Multiple Signals Induce Endoplasmic Reticulum Stress in Both Primary and Immortalized Chondrocytes Resulting in Loss of Differentiation, Impaired Cell Growth, and Apoptosis*

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The endoplasmic reticulum is the site of synthesis and folding of secretory proteins and is sensitive to changes in the internal and external environment of the cell. Both physiological and pathological conditions may perturb the function of the endoplasmic reticulum, resulting in endoplasmic reticulum stress. The chondrocyte is the only resident cell found in cartilage and is responsible for synthesis and turnover of the abundant extracellular matrix and may be sensitive to endoplasmic reticulum stress. Here we report that glucose withdrawal, tunicamycin, and thapsigargin induce up-regulation of GADD153 and caspase-12, two markers of endoplasmic reticulum stress, in both primary chondrocytes and a chondrocyte cell line. Other agents such as interleukin-1β or tumor necrosis factor α induced a minimal or no induction of GADD153, respectively. The endoplasmic reticulum stress resulted in decreased chondrocyte growth based on cell counts, up-regulation of p21, and decreased PCNA expression. In addition, perturbation of endoplasmic reticulum function resulted in decreased accumulation of an Alcian Blue positive matrix by chondrocytes and decreased expression of type II collagen at the protein level. Further, quantitative real-time PCR was used to demonstrate a down-regulation of interleukin-1, and link protein in chondrocytes exposed to endoplasmic reticulum stress. The chondrocyte is responsible for both synthesis and turnover of the abundant extracellular matrix, and may be sensitive to endoplasmic reticulum stress. Here we report that glucose withdrawal, tunicamycin, and thapsigargin induce up-regulation of GADD153 and caspase-12, two markers of endoplasmic reticulum stress, in both primary chondrocytes and a chondrocyte cell line. Other agents such as interleukin-1β or tumor necrosis factor α induced a minimal or no induction of GADD153, respectively. The endoplasmic reticulum stress resulted in decreased chondrocyte growth based on cell counts, up-regulation of p21, and decreased PCNA expression. In addition, perturbation of endoplasmic reticulum function resulted in decreased accumulation of an Alcian Blue positive matrix by chondrocytes and decreased expression of type II collagen at the protein level. Further, quantitative real-time PCR was used to demonstrate a down-regulation of interleukin-1, and link protein in chondrocytes exposed to endoplasmic reticulum stress-inducing conditions. Ultimately, endoplasmic reticulum stress resulted in chondrocyte apoptosis, as evidenced by DNA fragmentation and annexin V staining. These findings have potentially important implications regarding consequences of endoplasmic reticulum stress in cartilage biology.

Cartilage is a connective tissue that serves multiple functions in the developing embryo and in postnatal life. The chondrocyte is the only resident cell found in cartilage and is responsible for both synthesis and turnover of the abundant extracellular matrix (ECM).1 During development, chondrocytes proliferate and secrete ECM forming the cartilage template of the skeleton (1). In the adult, the chondrocytes secrete and maintain ECM that plays a role in growth, mechanical support, and the function of diarthrodial joints between bones (2, 3). The chondrocytes reside in an ECM that is neither vascularized nor innervated, therefore nutrient and waste exchange occurs through diffusion under both normal and pathological conditions.

The cartilage ECM is composed of collagenous proteins, mainly type II in hyaline cartilage, glycoproteins such as link protein, proteoglycans such as aggrecan, and glycosaminoglycans including chondroitin sulfate, heparin sulfate, and hyaluronic acid (4). The precursors of these macromolecules are synthesized in the endoplasmic reticulum (ER), modified and matured in the Golgi, and secrete and assembled into the ECM (5–7). A malfunction in this process because of internal or external problems may harm the chondrocyte and/or result in the production of a defective ECM that causes skeletal abnormalities (8). For example, pseudoachondroplasia (PASCH) and multiple epiphyseal dysplasia (MED) are autosomal dominant skeletal dysplasias resulting from mutations in the gene coding for cartilage oligomeric protein (COMP), a glycoprotein found in the ECM (9, 10). PASCH and MED patients are characterized by short stature, joint pain, and early onset of osteoarthritis (11). At the ultrastructural level there are enlarged ER vesicles containing accumulated COMP along with type IX collagen and aggrecan (12, 13). Another example is Nanomelia, a lethal autosomal recessive defect in chickens resulting from production of defective aggrecan which accumulates in the ER and disrupts normal chondrocyte metabolism (6, 8). These studies highlight the importance of the ER-Golgi system for the normal function of chondrocytes.

In eukaryotic cells, the ER is a major storage organelle for calcium and site of synthesis and folding of secretory proteins, cell membrane proteins, and lysosomal proteins, prior to their transport to the Golgi for further modification (14). Under physiological and pathological conditions, such as nutrient deprivation and oxidative stress, the function of the ER is impaired resulting in “ER stress” (15). During ER stress, cells may undergo metabolic arrest and activate specific pathways to survive (16). If the functions of the ER are severely impaired, apoptosis may occur to eliminate the cells from the organism (16, 17).

ER stress likely plays an important role in cartilage biology. Chondrocytes experience a variety of stresses such as osmotic stress (18), oxidative stress (19), and mechanical stress (20), which may act on matrix synthesis (21, 22). In addition, the avascular condition of cartilage renders the chondrocytes sensitive to potential depletion of nutrients, such as glucose and oxygen. Despite its obvious importance, little is known about the ER stress response in chondrocytes or the effects of ER
stress on chondrocyte proliferation and matrix gene expression. A recent report suggests that multiple outcomes may exist following induction of ER stress in chondrocytes (23). However, this study used a single immortalized chondrocyte cell line and did not report a clear consensus regarding the potential of different agents to induce ER stress in this cell model. Additional studies are needed to fully understand the action of various inducers of ER stress on chondrocytes and the consequences of ER stress.

Here we show that a variety of agents, including glucose deprivation, induce ER stress markers in primary and immortalized chondrocytes. Further, we demonstrate that ER stress inhibits chondrocyte growth, causes down-regulation of cartilage matrix expression at both the protein and mRNA level, and ultimately induces chondrocyte apoptosis.

MATERIALS AND METHODS

Cell Culture and Treatments—Immortalized rat chondrocytes (IRC) were maintained in culture as previously described (24). These cells have a well-characterized phenotype that is similar to articular chondrocytes. Experiments were performed with cells seeded in monolayer culture at a density of 0.6 × 10^6 cells/well of 6-well plates. Articular chondrocytes were isolated from femoral heads of 6-day-old Sprague-Dawley rats (Charles Rivers). Articular cartilage was aseptically dissected and minced and digested with gentle stirring for 2 h at 37 °C in HBSS containing 0.4% (w/v) collagenase (Worthington Biochemical Corporation, Lakewood, NJ). Cells were then washed and seeded in T-75 culture flasks. Experiments were performed with second passage cultures at a density of 0.6 × 10^6 cells/well of 6-well plates.

The cells were cultured in Ham’s F-12 medium (Invitrogen, Life Technologies, Inc.) +10% fetal bovine serum, with or without glucose. The following treatments were used where indicated: 1 µg/ml tunica-vinmycin (TN, Sigma), 0.5 µg thapsigargin (TG, Sigma), 3 µg/ml retinoic acid (Sigma), 10 ng/ml TNF-α (Genzyme, Ann Arbor, MI), 1 ng/ml IL-1β (Genzyme, Boston), 100 µM sodium nitroprusside (SNP, Sigma), 10 nM STP (staurosporine, Sigma), and 50 µM ZVAD-fmk (Alexis Biochemicals, San Diego, CA).

Cell Proliferation Assay—A CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega) was used to determine the viability of the cells, according to the instructions of the manufacturer. Briefly, 1 × 10^5 cells were seeded per well in 96-well plates, and at 24, 48, or 72 h, 20 µl of the aqueous solution were added into each well of control and experimental cultures and incubated at 37 °C for 2 h. Cell viability was measured using a colorimetric 96-well plate reader.

Western Blot Assay—Cells were collected by centrifugation and suspended in radioimmunoprecipitation assay buffer with protease inhibitors as previously described (25). After vortexing, the lysates were clarified by centrifugation, and supernatants were collected. The protein concentration was determined using the BCA assay reagent kit (Pierce). Equal amounts of cell protein were resolved on 12% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride (PVDF), using the Invitrogen X cell electrophoresis and blotting system (Invitrogen). The membranes were blocked in 5% (w/v) nonfat milk 0.1% Tween TBS buffer for 2 h, and were then incubated with the primary antibody overnight. Mouse monoclonal anti-GADD153 antibody (Santa Cruz Biotechnology) was used at 1:500 dilution; rat monoclonal anti-caspase-12 (Santa Cruz Biotechnology) was used at 1:1000 dilution; mouse monoclonal anti-p21 (Santa Cruz Biotechnology) was used at 1:500; goat anti-collagen II (Santa Cruz Biotechnology) was used at 1:1000 dilution; goat monoclonal anti-actin (Santa Cruz Biotechnology) was used at 1:1000 dilution; rabbit polyclonal anti-caspase-3 (Cell Signaling, Beverly, MA) was used at 1:1000 dilution. After four washes with 0.1% Tween TBS buffer, the membranes were incubated with horseradish peroxidase-linked anti-mouse, anti-rat, anti-rabbit, or anti-goat secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The peroxidase activity was visualized using an ECL system (Pierce).

Quantitative RT-PCR—RNA isolation and reverse transcription were carried out as previously described (26). Briefly, total RNA was isolated by using TRIzol reagent (Invitrogen). Genomic DNA was removed by treating with DNase (Invitrogen) according to the manufacturer’s instructions. RNA was reverse-transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. Reverse transcription was performed at 37 °C for 60 min.

Primers for quantitative real-time PCR were designed as previously described (26) and synthesized by Fisher Scientific. The primer sequences are as follows: Collagen IIα1 forward primer 5′-GAGTTGAAGACCGGAGACTCTG-3′, Collagen IIα1 reverse primer 5′-CTCATGTT-TGCAAAGACTTCCA-3′; Aggrecan forward primer 5′-CTAGCTGCT-TAGCGGATACAGC-3′, Aggrecan reverse primer 5′-TGACCCTGCA-AAGCTGAAAC-3′; Link protein forward primer 5′-CTGGTA TTGTT-GCAATGCT-3′, Link protein reverse primer 5′-AGGCTCATGCTG-TGGCGAGCTC-3′; FGF-2 forward primer 5′-CCAGGGTCCATAC3′-; PCNA reverse primer 5′-CCACGAGGCTCTTCACTGAT-3′; 18S forward primer 5′-AGTCTCGTCCTTGTTCACA-3′, 18S reverse primer 5′-GATCCAGGCGCCTCATAAAC-3′. 18S primers were used at 50 ng, other primers were used at 100 ng, and 18S was used as the normalizer. All primers were verified to have a similar efficiency of amplification according to the manufacturer.

The quantitative PCR was performed as previously described (26). Briefly, the 12-well plates in standard conditions as described above were transfected with Col2α1-luciferase construct (generous gift from Dr. V. Lefebvre) (1.5 µg/well) and pSV-β-galactosidase control vector (0.5 µg/ well using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Transfected cells were maintained in complete medium for 24 h, then treated with glucose withdrawal, 1 µg/ml tunica-vinmycin, and 0.5 µg thapsigargin for 24 h, and collected for analysis. Luciferase and β-galactosidase activity were detected with Promega Reporter assay system.

Immunocytochemistry—Chondrocytes were cultured in 4-well chamber slides at 0.2 × 10^5 cells/well, and annexin V-FITC (ApopTect Detection kit, CalbioChem). For detection of collagen II, IRC chondrocytes grown on chamber slides were washed with PBS and fixed with 5% formalin for 20 min followed by 0.2% Triton X-100 for 30 min at room temperature. The fixed and permeabilized cells were washed with PBS and blocked with 1% bovine serum albumin for 1 h at room temperature. After washing with PBS, cells were incubated with an anti-collagen II antibody (Santa Cruz Biotechnology) for 2 h followed by a fluorescein isothiocyanate-conjugated secondary antibody. Next, the cells were incubated at room temperature with a 1:1000 dilution of 2 µg/ml of anti-collagen type II blocking peptide (Santa Cruz Biotechnology) was used as negative control after incubated with anti-collagen type II antibody for 2 h at room temperature at 10:1 dilution. The stained cells were examined under a confocal laser microscope (Olympus, Fluoview FV300).

Nitric Oxide Assay—Analysis of nitric oxide (NO) levels was performed as previously described (25). 100 µl of medium was removed from each well at the time points indicated. NO levels were determined using the Griess reaction. The OD of the samples was compared with a standard curve generated by diluting a 20 mM stock of NaNO_2 in Ham's F-12 medium.
GADD153 expression level was recovered after glucose withdrawal at 72 h (Fig. 1A). However, glucose deprivation resulted in a strong induction of GADD153 at 24 h, which remained elevated at 48 h. The expression of procaspase-12 and cleaved (active) caspase-12 was detected by Western blot analysis. Active caspase-12 is expressed as a doublet of ~35 kDa. Glucose deprivation resulted in an increase in both the pro- and cleaved form of caspase-12. In addition to glucose withdrawal, TN and TG also induced the expression of both the pro- and cleaved form of caspase-12. The expression level of β-actin was used as a loading control.

RESULTS

Induction of ER Stress in Chondrocytes—The initial hypothesis under study was that disruption of ER function in chondrocytes by multiple signals would result in a typical ER stress response. This hypothesis was tested using the well-known ER stress marker, growth arrest, and DNA-damage inducible protein 153 (GADD153) (30). IRC cells maintained in medium containing glucose did not express GADD153 for up to 48 h in culture (Fig. 1A). However, glucose deprivation resulted in a strong induction of GADD153 at 24 h, which remained elevated at 48 h (Fig. 1A).

We extended this study to test other potential ER stress-inducing agents. Tunicamycin is a pharmacological inhibitor of N-linked glycosylation that causes defects in glycoprotein trafficking between the ER and Golgi, producing ER stress and inducing apoptosis in some cell types (31). Thapsigargin is a selective inhibitor of the Ca\(^{2+}\)-ATPase (SERCA) of the ER, which potentially induces ER stress by disturbing ER calcium (32). Both tunicamycin and thapsigargin resulted in induction of GADD153 in chondrocytes to a greater extent than that with glucose withdrawal at 72 h (Fig. 1B).

Select immortalized chondrocyte lines may be good models for studying ER stress; however, it is critical to first establish that an identical response can be observed in primary chondrocytes. Primary rat chondrocytes were subjected to the same treatments and an identical pattern of induction of GADD153 was observed as compared with the IRC chondrocytes (Fig. 1C).

Caspase-12 is a murine caspase, which is specifically activated in the cells subjected to ER stress (16). In order to confirm the induction of ER stress in chondrocytes, we examined caspase-12 expression. As shown in Fig. 2A, glucose deprivation up-regulated both procaspase-12 and activated caspase-12 in chondrocytes. Compared with 24 h, there was more procaspase-12 cleaved into activated caspase-12 at 48 h. In addition to glucose withdrawal, TN and TG also up-regulated both pro- and active caspase-12 in chondrocytes (Fig. 2B).

Cytokines such as TNF-α and IL-1β have a wide range of effects on chondrocytes including suppression of cartilage matrix synthesis and stimulation of matrix metalloproteinase expression, perhaps through the production of nitric oxide (21). In this context, we compared the ability of nitric oxide to induce ER stress, as measured by GADD153, to agents that directly perturb ER function. Treatment of chondrocytes with TNF-α, IL-1β, or SNP stimulated nitric oxide production ~10–30-fold over control cells, whereas there was no induction of NO in cells treated with the three ER stress inducers (data not shown). No induction of GADD153 was observed with TNF-α or SNP, and only a minimal response was observed with IL-1β (Fig. 3).

ER Stress Results in Decreased Matrix Expression in Chondrocytes—The ER is a principle site of protein synthesis and folding, as well as a site of biosynthesis for cartilage matrix by chondrocytes. Therefore we postulated that ER stress might be accompanied by effects on extracellular matrix synthesis. IRC cells were exposed to glucose withdrawal, TN and TG as described previously. At 24, 48, and 72 h, the cell monolayer was stained with Alcian Blue, and the cell-associated proteoglycan was extracted and quantified. Under control conditions, there was a progressive increase in cell-associated proteoglycan (Fig. 4A). In contrast, there was no accumulation of proteoglycan...
under ER stress-inducing conditions (Fig. 4A). Along with the decreased matrix accumulation, the chondrocytes exposed to ER stress-inducing conditions also showed morphological changes and accumulated less pericellular matrix than control cells (Fig. 4, B–E). Identical results were obtained with primary rat chondrocytes (data not shown). The results using Alcian Blue staining could be due, in part, to a reduction in total cell number. Therefore, we carried out studies to look at matrix gene and protein expression normalized to individual chondrocytes. First, total RNA from IRC chondrocytes and primary rat chondrocytes was isolated, and used to carry out quantitative real-time PCR with primers for collagen II, link protein and aggrecan. By 48 h we began to see decreased matrix gene expression (Fig. 5A), and by 72 h the mRNA coding for all three proteins was greatly decreased (Fig. 5B). At both time points, the expression of collagen II mRNA was preferentially inhibited compared with aggrecan and link protein mRNA. Most significantly, the primary rat chondrocytes showed a similar response with regard to down-regulation of matrix gene expression including the preferential effect on collagen II (Fig. 5C). The expression level of 18 S mRNA showed no significant change with the different treatments at either time point (Fig. 5D).

Because all the three treatments exerted a greater effect on collagen type II mRNA expression compared with aggrecan and link protein, we further examined the transcriptional activity of collagen type II under ER stress condition. Sox9 is a master transcription factor, which regulates cartilage-specific gene expression, including col2a and aggrecan (33). Sox9-dependent collagen type II promoter activity was detected by using a collagen type II promoter, which has collagen type promoter and four tandem repeats of Sox9 binding site in the enhancer site (33). All three ER stress treatments resulted in decreased Sox9-dependent collagen type II promoter activity after 24 h (Fig. 5E) compared with control, with glucose withdrawal and TG having more significant effects. Finally, immunoocytochemical staining and Western blot analysis revealed that type II collagen expression and/or accumulation was greatly decreased in chondrocytes after 48 h of exposure to ER stress inducers (Fig. 6, A–E), whereas there was no positive signal in control cells stained by collagen type blocking peptide (data not show). Taken together, these results indicate that ER stress results in an inhibition of matrix expression at the level of mRNA that results in less matrix produced by individual chondrocytes.

**ER Stress Results in Chondrocyte Growth Arrest**—The proper function of the ER is not only essential to the quality and quantity of protein, but also to overall cell behavior. Thus, we determined the effect of induction of ER stress on the growth and viability of chondrocytes. IRC and primary rat chondrocytes were incubated for 24 h, followed by 24, 48, and 72 h treatments with glucose withdrawal, 1 μg/ml TN, and 0.5 μM TG. Control chondrocytes showed an increase in cell number, whereas both the IRC and primary chondrocytes subjected to ER stress were either static or declined with respect to cell number (Fig. 7). To determine if cell cycle arrest is partially responsible for the growth arrest, the level of expression of p21 in chondrocytes was examined. There was a clear up-regulation of p21 in IRC chondrocytes under ER stress conditions induced by all three ER stress inducers (Fig. 8A). In
FIG. 5. ER stress results in down-regulation of cartilage matrix gene expression in both IRC and primary rat chondrocytes. A and B, IRC chondrocytes were cultured under control conditions or exposed to the ER stress-inducing treatments of glucose withdrawal ($-\text{Glu}$) or TN or TG for 48 h (A) or 72 h (B). Quantitative RT-PCR was performed on reverse-transcribed RNA isolated from each condition (see text for details). The results are presented as relative expression of mRNA coding for type II collagen, aggrecan, or link protein (LP) in treated versus control chondrocytes. At 48 h there is a $\geq 90\%$ decrease in mRNA coding for collagen II with all three treatments compared with control cells. The level of mRNA coding for aggrecan and LP is also suppressed at this time point, but to a lesser degree than collagen II. By 72 h, the steady state level of mRNA coding for all three matrix proteins is down-regulated by 85% or greater compared with control chondrocytes. Data shown are relative expression levels of mRNA calculated relative to the expression level in control cells for each mRNA transcript (see text for details and controls).

C, inhibition of steady state mRNAs coding for collagen II, aggrecan, and LP was also observed with all three ER stress-inducing agents in primary rat chondrocytes. D, there was no significant change in the expression level of the gene coding for 18 S ribosomal subunit with any treatment compared with control cells. Data shown are the mean $\pm$ S.D. of three independent experiments. E, ER stress results in decreased Sox9-dependent collagen type II promoter activity. IRC chondrocyte were grown in 12-well plates in normal medium for 24 h, then transfected with 1.5 $\mu$g/well Col2α1-luciferase construct and 0.5 $\mu$g/well pSV-β-galactosidase control vectors. After 24 h, the transfected cells were treated with glucose withdrawal ($-\text{Glu}$), TN or TG for 24 h. Luciferase activity was normalized against β-galactosidase activity. The data are shown as the relative luciferase activity of treated cells compared with control which is set at 1.0. All values are expressed as mean $\pm$ S.D. of three experiments carried out in duplicate.
addition, the expression of the p21 partner, PCNA, was decreased at 48 and 72 h under ER stress conditions (Fig. 8B).

ER Stress Affects Chondrocytes Survival—Prolonged ER stress can result in apoptosis (17). To detect whether ER stress can induce apoptosis in chondrocytes, we first performed a DNA fragmentation assay. IRC cells were treated with glucose withdrawal, TN, or TG for 24, 48, and 72 h. It was reported previously (34) that retinoic acid (RA) induces apoptosis in IRC chondrocytes; therefore, we used RA as a positive control. All three ER stress-inducing agents stimulated the chondrocytes to carry out DNA fragmentation (Fig. 9). It is necessary to confirm apoptosis with multiple endpoints. Therefore, we also examined annexin V-PI staining as a confirmation of the DNA ladder results. Compared with control IRC cells, chondrocytes exposed to all three ER stress inducers showed annexin V staining with varying degrees of PI labeling (Fig. 10, A–E).

Taken together, these results strongly suggest that ER stress in chondrocytes induced by glucose withdrawal as well as pharmacological agents ultimately can result in apoptosis.

Sequential Relationship of ER Stress to Apoptosis—It was hypothesized that early consequences of ER stress would precede apoptosis. A time course study was carried out to determine the time point following induction of ER stress when chondrocytes were undergoing apoptosis. No significant evidence of apoptosis was observed until at least 12 h after treatment with glucose withdrawal or TG (Fig. 11A). Tunicamycin treatment did not result in DNA fragmentation until at least 18 h of treatment (data not shown). In addition, the effector caspase, caspase-3, did not show significant activation until 12 h of exposure to the ER stress-inducing agents (Fig. 11B).

Next, to separate the early ER stress response from end stage apoptosis, we examined expression of ER stress markers at two time points prior to the onset of apoptosis. At 6 h, there was increased pro-caspase-12 expression in treated cells compared with control and by 9 h, there was an obvious increase in the level of activated caspase-12 compared with control (Fig. 12A). Expression of GADD153 was observed in chondrocytes exposed to glucose withdrawal and TG by 6 h, and at 9 h all three agents induced GADD153 expression compared with control (Fig. 12A). The expression of type II collagen was clearly down-regulated well before any evidence of chondrocyte apoptosis and showed a correlation with the ER stress response. Specifically, there was almost complete loss of collagen II expression by 6 h of treatment with glucose withdrawal and TG, with a slower time course for loss of collagen II observed.
following treatment with TN (Fig. 12A).

A second approach was used to test the hypothesis that ER stress resulted in loss of matrix expression independent of full apoptosis. Here chondrocytes were exposed to ER stress-inducing agents in the presence of a general caspase inhibitor, zVAD. The presence of zVAD fully inhibited the formation of a DNA ladder at 24 h (data not shown). However, even in the absence of full apoptosis there was still up-regulation of GADD153 and suppression of collagen II expression. Taken together, these results suggested that glucose withdrawal, TN, and TG induce ER stress with a proximal effect on down-regulation of matrix expression followed by apoptosis.

DISCUSSION

The mammalian stress response is an evolutionarily conserved mechanism that protects and allows cells to respond to a variety of environmental and metabolic conditions. ER stress occurs under physiological conditions, and the ER stress response plays an essential role in cell growth and differentiation (30, 35). Takao et al. (36) used a transgenic mouse model to monitor ER stress in vivo, and demonstrated that ER stress occurred in a variety of organs such as liver, spleen, brain, and kidney. However, little is known about the role of ER stress in cartilage. Here we present the first demonstration that a physiologically relevant signal such as decreased glucose can induce ER stress in primary rat chondrocytes as well as a chondrocyte cell line. The ER stress response was similar to that induced by pharmacological agents such as tunicamycin and thapsigargin in that both GADD153 and caspase-12 were up-regulated.

![Figure 8](http://www.jbc.org/) Increased p21 and decreased PCNA expression during ER stress in chondrocytes. A, p21 expression level was detected by Western blot analysis in IRC chondrocytes cultured under control conditions or exposed to ER stress-inducing agents for 48 or 72 h. There was a clear up-regulation of p21 with all three ER stress-inducing treatments. B, steady state level of mRNA coding for PCNA was determined by quantitative real-time PCR in control and experimental IRC chondrocytes. All three ER stress-inducing agents produced a down-regulation of PCNA mRNA. Data shown are the mean ± S.D. of three independent experiments.

![Figure 9](http://www.jbc.org/) DNA fragmentation is induced in chondrocytes exposed to ER stress-inducing treatments. All three ER stress-inducing treatments resulted in clear DNA fragmentation. There was no evidence of DNA fragmentation observed in control IRC chondrocytes for up to 72 h of culture. The analysis was performed on cytosolic DNA fractionated on a 1.8% agarose gel.

![Figure 10](http://www.jbc.org/) ER stress in chondrocytes results in externalization of annexin V. A, control IRC cells show minimal staining for annexin V or PI. B–D, IRC chondrocytes cultured in the absence of glucose (B) for 48 h showed abundant staining with annexin V and minimal staining with PI. Similar results were observed with exposure to TN (C) and TG (D). Each panel is shown as an overlay image of annexin V and PI double staining.

FIG. 10. ER stress in chondrocytes results in externalization of annexin V. A, control IRC cells show minimal staining for annexin V or PI. B–D, IRC chondrocytes cultured in the absence of glucose (B) for 48 h showed abundant staining with annexin V and minimal staining with PI. Similar results were observed with exposure to TN (C) and TG (D). Each panel is shown as an overlay image of annexin V and PI double staining.
GADD153 is a transcription factor expressed at low levels under homeostatic conditions and is highly induced by a variety of signals that result in ER stress (30). Caspase-12 is an ER-localized caspase, which is specifically induced by ER stress and not activated by other types of stress (37). A recent report indicated that the pharmacological agent tunicamycin could up-regulate GADD153 in an immortalized chondrocyte cell line and result in apoptosis (23). However, no experiments were conducted either with nontransformed cells or with physiological signals such as glucose availability. Adult cartilage is an avascular tissue, and chondrocytes are sensitive to potential depletion of nutrients, such as glucose, and oxygen. As the only resident cells, chondrocytes also experience a variety of other stresses, including oxidative stress and mechanical stress (19, 20). The induction of NO results in a variety of effects on the chondrocyte including oxidative stress and metalloproteinase expression (21). We did not observe a dramatic up-regulation of GADD153 either with cytokine treatments or by direct stimulation of NO production using the NO donor, SNP. These results are consistent, to some extent, with a recent report suggesting that IL-1 does not induce GADD153 but does up-regulate the expression of GRP78 (38), an ER chaperone through the production of NO (23). However, in that study the induction of GRP78 was only observed with semi-quantitative PCR and no induction was observed at the protein level. Whereas additional studies are needed, it is hypothesized that under physiological conditions, disruption of glucose homeostasis may be a major cause of ER stress in chondrocytes. This response is not restricted to chondrocytes since a previous study showed that glucose deprivation led to an up-regulation of GADD153 in an adipocyte cell line (39).

The high matrix to cell volume ratio in cartilage makes the synthetic activity of individual chondrocytes very important (3). Here we demonstrate that ER stress is associated with decreased expression of collagen II at the protein level, by both immunocytochemistry and Western blot analysis. Further, we demonstrated that ER stress in chondrocytes is associated with down-regulation of steady state levels of mRNAs coding for collagen II as well as aggrecan and link protein. It is likely that there are general effects on chondrocyte metabolism during ER stress. However, the lack of change in level of 18 S mRNA or the cytoskeletal protein actin, suggest that ER stress is preferentially affecting the expression of the genes coding for cartilage matrix proteins. To understand the mechanism underlying the effect of ER stress on expression of collagen II by chondrocytes, we determined the level of Sox9-dependent collagen type II promoter activity. Based on the observed decrease in Sox9-dependent collagen type II promoter activity, it is reasonable to speculate that ER stress has a negative feedback effect on transcription of the collagen II gene and perhaps other

**FIG. 11.** Time course studies for determination of apoptosis response. IRC chondrocytes were cultured in control conditions (CTRL) or exposed to glucose withdrawal, TN, or TG for 6, 9, or 12 h. A, DNA ladder assay was performed to detect the present of apoptosis. B, expression level of caspase-3 was detected by Western blot analysis at 9 and 12 h. No evidence of apoptosis was detected before 12 h based on the presence of DNA ladder formation. Significant activated caspase-3 was not observed until 12 h with all three treatments.

**FIG. 12.** ER stress response and down-regulation of collagen II expression occurred prior to the onset of apoptosis. IRC chondrocytes were cultured in control conditions (CTRL) or exposed to glucose withdrawal (−Glu), TN, or TG for 6 and 9 h. A, expression levels of caspase-12, GADD153, and collagen type II were detected by Western blot analysis. B, expression levels of collagen type II and GADD153 were detected by Western blot analysis, in the absence or in the present of 50 μM zVAD-fmk.
ER stress can result in cell cycle arrest (40). This cell cycle arrest may allow cells to reestablish homeostasis or to commit to apoptosis. It is clear from our data that the overall increase in cell number that was observed with cultured control chondrocytes did not occur when the cells were subjected to ER stress. In addition, this response was not unique to the IRC cell line but also occurred in primary chondrocytes. Up-regulation of p21 and decreased PCNA expression both suggest that cell cycle arrest is a component of the growth inhibition. The transcription factor GADD153 has been shown to induce cell cycle arrest in other cell types (41). On the other hand, GADD153 can form stable heterodimers with the C/EBP family of transcription factors to activate certain target genes (42). Moreover, it has been reported that C/EBP can interact with p21 and Rb family proteins to mediate cell cycle regulation (43). These studies imply that upon ER stress, induction of GADD153 might result in cell cycle arrest through p21. Currently, one model suggests that another GADD family protein-GADD34 is important for the up-regulation of p21 and subsequent cell cycle arrest (44) by inducing p53 phosphorylation and p21/WAF1 transcription. In future studies we will examine the expression of additional modulators of the ER stress response in chondrocytes such as GADD34, and the mechanism by which GADD153 induces cell cycle arrest.

Prolonged ER stress can lead to cell death (17). Here we report that the initial response of chondrocytes to ER stress is to reduce extracellular matrix expression. Longer term exposure to ER stress leads to apoptosis in chondrocytes as detected both by DNA ladder formation and annexin V staining. In addition, we have evidence for the mechanism by which ER stress activates apoptosis in chondrocytes. GADD153 and caspase-12 are not only ER stress markers, but they also are potential modulators of cell death. GADD153-deficient mice show reduced apoptosis in response to ER stress (45), and overexpression of GADD153 leads to inhibition of cell growth and apoptosis (46). Studies with caspase-12-deficient mice have shown that caspase-12 is required for ER stress-induced apoptosis (37). Moreover, through its function in inhibiting cyclin-dependent kinases, p21 is also a positive regulator of apoptosis in some cases (47). However, ER stress occurred in chondrocytes in the absence of apoptosis, and induction of GADD153 and down-regulation of matrix expression were not recovered when full apoptosis was blocked using the caspase inhibitor-zVAD. These results further support the hypothesis that the induction of ER stress occurs earlier than apoptosis, and the down-regulation of matrix expression was not simply because of loss of cell viability. Our study is the first report of a physiologically relevant signal, decreased glucose, activating ER stress in chondrocytes leading to decreased matrix synthesis, growth inhibition, and eventually apoptosis. In addition, our results with tunicamycin and thapsigargin, along with a recent study (23), also indicate that pharmacological manipulation of ER function in chondrocytes can lead to ER stress and similar consequences.

The cross-talk between ER stress-mediated cell death and mitochondrial apoptosis signaling has been established (16). It has been shown that caspase-9 is a substrate of caspase-12 and leads to the triggering of a specific cascade involving caspase-3 in a cytochrome c-independent manner (48). GADD153 has been recently shown to down-regulate Bcl-2 expression at the transcriptional level contributing to an apoptotic response (46). In this work, we observed that the activation of caspase-3 occurred later than induction of both activated caspase-12 and GADD153. Therefore, it is likely that caspase-3 is part of the downstream response leading to apoptosis. Future work will examine whether cytochrome c release from the mitochondria is observed which will help determine if mitochondria are involved.

Disruption of normal ER function is associated with skeletal diseases. Accumulation of misfolded collagen in the ER, or an overload of abnormal protein in the ER results in ER storage diseases such as Pseudoachondroplasia (PASCH), multiple epiphyseal dysplasia (MED) (9,10), osteogenesis imperfecta (OI), and procollagen type I, II, IV deficiency (49,50). Cartilage aging and age-associated cartilage diseases such as osteoarthritis are associated with increased apoptosis (51). In addition, abnormal glucose metabolism has been implicated in the pathogenesis of osteoarthritis (52, 53). Therefore, it is reasonable to predict that abnormal matrix synthesis, and chondrocyte apoptosis during cartilage aging and diseases might be linked to ER stress.

In summary, we present the first demonstration of a physiologically relevant signal activating a specific molecular pathway related to ER stress in chondrocytes. In addition, we link the consequences of the ER stress response in chondrocytes, namely decreased cartilage matrix expression, decreased chondrocyte growth, and chondrocyte apoptosis, to specific molecular mediators. These findings have broader implications in skeleton development, cartilage aging, cartilage diseases and cartilage degenerative disease. In addition, the chondrocyte is now an excellent model to work out fundamental mechanisms linking ER stress to cell behavior.

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Multiple Signals Induce Endoplasmic Reticulum Stress in Both Primary and Immortalized Chondrocytes Resulting in Loss of Differentiation, Impaired Cell Growth, and Apoptosis

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