Interleukin-1 β induces death in chondrocyte-like ATDC5 cells through mitochondrial dysfunction and energy depletion in a reactive nitrogen and oxygen species-dependent manner

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IL-1 (interleukin-1) acts as a key mediator of the degeneration of articular cartilage in RA (rheumatoid arthritis) and OA (osteoarthritis), where chondrocyte death is observed. It is still controversial, however, whether IL-1 induces chondrocyte death. In the present study, the viability of mouse chondrocyte-like ATDC5 cells was reduced by the treatment with IL-1 β for 48 h or longer. IL-1 β augmented the expression of the catalytic gp91 subunit of NADPH oxidase, gp91^{phox}, as well as inducible NO synthase in ATDC5 cells. Generation of nitrated guanosine and tyrosine suggested the formation of reactive nitrogen species including ONOO⁻ (peroxynitrite), a reaction product of NO and O₂⁻, in ATDC5 cells and rat primary chondrocytes treated with IL-1 β . Death of ATDC5 cells after IL-1 β treatment was prevented by an NADPH-oxidase inhibitor, AEBF [4-(2-aminoethyl)benzenesulphonyl fluoride], an NO synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester), and a ONOO⁻ scavenger, uric

acid. The viability of ATDC5 cells was reduced by the ONOO⁻-generator 3-(4-morpholinyl)sydnonimine hydrochloride, but not by either the NO-donor 1-hydroxy-2-oxo-3-(N-methyl-2-amino-propyl)-3-methyl-1-triazene or S-nitrosoglutathione. Disruption of mitochondrial membrane potential and ATP deprivation were observed in IL-1 β -treated ATDC5 cells, both of which were restored by L-NAME, AEBF or uric acid. On the other hand, no morphological or biochemical signs indicating apoptosis were observed in these cells. These results suggest that the death of chondrocyte-like ATDC5 cells was mediated at least in part by mitochondrial dysfunction and energy depletion through ONOO⁻ formation after IL-1 β treatment.

Key words: interleukin-1 β , NADPH oxidase, nitric oxide, nitric oxide synthase, peroxynitrite, superoxide.

INTRODUCTION

Degradation of articular cartilage is a common pathogenic change seen in the degenerative joint diseases, such as RA (rheumatoid arthritis) and OA (osteoarthritis). It is known that IL-1 (interleukin-1) plays pivotal roles in the pathogenesis of OA as well as RA. One of the major roles of IL-1 is considered to be the induction of catabolic processes that affect cartilage matrices. Namely, it is reported that IL-1 not only stimulates the release of degenerative enzymes, including matrix metalloproteinases, from chondrocytes and synoviocytes, but also inhibits the synthesis of extracellular matrix proteins by chondrocytes [1]. It has also been suggested that IL-1 is involved in the osteoclastogenesis and bone resorption that is increased in RA joints [2]. Reduced cellularity or apoptotic chondrocyte death in articular cartilage is another feature that is observed in clinical specimens from RA and OA cartilages [3–5] and those from animal models of these diseases [6]. The mechanism of chondrocyte death in RA and OA, however, has not been fully elucidated.

In these joint diseases, expression of iNOS (inducible NO synthase) and overproduction of NO were detected in synovial tissue and articular cartilage [7–10]. NO is a gaseous free radical produced by the members of the NOS (NO synthase) family

including iNOS. NO serves as a key signalling molecule in various physiological processes. NO synthesis has also been implicated as a causal or contributing factor to pathological conditions including RA and OA [7–10]. The effects of NO on cells depend on many complex conditions, such as the rate of NO production, its diffusion and the concentration of potential reactants, such as molecular oxygen, O₂⁻ and thiols [11,12]. A number of reports are available that describe the involvement of NO in the death and survival of various types of cells [13]. It has also been reported that NO takes part in the induction of apoptosis in chondrocytes *in vitro* [14–16], *ex vivo* [17] and *in vivo* [4,6]. On the other hand, the requirement of ROS (reactive oxygen species) is reported for NO-mediated chondrocyte death *in vitro* [18]. iNOS expression in chondrocytes is induced by a single stimulation of IL-1 or tumour necrosis factor [19], while multiple cytokines are required for the induction of iNOS in most of the other types of cells. In this context, IL-1 might cause apoptotic death of chondrocytes via overproduction of NO. Pro-inflammatory cytokines, including IL-1 α , induced apoptosis in bovine chondrocytes *in vitro* [20]. Increased apoptosis was observed in human chondrocytes from normal and OA cartilages after incubation with human IL-1 β [5]. IL-1-induced apoptosis was observed under the conditions where ROS-mediated necrosis was suppressed [14]. Conversely,

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; DePsipher™, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo-cyanine iodide; DMEM/F12, a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; FCS, foetal calf serum; γ -GCS, γ -glutamylcysteine synthase; gp91^{phox}, catalytic gp91 subunit of NADPH oxidase; HBSS, Hanks balanced salt solution; IL-1, interleukin-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; D-NAME, N^G-nitro-D-arginine methyl ester; L-NAME, N^G-nitro-L-arginine methyl ester; NBT, Nitro Blue Tetrazolium; NOC-7, 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene; (i)NOS, (inducible) NO synthase; OA, osteoarthritis; RA, rheumatoid arthritis; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, reverse transcriptase; SIN-1, 3-(4-morpholinyl)sydnonimine hydrochloride; SOD, superoxide dismutase; XO, xanthine oxidase.

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it has also been reported that deletion of the gene for either IL-1 β or iNOS increased the number of apoptotic cells in the articular cartilage of OA model mice [21]. In addition, IL-1 β and NO are also known to function as anti-apoptotic mediators in various types of cells, including chondrocytes [22,23]. Therefore it remains controversial whether IL-1 or NO contributes to chondrocyte death.

In the present study, we found that mouse chondrocyte-like ATDC5 cells died after exposure to IL-1 β via energy depletion in an RNS (reactive nitrogen species)-dependent mechanism.

EXPERIMENTAL

Reagents

Recombinant mouse IL-1 β was obtained from TECHNE Co. (Minneapolis, MN, U.S.A.). L-NAME (*N*^G-nitro-L-arginine methyl ester), D-NAME (*N*^G-nitro-D-arginine methyl ester), AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride] and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Allopurinol, uric acid and NBT (Nitro Blue Tetrazolium) were obtained from Wako Pure Chemical Co. (Osaka, Japan). The NO-donor NOC-7 [1-hydroxy-2-oxo-3-(*N*-methyl-3-amino-propyl)-3-methyl-1-triazene], having a half-life of 5 min, *S*-nitrosoglutathione, peroxyxynitrite (ONOO⁻), SIN-1 [3-(4-morpholinyl)sydnimine hydrochloride] and Hoechst 33258 dye were from Dojindo Laboratories (Kumamoto, Japan). Bovine Cu,Zn-SOD (where SOD is superoxide dismutase) was from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). All other reagents were of analytical grade from commercial sources.

Cell culture

ATDC5 cells [24] were supplied by The Riken BioResource Center of The Institute of Physical and Chemical Research (Tsukuba, Japan). Cultures of undifferentiated ATDC5 cells were maintained in DMEM/F12 (a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) (Sigma-Aldrich) supplemented with 5% (v/v) FCS (foetal calf serum) (Trace Biosciences Pty, Castle Hill, NSW, Australia), 100 units/ml penicillin G, 100 μ g/ml streptomycin and 2.5 μ g/ml amphotericin B. Once the culture was confluent, the medium was changed to DMEM/F12 with FCS and antibiotics (as described above), additionally supplemented with ITS (10 μ g/ml insulin, 5.5 μ g/ml transferrin and 6.7 ng/ml sodium selenite) (Invitrogen, Carlsbad, CA, U.S.A.) and the cells were cultured for 3 weeks to induce differentiation into chondrocyte-like cells [24]. Differentiation into chondrocyte-like cells was confirmed by the expression of mRNAs for type II and type X collagens and osteopontin, as well as the production of glycosaminoglycan visualized by Alcian Blue staining. Calcium deposition evaluated by Alizarin Red staining was negative in the cell culture that was used in the present study.

We also used primary rat chondrocytes isolated from the ribcages of 1-day-old Sprague-Dawley rats by successive digestion with collagenase D from *Clostridium histolyticum* (Roche Diagnostics GmbH, Mannheim, Germany). The rat chondrocytes were also cultured in DMEM/F12 supplemented with 5% FCS and antibiotics. Isolation of rat chondrocytes was carried out according to the protocol approved by the Ethical Board for Animal Experiments at Showa University (Approval No. 14060).

Cell viability

The cells cultured in 96-well plates were treated with the various reagents, including IL-1 β , in the complete medium containing

5% (v/v) FCS unless otherwise mentioned. The viability of the cells was examined by MTT assay. The MTT-formazan was solubilized and determined by reading the absorbance at 562 nm using a microplate reader (Model 3550, Bio-Rad, Hercules, CA, U.S.A.). Cell viability was also evaluated by counting the number of cells with a normal-shaped nucleus under a microscope after staining with 0.1 mg/ml Hoechst 33258 dye.

Production of NO and O₂⁻ by the cells

The NO production was evaluated by measuring NO₂⁻ and NO₃⁻ in the culture medium using NO₂/NO₃ Assay Kit-C II (Dojindo Laboratories). Briefly, the cells grown in 96-well plates were incubated with IL-1 β in the presence or absence of 5 mM L-NAME in the complete medium without Phenol Red. The supernatants of the culture medium obtained at 0, 12, 24, 48, 72 and 96 h after addition of IL-1 β were subjected to the determination of NO₂⁻ plus NO₃⁻.

Evaluation of O₂⁻ generation was performed as follows. ATDC5 cells were re-plated sparsely and treated with 1 ng/ml IL-1 β for 12, 24 and 48 h, and were washed three times with HBSS (Hanks balanced salt solution). Then the cells were incubated for 30 min at 37°C with 0.05% NBT in HBSS in the presence or absence of 1 ng/ml IL-1 β , 0.1 mM allopurinol, 0.3 mM AEBSF or 10000 units/ml Cu,Zn-SOD. After washing once with HBSS, the number of cells with and without deposition of NBT-formazan was counted under a light microscope.

Mitochondrial membrane potential

After treatment with 1 ng/ml IL-1 β , the adherent ATDC5 cells were harvested by digestion with 0.2% collagenase D and 0.4% dispase (Godo Shusei Co., Tokyo, Japan) in the complete medium and washed three times with PBS. The change in mitochondrial membrane potential was analysed by staining the cells with DePsipher™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyli carbocyanin iodide) (Trevigen, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions. In brief, DePsipher™ dye accumulates in the transmembrane space of normally polarized mitochondria to emit a red/orange fluorescence, but it cannot access the depolarized mitochondria and shows green fluorescence. The cells stained with DePsipher™ dye were analysed using a flow cytometer (FACSCaliber, Becton Dickinson, San Jose, CA, U.S.A.).

In another set of experiments, the cells were observed under a fluorescence microscope after staining with DePsipher™ dye. Briefly, the cells grown in the medium containing ITS were re-plated on the culture slides (Falcon No. 354108; BD Biosciences, Bedford, MA, U.S.A.) and incubated for 48 h with 1 ng/ml IL-1 β in the presence or absence of 5 mM L-NAME, 0.3 mM AEBSF or 0.7 mM uric acid. After staining with DePsipher™ dye for 30 min, the cells were subjected to microscopical observation.

Cellular ATP levels

ATP content in the cells was determined using an ATP Bioluminescence Assay kit (Roche Diagnostics GmbH). The ATP-dependent generation of chemiluminescence was measured using a Microplate Luminometer LB96 (EG&G Berthold, Bad Wildbad, Germany).

RT (reverse transcriptase)-PCR

Total RNA was extracted from the cells grown in 60-mm-diameter dishes using TRIzol® solution (Invitrogen), according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA using an Omniscript RT kit (Qiagen,

Table 1 Primers used for RT-PCR analyses of the genes involved in the production and elimination of RNS and ROSGclc, catalytic subunit of γ -GCS; Gclm, modifier subunit of γ -GCS.

Gene	Sense (S) and antisense (A) primers	Amplicon size (bp)
iNOS	(S) 5'-ACGCTTGGGTCTTGTCTCACT-3' (A) 5'-GTCTCTGGGTCCTCTGGTCA-3'	468
XO	(S) 5'-GCCAAGGATAAGGTACTTGT-3' (A) 5'-CTCCAGGTAGAAGTCTCTTG-3'	268
gp91 ^{phox}	(S) 5'-GAGGGTTTCCAGCCAGCACTTTGGT-3' (A) 5'-TGAAGGGGGCTGTATGTGG-3'	349
Mn-SOD	(S) 5'-CTTCAGCGTGCAGTGAAGTTCAT-3' (A) 5'-CTGAAGGTAGTAAGCGTGCTCCC-3'	327
Cu,Zn-SOD	(S) 5'-GCCACGAAGGCCGTGTGCGTGC-3' (A) 5'-ACTTCTTCATTTCCACCTTTGCC-3'	399
Gclc	(S) 5'-CACTGAGCTGGGAAGAGACC-3' (A) 5'-CTGGGTTGGGTCTGTGTCT-3'	468
Gclm	(S) 5'-TGGAGCAGCTGTATCAGTGG-3' (A) 5'-TGGAGTTAAGAGCCCTCCT-3'	316

Valencia, CA, U.S.A.) and was subjected to PCR amplification with Taq polymerase (TaKaRa Bio, Otsu, Japan). The primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The other primers were synthesized and supplied by Invitrogen. Sequences of the primers used are listed in Table 1. PCR products were separated on a 1.5% (w/v) agarose gel and were stained with ethidium bromide.

Western blot analysis

The cell lysates (25 μ g of protein) were subjected to SDS/PAGE (10% polyacrylamide gel) under reducing conditions. After electrophoresis, proteins were transferred on to PVDF membranes. The membranes were incubated for 1 h with the primary antibody against iNOS, XO (xanthine oxidase), gp91^{phox} (the catalytic gp91 subunit of NADPH oxidase) or the catalytic subunit of γ -GCS (γ -glutamylcysteine synthase) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), followed by incubation with a horseradish-peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences, Little Chalfont, Bucks., U.K.). The immunoreactive bands were visualized by the enhanced chemiluminescence reaction using ECL[®] Western Blotting Detection Reagent (Amersham Biosciences). The density of each band was measured by using the ImageJ software released on the Internet by National Institutes of Health (Bethesda, MD, U.S.A.).

Immunocytochemistry

The cells were fixed with ethanol/ethanoic (acetic) acid (19:1, v/v) and Zamboni's solution [0.1 M sodium phosphate buffer, pH 7.4, containing 4% (w/v) paraformaldehyde and 2 mg/ml 2,4,6-trinitrophenol (picric acid)] for immunostaining of 3-nitrotyrosine and 8-nitroguanosine respectively. For detection of 3-nitrotyrosine, fixed cells were incubated overnight with 100-fold-diluted anti-3-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, Charlottesville, VA, U.S.A.), and then for 1 h with Histofine Simple Stain MAX-PO (MULTI) (Nichirei Co., Tokyo, Japan). Peroxidase activity associated with the secondary antibody was visualized by a reaction with Histofine Simple Stain AEC substrate solution (Nichirei Co.). For detection of 8-nitroguanosine, the fixed cells were incubated overnight with 6.7 μ g/ml anti-8-nitroguanosine monoclonal antibody (Dojindo Laboratories) as reported previously [25]. The cell-bound

Figure 1 NOS-activity-dependent death of ATDC5 cells induced by IL-1 β

(A) ATDC5 cells were incubated for various periods with (○) or without (●) 1 ng/ml IL-1 β , stained with Hoechst 33258, and counted under the microscope. Each point indicates the percentage of viable cells relative to the initial number of viable cells. Results are the means $\pm$ S.D. for four independent experiments. *, Values were significantly lower than control without IL-1 β ($P < 0.01$). (B) ATDC5 cells were treated with 1 ng/ml IL-1 β for 72 h in the presence of 5 mM L-NAME (●) or D-NAME (○), and their viability was determined by MTT assay. Results are the means $\pm$ S.D. for four experiments. *, Values were significantly higher than the value obtained without L-NAME ($P < 0.01$).

primary antibody was visualized under a fluorescence microscope after incubation with Cy3-linked anti-mouse-IgG goat antibody (Amersham Biosciences).

RESULTS

Death of ATDC5 cells after exposure to IL-1 β

The mouse chondrocyte-like ATDC5 cells were incubated with 1 ng/ml recombinant mouse IL-1 β in the complete medium containing 5% (v/v) FCS. After exposure to IL-1 β , the number of adherent ATDC5 cells declined in a time-dependent manner: the number of adherent ATDC5 cells at 24 and 48 h after addition of IL-1 β was 90 and 37% of the initial number respectively (Figure 1A). Approx. 90–96% of the adherent cells had normal-shaped nuclei, and the apoptotic changes were not detected in these cells as far as we examined. More specifically, the following indices were not changed after IL-1 β treatment: cell membrane alteration visualized by annexin V binding, activation of caspase 3, expression of Bcl-2 family including Bcl-2, Bcl-X_L and Bax. The expression of CHOP, a transcription factor that is involved in the process of apoptosis associated with the endoplasmic reticulum stress, or Bip, a marker of endoplasmic reticulum stress, was not affected by IL-1 β either. In addition, either Z-DQMD-FMK (benzyloxycarbonyl-Asp-Gln-Met-Asp-fluoromethylketone) (Calbiochem–Novabiochem) a specific inhibitor for caspase 3 or Ac-YVAD-CMK (acetyl-Tyr-Val-Ala-Asp-chloromethylketone) (Peptide Institute, Osaka, Japan), a broad-spectrum inhibitor for caspases, did not ameliorate the survival of ATDC5 cells treated with IL-1 β . On the other hand, the cells severely burst after exposure to IL-1 β for more than 48 h (results not shown).

Figure 1(B) shows the concentration-dependent effects of IL-1 β to reduce the viability of ATDC5 cells assessed after 72 h of exposure to the cytokine. The NOS inhibitor L-NAME, but not

Figure 2 Expression of iNOS and the oxidative-stress-related enzymes in ATDC5 cells after treatment with IL-1 β

(A) Expression of mRNAs for iNOS, XO, gp91^{phox}, Mn-SOD, Cu,Zn-SOD, Gclc (catalytic subunit of γ -GCS), Gclm (modifier subunit of γ -GCS) and GAPDH in ATDC5 cells after incubation with 1 ng/ml IL-1 β was analysed by RT-PCR using the primers shown in Table 1. -RT is the negative reverse transcription control using GAPDH primers. (B) Western blot analyses of iNOS, gp91^{phox}, XO and Gclc expressed by ATDC5 cells at various time points after addition of 1 ng/ml IL-1 β to the culture. See text for details. The lower panel shows the changes in the density of the immunoreactive bands for iNOS (●), gp91^{phox} (■) and XO (▲) normalized by that of Gclc which was unaffected by IL-1 β . Expression level of each protein at each time point was shown as the ratio to that at 48 h. Results are the mean values for two experiments. (C) NO₂⁻ plus NO₃⁻ in culture medium of ATDC5 cells incubated with 1 ng/ml IL-1 β in the presence (○) or absence (●) of 5 mM L-NAME was quantified. Results are the means $\pm$ S.D. for four experiments. (D) O₂⁻ production by ATDC5 cells was assessed by the reduction of NBT. After treatment with 1 ng/ml IL-1 β for 12, 24 and 48 h, ATDC5 cells were incubated for 30 min at 37 °C with 0.05 % NBT in HBSS in the presence of 1 ng/ml IL-1 β with (○) or without 10 000 units/ml Cu,Zn-SOD (●). The cells were observed under a microscope. Part of the cells treated for 48 h with IL-1 β were incubated with 1 ng/ml IL-1 β and 0.05 % NBT in the presence of 0.1 mM allopurinol (■) or 0.3 mM AEBSF (▲). Each point shows the percentage of the number of cells with deposition of NBT-formazan to the total number of the cells. Results are the means $\pm$ S.D. for four experiments. * and **, Values obtained in the presence of inhibitors were significantly lower than that obtained without inhibitor (●) (* P < 0.05, ** P < 0.01). #The value of the AEBSF group (▲) was significantly lower than that of allopurinol group (■) (P < 0.01).

its inactive isomer D-NAME, exhibited marked protection against IL-1 β -induced cell death, indicating that NOS activity is essential for the death of ATDC5 cells observed after exposure to IL-1 β (Figure 1B).

Expression of iNOS and the genes related to regulation of oxidative stress in ATDC5 cells

It is well known that iNOS expression in chondrocytes is induced by IL-1 β [19]. We next analysed the mRNA and protein expression of iNOS and several other genes related to the metabolism of ROS in ATDC5 cells. Consistent with the up-regulation of iNOS mRNA level (Figure 2A), iNOS protein content was increased in IL-1 β -treated ATDC5 cells (Figure 2B). The accumulation of NO₂⁻ and NO₃⁻, stable end products of NO, in the culture medium indicated the constant production of NO by ATDC5 cells at least until 48 h after IL-1 β treatment (Figure 2C).

IL-1 β also augmented the mRNA and protein levels of XO and gp91^{phox} (Figures 2A and 2B). Low-level gp91^{phox} protein was also detected in the cells before addition of IL-1 β , and increased

gradually after treatment with the cytokine. XO and NADPH oxidase are a well-known enzyme and an enzyme system both of which produce O₂⁻ and H₂O₂. As shown in Figure 2(D), the Cu,Zn-SOD-inhibitable reduction of NBT by ATDC5 cells, which reflects the amount of O₂⁻, was detected after exposure to IL-1 β for 48 h. While both the NADPH oxidase inhibitor AEBSF [26] and the XO inhibitor allopurinol partially inhibited NBT reduction, the inhibitory potency of the former was much greater than that of the latter, indicating that the major source of O₂⁻ production was NADPH oxidase (Figure 2D).

Expression of mRNA for Mn-SOD, an O₂⁻-catabolizing enzyme distributed in mitochondria, was slightly up-regulated by IL-1 β , which is consistent with the earlier report of the up-regulation of Mn-SOD expression by IL-1 β in OA chondrocytes [27]. In contrast, expression of mRNA for Cu,Zn-SOD or that for either Gclc or Gclm, the two subunits of γ -GCS (catalytic and modifier subunits respectively), the rate-limiting enzyme for glutathione synthesis, was marginally affected by IL-1 β (Figure 2A). Increase in the expression of ROS-generators, such as XO and gp91^{phox}, and little change in that of ROS-scavenging systems, such as SODs

Figure 3 Involvement of ONOO⁻ in IL-1 β -induced death of ATDC5 cells

(A) Cytotoxicity of NO, S-nitrosoglutathione, and ONOO⁻ to ATDC5 cells. ATDC5 cells were incubated for 2 h with 1 mM NOC-7 (NO-donor), 1 mM S-nitrosoglutathione (GS-NO) or 1 mM SIN-1 (ONOO⁻-donor) in Krebs–Ringer phosphate buffer (15.6 mM sodium phosphate buffer, pH 7.4, containing 0.12 M NaCl, 4.8 mM KCl, 0.54 mM CaCl₂, 1.2 mM MgSO₄ and 11 mM glucose), followed by additional incubation for 2 days in the complete medium without NO-related compounds. Cytotoxicity of SIN-1 was examined in the presence or absence of 0.7 mM uric acid. The viability of the cells was determined by MTT assay. Results are the mean values for duplicated experiments. (B) Protective effect of uric acid on the IL-1 β -induced death of ATDC5 cells. The cells were incubated for 48 h with 1 ng/ml IL-1 β in the presence or absence of 0.7 mM uric acid, and the MTT assay was performed. Results are the means $\pm$ S.D. for four experiments. *, Values significantly higher than the IL-1 β group (left-hand column) ($P < 0.01$). (C) Immunocytochemical detection of 8-nitroguanosine (8-NO₂-Guo) and 3-nitrotyrosine (3-NO₂-Tyr) in ATDC5 cells after 48 h of treatment with 1 ng/ml IL-1 β in the presence of 5 mM L- or D-NAME. The specific antibody against 8-nitroguanosine was visualized with Cy3-labelled secondary antibody (red), and that for 3-nitrotyrosine with peroxidation reaction of diaminobenzidine and horseradish peroxidase linked to the secondary antibody (red/brown). (D) Effects of Cu,Zn-SOD (SOD), the inhibitors for NADPH oxidase (AEBSF) and XO (allopurinol), and uric acid on IL-1 β -induced death of ATDC5 cells. Cells were treated with 1 ng/ml IL-1 β for 72 h in the absence or presence of 500 units/ml Cu,Zn-SOD, 0.3 mM AEBSF, 0.1 mM allopurinol or 0.7 mM uric acid. Allopurinol, AEBSF or uric acid was added to the cells simultaneously with IL-1 β . Cu,Zn-SOD was added 24 h after IL-1 β . Cell viability was determined by the MTT assay. Results are the means $\pm$ S.D. for four experiments. *, Values significantly higher than the IL-1 β group (left-hand column) ($P < 0.01$).

and γ -GCS (Figure 2A), indicated the augmented production of ROS in the cells treated with IL-1 β .

Effects of NO and NO metabolites on the viability of ATDC5 cells

To examine whether NO or its metabolites is able to kill ATDC5 cells, the cells were incubated with 1 mM NOC-7, S-nitrosoglutathione or SIN-1 (Figure 3A). NOC-7 spontaneously liberates two molecules of NO with a half-life of 5 min in neutral milieu. The inability of NOC-7 or S-nitrosoglutathione to kill ATDC5 cells indicated that NO itself or nitrosothiols was unlikely to be responsible for the NOS-dependent demise of the cells observed after addition of IL-1 β . On the contrary, SIN-1, a donor of NO and O₂⁻, and hence a generator of ONOO⁻, produced cell death in ATDC5 cells, which was nullified by 0.7 mM uric acid, a known scavenger of ONOO⁻ (Figure 3A).

Involvement of RNS in the IL-1 β -induced death of ATDC5 cells

IL-1 β -induced ATDC5-cell death was also inhibited by uric acid (Figure 3B), indicating the possible involvement of ONOO⁻, a

reaction product of NO and O₂⁻, in the process of the cell death. It is known that ONOO⁻ and NO₂⁻ plus H₂O₂ in the presence of peroxidases leave nitrated aromatic compounds as their footprints [25,28–32]. As shown in Figure 3(C), we detected 8-nitroguanosine in ATDC5 cells incubated with IL-1 β in the presence of D-NAME, which was suppressed by L-NAME. In addition, 3-nitrotyrosine was also detected in the cells and the extracellular matrix of ATDC5 cells treated with IL-1 β (Figure 3C). Again, the formation of 3-nitrotyrosine decreased in the presence of L-NAME. These results show clearly that the nitrative stress from ONOO⁻ and/or other RNS was loaded on the cells after stimulation with IL-1 β .

The nitration reactions require the production not only of NO, but also O₂⁻ or H₂O₂, as described above. The death of ATDC5 cells induced by IL-1 β was inhibited by Cu,Zn-SOD, indicating that O₂⁻, but not H₂O₂, was involved in the cell death (Figure 3D). The amelioration of the survival rate by AEBSF, but not by allopurinol, suggested that most of the O₂⁻ involved in the cell death after treatment with IL-1 β was produced by NADPH oxidase (Figure 3D), which was consistent with the results shown in

Figure 4 NOS-activity-dependent formation of nitrated compounds and cell death in rat primary chondrocytes after exposure to rat IL-1 β

(A) The adherent rat primary chondrocytes having normal-shaped nuclei were counted after Hoechst 33258 staining following incubation for the indicated periods in the presence (○) or absence (●) of 1 ng/ml rat IL-1 β . Results are the means $\pm$ S.D. for four experiments. * and **, Values significantly lower than the control without IL-1 β (* P < 0.05, ** P < 0.01). (B) Rat chondrocytes were treated with 1 ng/ml rat IL-1 β for 72 h in the presence of 5 mM L-NAME (●) or D-NAME (○), and their viability was determined by the MTT assay. Results are the means $\pm$ S.D. (n = 4). *, Values significantly higher than the D-NAME control (○) (P < 0.05). (C) Effects of Cu,Zn-SOD (SOD) or AEBSF on IL-1 β -induced death of rat primary chondrocytes. Cells were treated with 1 ng/ml IL-1 β for 72 h in the absence and presence of 500 units/ml Cu,Zn-SOD or 0.3 mM AEBSF. Cu,Zn-SOD was added 24 h after IL-1 β . Cell viability was determined by the MTT assay. Results are the means $\pm$ S.D. for four experiments. * and **, Values significantly higher than the IL-1 β group (left-hand column) (* P < 0.05, ** P < 0.01). (D) Immunocytochemical detection of 8-nitroguanosine (8-NO₂-Guo; white) and 3-nitrotyrosine (3-NO₂-Tyr; black) in rat chondrocytes after 48 h of treatment with 1 ng/ml rat IL-1 β in the presence of 5 mM L- or D-NAME.

Figure 2(D): AEBSF was more effective than allopurinol to inhibit the reduction of NBT by IL-1 β -treated cells.

NO- and O₂⁻-dependent death of rat primary chondrocytes after exposure to IL-1 β

Similar results were obtained in the rat primary chondrocytes obtained from the ribcages of 1-day-old Sprague–Dawley rats (Figure 4). Namely, a time-dependent decline in cell viability was observed after exposure to recombinant rat IL-1 β (Figure 4A), which was rescued by 5 mM L-NAME, 0.3 mM AEBSF or 500 units/ml Cu,Zn-SOD (Figures 4B and 4C). The formation of 8-nitroguanosine and 3-nitrotyrosine in the IL-1 β -treated rat primary chondrocytes indicated the production of RNS, including ONOO⁻, in or around the cells (Figure 4D).

Disturbed mitochondrial membrane potential and lowered energy status in ATDC5 cells treated with IL-1 β

ATDC5 cells incubated for 24, 48 and 72 h in the presence and absence of 1 ng/ml IL-1 β were stained with DePsipherTM and analysed by flow cytometry. As shown in Figure 5(A), IL-1 β inhibited the membrane-potential-dependent accumulation of the dye in the transmembrane space of mitochondria, as indicated by a decrease in red/orange fluorescence and an increase in green fluorescence compared with that of the control cells. The fraction of cells with disrupted mitochondria showing less orange and greater green fluorescence increased time-dependently in ATDC5 cells cultured in the presence of IL-1 β (Figure 5A). Figure 5(B) shows the protective effects of L-NAME, AEBSF or uric acid on the mitochondrial membrane integrity against the detrimental

process seen after IL-1 β treatment. It is therefore suggested that the mitochondrial dysfunction was dependent mainly on the generation of ONOO⁻ derived from the reaction of NO and O₂⁻ produced by NOS and NADPH oxidase respectively.

Since a disturbance of mitochondrial function might lead to energy depletion in the cells, we quantified the amount of ATP in ATDC5 cells after treatment with IL-1 β . The ATP content of the control cells cultured for 48 h without IL-1 β was 10 pmol/10⁶ cells. In contrast, ATP was greatly reduced to 0.04 pmol/10⁶ cells by IL-1 β (Figure 6A). Inhibition of NOS and NADPH oxidase significantly restored the ATP level of the IL-1 β -treated cells. In addition, both uric acid and the combined use of L-NAME and Cu,Zn-SOD were more effective than the single use of L-NAME or AEBSF to maintain ATP level in IL-1 β -treated cells (Figure 6A). To examine the possibility that energy depletion caused the demise of ATDC5 cells, 3 mg/ml D-glucose was added twice to the culture medium of ATDC5 cells at 24 and 48 h after IL-1 β treatment. As shown in Figure 6(B), D-glucose partially rescued ATDC5 cells from death at 72 h after the addition of IL-1 β . Therefore, in our experimental set-up using mouse chondrocyte-like ATDC5 cells, RNS, especially ONOO⁻, formed after IL-1 β treatment caused mitochondrial dysfunction and hence energy depletion, which resulted directly in a decline in cellular viability.

DISCUSSION

There has not been conclusive evidence that IL-1 β induces chondrocyte death. In the present study, we demonstrated the death

Figure 5 Mitochondrial membrane potential of the cells treated with IL-1 β in the presence or absence of L-NAME, AEBSF or uric acid

(A) Flow-cytometric analyses of the mitochondrial membrane potential after incubation for 24, 48 and 72 h in the absence (upper panels) or presence (lower panels) of 1 ng/ml IL-1 β . A photomultiplier was used to detect the DePsipherTM monomer as green fluorescence and DePsipherTM aggregated in the mitochondrial transmembrane space as orange fluorescence. Note that the cells with weak green and intense orange fluorescence and those with intense green and weak red/orange fluorescence represent cells having polarized healthy mitochondria and those having mitochondria with disrupted membrane potential respectively. (B) Observation of the cells under a fluorescence microscope. ATDC5 cells were incubated for 48 h with 1 ng/ml IL-1 β in the presence or absence of 5 mM L-NAME, 0.3 mM AEBSF or 0.7 mM uric acid. After staining with DePsipherTM dye, mitochondria with polarized membrane potential gave red/orange fluorescence.

of rat primary chondrocytes as well as mouse chondrocyte-like ATDC5 cells following IL-1 β treatment (Figures 1 and 4). Progressive cell death (Figure 1) as well as mitochondrial dysfunction and energy depletion observed after 48 h of incubation with IL-1 β was avoided by inhibiting NOS or NADPH oxidase (Figures 1, 3 and 4), and by scavenging ONOO⁻ with uric acid (Figures 5 and 6).

It has been reported that NO triggers apoptotic changes in various types of cells, including chondrocytes: i.e. sodium nitroprusside at mM concentrations induced apoptosis in human and rabbit chondrocytes [14–16]. As the death of ATDC5 cells after exposure to IL-1 β was dependent on NOS activity (Figure 1B), we examined whether NO itself triggered the cell death using NOC-7, which spontaneously liberates NO in neutral solution, since it is reported that sodium nitroprusside is not exactly a simple NO-donor [33]. Exposure of ATDC5 cells to 1 mM NOC-7 for 2 h showed marginal effects on the cell viability (Figure 3A). It was theoretically considered that NOC-7 was decomposed almost completely, and 2-fold amount of NO to original NOC-7 on a molar basis was liberated during this period because of its short half-life (5 min). It is therefore conceivable that NO alone could not kill the cells, and the metabolism of NO into more toxic compounds by the cells seemed to be important in the cell death after IL-1 β treatment.

While we detected approx. 1–2 μ M nitrosothiols in the culture medium after exposure to IL-1 β (results not shown), the con-

tribution of nitrosothiols to the death of ATDC5 cells would be small, since S-nitrosoglutathione at 1 mM did not have any toxic effects on this cell line (Figure 3A). On the other hand, SIN-1 and chemically synthesized ONOO⁻ killed ATDC5 cells (Figure 3A, and results not shown). The protective effect of uric acid against the death of ATDC5 cells induced by IL-1 β indicated the possible involvement of ONOO⁻ in the process of the cell death (Figure 3B).

The death of ATDC5 cells proceeded approx. 48 h after addition of IL-1 β (Figure 1A), despite the earlier induction of NO production (Figure 2C). It could be explained by the augmented production of O₂⁻ after 48 h (Figure 2D). NADPH oxidase appeared to be a major source of O₂⁻ which contributed to the cell death (Figure 3D). It is known that IL-1 β stimulates phagocytes to produce O₂⁻/H₂O₂ via activation of NADPH oxidase [34,35]. The stimulation of chondrocytes to produce H₂O₂ by IL-1 α has also been reported [36]. Even though the ROS are now regarded as one of the intermediary molecules for IL-1 signalling in various cells, including chondrocytes [37–39], the role of NADPH-oxidase in chondrocyte death has not yet been fully elucidated. On the other hand, this is the first report, to our knowledge, that describes the up-regulation of gp91^{phox} expression by IL-1 β , while NADPH oxidase or its homologous system has been detected in chondrocytes [37,40]. The increase in the content of gp91^{phox} protein was obvious at 6 h after the addition of IL-1 β , and its level kept increasing thereafter (Figure 2B). Further studies are required

Figure 6 ATP levels in ATDC5 cells treated with IL-1 β in the presence or absence of L-NAME, AEBSF, SOD or uric acid

(A) Cellular ATP was extracted from the cells incubated for 48 h with the reagents indicated and quantified using a luciferin/luciferase reaction. Results are the means $\pm$ S.D. for four experiments. *, Values significantly higher than the ATP content in the cells treated with IL-1 β (second column from the left) (* P < 0.01). #, Values significantly higher than the IL-1 β + L-NAME group (third column from the left) and AEBSF groups (the fourth column from the left) (# P < 0.01). (B) Effects of D-glucose on the viability of ATDC5 cells treated with IL-1 β . The cells were incubated for 72 h with 1 ng/ml IL-1 β in the absence or presence of 3 mg/ml D-glucose (D-glucose was added at 24 and 48 h after IL-1 β). The viability of the cells was determined by the MTT assay. Results are the means $\pm$ S.D. for four experiments. *, Values significantly higher than the IL-1 β group (second column from the right) (* P < 0.05).

to understand the augmented production of O₂⁻ at 48 h after the addition of IL-1 β by an AEBSF-inhibitable system, highly likely by NADPH oxidase. Especially, the activating mechanism of NADPH oxidase and the regulation of expression of the other NADPH oxidase components, such as p67^{phox}, p47^{phox}, p40^{phox} and Rac by IL-1 β have to be investigated.

One of the targets for NO and ONOO⁻ in the cell is the mitochondrion, a key organelle in ATP production as well as in the determination of the fate of a cell [41]. It is reported that decreased respiratory activity and lowered energy status in chondrocytes was involved in the pathogenesis of OA [42]. Disruption of mitochondrial membrane potential in ATDC5 cells exposed to IL-1 β and its restoration by L-NAME, AEBSF or uric acid (Figure 5B) indicated that one of the main paths to the death of ATDC5 cells was that through mitochondrial dysfunction induced by ONOO⁻. Similar changes were observed in the cells exposed to an ONOO⁻-generator: 1 mM SIN-1 induced loss of mitochondrial membrane potential and nearly complete depletion of ATP in ATDC5 cells, and they were completely restored by 0.7 mM uric acid (results not shown). On the other hand, the inhibitory potency of AEBSF was higher or tended to be higher than that of L-NAME against mitochondrial dysfunction (results not shown), ATP loss (Figure 6A) and cell death (Figures 1B and 3D, and results not shown) induced by IL-1 β . Therefore there remains the possibility

that the ROS other than ONOO⁻ produced from NADPH-oxidase-derived O₂⁻/H₂O₂ is partially involved in the series of events.

It is known that disturbance of mitochondria leads to apoptosis through the activation of the caspase cascade. Recently, it was also reported that ONOO⁻ induced calpain-dependent and caspase-independent apoptosis in human chondrocytes via mitochondrial damage [43]. However, the death of IL-1 β -treated ATDC5 cells was not suppressed by a broad-spectrum caspase inhibitor, a caspase-3-specific inhibitor or a calpain inhibitor acetyl-Leu-Leu-norleucinal (results not shown). While mitochondrial dysfunction, in general, can cause not only apoptosis, but also necrotic cell death, ATP depletion suppresses apoptosis and steers the cell to necrosis [44,45]. Under our experimental conditions, a nearly complete depletion of ATP was observed in the IL-1 β -treated ATDC5 cells (Figure 6A), which might cause the loss of a diverse array of cellular functions and the viability. Therefore the cell death observed in the present study appeared more necrotic, even though we cannot foreclose the involvement of apoptosis through any undetermined route.

In earlier reports, increased oxidative stress in chondrocytes with aging [46], the increased formation of 3-nitrotyrosine in aged and OA cartilages [10] and reduced mitochondrial activity in OA cartilages [42] were described. The results obtained in the present study might be related to these phenomena and explain at least part of the pathological mechanism of degenerative joint diseases.

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