Glutamate Inhibits Chondral Mineralization through Apoptotic Cell Death Mediated by Retrograde Operation of the Cystine/Glutamate Antipporter*

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Although we have previously demonstrated the functional significance of excitatory amino acid transporters as well as glutamate (Glu) receptors (GluRs) expressed by chondrocytes, little attention has been paid to the possible expression of the cystine/Glu antiporter responsible for the bidirectional transmembrane transport of Glu in chondrocytes to date. In organotypic cultured mouse embryonic metatarsals isolated before vascularization, the chondral mineralization was significantly decreased in the presence of Glu at a high concentration. Apoptotic cells were detected within the late proliferating and prehypertrophic chondrocytic layers in metatarsals cultured in the presence of Glu. A group III metabotropic GluR (mGluR) antagonist partially, but significantly, prevented the inhibition of mineralization by Glu in metatarsals without affecting the number of apoptotic cells. Both decreased mineralization and apoptosis by Glu were significantly prevented by the addition of the cystine/Glu antiporter inhibitor homocysteic acid, as well as reduced glutathione (GSH) and cystine. Expression of mRNA for xCT and 4F2hc subunits, which are components of the cystine/Glu antiporter, was seen in both cultured mouse metatarsals and rat costal chondrocytes. In chondrocytes cultured with Glu, a significant decrease was seen in intracellular GSH levels, together with increases in the number of apoptotic cells and the level of intracellular reactive oxygen species. These results suggest that Glu could regulate chondrogenic differentiation toward mineralization through a mechanism associated with apoptosis mediated by the depletion of intracellular GSH after the retrograde operation of the cystine/Glu antiporter, in addition to the activation of group III mGluR, in chondrocytes.

In the vertebrate central nervous system, glutamate (Glu) is one of the most abundant free amino acids with a neurotransmitter role involving signaling machineries that include Glu receptors (GluRs)3 and Glu transporters (1, 2). In the glutamatergic synapses, Glu is condensed into synaptic vesicles through vesicular Glu transporters for subsequent exocytotic release into synaptic clefts upon stimulation. Glutamate is supposed to mediate the excitatory neurotransmission through GluRs categorized into two major groups. One is ionotropic Glu-gated ion channels (iGluRs) that are further classified into δL-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainite, and N-methyl-D-aspartate subtypes, whereas the other is G-protein-coupled metabotropic receptors (mGluRs) classified into the three different subtypes, group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, mGluR6, mGluR7, and mGluR8) (1, 2). The group I subtype stimulates the hydrolysis of membrane phospholipids in association with Gα11 protein, whereas both the group II and III subtypes inhibit the formation of cAMP with the aid of Gi/o protein.

However, several independent lines of evidence indicate that Glu may act as a “cytokine” rather than a “neurotransmitter” to affect a variety of cellular activities in different tissues (3, 4). For example, recent studies have shown that Glu may be one of the endogenous paracrine (autocrine) factors used for intercellular communications through particular GluRs in cartilage (5–7) and bone (8, 9). In cultured rat costal chondrocytes where constitutive expression is seen with mRNA for the GluR3 subunit of the iGluR subtype AMPA receptors, the addition of AMPA markedly stimulates the release of endogenous Glu in a Ca2+-dependent manner (5). Moreover, in cultured mouse embryonic metatarsals isolated before vascularization, chondral mineralization was almost completely abolished in the presence of the group III mGluR agonist L-(1)-2-amino-4-phosphonobutyrate (L-AP4) in a manner sensitive to an antagonist, without inducing any apoptotic cell death (6).

On the other hand, Glu transporters are required for the termination of signal transduction mediated by Glu as well as for the prevention of neurotoxicity mediated by this endogenous excitotoxin in the central nervous system. These Glu transporters are classified into 5 different isoforms, including Glu aspartate transporter (EAAT1; excitatory amino acid transporter 1), Glu transporter-1 (EAAT2), excitatory amino acid transporter 2, excitatory amino acid transporter 3 (EAAT3), excitatory amino acid transporter 4 (EAAT4), and excitatory amino acid transporter 5 (EAAT5).
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carrier 1 (EAAT3), EAAT4 and EAAT5 so far (10, 11). In addition to these Glu transporters, the sodium-independent and chloride-dependent high affinity Glu uptake carrier, cystine/Glu antiporter, has been identified in a variety of tissues (12, 13). This antiporter is a heterodimeric complex between the ubiquitous CD98 heavy chain, also referred to as 4F2hc, and the xCT light chain responsible for the determination of substrate specificity. The view that extracellular cystine is incorporated into the cytoplasm through the bi-directional cystine/Glu antiporter as a substrate for the biosynthesis of intracellular glutathione (GSH) is prevailing (14, 15). Although we have demonstrated that extracellular Glu could be brought into intracellular locations through particular EAAT isoforms expressed by the rodent chondrocytes (7), little attention has been paid to the possible expression and functionality of the cystine/Glu antiporter in chondrocytes.

In this study we have investigated the role of the cystine/Glu antiporter in chondral differentiation and cell survival in cultures of primary chondrocytes, prepared from adult rat costal cartilage, and in murine embryonic metatarsals, isolated before vascularization. In these systems, cells differentiate from resting to proliferating, prehypertrophic, hypertrophic, and calcified chondrocytes without the involvement of osteoblasts, osteoclasts, and capillary invasion (16, 17).

EXPERIMENTAL PROCEDURES

Materials—A Quickprep Micro mRNA Purification Kit, a First-strand cDNA Synthesis kit, and a DYEnamic ET Terminator Cycle Sequencing Kit were purchased from Amersham Biosciences, and a Protein Assay Kit was purchased from Bio-Rad. Rabbit polyclonal antibody against the xCT subunit was from Transgenic (Kumamoto, Japan) and an anti-rabbit IgG antibody was supplied by DAKO A/S (Glostrup Denmark). An In Situ Cell Death Detection Kit was purchased from Roche Diagnostics GmbH and Taq DNA polymerase was obtained from Takara (Tokyo, Japan). (RS)-α-Cyclopentyl-4-phosphonophenylglycine (CPPG) was supplied by Tocris Cookson (Bristol, United Kingdom). DMEM and MEM were purchased from Invitrogen. Total Glutathione Quantification Kit was obtained from Dophin (Osaka, Japan). Dihydroudrochlorofluorescein diacetate (DCFDA) was provided by Molecular Probes. In situ hybridization as previously described (6). In brief, tibiae were dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections mounted on slide glasses were fixed with 4% paraformaldehyde, and successively treated with 0.2M HCl and 10 μg/ml proteinase K. Sections were then subjected to the acetylation in 0.1 M triethanolamine, 0.25% acetic anhydride. After pre-hybridization, sections were performed to detect apoptotic cells based on labeling of DNA strand breaks. Metatarsals cultured for 5 days were fixed with 10% formalin neutral buffer solution, followed by the decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C. Metatarsals were then dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections were then mounted in 50% glycerol, and photographs of sections were taken using an Olympus microscope.

Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Assay—TUNEL staining was performed to detect apoptotic cells based on labeling of DNA strand breaks. Metatarsals cultured for 5 days were fixed with 10% formalin neutral buffer solution, followed by the decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C. Metatarsals were then dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections were then subjected to the TUNEL assay with a TUNEL detection kit according to the manufacturer’s instructions.

In Situ Hybridization Analysis—On day 5 cultured metatarsal rudiments were harvested, followed by the fixation with 10% formalin and subsequent decalcification with 20% EDTA for in situ hybridization as previously described (6). In brief, tibiae were dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections mounted on slide glasses were fixed with 4% paraformaldehyde, and successively treated with 0.2 M HCl and 10 μg/ml proteinase K. Sections were then subjected to the acetylation in 0.1 M triethanolamine, 0.25% acetic anhydride. After pre-hybridization, sections were performed to detect apoptotic cells based on labeling of DNA strand breaks. Metatarsals cultured for 5 days were fixed with 10% formalin neutral buffer solution, followed by the decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C. Metatarsals were then dissected for frozen sections with a thickness of 5 μm in a cryostat. Sections were then subjected to the TUNEL assay with a TUNEL detection kit according to the manufacturer’s instructions.

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Determination of Intracellular Reactive Oxygen Species (ROS)—The estimation of intracellular ROS was done using DCFDA as a probe as described previously (18). Chondrocytes were cultured for 5 days, followed by the addition of 1 mM Glu 12 h before the end of culturing. Moreover, metatarsals cultured in the presence of 1 mM Glu for 5 days were also used as samples. Samples were rinsed with PBS twice and then incubated in respective serum-free media containing 10 μM DCFDA at 37 °C for 30 min in a 5% CO2 incubator. In principle, DCFDA diffuses readily into cells, followed by the hydrolysis of ester groups by intracellular esterases and subsequent release of the dichloro derivative. This derivative is then oxidized to the

Negative and positive controls were used. Negative controls were treated with 10 μM DCFDA without the addition of Glu. Positive controls were treated with 10 μM DCFDA and 1 mM Glu. In addition, negative controls were treated with 10 μM DCFDA and 1 mM Glu at 37 °C for 16 h. After being washed, sections were treated with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as a substrate for ALP for 1.5 h at 37 °C, followed by several rinses in phosphate buffer. Sections were finally mounted in 50% glycerol, and photographs of sections were taken using an Olympus microscope.

Histological Analysis—On day 5 cultured metatarsal rudiments were harvested and fixed with 10% formalin neutral buffer solution, followed by the decalcification with 20% EDTA and subsequent immersion in 30% sucrose overnight at 4 °C. Metatarsals were then dissected for sections with a thickness of 5 μm in a cryostat for histological analyses. Sections were stained with alizarin red, hematoxylin and eosin, and Alcian blue under standard procedures, respectively. The activity of alkaline phosphatase (ALP) was determined by the enzymatic histochemistry. In brief, mounted sections were preserved in PBS at room temperature, and then staining was developed by the incubation in ALP buffer (0.01 M Tris-HCl (pH 9.5), 0.1 M NaCl, 0.05 M MgCl2) supplemented with 100-fold diluted solution of nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as a substrate for ALP for 1.5 h at 37 °C, followed by several rinses in phosphate buffer. Sections were finally mounted in 50% glycerol, and photographs of sections were taken using an Olympus microscope.
fluorescent parent dye by intracellular ROS. Cells or metatar-
sals were then washed with PBS twice, followed by the determina-
tion of intracellular ROS under a confocal laser-scanning
microscope (LSM 510; Carl Zeiss) with excitation at 485 nm
and emission at 525 nm, respectively.

**RT-PCR Analysis**—cDNA was synthesized with the oli-
go(dT) primer and reverse transcriptase from extracted total
RNA. PCR amplification was performed using specific primers
(Table 1), and PCR products were subcloned into a TA cloning
vector for the determination of DNA sequences by ABI Prism
7500 Genetic Analyzer (PerkinElmer Life Sciences) using a cycle
sequencing kit. Semi-quantitative PCR analyses were per-
duced below 30 cycles, where a linear response was achieved,
using primers for the housekeeping gene glyceraldehyde-3-
phosphate dehydrogenase.

**Immunohistochemistry**—Sections were prepared from tibia
of newborn mice at 1 day after birth (P1), fixed with 4%
paraformaldehyde in PBS for 20 min, washed with PBS, treated
with 0.3% hydrogen peroxide (H2O2) in methanol for 30 min,
and washed with 70% ethanol for 5 min, respectively. After
being washed with PBS, sections were subjected to blocking
with PBS containing normal goat serum or bovine serum albu-
min and washed with 70% ethanol for 5 min. The pellets were sus-
fuged at 5000 g for 5 min. The supernatant fractions
were isolated from adult female Wistar rat ribs, followed by the
isolation of chondrocytes in DMEM containing 10% fetal bovine
serum and antibiotics, and then centrifuged at 500 × g for 5 min. The pellets were sus-
fuged in DMEM containing 10% fetal bovine serum. Cells
were plated at a density of 4 × 104 cells/cm², followed by cul-
turing at 37 °C under 5% CO2 for additional 6 days. Culture
medium was exchanged to DMEM supplemented with 10% fetal bovine serum and 50 µg/ml ascorbic acid for subsequent
cultivation for different periods of up to 28 days. Medium was
dchanged every 2 to 3 days.

**Glu Inhibits Chondral Mineralization by Apoptosis**—Chondrocytes were placed in 24-well plates and cultured for different periods of time up to 21 days. Cells were rinsed with PBS twice and then stained for 30 min with 1% Alcian blue 8GS dissolved in 3% acetic acid. Cells were washed with 3% acetic acid for 30 s three times, and then stained cells were dissolved in 0.1% SDS for subsequent quanti-
tification of the absorbance at 650 nm.

**Determination of ALP Activity**—Chondrocytes were placed
at a density of 4 × 104 cells/cm² in 24-well plates and cultured
for different periods of up to 28 days. Cells were washed twice
with cold PBS, and then sonicated in 0.1 M Tris-HCl buffer
(pH 7.5) containing 0.1% Triton X-100. Assay buffer composed
of 0.05 M 2-amino-2-methylpropanol, 2 mM MgCl2, and 10 mM
p-nitrophenylphosphoric acid was added at a volume of 200 µl
into 10-µl cell suspensions, followed by the reaction for 30 min
at 37 °C and subsequent immediate determination of the
absorbance of p-nitrophenol at 405 nm.

**Cell Viability**—Cell survival analysis was performed by the 3-(4,5-
dimethylthiazol-2-y1)-2,5-diphenyl-2H-tetrazolium bromide (MTT)
reduction assay to access the mitochondrial activity. Cultured
chondrocytes were washed once with PBS and incubated with
0.5 mg/ml MTT in PBS for 1 h at 37 °C, followed by the addition
of 0.04 M HCl in isopropyl alcohol to the well and subsequent
shaking of the mixture for 10 min to dissolve the formazan.
The dissolved suspension was subjected to an enzyme-linked
immunosorbent assay reader and the absorbance at a wave-
length of 550 nm was measured.

**Hoechst Staining**—Chondrocytes were placed in 24-well
plates and cultured for a period of up to 3 days, followed by the
addition of Glu 48 h before the end of culturing. Cells were rinsed
with PBS twice and then stained for 15 min with 10
µg/ml Hoechst 33342 dissolved in DMEM. Cells were cultured
with DMEM, and then observed with an Olympus IMT-2-21
dissecting microscope.

**Determination of Intracellular Total GSH**—Cells were rinsed
with PBS twice, and then collected in 500 µl of PBS. One hun-
dred µl of cell suspension was next treated with 5 µl of 10%
5-sulfosalicylic acid and homogenized at 4 °C, followed by cen-
trifugation at 20,000 × g for 10 min. The supernatant fractions
were analyzed with a total glutathione quantification kit
according to the manufacturer’s instructions.

**Data Analysis**—Results are all expressed as the mean ± S.E.
and the statistical significance was determined by the two-
tailed and unpaired Student’s t test or the one-way analysis of
variance with Bonferroni/Dunnnett post hoc test.

**TABLE 1**
| Markers      | Upstream (5‘-3’)                  | Downstream (5‘-3’)          | Estimated base pair |
|--------------|----------------------------------|----------------------------|-------------------|
| collagen II  | GAGCTCCTGGGTAAGAGAGG             | CAAGTCTCTGGTGTTACCAGAA     | 457               |
| sox9         | AGGCCGTCGCGACCCAGTA              | CAGCCCTGCTCGTCAAGATACAC    | 407               |
| runx2        | CGCTTCTCTCTACCCGATAT             | TATGCAATTGCGCTGCTTG        | 581               |
| aggrecan     | CTTCTCTGCAAGAAGTACG              | ACTCCTCTCTGCTCAAGG         | 1872              |
| PPARγ        | TATGCACTGCAAGGCTTGAA             | CGGAGAAAGACTTTATGTATG       | 315               |
| GAPDH        | GGATGAGCCTGGTGTTACCAGA           | GATGCCAAAGTTGTCATGGATACCC  | 502               |

| Antiporters | Upstream (5‘-3’)                  | Downstream (5‘-3’)          | Estimated base pair |
|-------------|----------------------------------|----------------------------|-------------------|
| xCT         | CTCGCCATTTGACGCTACAT             | TCAGATTGCTGCTGACCTTC       | 182               |
| 4F2hc       | CTCCAGAAGATTTAAGACCTCTT          | TGGATTTGGCTGCTACAGTCAG     | 141               |
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Effects of Glu on Chondral Mineralization in Cultured Mouse Metatarsals—To evaluate the possible role of Glu in cartilage, metatarsals before vascularization were isolated from embryonic mice at 15.5 days after gestation and cultured in either the presence or absence of Glu at a concentration range from 1 μM to 1 mM for 5 days and subsequent determination of the total length and the mineralization ratio as described in the text. Typical pictures are shown in a, whereas quantitative data are shown in b as the mean ± S.E. in 4 independent experiments. *, p < 0.05; **, p < 0.01, significantly different from each control value obtained in metatarsals cultured in the absence of Glu. c, metatarsals were cultured in the presence of different EAAT substrates at a concentration of 0.5 mM for 5 days, followed by determination of the total length and mineralization ratio with alizarin red staining. Values are the mean ± S.E. in 4 separate experiments. Cont, control.

RESULTS

Effects of Glu on Chondral Mineralization in Cultured Mouse Metatarsals—To further investigate the effect of Glu on chondral mineralization, mouse metatarsals were cultured in either the presence or absence of Glu at a concentration of 500 μM or 1 mM for different periods up to 7 days, followed by measurement of the mineralization ratio. Sustained exposure to Glu at 0.5 or 1 mM for 3 to 7 days from the initiation of cultivation resulted in significant inhibition of the mineralization in cultured metatarsals, whereas the mineralization ratio was not significantly changed following the incubation with Glu at 0.5 to 1 mM for 2 days (Fig. 2a). Glutamate was added at 0.5 or 1 mM on different days from 0 to 5 days to metatarsals, followed by incubation for up to 5 days and subsequent measurement of the mineralization ratio. The mineralization ratio was significantly decreased following sustained exposure to 0.5 mM Glu from 0 to 5 days and 1 to 5 days (Fig. 2b), whereas Glu did not significantly affect the mineralization ratio at 0.5 mM when exposed after 2 days until the day of alizarin red staining. The mineralization ratio was significantly decreased when metatarsals were treated with Glu at 1 mM from 0 to 5 days and 1 to 5 days in addition to 2 to 5 days. However, no significant alteration was seen with the mineralization ratio in metatarsals cultured in the presence of Glu at 0.5 to 1 mM from 3 to 5 days.

An attempt was next made to evaluate the possible involvement of perichondrium and/or bone collar in the decreased mineralization by Glu. Metatarsals were isolated from mouse embryos at 15.5 days post-gestation, followed by digestion with 0.3% collagenase at 37 °C for 30 min to remove perichondrium and bone collar as described previously (19) and subsequent cultivation in the presence of Glu at 0.5 and 1 mM for 5 days. Because the collagenase digestion induced obviously abnormal shapes in metatarsals, the mineralization ratio was calculated by the measurement of areas of the total rudiment and the middle mineralized part in place of the length. As seen in control unstripped metatarsals, the mineralization ratio was significantly inhibited by Glu at a concentration of over 0.5 mM even in cultured metatarsals treated with collagenase (Fig. 2c, lower panel), whereas no significant alteration was found in the total relative area in metatarsals cultured in the presence of Glu irrespective of the collagenase digestion (Fig. 2c, upper panel).

Histological Analysis in Cultured Mouse Metatarsals—To further investigate the effect of Glu on chondral mineralization, several histological analyses were conducted on sections prepared from metatarsals cultured in the presence of Glu. Mouse metatarsals were cultured in the presence of Glu at 1 mM for 5 days, followed by the dissection of frozen sections at 5 μm for the histological analysis by staining with hematoxylin and eosin and Alcian blue, in addition to ALP and TUNEL. Sustained
exposure to Glu apparently decreased the number of chondrocytes embedded within a calcified matrix (calcified chondrocytes), with a concomitant induction of abnormal cell shapes in both late proliferating and hypertrophic zones when determined by hematoxylin and eosin (Fig. 3a, left panels) and Alcian blue (Fig. 3a, middle left panels) staining. The ALP staining pattern was markedly altered in metatarsals cultured in the presence of Glu for 5 days (Fig. 3a, middle right panels), whereas apoptotic cells positive to TUNEL staining were clearly detected within late proliferating and prehypertrophic chondrocytic layers in cultured metatarsals exposed to 1 mM Glu for 5 days (Fig. 3a, right panels).

An attempt was next made to determine whether Glu affects the localization profile of mRNA for different markers selectively expressed by chondrocytes at distinct differentiation stages in cultured metatarsals using in situ hybridization techniques. Under the experimental conditions used, few cells were labeled by a cRNA probe for type I collagen, which is expressed by osteoblasts in layers of differentiating chondrocytes in cultured metatarsals, irrespective of the presence of Glu (Fig. 3b, left panels). However, sustained exposure to Glu apparently altered both the number and the distribution pattern of cells labeled by cRNA probes for type II collagen, which is highly expressed by proliferating to prehypertrophic chondrocytes (Fig. 3b, middle left panels) and for type X collagen, which is preferentially expressed by hypertrophic chondrocytes (Fig. 3b, middle right panels), respectively, when determined in metatarsals cultured for 5 days. In addition, an obvious decrease was seen in the expression of mRNA for osteopontin predominantly expressed by calcified chondrocytes in metatarsals cultured in the absence of 1 mM Glu, followed by incubation with DCFDA for the determination of intracellular ROS levels on fluorescence image. Under these experimental conditions, highly fluorescent cells were observed at proliferating to prehypertrophic chondrocytes in metatarsals cultured in the presence of Glu, but not in those cultured in the absence of Glu (Fig. 3e). These data suggest that Glu may induce apoptotic cell death and facilitate the ROS generation, in addition to inhibiting the mineralization, in chondrocytes.

Effect of Group III mGluR Antagonist on Decreased Mineralization and Apoptosis by Glu—We have previously demonstrated that the group III mGluR agonist l-AP4 drastically inhibited chondral mineralization in a manner sensitive to an antagonist, without inducing apoptotic cell death in cultured mouse embryonic metatarsals isolated before vascularization (6). We therefore investigated the possible involvement of the group III mGluR subtype in Glu-induced chondral apoptosis. Metatarsals were treated with 1 mM Glu in either the presence or absence of 1 mM Glu, followed by incubation with DCFDA for the determination of intracellular ROS levels on fluorescence image. Under these experimental conditions, highly fluorescent cells were observed at proliferating to prehypertrophic chondrocytes in metatarsals cultured in the presence of Glu, but not in those cultured in the absence of Glu (Fig. 3e). These data suggest that Glu may induce apoptotic cell death and facilitate the ROS generation, in addition to inhibiting the mineralization, in chondrocytes.

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FIGURE 2. Inhibition of chondral mineralization by Glu in cultured metatarsals. a, metatarsals were cultured in the presence of Glu at 0.5 or 1 mM for different durations up to 7 days, followed by determination of mineralization ratios. b, glutamate was added at 0.5 or 1 mM on different days from 0 to 3 days in cultured metatarsals, followed by the additional culture for 5 days and subsequent determination of the mineralization ratio. c, metatarsals were digested with 0.3% collagenase at 37 °C for 30 min, followed by cultivation in the presence of Glu at 0.5 or 1 mM for 5 days and subsequent determination of the total area and the mineralization ratio. Values are the mean ± S.E. in separate experiments shown in each figure. *, p < 0.05; **, p < 0.01, significantly different from each control value obtained in metatarsals cultured in the absence of Glu.
metatarsals cultured in the presence of 1 mM Glu (Fig. 4b, upper panels). Quantitative analysis revealed that CPPG did not significantly affect the number of TUNEL-positive cells in cultured metatarsals irrespective of the presence of 1 mM Glu (Fig. 4b, lower panel). Therefore, it is unlikely that the group III mGluR subtype is at least partially involved in apoptotic cell death in metatarsals cultured in the presence of Glu at a high concentration. 

Role of Cystine/Glu Antiporter in Decreased Mineralization and Apoptosis by Glu—An attempt was made to elucidate underlying mechanisms for the decreased chondral mineralization and apoptosis by Glu in cultured metatarsals. Metatarsals were exposed to 1 mM Glu in either the presence or absence of GSH and cystine at a concentration range from 10 μM to 1 mM for 5 consecutive days, followed by alizarin red staining to measure the mineralization ratio in addition to TUNEL staining. The addition of GSH at 1 mM significantly prevented the decrease in the mineralization ratio in metatarsals cultured with 1 mM Glu without a marked effect alone (Fig. 5a, upper panel), whereas GSH significantly decreased the number of TUNEL-positive cells in a concentration-dependent manner at concentrations of 0.01 to 1 mM in metatarsals cultured in the presence of 1 mM Glu (Fig. 5a, lower panel). Similarly, the cystine/Glu antiporter substrate cystine was also effective in significantly preventing the Glu-induced inhibition of mineralization at concentrations above 0.5 mM (Fig. 5b, upper panel), but significantly abolished the increased number of TUNEL-positive cells at concentrations over 0.1 mM in metatarsals cultured in the presence of 1 mM Glu (Fig. 5b, lower panel). Moreover, the cystine/Glu antiporter inhibitor homocysteic acid significantly prevented both the decreased mineralization ratio (Fig. 5c, upper panel) and the increased number of TUNEL-positive cells (Fig. 5c, lower panel) in a concentration-dependent manner at concentrations used in metatarsals cultured in the presence of 1 mM Glu without significantly affecting both indices alone.

Possible Expression of Cystine/Glu Antiporter in Chondrocytes and Effects of GSH Depletion on Chondral Mineralization—To analyze the possible expression of mRNA for the cystine/Glu antiporter, mRNA was extracted from mouse metatarsals and rat costal chondrocytes for subsequent RT-PCR using specific primers for the antiporter subunits xCT and 4F2hc. Rat and mouse whole brains exhibited marked expression of mRNA for both xCT and 4F2hc subunits, whereas mRNA was invariably expressed for both xCT and 4F2hc subunits in metatarsals cultured for 1 and 5 days as well as in chondrocytes cultured for 4 and 21 days (Fig. 6a).
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Sequence analysis on amplified PCR products clearly confirmed the identity of mRNA for the corresponding xCT and 4F2hc subunits (data not shown). Metatarsals cultured for 5 days were dissected for frozen sections to evaluate distribution profiles of the xCT subunit in particular chondral cell types. In these sections, expression of the xCT subunit was invariably and widely detected from proliferating to hypertrophic chondrocytes (Fig. 6b). In sections not treated with the primary antibody, no marked immunoreactivity was detected in any chondral cells seen in cultured metatarsals (data not shown).

To further investigate underlying mechanisms for the inhibition by Glu of the chondral mineralization and apoptosis in cultured metatarsals, metatarsals were cultured in either the presence or absence of 1 mM Glu along with group III mGluR antagonist CPPG at 10 μM for 5 days, followed by the determination of the total length and the mineralization ratio. Metatarsals were similarly cultured as described above for subsequent TUNEL staining. Typical pictures are shown in the upper panel, whereas the number of positive cells is shown in the lower panel. Values are the mean ± S.E. in 4 separate experiments. *p < 0.05; **p < 0.01, significantly different from each control value obtained in metatarsals cultured in the absence of any regents. # p < 0.05, significantly different from the value obtained in metatarsals cultured in the presence of Glu alone.

A similar decrease was induced in the ratio of the middle mineralized part determined by alizarin red staining following sustained exposure to cyclohexene-1-one at a concentration of over 5 μM or to l-buthionine-[S,R]-sulfoximine at a concentration of over 50 μM in cultured metatarsals (Fig. 6c, middle panel), with the total length of the cartilage rudiments being unchanged (Fig. 6c, left panel). Moreover, the number of

TUNEL positive cells was significantly increased by the addition of cyclohexene-1-one or l-buthionine-[S,R]-sulfoximine in a concentration-dependent manner at the concentrations used (Fig. 6c, right panel).

Effects of Glu on Chondrocyte Differentiation—Cartilage were isolated from adult female Wistar rat ribs, followed by the digestion with collagenase and collection of cells toward subsequent plating for culture. Expression of mRNA was seen for several chondral marker genes including type II collagen, sox9, runt-related gene 2 (runx2), and aggrecan, but not for the adipocyte marker gene peroxisome proliferator-activated receptor-γ, in costal chondrocytes cultured for 4 to 28 days (Fig. 7a). Expression of mRNA was markedly decreased for type II collagen, sox9, and runx2 in metatarsals cultured in the presence of 1 mM Glu. Semi-quantitative RT-PCR revealed that expression of mRNA was drastically increased for runx2 during culturing from 7 to 14 days with a gradual increase thereafter up to 28 days, whereas a transient increase was seen in mRNA expression for both type II collagen and sox-9 at 14 days in rat costal chondrocytes cultured for 4 to 28 days (Fig. 7b). Although expression of mRNA for aggrecan was not significantly changed in the presence of 1 mM Glu at any stages from 4 to 28 days, significant suppression was found in mRNA expression of sox9, runx2, and type II collagen in costal chondrocytes cultured in the presence of 1 mM Glu. Quantitative analysis on 4 different experiments clearly showed significant inhibition of the expression of mRNA for sox9 at 14 days, for runx2 from 14 to 28 days, and type II collagen from 7 to 21 days, respectively, in chondrocytes cultured in the presence of 1 mM Glu.

In proportion to increasing culture periods up to 18 days, a linear increase was found in Alcian blue staining used for the detection of acidic mucopolysaccharide in cultured rat costal chondrocytes when quantified after the extraction of the dye on spectrometry, whereas the staining intensity was significantly decreased in chondrocytes cultured in the presence of 1 mM Glu from 7 to 21 days (Fig. 7c). A marked increase was also seen in the activity of ALP in chondrocytes cultured for a period of 7 to 28 days with a plateau on 18 days, whereas the activity was significantly decreased in chondrocytes cultured in the presence of 1 mM Glu from 11 to 28 days (Fig. 7d). Cultured metatarsals were next exposed to 1 mM Glu at different days of culture from 0 to 21 days, followed by the determination of ALP activity at 28 days. The ALP activity was significantly decreased following sustained exposure to 1 mM Glu from 3 to 28 days and
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Effects of Glu on Survival of Chondrocytes—To evaluate the possible effect of Glu on cell survival, chondrocytes prepared from rat ribs were cultured in either the presence or absence of Glu at concentrations below 1 mM for 4 consecutive days, followed by the determination of cell survival by the MTT assay as well as microscopic observation. Sustained exposure to Glu at a concentration of over 300 μM led to a marked decrease in the number of living cells on phase-contrast microscopy (Fig. 8a), whereas MTT assays clearly demonstrated a significant concentration-dependent decrease in the MTT reduction activity in costal chondrocytes cultured in the presence of Glu at 200 μM to 1 mM for 4 days (Fig. 8b). Costal chondrocytes cultured for 3 days were then exposed to Glu at 500 μM for subsequent Hoechst 33342 staining 2 to 48 h later. The number of apoptotic cells determined by Hoechst 33342 staining was significantly increased in chondrocytes treated with Glu for 6 to 48 h (Fig. 8c). To next evaluate the possible involvement of either EAATs or GluRs in decreased cell survival in primary cultured chondrocytes, cells were cultured in either the presence or absence of different EAAT substrates and GluR agonists for 4 consecutive days, followed by the determination of cell survival by MTT assay. Several EAAT substrates, such as d-Glu, L-aspartate, and L-aspartate, did not significantly change the cell survival determined by the MTT assay at 500 μM (Fig. 8d). Moreover, no significant change was induced by the cultivation for 4 days with different agonists for iGluRs and mGluRs in the MTT reduction activity in cultured chondrocytes. These included AMPA, AMPA plus cyclothiazide, N-methyl-d-aspartate, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine, and L-AP4 (Fig. 8e).

Effects of Glu on Bax/Bcl-2, ROS, Nitric Oxide, and GSH in Chondrocytes—An attempt was first made to determine whether Bax/Bcl-2 family members are involved in the Glu-induced chondral apoptotic cell death. Chondrocytes were cultured with 50 μg/ml ascorbic acid for 4 days, followed by a further cultivation in either the presence or absence of 1 mM Glu for 4 h and subsequent extraction of mRNA for RT-PCR. Semi-quantitative RT-PCR revealed that expression of mRNA was significantly increased for both bax and p53 after brief exposure to 1 mM Glu for 4 h, whereas slight but significant suppression was seen in mRNA expres-

![FIGURE 5. Role of cystine/Glu antipporter in mineralization and apoptosis in cultured metatarsals.](image)

6 to 28 days in addition to 0 to 28 days, whereas Glu did not significantly affect the ALP activity when exposed after 9 days until the day of the determination of ALP activity (Fig. 7e).

**DISCUSSION**

The main finding of this study is that Glu markedly suppresses mineralization and induces apoptosis in association with increased ROS generation and GSH depletion in a mechanism involving the cystine/Glu antipporter. To our knowledge,
This is the first direct demonstration of a functional role for the cystine/Glu antiporter in cultured mouse metatarsals comprising almost entirely of chondrocytes at different maturation stages, in addition to cultured rat costal chondrocytes. Although we have previously demonstrated the functional expression by chondrocytes of different Glu signaling machineries responsible for cellular differentiation (5–7), no direct evidence for a pivotal role of the cystine/Glu antiporter in mechanisms related to the maintenance of the chondrocytic viability in cartilage is available in the literature. Our studies suggest that the neurotoxic excitatory amino acid Glu could also be cytotoxic to chondrocytes at particular differentiation stages leading to the inhibition of mineralization toward the developmental maturation as a consequence of the depletion of cytoprotective GSH through the facilitation of the retrograde transmembrane transport of the substrate cystine in cartilage. However, the exact mechanism as well as pathological significance of the response to Glu of cells located within late proliferating to prehypertrophic chondrocyte layers remains to be elucidated. That some of the effects on mineralization might be a consequence of the calcium-binding properties of Glu is unlikely given the efficient prevention by a group III mGluR antagonist (CPPG), a substrate (cystine), and an inhibitor (homocysteic acid) of the cystine/Glu antiporter, and an endogenous antioxidant (GSH) against the Glu-induced inhibition of mineralization.

The metatarsal culture system used in this study is well known to be an ex vivo model of chondrocyte developmental maturation, which undergoes normal patterns of both proliferation and differentiation for at least 5 days in culture (20). This allowed us to investigate the effects of Glu in an experimental system where chondrocytes retain their normal architecture of round and columnar prehypertrophic and hypertrophic zones. The absence of mesenchymal cells, other than chondrocytes, in any of the cell layers, except those in bone collar or perichondrium of cultured metatarsals, was confirmed by in situ hybridization using a probe for the osteoblastic cell differentiation marker type I collagen. Furthermore, in primary cultured chondrocytes isolated from rat ribs, no osteoblasts were found by RT-PCR using specific primers for type I collagen, as shown in our previous study (6). Taken together, the possibility that the effect of Glu may be mediated by functional glutamatergic signaling molecules expressed by osteoblasts in the organotypic cultured mouse metatarsals and primary cultured rat costal chondrocytes is, therefore, ruled out.

The cystine/Glu antiporter is a crucial determinant of the regulation of intracellular GSH concentrations in a variety of eukaryotic cells (14, 15). Under the condition of high extracel-

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**FIGURE 6. Possible expression of cystine/Glu antiporter in chondrocytes and effects of GSH depletion on chondral mineralization and apoptosis in cultured metatarsals.**

(a) mRNA was extracted from primary chondrocytes cultured for 4 and 21 days and metatarsals cultured for 1 and 5 days for subsequent RT-PCR using primers specific for xCT and 4F2hc. Typical pictures are shown in the figure with similar results in three separate determinations. (b) Metatarsals were cultured for 5 days, followed by fixation with formalin and subsequent dissection of frozen sections for detection of the xCT subunit on immunohistochemical analysis. Typical micrographs are shown in this figure, whereas similar results were invariably obtained in at least three independent determinations. (c) Metatarsals were cultured in the presence of cyclohexene-1-one (CHX) at a concentration range from 1 to 10 μM or l-buthionine(S,R)-sulfoximine (BSO) at a concentration range from 10 to 100 μM for 5 days, followed by the determination of the total length and the mineralization ratio in addition to TUNEL staining. Values are the mean ± S.E. in four separate experiments. *, p < 0.05; **, p < 0.01, significantly different from control value obtained in metatarsals cultured in the absence of any added regents.
lular Glu levels, extracellular Glu could be taken up in exchange for intracellular cystine through the cystine/Glu antiporter, followed by GSH depletion attributable to the decreased cystine level in the cytoplasm and subsequent cell death due to GSH depletion (14, 15). We have previously demonstrated that the group III mGluR agonist L-AP4 drastically inhibits chondral mineralization in a manner sensitive to an antagonist, without inducing apoptotic cell death, in cultured mouse embryonic metatarsals isolated before vascularization (6). Thus, it is conceivable that extracellular Glu at a high concentration could induce apoptosis through a mechanism associated with the depletion of intracellular GSH as a result of retrograde operation of the cystine/Glu antiporter, but not related to the possible excitotoxicity mediated by particular GluRs, in chondrocytes. Indeed, in the present study, the group III mGluR antagonist CPPG failed to completely abolish inhibition of mineralization by Glu in cultured metatarsals without significantly affecting the Glu-induced apoptosis. Our findings that both decreased mineralization and apoptosis by Glu were significantly prevented by the addition of a cystine/Glu antiporter inhibitor and a substrate, in addition to antioxidants such as GSH, in cultured metatarsals support this proposal. Therefore, Glu could negatively regulate the developmental differentiation toward mineralization through a mechanism associated with the apoptosis of late proliferating to prehypertrophic cells after the depletion of intracellular GSH due to the massive transmembrane efflux of intracellular cystine by the retrograde operation of the cystine/Glu antiporter in chondrocytes.

Both degradation and apoptosis of chondrocytes in articular cartilage are common pathogenic changes seen in different degenerative joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA) (21–23). The Bcl-2 family is shown to modulate cell survival, whereas some members (e.g., Bcl-2 and Bcl-XL) attenuate apoptosis with others (e.g., Bax, Bad, and Bcl-XS) facilitating apoptosis (24, 25). Consequently, the ratio of Bax/Bcl-2 is believed to be an important determinant of the incidence of apoptotic cell death (26, 27). It has been reported that the Bcl-2 family is involved in controlling the maturation and apoptosis of chondrocytes in the growth plate (28), moreover, that the Bcl-2 family might be responsible for the pathogenesis of RA as well as OA. Indeed, Bcl-2 expression is significantly lower in cartilage with both RA (22) and OA (29) than in normal cartilage. In this study, we have demonstrated that Glu markedly increased the ratio of Bax/Bcl-2 (1.0 versus 2.1) in association with the induction of apoptosis at a high concentration in cultured chondrocytes.

Previous studies have demonstrated a drastic increase in endogenous Glu levels (6.25 versus 326 μM) in the synovial fluid.
obtained from patients with rheumatoid arthritis (30), whereas the elevation of Glu in synovial fluid is shown to be relevant to the increased edema and the sensitization to thermal hyperalgesia in experimental arthritis models (31, 32). We have also shown a marked increase in the activity of $[^3H]$Glu accumulation sensitive to different EAAT inhibitors, but not to cystine or homocysteic acid, in synovial fibroblasts of rats with collagen-induced arthritis (33). In particular pathological situations such as OA and RA, therefore, extracellular Glu would reach the levels required for the retrograde operation of the cystine/Glu antiporter around chondrocytes in articular cartilage. It should be also noted that serum Glu levels vary from a low micromolar range in humans (34) to a high micromolar range in rats (35) under physiological conditions. The cytotoxicity shown in the present investigation argues in favor of an idea that extracellular Glu would play a pivotal role in mechanisms underlying articular chondral abnormalities observed in humans with degenerative joint diseases, such as OA and RA, at high concentrations. A possible speculation is that apoptotic cell death of proliferating to prehypertrophic chondrocytes would lead to the prevention of subsequent cellular differentiation to both hypertrophic and calcified chondrocytes responsible for the maintenance of the normal architectural structure and functionality of the articular cartilage in a particular situation.

Nevertheless, the involvement of the group III mGluR subtype in mechanisms underlying the decreased mineralization by Glu, but not apoptotic cell death, is conceivable as described elsewhere (6). The fact that a group III mGluR antagonist significantly but partially prevented the decreased mineralization by Glu without affecting apoptosis gives support to the latter idea. In our previous paper (6), the group III mGluR agonist L-AP4 drastically abolished chondral mineralization, without inducing cell death, in a manner sensitive to the antagonist CPPG in cultured mouse metatarsals. Therefore, it is likely that decreased mineralization is at least in part mediated by both activation of the group III mGluR subtype and the reversal drive of the cystine/Glu antiporter toward apopto-
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![Diagram depicting the regulatory effects of Glu on chondrocytes.](image)

**FIGURE 10.** Diagram depicting the regulatory effects of Glu on chondrocytes. The concentration of extracellular Glu is a crucial determinant for the direction of transport by the bi-directional cystine/Glu antiporter expressed by chondrocytes. Under particular physiological or pathological conditions with high Glu levels in the extracellular circumstance, the antiporter could be driven to the retrograde direction leading to the inhibition of mineralization through apoptotic cell death after the depletion of the intracellular antioxidant GSH in chondrocytes.

The pathways linking the Glu effects on apoptosis and chondral mineralization through the involvement of retrograde operation of the cystine/Glu antiporter are summarized in Fig. 10. In chondrocytes, mRNA expression is seen for different glutamatergic signaling machineries; the vesicular glutamate transporter-1 (5), required for the signal output, both the iGluRs and mGluRs (6), required for the signal input, and the EAATs (7), required for the signal termination. Upon stimulation, Glu is released in a Ca\(^{2+}\)-dependent manner from chondrocytes (5), whereas Glu activates the group III mGluR subtype linked to the inhibition of cAMP formation, at a relatively low concentration, for the subsequent prevention of cell differentiation toward mineralization (6). EAATs are usually responsible for the regulation of the environmental concentration of extracellular Glu in chondrocytes (7), whereas their malfunction results in a massive increase in extracellular Glu levels. Extracellular Glu at a high concentration could then reverse the direction of transport by the bi-directional cystine/Glu antiporter, which is required for the anterograde incorporation of extracellular cystine into the cytoplasm for the biosynthesis of the intracellular antioxidant GSH in exchange for intracellular Glu. This retrograde operation of the antiporter leads to the depletion of intracellular GSH for subsequent apoptotic cell death, which finally induces disturbed cell differentiation and maturation toward mineralization. The concentration of extracellular Glu is quite important for the determination of the direction of transport by the bi-directional cystine/Glu antiporter expressed in chondrocytes. Moreover, a drastic increase is seen in \(^{3}H\)Glu accumulation mediated by EAATs in synovial fibroblasts of rats with collagen-induced arthritis (33). However, the up-regulation of EAATs could be insufficient to fully compensate the drastic elevation of both Glu (54-fold) and aspartate (28-fold) levels in the extracellular environment of chondrocytes in arthritic cartilage (30). Levels specifically in cartilage and/or the physiological relevance need to be more clearly established to validate the model presented.

We have also shown that activation of the group III mGluR subtype by \(\alpha\)-AP4 significantly inhibits cAMP formation by both forskolin and parathyroid hormone (PTH) in a CPPG-sensitive manner in cultured mouse metatarsals (6). As PTH is known to inhibit chondral mineralization through a mechanism related to the increased formation of AMP after activation of PTH receptors expressed at cell surfaces (20), however, the reason why decreased mineralization is similarly brought about by the two transmembrane signaling systems oppositely directed to intracellular cAMP levels in metatarsals is not clarified so far. Our preliminary data that PTH induced a marked bending as well as decreased mineralization of chondral rudiments could at least in part give us a clue for the explanation of the paradoxical data between the inhibition of mineralization by PTH and Glu in cultured metatarsals.

It thus appears that Glu inhibits chondral mineralization through a mechanism related to the reversal drive of the cystine/Glu antiporter for apoptosis in addition to activation of the group III mGluR subtype. The activity as well as the direction of the cystine/Glu antiporter expressed at cell surfaces may be a crucial determinant of the cellular survival and developmental differentiation through modulation of the endogenous level of GSH that plays a pivotal role as a predominant antioxidant in chondrocytes. Therefore, the cystine/Glu antiporter would be a novel target for the discovery and development of strategies useful for the therapy and treatment of a variety of degenerative joint diseases such as OA and RA.

**REFERENCES**

1. Hollmann, M., O'Shea-Greenfield, A., Rogers, S. W., and Heinemann, S. (1989) *Nature* **342**, 643–648
2. Yoneda, Y., Kuramoto, N., Kitayama, T., and Hinoi, E. (2001) *Prog. Neurobiol.* **63**, 697–719
3. Skerry, T. M., and Genever, P. G. (2004) *Trends Pharmacol. Sci.* **22**, 174–181
4. Hinoi, E., Takarada, T., Ueshima, T., Tsuchihashi, Y., and Yoneda, Y. (2004) *Eur. J. Biochem.* **271**, 1–13
5. Wang, L., Hinoi, E., Takemori, A., and Yoneda, Y. (2005) *Biol. Pharm. Bull.* **28**, 990–993
6. Wang, L., Hinoi, E., Takemori, A., Takarada, T., and Yoneda, Y. (2005) *Br. J. Pharmacol.* **146**, 732–743
7. Hinoi, E., Wang, L., Takemori, A., and Yoneda, Y. (2005) *Biochem. Pharmacol.* **70**, 70–81
8. Chenu, C., Serre, C. M., Raynal, C., Burt-pichat, B., and Delmas, P. D. (1998) *Bone* **22**, 295–299
9. Genever, P. G., and Skerry, T. M. (2001) *FASEB J.* **15**, 1586–1588
10. Danbolt, N. C. (2001) *Prog. Neurobiol.* **65**, 1–105
11. Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9394–9398
Acad. Sci. U. S. A. 89, 10955–10959
12. Bannai, S. (1986) J. Biol. Chem. 261, 2256–2263
13. Sato, H., Tamba, M., Ishii, T., and Bannai, S. (1999) J. Biol. Chem. 274, 11455–11458
14. Kato, S., Negishi, K., Mawatari, K., and Kuo, C. H. (1992) Neuroscience 48, 903–914
15. Sato, H., Fujiwara, K., Sagara, J., and Bannai, S. (1995) Biochem. J. 310, 547–551
16. Klement, B. J., and Spooner, B. S. (1993) J. Exp. Zool. 265, 285–294
17. MacLean, H. E., Guo, J., Knight, M. C., Zhang, P., Cobrinik, D., and Kronenberg, H. M. (2004) J. Clin. Invest. 113, 1334–1343
18. Moriguchi, N., Hinoi, E., Tsuchihashi, Y., Fujimori, S., Iemata, M., Takarada, T., and Yoneda, Y. (2006) Histol. Histopathol. 21, 969–977
19. Haaijman, H., Karperien, M., Lanske, B., Hendriks, J., Lowik, C., Bronckers, A., and Burger, F. (1999) Bone 25, 397–404
20. Guo, J., Chung, U. I., Kondo, H., Bringhurst, F. R., and Kronenberg, H. M. (2002) Dev. Cell 3, 183–194
21. Hashimoto, S., Ochs, R. L., Komiyai, S., and Lotz, M. (1998) Arthritis Rheum. 41, 1632–1638
22. Kim, H. A., and Song, Y. W. (1999) Arthritis Rheum. 42, 1528–1537
23. Héraud, F., Héraud, A., and Harmand, M. F. (2000) Ann. Rheum. Dis. 59, 959–965
24. Reed, J. C. (2000) Am. J. Pathol. 157, 1415–1430
25. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
26. Vaux, D. L., Cory, S., and Adams, J. M. (1988) Nature 335, 440–442
27. Korsmeyer, S. J. (1992) Blood 80, 879–886
28. Amling, M., Neff, L., Tanaka, S., Inoue, D., Kuida, K., Weir, E., Philbrick, W. M., Broadus, A. E., and Baron, R. (1997) J. Cell Biol. 136, 205–213
29. Kim, H. A., Lee, Y. J., Seong, S. C., Choe, K. W., and Song, Y. W. (2000) J. Rheumatol. 27, 455–462
30. McNearney, T., Speegle, D., Lawand, N., Lisse, J., and Westlund, K. N. (2000) J. Rheumatol. 27, 739–745
31. Lawand, N. B., Willis, W. D., and Westlund, K. N. (1997) Eur. J. Pharmacol. 324, 169–177
32. Lawand, N. B., McNearney, T., and Westlund, K. N. (2000) Pain 86, 69–74
33. Hinoi, E., Ohashi, R., Miyata, S., Kato, Y., Iemata, M., Hojo, H., Takarada, T., and Yoneda, Y. (2005) Biochem. Pharmacol. 70, 1744–1755
34. Plaitakis, A., Berl, S., and Yahr, M. D. (1982) Science 216, 193–196
35. Herlin, P. M., James, J. H., Joffe, S. N., Kulneff-Herlin, A. E. A., and Fischer, J. E. (1982) J. Neurochem. 38, 1170–1173

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