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cDNA array reveals mechanosensitive genes in chondrocytic cells under hydrostatic pressure

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Abstract

Hydrostatic pressure (HP) has a profound effect on cartilage metabolism in normal and pathological conditions, especially in weight-bearing areas of the skeletal system. As an important component of overall load, HP has been shown to affect the synthetic capacity and well-being of chondrocytes, depending on the mode, duration and magnitude of pressure. In this study we examined the effect of continuous HP on the gene expression profile of a chondrocytic cell line (HCS-2/8) using a cDNA array containing 588 well-characterized human genes under tight transcriptional control. A total of 51 affected genes were identified, many of them not previously associated with mechanical stimuli. Among the significantly up-regulated genes were immediate-early genes, and genes involved in heat-shock response (hsp70, hsp40, hsp27), and in growth arrest (GADD45, GADD153, $p21^{Cip1/Waf1}$, tob). Markedly down-regulated genes included members of the Id family genes (dominant negative regulators of basic helix-loop-helix transcription factors), and cytoplasmic dynein light chain and apoptosis-related gene NIP3. These alterations in the expression profile induce a transient heat-shock gene response and activation of genes involved in growth arrest and cellular adaptation and/or differentiation.

Keywords: Chondrocytic cell; Hydrostatic pressure; Expression analysis; mRNA stabilization

1. Introduction

Chondrocytes derived from mesenchymal cells are involved in matrix production of various cartilaginous tissues. These cells are constantly influenced by mechanical stress when forces are transmitted across joints [1]. Mechanical force transmitted upon the cartilage tissue and chondrocytes acts in several ways such as by cellular deformation, hydrostatic pressure (HP), streaming potentials and change in tissue osmolality [2]. HP is one of the forces that changes momentarily, e.g., during locomotion. Chondrocytes respond to HP changes by altering their synthetic capacity depending on the type, timing and mode of pressure [3–8]. For example, the expression of aggrecan increases during relatively low, intermittent pressure and decreases under more strenuous and static pressure [4,9]. Type II collagen and aggrecan mRNA expression can have different expression patterns depending on the loading profile, suggesting that specific mechanical loading protocols may be required to optimally promote synthesis and repair in, for example, injured cartilage [10]. Recently, it was reported that reduced pO_2 and different settings of intermittent HP increased the proliferation, collagen secretion and phenotype stability of chondrocytes [11].

The mechanisms how chondrocytes respond to mechanical factors are still poorly understood. Increases in cyclic AMP and hyperpolarization of chondrocyte plasma membrane occur under intermittent HP, due to opening of small conductance Ca^{2+}-dependent K^+ channel [12]. This hyperpolarization response was dependent on α5β1 integrins [13], leading to release of interleukin 4 [14]. Intra- and intercellular calcium signaling has been shown in mechanically stimulated chondrocytes [15–17], and calcium signaling was required for ultrasound stimulation of proteoglycan synthesis [18]. Myo-inositol 1,4,5,-triphosphate and Ca^{2+}/calmodulin system were shown to mediate increased aggrecan mRNA levels in compressed chondrocytes [19]. Nitric oxide also appears to

Abbreviations: HP, hydrostatic pressure; GADD, growth arrest and DNA damage-inducible gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCS-2/8, human chondrosarcoma cell line; Hsp, heat-shock protein

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participate in regulation of mechanotransduction, controlling, for instance, proteoglycan synthesis in dynamically compressed chondrocytes enbedded in agarose [20]. Fluid flow-induced mechanotransduction has been shown to involve an activation of mitogen-activated protein kinases [21].

Continuous HP at physiological levels generally stimulates proteoglycan synthesis in chondrocytes, while higher pressures are inhibitory [9]. High continuous HP results in specific cellular responses, too. Continuous 30-MPa pressure induces a specific stress response by increasing cellular hsp70 mRNA and protein levels in chondrocytic cells [22,23]. The accumulation of hsp70 mRNA occurs due to stabilization of hsp70 mRNA, without induction of the gene itself [22], and protein synthesis is required for the stabilization [24]. Surprisingly, bovine primary chondrocytes were resistant to pressure-induced expression of hsp70, while synovial cells from the same animal and human skin fibroblasts were pressure-sensitive accumulating hsp70 mRNA and protein [25]. However, the synthetic activity, measured as proteoglycan synthesis, returned to control level in primary chondrocytes within 48 h after release of the pressure [26]. High pressure also increased mRNA levels of interleukin 6 and tumour necrosis factor α [27]. Interleukin 6 may be connected with the increased synthesis of Hsp90 observed in human chondrocytic T/C28a4 cell line [28], since interleukin 6 is known to activate hsp90β gene [29].

2. Materials and methods

2.1. Cell culture and exposure to HP

HCS-2/8 human chondrosarcoma cells [30] and T/C28a4 human immortalized chondrocytes [31] 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 monolayers [30] was confirmed in our Northern blotting and RT-PCR analyses, too (data not shown). Cells were grown to a subconfluent state before the experiments. Before exposure to HP, the medium was changed and 15 mM Hepes (pH 7.3) was added. To expose the cells to pressure, the culture dishes were filled with the medium described above and sealed with a plastic membrane. 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 [4]. Cells were exposed to continuous 30 MPa HP for 3, 6 and 12 h. Cells from two culture dishes ($5-6 \times 10^6$ cells) were used for the RNA extractions, and cells from a single dish ($2.5-3 \times 10^6$ cells) for the protein extractions.

2.2. Expression profiling with a cDNA array

The Atlas™ human cDNA expression array kit was purchased from Clontech Laboratories (Palo Alto, CA). All procedures for labeling and purifying the probes were accomplished by following the manufacturer's recommendations. [α^{32}P]-dCTP-labeled cDNA probes were generated by reverse transcription of mRNA from untreated and pressurized monolayer cultures. The probes were purified by column chromatography (ChromaSpin™, Clontech Laboratories) and met or exceeded the manufacturer's recommendation for specific activity. The membranes were hybridized in ExpressHyb™ solution (Clon tech Laboratories) overnight at 68 °C, then washed twice (20 min with 0.1× SSC/0.5% sodium dodecyl sulfate (SDS)). Autoradiography signals were quantified using a Storm™ phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA), and the values obtained were normalized against the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 23-kDa highly basic protein, α-tubulin, β-actin, human leukocyte antigen C4, and 40S ribosomal protein spotted on the membranes.

2.3. Ribonuclease protection assay

In order to confirm the results obtained from the cDNA array, 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 cellular RNA was isolated using TRIzol® reagent (Life Technologies, Paisley, UK). The probes were labeled with [α^{32}P]-UTP and hybridized 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 normalized against GAPDH. Total RNA from HeLa cells was used as positive control.

2.4. Northern blot analysis

Total cellular RNA was isolated using TRIzol® reagent (Life Technologies). RNA was separated on a 1% agarose/formaldehyde gel, and transferred to Hybond-N nylon membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden). The probe was labeled with [α^{32}P]-CTP using the random prime method and hybridized with 20 μg of total RNA. The probe specific for GADD45α was made by RT-PCR using RNA from the HCS-2/8 cell line with the following primers: 5′ -GAC GAC GAC AGA GAT GTG G-3′ and 5′ -GTT TTC CTT CCT GCA TGG TTC-3′. Autoradiography signals were quantified using the Storm™ phosphorimager (Molecular Dynamics) and the values obtained were normalized against GAPDH [32].

2.5. Western blot analysis

Whole-cell extracts were prepared in RIPA buffer (1× 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 × *g* for 10 min at 4 °C. For the determination of protein concentration, the Bradford method [33] was used and protein extracts (30 μg per lane) were electrophoresed on 12.5% SDS-PAGE gels and transferred onto Protran® nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) by electroblotting. Even loading was confirmed by staining the membranes with Ponceau-S. Polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizing the Id-1 (sc-488), Id-2 (sc-489), Id-3 (sc-490) and horseradish peroxidase-conjugated secondary antibodies (Zymed, South

Table 1
Genes detected on cDNA array whose expression are change due to the 30 MPa static hydrostatic pressure treatment at indicated time points

Gene	Fold of change			Function	GB access
	3 h	6 h	12 h		
Heat shock protein 40	1.8	3.1	0.4	stress response	D49547
Heat shock 27-kDa protein 1	0.9	2.9	1.0	stress response	X54079
Heat shock 70-kDa protein 1	4.5	8.6	4.0	stress response	M11717
Growth arrest and DNA-damage-inducible protein GADD45	2.0	5.7	7.4	stress response/cell cycle control	M60974
Growth arrest and DNA-damage-inducible protein GADD153	1.8	5.0	4.6	stress response/cell cycle control	S40706
tob	1.1	2.0	1.0	cell cycle control	D38305
Cyclin-dependent kinase inhibitor 1A (p21; Cip1; WAF)	1.0	3.2	2.9	cell cycle control	U09579
40S ribosomal protein S19	0.6	1.0	0.8	cell cycle control	M81757
Prothymosin alpha	0.5	1.3	0.9	cell cycle control	M26708
p55CDC (cell division control protein 20)	0.3	1.1	0.0	cell cycle control	U05340
c-jun proto-oncogene	9.9	9.8	6.6	transcription factor	J04111
Endothelial transcription factor GATA-2	4.1	8.9	4.3	transcription factor	M68891
Early growth response protein 1/transcription factor ETR103	1.7	2.7	1.1	transcription factor	M62829
cAMP-dependent transcription factor ATF-4 (TAXREB67)	1.5	2.6	1.8	transcription factor	D90209
fra-1 (fos-related antigen 1)	0.7	1.8	2.6	transcription factor	X16707
Homeobox C1 protein	0.7	1.1	0.4	transcription factor	M16937
Transcription factor AP-2	0.2	0.7	0.7	transcription factor	M36711
DNA-binding protein Id-1	0.6	0.7	0.1	proliferation/differentiation	D13889
DNA-binding protein inhibitor Id-2	1.3	0.9	0.4	proliferation/differentation	M97796
DNA-binding protein inhibitor Id-3	0.8	0.7	0.1	proliferation/differentiation	X69111
NIP3	1.1	0.4	0.2	apoptosis	U15174
Cytoplasmic dynein light chain 1 (HDLC1)	0.4	0.4	0.5	apoptosis/intracellular transport	U32944
Heparin binding EGF-like growth factor	>10	>10	>10	growth factor	M60278
Vascular endothelial growth factor, VEGF	2.5	4.9	1.5	growth factor	M32977
Connective tissue growth factor	0.5	1.4	0.2	growth factor	M92934
Heregulin alpha	0.3	1.1	0.9	growth factor	U02326
EB1 (protein that binds to APC)	0.4	1.1	0.6	microtubular dynamics	U24166
ATP-dependent DNA helicase II (thyroid antigen 70 kDa)	1.1	0.2	0.1	DNA repair	M32865
UV excision repair protein RAD23 (p58/HHR23B)	0.7	0.7	0.5	DNA repair	D21090
Superoxide dismutase	0.6	1.1	0.5	DNA repair/antioxidation	K00065
UV excision repair protein RAD23 (HHR23A)	0.5	0.9	0.7	DNA repair	D21235
DNA-repair protein XRCC1	0.4	0.5	0.5	DNA repair	M36089
Integrin alpha-6	0.7	1.4	0.1	cell adhesion	X53586
Alpha-catenin	0.5	1.1	1.2	cell adhesion	D13866
Cation-independent M-6-P receptor (IGFR-2)	1.4	2.7	1.8	signaling	Y00285
Basic fibroblast growth factor receptor	1.3	2.7	0.6	signaling	M37722
cAMP-dependent protein kinase alpha-catalytic subunit	0.5	0.7	0.4	signaling	X07767
Tyrosine-protein kinase receptor ufo (sky)	0.4	1.2	0.7	signaling	D17517
Tyrosine-protein kinase CAK (EDDR1; TRK E)	0.4	0.7	0.6	signaling	X74979
Activated p21cdc42Hs kinase (ack)	0.3	0.9	0.5	signaling	L13738
Transducin beta-2	0.3	0.9	0.4	signaling	M36429
MAP kinase kinase 3 (MMK3)	0.3	0.6	0.4	signaling	L36719
Transducin beta-1	0.3	0.5	0.4	signaling	M36430
MAP kinase	0.2	0.8	0.3	signaling	L05624
Tyrosine-protein kinase receptor eph-3	0.1	0.3	0.3	signaling	X75208
Prostaglandin E2 receptor EP4 subtype	0.1	0.9	1.2	signaling	L28175
Protein kinase MLK-3	0.0	0.4	0.0	signaling	L32976
Glucose-6-phosphate isomerase	1.2	1.9	0.3	metabolism	K03515
Glutathione S-transferase P	0.5	0.6	0.4	metabolism/apoptosis	X15480
NADH-ubiquinone oxidoreductase B18 subunit (SQM1)	0.4	0.7	0.3	metabolism	M33374
Dioxin-inducible cytochrome *P*450 (CYP1B1)	0.2	0.5	0.2	metabolism/detoxification	U03688

Control has been adjusted to have a value of 1.

San Francisco, CA, USA) were used for the analysis. The autoradiography signal was developed with the Super-Signal® West Pico enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA).

3. Results

Gene expression profiling of each array membrane was performed for samples exposed to continuous HP for 0, 3, 6

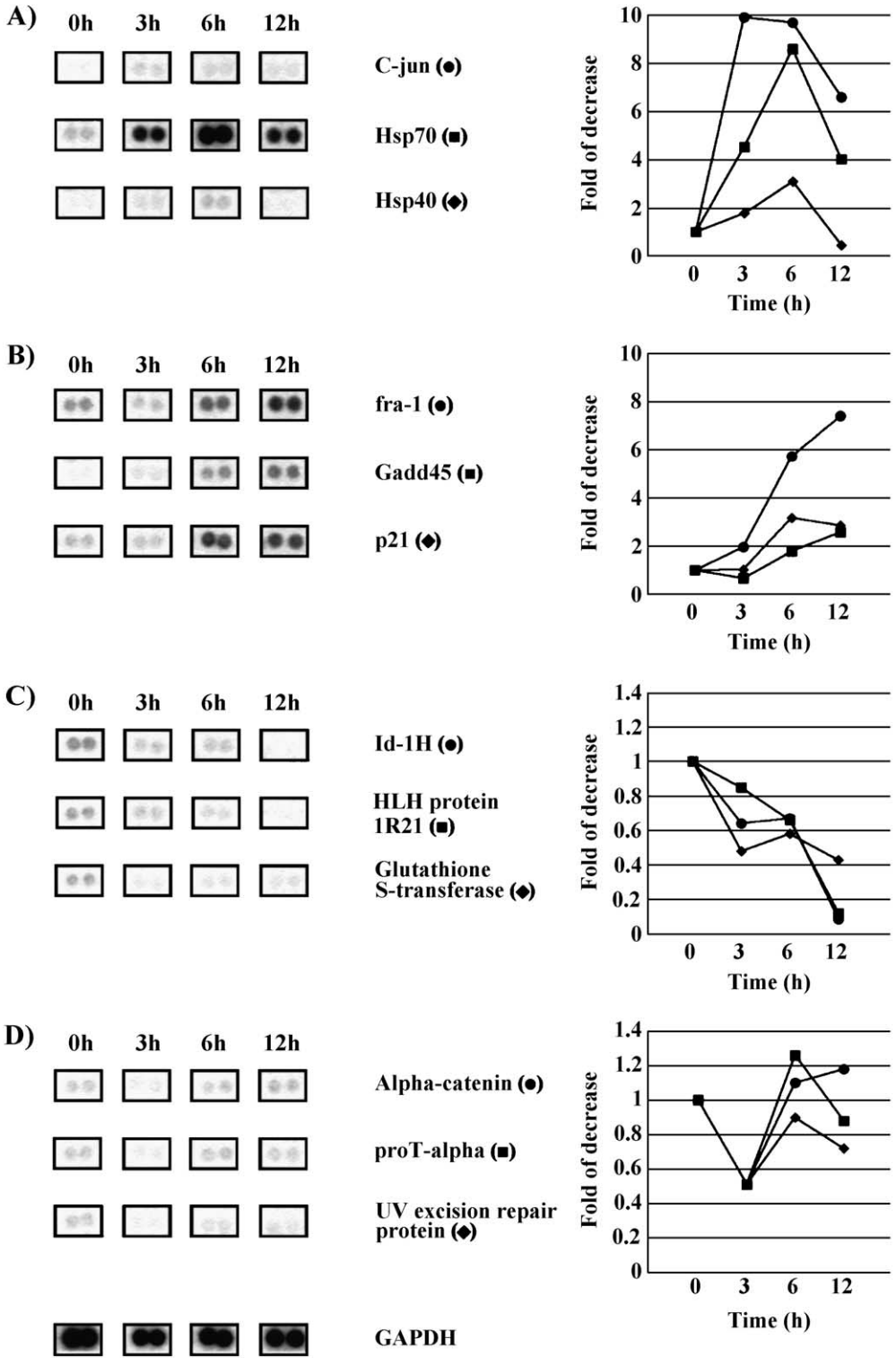

Fig. 1. Various genes analyzed by cDNA microarray grouped into categories according to their expression kinetics. (A) Increased and subsequently decreased mRNA levels during pressure treatment. (B) Solely increased mRNA levels. (C) Constantly decreased or (D) temporarily decreased mRNA levels under pressure treatment. The GAPDH mRNA served as a normalization control. The cDNA fragments have been spotted in duplicate on hybridization membrane.

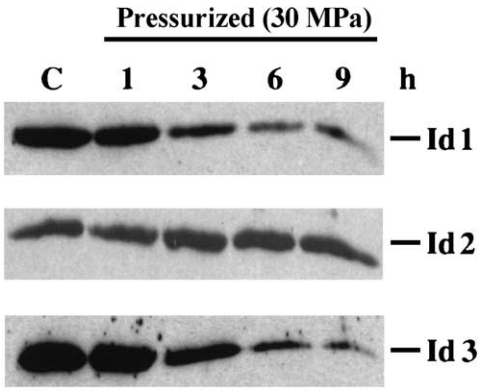

Fig. 2. Western blot analysis with antibodies against Id-proteins. Whole cell extracts were collected from nonstressed control cells, and from cells exposed to 30 MPa static hydrostatic pressure for 1–9 h. Experiments were repeated two times.

and 12 h. After normalization based on expression level of several housekeeping genes, the genes whose mRNA expression ratio compared with that of the control sample was 2.0 or more in one or several time point(s) during the follow-up were considered up-regulated, and genes whose expression ratio was 0.5 or below were considered down-regulated. Our findings show that a total of 51 genes were markedly affected at one or several time point(s) (Table 1).

Increased expression of hsp70, hsp40 and hsp27 mRNAs was observed as a group of genes having a common function related to stress response. The most intensive increase for hsp70 mRNA was with a ratio (experiment/control) of 8.6 at 6 h (Table 1, Fig. 1A). The expression levels for the growth arrest and DNA damage-inducible genes (GADD45 and GADD153) also increased constantly under pressure (Table 1). Similarly, anti-proliferative proteins tob (transducer of ErbB-2) and $p21^{Cip1/Waf1}$ (inhibitor of cyclin-dependent kinases) genes were up-regulated. In contrast, ribosomal protein S19, prothymosin alpha and p55CDC, genes associated with cell proliferation, were all down-regulated (Table 1).

Differences were noticed in the behavior of genes coding for various transcription factors. A clear increase in the

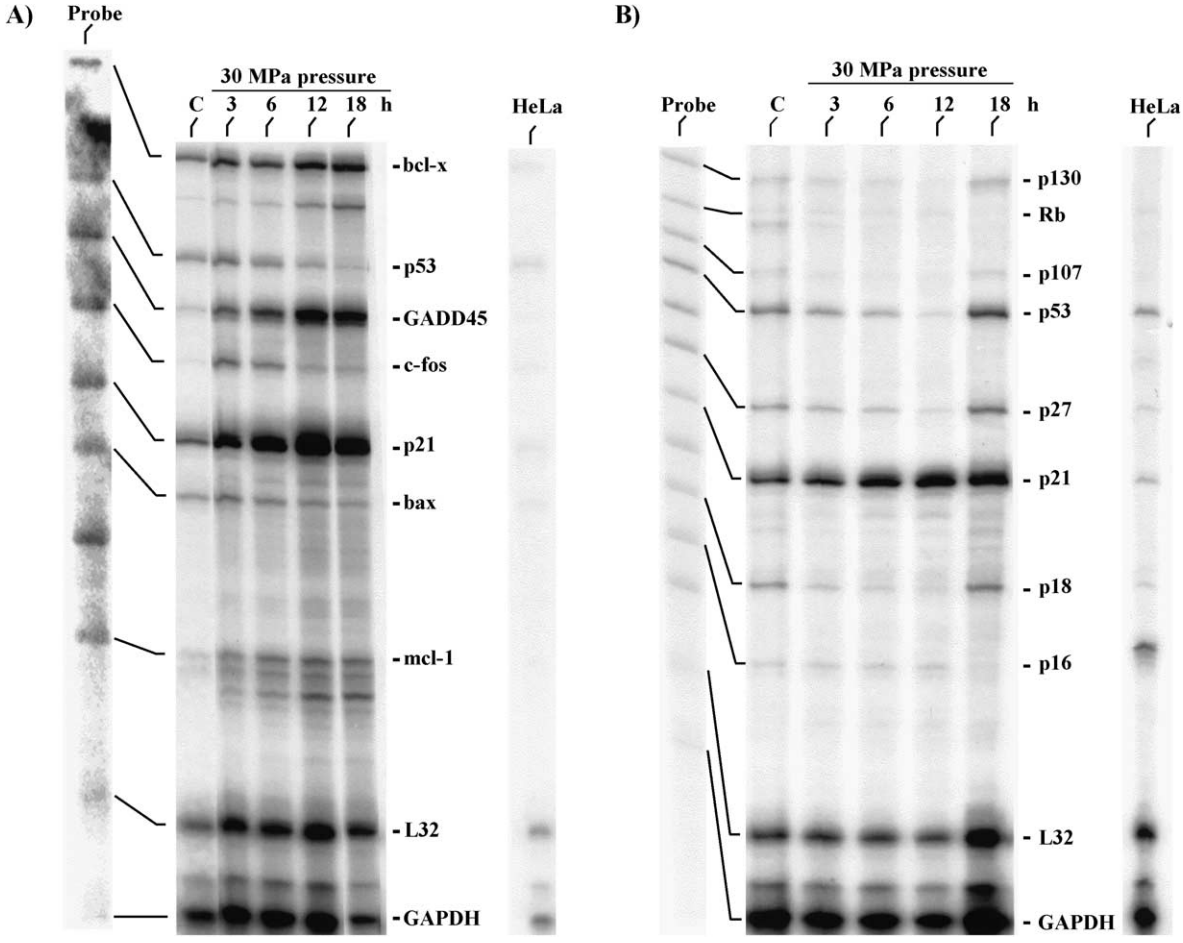

Fig. 3. Semiquantitative expression analysis by the ribonuclease protection assay. Total cellular RNA (15 μg) from HCS 2/8 cells exposed to 30 MPa continuous hydrostatic pressure (3–18 h) was loaded per lane. (A) Analysis of stress-related expression products: bcl-X, p53, GADD45, c-fos, p21, bax, and mcl-1. (B) Analysis of expression products involved in the regulation of the cell cycle: p130, Rb, p107, p53, p27, p21, p18, and p16. Housekeeping genes: L32 and GAPDH. Unprotected probe and positive control (HeLa RNA) are also illustrated. Densitometric analysis of mRNA levels, relative to GAPDH mRNA, was performed using a phosphorimager.

expression level of c-jun mRNA was observed (Table 1). Furthermore, GATA-2, early growth response gene (egr-1), activating transcription factor (ATF)-4 and fra-1 genes were up-regulated (Table 1), while genes coding for homeobox CI and AP-2 were down-regulated (Table 1). Inhibitors of differentiation/DNA binding proteins (Id1, Id2 and Id3) were uniformly decreased in expression during the pressurization, the fold of change being 0.1, 0.4 and 0.1 at 12-h time point, respectively. The Western blot analysis revealed a steady decrease in Id1 and Id3 proteins under high HP, confirming the results obtained from the cDNA array analysis (Fig. 2). However, no change was noticed in the amount of Id2 protein level within 9 h. The apoptosis-related gene NIP3 and cytoplasmic dynein light chain 1 (HDLC-1) gene expressions decreased upon HP loading. Genes coding for growth factors were specifically affected. Thus, vascular endothelial growth factor (VEGF) and EGF-like growth factor 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 gene expression kinetics were observed (Fig. 1).

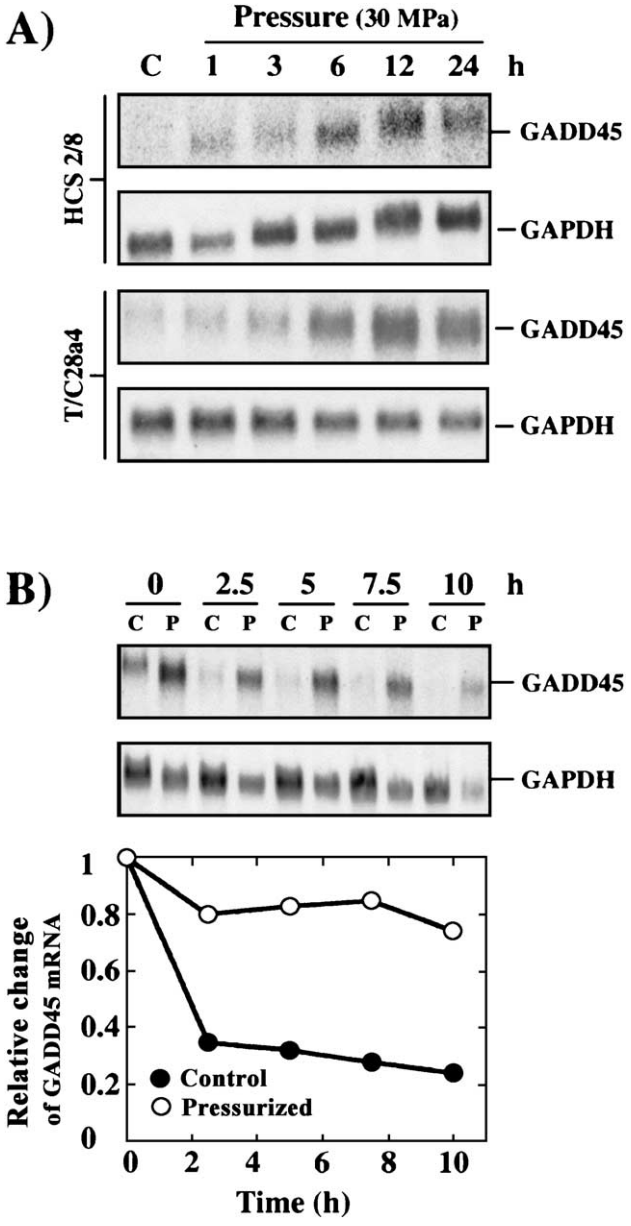

Fig. 4. (A) Analysis of steady-state level of GADD45 mRNA by Northern blot hybridization. Total RNA samples (20 μg) were hybridized with GADD45 and GAPDH probes. The GAPDH mRNA served as a normalization control. The total RNA was extracted from HCS 2/8 and T/C28a4 cells pressurized up to 24 h. RNA samples isolated from unpressurized cells served as a control (C). (B) Analysis of GADD45 mRNA stability by Northern blot hybridization. The T/C28a4 cells pressurized for 6 h and nonstressed control cells were incubated in the presence of 10 μM actinomycin D for 0–10 h. The total RNA samples were hybridized with GADD45 and GAPDH probes. Quantitative analysis of GADD45 mRNA levels, relative to GAPDH mRNA, was performed using a phosphorimager. Experiments were repeated two times.

Our cDNA microarray analysis showed that, in response to high HP, several changes in gene expressions were involved with the genes regulating cell cycle or stress response. For this reason, we analyzed a set of genes associated with these functions using a ribonuclease protection assay. The up-regulation of the expression of GADD45 and $p21^{Cip1/Waf1}$ was confirmed and, in addition, down-regulation of p53 and p18 was evident after HP exposure. However, no marked changes in the expression profiles of other cell cycle or stress-associated genes included in the assay were observed (Fig. 3).

Northern blot analysis was used to further analyze the induction of GADD45 mRNA, the growth arrest-specific protein, upon loading. In addition to the HCS-2/8 cell line, we used another human chondrocytic cell line (T/C28a4) to determine whether there were differences in the pressure response. Elevated levels of GADD45 mRNA were observed in both cell lines with similar kinetics (Fig. 4A). It has been shown that T/C28a4 cells respond to high HP by accumulating Hsp70 mRNA via stabilization of its mRNA, without transcriptional activation [22]. GADD genes have also been shown to be posttranscriptionally regulated by glutamine deprivation, which increases accumulation of GADD45 and GADD153 proteins primarily by mRNA stabilization [34]. Interestingly, Northern blot analysis demonstrated increased stability of GADD45 mRNA during 10-h followup in the presence of actinomycin D after 6-h pressure treatment (Fig. 4B), indicating the changes in posttranscriptional regulation.

4. Discussion

Altogether, 51 out of 588 genes analyzed were significantly affected by the high HP during 12-h treatment. Genes related to heat-shock response, growth arrest and differentiation were distinctively involved in the pressure response. We have previously reported an accumulation of Hsp70 in the presence of 30-MPa continuous HP [22]. This

took place through mRNA stabilization without transcriptional activation of the corresponding gene in the chondrocytic cells (T/C28a4), while cyclic 30-MPa pressure did not activate a heat-shock response. Interestingly, the heat-shock response seems to be dependent on cell type, since primary bovine chondrocytes did not respond to high continuous pressure by increasing Hsp70 protein level, while in primary bovine synovial cells and primary human fibroblasts a remarkable Hsp70 response was seen [25]. Also, a preconditioning with pressure rendered primary fibroblasts baroresistant as regards Hsp70 response [25]. The increased expression of hsp70 gene in this microarray for pressurized HCS2/8 cells is consistent with previous findings [28] and probably indicates an adaptive response against external stress. Hsp40 has been shown to co-operate with Hsp70 and is involved in the chaperone function and formation of thermotolerance [35]. Hsp27 has been shown to be involved in intracellular signaling and drug resistance, in addition to thermotolerance [36]. Many heat-shock genes were induced in pressurized HCS-2/8 cells, which may indicate the presence of misfolded proteins generated by the high pressure. Interestingly, an altered mobility of secreted aggrecans on agarose gels was previously observed with bovine primary chondrocytes [5].

HP in the range of 15 to 25 MPa has been shown to cause arrest of the cell cycle in G1 phase in an exponentially growing culture of *Saccharomyces cerevisiae*, whereas a pressure of 50 MPa did not [37]. Significant alterations in the expression of genes affecting the cell cycle were also noticed in our experimental setup. The anti-proliferative gene $p21^{Cip1/Waf1}$, up-regulated by HP, is a cyclin-dependent kinase inhibitor which has been shown to inhibit proliferation both in vivo and in vitro, resulting in cell cycle arrest in G1 phase [38]. Induction of $p21^{Cip1/Waf1}$ gene is involved in terminal differentiation of, for example, the megakaryoblastic leukemia cell line CMK [39] and the myelomonocytic cell line U937 [40]. $p21^{Cip1/Waf1}$ expression also increased during the differentiation of chondrogenic MCT cells, obviously via signaling through the Raf-1 pathway [41]. The gene is widely expressed in permanent cartilage, and in cartilage undergoing endochondral ossification, and it is associated with hypertrophic differentiation of cartilage [42]. Several stress stimuli, including oxidants, genotoxins, metabolic deficiencies and irradiation, have been shown to induce expression of the $p21^{Cip1/Waf1}$ gene [43]. The up-regulation of $p21^{Cip1/Waf1}$ under pressure conditions seems to be specifically regulated, since no other genes regulating the cell cycle were up-regulated. p53 and another cyclin-dependent kinase inhibitor, p18, were down-regulated.

Growth arrest and DNA-damage inducible proteins GADD45 and GADD153 were up-regulated by high HP. Genes coding for these proteins are activated due to various stress signals such as hypoxia, irradiation, genotoxic drugs and withdrawal of growth factors [44]. The $p21^{Cip1/Waf1}$ and GADD45 genes exhibit independently their growth-suppressive activity. The GADD45 gene interacts with $p21^{Cip1/Waf1}$ [45], proliferating cell nuclear antigen [46], MTK/MEKK4 kinase [47] and Cdc2 kinase [48]. The GADD genes are posttranscriptionally regulated by glutamine deprivation, which induces the expression of GADD45 and GADD153 primarily by mRNA stabilization [34]. Similarly to our previous report on hsp70 mRNA [22], we show here a stabilization of GADD45 mRNA in HCS-2/8 cells after exposure to HP. This may indicate an involvement of more extensive use of posttranscriptional regulation in response to cellular adaptation against stress.

Yet another anti-proliferative gene, tob, was up-regulated under pressure treatment. This gene is ubiquitously expressed and belongs to the Btg family of anti-proliferative gene products (also known as APRO genes), whose members have the Btg1 homology domain in their amino-terminal region. APRO genes are involved in the negative control of the cell cycle in particular during cell differentiation [49]. Ribosomal protein S19 as a part of translational machinery [50], prothymosin alpha as histone binding nuclear protein [51], and p55CDC as an activator of the mitotic spindle assembly checkpoint [52,53], are genes required in cell proliferation and were all down-regulated during the pressure treatment. These findings are in agreement with up-regulation of anti-proliferative genes and with initiation of possible adaptation process.

Cellular adaptation to external stimuli is initiated by expression of immediate-early genes and transcription factors in order to recruit a new set of genes. Increased expression for c-jun, a component of AP-1 heterodimer, was seen after 3-h pressure being rather sustained along the course of the experiment. A more delayed increase in expression was seen with GATA-2, egr-1, fra-1 and AFT-4 genes. egr-1 is a zinc finger transcription factor that is expressed in response to mediators associated with growth and differentiation. In human umbilical vein endothelial cells, egr-1 and c-Fos have been shown to be activated by a constant mechanical stretch [54]. Egr-1 mediates fluid shear stress induction of the tissue factor in human endothelial cell [55]. ATF-4 has a bZip DNA binding domain that binds to the consensus ATF/CRE site on a promoter. This factor can be either a transcriptional activator or repressor that has been shown to form heterodimers with Fos, Jun, JunD and several C/EBP proteins. Previous studies show that ATF-4 can be up-regulated by a variety of extracellular signals (hypoxia, calcium ionophore, heregulin or homocysteine) in different cell types [56]. In contrast, AP-2 was significantly down-regulated after the onset of pressurization. AP-2 is one of the specific activators of the $p21^{Cip1/Waf1}$ promoter [57]. Thus, the up-regulation of $p21^{Cip1/Waf1}$ in our experimental setup may be mediated by some other transcription factor.

Inhibitors of differentiation/DNA binding proteins (Id1, Id2 and Id3) were markedly down-regulated in pressurized HCS-2/8 cells. Id-family members act as dominant negative regulators of basic helix-loop-helix proteins, and are important for the maintenance of nondifferentiated phenotype, as well as for vigorous cell division by inhibiting the binding

of phenotype-specific transcription factors [58]. Id proteins form nonfunctional heterodimers with functional monomeric transcription factors. Thus, decreased amounts of Id proteins allow a cell to recruit a new set of functional dimeric transcription factors which are capable of activating transcription specific for a certain cell type [59]. The Id1(+/−)–Id3(−/−) double knockout mice display premature withdrawal of neuroblasts from the cell cycle and expression of neural-specific differentiation markers indicating premature differentiation [60]. Id1, and probably other Id family members, can activate the cell cycle by inhibiting E2A bHLH-regulated expression of the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ [61]. Here we show that down-regulation of Id proteins under pressure treatment in chondrocytic HCS-2/8 cells coincides with increased expression of $p21^{Cip1/Waf1}$, a result obtained in the array analysis (Table 1). When mitogenic signal is introduced, Id proteins down-regulate the expression of immediate-early genes c-fos and egr-1 by antagonizing the ETS domain proteins SAP-1 and Elk-1 responsible for induction of these genes [62]. This is consistent with the observed up-regulation of immediate-early genes and concurrent decrease in expression of Id genes under pressure.

Extracellular stimuli that cannot be tolerated by a cell may induce an apoptotic process leading to cell death. In this array analysis, there was no evident induction of genes involved in apoptosis within the time frame investigated. Nip3 (19-kDa interacting protein-3) is an apoptosis-inducing dimeric mitochondrial protein [63] that was significantly down-regulated during the pressure treatment. The pro-apoptotic gene, bax beta, was neither affected by the pressure. Human cytoplasmic dynein light chain gene (HDLC-1) was also down-regulated; the precise function of this protein is unknown, but its ubiquitous expression and conservation suggest a critical role in the function of the dynein motor complex. Cytoplasmic dynein, a large minus-end-directed microtubule motor, performs multiple functions during the cell cycle. In interphase, dynein moves membrane organelles, while in mitosis it moves chromosomes and helps to form the mitotic spindle. Previously reported reversible alterations of Golgi morphology under 30-MPa pressure [64] may be associated with reduced amount of a HDLC. Down-regulation of its critical subunit may be related to pressure-induced arrest of the cell cycle.

The use of cDNA array analysis revealed several functionally related genes that are affected by continuous HP. Among these, there are several genes not previously known to be affected by physical stress. Thus, this kind of an approach allows us to elucidate the intracellular events taking place in cells under mechanical stress.

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