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Review

Cell death in cartilage

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Introduction

Osteoarthritis (OA) is an aging-related disease that affects not only hyaline articular cartilage but all structures of weight-bearing joints. The disease process involves changes in the subchondral bone[1,2] and is often associated with an inflammatory response[3,4]. The central pathogenic events in OA are loss and abnormal remodeling of cartilage extracellular matrix. Chondrocytes constitute the only cell type of the articular cartilage. They maintain tissue homeostasis, respond to injury and perform the cartilage remodeling process that characterizes OA. Previous concepts on OA pathogenesis focused on the role of chondrocytes in the degradation of the extracellular matrix. More recent findings suggest that chondrocyte death and survival are closely linked to cartilage matrix integrity. This review discusses mechanisms and consequences of chondrocyte death.

Forms of cell death

Cell death occurs in a variety of morphologic and molecular manifestations. Attempts have been made to classify different forms of cell death and to reconcile descriptive terminologies, which are either based on morphologic observations or mechanism-oriented experimental findings. Apoptosis and necrosis have been contrasted as mechanistically and morphologically distinct types of cell death, but it is now clear that they share common features and delineate a continuous spectrum of cell death modalities. Apoptosis is a form of programmed cell death (PCD) and is either physiologic (as during development) or pathologic.

Necrosis as the conceptual counterpart is the consequence of a pathologic incident. Apoptosis and necrosis comprise a partially overlapping spectrum of cellular events[5–8]. Cell death can be initiated as apoptosis but be diverted into pathways that create features of necrosis; this has been termed aborted apoptosis. For example, depending on intracellular energy levels apoptosis induced by the death receptor Fas (CD95) can become necrosis-like[11,12]. However, apoptosis is regulated by cellular signaling systems that lead to the orderly disintegration of individual cells. The cell remnants are packaged into small vesicles or apoptotic bodies that are generally removed by phagocytosis[13]. Unlike apoptosis, necrosis does not require activation of specific intracellular signaling cascades, and is a form of non-programmed or accidental cell death. A distinctive feature of apoptotic cell death is the activation of caspases, a class of cysteine proteases with specificity for aspartic acid residues[14]. Although not all forms of PCD depend on caspases, these proteases play pivotal roles in the initiation as well as the execution phase of apoptotic programs[15]. Criteria that distinguish apoptosis and necrosis are summarized in Table I.

Oncosis has been proposed as a distinct form of cell death. Although many of its features, such as increased membrane permeability or cell and organelle swelling, are similar to necrotic death it has been suggested to be a form of PCD[16,17]. Conceptually, necrosis is defined as the final stage of any form of cell death including oncosis and apoptosis. The mechanisms leading to oncosis have not been investigated in detail but some evidence suggests that failure of ionic pumps and ATP depletion may be among the initiators of oncosis[16,18]. A role for phospholipase A_2 has also been suggested[19,20]. In a recent study crosslinking of porimin, a 110 kDa cell surface molecule was shown to induce oncosis in lymphoid cells indicating that oncosis, similar to certain forms of apoptosis, can be mediated by receptor-elicited signal transduction events[21,22]. Internucleosomal DNA fragmentation does not occur during oncosis. Cell death resembling oncosis has been observed in atherosclerotic lesions[23] and in ischemic heart disease[24] and may also occur in bone and cartilage[25].

Structure-forming or developmental PCD can also be the consequence of autophagy, a type of cell death that appears to be mechanistically distinct from apoptosis and dependent on vacuolar proteolysis. This type of cell death may or may not involve caspase activation and DNA degradation, and it has been speculated that autophagy occurs in cells which contain a protein degradation machinery similar to that usually associated with the lysosomes of phagocytic cells. The molecular mechanisms of

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Table I
Distinguishing features of apoptosis and necrosis

Necrosis	Apoptosis
Morphologic Features	
• Swelling of cytoplasm and mitochondria	• Shrinking of cytoplasm and chromatin condensation
• Non-specific karyolysis	• Nuclear fragmentation
• Total cellular disintegration without formation of vesicles	• Formation of membrane enclosed structures (apoptotic bodies)
Physiologic Characteristics	
• Induced by non-physiologic stimuli	• Induced by physiologic stimuli
• Early loss of membrane integrity	• Tightly regulated signaling events
• No energy requirement	• Energy dependent
• Random digestion of nuclear DNA (leading to smear in agarose gelelectrophoresis)	• Enzymatically catalyzed changes of cell membrane (annexin V binding)
• DNA degradation occurs after membrane permeabilization	• Orderly fragmentation of chromosomal DNA (DNA laddering)
	• Activation of caspases
	• Late loss of membrane integrity
Consequences	
• Affects groups of cells in a tissue	• Affects individual cells
• Associated with an inflammatory response	• Phagocytosis by adjacent cells or macrophages
• Disintegrating cells are phagocytosed by leukocytes	• Typically not associated with an inflammatory response

autophagy have been investigated in yeast and homologues of some of the involved genes are found in higher organisms including humans (for a recent review see[26]).

Summary: cell death occurs in a wide variety of morphologic manifestations. PCD can have features of necrosis or apoptosis or both. So far, only the mechanistic details of apoptosis have been characterized while relatively little information is available on the mechanisms of necrosis, oncosis and autophagy.

Mechanisms of apoptosis

Apoptosis can be divided into an initiation and execution phase (Fig. 1). Diverse stimuli that affect either mitochondrial function or death receptor activity or that cause endoplasmic reticulum (ER) stress can initiate apoptosis. During the early stages of apoptosis the activation of initiator caspases are activated. The execution phase results in the structural disintegration of the cell with cytoplasmic and nuclear material being packaged into apoptotic bodies.

The regulated degradation of nuclear DNA as a hallmark of apoptosis is an enzymatic process catalyzed by a magnesium-dependent deoxyribonuclease called caspase-activated deoxyribonuclease (CAD)[27] or DNA fragmentation factor 40 (DFF40)[28] and possibly by DNase I[29]. CAD cleaves nuclear DNA within the histone H1-bound spacer regions between nucleosomes, generating double-stranded DNA fragments of multiples of 180 base pairs[30]. Upon separation by gel electrophoresis these fragments form the characteristic DNA ladder. Larger DNA fragments of 50 to 300 kilobase pairs are also generated during apoptosis[31]. This so-called domain sized DNA fragmentation can occur in the absence of internucleosomal DNA cleavage and may suggest the presence of at least one additional nuclease involved in the fragmentation of genomic DNA during apoptosis.

Another key feature of apoptotic programs is the activation of caspases. Caspase activity can be blocked by inhibitor peptides specific for individual caspases or by pan-caspase inhibitors such as z-VAD.fmk. Typically, cleavage of DNA into nucleosomal DNA fragments can be blocked by caspase inhibitors, although cells are not always prevented from undergoing alternative forms of PCD[32]. In the nucleus latent CAD forms an inhibitory complex with the inhibitor of caspase-activated deoxyribonuclease (ICAD) protein, also called DNA fragmentation factor 45 (DFF45). Activated caspase-3 cleaves ICAD and facilitates the assembly of CAD into its active form leading to the production of nucleosomal DNA fragments[33].

Recent evidence suggests that the digestion of ICAD and apoptotic DNA laddering as well as cell death are not always dependent on caspase activity as these events can also be catalyzed in a caspase-independent manner by granzyme B, a serine protease with specificity for aspartic acid residues[34]. Caspase-independence of DNA fragmentation has also been described for cell death mediated by apoptosis inducing factor (AIF), a mitochondrial flavoprotein that, upon induction of apoptosis, translocates to the nucleus and induces stage I chromatin condensation and domain sized DNA fragments[35–37]. The release of mitochondrial AIF can, for example, be triggered by poly-ADP ribose polymerase (PARP) activation in response to DNA-damaging agents[38]. Several other studies demonstrate that apoptosis-like PCD can occur without internucleosomal DNA cleavage[9–11,31,32,39,40]. B cell lymphomal/leukemia-2 (Bcl-2) inhibits cell death in situations where PCD proceeds in a caspase-independent manner, suggesting mitochondrial regulation or an involvement of the endoplasmic reticulum (ER) in this type of cell death[41,42]. A novel cell death pathway has been described that leads specifically to the activation of caspase-12 localized to the ER[43]. This caspase is activated upon ER stress, possibly by calpain in a calcium-dependent manner[44]. A role for TNF receptor-associated factor-2 (TRAF-2), which interacts with procaspase-12 and induces its clustering in response to ER stress has also been suggested[45]. To date, the downstream effector molecules of caspase-12 have not been identified. However, PARP cleavage and chromatin condensation as a consequence of ER stress have been demonstrated[43].

Summary: these findings suggest that in some PCD systems caspase activity and internucleosomal DNA fragmentation are dispensable downstream occurrences with

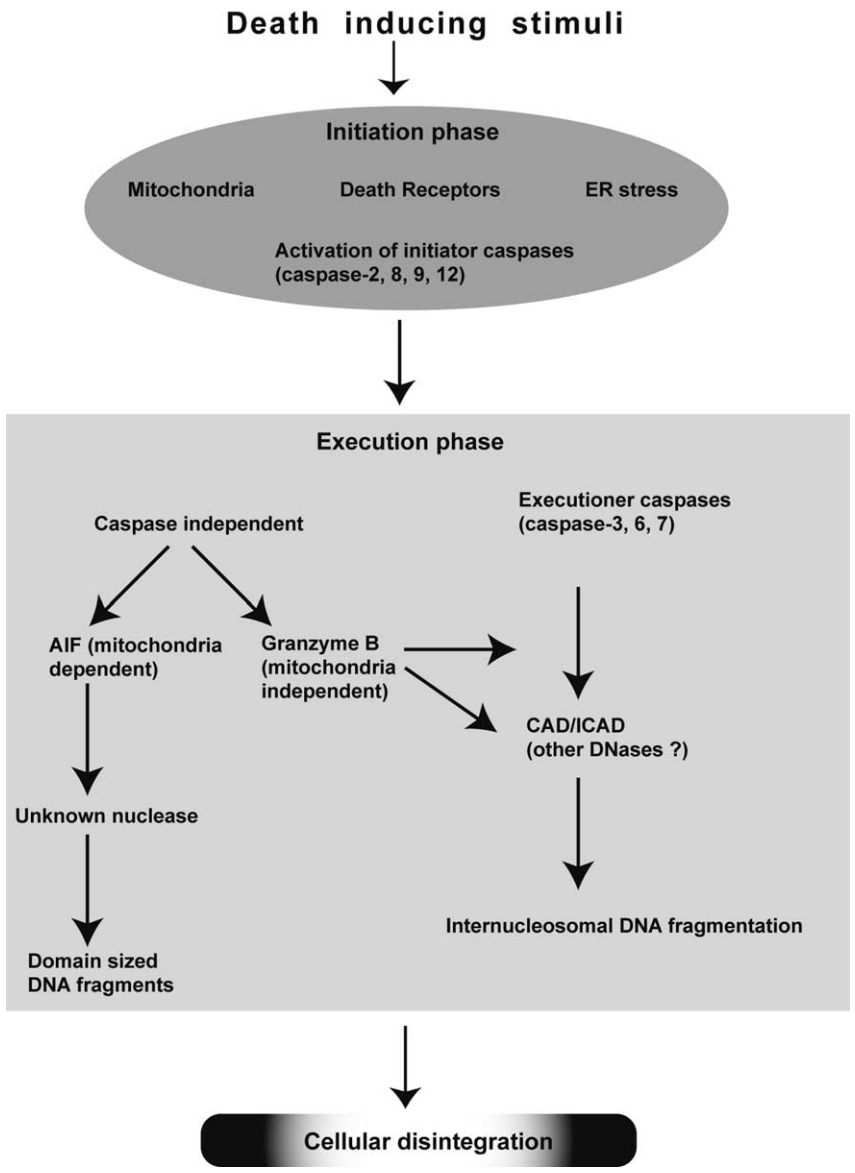

Fig. 1. The initiation phase of PCD is triggered when death stimuli activate at least one of three distinct cell death pathways: the mitochondrial pathway, the death receptor pathway or the ER stress pathway. These pathways generally lead to the activation of initiator caspases. Caspase-dependent PCD is based on executioner caspases that cleave downstream substrates including ICAD. This causes the activation of CAD and the cleavage of chromosomal DNA into small fragments, which can be detected by agarose gel electrophoresis as a 'ladder'. However, in certain instances the mitochondrial pathway can also cause cell death when caspase activity is blocked. In these cases the execution phase of apoptosis is caspase-independent. For example: a caspase independent execution phase is triggered upon release of apoptosis inducing factor (AIF) that is located between the inner and outer membrane of the mitochondria. Upon translocation to the nucleus an unknown signaling cascade causes chromatin condensation and large scale degradation of chromosomal DNA and subsequently cell death that is not inhibited by z-VAD.fmk. Another caspase independent execution pathway is based on granzyme B. Granzyme B generally activates caspases but direct cleavage of ICAD has been demonstrated suggesting that the caspase-dependent amplification loop is dispensable in granzyme B induced apoptosis. All pathways lead to the orderly disintegration of the cell.

respect to the induction of cell death. In many cases, however, these two events are tightly linked features of apoptosis execution.

Methods for cell death detection

Cell death is usually assessed by measuring parameters such as DNA degradation, disintegration of lipid bilayers, mitochondrial activity or, in the case of adherent cells, cell detachment (Table II). In tissues electron microscopy represents a reliable tool for the identification and ultrastructural characterization of apoptotic cells but it is not suitable for quantification of apoptosis. Application of a single technique is often not sufficient to definitively distinguish between apoptosis and necrosis. For example, changes in membrane integrity that are associated with the release of cytosolic components occur during late

Table II
Methods for the characterization of cell death

Cell death parameter	Method	Specific detection of apoptosis
• Externalization of phosphatidyl serine	Annexin V binding	no
• Release of cytosolic compounds	^{51}Cr release, ^{3}H-labeled proteins, enzymatic activities in culture supernatants	no
• Uptake of dyes	Vital dyes (counting of cells) or fluorescent dyes for FACS	no
• DNA laddering	Agarose gel electrophoresis	yes
• *In situ* DNA cleavage	TUNEL	no
• Nuclear condensation and fragmentation	DAPI	yes
• DNA degradation	DNA content in sub G1 cells (FACS), agarose gel electrophoresis, DNA fragmentation ELISA	no
• Internucleosomal DNA fragmentation	DNA fragmentation ELISA, agarose gel electrophoresis	yes
• Oxidative phosphorylation	MTT, Alomar Blue	no
• Mitochondrial membrane polarization	Aggregation, uptake, sequestration of fluorescent dyes	no
• Caspase activity	Conversion of fluorogenic substrates	yes
• Caspase processing	Western blot, immunhistochemistry	yes
• Cleavage of caspase substrates	Western blot, immunhistochemistry	yes
• Loss of cell attachment	Staining of cells with crystal violet or fluorescent DNA-binding dyes	no
• Cellular ultrastructure	Electron Microscopy	yes

apoptosis as well as during the early stages of necrosis[46]. The binding of annexin V to phosphatidylserine residues can be seen in apoptotic as well as necrotic or oncotic cells with partially disintegrated plasma membranes[47,48]. DNA fragmentation is typically detected by conventional agarose gel electrophoresis of purified nuclear DNA or by pulse field electrophoresis. CAD-mediated internucleosomal DNA cleavage leads to the characteristic electrophoretic pattern referred to as 'DNA laddering', which distinguishes apoptosis from the random DNA degradation that occurs during necrotic cell death.

While the isolation of DNA of chromosomal origin and the detection of DNA laddering do not pose a technical problem in cultured cells this is often difficult or impossible for tissues such as cartilage. DNA cleavage in tissue sections is detected by TdT-mediated dUTP nick end-labeling (TUNEL). This immunhistochemical method is based on the enzymatic labeling of 3′-hydroxyl groups of deoxyribose using terminal deoxynucleotidyl transferase (TdT) and deoxyuridine triphosphate (dUTP) coupled to biotin or digoxygenin for antibody detection. However, free 3′-DNA hydroxyl groups are also generated during random necrotic DNA degradation. TUNEL is therefore not suitable to definitively distinguish between apoptosis and necrosis[49–52]. In addition, in cartilage sections the TUNEL technique may yield false positive signals leading to an overestimation of cell death[53]. Antibodies specific for active caspase-3 or neoepitopes in cleaved caspase substrates such as the 85 kDa fragment of PARP have been used to identify apoptotic cells in tissues, including cartilage[54]. This may be a valuable tool for identifying apoptosis in cartilage when used in combination with TUNEL.

Mitochondrial respiratory activity is an indirect indicator of cell viability. The widely used 3-(4,5-dimethylthiazol-2y1)-2-5-diphenyltetrazolium bromide (MTT) assay provides a colorimetric assessment of mitochondrial dehydrogenase activity in cultured cells and is based on the conversion of tetrazolium salts into formazan. Impairment of oxidative phosphorylation does, however, not necessarily lead to cell death. On the other hand, some pro-apoptotic and anti-proliferative stimuli do not cause a reduction of mitochondrial dehydrogenase activity[55–58]. However, in combination with other viability assays or cell counting the measurement of mitochondrial activity is useful for assessing cell viability.

Summary: most commonly used cell death assays do not distinguish between the various cell death modalities since they measure variables common to different forms of cell death. In order to reliably characterize the type of cell death for a given cell-stimulus combination or tissue it is necessary to apply a combination of methods measuring morphological as well as mechanistic cell death parameters.

Regulators of chondrocyte death

EXTRACELLULAR MATRIX COMPONENTS

Cell-matrix interactions have been shown to mediate survival in anchorage-dependent cell types[59]. Apoptosis resulting from a lack of cell adhesion was first demonstrated for endothelial cells and termed 'anoikis' (the Greek word for homelessness)[60]. This type of cell death may be mediated by the CD95/CD95 ligand death receptor system[61–63] or by cytoskeletal reorganization leading to the release of the pro-apoptotic BH3-only protein Bcl-2 modifying factor (Bmf)[64]. Studies on chondrocyte death pathways have been conducted with animal and human chondrocytes in monolayer culture or embedded into agarose or alginate matrices. Loss of certain matrix components by itself can cause chondrocyte death. In cartilage of mice homozygous for targeted inactivation of the collagen type IIA1 gene, a decrease in cell density was associated with ultrastructural features similar to apoptosis[65]. Increased TUNEL signals and degradation of DNA isolated from knee cartilage were also observed, supporting the notion that type II collagen is important for chondrocyte survival *in vivo*. The survival-promoting effect of extracellular matrix is in part mediated by integrins. The $\alpha1\beta1$ integrin is a major collagen receptor that is expressed in chondrocytes. In mice inactivation of the integrin $\alpha1$ gene was associated with more severe cartilage degradation, glycosaminoglycan depletion and synovial hyperplasia when compared to wild type mice. In these mice cartilage cellularity was reduced and the frequency of apoptotic cells

was increased[66]. Death of chondrocytes maintained in suspension could be prevented by collagen and was associated with integrin β1-dependent cell aggregation[67]. In chicken sterna type X collagen deposition and chondrocyte survival were dependent on integrins containing the α2 and α3 or β1 subunits, indicating that integrin-matrix interactions are required for chondrocyte developmental programs as well as survival[68]. Antibodies to the integrin α5-subunit induced death in freshly isolated human chondrocytes[69] and peptides containing the sequence arginine-glycine-aspartic acid (RGD peptides) that interfere with integrin-mediated matrix binding reduce MTT-oxidizing activity in cultured chicken chondrocytes[70]. These findings suggest a direct involvement of integrin-ligand interactions in chondrocyte death. Hyaluronan also protects chondrocytes from death receptor induced apoptosis and this is mediated via the CD44 receptor[71].

In OA and aging cartilage extracellular matrix can be enzymatically degraded or undergo posttranslational modifications such as advanced glycation[72,73] or tyrosine nitrosylation[74]. It has been demonstrated that such altered matrix induces chondrocyte responses that are not activated by the native intact molecules. Examples include the 29 kd fragment of fibronectin[75] and advanced glycation end products[76], which induce inflammatory responses.

Summary: chondrocyte survival is regulated by the interaction of integrins with native cartilage matrix components. While it is possible that chondrocyte survival is exclusively promoted by the presence of native extracellular matrix the role of altered extracellular matrix or fragments of matrix proteins in promoting chondrocyte survival or inducing cell death has yet to be investigated.

MECHANICAL INJURY

Mechanical stimuli represent important regulators of chondrocyte function and induce inflammatory mediators such as IL-1β[77] and NO[78], matrix metalloproteinases[79] or the release of proteoglycans from cartilage explants[80]. Certain types of mechanical injury can also induce chondrocyte death[81]. The average peak pressures generated at the tibial articular surface during normal walking have been reported to be approximetely 4 MPa. This suggests a physiological range of mechanical stress for articular cartilage[81] and is borne out by the report that cyclic loading at 6.9 MPa produced rapid cartilage damage (by 250 cycles), while less than 3.5 MPa did not produce any mechanical damage even after 120,000 cycles of compression[82]. The lower limit of pressures that caused significant chondrocyte death after loads simulating single impact has been reported to be between 15 MPa and 20 MPa[81,83]. At these impact loads a linear correlation is seen with impact injury and percent chondrocyte death[84].

The rate at which mechanical stress is applied also determines the type and degree of cartilage damage and chondrocyte death. For the same level of applied mechanical stress, higher rates of mechanical stress produced more cartilage cracks with chondrocyte death mainly localized to the cracks, whereas low rates of mechanical stress produced a more diffuse distribution of chondrocyte death without significant cracks[80]. The threshold for inducing chondrocyte death by mechanical stress drops significantly when the loading is cyclic. Cyclic loads produced significant cell death above 3.5 MPa in human patellar explants[82], and 6 MPa in bovine explants[85]. Again, a strong correlation was observed between chondrocyte death and stress levels above 6 MPa[85].

Attempts have been made to distinguish the type of chondrocyte death in response to cartilage injury. A study in which *ex vivo* bovine articular cartilage was assessed for apoptotic and necrotic cell death following holmium:YAG laser treatment suggests that TUNEL positive cells are not necessarily apoptotic as plasma membrane disintegration occurred before DNA fragmentation[86]. Tew *et al*. demonstrated apoptosis and necrosis at the edges of surgically created wounds in cartilage explants obtained from mature and immature bovine metatarsal joints[87]. Loening *et al*. reported apoptosis in calf cartilage explants in response to injurious compression with stress levels as low as 4.5 MPa when applied for six cycles of 25 min compression. A dose response was seen with an increasing percentage of cells staining positive for TUNEL with increasing stress levels. This correlated with decomposition of biomechanical properties of the injured cartilage[88]. A similar stress level-dependent increase in the percentage of TUNEL positive cells was reported in mature bovine femoral articular cartilage[89] and human tibial and femoral articular cartilage[90]. These *in-vitro* findings were substantiated by *in-vivo* reports of apoptosis after impact injury in rabbit patellae[89] and clinically, in arthroscopic cartilage biopsies of patients with acute knee injury[91].

A time course of progressive apoptosis has also been reported after mechanical injury. Within 24 h after surgical wounding TUNEL positive cells were seen in a band adjacent to the edge of the wound in immature cartilage[87]. The number of TUNEL positive cells increased up to the fifth day after wounding. In mature cartilage, TUNEL positive cells did not appear in response to wounding until the fifth day after injury. In human cartilage explants, the earliest signs of apoptosis due to mechanical stress appeared around 6 h after injury and the percentage of apoptotic cells was shown to increase up to 7 days after injury[90]. A pattern of progressive apoptosis may open a window for therapeutic modulation of cell death.

Summary: in animal models as well as in human cartilage injurious compression causes chondrocyte death. TUNEL positive cells can be detected shortly after injury. This indicates that impact-induced chondrocyte death is associated with DNA degradation.

NITRIC OXIDE

Nitric oxide (NO) is a modulator of several chondrocyte functions and is spontaneously produced by OA cartilage explants[92]. High levels are also released from cytokine-activated normal cartilage and isolated chondrocytes[93]. NO production has been linked to the induction of chondrocyte death. Blanco *et al*. first reported that high concentrations of the NO donor sodium nitroprusside (SNP) induced apoptosis-like cell death in cultured human chondrocytes[94]. However, IL-1β, a stimulus of inducible nitric oxide synthase (iNOS) expression and NO production in chondrocytes did not cause cell death[94,95]. But in combination with an oxygen radical scavenger hypoploidy and DNA fragmentation were observed. These effects were abrogated by a specific inhibitor of iNOS activity indicating that apoptosis induced by IL-1β was dependent on endogenous NO generation and that the proapoptotic effect of NO could be blocked by reactive oxygen species (ROS)[94]. It was proposed that the balance between intracellular NO and ROS may determine the type of chondrocyte death, with a low concentration of ROS promoting apoptosis in the presence of NO and a high concentration of ROS promoting necrosis.

In cultured human chondrocytes IL-1β induces binding of annexin V but cell death or a causal relationship between NO generation and annexin V binding was not demonstrated[96]. SNP increased caspase-3 activity about 2.5-fold in human OA chondrocytes. A caspase-3 specific inhibitor peptide caused a partial inhibition of nucleosomal DNA fragmentation as analyzed by ELISA suggesting that cell death and cleavage of chromosomal DNA induced by exogenous NO in cultured chondrocytes may depend in part on active caspase-3[97]. However, caspase-3 processing in response to SNP was not detected by immunoblotting and a caspase-3-specific inhibitor peptide failed to inhibit DNA degradation in cultured human chondrocytes[98]. It was also observed that IL-1β-induced NO can partially inhibit internucleosomal DNA fragmentation and caspase-3 processing induced by CD95 activation and simultaneous treatment with proteasome inhibitors[99]. This effect of endogenous NO was mimicked by SNP. However, cell death was not blocked suggesting that NO specifically interferes with apoptosis execution but does not prevent chondrocytes from undergoing a form of cell death that does not require caspase-3 activation or internucleosomal DNA fragmentation[99].

In rabbit chondrocytes SNP induced p38 mitogen-activated protein kinase-dependent cell death and this was associated with enhanced caspase-3 activity, suggesting apoptosis as the cell death modality[100,101]. However, NO production as a result of adenovirus-mediated overexpression of iNOS did not cause cell death in rabbit chondrocytes[102]. There are no reports on the induction of apoptosis by endogenous NO or NO donors in cartilage explants. An *in-vivo* study in a canine model of OA showed that oral application of the iNOS inhibitor L-NIL significantly reduced the number of TUNEL positive cells in femoral condyles[54] but it is not clear whether this is directly related to NO effects on cell survival or the result of protective effects of L-NIL against cartilage degradation.

In certain cell types NO inhibits apoptosis through S-nitrosylation of cysteine residues present in the catalytic center of caspases as well as through a variety of additional mechanisms[103] while in other cell types exogenous or endogenous NO are proapoptotic[104]. The mechanisms responsible for these dual actions of NO in regulating apoptosis are poorly defined.

In human chondrocytes the effects of NO-donors on cell death are age-dependent: chondrocytes from older donors show an increased ratio of oxidized glutathione to reduced glutathione when compared to cells from younger donors. This may cause cells from older donors to be more susceptible to oxidant stress and explain why chondrocytes from older donors are more sensitive to nitric oxide donor-induced death[105].

Summary: in chondrocytes, NO is produced in response to cytokines such as IL-1 but conclusive evidence for cell death as a consequence of endogenous NO-production has not been reported. In vitro studies using NO-donors have demonstrated induction of cell death. However, the mechanisms of NO donor-induced chondrocyte death vary with experimental conditions.

DEATH RECEPTORS

Members of the TNF-receptor family are transmembrane receptors that contain intracellular death domains and activate apoptosis signaling pathways. Fas (CD95) is expressed on the cell surface of cultured chondrocytes[106] and its expression levels are modulated by the density of the cell cultures[107]. Fas expression was demonstrated in cartilage from normal as well as OA donors[106,108]. Fas ligand (FasL, CD95L) expression in cartilage was not detected by immunhistochemistry or reverse transcriptase-polymerase chain reaction (RT-PCR)[106]. Fas activation by an agonistic anti-Fas antibody (CH-11) leads to low levels of apoptosis in cultured chondrocytes. In cartilage tissue culture, antibody to Fas does not cause cell death, most likely because the extracellular matrix prevents the antibody from interacting with the receptor. Alternatively, chondrocytes may not respond to Fas stimulation when anchored in native cartilage matrix as they are protected from Fas-dependent apoptosis through survival signals generated by the interaction of integrins or other cell membrane receptors with extracellular matrix ligands. Although this suggests that the Fas/FasL system by itself is not a potent inducer of chondrocyte apoptosis, Fas may trigger apoptosis in cartilage where matrix is degraded, or effectively enhance chondrocyte death in combination with other apoptosis promoting factors. When survival-promoting activities were neutralized by proteasome inhibitors *in vitro*, the Fas pathway induced apoptosis in a greater number of chondrocytes[109].

The TNF receptor and Fas are members of the same gene family and TNF-α can induce apoptosis in certain cell types. TNF-α mediated chondrocyte death has not been conclusively established. Hypertrophic and non-hypertrophic chondrocytes from chicken sterna appear to be uniquely responsive to TNF-α. When challenged with TNF-α these cells underwent death and activation of interleukin-1β-converting enzyme (ICE), a caspase not directly related to apoptosis[110]. TNF-α stimulation of cultured human chondrocytes also led to a small increase in the number of TUNEL positive cells[111]. Other studies detected nucleosomal DNA fragmentation or large scale DNA degradation in response to TNF-α when the chondrocytes were simultaneously treated with proteasome inhibitors[109,112]. TNF-α on its own had no effect. In a chondrocytic cell line TNF-α induced internucleosomal DNA cleavage and cell death but only in the absence of serum[113]. These data suggest that TNF-α induces apoptosis in chondrocytes only in the presence of additional pro-apoptotic stimuli or the absence of survival-promoting factors.

Summary: Fas-activation induces limited apoptotic death in cultured human chondrocytes while TNF-α on its own does not appear to affect human chondrocyte viability. Apoptosis mediated by Fas or TNF receptor can be enhanced when chondrocytes are simultaneously treated with inhibitors of cell survival functions.

MITOCHONDRIA

Mitochondria are central regulators of apoptosis (for reviews see[114–116]). In rat OA cartilage as well as in human OA, mitochondria undergo ultrastructural changes that can be linked to different stages of cell death[117]. During bone development mitochondria may also be involved in chondrocyte death. In the avian growth plate mitochondria show a maturation-dependent reduction of oxidative phosphorylation[118] but a causal relation between reduced energy metabolism and chondrocyte death was not demonstrated. The activity of respiratory chain complexes II and III as well as the mitochondrial membrane potential are significantly reduced in cultured human chondrocytes from OA donors when compared to normal donors[119]. In cultured rabbit chondrocytes NO donor treatment induced a loss of

mitochondrial membrane potential and inhibition of ATP synthesis. However, this functional impairment was not associated with cell death suggesting that NO affects energy metabolism and other chondrocyte functions but not necessarily cell viability[120]. It is, of course, possible that energy-compromised chondrocytes become sensitized to other death inducers. Loss of mitochondrial function may also be linked to NO production induced by inorganic phosphate (Pi)[121]. A causal relationship between Pi, NO production, and mitochondrial dysfunction in avian growth plate chondrocytes has recently been suggested[122] but no data are available regarding this possible relationship in OA.

Summary: in OA as well as in hypertrophic growth plate chondrocytes the functionality of mitochondria is impaired and this may contribute to chondrocyte death.

P53 AND C-MYC

Several studies have analyzed the expression of the apoptotic regulators p53 and c-myc in cartilage and their correlation with the presence of apoptotic cells. Expression of p53 was detected in mice in the hypertrophic zone of Meckel's cartilage[123]. No direct spatial or temporal correlation was observed between the expression of p53 and the presence of TUNEL positive cells. In p53 knockout mice no significant effect on cell death in cartilage was detected[124] although bone development was affected. However, immobilized knees of wildtype mice showed enhanced cartilage degeneration and increased cell death when compared to p53 knockout mice. These findings suggest a correlation between cartilage degeneration, cell death and p53 expression under conditions of impaired joint mobility[125]. p53 expression was not detected in costosternal growth plate or bone of neonatal infants with a gestation period of approximately 26 weeks[126]. In OA and RA cartilage lesions the frequency of *in situ* nick end labeling (ISNEL) positive cells correlated with p53 expression[127]. In cultured human chondrocytes hydrostatic pressure induced apoptosis and this was associated with increased p53 mRNA and protein expression[128]. In cultured rabbit chondrocytes NO donor treatment caused induction of p53 via p38 MAP kinase and NFkappaB. Ectopic expression of p53 enhanced NO donor-induced cell death indicating that p53 might influence chondrocyte survival functions in the presence of NO[100].

In rat growth plates higher levels of c-myc were detected in hypertrophic than in proliferating chondrocytes, indicating a role for c-myc in terminal chondrocyte differentiation[129]. Subcellular localization also changed and c-myc immunoreactivity was found in the nuclei of proliferating chondrocytes. It decreased in the nuclei of mature chondrocytes and appeared in the cytoplasm. The s-myc protein was expressed in rat embryo cells committed to undergo differentiation into hypertrophic chondrocytes[130]. c-myc immunoreactivity increased in fully differentiated hypertrophic chondrocytes[131]. In rabbit growth plates c-myc staining frequently colocalizes with cells showing DNA strand breaks. In chicken overexpression of c-myc interfered with limb development but increased apoptosis was not observed[132]. In a canine model of OA intense staining for c-myc was found in areas of cartilage erosion[133]. In the lesions of RA and OA cartilage c-myc expression correlated with ISNEL signals and the degree of cartilage destruction[127]. In cultured human chondrocytes apoptosis and c-myc expression could be induced by hydrostatic pressure[128].

Summary: p53 may be involved in the regulation of developmental and disease-related chondrocyte death but the precise mechanisms remain to be defined. There is no direct evidence that c-myc influences chondrocyte survival or death.

APOPTOSIS INHIBITORS

The anti-apoptotic protein Bcl-2 is expressed in cartilage and cultured chondrocytes. In normal cartilage Bcl-2 was found in mid-zone chondrocytes while OA cartilage showed increased staining adjacent to fibrillations[134]. Immunhistochemical analysis demonstrated that Bcl-2 expression was more prominent in normal than in OA cartilage[108]. Overexpression of Bcl-2 protected rat chondrocytic cells from undergoing apoptosis induced by retinoic acid or serum withdrawal[135]. In cultured human chondrocytes Bcl-2 expression is induced by IL-1β and this may in part account for the anti-apoptotic effects of this cytokine[95]. In OA chondrocytes Bcl-2 expression was blocked by NO donors and this might render these cells suceptible to apoptosis involving the mitochondrial pathway[97]. Transgenic mice overexpressing parathyroid hormone-related peptide (PTHRP) have reduced levels of Bcl-2 expression and show abnormal bone development, suggesting a relation between PTHRP-dependent Bcl-2 expression and normal skeletal development[136]. Cartilage from collagen type II deficient mice also shows a marked decrease in Bcl-2 expression and increased cell death[65].

Several other intracellular inhibitors of apoptosis signaling cascades may mediate protective effects in chondrocytes including inhibitors of the catalytic activation of caspases such as the inhibitor of apoptosis proteins (IAPs, reviewed in[137]). Despite substantial expression of Fas in cultured chondrocytes, activation of this death receptor leads to only marginal and incomplete caspase-3 processing[109]. This limited processing of caspase-3 is probably due to potent inhibitory mechanisms blocking transduction of the death signal upstream of or at the level of caspase-3 activation. Some evidence suggests that low expression of caspase-8 and expression of Fas-associated death domain-like interleukin-1beta-converting enzyme (FLICE) inhibitory protein (FLIP) may in part be responsible for this impairment of Fas signaling[109,138].

In one study, specific inhibition of mitogen-activated protein kinase kinase (MEK) led to a dose-dependent increase in caspase-3 processing and apoptosis-like cell death indicating that the MAP kinase extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway may have apoptosis inhibitory functions in chondrocytes[139].

Cytokines and growth factors also regulate chondrocyte apoptosis and survival. Autocrine production of insulin-like growth factor (IGF)-1 and -2 promoted survival of alginate-embedded human chondrocytes in serum-free medium. When the interaction of IGF with its receptor was blocked by addition of an IGF receptor-specific antibody increased caspase-3 activity and apoptosis were observed[140]. However, the effect of caspase inhibition on cell death was not investigated in this study. Other cytokines also protect chondrocytes from apoptosis, possibly through NF-kappaB activation. This transcription factor is known to interfere with apoptosis induction by TNF-α[141]. In chondrocytes NFkappaB is strongly activated by certain cytokines and studies using inhibitors of NF-kappaB provide evidence that it is, at least partially, involved in chondrocyte protection from Fas and NO donor induced death[95,97,142]. For example, IL-1β has anti-apoptotic activity with respect

to Fas-induced apoptosis. This effect, which was abrogated by inhibitors of tyrosine phosphorylation appears to depend partly on the activation of NF-kappaB[95]. TNF-α was shown to mediate protection of chondrocytes from SNP-induced cell death possibly through NF-kappaB and cyclooxygenase 2 activity[142].

Summary: *in vitro* studies indicate that anti-apoptotic modulators such as NF-kappaB interfere with the induction of cell death by Fas, TNF receptor, and NO donors. In cultured chondrocytes, expression of the anti-apoptotic protein Bcl-2 is influenced by inflammatory mediators while the analysis of knockout mice suggests a role for PTHRP as well as collagen type II in the regulation of Bcl-2 expression. In addition, cytokines can have anti-apoptotic functions in certain experimental settings, probably through effects on expression and/or activation of intracellular apoptosis regulators.

Apoptosis in joint development

PCD of chondrocytes is an essential process in normal skeletal development. In chicken embryos, cell death was detected by TUNEL at very early stages of joint formation in the developing anlagen[143]. In several electron and light microscopic studies features of apoptosis were reported in growth plate at the chondro-osseous junction[144,145]. In chicken tibial growth plate, evidence of apoptotic cell death was detected in the hypertrophic as well as the proliferative zone by TUNEL and ultrastructural analysis[146].

The type of this developmentally restricted cell death may be apoptosis but this has been challenged by the discovery of 'dark' chondrocytes, which do not display the typical ultrastructural features of apoptotic cells. In the avian epiphysis 'dark' chondrocytes undergo a hybrid form of cell death, which has ultrastructural characteristics of apoptosis as well as necrosis. This type of cell death may be associated with abnormalities in the cell cycle[147,148]. Another unusual type of epiphyseal chondrocytes are 'paralyzed' cells, which also display ultrastructural features that are different from apoptosis[149]. Dark chondrocytes and paralyzed chondrocytes have also been observed in the chondroepiphysis of rabbits[150]. These findings suggest that developmental chondrocyte death may represent a modified form of cell death distinct from apoptosis or necrosis, which may have developed as an adaptation to the specific conditions of cartilage tissue.

Chondrocyte death in growth plate is part of the developmental extracellular matrix remodeling process. Deposition of calcified matrix adjacent to the hypertrophic zone[151,152] was accompanied by vascular invasion and chondrocyte death suggesting a causal link between local elevation of calcium levels and developmental chondrocyte death. Matrix vesicles are membrane-enclosed particles released by differentiating growth plate chondrocytes at sites of initial calcification[153]. Based on functional similarities between matrix vesicles and apoptotic bodies[154], it is possible that the generation of matrix vesicles in growth plates is at least in part related to programmed chondrocyte death. A link between chondrocyte death and vascular invasion has also been suggested. In mice with targeted inactivation of the gelatinase B (MMP-9) gene, vascularization of the growth plate and apoptosis of hypertrophic chondrocytes were delayed. This indicates that chondrocyte apoptosis during endochondral bone formation may be regulated by MMP-9-dependent angiogenesis. Alternatively, MMP-9-dependent matrix degradation may cause chondrocyte apoptosis, which triggers vascular invasion[155].

Several studies have addressed potential regulators of chondrocyte death in growth plate. Terminally differentiated growth plate chondrocytes were shown to undergo a maturation-dependent loss of mitochondrial function prior to cell death[118]. Pi induced cell death in terminally differentiated chondrocytes from chicken growth plates[156]. These cells were uniquely susceptible since sternal chondrocytes representing earlier developmental stages did not undergo cell death in response to Pi. A similar selective sensitivity was demonstrated for hypertrophic chondrocytes from chicken tibia, which underwent cell death when overexpressing plasma transglutaminase, an enzyme with protein-crosslinking activity. Non-hypertrophic chondrocytes transfected with the same construct did not undergo cell death[157]. Mice with targeted inactivation of galectin 3, a beta-galactoside-binding protein, showed increased numbers of empty lacunae at the chondrovascular junction as well as condensed chondrocytes in the hypertrophic zone. This finding indicates a role for galectin 3 in the regulation of epiphyseal chondrocyte death and vascularization[158].

Summary: chondrocyte death that is associated with DNA degradation occurs in the growth plate as evidenced by TUNEL staining. Ultrastructural analysis suggests a form of cell death distinct from apoptosis or necrosis. Chondrocyte death in the growth plate is linked to matrix mineralization and vascular invasion. In vitro studies suggest that Pi is an inducer of chondrocyte death.

Apoptosis in OA cartilage

Several studies have examined cell death in human articular cartilage affected by OA[53,96,108,127,159,162] or in experimental OA models[163]. While most studies found increased apoptotic cell death in OA cartilage, there was a wide range in the reported frequencies of apoptotic cells. An age-related increase in chondrocyte apoptosis has also been reported[164] and may account for the reduced cartilage cellularity that had been observed earlier[165–167]. In human OA cartilage electron microscopy revealed nuclear and cytoplasmic features consistent with apoptotic cell death. The superficial zone of OA cartilage contained some empty lacunae, lysosome-like structures, matrix vesicle-like structures, fragmented chondrocytes, and nuclear condensation[159]. In addition to cells with ultrastructural features of apoptosis human OA cartilage also contains cells that appear necrotic (Fig. 2). In OA, chondrocyte death was correlated with age and disease severity. Within the same joint, TUNEL positive cells were more frequent in cartilage lesions than in non-lesional areas[108] and cartilage areas that contained TUNEL positive cells showed proteoglycan depletion[160]. Since cartilage does not contain mononuclear phagocytes and is avascular, dead cells or apoptotic bodies are not removed but remain in the lacunae, where they disintegrate and release their contents. Ultrastructural evidence suggests that disintegration of chondrocytes in articular cartilage may lead to the formation of membrane-enclosed structures resembling matrix vesicles[154,159,162]. These structures, which are remnants of dead cells may in fact be apoptotic bodies and may contribute to matrix mineralization or degradation in OA.

Apoptotic chondrocytes in the upper zone of osteoarthritic cartilage were associated with mineral deposits. These needle-like mineral crystals were often associated with matrix vesicles as seen in calcifying growth plate cartilage suggesting that osteoarthritic chondrocytes may

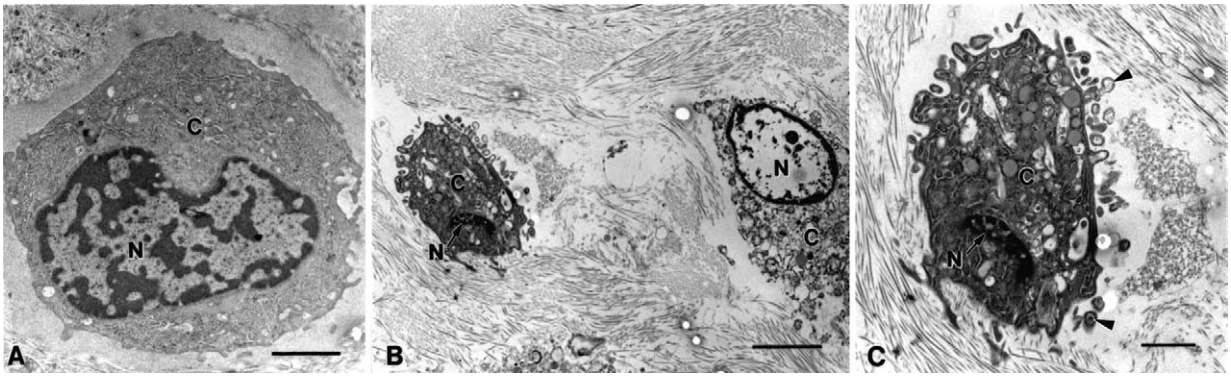

Fig. 2. Chondrocyte death in OA cartilage. (A) The normal chondrocyte has a relatively large nucleus and small cytoplasmic volume. The cell and cellular organelles are surrounded by intact membranes. The chromatin within the nucleus is evenly distributed. (B) The right cell shows irregular chromatin with luscent vacuoles. Intact cellular organelles cannot be detected and the cytoplasma membrane is deteriorating but there is no nuclear/cellular shrinkage. The chromatin is marginalized but not condensed or fragmented indicating necrosis-like cell death. In contrast, the left cell (see also higher magnification in (C)) displays a highly condensed nucleus (arrow) and cell shrinkage indicative of apoptosis. However, necrotic changes such as the formation of vacuoles, disruption of cellular organelles and cytoplasmic protrusions (cones) are also evident. N, nucleus, C, cytoplasm.

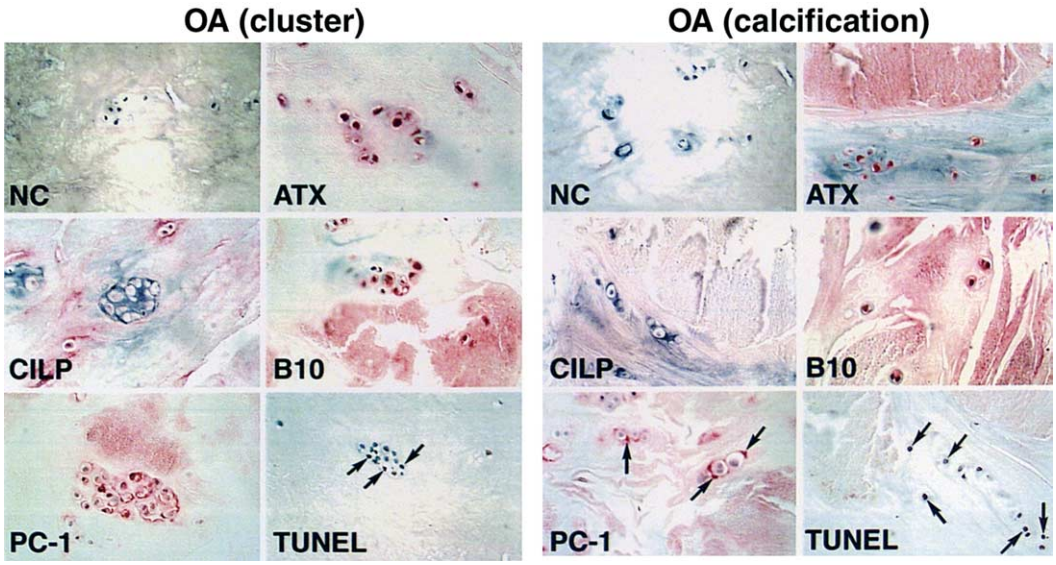

Fig. 3. Localization of TUNEL-positive cells, calcium deposits and pyrophosphate-generating enzymes in menisci from OA-affected human knees. The left panel shows apoptotic cells, many in clusters, in the vicinity of (alizarin red-positive) calcified areas. The right panel shows cells immediately bordering calcifications. Staining for the pyrophosphate-producing enzymes PC-1, ATX and B10 is also prominent at sites of calcification and in areas with TUNEL-positive cells.

enter a differentiation state reminiscent of hypertrophy[161]. A link between chondrocyte apoptosis and calcification has also been observed in menisci from human OA joints[168] where TUNEL positive cells were co-localized with strong expression of enzymes that mediate calcium pyrophosphate dihydrate deposition (Fig. 3). In a rabbit model, in which OA-like changes in cartilage were induced by anterior cruciate ligament transection chondrocyte death correlated with extracellular matrix degradation and NO production[163]. In a canine OA model caspase-3 levels and the number of TUNEL positive chondrocytes in tibial plateus were increased significantly when compared to untreated controls[54]. This also correlated with the histologic severity of the lesions. A similar correlation of caspase-3 expression, OA grade and cell death has been reported for human OA[169].

Summary: cell death with features of apoptosis has been detected in OA cartilage as well as animal models of OA. This was associated with matrix degradation and calcification, suggesting a role for cell death in OA pathogenesis. Inducers and mechanisms of chondrocyte death in OA cartilage have not been conclusively identified.

Prevention of chondrocyte apoptosis

Several applications of the therapeutic use of anti-apoptotic agents have already been explored in neurodegenerative diseases, ischemia/reperfusion injury, and autoimmune disorders. The key role that caspases play in initiating and executing apoptosis make them prime targets for apoptosis modulation. Some of the early reports of

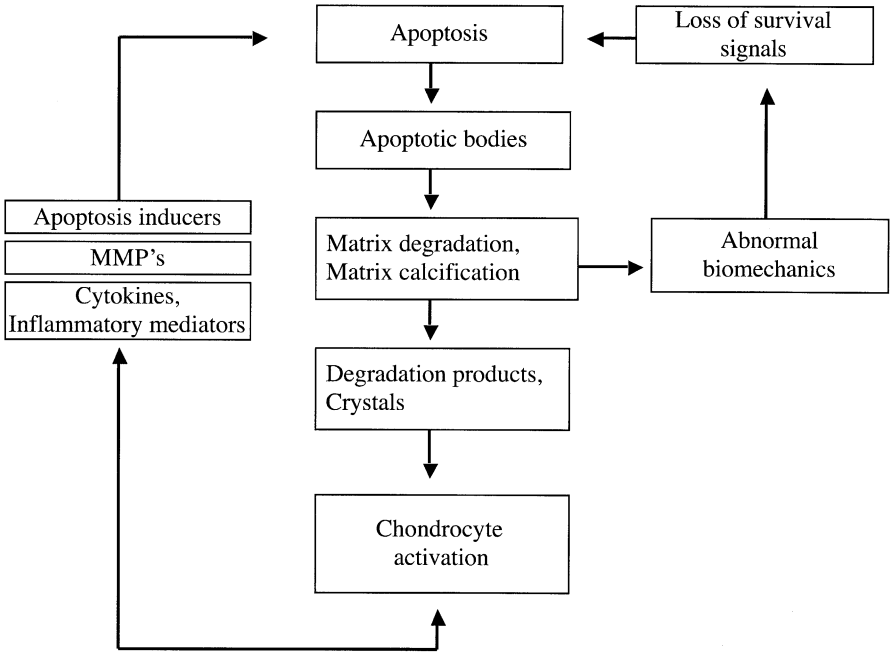

Fig. 4. Linkage of cell death, inflammation, and matrix degradation. Cell death can lead to altered extracellular matrix structure and abnormal mechanical function. Cytokines induce matrix metalloproteinases which generate matrix degradation products that can contribute to the activation of catabolic responses in chondrocytes. Degraded cartilage matrix is also compromised in its survival promoting effects for chondrocytes.

apoptosis inhibition were (1) expression of p53 to block retinal cell apoptosis in Drosophila mutants[170], (2) inhibition of caspases to decrease neuronal cell death in mice[171], (3) and reduce ischemia/reperfusion induced damage of cardiomyocytes in rats[172]. Several reports of varying success with inhibition of apoptosis have been published in models of various diseases affecting the central nervous system[173–176], liver[177,178], heart[179,180], lung[181] and kidneys[182].

Fewer reports are available regarding prevention of apoptosis in chondrocytes. Nuttall *et al.* induced apoptosis in immortalized human chondrocytes (via TNF-α, staurosporine, okadaic acid, and reduced serum) and reported that non-specific caspase inhibition by the pan-caspase inhibitor Z-VAD.fmk as well as specific inhibition of caspase-3 by Ac-DMQD-CHO blocked apoptosis[113]. D'Lima *et al.* reported chondrocyte apoptosis after mechanical injury to bovine and human articular cartilage explants[89,90]. Caspase inhibition with Z-VAD.fmk significantly reduced the percentage of cells undergoing apoptosis[183].

While caspase inhibition may prevent or reduce apoptosis in chondrocytes, it has been shown that the rescued cells are not always fully functional. Caspase inhibition of spontaneous and drug-induced apoptosis in B lymphocytes prevented apoptosis but resulted in cell necrosis rather than cell survival[184]. In a model of Parkinson's disease, caspase inhibition via Z-VAD.fmk blocked caspase activation and prevented apoptotic cell death but did not restore neuronal functionality[185]. Caspase activity was blocked by Z-VAD.fmk in neurons exposed to colchicine, but the neurons underwent delayed cell death[186].

In contrast to these findings apoptosis inhibition was associated with improved functional outcome in a variety of disease models. Anti-apoptotic treatment prevented blindness in Drosophila retinal degeneration mutants[170], reduced amebic liver abscess size[178], improved myocardial function in rat model of sepsis[187], reduced myocardial infarct size[179,188], limited cerebral infarct size in mice[174], improved neurologic outcome in rats with cerebral ischemia[189], prolonged survival rates of mice with acute lung injury[181], and prevented inflammation after renal ischemia-reperfusion injury[182]. Specific to chondrocytes, the findings of Nuttal *et al.* suggest that caspase inhibition retains functionality of the type II collagen promoter after challenge with camptothecin or tumor necrosis factor-alpha plus cycloheximide, two potent inducers of caspase-dependent chondrocyte death. Reduction of cartilage degeneration following caspase inhibition has also been reported for a rabbit model of OA suggesting the possibility that caspase inhibitors may have beneficial effects in human OA[183].

Summary: *in-vitro* studies suggest that caspase inhibition does not always rescue cells from undergoing cell death even in situations where caspase activation is involved. On the other hand, some experimental models demonstrate that caspase inhibition can lead to improved disease outcome. Caspase inhibitors are potentially of therapeutic value for the treatment of injury-induced OA.

Conclusion

Research on chondrocyte apoptosis has revealed a histologic association between cell death and extracellular matrix degradation. Mechanisms can be proposed (Fig. 4) to explain how cell death and structural damage are linked and contribute to the chronic matrix remodeling process that characterizes OA. More research is required to fully characterize the frequency and types of cell death in aging and OA cartilage. Inducers of chondrocyte death and

intracellular signaling pathways are yet to be analyzed in detail. Potential therapeutic applications of apoptosis inhibition appear feasible and should be pursued for acute cartilage injury as well as OA.

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