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Metabolic loading of guanosine induces chondrocyte apoptosis *via* the Fas pathway

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Abbreviations: Fas-Fc, Fas-Fc chimeric protein; FasL, Fas ligand

Abstract

Although the apoptosis of chondrocytes plays an important role in endochondral ossification, its mechanism has not been elucidated. In this study, we show that guanosine induces chondrocyte apoptosis based on the results of acridine orange/ethidium bromide staining, caspase-3 activation, and sub-G1 fraction analysis. The potent inhibitory effect of dipyridamole, a nucleoside transporter blocker, indicates that extracellular guanosine must enter the chondrocytes to induce apoptosis. We found that guanosine promotes Fas-Fas ligand interaction which, in turn, leads to chondrocyte apoptosis. These findings indicate a novel mechanism for endochondral ossification via metabolic regulation.

Keywords: apoptosis; chondrocytes; ossification; Fas ligand; guanosine

Introduction

Endochondral ossification is a process by which a mesenchymal cartilagenous template is replaced by mineralized skeletal elements (Wright *et al.*, 1995). This process is driven by the proliferation and differentiation of growth plate chondrocytes that undergo a number of profound physical and biochemical changes as they mature (Zuscik *et al.*, 2002). Initially, growth cartilage cells proliferate, becoming mature chondrocytes that can form an extracellular matrix (ECM) composed of aggrecan and type II collagen (Poole, 1991). The cells then transform into hypertrophic chondrocytes that are capable of producing type X collagen and alkaline phosphatase (ALPase). The matrix incorporates minerals and blood vessels so that hematopoietic osteoclast-precursor cells and perivascular osteoblast-progenitor cells can be recruited. The cartilage is replaced by bone (Poole, 1991) in the final step of the process. Accompanying these changes is an increase in the number of apoptotic cells. A number of studies support the hypothesis that the terminally differentiated chondrocytes of the growth plate undergo programmed cell death (Gibson, 1998). How apoptosis is initiated and regulated remains to be elucidated.

Another notable change occurring in the cartilage is a significant increase in the guanosine level in the hypertrophic zone and in the calcified cartilage (Matsumoto *et al.*, 1988). At present, the role of guanosine in chondrocytes has not been identified. Nonetheless, natural nucleosides are important metabolites and are the main source for nucleic acid synthesis. Nucleosides also have a myriad of physiological functions in various organs and systems, for example, acting as an anti-inflammatory agent and inhibiting lipolysis in fat cells (Griffith and Jarvis, 1996). The elevation of the guanosine level in the growth plate indicates a potentially significant physiological function for guanosine in chondrocytes. We present evidence that guanosine plays an important role in the apoptotic cell death of the growth plate. Our results indicate that elevation of the guanosine level in chondrocytes induces Fas ligand induction and eventually leads to cellular apoptosis.

Materials and Methods

Chemicals and reagents

Pepstatin A, leupeptin, phenylmethylsulfonyl fluoride, and aprotinin were purchased from Roche Molecular Biochemicals (Basel, Switzerland). Human Fas-Fc and a Cpp32/caspase-3 colorimetric protease kit were obtained from R&D systems (Minneapolis, MN). FBS was a Gibco-BRL product (Grand Island, NY), and MTT, acridine orange, ethidium bromide, and dipyridamole were purchased from Sigma (St. Louis, MO).

Chondrocyte culture

A primary culture of chondrocytes was prepared from rat costal cartilage according to the procedure previously described by Izumi *et al.* (1995). Briefly, 1-week-old Sprague-Dawley rat costal cartilage was placed in DMEM containing 4.5 g/L glucose. Cartilage slices were digested in 1% trypsin followed by 0.1% collagenase. After rinsing with growth medium (DMEM, 10% FBS, 50 μg/ml penicillin/streptomycin), a single cell suspension was obtained. Analyses with chondrocytes were peformed by culturing the cells in growth medium for various time periods. The effects of guanosine and other reagents were examined, using the culture medium without the reagent as a control system.

Staining cells with acridine orange/ethidium bromide

A cover slip was placed in each well of a 6-well culture plate (Nunc, Naperville, IL) and coated with 3% gelatin. Rat chondrocytes were seeded at 1.0×10^5 cells per well in 3 ml of 10% FBS-DMEM by incubating for 24 h, as described by Folkman *et al.* (1979). Cells were then cultured in the absence or presence of guanosine for 48 h. The culture medium was removed and 100 μl per well of a dye mixture (100 μg/ml acridine orange, 100 μg/ml ethidium bromide in PBS) was added to the cells. Subsequently, the cover slips were mounted onto glass slides and visualized using a Bio-Rad 2100 MP confocal system (Bio-Rad, Hercules, CE).

Measurement of caspase-3 activity

The caspase-3 activity was measured using a Cpp32/caspase-3 colorimetric protease kit following the manufacturer's instruction. Briefly, 1×10^7 cells were lysed on ice for 30 min with 100 μl of chilled 'cell lysis buffer' included in the kit. After centrifugation at 12,000 × *g* for 15 min at 4°C, the protein concentration in the supernatant was determined by the BCA method. Then, 150 μg of protein in the supernatant was diluted with 50 μl of 'dilution buffer' and mixed with 50 μl of 2 × 'reaction buffer' (containing 10 mM DTT) together with 5 μl of 4mM Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA). After incubation for 2 h at 37°C, the amount of released pNA was measured by absorbance at 405 nm on an ELISA reader.

Reverse transcription (RT)-PCR analysis of total RNA

Total RNA of rat chondrocytes was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) following the protocol recommended by the manufacturer. Three hundred ng of RNA was reverse transcribed and the resulting cDNA was amplified using a one-step RT-PCR kit (Qiagen, Stanford Valencia, CA). Reverse transcription was performed using a thermal program of 50°C for 1 h, coupled with 95°C for 15 min. PCR reactions were carried out using a thermal cycle program of 94°C for 30 s, 52°C for 60 s, and 72°C for 60 s for 30 repeating cycles. GAPDH was used as a reference for the semiquantitative analysis of gene expression. The nucleotide sequences of the primers were: Fas forward primer: 5'-GAATGCAAGGGACTGATAGC-3', Fas reverse primer: 5'-TGGTTCGTGTGCAAGGCTC-3', FasL forward primer: 5'-GGAATGGGAAGACACATATGGAACTGC-3', FasL reverse primer: 5'-CATATCTGGCCAGTAGTGCAGTAATTC-3', collagen type II forward primer: 5'-AGGAGGCTGGCAGCTG-3', collagen type II reverse primer: 5'-CACTGGCAGTGGCGAG-3', GAPDH forward primer: 5'-CAAGCTCCTACCATTCATGC-3', GAPDH reverse primer: 5'-TTCACACCGACCTTCACC-3'. PCR products were resolved by agarose gel (2%) electrophoresis and visualized with ethidium bromide under UV light.

Cell viability test

The viability of chondrocytes was determined using an MTT assay to assess non-viable cells, *i.e.* apoptosis (Mansfield *et al.*, 1999). The assay is based on the enzymatic activity of mitochondrial dehydrogenases reacting with pale yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), producing a dark blue formazan product. To perform the analysis, MTT (120 μg/ml) was added to the serum-free medium and the cells were cultured for 3 h at 37°C. After removing the culture medium, the product was solubilized in dimethyl sulfoxide (DMSO) and the absorbance at 540 nm was read on an ELISA reader.

Flow cytometry

To investigate the effect of guanosine on the

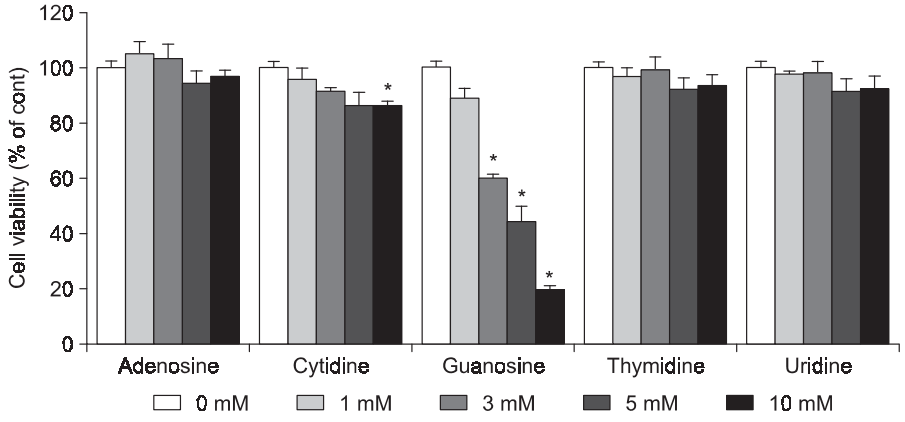

Figure 1. The effects of guanosine on the growth of chondrocytes in culture evaluated by cell viability. Rat chondrocytes (1×10^4 cells per well) were cultured for 48 h in a 96-well culture plate in the absence or presence of guanosine. The dose-dependent effects of guanosine were assessed by an MTT assay. The mean values and SDs from three independent analyses are shown. *$P < 0.01$.

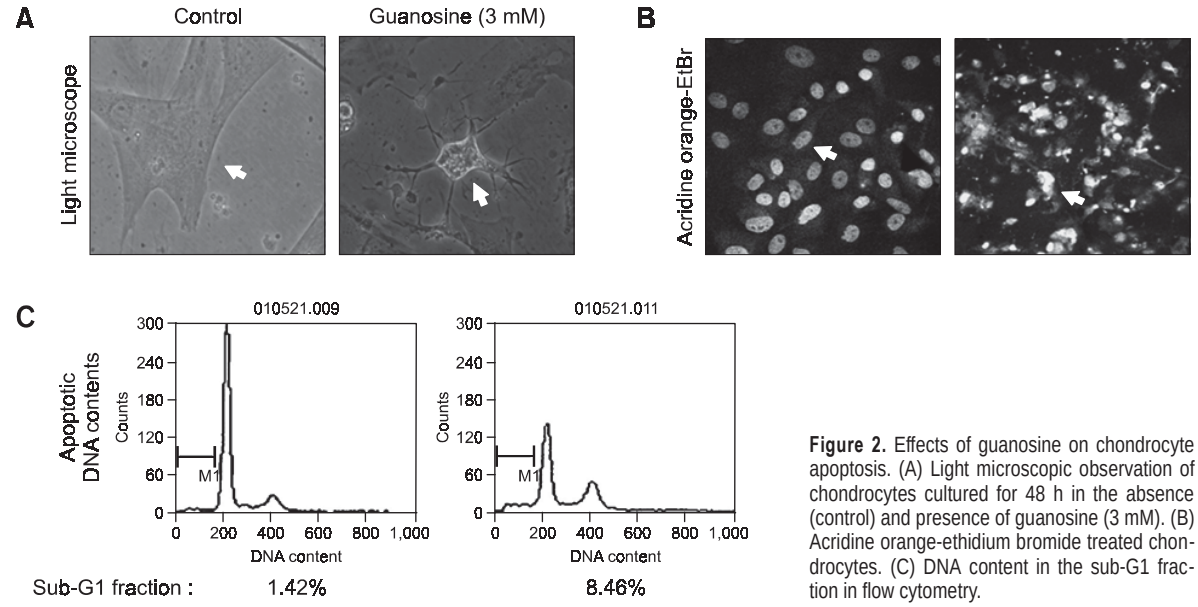

Figure 2. Effects of guanosine on chondrocyte apoptosis. (A) Light microscopic observation of chondrocytes cultured for 48 h in the absence (control) and presence of guanosine (3 mM). (B) Acridine orange-ethidium bromide treated chondrocytes. (C) DNA content in the sub-G1 fraction in flow cytometry.

apoptosis of chondrocytes, the DNA content of the cells at a given stage of the cell-cycle was determined by flow cytometry, according to the method previously described by Noguchi *et al.*, (1981). Chondrocytes were cultured with a desired concentration of guanosine for 48 h and fixed with 70% ethanol. The cells were then treated with 0.25 μg/ml of RNase and stained with 50 μg/ml of propidium iodide. The DNA content of the chondrocytes was then measured using a FACScaliber system (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Statistical analyses were performed using Student's paired *t*-test (*P* values are reported in the figure legends). At least 3 independent analyses were performed for each test.

Results

Induction of chondrocyte apoptosis by guanosine

We investigated the effect of guanosine on the growth of chondrocytes by means of an MTT assay. As shown in Figure 1, an increase in the concentration of guanosine resulted in a dose-dependent decrease in the number of viable chondrocytes, indicating significant cell death within 48 h. On the other hand, adenosine, cytidine, thymidine and uridine at various concentrations did not appear to affect the growth of chondrocytes.

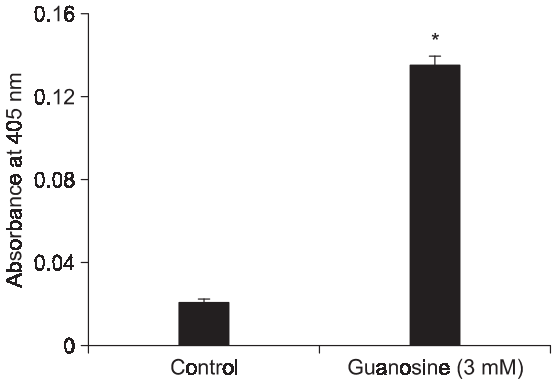

Figure 3. Effects of guanosine on the apoptotic process of chondrocytes evaluated by a caspase-3 activity assay. Chondrocytes were cultured for 36 h in the absence or presence of guanosine. The cell lysates were incubated with Ac-DEVD-pNA for 4 h at 37°C. *$P < 0.05$.

Condrocytes cultured in the absence of guanosine maintained their original nuclear morphology throughout the culture period (Figure 2A and B). Staining of dead cells with acridine orange-ethidium bromide revealed condensation and fragmentation of the nuclei, which are typical manifestations of apoptosis (Figure 2B). Subsequent FACS analysis of the cells showed an increasingly larger sub-G1 fraction with presence of 3 mM guanosine in the culture (Figure 2C).

Since caspase-3 is an executioner of apoptosis by a variety of stimuli (Earnshaw *et al.*, 1999), we investigated the possibility that caspase-3 is involved in guanosine-induced apoptosis of chondorycytes. Culturing chondrocytes for 36 h in 3 mM guanosine increased the caspase-3 activity in the chondrocytes 7-fold compared to a control system (Figure 3).

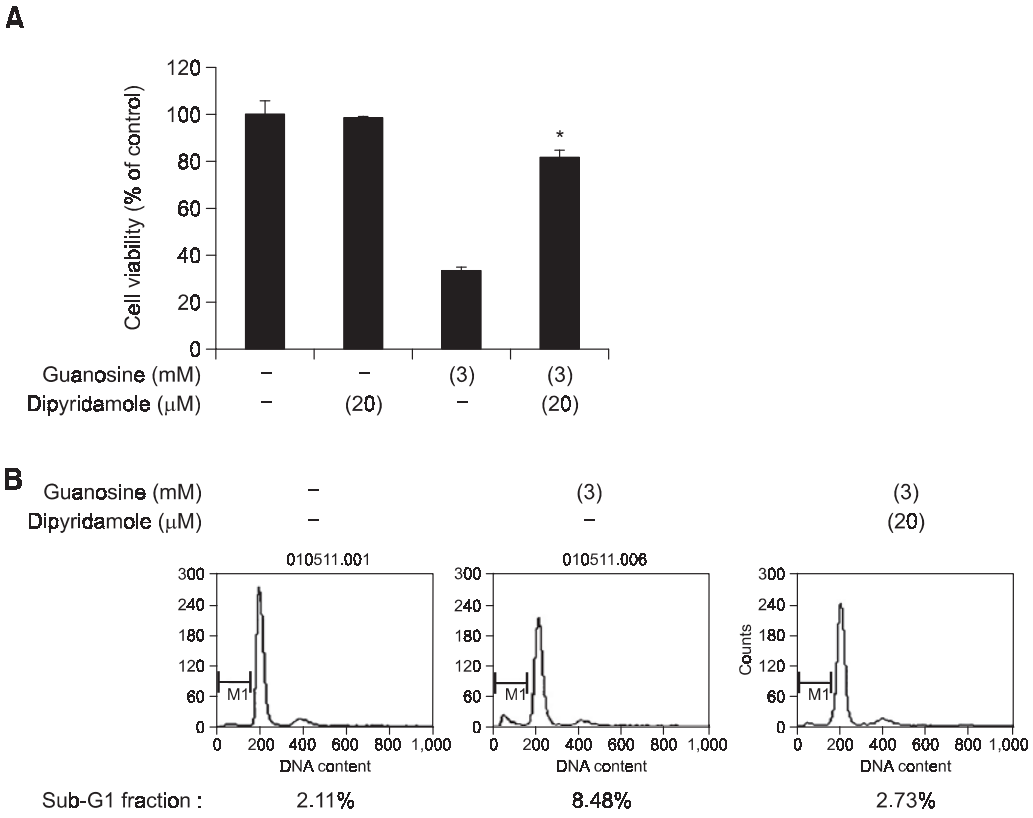

Figure 4. Effects of a transporter blocker on guanosine-induced chondrocyte apoptosis. (A) Dipyridamole inhibits guanosine-induced cell death, as determined by a cell viability assay, (B) DNA content analysis of the sub-G1 fraction by flow cytometry. Cells were pretreated with dipyridamole (20 μM) for 1 h, followed by coincubation with guanosine for an additional 48 h. *$P < 0.01$.

Intracellular induction of guanosine in chondrocyte apoptosis

To determine whether the apoptosis of chondrocytes is mediated by guanosine as a transporter or as a receptor, guanosine-treated chondrocytes were incubated with dipyridamole, which is a nucleoside analog capable of specifically inhibiting transporter activity. A cell viability assay and flow cytometry analysis revealed significant suppression of guanosine-induced apoptosis by dipyridamole (Figure 4A and B).

Induction of Fas ligand by guanosine

To understand the mechanisms by which guanosine induces apoptosis in chondrocytes, we examined the expressions of apoptosis regulating genes by the RT-PCR method. Fas/Fas ligands are regarded as autocrine receptor-ligands associated with cell death in their signal transduction pathway (Belka *et al.*, 1998). Culturing chondrocytes in the presence of guanosine produced transient expression of the Fas ligand within chondrocytes such that maximal expression of the Fas ligand was observed after 4 h. Fas ligand induction was not observed in the control system over the same time period (Figure 5A). Fas mRNA, however, was expressed constitutively in cultures both with and without guanosine (Figure 5A). Thus, the secretion and autocrine engagement of the Fas ligand with Fas at the cell surface are probably linked to induction of chondrocyte apoptosis by guanosine. As a test, cells were preincubated prior to addition of guanosine with the fusion molecule Fas-Fc, which is known to interfere with the Fas-Fas ligand interaction and thereby inhibit the signals originating from Fas (Yamaoka *et al.*, 2000). Fas-Fc (10 μg/ml) was able to block guanosine-induced chondrocyte apoptosis (Figure 5B).

Discussion

Endochondral ossification involves an ordered progression from cell division through hypertrophic differentiation to cell death (Gibson, 1998). However, the mechanism of cell death in cartilage has not been elucidated owing to the characteristic of cartilage as a single cell type that is not innervated, vascularized, or penetrated by lymphatic vessels

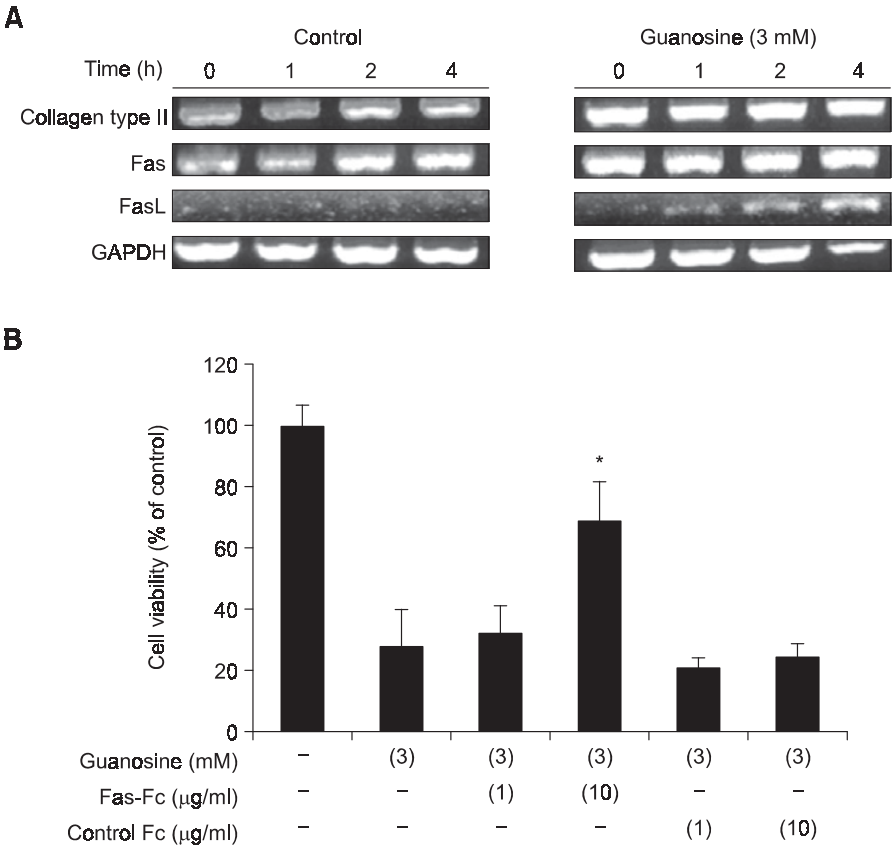

Figure 5. Induction of the Fas ligand in guanosine-induced chondrocyte apoptosis. (A) RT-PCR analysis of FasL mRNA levels in chondrocytes cultured in the absence (control) or presence of guanosine (3 mM) and harvested at various times during the culture. The analysis compared the effects of collagen type II, Fas, FasL, and GAPDH. (B) Inhibition test of FasL-induced apoptosis of chondrocytes by Fas-Fc measured in terms of cell viability. The chondrocytes were incubated in the presence of guanosine (3 mM) for 48 h. Fas-Fc at various concentrations or control Fc at corresponding concentrations were added 30 min prior to addition of guanosine. The results shown are the mean values of three independent analyses. *$P < 0.05$.

(Ishizaki *et al.*, 1994). Matsumoto *et al.* reported that guanosine concentrations were substantially increased in the hypertrophic zone and in calcified cartilage (1988). We believe that the high levels of guanosine in those areas are linked with chondrocyte physiology, especially apoptosis. Based on the results of an MTT assay (Figure 1), guanosine induced the cell death of chondrocytes in a dose-dependent manner. The effect of guanosine for induction of apoptosis was confirmed by light microscopy, fluorescence microscopy with acridine orange/ethidium bromide dual-staining, FACS analysis, and a caspase-3 activity assay (Figure 2 and 3). Additionally, the effect of guanosine for induction of apoptosis was confirmed by dipyridamole, a guanosine transporter blocker that caused inhibition of guanosine induced chondrocyte apoptosis (Figure 4). Thus, guanosine can induce apoptosis within chondrocyte cells by activating the signal pathway leading to apoptosis.

We investigated whether or not guanosine activated of the extrinsic death pathway rather than the intrinsic pathway as a mechanism for chondrocyte apoptosis. Fas/FasL was used as a candidate extrinsic cell death system because the autocrine Fas ligand was reported to promote apoptosis in peripheral lymphocytes (Belka *et al.*, 1998). Under normal conditions, other cell types do not enter cartilage and chondrocytes do not migrate within the matrix of the cartilage (Hashimoto *et al.*, 1998). The presence of guanosine in the culture increased FasL levels in chondrocytes, concomitant with the induction of apoptosis (Figure 5). Apparently guanosine induces chondrocyte apoptosis by activating the Fas/FasL pathway.

We reported in a previous study that purine nucleoside-induced apoptosis in HL-60 human leukemia cells was modulated by addition of pyrimidine nucleosides (Kim *et al.*, 1998). Although the effect of an unbalanced nucleoside was not fully explainable; some changes were attributed to induction of Nm23 and/or myc (Kim *et al.*, 1998). The importance of a nucleoside balance at the extreme upstream position of the apoptotic pathway has also been suggested for modulating apoptosis induction (Kang *et al.*, 2001). These reports indicate that a metabolic disturbance of nucleosides may lead to apoptosis. The results of this study provide the first evidence that an intermediate metabolic product participates in the control process of endochondral ossification. The present findings should stimulate research leading to intervention or treatment of age-associated osteoarthritis by manipulating the guanosine content in chondrocytes.

In conclusion, metabolic regulation by the hypertrophic and calcified zone of cartilage generates guanosine, which plays an important role in controlling the apoptotic process in chondrocytes. Condrocyte apoptosis may be mediated by the FasL pathway. The potential for using guanosine as a target for development of novel treatment modalities for osteoarthritis should be investigated.

Acknowledgement

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