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The role of adenosine in chondrocyte death in murine osteoarthritis and in a murine chondrocyte cell line

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Summary

Objective: To investigate the role of adenosine in chondrocyte death in murine osteoarthritis (OA).

Methods: 5′-Nucleotidase (5′NT) generates adenosine. Enzyme activity was measured histochemically in normal murine and osteoarthritic STR/ort strain tibial cartilage. Adenosine-mediated cell death was investigated in MC615 chondrocyte cultures. Adenosine receptors (ARs) were assessed by reverse transcriptase polymerase chain reaction (RT-PCR). Cellular uptake of [^{3}H] adenosine was measured with or without the inhibitor, nitrobenzylthioinosine (NBTI). Cell death was assessed by cell counting and DNA laddering following selective receptor stimulation, or after modulating adenosine metabolism with adenosine deaminase (ADA) or adenosine kinase (AK) inhibitors [erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and Iodotubericidin (Itub)], or with homocysteine (HC). Markers of apoptosis were assessed by Western blotting. Cell studies were validated by incubating normal murine knee joints in a medium containing adenosine and metabolic inhibitors. Apoptotic chondrocytes were identified with the TUNEL reaction.

Results: 5′NT activity in STR/ort tibial cartilage increased with development of OA, especially close to OA lesions. Adenosine induced MC615 cell death in the presence of ADA inhibition (100 μM EHNA), or 1 mM HC, or both. Adenosine uptake, mediated by NBTI-sensitive adenosine transporters, was required for cell death. ARs were expressed (A2b > A2a > A1) but were not involved in mediating cell death. Cell death involved the activation of caspase-3 and DNA fragmentation and was prevented by inhibiting caspase activity. However, neither caspase-8 nor caspase-9 was detected. Adenosine + EHNA induced chondrocyte apoptosis in normal murine knee joints.

Conclusion: Increased adenosine production may induce chondrocyte apoptosis and play a role in OA in STR/ort mice.

Key words: Osteoarthritis, Adenosine, 5′-Nucleotidase, Chondrocyte, Cell death, Apoptosis, Animal model.

Introduction

Purines are important cellular metabolites, which are involved in a wide variety of processes. They are generated by 5′-nucleotidases (5′NT), a family of enzymes which dephosphorylate ribo- and deoxyribonucleoside monophosphates (e.g., adenosine monophosphate (AMP)) to yield their respective nucleoside (e.g., adenosine). In human tissues there are five cytosolic, one mitochondrial and one extracellular 5′-nucleosidase. The latter, ecto-5′nucleotidase (ecto-5′NT), is a glycosylphosphoinositol-membrane linked enzyme. These enzymes have many important physiological functions ranging from involvement in maintaining balanced nucleotide triphosphate pools in energy metabolism to roles in cellular signalling[1]. Adenosine has an important physiological signalling role in the peripheral and central nervous systems[2]. However, excessive adenosine levels are cytotoxic[3–6], although the mechanism of adenosine-mediated cell death seems to vary in different cell types.

Two mechanisms of cytotoxicity exist, and are initiated either extracellularly or intracellularly, depending on the mode of action (Fig. 1). Extracellular adenosine can interact with purine receptors. Investigation of the role of these receptors in cytotoxic effects remains incomplete, since few earlier studies used selective receptor agonists[7]. Szondy[8] treated human thymocytes with the adenosine analogue 2-chloroadenosine (2-CA) and various adenosine receptor (AR) agonists. 2-CA was the most potent at selectively depleting thymocyte subpopulations. The mechanism was Ca^{2+}-mediated and appeared to involve cyclic AMP (cAMP). In other studies, the cytotoxic effects of adenosine were found to be mediated via various AR subtypes[9,10]. However, it is noteworthy that some studies have shown that activation of AR subtypes by adenosine can have a protective effect on the cells. This is largely due to its inhibitory effect on NO release[11,12].

Dawicki *et al.*[5] showed that adenosine had a cytotoxic effect on cultured pulmonary artery endothelial cells and that for it to exert this effect, it had to enter the cell. Other studies have shown that the main route of adenosine uptake is via a series of adenosine transporters[13]. Once in the cell,

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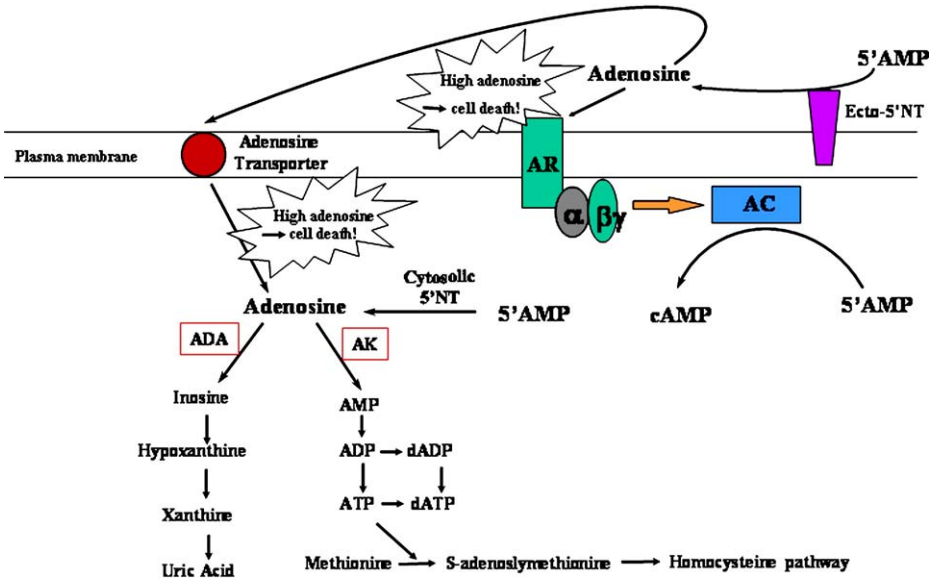

Fig. 1. Overview of purine metabolic pathways. Adenosine is produced from 5′AMP, either extracellularly by ecto-5′NT, or intracellularly by cytosolic-5′NT. Extracellular adenosine can signal via ARs which are coupled to G-proteins ($\alpha\beta\gamma$ subunits) regulating the activity of AC and the production of cAMP, or it can enter the cell via the adenosine transporter. In the cell, adenosine is metabolised to uric acid after conversion to inosine by ADA, or converted to AMP via AK. Alternatively, adenosine can be diverted into the HC pathway. High levels of adenosine can induce cell death by mechanisms dependent on either interaction with ARs or cellular uptake via the adenosine transporter, depending on the cell type (for details see the text). Figure modified from Ceruti *et al.*[3].

adenosine can be metabolised by multiple pathways[14] (Fig. 1), a degradatory pathway converts adenosine to inosine and then to uric acid. The conversion to inosine is mediated by adenosine deaminase (ADA). Errors in this pathway are clinically manifested in severe combined immunodeficiency syndrome (SCIDS) patients. These patients either lack ADA or the enzyme is grossly inhibited. As a result, they have massive thymocyte deletion and overwhelming immunodeficiency[15]. Another disorder associated with this pathway is gout, in which the accumulation of uric acid crystals within the joint is the major feature[16]. This disorder is due to excessive activity of the pathway. A second pathway available to adenosine is the adenosine kinase (AK) pathway. In this, adenosine is converted to AMP by AK. AMP is further metabolised to adenosine triphosphate (ATP) and dATP. The balance between ATP and dATP is important in maintaining efficient DNA repair.

A third pathway is the homocysteine (HC) pathway (see Fig. 1) in which *S*-adenosylmethionine (SAM) is converted to *S*-adenosylhomocysteine (SAH), where SAM acts as a methyl donor to methyl transferases. Alterations in the equilibrium of this reaction have been reported in several pathologies including atherosclerosis[17], stroke[18] and thrombosis[19]. A possible link with Alzheimer disease has also been proposed[20].

Elevated levels of 5′NT have been found in the synovial fluid of both osteoarthritis (OA) and rheumatoid arthritis (RA) patients[21,22]. Moreover, addition of adenosine to chondrocyte cultures was reported to have cytotoxic effects[21]. This led to our hypothesis that the generation of adenosine may trigger chondrocyte apoptosis in arthritis. Chondrocyte apoptosis is a well established feature of both human[23–25] and murine[26] OA. Thus we investigated 5′NT activity in tibial cartilage of STR/ort mice, a naturally occurring model of OA[27], and the mechanism of adenosine-mediated cell death in murine MC615 chondrocytes.

Methods

IN VIVO EXPERIMENTS

Inbred male STR/ort mice from the Imperial College colony were sacrificed at 6, 12, 20 and 40 weeks of age. Sex and age-matched CBA mice, a strain which has not been shown to develop OA at the ages studied, were purchased from Charles River Laboratory (Margate, Kent, UK). Mice were killed by cervical dislocation. The knee joints were dissected out and prepared for sectioning, as described in detail previously[26]. After chilling to $-70\,^{\circ}C$ in *n*-hexane cooled by industrial methylated spirits and solid carbon dioxide, frozen knee joints were mounted on metal chucks (with the patella facing up) for sectioning. Sectioning was carried out in the anterior to the posterior direction. Serial 10 μm sections were cut through the whole joint and mounted onto chrome-alum coated microscope slides. Sections not immediately required were stored at $-80\,^{\circ}C$ for future use.

The TUNEL assay (Apoalert DNA Fragmentation Assay, Clontech Inc, Palo Alto, CA, USA) was performed, as previously[26], following the manufacturer's protocol. Briefly, sections fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) were washed in PBS and subjected to brief proteinase K digestion (10 μg/ml) (5 min, room temperature). Sections were again fixed, washed and incubated with equilibrium buffer, before application of 50 μl of reaction mixture (45 μl labelling solution, 4 μl equilibrium buffer, 1 μl transferase enzyme) to each slide. Following incubation (37 °C for 60 min), the slides were treated with 1× saline-sodium citrate (SSC) solution before mounting with a fluorescence mounting media (Vector Laboratories, Paisley, UK). Nuclei of apoptotic cells were stained green by this procedure whilst the intact DNA of normal cells was stained red by propidium iodide in the medium.

The 5′NT activity of STR/ort and CBA mouse knee joint cartilage chondrocytes was detected in unfixed cryostat

sections using a histochemical method[28]. Sections were incubated for 5 min at 37°C in medium containing 4 mM AMP as a substrate after which they were washed and treated with 0.1% ammonium sulphide solution. 5′NT activity was detected by the formation of a black/brown precipitate of lead sulphide. Some sections were incubated in the presence of the 5′NT inhibitor nickel chloride (10 mM), as a negative control[28].

CELL CULTURE EXPERIMENTS

An immortalised murine chondrocyte cell line (MC615) was a kind gift from Dr Bjorn Olsen (Harvard Medical School, Boston). Cells (passages 19–23) were maintained in Dulbecco's Modified Essential Medium (DMEM)/Ham's F12 (50:50) (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum, penicillin, streptomycin, fungizone (PSF), and 10 mM HEPES. Media were changed every 2–3 days. For experiments, cells were plated at 1.6×10^6 cells/well in 6-well plates, unless specified otherwise. Cells were allowed to settle for 24 h, after which the medium was changed. Treatment of the cells was carried out on the following day.

To investigate the effect of inhibiting adenosine metabolic pathways, cells were pre-incubated for 30 min at 37°C with whole medium containing either the ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 10 μM and 100 μM), or the AK inhibitor, Iodotubericidin (Itub, 1 μM and 10 μM). Following pre-incubation, cells were treated with 5 mM adenosine and/or 1 mM HC in the presence or absence of the compounds for 24 h at 37°C. Treatment was stopped by aspirating the medium and washing the cell layer, after which the cells were counted. The cell layers were washed twice with sterile PBS before addition of 1 ml of trypsin/EDTA (Invitrogen). Cells were trypsinised for 3 min at 37°C. The trypsinised cells were transferred to a tube containing an excess of medium and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of medium. Cells were counted using a haemocytometer. Dishes were examined under the microscope to ensure that all cells had been removed.

To investigate adenosine uptake, MC615 cells were plated at 2×10^5 cells/well in 24-well plates. The cells were allowed to settle for 24 h before washing each well with 3×1 ml Hank's Balanced Salt Solution (HBSS). All wells were pre-incubated for 15 min at 37°C, with medium containing 100 μM EHNA (Sigma–Aldrich, Poole, Dorset, UK) in the presence or absence of the adenosine transporter inhibitor, nitrobenzylthioinosine (NBTI, 1.0–100 μM). To initiate uptake, [^{3}H] adenosine was added to a final concentration of 10 μCi/ml and the cells incubated for a further 5 min. Each treatment was conducted in duplicate. Following incubation the medium was collected and the cell layer washed extensively three times with ice-cold HBSS. A 0.5 ml of 1 M NaOH was added to each of the wells and the cell layer left to solubilise overnight at 37°C. Radioactivity levels were measured by adding 100 μl of the radioactive sample to 4 ml of scintillation fluid (Ecoscint; National Diagnostics, Atlanta, GA, USA) and the counts measured using a Beckman LS6000SC scintillation counter. In control experiments, inulin [^{14}C]carboxylic acid, which is impermeable to cells, was used as a negative control.

A DNA fragmentation assay was used to assess the effect of adenosine on apoptosis in MC615 chondrocytes. This was performed using the DNA ladder isolation kit from Oncogene Research Products (CN Biosciences, Nottingham, UK), following the manufacturer's protocol. Cell cultures were treated with compounds for 9 h after which the media were transferred into separate tubes and the cell layers washed three times with sterile PBS. The aspirated media fractions were centrifuged to collect any floating cells. The resulting cell pellet was treated in the same manner as the cell layers. Five hundred microlitres of extraction buffer was added directly to the monolayer in each well and the plate rocked for 10 min at room temperature. The cell lysate was transferred into sterile eppendorfs and left on ice for 30 min before being centrifuged (room temperature, 5 min, 5000 rpm). The supernatant was transferred to a clean eppendorf and 20 μl of solution 2 was added before incubating for 60 min at 37°C. Following incubation, 25 μl of solution 3 was added to each of the tubes, after which the samples were incubated overnight at 50°C. To precipitate DNA, 2 μl Pellet Paint™ Co-precipitant, 60 μl sodium acetate (pH 5.2) and 662 μl isopropanol were added and the samples incubated for 2 min at room temperature. Samples were then centrifuged at 14,000 rpm at 4°C, after which the supernatants were removed and the DNA pellets washed with 75% ethanol, followed by 100% ethanol. After the last wash, the supernatant was discarded and the samples were air dried before being dissolved in resuspension buffer. To visualise DNA laddering, samples were mixed with 6× gel loading buffer and loaded onto a 1.5% agarose gel. The gel was stained with 0.5 μg/ml ethidium bromide and laddering visualised using an UV illuminator.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

To investigate AR expression by MC615 chondrocytes, RNA was isolated from cells with RNAzol B reagent (Ambion Ltd, Huntingdon, UK), following the manufacturer's protocol, after which it was resuspended in diethyl pyrocarbonate (DEPC)-treated water and the concentration measured spectrophotometrically at 260 nm. Total RNA (5 μg) was reverse transcribed using Superscript II and random hexamers (Invitrogen, Paisley, UK) and 2 μl of the transcribed product was used in a 100 μl PCR reaction mix containing 1× PCR buffer, 10 mM dNTP mixture, 10 μM primer pair, the appropriate concentration of $MgCl_2$ and 1 μl of *Taq* DNA polymerase (Invitrogen). PCR reactions were carried out in a Hybaid Omnigene thermocycler. Following the initial denaturation step (94°C for 5 min), each PCR cycle comprised denaturation (94°C for 45 s), annealing (45 s) and extension (72°C for 45 s). The annealing temperature was optimised for each primer pair. A1, A2a and A3 purine receptor primers required 60°C, whereas the A2b receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers required 56°C. The $MgCl_2$ concentrations for PCR reactions were also optimised, receptors A1, A2a, and A3, and GAPDH requiring 1.5 mM and receptor A2b requiring 2 mM. A final cycle of 72°C for 10 min was performed to complete any partially amplified products. A total of 40 PCR cycles were performed for each AR primer pair, but only 25 cycles were required for GAPDH. The PCR conditions were chosen to be non-saturating in all cases. PCR products were electrophoresed on 1.5% agarose gels and visualised using an UV transilluminator. The band densities were quantified using the NIH Image program.

Primer sequences were designed using Genejockey and were as follows: A1 AR, sense: GCC TGT CCT GTC CTC ATC CTC ACC; antisense: CAG ACG AAG AAG TTG

AAG TAG ACC. A2a AR, sense: TTC TTC TGC TCC ACG TGC CAG C; antisense: TGG TGC TCC TGG GTA AGA AGC. A2b AR, sense: AGA ATG TGG TCC CCA TGA GC; antisense: ATG GGG TTG ACA ACT GAA TTG G. A3 AR, sense: GTG TGA GGT GGC TGT CTA TCC TGG; antisense: TCT TCC AGA GAC CAT ACC ACC ACG. GAPDH, sense: ACC ACA GTC CAT GCC ATC AC; antisense: TCC ACC ACC CTG TTG CTG TA.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Chondrocyte cell layer samples were extracted in radioimmunoprecipitation (RIPA) buffer (0.5% sodium deoxycholate, 1% NP40 and 0.1% sodium dodecyl sulphate (SDS)) and transferred to an eppendorf. The cell lysate was drawn through a 21-gauge needle to achieve a uniform suspension. The protein concentration was measured with the Bradford assay and known protein concentrations of each sample were mixed with 3× sample buffer (containing β-mercaptoethanol and 1× protease inhibitors) and electrophoresed on a 7.5% or 15% SDS-polyacrylamide gel, prior to transfer to an activated polyvinylidene fluoride (PVDF) membrane in transfer buffer containing 20 mM Tris, 390 mM glycine and 40% v/v methanol. Following transfer the membrane was washed in PBS and blocked using 5% non-fat milk (diluted in Tris buffered saline (TBS)-Tween) at room temperature for 60 min. Blots were probed with primary antibodies to caspase-3 (Research Diagnostics Inc, Flanders, NJ, USA; 1:400) or poly adenosine diphosphate (ADP)-ribose-polymerase (PARP) (Serotec, Oxford, UK; 1:5000) overnight at 4°C after which they were washed with TBST and incubated with an horse radish peroxide (HRP)-conjugated secondary antibody (DAKO, Ely, Cambridgeshire, UK; 1:2000) for 1 h at room temperature. Antibody binding was visualised with the ECL kit (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK).

IN VIVO VALIDATION OF ADENOSINE-INDUCED CHONDROCYTE DEATH

Organ culture experiments were carried out on disarticulated knee joints from 8-week-old CBA mice. Joints were incubated at 37°C for 24 h in media containing 100 μM EHNA or 5 mM adenosine + 100 μM EHNA. Following treatment the joints were snap frozen, sectioned and the sections analysed using the TUNEL assay.

STATISTICAL ANALYSIS

Results were assessed using analysis of variance (ANOVA) for multiple comparisons with Bonferroni corrections.

Results

5′NT ACTIVITY IN NORMAL AND OSTEOARTHRITIC ARTICULAR CARTILAGE

5′NT activity was assessed in the articular cartilage of the stifle (knee) joints of STR/ort mice of different ages, which exhibited varying degrees of OA damage. The latter was graded on a scale of 0–6, as described previously[27], where 0 corresponds to normal cartilage and 6 corresponds to 80%, or greater, loss of the articular cartilage. Age-matched CBA mice were used as controls. Enzyme activity in STR/ort mice varied both with age and lesion severity. Young STR/ort mice (6 weeks old) had no OA lesions and showed very little enzyme activity in the tibial plateau cartilage except in some superficial zone chondrocytes on the medial side [Fig. 2(A, B)]. Control sections which had been treated with the 5′NT enzyme inhibitor nickel chloride showed significantly lower levels [Fig. 2(C)]. Some degree of activity is expected as nickel chloride only inhibits 80% of the total activity[28]. 5′NT activity in tibial cartilage of young CBA mice [Fig. 2(D)] was also associated predominantly with superficial zone chondrocytes on the medial side, as in STR/ort mice.

By 20 weeks of age most STR/ort mice develop focal OA lesions of varying severity in the medial tibial plateau whilst the lateral plateau is almost always spared[27]. 5′NT activity is prominent at this age in the chondrocytes of the medial plateau where low grade histopathological lesions have developed and is found in cells throughout the full depth of the cartilage [Fig. 3(A)]. A similar level of activity and distribution is seen in cartilage of older mice with low grade lesions [Fig. 3(B)]. Similarly chondrocytes in cartilage adjacent to more advanced lesions show evidence of 5′NT activity throughout the full depth of the cartilage [Fig. 3(C)] although those on the margin of the lesion frequently show little or no

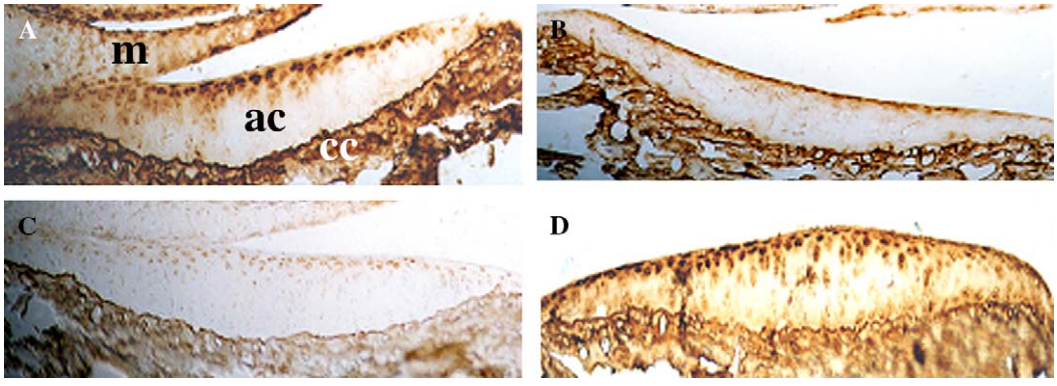

Fig. 2. Histochemical detection of 5′NT activity in articular cartilage of young STR/ort and CBA mice. (A) Medial and (B) lateral tibial plateau in 6-week-old STR/ort mice. Only low levels of enzyme activity were detected, mainly in the superficial zone. The histology of the cartilage is normal. (C) A 6-week-old STR/ort medial tibial plateau. 5′NT activity was inhibited by incubating sections with the 5′NT inhibitor nickel chloride. (D) Age-matched CBA mouse medial tibial plateau showing a similar distribution of 5′NT activity. ac, articular cartilage; cc, calcified cartilage; and m, meniscus. Original magnification, ×37.

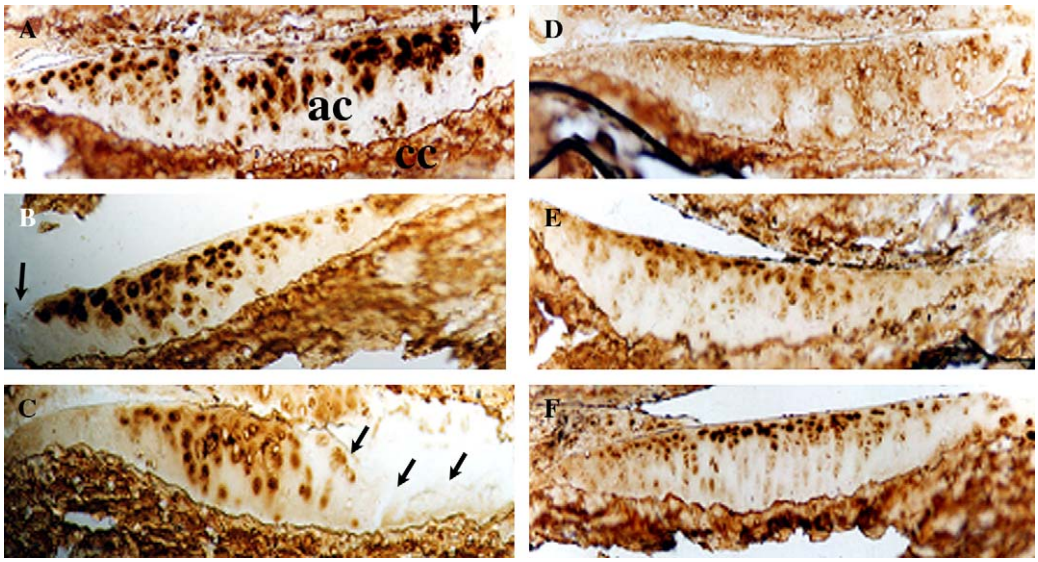

Fig. 3. Histochemical detection of 5′NT activity in articular cartilage of older STR/ort mice with OA lesions of varying severity. (A) A 20-week-old STR/ort mouse, medial tibial plateau, grade 2 OA lesion (arrow). High levels of enzyme activity are associated with chondrocytes throughout the full depth of the cartilage and are particularly prominent in the superficial and mid zones adjacent to the lesion. (B) A 40-week-old STR/ort mouse, medial tibial plateau, grade 2 lesion (arrow) showing a similar distribution and high level of 5′NT activity as in the younger mouse with a grade 2 lesion (panel A). (C) A 40-week-old STR/ort mouse, medial tibial plateau, grade 4 lesion (arrows indicate the cartilage surface at the lesion site). 5′NT activity is associated with chondrocytes throughout the full depth of the cartilage close to the lesion but is low or absent in some lacunae situated at the margins of the lesion, possibly due to chondrocyte death in this location. (D) Chondrocytes in the same medial cartilage, but located at some distance from the focal lesion area show only low levels of 5′NT activity. (E) The lateral plateaux at all ages show only modest levels of activity, mainly in the superficial zone. (F) A 20-week-old CBA mouse medial tibial cartilage. Older CBA mice show a similar distribution and level of 5′NT activity to that seen in young CBA mice. ac, articular cartilage; cc, calcified cartilage; and m, meniscus. Original magnifications, A–C, ×45; and D–F, ×40.

activity [Fig. 3(C)]. These same cells also show loss of expression of aggrecan and other proteins and may be pre-apoptotic, or have already died, as discussed previously[27]. Chondrocytes in cartilage located at some distance from the lesion show only low levels of 5′NT activity [Fig. 3(D)]. Similarly chondrocytes in the lateral tibial plateau have only modest levels of 5′NT activity, mainly confined to the superficial zone [Fig. 3(E)]. 5′NT activity in the medial cartilage of CBA control mice is also largely confined to chondrocytes of the superficial zone [Fig. 3(F)] and levels are similar to those found in younger CBA mice [Fig. 2(D)]. In all cases 5′NT activity detected in either older STR/ort or older CBA mouse cartilage was greatly reduced in the presence of the enzyme inhibitor nickel chloride. Thus, in summary, the development of focal osteoarthritic lesions in the medial tibial plateau cartilage of the STR/ort mouse is accompanied by increased 5′NT activity in chondrocytes throughout the full depth of cartilage adjacent to the lesional area.

AR EXPRESSION IN MURINE MC615 CHONDROCYTES

The expression of AR subtypes in murine chondrocytes (MC615) was investigated by RT-PCR. The specific primers to each receptor were checked initially against a total RNA panel (Clontech, Palo Alto, CA, USA) containing RNA from a series of mouse tissues and the previously reported pattern of expression was found (data not shown). Analysis of mRNA from MC615 cells showed the expression of three of the four receptor subtypes. The A2b receptor is the most abundantly expressed, with the A1 and A2a receptors being expressed at a lower level. A3 transcripts were absent [Fig. 4(A)]. These differences, when represented graphically, show a 6-fold difference between A1 and A2b and a 3-fold difference between A2a and A2b [Fig. 4(B)].

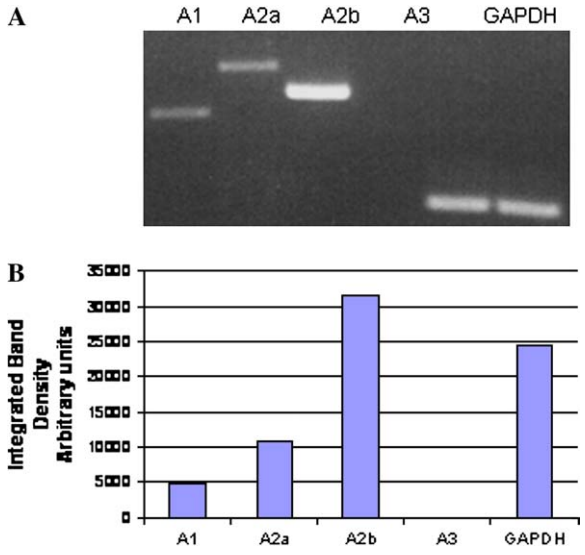

Fig. 4. Differential expression of AR subtype transcripts in murine chondrocytes (MC615 cells). (A) Total RNA from MC615 cells (MC) was reverse transcribed and used for PCR. The PCR products were electrophoresed on a 1.5% gel to assess comparative levels of the AR subtype expression. GAPDH was used as a loading control. Band sizes: A1, 316 bp; A2a, 391 bp; and A2b, 345 bp. (B) Quantification of PCR bands using densitometry analysis and NIH Image software. Two further independent experiments gave very similar results.

In a separate set of experiments, chondrocytes were treated for 1 h with selective and non-selective agonists of these receptors (C6-cyclopenyladenosine (CPA) for A1, CGS 21860 for A2a, 5′-(Nethylcarboxamido) adenosine (NECA) and adenosine which is non-selective for A1/A2 receptors) after which cAMP levels and DNA fragmentation were measured. Whilst the anticipated changes in cAMP levels occurred, as detected by a cAMP enzyme immunoassay (Amersham Biosciences, Chalfont St. Giles, Bucks, UK), no DNA fragmentation was detected in response to any of these agonists (data not shown).

ADENOSINE UPTAKE BY MC615 CELLS

Adenosine can enter cells via adenosine transporters. The presence of these transporters on the surface of chondrocytes had not been reported previously so experiments were carried out to determine whether they exist on murine chondrocytes. Chondrocyte cultures were treated with EHNA to block intracellular adenosine metabolism by ADA (see below) and the uptake of radioactive adenosine was measured, in the presence and absence of the transporter inhibitor, NBTI. Control cells, which had not been treated with any radioisotope, showed only background levels of radioactivity (Fig. 5). Incubation of MC615 cells for a period of 5 min with 10 μCi of [^{3}H] adenosine resulted in uptake of the nucleotide into the cell (Fig. 5). This uptake accounted for 1.4% of the total radioactivity present in the medium (containing 100 μM EHNA). Treatment of cells with 1 μM NBTI resulted in a 79.5% reduction in adenosine uptake, 10 μM NBTI, an 87.6% reduction and 100 μM NBTI, a 97.5% reduction ($P = 0.02$). The morphology of the cells remained normal indicating that these effects were not simply due to cytotoxicity of NBTI. There was no uptake of [^{14}C] inulin into the cells in either the presence or absence of NBTI (data not shown).

THE EFFECT OF INHIBITORS OF PURINE METABOLISM ON CELL NUMBER IN MC615 CELLS

To investigate the involvement of the ADA, AK and HC pathways in adenosine-mediated cell death, cells were treated with selective and non-selective inhibitors to ADA (EHNA) and AK (Itub) and with HC, in the presence and absence of 5 mM adenosine, for 9 h. Preliminary experiments carried out using various concentrations of each of the compounds showed a dose dependent effect on the number of adherent cells remaining after each treatment. From these experiments, the optimal concentration of each compound which could be used without causing reduction in adherent cell number was deduced and used for subsequent experiments. Untreated cells showed the expected doubling of the cell number from 1.6×10^6 cells/well to 3.2×10^6 cells/well over the course of the experiment (Fig. 6). Adenosine (5 mM) treatment resulted in a small decrease in the cell number compared to the control but this was not statistically significant. A similar effect was seen for 100 μM EHNA treatment. However, incubation of the cells with adenosine + EHNA resulted in a much greater decrease in cell number. Visually the cells appeared degenerate, with loss of normal rounded morphology. Increased detachment of cells and cellular debris in the medium was also detected. The effects of adenosine + EHNA were prevented if cells were treated with the adenosine transporter inhibitor NBTI (result not shown), indicating that they were dependent on cellular uptake of adenosine. Treatment of cells with the AK inhibitor, Itub, resulted in an 81% decrease in the adherent cell number as compared to controls. The morphology of the cells resembled that seen for adenosine + EHNA treatment. However, treatment with 10 μM Itub and adenosine together decreased cell loss. Surprisingly, treatment of cells with 1 mM HC resulted in a 13% increase in cell number, whereas HC + adenosine induced a 76% decrease in cell count and widespread cell detachment.

To investigate the interaction between the ADA, AK and HC pathways, cells were incubated with each of the inhibitors in the presence of HC. In each case the reduction in cell

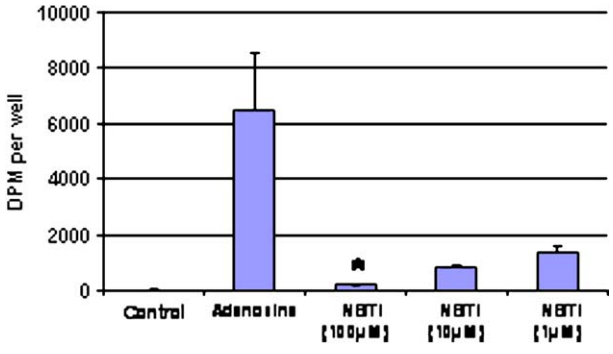

Fig. 5. Uptake of [^{3}H] adenosine following treatment of cells with varying concentrations of the adenosine transporter inhibitor, NBTI. Cells were pre-treated with 100 μM EHNA + 1.0−100 μM NBTI. Adenosine uptake was assessed following addition of 10 μCi [^{3}H] adenosine in the presence of the inhibitors for 5 min at 37°C. A dose responsive inhibition of uptake was observed in cells treated with 100 μM NBTI. DPM, disintegrations per minute. *$P = 0.02$, $n = 6$ (unpaired t-test). Two further independent experiments gave very similar results.

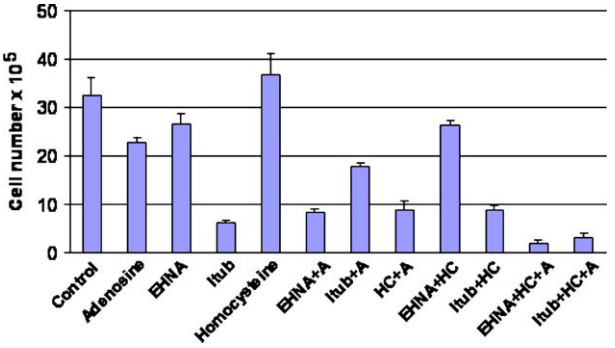

Fig. 6. The effect of inhibitors of adenosine metabolism and HC on chondrocyte proliferation in the presence or absence of adenosine. Cells were pre-treated with 100 μM EHNA, 10 μM Itub, or 1 mM HC, or with combinations of these, for 30 min prior to incubation with medium containing the respective compound(s), with or without 5 mM adenosine, for 24 h. Cells treated with HC alone or with HC + adenosine were not pretreated. Cells were trypsinised and counted. Results are shown as mean cell number $\times 10^5 \pm$ S.E.M., $n = 3$ wells/treatment, P values: Control vs adenosine = NS, Control vs EHNA = NS, Control vs Itub = <0.001, Control vs HC = NS, EHNA + adenosine vs EHNA = <0.001, Itub + adenosine vs Itub = NS, HC + adenosine vs HC = <0.001, Itub + HC vs HC = <0.001, EHNA + HC + adenosine vs EHNA + HC = <0.001, EHNA + HC + adenosine vs EHNA + adenosine = NS, Itub + HC + adenosine vs Itub + adenosine = <0.05, Itub + HC + adenosine vs HC or adenosine = <0.001, and Itub + HC + adenosine vs Itub + HC = NS. (ANOVA) NS = not significant. The results are representative of two further independent experiments.

number was found to be similar to that seen for inhibitor treatment alone. However, in the presence of adenosine these combinations were found to be much more detrimental to cell number (Fig. 6).

CHARACTERISATION OF THE CELL DEATH PATHWAY BY THE DETECTION OF PROTEIN MARKERS OF APOPTOSIS

To investigate which pathway of apoptosis may be operational in cells treated with adenosine and EHNA or HC, the protein expression of three caspases (caspase-3, -8 and -9) and the DNA repair enzyme PARP was tested. The antibody used to detect caspase-3 recognises both the inactive proenzyme (32–33 kDa) and the active forms (20 kDa fragment and 12–13 kDa fragment) of the enzyme. Control, adenosine-treated, EHNA-treated and HC-treated cells exhibited the inactive 33 kDa protein. Treatment of cells with 5 mM adenosine + 100 μM EHNA, 5 mM adenosine + 1 mM HC, or 5 mM adenosine + 100 μM EHNA + 1 mM HC, resulted in the appearance of 20 kDa and 13 kDa fragments, indicating activation of caspase-3 [Fig. 7(A)]. EHNA + HC gave a similar result to controls. Neither caspase-8 nor caspase-9 expression was detected in both the treated and the untreated MC615 cell lysate samples (data not shown).

Further evidence of caspase-3 activity was detected by the presence of the inactivated form of the caspase-3 substrate, PARP. Cleavage of PARP to an 85 kDa fragment was detected in adenosine + EHNA, adenosine + HC, and adenosine + EHNA + HC-treated cultures [Fig. 7(B)].

DNA FRAGMENTATION AFTER TREATMENT WITH ADENOSINE AND EHNA OR HC

Untreated, adenosine-treated, EHNA-treated, and HC-treated cells showed no evidence of DNA laddering. In these cases, a tight high molecular weight DNA band was observed. Laddering was clearly detected in adenosine + EHNA-treated and in adenosine + HC-treated cells. This laddering was prevented when the caspase inhibitor, Z-VAD-FMK, was included. However, in this case a much broader high molecular weight DNA band was seen (Fig. 8). Treatment with the caspase inhibitor alone, or in

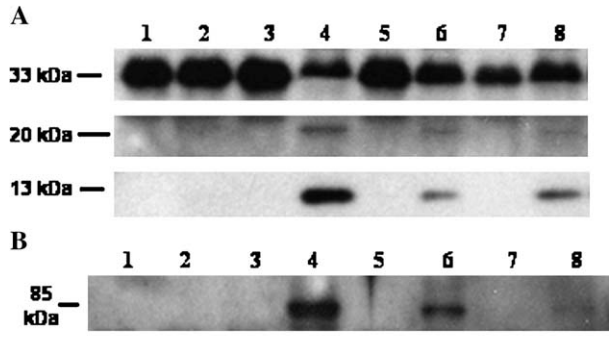

Fig. 7. Conversion of procaspase-3 to caspase-3 following treatment of MC615 cells with adenosine and inhibitors of adenosine metabolism or with HC. Cells were treated for 9 h at 37°C, after which they were processed for Western blot analysis for (A) procaspase-3 (33 kDa) and caspase-3 (20 kDa and 13 kDa) and (B) PARP (33 kDa). Treatments were as follows: lane 1, no treatment; lane 2, adenosine; lane 3, HC; lane 4, HC + adenosine; lane 5, EHNA; lane 6, EHNA + adenosine; lane 7, EHNA + HC; and lane 8, EHNA + HC + adenosine. The 20 kDa and 13 kDa cleavage products of activated caspase-3 were detected in cultures treated with HC + adenosine (lane 4), EHNA + adenosine (lane 6) and EHNA + HC + adenosine (lane 8). Proteolytic processing of PARP to an inactive 85 kDa product was also detected with these treatments. The blots are representative of duplicate cultures for each condition. Two further independent experiments gave the same result.

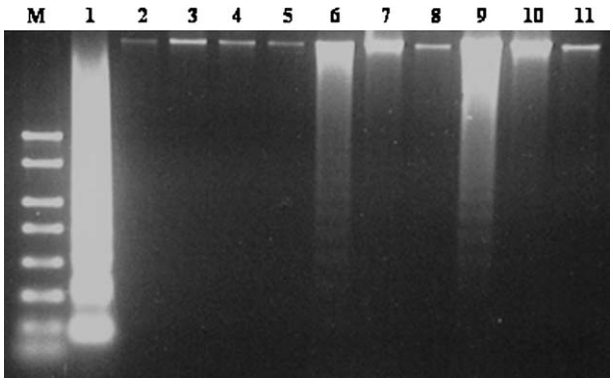

Fig. 8. DNA laddering in cells treated with EHNA + adenosine and HC + adenosine and the effect of the caspase inhibitor, Z-VAD-FMK. Cells were treated for 9 h with 5 mM adenosine and 100 μM EHNA or 1 mM HC, in the presence or absence of the caspase inhibitor (75 μM). DNA fragmentation was assessed as described in the Methods. Lane M, DNA size marker; lane 1, DNA fragment positive control supplied with kit; lane 2, untreated control; lane 3, caspase inhibitor alone; lane 4, adenosine alone; lane 5, adenosine + caspase inhibitor; lane 6, adenosine + EHNA; lane 7, adenosine + EHNA + caspase inhibitor; lane 8, EHNA alone; lane 9, adenosine + HC; lane 10, adenosine + HC + caspase inhibitor; and lane 11, HC alone. Laddering is present after treatment with either adenosine + EHNA (lane 6) or adenosine + HC (lane 9). Both effects are counteracted by co-incubation with the caspase inhibitor (lanes 7 and 10). The gel is representative of duplicate cultures for each treatment. Two further independent experiments gave the same result.

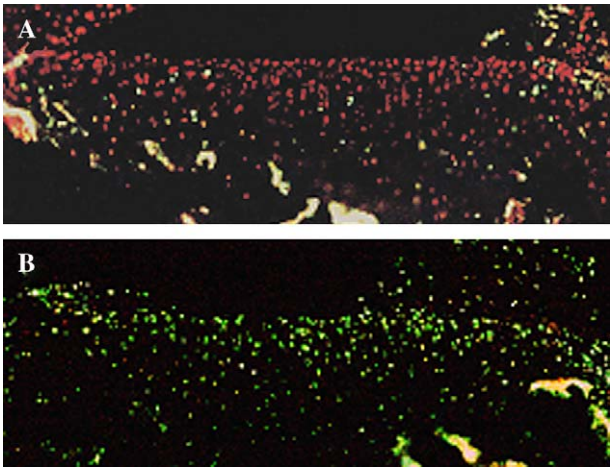

Fig. 9. The effect of adenosine on chondrocytes *in vivo* in CBA murine knee joints. The 8-week-old disarticulated CBA knee joints were cultured in media containing 5 mM adenosine in the presence or absence of 100 μM EHNA. Control knee joints treated with EHNA alone show very few TUNEL positive cells (A) (×25). Co-incubation with 5 mM adenosine results in a large increase in the number of TUNEL positive cells (B) (×30).

combination with adenosine, had no effect on DNA. These results are consistent with the cell count data.

VALIDATION OF THE EFFECTS OF ADENOSINE ON CHONDROCYTE CELL DEATH IN CARTILAGE

To validate the effect of adenosine on chondrocyte cell death *in situ*, 8-week-old CBA mouse knee joints were cultured in medium containing adenosine (5 mM) and EHNA (100 μM) and the effect on cell death was observed by detecting TUNEL positive cells. Very few TUNEL positive cells were detected in the control joints (Fig. 9). Any cells that were present were randomly located in the articular cartilage. No difference was seen between the chondrocytes of the medial and lateral tibial plateaux in this respect. In contrast, results obtained for treated joints were quite different. TUNEL positive cells were observed throughout the full depth of the cartilage, with virtually all cells staining positive (Fig. 9). This phenomenon was not specific to the medial compartment.

Discussion

Our results show that in the locality of OA lesions in STR/ort mice, 5′NT activity is increased in chondrocytes throughout the full depth of the medial tibial cartilage, when compared with non-lesional areas. The distribution of the increased 5′NT activity in damaged areas corresponds closely to the distribution of chondrocytes which stain positively for TUNEL and which show ultrastructural features indicative of cell death in the STR/ort medial tibial plateau[26]. Any 5′NT activity detected in young pre-lesional mice, or in non-lesional areas of older STR/ort mice, or in non-arthritic CBA mice, was localised predominantly in the superficial zone of the articular cartilage. Why superficial zone cells constitutively express 5′NT activity in normal cartilage is not known, but these cells are specialised, as shown by their synthesis of a specific matrix protein exclusive to this zone[29].

Increased 5′NT activity in osteoarthritic cartilage would result in increased production of adenosine, a factor known to induce cell death in some cell types. Chondrocyte apoptosis is a feature of murine, as well as of human OA[26]. To investigate whether high concentrations of adenosine induce cell death in murine chondrocytes and by what mechanism, we carried out experiments on the murine chondrocyte cell line, MC615. This cell line is exceptionally well differentiated and expresses classic markers of the chondrocytic phenotype including types II, IX and XI collagen, aggrecan and link protein[30]. The requirement for large numbers of chondrocytes for our *in vitro* experiments precluded the use of primary chondrocytes isolated from murine articular cartilage which cannot be obtained in sufficient numbers for this. Extracellular adenosine signals cells via transmembrane ARs which couple intracellularly to adenyl cyclase (AC). The expression of ARs varies depending on tissue type. Human articular chondrocytes strongly express the A2b receptor subtype and weakly express the A2a receptor[31]. The A1 and A3 receptors are not expressed. There is currently no published information about AR expression in primary murine chondrocytes. However, RT-PCR showed that the murine MC615 chondrocytes express the A2b subtype prominently, whilst only weakly expressing A2a. They also express the A1 receptor very weakly, like human chondrocytes, but not A3. Further proof for the presence of active A1 and A2 receptor subtypes in MC615 cells was obtained by stimulating the cells with selective and non-selective AR agonists. However, whilst selective receptor stimulation modulated cAMP levels it failed to induce DNA fragmentation in MC615 cells. Thus, it is unlikely that adenosine induces chondrocyte cell death through cell surface receptors. Nevertheless, it is noteworthy that stimulation of the A2 subtype in rat mesangial cells by adenosine stimulates the activity of 5′NT[32].

Adenosine uptake is mediated via adenosine transporters in most of the tissues[33] and uptake is required for its cytotoxic effect in mouse neuroblastoma cells[4]. We found that adenosine is taken up by MC615 cells and that this can be inhibited by the transporter inhibitor NBTI. The presence of adenosine transporters in murine chondrocytes has not been established previously. Incubation of MC615 chondrocytes with 5 mM adenosine alone induced a 30–50% reduction in the number of adherent cells but was not sufficient to induce cell death, as indicated by a lack of DNA fragmentation, caspase-3 activation or PARP cleavage. Very few floating cells were detected in the medium in these cultures, implying that the reduction in cell number might be due to a decrease in cell proliferation. However, treatment of MC615 with adenosine in the presence of the ADA inhibitor, EHNA, resulted in a far greater reduction in cell number accompanied by DNA fragmentation, caspase-3 activation and PARP cleavage. EHNA treatment alone did not have this effect. Thus it seems likely that when adenosine is taken up by the cell and its degradation blocked by EHNA, its intracellular concentration increases sufficiently to induce apoptosis. This interpretation is supported by the finding that equine chondrocytes treated with EHNA accumulate adenosine[34]. Treatment of the cells with the AK inhibitor, Itub, was cytotoxic, presumably due to a loss of ATP generating capacity, but could be partly rescued by exogenous adenosine. We speculate that, *in vivo*, high 5′NT activity in osteoarthritic STR/ort chondrocytes would likely increase extracellular adenosine levels. A physiological age-related decline in the ADA activity may reduce these cells' capacity to remove increased adenosine taken up via the transporter, resulting in high intracellular levels, inducing apoptosis. The specific activity of rat myocardial ADA decreases with age[35].

Adenosine can also be metabolised through the HC pathway (Fig. 1). The function of this pathway is to provide a source of activated methyl groups for methylation reactions in the cell. SAM is converted to SAH with the release of active methyl groups. The SAH is then hydrolysed to HC which, on addition of a methyl group from N^5-methyltetrahydrafolate, regenerates methionine. During pathological situations, SAH accumulates and has been reported to aggravate adenosine-mediated cell death[6]. Treatment of MC615 cells with 1 mM HC resulted in an increase in cell number, as it did also in rat aortic smooth muscle cells in which DNA synthesis was stimulated by 4.5-fold[36]. However, human umbilical vein endothelial cells show a dose dependent decrease in DNA synthesis in response to HC[17].

Treatment of MC615 cells with 5 mM adenosine and 1 mM HC together induced a large reduction in the number of adherent cells, DNA fragmentation and activation of caspase-3, indicating induction of apoptosis, an effect not observed with either agent alone. Moreover this combination supplemented by either of the inhibitors (EHNA or Itub) further increased cell death. SAM can also serve as a propylamine donor in the synthesis of the polyamines spermidine and spermine. This normally accounts for not more than 5% of SAM metabolism[37]. However, when both exogenous HC (a precursor of methionine) and adenosine are present, increased levels of SAM and its products, such as

polyamines, may be generated in the cells. A link between increased polyamines and induction of apoptosis has been established recently in some cell types[38,39] and spermine triggers the activation of caspase-3 in both cell and cell free systems[40]. Recent evidence indicates that polyamines are important in tumour necrosis factor (TNF)-induced apoptosis in chondrocytes since inhibiting their synthesis markedly reduced DNA fragmentation and caspase-3 activation induced by the cytokine[41].

Procaspase-3 activation and cleavage of the DNA repair enzyme PARP occurred only in the adenosine + EHNA and adenosine + HC-treated cultures and provide firm evidence of chondrocyte apoptosis in these conditions. In contrast, caspase-8 and caspase-9 expression was not seen. Kühn and Lotz[42] detected no procaspase-8 processing by immunoblotting after stimulating human chondrocytes with CD95 antibody, although the mRNA transcript of caspase-8 was detected. It may be that the protein levels of some caspases are very low or their half lives very short, making detection difficult. Alternatively the activation of caspase-3 in chondrocytes may not be mediated through any of the classical pathways. For example, caspase-8 is thought to have an obligatory role in apoptosis initiated by death receptors, but Kischkel *et al.*[43] found that caspase-10 could trigger this pathway, independent of caspase-8.

Finally the finding that many TUNEL positive chondrocytes were present through the full depth of the cartilage when disarticulated CBA mouse knee joints were incubated in media containing adenosine + EHNA indicates that high levels of adenosine can directly induce cell death in murine articular cartilage. This adds further support to the proposal that chondrocyte death in the STR/ort mouse model of OA[26] may be driven, at least in part, by increased adenosine production by 5′NT. It seems likely that for normal cartilage function, extracellular adenosine levels must be quite tightly regulated since depletion leads to increased glycosaminoglycan (GAG) release and the production of matrix metalloproteinase (MMP)-3, MMP-13, prostaglandin E_2 (PGE_2) and nitric oxide (NO)[44], whilst increases may trigger cell death.

Acknowledgments

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