode of pressure is important for proper organelle structure and cellular function. Pressure also affects the stress fibres, since high static pressure (30 MPa) caused reversible disappearance of stress fibres of bovine chondrocytes, while 15-30 MPa cyclic pressure has only moderate effects on these intracellular fibres (Parkkinen et al. 1995). Such involvement of cytoskeleton in pressure response is in accordance with the findings of the studies concerning the pressure effects on membrane hyperpolarisation mentioned above.

Various environmental factors may lead to cellular stress response involving evolutionary highly conserved heat shock proteins (Hsps). These stress factors include toxic agents, changes in temperature or osmolarity, radiation, ischemia and oxygen radicals. Hydrostatic pressure is also a potent inducer of heat shock response depending on the mode and magnitude of the pressure applied. High static pressure-induced (30 MPa) accumulation of Hsp70 mRNA and protein (Takahashi et al. 1997, Kaarniranta et al. 1998, Elo et al. 2000), whereas high cyclic pressure had no effect on heat shock response (Kaarniranta et al. 1998). Interestingly, the accumulation is caused by mRNA stabilization rather than transcriptional activation (Kaarniranta et al. 1998). Such regulation is not known to be involved in heat shock responses directed against any other stressful factor. Furthermore, primary bovine chondrocytes resisted hydrostatic pressure-induced stress, whereas in the primary synovial cells and fibroblasts an accumulation of Hsp70 mRNA and protein was observed (Kaarniranta et al. 2001). Thus, pressure-

induced metabolic stress responses may be restricted to a certain cell type, and other cell types are adapted to pressurized environment.

High hydrostatic pressure (>20-25 MPa) is presumably stressful for isolated cells. However, the viability of chondrocytic cells is not altered by such pressure. For example, very high (50 MPa) pressure did not affect the cellular viability of human chondrosarcoma cells (Takahashi et al. 1998). Expression of tumour necrosis factor α and interleukin 6 (IL-6), both reported to be up-regulated in the cartilage of an experimental osteoarthritis (OA) model and osteoarthritis patients (Kammermann et al. 1996, Mohtai et al. 1996), showed a transient pressure dependent enhancement of expression (Takahashi et al. 1998). Thus, high pressure may enhance expression of specific genes associated with degenerative disease, even if the overall viability is not changed. Excessive load is one of the factors presumably involved in the pathogenesis of OA (Lequesne et al. 1997), and mechanical overload is a risk factor for the development of secondary OA (Aigner et al. 2002). The cellular viability under load may be dependent on the degree of the pathological process, since isolated human chondrocytes from OA cartilage showed induction of apoptosis under 5 MPa cyclic pressure through up-regulation of apoptosis related genes (Islam et al. 2002). The expression of TNF-receptor I was also enhanced in chondrocytes derived from OA cartilage, but not in healthy cells or in the chondrocytic cell line (Westacott et al. 2002).

Extracellular matrix in articular cartilage responds to loading and is under constant turnover around the cells. Running tests *in vivo* have shown that cartilage is capable of remodelling and can change its composition according to the prevailing forces (Kiviranta et al. 1994). Such controlled matrix turnover involves a delicate balance between a set of

matrix degrading enzymes and their inhibitors. The dominantly expressed matrix metalloproteinase (MMP) in normal articular cartilage is stromelysin 1 (MMP-3), which plays a crucial role in PG, especially aggrecan, turnover (Cawston et al. 1999). This enzyme degrades PGs *in vitro* and *in vivo* (Hughes et al. 1991) and is up-regulated by IL-1 and TNF-α (MacNaul et al. 1990). Type II collagen degradation is thought to be achieved by the action of the collagenases, which are capable of cleaving the triple helical portion of the fibrillar collagen. All known collagenases are also capable of cleaving aggrecan (Hughes et al. 1991, Fosang et al. 1994, Fosang et al. 1996). Collagenase 3 (MMP-13) is expressed in chondrocytes (Reboul et al. 1996) and has high specific activity against type II collagen (Knauper et al. 1996). The coordinate action of MMPs involves a set of specific inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs) (Nagase et al. 1999). Cyclic hydrostatic pressure increases the expression of TIMP-1 but decreases the expression of MMP-13, and has no effect on amount of TIMP-2 mRNA (Wong et al. 2003). Static pressure has no effect on the TIMP-1 or stromelysin mRNA production (Takahashi et al. 1998). Thus, pressure causes changes in the expression patterns of the ECM molecules, and plays a regulatory role in the synthesis of molecules affecting the breakdown rate of ECM.

3. AIMS OF THE STUDY

Articular cartilage is an essential part of the human musculoskeletal system, providing a system that transmits forces in the synovial joints. The degenerative diseases of articular cartilage (e.g., osteoarthritis) weaken the invidual's mobility, and can seriously impair the quality of life. The molecular mechanisms involved in the regulation of chondrocyte metabolism under different loading patterns in the tissue are not well understood. Hydrostatic pressure has been shown to affect the production of various ECM molecules by chondrocytes. However, little is known about the overall regulation of gene expression under these conditions. Earlier studies have shown the importance of the magnitude of joint loading for the maintenance of functional cartilage and for the response of chondrocytes under excess hydrostatic loading. The objectives of this research were the following:

1. To identify gene expression patterns of chondrocytic cells under high hydrostatic pressure by differential display (mRNA fingerprinting) and cDNA array methods.

2. To study the effects of different loading patterns (5 MPa static and cyclic pressure, cyclic mechanical strain) on chondrocytic gene expression and mRNA stability.

3. To characterize the expression patterns and intracellular localization of an up-regulated, mechanosensitive gene expression product (RTN4-B), identified by DDRT-PCR.

4. MATERIALS AND METHODS

4.1. Cell culture and treatments (I-IV)

HCS-2/8 human chondrosarcoma cells (I-IV) (provided by Professor M. Takigawa (Takigawa et al. 1989)) and T/C28a4 human chondrocytic cells (I) (provided by Dr. M.B. Goldring (Goldring et al. 1994)) were cultured in a humidified 5% CO_2/95% air atmosphere at 37°C in DMEM with 10% fetal calf serum, penicillin (50 units/ml), streptomycin (50 units/ml) and 3 mM glutamine. The expression of chondrocyte-specific genes aggrecan and type II collagen in HCS-2/8 cell monolayer (Takigawa et al. 1989) was confirmed by RNA hybridization analysis and RT-PCR (data not shown). This cell line has been shown to express aggrecan and several collagens typical of cartilage as well as SOX9 (cartilage-specific transcriptional regulator) transcripts according to the cDNA array profiling (Saas et al. 2004). Cells were grown to a subconfluent state before the experiments. Before hydrostatic pressure treatments, the medium was changed and 15 mM Hepes (pH 7.3) was added. Primary bovine chondrocyte cell cultures were isolated from 1- to 2-year-old animals (n = 2). The articular cartilage from patellar surface of the femur was cut into 1-2 mm^3 pieces, washed twice with PBS, and digested with hyaluronidase (0.5 mg/ml, Sigma, St. Louis, MO, USA), supplemented with gentamycin (0.1 mg/ml, PAA, Linz, Austria), Fungizone® (2.5 µg/ml, PAA), ascorbic acid (0.5 µg/ml, Sigma) in Dulbecco's MEM Nut Mix F-12 medium (Gibco) at 37°C for 30 min with continuous shaking. The medium was then changed to collagenase (3 mg/ml, Sigma) digestion solution, supplemented with 10 % fetal calf serum (FCS), DNase (0.2 mg/ml, Sigma), 2 mM glutamine (PAA), Fungizone® (10 µl/ml, PAA), penicillin 50 units/ml

(PAA), streptomycin 50 units/ml (PAA), ascorbic acid (0.5 μg/ml, Sigma) in Dulbecco's MEM Nut Mix F-12 medium (Gibco), and kept at 37°C in a periodic magnetic stirring overnight. After extracellular matrix digestion, chondrocytes were cultured in Dulbecco's MEM Nut Mix F-12 medium including 10 % FCS, penicillin, streptomycin and glutamine. The cells were plated at 1 x 10^6 /ml, and used 2-3 days after plating for the experiments.

To expose the cells to pressure (I-IV), the culture dishes were filled with the growth medium including 15 mM HEPES (pH 7.3) to stabilize pH during pressure treatments, and sealed with plastic membranes. The dishes were then placed into the pressurization chamber (Fig. 4). Both the effects on pH, pO_2 and pCO_2 in this culture system and the apparatus for hydrostatic pressurization of the cells have been previously described in detail (Parkkinen et al. 1993). Cells were exposed to cyclic (0.5 Hz) 5 MPa and static 5 or 30 MPa pressure for various time periods, indicated in the results. During normal walking, a 5 MPa pressure cycle takes place in normal articular cartilage (Hodge et al. 1986). The 0.5 Hz frequency was chosen to represent a regular walking cycle. Furthermore, 30 MPa static pressure is known to be stressful for chondrocytes (Kaarniranta et al. 1998) and was chosen to represent a high pressure load. Exposure to the cyclic mechanical stretching (III) was performed using the High Strain Cell Stretching Apparatus (University of Ulm, Germany). The specifications of this apparatus have been published previously (Neidlinger-Wilke et al. 1994). Two to four days before the stretching experiments, cells were plated on special dishes made of silicone rubber (Elastosil 601-Me, Wacker Chemie, Munich, Germany). Prior to plating, the dishes were treated with 10 μg/ml poly-D-lysine (Sigma St. Louis, MO, USA) solution for 6 hours to

increase cell adherence onto the dishes. Cells were exposed to cyclic stretch (8% of their original length) at a stretching frequency of once every two seconds (0.5 Hz) up to 24 hours. Control cells were treated similarly except for the stretching exposure.

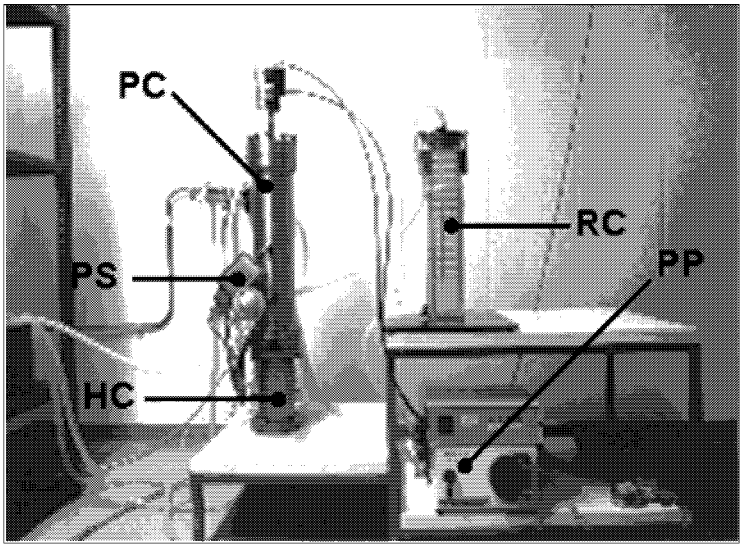

Figure 4. The apparatus for hydrostatic pressurization of the cultured cells (Parkkinen et al. 1993). The sealed Petri dishes are placed inside a specially designed rack which is then placed into a stainless steel pressure chamber (PC). There is a hydraulic cylinder (HC) under the pressure chamber. The oil pressure generated by the hydraulic pump is conducted into the hydraulic cylinder. The pressure valves are controlled by a computer. The hydraulic cylinder transfers pressure into the aqueous space of the pressure chamber. A peristaltic pump (PP) is used to fill the chambers with pre-warmed water (37°C). Control samples are placed into the reference chamber (RC), which is filled with pre-warmed water. A pressure sensor (PS) is attached into the pressure chamber.

For heat shock treatment (IV), the culture plates were filled with medium and sealed with the same plastic membranes as in the pressure treatment. The plates were then submerged in a water bath for 1 hour at 43°C (Kaarniranta et al. 1998). For heavy metal treatment (IV), 0.5 mM $CdCl_2$ was added to the culture medium, and for the recovery period, fresh medium was changed. Exposure to UVB irradiation (IV) was done with four

Philips TL 20W/12 lamps (peak at 308 nm, dosage 153 mJ/cm^2) for 1 minute after the medium had been changed to PBS. Radiation was carried out at room temperature (20-23°C) at a target distance of 30 cm. After exposure, the culture medium was replaced with fresh medium for the recovery period.

4.2. Profiling of gene expression

4.2.1 Differential display analysis (mRNA fingerprinting, IV)

In order to identify mRNA species for differentially expressed genes differential display RT-PCR analysis was performed as previously described elsewhere (Liang et al. 1992). Briefly, total cellular RNA was isolated using TRIzol® reagent (LifeTechnologies). Three degenerate anchored oligo(dT) primers (5'-T_{12}A/C/G-3') were used in separate reverse transcription reactions. PCR amplifications were performed using the three cDNA pools from control and pressurised samples and altogether 13 different arbitrary decamers and radioactive nucleotide ([α^{33}P]-ATP) were used. The radioactively labelled PCR products representing a subpopulation of mRNAs defined by the given primer were separated on a 6% denaturing polyacrylamide / urea gel. The gel was dried onto filter paper, and autoradiography was performed. After side-by-side comparison the bands of interest were cut out from the gel and reamplified. The resulting PCR products were cloned and sequenced for comparison against the sequence database.

4.2.2. cDNA array analysis (I-III)

The Atlas™ human cDNA expression array kit was purchased from Clontech Laboratories (Palo Alto, CA, USA). All the procedures for labelling and purifying the

probes were carried out following the manufacturer's recommendations. [α^{32}P]-dCTP-labelled cDNA probes were generated by reverse transcription of mRNA from untreated and pressurised monolayer cultures. The probes were purified by column chromatography (ChromaSpin™, Clontech Laboratories). The membranes were hybridized in ExpressHyb™ solution (Clontech Laboratories) overnight at 68°C, and washed twice (20 min with 0.1x SSC/0.5% sodium dodecyl sulphate). Autoradiography signals were quantified using a Storm™ phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA), and the values obtained were normalised against housekeeping genes (GAPDH, 23-kDa highly basic protein, α-tubulin, β-actin, 40S ribosomal protein) as an internal control.

4.3. RNA hybridization analysis, analysis of mRNA stability and protection assay

Total cellular RNA was isolated (I-IV) using TRIzol® reagent (LifeTechnologies). RNA was separated on a 1% agarose/formaldehyde gel, and transferred to a Hybond-N nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden). The probes were labelled with [α^{32}P]-CTP using the random prime method and hybridized with 10-20 μg of total RNA. Cells from two culture dishes (5-6 x 10^6 cells) were used for the RNA extractions. Autoradiography signals were quantified using the Storm™ phosphorimager (Molecular Dynamics), and the values obtained were normalised against GAPDH. Tissue specific expression of human reticulon 4 mRNA was analysed using Multiple Tissue Northern and Human Cancer Cell Line Blot II (MTN®, Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's recommendations.

To study mRNA stability (I, II), actinomycin D (Sigma) was applied to cultures at a final concentration of 10 μM. Total RNA samples were collected and treated as in the RNA hybridization analysis described above.

In order to confirm the results obtained from the cDNA array (I), the RiboQuant™ Multi-probe RNase Protection Assay System involving human stress-related (hStress-1) and cell cycle-related genes (hCC-2) was used (PharMingen, San Diego, CA, USA). Total RNA was isolated using TRIzol® reagent (LifeTechnologies, Paisley, UK). The probes were labelled with [α^{32}P]-UTP and hybridised with 15 μg of total RNA. To remove RNA and proteins, RNase and proteinase K treatments were carried out. The protected bands were separated on 5% PAGE gel. Autoradiography signals were quantified using the Storm™ phosphorimager (Molecular Dynamics) and the values obtained were normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA from HeLa cells was used as a positive control.

4.4. Immunoblotting analysis (I)

Whole cell extracts were prepared in RIPA buffer (1 x PBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.1 mg/ml PMSF, 30 μl/ml aprotinin, 1 mM sodium orthovanadate) and centrifuged at 10 000 x and 4° C for 10 min at according to antibody supplier's recommendations. Protein concentration was determined by the Bradford method (Bradford 1976) and protein extracts were electrophoresed on 12.5% SDS-PAGE gels, then transferred onto Protran® nitrocellulose membranes (Schleicher & Schuell GmbH, Dassel, Germany) by electroblotting. Polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizing the Id1 (sc-488), Id2 (sc-489), Id3

(sc-490) and horseradish peroxidase-conjugated secondary antibodies (Zymed, South San Francisco, CA, USA) were used for the analysis. The autoradiography signal was developed with the SuperSignal® West Pico enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA).

4.5. Construction of fluorescent fusion proteins (IV)

The total coding region of human reticulon 4-C was obtained by RT-PCR from the cDNA library of HCS-2/8 cells. The PCR product amplified with forward primer 5'-ATATATATATGAATTCTATGGACGGTCAGAAGAAAAATTGG-3' and reverse primer 5'-ATATATATATGGATCCTTCAGCTTTGCGCTTCAATCCAG-3' was cloned into the C-terminus of pEGFP-C1 expression vector (excitation maximum 488 nm / emission maximum 507 nm, Clontech Laboratories, Palo Alto, CA). The former primer and a primer 5'-TATTATTATGGATCCTATTAT**ATG**TAT**GCG**CAATCCAGGGA-TTTTTGC-3' was used to obtain a construct with the double mutated ER-retention signal Lys^{-5} (195) to Arg and Lys^{-3} (197) to His (the primers have additional sequences for restriction and directed cloning, mutated codons are indicated in bold and underlined). Arginine and histidine are positively charged residues that are not able to form a functional retention signal (Jackson et al. 1990), although the total charge is maintained. This PCR product was also cloned into pEGFP-C1 vector to study subcellular localization of RTN4-C. The expected DNA sequences of the normal and mutated clones were verified with DNA sequencing.

4.6. Confocal microscopy (IV)

Transfections of HCS-2/8 cells were made using Fugene 6™ reagent (Roche, Basel, Switzerland) according to the manufacturer's recommendations. Visualization of mitochondria and endoplasmic reticulum was performed by transfecting the cells with pEYFP-Mito™ and pEYFP-ER™ vectors according to the manufacturer's recommendations (BD Living Colors™, BD Biosciences, Palo Alto, CA, USA). Micrographs were obtained approximately 24 hours after transfection using an Ultraview® confocal microscope (Perkin Elmer Life Sciences, Wallac-LSR, Oxford, UK) on a Nikon Eclipse TE300 microscope and a 100x oil immersion objective (Nikon Plan Fluor, NA 1,3). Cells were visualized in PBS at 37°C. The deconvolution was performed with a DeconWIZARD deconvolution software package (Wallac, UK). Single images obtained from the confocal scan were analysed using a linear estimation algorithm for 2D images in order to reduce out-of-focus light from images. The scale was assessed using a FocalCheck™ 6 μm fluorescent microsphere (Molecular Probes, Oregon, USA) mounted on a slide.

5. RESULTS

5.1. The effect of high hydrostatic pressure on the expression profile of HCS-2/8 cells (I)

High continuous 30 MPa pressure decreases the synthesis of ECM components and to induce a heat shock response in immortalized chondrocytes. Hence, pressure of this magnitude probably constitutes a stressful environment for chondrocytes. The expression profile of human chondrosarcoma cells was assessed using a cDNA array containing 588 well-characterized human genes under tight transcriptional control. A total of 51 genes (8.7%) were identified, many of them not previously associated with mechanical stimuli. Normalization based on the measured signals of several housekeeping genes. The genes whose mRNA expression ratio (compared with that of the housekeeping genes) was 2.0 or more at least at one time point (0, 3, 6 and 12 hours) during the follow-up were considered up-regulated, and genes whose expression ratio was 0.5 or below were considered down-regulated. For validation of the cDNA array technology, a series of four membranes was used to hybridize samples at four time points (0, 3, 6 and 12 hours) (I). After identification of candidate genes, the changes in expression levels were confirmed using alternative methods (protection assay, mRNA hybridization analysis, immunoblotting analysis). In addition, the expression profiles obtained from sequential studies (I, II and III) showed similar basic expression patterns (data not shown). Thus, expressions of cartilage-specific proteoglycans (aggrecan, biglycan and decorin) were detected. Expression of collagen type II was not detected in cDNA array, but was observed using RT-PCR (data not shown). In addition, TIMP 1-3 and MMP-3

(stromelysin 1) MMP-11, MMP-14 and MMP-17 expression was observed. This is consistent with findings of previous analyses of human chondrocyte expression patterns (Aigner et al. 2001, Aigner et al. 2003). Many of the spotted genes did not reveal a positive signal, including, for example, cytokeratins and major T cell surface antigens. Thus, these genes served as internal negative controls. The genes known to be expressed by most cells (e.g., housekeeping genes) and by chondrocytes (e.g., aggrecan) were considered as internal positive controls.

Increased expression of mRNAs representing heat shock proteins 70, 40 and 27 were observed, indicating the involvement of stress response. The most intense signal was observed six hours after the beginning of pressurization (I, Table 1, Fig. 1A). The expression of the growth arrest and DNA damage-inducible genes (GADD45 and GADD153) also increased constantly under pressure (I, Table 1, Fig. 3A). The up-regulation of GADD45 was confirmed using a ribonuclease protection assay (RPA) (I, Fig. 3A). Similarly, anti-proliferative tob (transducer of ErbB-2) and $p21^{Cip1/Waf1}$ (inhibitor of cyclin-dependent kinases) genes were up-regulated. Again, up-regulation of $p21^{Cip1/Waf1}$ was confirmed using RPA (I, Fig. 3A and 3B). In contrast, ribosomal protein S19, prothymosin alpha and p55CDC, genes associated with cell proliferation, were all down-regulated (I, Table 1). Furthermore, inhibitors of differentiation/DNA binding proteins Id1-3 were markedly down-regulated in pressurised HCS-2/8 cells. (I, Table 1, Fig. 2). The immunoblotting analysis revealed a steady decrease in the amount of Id1 and Id3 proteins under pressure, confirming the results obtained from the cDNA array analysis (I, Fig. 2). However, no change was observed in the amount of Id2 protein within 9 hours.

Differences were noticed in the behaviour of genes encoding for various transcription factors. A significant increase in expression of c-jun mRNA was observed (I, Table 1). Furthermore, GATA-2, early growth response gene (egr-1), activating transcription factor (ATF-4) and fra-1 genes were up-regulated (I, Table 1). The apoptosis-related gene NIP3 and cytoplasmic dynein light chain 1 (HDLC-1) gene expression decreased upon loading. Furthermore, genes coding for growth factors were variably affected: vascular endothelial growth factor (VEGF) and EGF-like growth factors were up-regulated at all time points, unlike connective tissue growth factor (CTGF) and heregulin alpha. In addition to the results described above, a number of other genes showing constant or transient changes in expression kinetics were observed (I, Table 1, Fig. 1).

5.2. The effects of hydrostatic pressure on mRNA stability in HCS-2/8 cells (II)

Mammalian gene expression is regulated on transcriptional, translational and post-translational levels. One type of regulation is to control the stability of the mRNA species: the longer the half-life of an mRNA molecule, the more protein molecules can be produced by translational machinery. In this study the aim was to determine whether changes in mRNA stability are a general phenomenon or confined to certain mRNA species under high pressure conditions. A cDNA array analysis containing 1200 well-characterized human genes was used to analyze RNA samples of actinomycin-treated and non-treated cells collected after continuous (30 MPa) pressurization (II). Total RNA samples were collected after 6 hours pressurization, and the relative mRNA stability of the control sample (actinomycin-treated control vs. control) and pressurized sample

(actinomycin-treated pressurized sample vs. pressurized sample) was assessed (II, Tables 1 and 2).

A number of immediate-early genes, regulating cell cycle and growth were up-regulated due to high pressure. Decreases in osteonectin, fibronectin and collagen types VI and XVI mRNAs were observed. Bikunin, cdc37 homologue and Tiam1, genes linked to hyaluronan metabolism, were also down-regulated. Only three mRNA species, tyrosine-protein kinase ABL2, MCL-1 (myeloid cell leukaemia-1 protein) and BMP-4 (bone morphogenetic protein 4), showed moderate increases in relative stability (II, Table 1). The relative stability of down-regulated mRNA species appeared to increase moderately (II, Table 2). In addition, the mRNA stability of certain immediate-early genes, such as c-jun, jun-B and c-myc, seemed to decrease under pressure treatment (II, Table 1).

The overall gene expression profile was similar to that observed in the first study. Immediate-early genes c-jun and fra-1 were up-regulated. In addition, c-myc, jun-B and fra-2, genes included in this larger scale array, were significantly up-regulated indicating the recruitment of new sets of genes after the beginning of pressurization (I, Table 1 and II, Table 1). Similarly, $p21^{Cip1/Waf1}$ and VEGF were up-regulated. The results of the array analyses are not entirely comparable, since the gene composition of the arrays was not the same.

5.3. Effects of low static and cyclic hydrostatic pressure on the expression profiles (III)

A cDNA array analysis containing 1176 characterized human genes was used to analyze RNA samples from pressure-treated and non-treated cells collected after continuous and cyclic (5 MPa) pressurization (III). Relatively few genes were found to alter their expression rates (1.3 % after cyclic pressurization, and 1.7 % after static pressurization. III, Table 2). Different set of genes were affected depending on the mode of pressure. Thus, for example, 5 MPa continuous pressure down-regulated the expression of the genes involved in intracellular signalling and cell division (mitogen-activated protein kinase 9 and proliferating cell nuclear antigen), cell adhesion (integrins) and cytoskeletal assembly (vimentin, zyxin 2) (III, Table 2). Only γ-catenin (also known as plakoglobin and desmoplakin III) was found to be up-regulated by continuous and cyclic 5 MPa pressure.

Cyclic 0.5 Hz physiological (5 MPa) pressure affected several genes which were not affected by continuous pressure (III: Table 2). For example, genes involved in cell adhesion and differentiation (L1 cell adhesion molecule, polycystin, Notch4, and polyhomeotic 2 homolog) were up-regulated, but not affected by continuous pressurization. Furthermore, cell cycle protein P38-2G4, histone 3 and E2F dimerization partner were down-regulated. These genes participate in cell division and DNA packaging, and also act as a transcription factor. In addition to the above-mentioned γ-catenin, insulin-like growth factor I receptor (IGF1R) and Notch protein homolog 4 (Notch4) were notably up-regulated during cyclic load (III: Table 2).

In this study the cells were also mechanically stretched (III, Table 1) to compare the expression profile with the profile of the pressurized cells. Various genes encoding ECM molecules, matrix turnover factors and intracellular signalling proteins or transcription factors, for example, were affected. Furthermore, the overall expression pattern was different from the one obtained from the pressurized cells (Table 3).

5.4. DDRT-PCR analysis of pressurized HCS-2/8 cells (IV)

The differential gene expression of HCS-2/8 cells under pressure was screened by the differential display PCR method (mRNA fingerprinting). This is a method parallel to the cDNA array technique, involving the use of a set of arbitrary primers that hybridize with the cDNA pool made of the mRNA of cells under study (Fig. 5). Using this technique, reticulon 4-B, a member of the reticulon gene family, was found to be up-regulated under high 30 MPa static hydrostatic pressure. The up-regulation was confirmed with RNA hybridization analysis, and was found to take place also in primary bovine chondrocytes. However, in primary chondrocytes the induction of gene activity took place earlier than in HCS-2/8 cells (IV, Fig. 3A and 3B). Two other forms of the RTN4 subfamily, RTN4-A and RTN4-C, were also expressed by both cell types (IV, Fig 2), but their expression did not change during pressure treatment (unpublished data). For comparison, HCS-2/8 cells were cyclically stretched, but no induction of RTN4-B was evident on the mRNA level (IV, Fig 3C). In addition, the effect of several potentially stressful factors on the expression kinetics of RTN4-B in HCS-2/8 cells was tested. However, heat shock

treatment, UV-B irradiation or the addition of heavy metal to the growth medium had no effect on the activity of the RTN4-B gene (IV, Fig 4A-C).

All forms of RTN4 (A-C) have a so-called reticulon homology domain (RHD), consisting of two transmembrane domains, and a potential di-lysine ER-retention motif (KxKxx-COOH). The sequence structure of RHD is identical between RTN4-A, RTN4-B and RTN4-C (IV, Fig 1). The N-termini have variable sequences and lengths. In this study, the intracellular distribution of RTN4-C, the shortest member of this subfamily, was elucidated. This isoform contains the C-terminus with the putative ER-retention motif. The full-length coding sequence cloned into a commercial vector containing fluorescent protein was transfected into HCS-2/8 cells. A reticular distribution pattern, typical of ER-associated reticulons, was visualized by a confocal microscope (IV, Fig 6A and 6D). The reticular pattern was enhanced by convolution using a linear estimation algorithm, and was found to be similar to that revealed by a commercial ER specific fluorescent construct (IV, Fig 6D and I). The significance of the putative ER-retention signal was studied using an expression vector with a fluorescent-tagged RTN4-C coding sequence and a mutated ER-retention signal (IV, Fig 1). The intracellular distribution pattern was similar to that formed by the native RTN4-C coding sequence (IV, Fig 6B, 6E and 6I). The fluorescent construct without insert produced an even distribution signal in the cytoplasm and nucleus (IV, Fig 6G).

Tissue distribution and mRNA expression of the reticulon 4 subtypes in several malignant cell lines were also studied. The original DDRT-PCR clone was used as a probe. It is derived from the 3' end of the coding region (the common RHD domain) and part of the 3' UTR. All 12 human tissues included in the assay expressed a 2.6 kb

transcription product presenting RTN4-B (IV, Fig 5A). Similarly, all human cancer cell lines expressed a common ~ 2.6 kb product. Liver, kidney, skeletal muscle and brain tissue showed a hybridization signal for ~ 1.7 kb product presenting RTN4-C. Brain tissue also revealed a ~ 4.6 kb message probably presenting RTN4-A (IV, Fig 5A). However, other isoforms (RTN4A or C) were not detectable in most of the malignant cell lines. In promyelocytic leukaemia cells, the ~ 1.7 kb product was evident, and in chronic myeloid leukaemia cells a ~ 4.3. kb unidentified expression product was found (IV, Fig 5B).

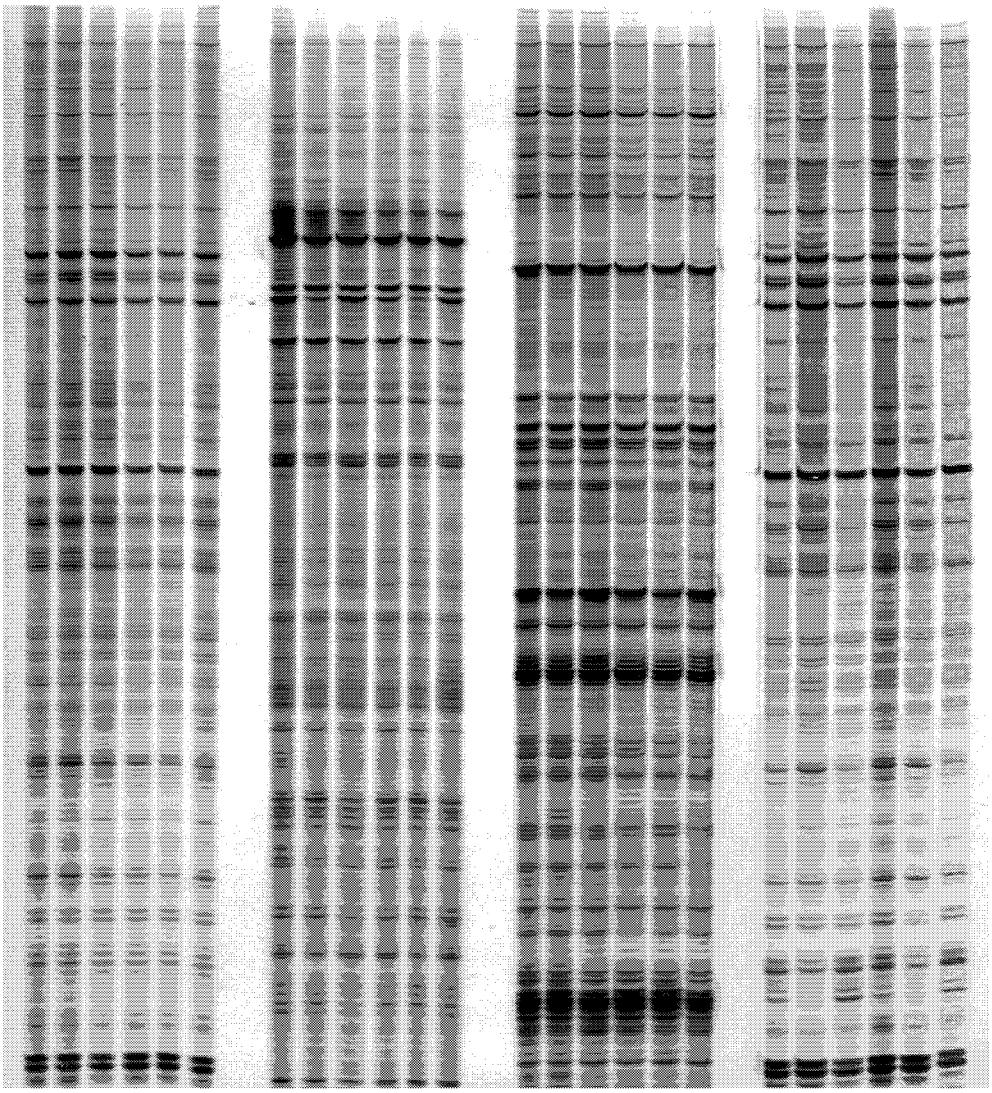

Figure 5. Example of an autoradiography of radioactively labelled differential display PCR products separated on PAGE gel. Lanes are in six-lane sets, each set corresponding to a particular arbitrary primer combination with degenerate anchored primers (5'-T_{12}G-3', 5'-T_{12}C-3', 5'-T_{12}A-3'). Control samples are shown in the first two lanes of each set and the rest are samples from pressurized cells. PCR control reactions not shown.

6. DISCUSSION

6.1. High pressure effects on the expression profile of HCS-2/8 cells (I, II)

The human chondrosarcoma cell line HCS-2/8 was used in studies I-IV as the model of chondrocytes. These cells are well characterized and they have been used to study the effects of hydrostatic pressure on cellular functions (Takigawa et al. 1989, Takahashi et al. 1997, Takahashi et al. 1998). They produce cartilage-specific proteoglycans and type II collagen. They have also been used in several other studies concerning cellular metabolism of chondrocytes (Tsuji et al. 1996, Ishida et al. 1997, Inoue et al. 1998, Liu et al. 1998, Nakanishi et al. 2000, Eguchi et al. 2001, Yosimichi et al. 2001, Nishida et al. 2002,Yosimichi et al. 2002, Hattori et al. 2003 , Kubota et al. 2003). The differential display and cDNA array techniques allow the systematic comparison of mRNAs expressed in various biological processes or as a response to experimental treatments.

The results obtained in studies I and II suggest that gene expression is focused on the adaptation to the new environmental stress under 30 MPa static pressure. A transient activation of heat shock response in the HCS-2/8 cell line was observed, which is in accordance with previously published data obtained with HCS-2/8 cells (Takahashi et al. 1997) and another chondrocytic cell line T/C28a4 (Kaarniranta et al. 1998). In addition, Hsp27 and Hsp40 are moderately induced during this period (I, table 1, Fig. 1). The amount of Hsp70 mRNA returned to almost the same as those under atmospheric pressure after 6-12 hours from the beginning of the pressurization. This is also consistent with the morphological changes of the cells under high pressure. During the first 0-12 hours under pressure, cells were predominantly rounded and more retracted compared

with control cells when visualized using phase contrast microscopy (data not shown). The cytoskeleton is probably rearranged during the first hours, and after approximately 12 hours the original flattened polygonal shape was achieved (data not shown). Such a change in cell shape under high static pressure has also been documented using primary bovine chondrocytes (Parkkinen et al. 1993).

In addition to heat shock response, high static pressure activated a set of genes that are involved in growth arrest (I, II). Up-regulation of genes coding for growth arrest and DNA-damage inducible proteins (GADD45 and GADD153) was observed. Activation of these genes is also associated with other stress signals such as hypoxia, irradiation, genotoxic drugs and withdrawal of growth factors (Sanchez et al. 1995). The anti-proliferative gene $p21^{Cip1/Waf1}$, a cyclin-dependent kinase inhibitor, and tob, a member of the Btg family of anti-proliferative genes, were also up-regulated. A set of genes required in cell proliferation (ribosomal protein S19, prothymosin alpha, p55CDC) were, in turn, down-regulated under high pressure treatment. Thus, the introduction of high pressure seems to be connected with activation of genes involved in anti-proliferative processes. The actual proliferation rate was also found to be low under high pressure during 72 hours follow-up (measured by hemocytometer count, data not shown).

Adaptation to a new environmental situation involves the recruitment of new genes. This is preceded by activation of immediate-early genes, i.e. transcription factors or transcriptional inhibitors, which turn other genes on or off. Introduction of high pressure up-regulated various genes encoding transcription factors (I, Table 1 and II, Table 1.), indicating the beginning of some adaptative process. Interestingly, inhibitors of differentiation/DNA binding proteins (Id1-3) were markedly down-regulated in

pressurized HCS-2/8 cells (I, Fig. 2). Id family members act as dominant negative regulators of basic helix-loop-helix proteins, and are important for the maintenance of a non-differentiated phenotype, as well as for vigorous cell division by inhibiting the binding of phenotype-specific transcription factors (Norton 2000). Thus, pressure may affect the differentiation of HCS-2/8 cells. However, the aspect of differentiation needs further characterization of the expressional behaviour of phenotype-specific genes under pressure.

The Human Cancer 1.2 cDNA array, used in this study, has also been utilized to determine the regulated gene expression in normal and osteoarthritic (OA) human articular cartilage (Aigner et al. 2001, Aigner et al. 2003). Interestingly, among the significantly regulated genes in OA cartilage are genes which are also regulated by high 30 MPa static pressure. Thus, GADD45 and anti-proliferative protein PC3 are up-regulated by pressure, but they are down-regulated in chondrocytes from OA patients. The under-expression of GADD45 is associated with the early phase of degeneration. In addition, cell cycle regulator $p21^{Cip1/Waf1}$, up-regulated by high pressure, is expressed in normal and early-phase OA, but not in late stage disease. Fibronectin, a component of the ECM, is expressed during early-stage OA, but expression was down-regulated by the pressure treatment. Production of the transcriptional regulators c-jun and c-myc is induced in early phase OA and by high pressure. Furthermore, DNA repair protein HHR23A is down-regulated both by pressure and OA (Aigner et al. 2001, Aigner et al. 2003). These findings suggest, that the physical environment of the chondrocytic cells affect the same genes involved in a degenerative process of OA. However, the changes in

the transcriptional activity of pressurized cells are mainly different from the ones observed in OA.

6.2. Effects of high pressure on mRNA stability assessed by cDNA array (II)

The effect of hydrostatic pressure on mRNA stability has been shown in the regulation of heat shock protein 70 gene expression. Thus, pressure treatment elevated the amount of heat shock protein 70 mRNA and protein without transcriptional induction of hsp70 gene in primary bovine chondrocytes. In contrast, increased expression of Hsp70 protein was mediated solely through the stabilization of hsp70 mRNA molecules. This kind of induction was a result of continuous 30 MPa pressure, but not of cyclic pressure (Kaarniranta et al. 1998). In study II, the actinomycin based mRNA stability analysis was applied to the cDNA array technique to screen possible changes of messenger half-lives on a large scale in HCS-2/8 cells under pressure.

High static pressure induced the expression of several genes, and only three of them, tyrosine-protein kinase ABL2, myeloid cell leukaemia-1 protein and bone morphogenetic protein 4, showed increases in relative stability compared with other genes in the pressurized sample. Most of the up-regulated genes showed, however, no significant changes or decreased mRNA stability. Thus, an increase in messenger stability seems to be restricted to certain up-regulated mRNA species, and seems not to be a general form of post-transcriptional regulation under pressure. Interestingly, many of the immediate-early genes (c-jun, c-myc, jun-B) seemed to be destabilized due to high pressure. This is probably involved in the cellular adaptation process, where rapid turn-over of key regulatory proteins is needed.

Surprisingly, the majority of genes down-regulated under pressure treatment showed a consistent increase in mRNA stability compared with control cells. This may be a compensatory mechanism to preserve the expression of transcriptionally down-regulated genes. However, this needs further investigation and the use of alternative techniques to confirm this finding.

6.3. Effect of loading patterns and magnitude on gene expression (I, II, III)

The mode, duration and magnitude of pressure can influence on the cellular response and synthetic capacity of ECM molecules (Table 1). In studies I and II, HCS-2/8 cells were pressurized statically for 3-12 hours using high static pressure (30 MPa). In study III, the cells were pressurized statically and cyclically for 6 hours using 5 MPa pressure. In addition, the HCS-2/8 cells were mechanically stretched to compare the expression profiles between different physical stimuli. The results are partly summarized in Table 3, which shows some of the affected molecules from different functionally distinctive molecular groups.

Low static and cyclic pressure influenced a different set of genes than did high static pressure. Only a few genes were found to increase expression due to 5 MPa pressure irrespective of the mode. The only gene induced by both pressure modes, γ-catenin, acts as a transcriptional regulator in the Wnt signalling pathway and is a major constituent of membrane cell-cell adhesion sites with β-catenin (Ueda et al. 2001, Shtutman et al. 2002). Interestingly, it was down-regulated by mechanical stretching. β-catenin and its homologue γ-catenin, play an important role in cadherin-based adherens junctions by linking cadherin receptors to the actin cytoskeleton. The previous study has shown

activation of α5β1-integrin/β-catenin signaling pathway in human articular chondrocytes during pressure-induced strain (Lee et al., 2000). Promyelocytic leukemia gene (PML) expression has been reported to be activated due to the overexpression of β-catenin or γ-catenin. PML is a nuclear protein localized in characteristic dots, known as nuclear bodies, or PML domains that contain a number of proteins involved in the regulation of transcription, apoptosis, and cell cycle progression (Shtutman et al. 2002). γ-catenin was also up-regulated by cyclic 5 MPa pressure (Table 3). The implication of γ-catenin in pressure response needs further characterization.

Both static and cyclic pressure caused down-regulation of the expression of several genes involved in intracellular signalling, cell adhesion and cytoskeletal assembly. Interestingly, the behaviour of some genes was reversed depending on the mode of pressure. Thus, STAT inhibitor 3, insulin-like growth factor binding protein 5 (IGFBP-5), insulin-like growth factor I receptor (IGF1R), interferon-inducible protein 9-27 and fas-activated serine/threonine kinase were down-regulated by low cyclic pressure, but up-regulated by low static pressure. STAT inhibitor 3 plays a role in negative feedback control of the JAK-STAT signalling pathway. The signal transducer and activator of transcription (STAT) is phosporylated by JAK (Janus kinase) after cytokine activation. STAT influences specific gene expression, cell proliferation, differentiation and apoptosis (Minamoto et al. 1997). The IGF1R on the plasma membrane has tyrosine kinase activity and is activated by insulin-like growth factors I and II and is coupled to several common intracellular second messenger pathways (Butler et al. 2001). Fas-activated serine/threonine kinase is a component of a molecular cascade involved in Fas-mediated apoptosis (Tian et al. 1995).

Furthermore, some genes were affected only by cyclic pressure or by static pressure. Thus, for example, Notch protein homolog 4 was up-regulated by cyclic pressure, but not affected by static pressure. Vascular endothelial growth factor receptor 1, zyxin 2 and Notch were down-regulated by static pressure, but not affected by cyclic pressure. Notch4 belongs to the Notch protein family of transmembrane receptors. Activation of Notch protein leads to the release of an intracellular domain, which translocates into the nucleus and interacts with a DNA binding factor, RBP-J. This complex acts as a transcriptional repressor of other target genes (MacKenzie et al. 2003). This indicates the recruitment of specific sets of genes depending on the mode and magnitude of pressure (I, II, III).

Yet another kind of genetic response was observed when the HCS-2/8 cells were mechanically stretched (III, Table 1, Table 3). Thus, for example, proliferating nuclear antigen (PCNA) was up-regulated by mechanical stretching, but down-regulated by 5 MPa pressure treatments. Cyclin D1, associated with cell cycle, was also up-regulated. Furthermore, stretching activated another set of immediate-early genes than those activated by the pressure treatments, and the expression of stromelysin 1 (MMP-3), the major metalloproteinase in cartilage, was induced. Interestingly, the expressions of γ-catenin and N-CAML1, involved in cell adhesion, were down-regulated by stretching and up-regulated by physiological levels (5 MPa) of static or cyclic pressure (Table 3). The hypoxia-inducible factor 1, up-regulated by stretching, has been shown to be expressed by differentiated human fetal chondrocytes (Stokes et al. 2002) and its expression is also increased in early- and late-stage OA (Aigner et al. 2001, Aigner et al. 2003). Thus, the

Table 3. A summary of the cDNA array results showing some of the observed changes in gene expression under the different mechanical conditions (I-III). The indicated molecules are from various functional groups showing the differential effects of mechanical stimuli (↓= inhibition, ↑= stimulation, - = no effect, ↓↑ in OA = previously associated with osteoarthritis).

	Biomechanical factor			
Functional group	30 MPa static pressure	5 MPa static pressure	5 MPa cyclic pressure (0.5 Hz)	Mechanical stretching (0.5 Hz)
Stress response	Hsp 70 ↑	-	-	-
	Hsp 40 ↑	-	-	-
	Hsp 27 ↑	-	-	-
Control of cell cycle	GADD45 ↑ (↓ in OA)	-	-	-
	GADD153 ↑	-	-	-
	$p21^{waf1}$ ↑	-	-	-
	-	PCNA ↓	PCNA ↓	PCNA ↑
	-	-	-	Cyclin D1 ↑
	Anti-proliferative protein PC3 ↑ (↓ in OA)	-	-	-
Transcriptional control	c-jun ↑	-	-	-
	c-myc ↑	-	-	-
	fra-1 ↑	-	-	-
	TXBP151 ↓	-	-	TXBP151 ↑
	-	E2F ↓	-	-
	GATA-2 ↑	-	-	-
	-	-	-	Elf 1 ↑
	-	-	-	HIF-1α ↑ (↑ in OA)
	-	-	-	HMG17 ↑
ECM components	Fibronectin ↓ (↑ in OA)	-	-	-
	Collagen α 1(VI) ↓	-	-	-
	Collagen α 1(XVI) ↓	-	-	-
	Collagen α 3 (VI) ↓	-	-	Collagen α 3 (VI) ↑
ECM metabolism	TIMP-2 ↓	-	-	-
	-	-	-	MMP-3 ↑ (↑ in OA)
Growth factors and their receptors	VEGF ↑	-	-	-
	CTGF ↓	-	-	-
	EGF-like GF ↑	-	-	-
	-	-	-	PDGF-B ↑
	-	-	-	Pleiotrophin ↑
	-	IGFBP-5 ↑	IGFBP-5 ↓	-
	-	IGF1R ↑	IGF1R ↓	IGF1R ↓
	TGF-α ↓	-	-	TGF-α ↓
Cellular adhesion	Integrin α_6 ↓	-	-	Integrin α_6 ↑
	-	Integrin α_E ↓	Integrin α_E ↓	-
	-	Integrin β_8 ↓	Integrin β_8 ↓	-
	-	γ-catenin ↑	γ-catenin ↑	γ-catenin ↓
	-	N-CAML1 ↑	-	N-CAML1 ↓
	-	Polycystin ↑	-	-
Differentiation	Id1-3 ↓	-	-	-
	-	-	Notch ↓	-
	-	-	-	RAR- α ↓
	-	-	-	Wnt8B ↓
	-	-	-	BMP4-R ↑
Cytoskeletal components	-	-	Zyxin 2 ↓	-
	-	Vimentin ↓	Vimentin ↓	-
	HDLC-1 ↓	-	-	-
DNA repair	HHR23A ↓ (↓ in OA)	-	-	-
	HHR23 B ↓	-	-	-
	-	Rad6 ↓	Rad6 ↓	-

biomechanical response on the genetic level varies depending on the nature of the overall load. This kind of screening of differential expression enables the discovery and further characterization of genes which have not previously been associated with biomechanical factors.

6.4. The barosensitivity and intracellular localization of Reticulon 4-B (IV)

In addition to cDNA array analysis, differential display RT-PCR was the method used to screen differential expression in HCS-2/8 cells under 30 MPa hydrostatic pressure. This method is based on random hybridization of arbitrary primers in cDNA molecules in PCR reactions. The labelled and amplified DNA molecules are separated on a polyacrylamide gel and the products are compared side by side after autoradiography. The differential display technique can be used to find novel previously unsuspected genes. It is also useful when interest is focused on expression patterns of organisms whose genome is not well sequenced.

Differential display analysis produced several differentially displayed bands, from which some with the most prominent difference in intensity were selected for further characterization. However, there was a considerable amount of heterogeneity in the PCR products, and in some cases a single strand conformation polymorphism analysis was needed to distinguish between PCR products having the same length but different sequences (data not shown). The RNA hybridization analyses were subsequently performed, and cloned PCR products were used as hybridization probes to confirm differential expression. However, in most cases differential expression detected in differential display gels could not be confirmed by RNA hybridization analysis. Thus,

DDRT-PCR is a relatively laborious method because of the high number of false positive bands and / or the heterogeneity of PCR products.

One of the differential display products showed a significant up-regulation under high pressure conditions assessed by RNA hybridization analysis. After cloning and sequencing, it was identified as reticulon 4-B (IV). It is a member of the reticulon (RTN) gene family, which is currently known to consist of four members (RTN1-4) that have, excluding RTN3, two or three splice variants. All RTN proteins have 70 % homology in a 200 residue region at the C-terminus (reticulon-homology domain). The RTN4 subfamily has three isoforms: RTN4-A, RTN4-B and RTN4-C. These contain the di-lysine ER-retention motif (KxKxx-COOH) at the C-terminus (GrandPre et al. 2000). The functions of these proteins are not currently known. Study IV showed for the first time the expression of members of the RTN4 family in cartilaginous cells, and high static pressure was shown to up-regulate the RTN4-B isoform expression during 12-24 hour pressurization in HCS-2/8 cells (IV). Similar induction of gene activity was found to take place in primary bovine chondrocytes under high pressure (IV). The significance of such activation needs further investigation, since the function of RTN4-B is unknown. RTN proteins are localized in endoplasmic membranes but the intracellular localization of RTN4-C was not altered by high pressure (IV). Cyclic stretching had no effect on the amount of RTN4-B mRNA. Thus, the RTN4-B gene may have a mechanosensitive response element which is activated by pressure, but is not responsive to stretching. In addition, other potentially stressful factors (heat shock, UV irradiation and heavy metal treatment) did not affect the expression of RTN4-B, indicating some degree of pressure sensitivity. Thus, RTN4-B seems not to be involved in the cellular stress response.

Since the intracellular location of RTN proteins in chondrocytic cells have not been previously studied, a human RTN4-C fluorescent fusion protein was constructed for microscopic study. RTN4-C, the shortest member of the RTN4 family, contains the putative ER-retention motif and is known to be localized reticularly in COS-7 monkey kidney cells, indicating a close association with ER (GrandPre et al. 2000). A similar reticular pattern was also found in HCS-2/8 cells after transfection of the fusion construct (IV, Fig. 6). This is in line with findings of previous studies concerning the intracellular localization of RTN4A-C (Morris et al. 1999, Oertle et al. 2003, Oertle et al. 2003). The putative ER-retention motif has been suggested to be the localization signal in members of RTN4 family according to database sequence analyses. A recent study indicates that a second hydrophobic region of RTN4-B (IV, Fig. 1) is responsible for ER association (Oertle et al. 2003). However, there are no studies showing the significance of the ER-retention signal in the intracellular localization. In study IV, this motif was mutated so that the original charges of the amino acid residues were unchanged despite the mutation of the critical di-lysine motif. Interestingly, despite the mutation, no alterations were observed in the intracellular localization (IV). This kind of mutation has previously been shown to abolish the guiding function of the motif in other ER-associated proteins (Jackson et al. 1990). Thus, there is another, unidentified motif in RTN4-C and presumably also in RTN4-A and RTN4-B, which is responsible for intracellular guidance.

7. SUMMARY AND CONCLUSIONS

These experiments were undertaken to study the effects of hydrostatic pressure on the gene expression profile of human chondrocytic cells *in vitro*. Pressure is one of the main components of the overall load on human articular cartilage, especially in the weight-bearing joints. A previously well-characterized human chondrosarcoma cell line (HCS-2/8) was used as a chondrocytic cell model. The cells under study were subjected to high static, low static and cyclic pressure, and to cyclic mechanical strain. The main analysis methods for screening of pressure-sensitive genes were cDNA array and differential display RT-PCR techniques.

This study demonstrated that:

1. The gene expression of chondrocytic cells was selectively affected by high static (30 MPa) hydrostatic pressure. Altogether 51 genes out of the 588 genes under study showed marked alterations in gene activity. The transcriptional activation of the genes involved in the immediate-early response (transcription factors c-jun, c-myc and GATA-2), stress response (heat shock proteins), control of cell cycle (GADD-proteins, $p21^{Cip1/Waf1}$, PC3), and inactivation of the genes involved in inhibition of differentiation (Id1-3), DNA repair (HHR23A and HHR23B) and matrix formation (fibronectin) was observed. These changes in the transcriptional activity of pressurized cells are mainly different from the ones previously observed in osteoarthritic cartilage. Analysis of the gene expression

patterns also revealed many genes that have not yet been reported to be expressed by chondrocytic cells.

2. Physiological levels of hydrostatic pressure (5 MPa) which take place during walking and standing, influenced a different set of genes than did high pressure. Surprisingly, only a few genes were found to change their transcriptional activity due to this level of loading irrespective of the mode (static and cyclic). Transcriptional activations of genes involved in cell adhesion (γ-catenin, N-CAML1, polycystin) and down-regulation of vimentin (cytoskeletal protein) and integrins α_E and β_8 (cell adhesion) were observed to be due to cyclic and static 5 MPa pressure. In contrast, insulin-like growth factor binding protein 5 (IGFBP-5), insulin-like growth factor I receptor (IGF1R), signaling protein STAT inhibitor 3 and zyxin2 were differentially regulated depending on the mode of pressure. The mechanical stretching activated another set of immediate-early genes than did the pressure treatments, and the expression of stromelysin 1 (MMP-3), the major metalloproteinase in cartilage, was induced. In contrast, the expression of γ-catenin and N-CAML1 was down-regulated.

3. The stability of mRNA molecules was selectively regulated by hydrostatic pressure: three mRNA species, tyrosine-protein kinase ABL2, myeloid cell leukaemia-1 protein (MCL-1) and bone morphogenetic protein 4 (BMP-4), showed moderate increases in relative stability. The previously observed intense increase in the stability of Hsp70 mRNA under high pressure seems not to be the general form of regulation of the gene expression in chondrocytic cells.

4. The expression of reticulon 4-B (RTN4-B) in HCS-2/8 cells is regulated by high hydrostatic pressure. This is the first report describing the expression and intracellular distribution of reticulon 4 proteins in chondrocytic cells. Reticulons 4-A, 4-B and 4-C are endoplasmic reticulum-associated proteins and members of the reticulon gene family. Their molecular functions are currently unknown. The RTN4-B gene was shown to be activated by 30 MPa static pressure, but not by other kinds of stressful factors. RTN4-C, the shortest isoform, was shown to be associated with endoplasmic reticulum. This association was unaltered by high hydrostatic pressure. These results demonstrate the existence of a novel mechanosensitive gene in cells of cartilaginous origin.

In conclusion, the results offered in this study demonstrate the profound change in the pattern of gene expression that can occur when chondrocytic cells are subjected to mechanical loading. The effects of a biomechanical environment on the overall gene expression in articular cartilage are currently poorly understood. The cDNA technology offers a powerful tool for investigating the functions of a large number of different genes simultaneously, and it allows the analysis of entire biologic systems instead of single aspects of them. Such an approach reveals novel functions of genes, which have not previously been associated with chondrocytes.

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