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Differential Expression of GADD45 β in Normal and Osteoarthritic Cartilage:

Potential Role in Homeostasis of Articular Chondrocytes

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Abstract

Objective—Our previous study suggested that growth arrest and DNA damage–inducible protein 45 β (GADD45 β) prolonged the survival of hypertrophic chondrocytes in the developing mouse embryo. This study was undertaken, therefore, to investigate whether GADD45 β plays a role in adult articular cartilage.

Methods—Gene expression profiles of cartilage from patients with late-stage osteoarthritis (OA) were compared with those from patients with early OA and normal controls in 2 separate microarray analyses. Histologic features of cartilage were graded using the Mankin scale, and

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AUTHOR CONTRIBUTIONS

Dr. Goldring had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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GADD45 β was localized by immunohistochemistry. Human chondrocytes were transduced with small interfering RNA (siRNA)-GADD45 β or GADD45 β -FLAG. GADD45 β and COL2A1 messenger RNA (mRNA) levels were analyzed by real-time reverse transcriptase-polymerase chain reaction, and promoter activities were analyzed by transient transfection. Cell death was detected by Hoechst 33342 staining of condensed chromatin.

Results—GADD45 β was expressed at higher levels in cartilage from normal donors and patients with early OA than in cartilage from patients with late-stage OA. All chondrocyte nuclei in normal cartilage immunostained for GADD45 β . In early OA cartilage, GADD45 β was distributed variably in chondrocyte clusters, in middle and deep zone cells, and in osteophytes. In contrast, COL2A1, other collagen genes, and factors associated with skeletal development were up-regulated in late OA, compared with early OA or normal cartilage. In overexpression and knockdown experiments, GADD45 β down-regulated COL2A1 mRNA and promoter activity. NF- κ B overexpression increased GADD45 β promoter activity, and siRNA-GADD45 β decreased cell survival per se and enhanced tumor necrosis factor α -induced cell death in human articular chondrocytes.

Conclusion—These observations suggest that GADD45 β might play an important role in regulating chondrocyte homeostasis by modulating collagen gene expression and promoting cell survival in normal adult cartilage and in early OA.

Osteoarthritis (OA) is defined largely as a disease of cartilage, since chondrocytes, which constitute the unique cellular component of adult articular cartilage, are able to respond to mechanical injury, joint instability due to genetic factors, and biologic stimuli such as cytokines and growth and differentiation factors. In young individuals without genetic abnormalities, biomechanical factors due to trauma are strongly implicated in initiating the OA lesion (1,2). Mechanical disruption of cell-matrix interactions may lead to aberrant chondrocyte behavior that is reflected in the appearance of fibrillations, cell clusters, and changes in quantity, distribution, or composition of matrix proteins (3). In the early stages of OA, a transient increase in chondrocyte proliferation is associated with increased synthesis of catabolic cytokines and matrix-degrading enzymes. Local loss of proteoglycans and cleavage of type II collagen occur initially at the cartilage surface, resulting in an increase in water content and a loss of tensile strength in the cartilage matrix as the lesion progresses.

A number of studies have demonstrated enhanced biosynthesis and increased global gene expression of aggrecan and type II collagen in human OA cartilage (4,5). The increased levels of anabolic factors such as bone morphogenetic protein 2 (BMP-2) and inhibin β A/activin suggest a possible mechanism of cartilage anabolism (5–7). Nevertheless, Aigner and coworkers have shown that expression of the type II collagen gene (COL2A1) is suppressed in upper zones with progressing matrix destruction, whereas global COL2A1 gene expression is increased in late-stage OA cartilage compared with normal and early degenerative cartilage (8–10). Phenotypic modulation, with expression of collagens normally absent in adult articular cartilage or at atypical sites in OA cartilage, has been proposed (11). Once the cartilage is severely degraded, the chondrocyte is unable to replicate the complex arrangement of collagen laid down during development. Furthermore, the chondrocyte stress response may result in the loss of viable cells due to apoptosis or senescence (12).

Growth arrest and DNA damage-inducible protein 45 β (GADD45 β) is a member of a family of small (18-kd) proteins that respond to genotoxic stress. Initially, GADD45 β , encoded by myeloid differentiation factor 118, was identified as a primary response gene activated in murine myeloid leukemia cells by interleukin-6 during terminal differentiation (13,14). Microarray studies have shown that another family member, GADD45 α , is expressed at higher levels in normal cartilage than in OA (9) and is induced by hydrostatic pressure (15). Recently, we identified GADD45 β as a BMP-2-induced early gene in chondrocytes and as

an essential mediator of *Col10a1* and *Mmp13* gene expression in late-stage hypertrophic chondrocytes in the mouse embryo (16). Since the hypertrophic zone was compressed in *Gadd45b*-deficient embryonic growth plates, we hypothesized that GADD45 β may act as a cell survival factor during terminal differentiation (16).

In 2 separate global analyses of gene expression in cartilage, we showed in this study that chondrocytes express GADD45 β messenger RNA (mRNA) at higher levels in cartilage from normal donors and patients with early OA than in cartilage from patients with late-stage OA. However, the distribution of GADD45 β in chondrocyte clusters and in osteophytes in OA cartilage suggests different roles of GADD45 β in OA compared with normal cartilage, where GADD45 β is localized in the nuclei of all chondrocytes. Interestingly, overexpression of GADD45 β down-regulates COL2A1 promoter activity in chondrocytes, consistent with the reciprocal expression of the 2 genes in cartilage from patients with early and late OA. GADD45 β gene transcription is induced by NF- κ B, and endogenous levels of GADD45 β promote cell survival and protect against tumor necrosis factor α (TNF α)-induced cell death in chondrocytes. These results may explain how cartilage damage and other environmental stresses influence the survival and anabolic activities of chondrocytes in OA cartilage.

MATERIALS AND METHODS

Cartilage samples

Human articular cartilage samples were obtained from discarded surgical material or from cadavers, with the approval of the Institutional Review Boards of the Beth Israel Deaconess Medical Center (BIDMC), New England Baptist Hospital, the University of Erlangen–Nuremberg (Erlangen, Germany), the Graduate School of Medicine and Dentistry, Kagoshima University, and the Hospital for Special Surgery.

RNA isolation and microarray analysis

Cartilage samples were rapidly frozen in liquid nitrogen, and total RNA was isolated as previously described (17,18). Transcription profiling of RNA samples derived from cartilage from 3 patients with early OA (mean age 72 years) and 3 patients with late OA (mean age 78 years) was performed at the BIDMC Genomics Center, using the GeneChip Human Genome U133A array (Affymetrix, Santa Clara, CA) containing 22,283 genes. Array experiments were performed according to the recommendations of the manufacturer with 1 μ g of total RNA per sample. After polymerase chain reaction (PCR) amplification of each sample, complementary DNA fragments were hybridized with a pre-equilibrated Affymetrix chip, washed, stained, and scanned in the HP ChipScanner (Affymetrix), as previously described (19). Microarray analysis of RNA extracts from cartilage samples from 13 normal individuals (mean age 71.7 years [range 48–87 years]) and 12 patients with late-stage OA (mean age 64.7 years [range 60–84 years]) diagnosed according to the American College of Rheumatology criteria (20), was performed at the Münster University Genomics Center (Münster, Germany), using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array for >47,000 transcripts.

Analysis with dChip software

Scanned array images were analyzed using dChip (21), which has been shown to be more robust than Affymetrix Microarray Analysis Suite software, version 5.0, in signal calculation for ~60% of genes (22). In the dChip analysis, a smoothing spline normalization method was applied prior to obtaining model-based gene expression indices, also known as signal values. Single, array, and probe outliers were interrogated as described in dChip, where image spikes are treated as single outliers. Since no outlier chip was identified, all samples were used for subsequent analysis. To compare cartilage samples from patients with

early and late-stage OA, we used the lower confidence bound, which is a stringent estimate of the fold change and has been shown to be the better ranking statistic (23). We used the dChip method to assess differentially expressed genes according to the lower confidence bound, since it is superior to other commonly used approaches, such as the Affymetrix Microarray Analysis Suite 5.0 algorithm and Robust Multiarray Average (24,25). If the lower confidence bound of the fold change between the experiment and the baseline was >1.2 , we considered the corresponding gene to be differentially expressed. Studies using custom arrays and quantitative real-time reverse transcriptase (RT)-PCR have suggested that Affymetrix chips may underestimate differences in gene expression (26). Based on this work and others (27), a criterion of selecting genes that have a lower confidence bound >1.2 most likely corresponds to genes with an “actual” fold change of 3 in gene expression.

Real-time RT-PCR

Total RNA (1.0 μg) was reverse-transcribed in 20 μl containing final concentrations of 2.4 IU/ μl of murine leukemia virus reverse transcriptase, 2.5 μM of oligo(dT)₁₆, and 1 unit/ μl of RNase inhibitor, all obtained from Applied Biosystems (Foster City, CA). Amplifications were carried out using SYBR Green I-based real-time PCR on the MJ Research DNA Engine Opticon™ Continuous Fluorescence Detection System (MJ Research, Waltham, MA), as previously described (16,28). For each run, serial dilutions of GAPDH plasmids were used as standards for quantitative measurement of the amount of amplified DNA. All samples were run in triplicate, and the data were calculated as the ratio of GADD45 β or COL2A1 to GAPDH. The primers used for real-time PCR were as follows: for GADD45 β , 5'-TCGGATTTTGCAATTTCTCC-3' (sense) and 5'-GGATGAGCGTGAAGTGGATT-3' (antisense); for human GAPDH, 5'-CAAAGTTGTCATGGATGACC-3' (sense) and 5'-CCATGGAGAAGGCTGGGG-3' (antisense); and for human COL2A1, 5'-CAACACTGCCAACGTCCAGAT-3' (sense) and 5'-CTGCTTCGTCCAGATAGGCAAT-3' (antisense).

Immunohistochemistry

Articular cartilage samples were obtained from hip joints from 4 asymptomatic donors (mean age 81.5 years) undergoing joint replacement for femoral neck fracture and from knee joints from 10 patients (mean age 74 years) undergoing total knee arthroplasty because of symptomatic OA. Multiple areas of cartilage (0.5 \times 0.5 cm^2) were fixed in 4% paraformaldehyde for 4 hours at room temperature and embedded in paraffin. Serial sections of 8 μm were cut and stained with toluidine blue. Histologic features were assessed, and sections were assigned a grade on the Mankin scale (29). Sections of fixed and paraffin-embedded cartilage were deparaffinized and blocked with normal horse serum or protein block (Dako x0909; Dako, Carpinteria, CA). Immunohistochemical analysis for GADD45 β was performed as previously described (16,30), using goat polyclonal anti-GADD45 β (sc-8776; Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:400 dilution (0.5 $\mu\text{g}/\text{ml}$ final concentration), rabbit biotinylated anti-goat IgG (Sigma, St. Louis, MO), and the Vectastain Elite ABC kit (Vector, Burlingame, CA.). Sections were counterstained with hematoxylin. For negative controls, normal goat IgG (sc-2028) was used in place of the primary antibody against GADD45 β .

Cell culture

The immortalized human chondrocyte cell line, C-28/I2, was cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1/1 [volume/volume]) (Invitrogen, San Diego, CA) containing 10% fetal calf serum (FCS) (Biowhittaker, Walkersville, MD), as previously described (31,32). For experiments, subconfluent cultures were moved to medium containing 1% Nutridoma-SP (Roche, Indianapolis, IN) or 1% serum for 18 hours

for mild starvation. Primary chondrocytes were isolated by sequential digestion with Pronase and collagenase P (Invitrogen) from human articular cartilage obtained from intact regions of femoral condyles from patients undergoing total knee replacement surgery, and cultured to confluence in DMEM–Ham’s F-12 containing 10% FCS.

Transient transfections using luciferase reporter constructs and expression plasmids

The human GADD45 β promoter fragment spanning –1,604 to +141 bp was prepared by PCR using human genomic DNA (BD Clontech, Basing-stoke, UK) as template, as previously described (16). The –496 to +141 bp construct was prepared by PCR using the *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) and the –1,604 to +141 bp construct as template. The COL2A1 sequence spanning –577 to +3,428 bp was cloned into the pGL2-basic (pGL2-B) luciferase reporter gene vector (Promega, Madison, WI) to generate pGL2B-COL2 (33). The pcDNA3-GADD45 β -FLAG and pcDNA3-FLAG empty vector and the NF- κ B p65 and p50 expression vectors have been reported previously (16,34). The transduction of C-28/I2 cells with lentiviral–small interfering RNA (siRNA)–green fluorescent protein (GFP) (GFP–knockdown [KD]) or lentiviral-siRNA-GADD45 β (GADD45 β -KD) and tests for specificity and efficiency of knockdown have been described previously (16). Transient transfection experiments were carried out using C-28/I2 cells with Plus and Lipofectamine reagents (Invitrogen), as previously described (16,32). Luciferase activity was determined by the Dual Luciferase Assay using the Autolumat LB953 luminometer (EG&G Berthold, Oak Ridge, TN). Each experiment was repeated at least 3 times and each data point was calculated as the mean \pm SD of 3–6 wells per experiment.

Hoechst staining

Primary human articular chondrocytes, obtained from 5 patients (2 men and 3 women) with OA (age range 53–70 years) who underwent total knee replacement, were passaged at a density of 2.5×10^4 cells/cm² and transfected with 50 nM of siRNA oligos against GFP (sense GCAAGCUGACCCUGAAGUUCAU and antisense GAACUUCAGGGUCAGCUUGCCG) or against GADD45 β (Hs_GADD45B_6_HP siRNA; Qiagen, Chatsworth, CA) using Plus and Lipofectamine reagents. Transfection efficiency was assessed using siRNA GFP conjugated with rhodamine. The GADD45 β siRNA sequences were selected from among 5 sets tested at 48 and 72 hours, and knockdown was confirmed by real-time PCR in the Opticon 2 Real Time PCR Detector System (Bio-Rad, Richmond, CA) (see Supplementary Figure 1, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>). Seventy-two hours after transfection, cells were treated with 50 ng/ml TNF α in serum-free medium for 24 hours, and cell death was assessed by staining with 10 μ g/ml Hoechst 33342 dye (Invitrogen) to detect chromatin condensation. Cell death was defined by condensed and/or fragmented chromatin in the blue fluorescence–emitting nuclei. At least 200 cells from randomly selected fields were counted in each experiment. The significance of the differences was estimated using analysis of variance followed by Student’s *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Higher levels of GADD45 β expression in early OA than in late OA

Based on our previous study (16), we hypothesized that GADD45 β might be differentially expressed in adult articular cartilage. Our initial screen used total RNA isolated from cartilage samples from 3 patients with early OA and 3 patients with late OA. Microarray analysis on Affymetrix GeneChip U133A identified 358 transcript variants (lower confidence bound 1.2) that were highly expressed genes in cartilage from patients with early OA. GADD45 β was expressed 2.66-fold higher in early OA than in late OA (see

Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>).

Other genes up-regulated prominently in early OA encoded intracellular regulators of cell proliferation (DDX11, CCNG1, HRASLS3, CDC25C, CHEK1, H2AFN, H2AFB, RAB26), mitochondrial function (OXA1L, TOMM20, TIMM17A, CYP4B1, ACADL, COX7C, CTAG1), cytoskeletal function (TMOD, ROCK1, VCL, HINT1, TLN4), ion transport (SCNN1A, SCN2B, ACADL, CLDN4, NTT5), intracellular transport (LTF, GRM1, TFR2), signal transduction (PIK3R1, MAP2K5, SWAP1, PDE9A, SSR4, PIK4CB, GRAF, MAPK4, MSTIR, MAPK14), transcription (RREB1, ETV5, FOXG1A, SAP18, ZNF144, ZNF254, ZNF277, ZNF363, SRY, SOX17), and protein translation and modification (EIF4B, EIF4EBP2, RABBG-GTB, MAOA, ASB13). Genes associated with inflammation (SAA2, C3, TNFRSF8, IL1R1), angiogenesis (FLT1, TIE, EPHB4, NRP2, BAI3), cell adhesion (CDH7, GPC3, CELSR1, ITGA2, ITGA8, ITGB4, PCDH11), matrix degradation (CSNK2A2, ADAM11, ADAM23, MMP10), and skeletal development (FGF4, FGF5, FGF16, BMP7, MADHIP, FST, DVL2, PTCH, HOXA4, HOXB6, ALP1) were also up-regulated in early OA (Supplementary Table 1).

Real-time PCR confirmed the differential expression of *GADD45 β* mRNA in early and late, or severe, OA cartilage samples analyzed by microarray (Figure 1A). Interestingly, severe OA sample 3 was an autopsy specimen with histologic features consistent with a less severe stage of OA than sample 1 or sample 2. We decided to include these data in this report, since they illustrate the importance of uniform handling of material and because the Self Organizing Map analysis also indicated sample 3 as an outlier. The Self Organizing Map algorithm groups genes that have similar expression patterns within the same cluster.

As shown in Figure 1B, 30 distinct clusters were generated, of which 21 (c0–c11, c13–c17, and c20–c23) contained genes that were primarily up-regulated in severe OA sample 1 and severe OA sample 2, and 8 (c12, c18, c19, and c24–c28) contained genes that were up-regulated in early OA sample 1, early OA sample 2, and early OA sample 3. The cluster c29 contained genes that were only slightly up-regulated in early OA. In some, but not all, of the clusters, severe OA sample 3 was an outlier, particularly in clusters c0–c5. *GADD45 β* clustered in c25 with other intracellular mediators involved in transport, mitochondrial function, and cell cycle regulation. The analysis of all 824 differentially expressed genes indicated that many of the same genes were down-regulated in severe OA samples 1, 2, and 3, as shown for the top 54 differentially expressed genes in the heat map in Figure 1C. In severe OA sample 3, however, most of the genes up-regulated in severe OA sample 1 and severe OA sample 2 were down-regulated, as in the early OA samples.

Higher levels of *GADD45 β* expression in normal cartilage than in cartilage from patients with late-stage OA

To determine whether *GADD45 β* expression in normal cartilage was up- or down-regulated compared with OA cartilage, we analyzed a data set from Affymetrix GeneChip Whole Genome U133 Plus 2.0, in which total RNA extracts from cartilage samples from 13 normal donors and 12 patients with late-stage OA were compared. These data were subjected to bioinformatics analysis by dChip in the BIDMC Genomics Center (<http://www.bidmcgenomics.org/>), using lower confidence bound >1.2. As shown in the dChip analysis in Figure 2, all 3 transcripts of *GADD45 β* detected by the GeneChip were markedly down-regulated in OA compared with normal cartilage samples. Consistent with a previous report by Aigner et al (9), *GADD45 α* was also decreased in OA relative to normal cartilage, whereas *GADD45 γ* was expressed at low levels with a lower confidence bound close to 1.2 (Figure 2A).

Immunohistochemical analysis of GADD45 β expression in adult human articular cartilage

Since analysis of global gene expression in extracts of whole cartilage samples does not take into account the cellular distribution, we investigated the protein expression of GADD45 β in normal and OA cartilage at different stages to obtain some clues about its cellular localization and function. Immunohistochemistry demonstrated nuclear localization of GADD45 β in all chondrocytes where it was expressed. GADD45 β was present in chondrocytes throughout the superficial and middle zones of normal cartilage (Figure 3A). In early OA, some chondrocytes expressed GADD45 β strongly, but with variable distribution in the middle and superficial zones or in the deep zones. In late OA, few chondrocytes stained for GADD45 β (Figure 3A). GADD45 β was strongly expressed in chondrocyte clusters and also observed in cells throughout osteophytes (Figure 3B).

Up-regulation of COL2A1 and other extracellular matrix genes in cartilage from patients with late-stage OA

Analysis using Affymetrix GeneChip U133A identified 466 transcripts (lower confidence bound >1.2) that were highly expressed in late, or severe, OA (see Supplementary Table 2, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>). Hierarchical clustering analysis indicated that genes associated with extracellular matrix synthesis and anabolism were up-regulated in late OA (Figure 1C). COL2A1 was among the most highly expressed genes in clusters 0 and 1. The collagen genes, COL3A1, COL1A1, COL1A2, COL6A1, COL6A3, COL5A1, COL5A2, and COL15A1, which were often represented more than once in clusters 0, 1, 2, 3, 4, and 5, were all strongly up-regulated in cartilage from patients with late OA compared with cartilage from patients with early OA (Supplementary Table 2). COL9A3, COL11A1, COL9A2, and COL18A1 were found in clusters 6, 8, 20, and 22, respectively. Genes involved in posttranslational modification of collagens (PCOLCE, PLOD, P4HA2, LOXL1, and BMP1) and other matrix-associated genes such as those for HXB, TNXB, OGN (mimcan), BGN, FN1, LUM, CSPG2, CHAD, SDC1, and SPARC were also prominently represented.

Of interest were the other most highly expressed genes, including asporin (ASPN), matrilin 3 (MTN3), CILP, AQP1, S100A4, SGK, IGFBP7, IGFBP3, ANXA2, DKK3, WIF1, CTKSF1B1, POSTN, and LTBP1, that play a role in cartilage development and homeostasis. Other genes potentially involved in inflammation, angiogenesis, and stress responses, including PTGES, VCAM1, TNFSF11, TNFSF10, TNFRSF11B (osteoprotegerin), ANGPTL2, PLA2G4A, PRKCA, AXL, and GAS7, heat-shock proteins 22 (HSPB8), 27 (HSPB1), 47 (SERPINH1), 70 (HSPA1B), and 90 (HSPCA), and genes involved in tissue catabolism (SERPINA5, SERPINE1, PRSS23, CTSK, and MMP13) were also up-regulated. SDRG1, CRTAC1, FAP, MIA/CD-RAP, and NOTCH2 are of potential interest as markers in cartilage from patients with late OA (Supplementary Table 2).

Negative regulation of COL2A1 mRNA and promoter activity by GADD45 β

To further explore the reciprocal relationship between GADD45 β and matrix gene expression observed in our microarray analyses, we analyzed the influence of endogenous or overexpressed GADD45 β on COL2A1 mRNA levels and promoter activity in the human chondrocyte C-28/I2 cells transduced with lentiviral siRNA-GFP (GFP-KD control) or siRNA-GADD45 β (GADD45 β -KD) (16). The levels of COL2A1 mRNA, analyzed by quantitative real-time RT-PCR, and COL2A1 promoter activity were increased by GADD45 β silencing (Figure 4A). Overexpression of GADD45 β suppressed COL2A1 promoter activity in a dose-dependent manner. Cotransfection with GADD45 β -FLAG inhibited COL2A1 promoter activity compared with cotransfection with the control

pcDNA3-FLAG vector (Figure 4B). These results suggest that GADD45 β expression may be associated with low collagen turnover in cartilage in physiologic conditions.

GADD45 β is a survival factor in human articular chondrocytes

Since NF- κ B is known to induce GADD45 β during genotoxic and oxidative stress (28,35) and to play an important role in TNF α -induced apoptosis in chondrocytes, we hypothesized that the induction of GADD45 β by NF- κ B could represent a protective feedback mechanism for cell survival, as shown in previous studies of other cell types (36,37). In addition, several genes associated with apoptosis were differentially expressed in early and late OA, including apoptosis antagonizing transcription factor (AATF), caspase 10 (CASP10), BCL2-associated athanogene 3 (BAG3), apoptosis-related protein (APR), and apoptosis-related cysteine proteinase (Supplementary Tables 1 and 2).

As shown in Figure 5, cotransfection of the NF- κ B subunits p65 and p50 up-regulated GADD45 β promoter activity in C-28/I2 chondrocytes. Compared with the empty vector, p65 alone, but not p50, stimulated the activity of both promoter constructs. In cotransfections with both p65 and p50, the level of -1,604 to +141 bp promoter activity was >2-fold greater than in the presence of p65 or p50 alone. Of note, the activity of the shorter -496 to +141 bp promoter in the presence of p65 alone was not significantly different from that in the presence of both p65 and p50, consistent with the location of functional NF- κ B sites described previously (35). We then examined whether GADD45 β could serve as a cell survival signal, as reported for the NF- κ B response in other cell types (28,36,37). Knockdown of GADD45 β expression after transfection of siRNA-GADD45 β in cultured human articular chondrocytes isolated from 5 patients increased cell death, measured by Hoechst staining of condensed chromatin, in both the absence and the presence of TNF α (Figure 6). These results indicated that GADD45 β was a prosurvival signal in chondrocytes and that endogenous levels were sufficient to protect against cell death.

DISCUSSION

Based on our recent findings that highlighted a new function of GADD45 β in hypertrophic chondrocytes during endochondral ossification (16), we investigated whether this stress response factor might play a role in chondrocyte survival in adult articular cartilage. In our initial screen of a small number of samples of cartilage with well-characterized pathology, we found, surprisingly, a clear distinction between genes up-regulated in early and late OA, using GADD45 β and COL2A1, respectively, as “marker” genes. The down-regulation of GADD45 β in late (moderate to severe) OA was confirmed using a much larger data set from the U133 Plus 2.0 array containing the whole human genome. Along with the Self Organizing Map analysis showing that one of the late OA samples was an outlier, these data demonstrated the value of comparisons among individual samples. The consistency and statistical significance of differences in global gene expression were surprising because of the heterogeneity in proliferative and synthetic activities that we would expect at any given time among cells within individual human cartilage samples. Immunohistochemical analysis of individual cartilage samples also supported the assumptions derived from the microarray data. The expression of GADD45 β in all chondrocytes throughout normal articular cartilage, in addition to its localization in early OA cartilage at sites peripheral to the lesion and in chondrocyte clusters and osteophytes, along with our finding that endogenous levels protect against cell death in articular chondrocyte cultures, suggests a role of GADD45 β as a survival factor in chondrocytes in both health and disease.

Consistent with the low levels of COL2A1 in normal and early OA cartilage, GADD45 β down-regulated COL2A1 promoter activity and mRNA levels in chondrocyte cultures. In the mouse embryo, GADD45 β is required for expression of the type X collagen gene during

chondrocyte hypertrophy (16), and our present results suggest that it may also be associated with decreased COL2A1 transcription during terminal differentiation. The increased expression of the vascular endothelial growth factor receptors, FLT1 and NRP2, which mediate angiogenesis during endochondral ossification, and other genes associated with skeletal development in cartilage samples from patients with early OA, supports this concept. Thus, GADD45 β may be an important signal that serves as the molecular link common to pathways of cartilage remodeling in endochondral ossification and in OA.

Previous studies have shown that gene expression and synthesis of type II collagen are up-regulated in OA cartilage even though catabolic activity leads finally to the destruction of the matrix (38–40). Thus, the endogenous levels of GADD45 β in normal cartilage may reflect the low matrix synthetic activity in quiescent chondrocytes under low turnover conditions. The up-regulation of other matrix genes, including those for types I, III, and V collagens and MTN3, in late OA may reflect, however, an aberrant attempt at repair. Despite the high levels of COL2A1 in late OA cartilage, SOX9, the master switch for the COL2A1 phenotype, was not differentially expressed, consistent with findings that SOX9 mRNA is decreased near the lesions in OA cartilage (41), and that SOX9 and COL2A1 expression do not colocalize in adult articular cartilage (42). The differential up-regulation of transcription factors, such as MAF, SOX4, PMX1, FosB, RUNX1, DLX4, ELF2, NFAT5, and zinc-finger protein 42 (ZNF42), ZNF131, and ZNF137, suggests alternative mechanisms for regulating the other differentially expressed genes in cartilage in patients with late OA, whereas distinct transcription factors, including RREB1, ETV5, FOXG1A, SAP18, ZNF144, ZNF254, ZNF277, ZNF363, SRY, and SOX17, were up-regulated in early OA.

Although this is the first report of differential expression of GADD45 β in normal and OA human cartilage, our results confirmed previous findings of microarray studies of other genes, particularly matrix genes (10,43). Genome-wide scans in OA patients have identified polymorphisms in MTN3 (44), ASPN, which inhibits cartilage anabolism by binding to transforming growth factor β (45), and other genes (46) that were also up-regulated in late OA in our microarray analysis. Of interest were genes with prominent roles in anabolism, including CILP, AQP1, S100A4, IGFBP7, and ANXA2. The increased expression of the Wnt-associated genes, including DKK3, WIF1, and SFRP4, as well as CKTSF1B1, POSTN, LTBP1, TGFB114, FGF2, FGF18, Gli3, DLX4, and ACVR1B, suggests additional mechanisms due to modified biologic activities in the calcified cartilage and subchondral bone (47–49).

Other genes involved in regulating skeletal development were differentially up-regulated in early OA. We reported previously that RUNX2 is involved in the induction of GADD45 β , resulting in induction of MMP13 in hypertrophic chondrocytes (16). RUNX2 (data not shown) and MMP13 were expressed in early OA, although at higher levels in late OA, suggesting that RUNX2 is not the only requirement for GADD45 β expression in adult articular cartilage. The presence of GADD45 β in osteophytes, outgrowths from the periosteum that undergo endochondral ossification, also suggests its involvement in unsuccessful repair.

Regarding OA pathogenesis, few of the obvious candidates were identified. Genes associated with inflammation, angiogenesis, cell adhesion, and matrix degradation were up-regulated in early OA. Many of these genes were differentially expressed with relatively low fold change and lower confidence bound, reflected in the cluster analysis, suggesting that they may be expressed at early stages of OA and throughout the progression of the disease. For example, MMP10 has been implicated as a collagenase activator during cartilage degradation in arthritis (50). In late-stage OA, TNF superfamily members and heat-shock proteins may represent responses to stress and inflammation. Although serine proteinase

inhibitors, SERPINA5 and SERPINE1, are expressed along with few proteinases other than PRSS23 (serine protease 23), cathepsin K (a cysteine proteinase), and MMP13, the level of catabolic activity in late-stage OA cannot be predicted by these results. Other candidates for further study for which novel roles have been identified in cartilage include SDRG1 (51), CRTAC1 (52), FAP (53), MIA/CD-RAP (54), and NOTCH2 (55).

In adult articular cartilage, chondrocytes are quiescent, and cell survival and anabolic activity are essential for maintenance of this avascular tissue. Thus, chondrocyte apoptosis may have significant consequences in the pathogenesis of OA, although its importance in the slowly progressing disease in humans is controversial (12). Studies showing increased chondrocyte death in response to injurious compression support the idea of an association between apoptosis and increased stress (56). The presence of GADD45 β in the nuclei of nonmitotic chondrocytes and the differential expression of several apoptosis-related genes in OA cartilage are consistent with its described role in other tissues as an antiapoptotic molecule. GADD45 β is associated with G₂/M cell cycle arrest (57), and its interaction with MTK1/MEKK4 promotes activation of JNK, as well as p38 MAPK (58). However, GADD45 β interaction with MKK7, resulting in inhibition of TNF α -induced JNK activity by engaging the ATP-binding site, is responsible for the antiapoptotic activity of NF- κ B (36,37,59). Thus, inadequate levels of GADD45 β and sustained activation of JNK by mediators of inflammation may explain the proapoptotic response to NF- κ B in some cell types (60). Our finding that endogenous levels of GADD45 β protect against TNF α -induced cell death suggests a role for this survival factor as a feedback regulatory molecule during acute cell stress. Interestingly, the tumor suppressor CYLD, which is induced by TNF α and negatively regulates NF- κ B signaling (61) and adlican, which is involved in colon cancer progression (62), were both up-regulated in cartilage from patients with late-stage OA.

Overall, our results indicate that GADD45 β is a key factor contributing to physiologic cartilage homeostasis and to the imbalance in matrix remodeling in OA cartilage, as well as to chondrocyte survival after cell cycle arrest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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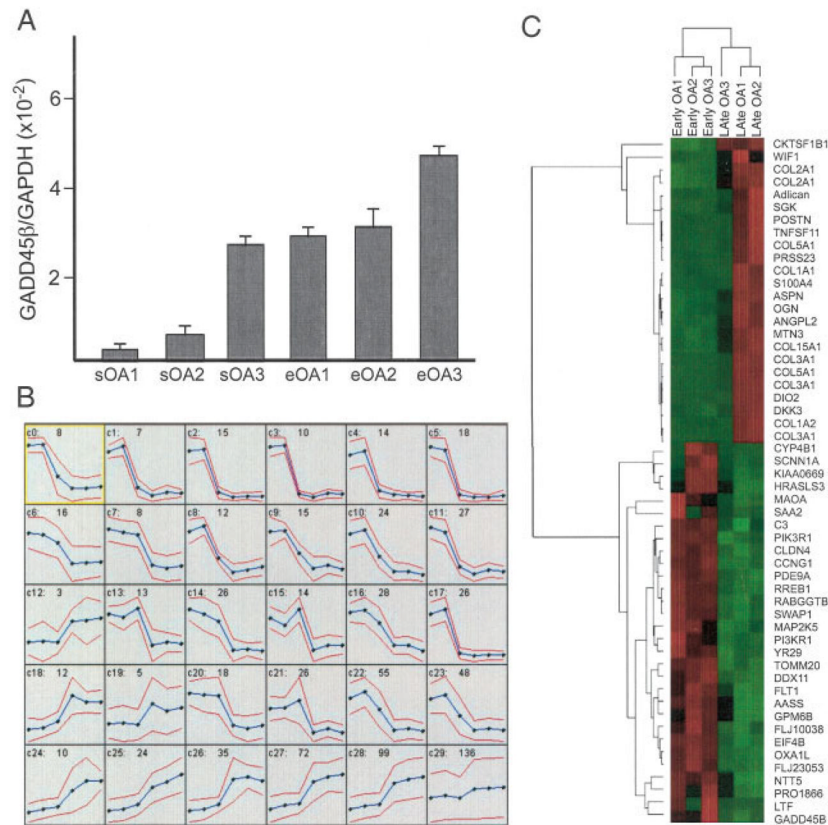
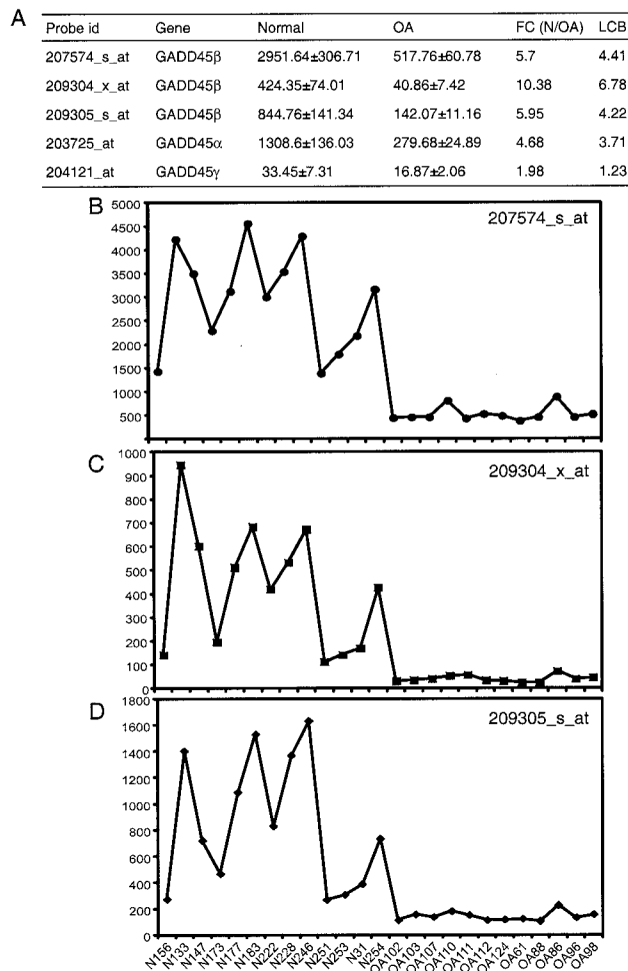


Figure 1. Comparison of gene expression patterns in cartilage samples from patients with early osteoarthritis (OA) and patients with late-stage OA. **A**, Growth arrest and DNA damage–inducible protein β (*GADD45 β*) expression in cartilage samples from patients with early OA and patients with late-stage OA. RNA extracts from cartilage samples obtained from 3 patients with early OA (eOA1–eOA3) and 3 patients with late, or severe, OA (sOA1–sOA3) were analyzed by quantitative real-time polymerase chain reaction. Each sample was analyzed in triplicate. Bars show the mean and SD ratio of *GADD45 β* to *GAPDH*. **B**, Self Organizing Map analysis of gene expression in cartilage samples from patients with early or severe OA. The algorithm generated 30 distinct clusters (c0–c29), each representing genes with a similar expression pattern. Each square in the grid corresponds to 1 cluster. The number in the top left of each square is the cluster ID. The yellow box around c0 marks the first cluster obtained in the analysis. The number in the top middle of each square is the number of genes in the cluster. Blue dots represent the average expression patterns of the genes in each cluster in (from left to right) severe OA sample 1, severe OA sample 2, severe OA sample 3, early OA sample 1, early OA sample 2, and early OA sample 3. Red lines represent the variation from the average expression pattern. Note that severe OA sample 3 is an outlier with respect to genes that are up-regulated in severe OA. **C**, Hierarchical clustering analysis of the microarray data from cartilage samples obtained from 3 patients with early OA and 3 patients with late OA, analyzed using Affymetrix GeneChip U133A. Up-regulated genes are shown in red; down-regulated genes are shown in green. Black indicates no change. *GADD45 β* gene expression was up-regulated in early OA, along with genes involved in cell cycle regulation, such as *DDX11*, *HRASLS3*, and *CCNG1*, and in mitochondrial function, such as *OXA1L* and *TOMM20*, as well as other genes involved in intracellular functions. The genes encoding types I, II, III, and V collagen were all up-

regulated in late OA compared with early OA, as were ASPN, MTN3, and the Wnt-associated genes, WIF1 and DKK3.



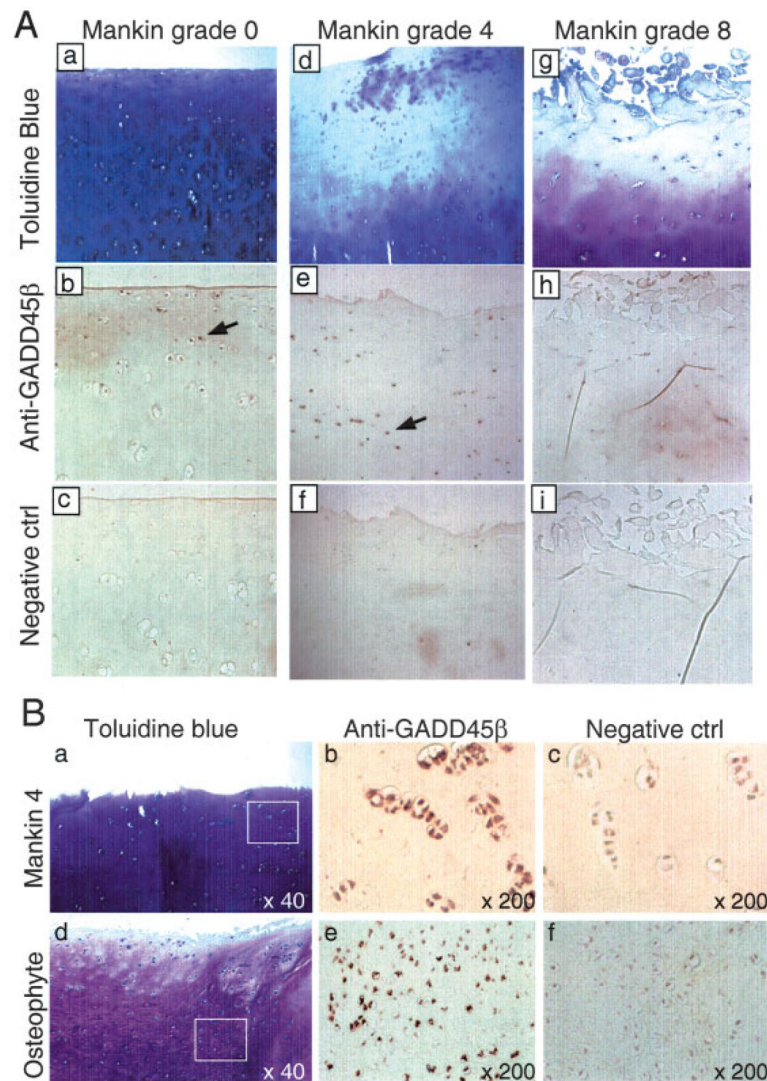


Figure 3. Immunohistochemical analysis of GADD45 β expression in representative specimens of human adult articular cartilage, including those used in the microarray analyses. **A**, Cartilage sections from a normal donor (Mankin grade 0) (a–c), a patient with early OA (Mankin grade 4) (d–f), and a patient with late-stage OA (Mankin grade 8) (g–i) were stained with toluidine blue or subjected to immunohistochemistry using anti-GADD45 β or normal goat IgG (negative control). **Arrows** in **b** and **e** show single cells with nuclear staining (original magnification $\times 40$). **B**, Sections with chondrocyte clusters in the middle zone from a patient with early OA (Mankin grade 4) (a–c) and an osteophyte from a patient with late OA (Mankin grade 8) (d–f) were stained with toluidine blue or immunostained with anti-GADD45 β or normal goat IgG. Boxed areas in **a** and **d** are shown at higher magnification in **b** and **e**. See Figure 1 for other definitions.

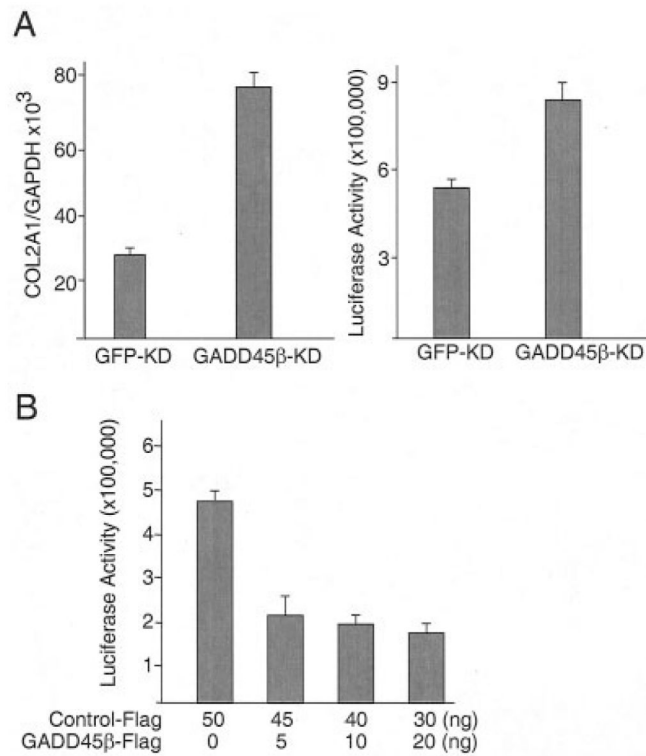


Figure 4. Increased COL2A1 mRNA and promoter activity after small interfering RNA (siRNA) blockade of endogenous growth arrest and DNA damage-inducible protein β (GADD45 β). **A**, Total RNA was extracted from cultures of C-28/I2 cells transduced with lentiviral siRNA–green fluorescent protein (GFP) (GFP–knockdown [KD]) or siRNA–GADD45 β (GADD45 β -KD) (left). Quantitative real-time reverse transcriptase–polymerase chain reaction analysis was performed to determine the levels of COL2A1 mRNA normalized to GAPDH mRNA. GFP-KD and GADD45 β -KD C-28/I2 cells were transfected with pGL2-B/4.0, and COL2A1 promoter–driven luciferase activity was measured (right). **B**, C-28/I2 cells were cotransfected with pGL2-B/4.0 (COL2A1, spanning –577 to +3,428 bp) and pcDNA3-FLAG (control) or increasing amounts of GADD45 β -FLAG, as indicated. Bars show the mean and SD of triplicate wells.

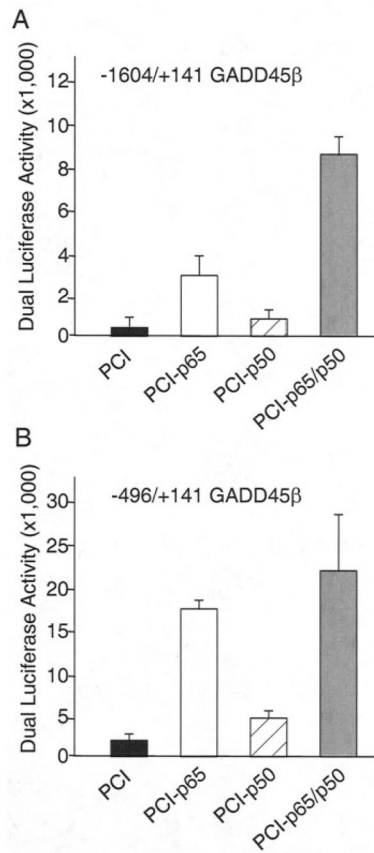


Figure 5.

Induction of growth arrest and DNA damage–inducible protein β (GADD45 β) by NF- κ B in chondrocytes. C-28/I2 cells were cotransfected with pGL2-GADD45 β promoter constructs –1,604 to +141 bp (A) and –496 to +141 bp (B), and the empty vector, pCI, or expression vector encoding the NF- κ B subunit p65 alone, the NF- κ B subunit p50 alone, or both p65 and p50. Bars show the mean and SD.

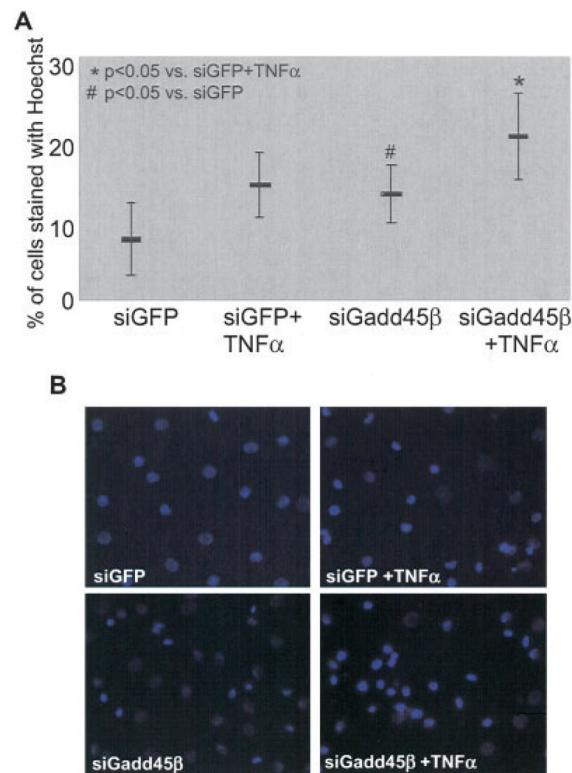


Figure 6.

Growth arrest and DNA damage-inducible protein β (GADD45 β) is a cell survival factor in human articular chondrocytes. Primary cultures were passaged at 2.5×10^4 cells/cm², grown to confluence, transfected with small interfering RNA (siRNA)-GFP (siGFP) or siRNA-GADD45 β oligos, and treated 72 hours later with tumor necrosis factor α (TNF α) for 24 hours. **A**, Cell death was evaluated by staining with Hoechst 33342 and counting the number of nuclei with brightly stained condensed chromatin. Bars show the mean \pm SD percentage of cells showing nuclear condensation and fragmentation relative to the total number of cells counted (200 cells per data point). Results are from 4 independent experiments, each performed in triplicate. **B**, Representative photomicrographs of Hoechst-stained cells from 1 set of cultures isolated from a single patient are shown.