Physiological cell death of chondrocytes in vivo is not confined to apoptosis

NEW OBSERVATIONS ON THE MAMMALIAN GROWTH PLATE

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Chondrocytes at the lower zone of the growth plate must be eliminated to facilitate longitudinal growth; this is generally assumed to involve apoptosis. We attempted to provide definitive electron-microscopic evidence of apoptosis in chondrocytes of physes and chondroepiphyses in the rabbit. We were, however, unable to find a single chondrocyte with the ultrastructure of 'classical' apoptosis in vivo, although such a cell was found in vitro. Instead, condensed chondrocytes had a convoluted nucleus with patchy chromatin condensations while the cytoplasm was dark with excessive amounts of endoplasmic reticulum. These cells were termed 'dark chondrocytes'. A detailed study of their ultrastructure combined with localisation methods in situ suggested a different mechanism of programmed cell death. In addition, another type of death was identified among the immature chondrocytes of the chondroepiphysis. These cells had the same nucleus as dark chondrocytes, but the lumen of the endoplasmic reticulum had expanded to fill the entire non-nuclear space, and all cytoplasm and organelles had been reduced to dark, worm-like inclusions. Since these cells appeared to be 'in limbo', they were termed 'paralysed' cells. It is proposed that 'dark chondrocytes' and 'paralysed cells' are examples of physiological cell death which does not involve apoptosis. It is possible that the confinement of chondrocytes within their lacunae, which would prevent phagocytosis of apoptotic bodies, necessitates different mechanisms of elimination.

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Physiological cell death refers to a process which occurs during the normal development or maintenance of tissue, i.e., without any pathological or accidental injury. It is synonymous with 'programmed cell death' and both may be abbreviated as PCD. The term 'programmed' may best be reserved for those instances of death which always appear at the same place and stage of development, for example the death of cells to form the interdigital spaces, whereas the term 'physiological' also includes the sporadic and non-predictable death of dysfunctional or imperfect cells, the survival of which would compromise normal growth.

The principal method of PCD is apoptosis which has a well-defined and very characteristic morphology and biochemistry.¹⁻⁴ The term 'apoptosis' is often used as a synonym of PCD. In recent years, several studies have investigated the importance of PCD in the development of cartilage, its ageing and some cartilage diseases.⁵ For example, apoptosis of articular chondrocytes was linked to the degradation of cartilage and the severity of osteoarthritis.⁶⁻⁸ The number of apoptotic chondrocytes in the growth plates of rabbits increased as the growth plates neared closure,⁹ and when apoptosis of terminal hypertrophic chondrocytes was delayed in MMP-9 knock-out mice, there was an eightfold lengthening of the growth plate.¹⁰ Clearly, cell death of chondrocytes is as important as proliferation and viability in overall growth and in the maintenance of cartilage generally.

The identification of apoptosis as a physiological method of cell death, distinct from the pathological necrosis, was initially deduced from morphological observations by electron microscopy.¹ Apoptotic cells have a characteristic and unmistakable morphology; the nuclear chromatin condenses into dark crescents, caps or spheres, the cell shrinks, buds into apoptotic bodies and the corpses are rapidly phagocytosed by other cells.¹ When it was found that the chromatin clumping was a consequence of DNA cleavage by an endonuclease into nucleosome-sized fragments, it became possible to detect apoptosis by electrophoresing the DNA, since the fragmented DNA appeared as a band on a DNA gel, i.e., as a characteristic 'ladder'.¹¹ Unfortunately, this method is only applicable to cells of which a relatively high percentage is simultaneously undergoing PCD. To detect individual cells undergoing apoptosis in situ, Gavrieli et al¹² developed the technique of Terminal-transferase

dUTP Nick End Labelling (TUNEL). This method has been widely used to survey many tissues to investigate the incidence of apoptosis.

Since the terminal hypertrophic chondrocytes of the growth plate (physis) have always been assumed to die, several studies were aimed at demonstrating apoptosis in those cells. Bronckers et al,¹³ using mice three to nine days old, identified very few TUNEL-positive cells in open lacunae at the chondro-osseous junction, suggesting that apoptosis took place after the release of chondrocytes into the vascular space. Other studies on rabbit or rat growth plates found a large proportion of TUNEL-positive chondrocytes within intact lacunae,^{9,14} but many of the labelled cells were in the upper hypertrophic region, not in terminal lacunae, as might have been expected.

In view of these conflicting findings, we have carried out a detailed investigation of PCD in the growth plates and chondroepiphyses of 0- to 20-week-old rabbits, initially with the aim of verifying the presence of apoptosis. Particular attention was paid to the ultrastructure of dying cells, since this was the most unambiguous way to identify apoptosis. In spite of examining many electron-microscopic sections, we have so far not identified chondrocytes with the morphology of classical apoptotic cells. Instead, two different types of chondrocyte were identified which shared some features with apoptotic cells, but differed in other crucial aspects. These chondrocytes were termed 'dark chondrocytes' and 'paralysed cells'. We then concentrated on identifying the characteristics of these unusual chondrocytes, using light, confocal and electron microscopy and now present our findings.

Materials and Methods

We dissected the heads from femora and humeri of 0- to 8-day and 5- and 20-week-old New Zealand White rabbits (eight in total). The endochondral bone below the physal growth plate was removed so that the specimen consisted of the chondroepiphysis, in which the secondary ossification centre had started to develop, and the physal growth plate. Only the 20-week samples were decalcified by 5% EDTA.

Incorporation of fixable fluorescent markers of live/dead cells. To distinguish dying from viable cells, ethidium homodimer-1 (EH-1) and 5-chloromethylfluorescein diacetate (CMFDA, CellTracker green) were loaded into explants of the femoral head by placing the tissue for five hours in medium containing 25 μ M of each marker. The former is an intercalating DNA fluorescent dye which has a very low membrane permeability,¹⁵ i.e., the dye can only reach the nucleus if the cell membrane is no longer intact. It will therefore identify cells with a leaky or damaged cell membrane. CellTracker green freely diffuses into cells where its chloromethyl moieties react with intracellular thiols and their acetate groups are cleaved by cytoplasmic esterases, releasing a green fluorescent product.¹⁵ It there-

fore labels the cytoplasm of viable cells. These markers survive fixation and processing into paraffin wax.¹⁶

Electron microscopy. The tissues from the femoral and humeral heads were fixed in 2% glutaraldehyde in 0.05M sodium cacodylate (with or without 0.7% Ruthenium hexamine trichloride) for 4 to 18 hours, postfixed in 1% osmium tetroxide, then dehydrated and embedded in Spurr resin. Semi-thin sections were cut at 1 μ m and stained with Toluidine Blue. Ultrathin sections were contrasted with lead citrate and uranyl acetate.

Light microscopy. Specimens were fixed for 5 to 18 hours either in 4% paraformaldehyde (in 0.1M phosphate-buffered saline, pH 7.4) or in periodate-lysine-paraformaldehyde (PLP), the latter fixative being optimal for preserving acid phosphatase.¹⁷ Fixation was carried out under rotation for six hours at room temperature, then overnight at 4°C. Sections 5 to 6 μ m thick were mounted on to poly-L-lysine coated slides.

TUNEL method. The method of Gavrieli et al¹² was used with d-UTP-biotin, terminal transferase (both from Roche; Molecular Biochemicals, Indianapolis, Indiana) and avidin-peroxidase with AEC (3-amino-9-ethylcarbazole), which resulted in a brown reaction product. No proteinase K digestion was carried out since this pretreatment had given inconsistent results and often led to positive staining of viable cells. Instead, nuclear proteins were removed by a limited microwave digestion technique.¹⁸ Positive and negative controls were carried out on slides from the same block. Positive controls were treated with DNase (2 U/ml) for 1 hour at room temperature. Negative controls were processed without terminal transferase. Only when more than 90% of all nuclei had been stained in positive controls and no nuclei stained in negative controls was the TUNEL staining considered to give reliable results.

For the co-localisation of TUNEL-positive cells with the fluorescent markers, the same section was scanned several times with a LEICA TCS laser confocal microscope. First, the section was scanned three times with transmitted light at wavelengths of 488, 568 and 647 nm. The three images were combined, using a look-up table of green-red-blue for the three different wavelengths. This resulted in a false-colour image in which the blue cartilage matrix appeared green and the brown TUNEL-positive cells were dark blue. The same section was then scanned, using the krypton/argon laser with simultaneous green/red fluorescence.

Acid phosphatase activity. Enzyme activity was localised by an azo-dye coupling method, using Naphthol AS-B1 phosphate, hexazotised pararosaniline and Fast Red.¹⁷ Sections were counterstained with 0.5% Alcian Blue 8GX (in 1% acetic acid) for 1 minute, then dehydrated and mounted in DPX.

Results

We have examined many micrographs of the physes and chondroepiphyses of rabbits of various ages but so far have

Fig. 1a

Fig. 1b

Fig. 1c

Different types of physiological cell death in chondrocytes. Figure 1a – Photomicrograph showing a condensed apoptotic chondrocyte containing five membrane-bound apoptotic bodies of a cultured chondroepiphysis from a four-day old rabbit ($\times 7800$). Figure 1b – Photomicrograph of the lower hypertrophic zone of an eight-day-old humeral physis showing a condensed 'dark chondrocyte' containing a convoluted nucleus, abundant rER and numerous vacuoles, some of which (arrows) are released into the extracellular space ($\times 7150$). Figure 1c – Photomicrograph of the chondroepiphysis of a humerus from a neonatal rabbit showing a 'paralysed' chondrocyte with a dark nucleus, which is surrounded by pale material containing dark, worm-like inclusions (arrows), but no cytoplasmic organelles ($\times 3900$).

failed to find an example of a classical apoptotic chondrocyte in vivo. Only in cultured explants of young chondroepiphyses was the occasional chondrocyte found which had the morphology of classical apoptosis. The chondrocyte shown in Figure 1a has these unmistakable features. The nucleus has segmented into apoptotic bodies, five of which are in the plane of section. Each contains a rounded mass of condensed chromatin and is individually surrounded by a membrane. The cell has shrunk away from the lacunar wall, leaving an empty space. Cell organelles are more closely packed together, but are otherwise nearly normal and the cell membrane appears intact. This chondrocyte and one other, shown in Roach and Clarke,¹⁸ are so far the only two 'classical apoptotic' chondrocytes which we have observed in our studies. Notably, in both cases, the cartilage containing the apoptotic chondrocytes had been cultured.

Instead of classical apoptotic cells, we found chon-

drocytes which contained a dark nucleus with condensed chromatin, consistent with cell death. Figures 1b and 1c show two types of such an atypical chondrocyte. That illustrated in Figure 1b will be referred to as a 'dark chondrocyte' because of its dark cytoplasm and nucleus, a term also used by Wilsman et al.¹⁹ whereas that shown in Figure 1c will be termed a 'paralysed chondrocyte' for reasons which will be made clear later. When comparing the morphology of these cells with that of the apoptotic chondrocyte, several crucial differences are immediately apparent:

1) There is no fragmentation into apoptotic bodies in 'dark' and 'paralysed' chondrocytes. Instead, the whole nucleus is irregularly shaped with a convoluted nuclear membrane. In addition, the pattern of chromatin condensation is different with small, irregular patches compared with the characteristic solid or half-moon-shaped masses of apoptotic cells.

Fig. 2a

Fig. 2b

Fig. 2c

Normal chondrocytes in the proliferative zone of the growth plate (physis). Figure 2a – Photomicrograph of the femoral head of a six-week-old rabbit showing typical proliferating chondrocytes which are active, flattened cells with a prominent nucleolus (arrows) and a high nucleus-to-cytoplasm ratio which characterises young cells ($\times 1300$). Figure 2b – Photomicrograph of the femoral head of a five-week-old rabbit showing combined staining for proliferating nuclear antigen (PCNA, brown) and DNA fragmentation by the TUNEL method (blue). As expected, many chondrocytes are dividing (brown nucleus), but occasional cells (arrows) are TUNEL-positive ($\times 845$). Figure 2c – Confocal microscopy of the femoral head of a two-day-old rabbit after preloading the specimen with 'live-dead' fluorescent markers. Most proliferative chondrocytes are viable cells with an intact cell membrane, with the exception of some cells in the central column ($\times 325$).

2) The cellular organelles in the apoptotic cell are tightly packed together, but otherwise nearly normal. In the dark chondrocyte there is an excessive amount of rough endoplasmic reticulum (rER) and many vacuoles while in the paralysed cell the dark nucleus is surrounded by a pale material containing dark, worm-like inclusions (arrows in Figure 1c).

3) The cell membrane remains intact in the apoptotic cells whereas in the dark chondrocyte the membrane is no longer intact and cellular material is extruded into the extracellular space (arrows in Figure 1b).

PCD in the proliferative zone of the physis. This zone contains columns of flattened, oblong cells (Fig. 2a). As expected, most chondrocytes were cells which were undergoing cell division as indicated by the positive immunostain-

ing for proliferating cell nuclear antigen (PCNA) (Fig. 2b). The cells had a prominent nucleolus (arrows in Figure 2a), indicating active protein synthesis, and were capable of converting the CellTracker Green into a green fluorescent product (Fig. 2c) demonstrating cell viability.

Much more rarely 'dark chondrocytes' were present (Fig. 3a) and the numbers and locations within the proliferative zone were extremely variable. Overall, only 0% to 2% of all proliferative chondrocytes were of the dark type. A column of chondrocytes either contained no dark cells (Fig. 2a) or one to two 'dark chondrocytes' (Fig. 3b). Much more rarely, nearly the whole column might consist of 'dark chondrocytes' (Fig. 3a).

These cells had probably originated from the light chondrocytes by asymmetrical cell division, since both types

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

Fig. 3e

'Dark chondrocytes' in the proliferative zone of the growth plate (physis). Figure 3a – Photomicrograph of the femoral head of an eight-day-old rabbit showing a column containing mostly 'dark chondrocytes'. There is extensive vacuolation and release of vacuoles (large open arrow), a dark nucleus and extensive rER ($\times 2600$). Figure 3b – Photomicrograph of the humeral head of an eight-day-old rabbit showing doublets consisting of one normal and one very flat dark chondrocyte, suggesting that both were derived from the same mother cell ($\times 6175$). Figure 3c – Photomicrograph of the humeral head of an eight-day-old rabbit showing spindly remnants of 'dark chondrocytes' which are only situated between enlarged typical chondrocytes ($\times 2600$). Figure 3d – Photomicrograph of the femoral head of a five-week-old rabbit showing combined staining for proliferating nuclear antigen (PCNA, brown) and DNA fragmentation by the TUNEL method (blue). In these columns, most chondrocytes are TUNEL-positive ($\times 845$). Figure 3e – Confocal microscopy of the femoral head of a two-day-old rabbit after preloading the specimen with 'live-dead' fluorescent markers. Most proliferative chondrocytes are viable cells with an intact cell membrane, with the exception of some cells in the central column ($\times 975$).

were sometimes found in a doublet (Fig. 3c). Such 'dark chondrocytes' contained abundant rER and a high number of vacuoles. Subsequently, the vacuoles were extruded into the extracellular space, as shown by the chondrocyte marked by a large open arrow in Figure 3a. The extremely flat, spindle-like cells seen in Figure 3c, in which nearly all

the cytoplasmic and nuclear material had disappeared, are likely to represent a near-end stage in the self-destruction of 'dark chondrocytes' in the proliferative zone. These observations suggest that these cells did not progress to the maturing and hypertrophic zones, but were eliminated within the proliferative zone.

Fig. 4a

Fig. 4b

Fig. 4c

'Dark chondrocytes' in the hypertrophic zone of the physis. Figure 4a – Photomicrograph of the hypertrophic zone of the physis of a femoral head from a four-day-old rabbit, as seen in semi-thin resin-embedded sections. The columns are not yet as well organised as in older growth plates. Most chondrocytes are large, hydrated cells, but several cells in the last three to four lacunae before the vascular front are condensed. Condensed cells are also present in the upper hypertrophic zone (arrows), but not as frequently ($\times 400$). Figure 4b – Photomicrograph of a 'dark chondrocyte' of the upper hypertrophic zone of an eight-day old rabbit. There is a prominent nucleolus (arrow), an enormous amount of rER and a new intralacunar matrix (asterisks) which is produced as the chondrocyte shrinks ($\times 4390$). Figure 4c – The area marked by the square in Figure 4b is shown in higher magnification. A most unusual feature is that the ribosomes appear to be present on the inside of the lumen, but this is due to an inversion in that the former rER lumen now forms the interconnecting spaces, whereas the apparent lumen contains cytoplasm and secretory vesicles (sv) ($\times 39\,000$).

With the combined PCNA + TUNEL method, some chondrocytes of the proliferative zone did contain DNA strand breaks (Fig. 3d), consistent with nuclear fragmentation in 'dark chondrocytes'. When the fluorescent markers were incorporated, the occasional nucleus was labelled with ethidium homodimer-1, indicating that the cell membrane was no longer intact (one cell in Figure 2c and the cells in Figure 3e). This would be consistent with the ultrastructure

of the dark chondrocytes, since blebbing and release of vacuoles suggested a non-intact cell membrane. The top three cells in Figure 3e showed a yellow fluorescence which indicated that the red and green fluorescence overlapped. Clearly, these cells were still viable, but had already lost integrity of the cell membrane suggesting that changes in the cell membrane were a relatively early event in the mode of cell death characterised by 'dark cells'.

Fig. 5a

Fig. 5b

Fig. 5c

Fig. 5d

'Dark chondrocytes' in the last few lacunae of the hypertrophic zone of the physis. Figure 5a – Photomicrograph of the femoral head of a six-week-old rabbit showing dark chondrocytes in the last lacunae of the growth plate. The cells have shrunk, but the vacated space has been infiltrated by a fine granular material. Lacunae which are open to the vascular space contain a much more coarse granular material. One ultimate closed lacuna (*) does not contain a cell ($\times 880$). Figure 5b – The 'dark chondrocyte' marked with an arrow in Figure 5a is shown at a higher magnification. There is very convoluted nucleus, abundant endoplasmic reticulum which no longer contains ribosomes, and numerous vacuoles and vesicles, some of which are extruded into the extracellular space (arrows) ($\times 7150$). Figure 5c – Photomicrograph of the femoral head of a six-week-old rabbit showing another example of the last closed lacuna containing either a condensed cell or no cell (asterisk). One opened lacuna of the vascular front (vf) can be recognised by its coarse granular matrix ($\times 1300$). Figure 5d – A higher magnification of the condensed chondrocyte of Figure 5c – Nearly all the cytoplasm has gone and only the nucleus with condensed chromatin remains ($\times 10\,725$).

Fig. 6a

Fig. 6b

Fig. 6c

Fig. 6d

Characteristics of hypertrophic chondrocytes of the physis. Figure 6a – Photomicrograph of the femoral head of a four-day-old rabbit showing tartrate-resistant acid-phosphatase activity (TRAP) in a PLP-fixed specimen. All the chondrocytes have some TRAP activity, but the strongest (arrows) is found in some terminal chondrocytes ($\times 400$). Figure 6b – Photomicrograph of the femoral head of a six-week-old rabbit showing TUNEL staining (blue) of chondrocytes in the upper (uhz) and lower (lhz) hypertrophic zones. TUNEL-positive chondrocytes were consistently present in the upper hypertrophic zone, whereas staining in the lower zone was more variable ($\times 400$). Figures 6c and 6d – Two confocal images of the same section, first viewed with transmitted light to give a false-colour image of the TUNEL-stained cells (c), and subsequently with fluorescent light which demonstrated the fluorescent markers (d). The TUNEL-positive chondrocytes (purple) near the top of the view show a green fluorescence (small arrows), indicating that they were viable cells with an intact cell membrane. Below these cells there are two chondrocytes which are TUNEL-positive and show green cytoplasm with a red-fluorescent nucleus (large arrows) indicating that these chondrocytes had a non-intact cell membrane but were still viable. In the very terminal cells, the nuclei show a red fluorescence, but no green fluorescence was present, indicating that the cells were no longer viable. There are also clumps of red/green cells in the vascular spaces, but it is impossible to identify them ($\times 400$).

Fig. 7a

Fig. 7b

Fig. 7c

Stages in the development of 'paralysed' chondrocytes. Figure 7a – Photomicrograph of the humeral head of a neonatal rabbit showing a pair of chondrocytes from the chondroepiphysis. The lumen of the rER has expanded and surrounded organelles, such as lysosomes (ly) and mitochondria (m). Regions of cytoplasm have become encircled and isolated, forming 'islands' (arrows) within a 'lake' of rER lumen. This stage is probably reversible ($\times 4725$). Figure 7b – Photomicrograph of a humeral head of a neonatal rabbit showing expansion of the rER lumen. The lake of rER lumen fills most of the non-nuclear space of the cell and contains numerous cytoplasmic islands (arrows). The nucleus shows first signs of chromatin aggregation ($\times 4725$). Figure 7c – Photomicrograph of the humeral head of a neonatal rabbit showing the final stage paralysed chondrocytes. In one cell, there is leakage of the cellular contents into the extracellular space (arrow). There is a dark nucleus, a vastly expanded former rER lumen, and absence of cellular organelles, except for the golgi apparatus (go) and some vacuoles (v) ($\times 3500$).

PCD in the hypertrophic zone of the physis. This zone is characterised by the considerable enlargement of the lacunae and hydration of the chondrocytes. In the terminal or lower hypertrophic zone, i.e., the last three to four closed lacunae before the vascular front, a number of condensed cells could clearly be distinguished from their paler neighbours (Fig. 4a). This section was selected because it showed a relatively high number of terminal condensed cells and was not representative of the incidence of condensed cells across the whole growth plate. Overall, only an estimated 6% to 10% of terminal lacunae contained a condensed cell; the others were either empty (5% to 10%) or contained a hydrated chondrocyte. In the region above the terminal zone, i.e., the upper hypertrophic zone, condensed chondrocytes were less common, but were seen occasionally (arrows).

There was some variation in the ultrastructure of the condensed cells, but all were 'dark chondrocytes', not classical apoptotic cells. By comparing the cellular features of 'dark chondrocytes' in the upper hypertrophic zone with those in the lower zone, stages in their formation could be identified.

Upper hypertrophic zone. 'Dark chondrocytes' in this zone still had a prominent nucleolus (large arrow) as well as an excessive amount of rER which filled virtually the whole cytoplasmic space (Figs 4b and 4c). Since the nucleolus is a ribosome-producing machine, a prominent nucleolus indicated a high rate of production of ribosomes which was borne out by the enormous number of ribosomes present on the endoplasmic reticulum. The secretory product of these 'dark chondrocytes' included proteoglycans, because it was stained by the Ruthenium hexamine trichloride added to the fixative, just like the cartilage matrix which surrounded the chondrocytes. This matrix infilled the space created by shrinkage of the dark chondrocyte.

Terminal zone. 'Dark chondrocytes' of this zone were condensed cells, shrunk to the centre of the lacunae, with the space between the cell and lacunar wall filled with a fine, granular material which was different in structure from the interlacunar matrix (Fig. 5a). Those lacunae which were open to the vascular space contained much coarser granular material (bottom lacunae in Figure 5a). It is not known what caused this sudden change in the intralacunar matrix, but it serves as a useful feature to distinguish open

from closed lacunae. The 'dark chondrocytes' of the terminal zone still had abundant endoplasmic reticulum, but ribosomes could no longer be recognised (Fig. 5b). In addition, the cells contained numerous vacuoles, some of which contained cellular material, and some were released into the extracellular space (arrows). There appeared to be no cell membrane and the nucleus was very convoluted with patchy chromatin condensations. Clearly, this cell was dying by a process which involved digestion of cellular components and release of cytoplasmic remnants. The chondrocyte shown in Figures 5c and 5d was located in the last closed lacuna, because the subjacent lacuna contained the coarse granular material of opened lacunae. This chondrocyte had shrunk even more and had lost nearly all its cytoplasm (Fig. 5d). Notably some last lacunae (asterisks in Figures 5a and 5c) which were still closed judging by the granularity of the intralacunar matrix, did not contain a cell. It was possible that the cell was out of the plane of the section, but equally possible that some last intact lacunae no longer contained a cell.

Possible markers of 'dark chondrocytes'. Three putative methods of identifying 'dark chondrocytes' were investigated: 1) tartrate-resistant acid phosphatase (TRAP), because increased activity of TRAP is known to accompany the autophagocytic mode of PCD;²⁰ 2) the TUNEL method, which is to date the most frequently used method to detect apoptotic cells in situ; and 3) the incorporation of ethidium homodimer-1 (EH-1), a marker for a leaky cell membrane.

1) To demonstrate TRAP activity in cells other than osteoclasts requires fixation with periodate-lysine-paraformaldehyde, as shown in a previous study.¹⁷ Although all the chondrocytes of the growth plate contained some TRAP activity, especially strong activity was found in some terminal chondrocytes (arrows in Figure 6a) in support of the idea of self-destruction as a mechanism of eliminating 'dark chondrocytes'. The differences in staining intensities, however, were not sufficient to serve as a marker for these cells.

2) With the TUNEL method, the results could be extremely variable. Contrary to expectations, there was generally better staining in chondrocytes of the upper hypertrophic zone, with more variable staining in the terminal cells (Fig. 6b). When the TUNEL method was applied to sections from an epiphysis which had been preloaded with the 'live-cell' marker CMFDA, it was possible to demonstrate that at least some TUNEL-positive cells of the upper hypertrophic zone were viable (Figs 6c and 6d). This suggested that not all DNA breaks were related to cell death, but that some were perhaps temporary, possibly due to active gene transcription.²¹

3) The EM studies had suggested that loss of membrane integrity was associated with 'dark chondrocytes'. Moreover, in plastic sections in which 'dark chondrocytes' could be distinguished unambiguously from their pale neighbours, Bott, Roach and Clarke²² had demonstrated that only

'dark chondrocytes' were labelled with EH-1. This suggested that those EH-1-labelled cells which were present within lacunae in Figures 6c and 6d were 'dark chondrocytes'. Taken together, the data suggest that loss of integrity of the cell membrane was an early feature of 'dark chondrocytes' and that EH-1 may be a suitable marker for these cells.

PCD in the chondroepiphyses: 'paralysed' chondrocytes. In rabbits, the secondary ossification centre develops just after birth so that the femoral heads from rabbits aged less than four days consisted mostly of non-calcified epiphyseal cartilage containing small, immature chondrocytes with just a small bony epiphysis.²³ Among these immature chondrocytes, a small percentage (~1% to 2%) of cells was present which had the radically different appearance of the type shown in Figure 1c.

In order to understand how the unusual morphology arose, we examined cells of intermediate morphologies. The likely sequence of cellular events is illustrated by the chondrocytes shown in Figure 7. The lumen of the rER expanded, probably due to retention of secreted proteins. This expanded lumen began to surround cellular organelles (such as lysosomes, ly, or mitochondria, m) which resulted in the formation of 'cytoplasmic islands' within a 'lake' of rER lumen. In the chondrocyte shown in Figure 7b, the rER lumen filled most of the cell and contained numerous cytoplasmic islands (arrows). Comparing Figure 7b with the 'end-stage' illustrated by the chondrocytes in Figure 7c suggested that the contents of the cytoplasmic islands were graded by as yet unknown mechanisms, so that the end-result would be the dark, worm-like inclusions seen in Figure 7c. Only some vacuoles (v) and a hypertrophied Golgi apparatus (go) remained, but otherwise all cellular organelles had disappeared. It is notable that the dark type of nucleus was only apparent in those cells in which the former rER lumen took up most of the non-nuclear space, suggesting that the cytoplasmic digestion processes preceded nuclear condensation.

Discussion

We have identified two types of chondrocyte in vivo, 'paralysed' and 'dark' chondrocytes, which appeared to be undergoing PCD which was different from classical apoptosis. As summarised in Table I, these cells had some characteristics in common with either apoptotic or necrotic cells, but other features unique to cell 'paralysis' and the death characterised by 'dark chondrocytes'. For example, the nuclear chromatin condensed in all modes of death, including necrosis, but only in apoptotic cells were large solid masses present and predominantly at the nuclear periphery. Movement of chromatin to the periphery required ATP²⁴ which was available during the early stages of apoptosis, but presumably not in necrosis, paralysis or 'dark chondrocytes'. On the other hand, the massive expansion of the endoplasmic reticulum was only found in

Table I. Comparison of the main features characteristic of the various modes of cell death

	Physiological cell deaths			Pathological cell death Necrosis
	Apoptosis	'Paralysis'	'Dark chondrocytes'	
Cytoplasmic digestion?	No digestion, except by phagocytosis in other cells	Digestion of cytoplasm + organelles within cell	Partial digestion of cytoplasm + organelles within cell	Initial swelling and bursting of cytoplasm + organelles, vacuolation
Timing of nuclear and cytoplasmic events	Chromatin condensation precedes or is coincident with cell shrinkage	Chromatin condensation after cytoplasmic digestion	Chromatin condensation after or coincident with secretion + vacuolation	Nuclear condensation (pyknosis) + partial collapse of cell
'Blebbing' v 'budding'*	Budding into apoptotic bodies	Late stage: blebbing	Extensive blebbing	Extensive blebbing
Pattern of chromatin condensation	Large, rounded or crescent-shaped masses at perimeter of rounded nucleus	Small patches throughout nucleus, nucleus has convoluted outline	Small patches throughout nucleus, nucleus has convoluted outline	Small patches throughout nucleus, nucleus usually rounded and condensed
Release of proteolytic enzymes?	No, lysosomes remain intact within apoptotic bodies	No, lysosomes digested within 'cytoplasmic' lumina	Probably No	Yes, uncontrolled release of lysosomal enzymes
Function	Elimination of individual cells without inflammation	?? 'neutralisation' of dysfunctional cells where no phagocytosis	?? secretive function before cell death	Unavoidable death of cells, inflammation

* the terms 'budding' and 'blebbing' have been used interchangeably by many authors. We prefer to follow Majno and Joris³ and draw a distinction between the pseudopodia sent out by apoptotic cells, which Kerr has aptly described as 'budding', and the much smaller, blister-like, vacuolated structures for which the term 'blebbing' should be reserved

'paralysed' and 'dark chondrocytes'. These features and the fact that cytoplasmic digestion preceded nuclear changes in paralysed and dark cells, whereas almost the opposite was found for apoptosis, suggested that 'paralysed' and 'dark chondrocytes' were not merely the early stages of apoptosis, but represented two hitherto unrecognised mechanisms of physiological cell death.

This is not the first report of such unusual chondrocytes. Many years ago, cells with the same morphology as 'paralysed' cells were observed in human fetal cartilage.²⁵ 'Dark chondrocytes' were described in the mandibular condyle of mice²⁶ and the physes of growing swine,¹⁹ confirming that 'dark' and 'paralysed' chondrocytes are not confined to the rabbit. Previously, however, the significance of these atypical chondrocytes was unknown because the concept of PCD had not yet been established.

Recently, several studies on apoptosis in mammalian growth plates^{14,27} or in osteoarthritic cartilage^{6,7,28} included electron micrographs showing condensed chondrocytes in support of apoptosis. In each case, the chromatin condensations were patchy, the nucleus was convoluted, there was abundant endoplasmic reticulum, vacuoles had formed and extensive blebbing had occurred, all features of 'dark chondrocytes' rather than of classical apoptotic cells. The blebbing had sometimes been wrongly interpreted as the formation of apoptotic bodies; apoptotic bodies 'bud', not bleb,³ and are comparatively large, not the size of matrix vesicles. In vitro, however, among isolated chondrocytes in culture,²⁹⁻³¹ cells with the morphology of classical apoptosis could be identified, in agreement with our findings. This suggests that either the chondrocytes in vivo received different death-inducing factors or that the cellular responses to the same death signal differed.

In either case, the mechanisms of executing PCD in chondrocytes in vivo differed from classical apoptosis.

Other studies have also drawn attention to the different morphologies of physiological cell death.^{3,32-35} Because apoptosis crucially depends on phagocytosis of apoptotic corpses by neighbouring cells, it may not be appropriate when large numbers of cells need to be eliminated so that the heterophagocytic mechanisms become overloaded or when there are no phagocytes to clear away the apoptotic corpses. If apoptotic bodies are not phagocytosed quickly, they undergo a secondary necrosis, presumably with uncontrolled release of lysosomal or matrix-degrading enzymes. Chondrocytes are separated from neighbouring cells by an extracellular matrix and therefore a mode of cell death which prevents negative effects on neighbouring cells but does not depend on phagocytosis would be required in order to neutralise or inactivate dysfunctional cells. Both 'cell paralysis' and the mechanism of death exemplified by 'dark chondrocytes' may represent solutions to this problem.

In 'cell paralysis', dysfunctional epiphyseal chondrocytes were rendered impotent in respect of damaging consequences by digesting the cytoplasm and all organelles within enclosed 'islands' formed by the expanded and hydrated lumen of endoplasmic reticulum. Once water was expelled from the rER lumen, an inactive cellular debris would remain in the tissue, possibly until the tissue containing the remnant was resorbed during growth.¹⁸

'Dark chondrocytes' appeared to eliminate themselves by forming vacuoles, digesting and expelling their cellular content. The present preliminary data suggest that lysosomal acid phosphatase played a role and that cytoplasmic digestion preceded any nuclear changes. Vacuolisation and partial autophagocytosis with delayed collapse of the nucleus were also characteristic features of the PCD during insect metamorphosis^{20,36} or ovarian atrophy.³⁷ This type of PCD, also called lysosomal or type-II PCD, predominat-

ed in situations in which vast numbers of cells needed to be eliminated simultaneously.^{3,32,33,35} The cell death exemplified by dark chondrocytes was thus similar, but not identical to the type-II mode of PCD.

As 'dark chondrocytes' of the proliferative zone eliminated themselves, their space would be taken up by the expansion of the matrix around neighbouring proliferating cells. In this way, any chondrocytes in which mistakes in DNA duplication occurred during mitosis would be eliminated before reaching the next stage of differentiation. As shown by Evan et al,³⁸ whenever cells undergo mitosis, they are at the same time primed for cell death by activation of the transcription factor c-Myc. This is expressed in most proliferating cells including chondrocytes,³⁹ supporting the notion that there are 'fail-safe' mechanisms for eliminating defective proliferative chondrocytes.

It is accepted that elimination of chondrocytes must occur in the lower zone of the growth plate to facilitate longitudinal growth. Once released into the vascular spaces, phagocytosis of apoptotic corpses would theoretically be possible. Nevertheless, the mechanism of death seems to involve 'dark chondrocytes' rather than classical apoptosis. In 'dark chondrocytes', cell death was preceded or accompanied by a massive expansion of the endoplasmic reticulum, suggesting an increased rate of protein synthesis and/or secretion. As the 'dark chondrocytes' shrunk to the centre of the lacunae, the space between the cell and the lacunar wall was infilled by a matrix which consisted, at least partly, of proteoglycans. This suggested that the shrinkage was a gradual process, allowing time for secretion of matrix. It is also probable that at least some 'dark chondrocytes' self-destructed before the opening of the lacuna so that some intact terminal lacunae no longer contained a cell. In our study, empty lacunae were observed, although a condensed cell may have been present out of the plane of the section. Farnum and Wilsman,⁴⁰ however, demonstrated that 5% of the last closed lacunae were indeed empty, as proven by examining the same lacuna in serial sections of growth plates of swine, providing convincing evidence that at least some chondrocytes must have been eliminated before vascular invasion.

Interpretation of TUNEL staining at the light-microscope level. Because ultrastructural analyses are time-consuming and only relatively small regions can be surveyed, most studies directed at quantifying PCD have either used the TUNEL method^{7,8,13,14,27} or carried out fluorescent-activated cell sorter (FACS) analysis after isolating the cells from the tissue.^{6,8} Our study confirmed previous work in identifying a relatively high percentage of TUNEL-positive chondrocytes not only in the hypertrophic, but also in the other zones of the growth plate. Generally, fewer cells were identified as apoptotic by FACS analysis. Since most TUNEL-positive chondrocytes were non-condensed cells, which were still viable, it must be questioned whether the TUNEL-positive chondrocytes in that zone were really dying cells. Hence any data relying on the TUNEL method

should be interpreted with care and should always be supported by morphological evidence, such as cellular condensation or the presence of apoptotic bodies. Positive TUNEL-staining in condensed cells would provide strong support for cell death, but not necessarily for apoptosis, since 'dark chondrocytes' also appeared as condensed cells under the light microscope and also contained DNA strand breaks.

Our findings suggest that it may be advisable to distinguish between the functional terms of programmed or physiological cell death and the descriptive term apoptosis, and avoid synonymising apoptosis with programmed cell death. This distinction would be important when considering the mechanisms of cell death. For example, it is likely that at some time in the future therapeutic measures will be developed to prevent inappropriate cell death by blocking its induction and/or execution, for instance, chondrodysplasias or osteoarthritis. Any measures dependent on classical apoptosis would probably not be appropriate for chondrocytes because their mode of PCD clearly differs from classical apoptosis.

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References

1. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-57.
2. Wyllie AH. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer Metastasis Rev* 1992;11:95-103.
3. Majno G, Joris I. Apoptosis, oncosis, and necrosis: an overview of cell death. *Am J Pathol* 1995;146:3-15.
4. Lockshin RA, Zakeri Z, Tilly JL. *When cells die: a comprehensive evaluation of apoptosis and programmed cell death*. New York: Wiley-Liss, 1998.
5. Horton WE, Feng L, Adams C. Chondrocyte apoptosis in development, aging and disease. *Matrix Biol* 1998;17:107-15.
6. Blanco FJ, Guitian R, Vazquez-Martul E, DeToro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis: a possible pathway for osteoarthritis pathology. *Arthritis Rheum* 1998;41:284-9.
7. Hashimoto S, Ochs RL, Komiya S, Lotz M. Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. *Arthritis Rheum* 1998;41:1632-8.
8. Hashimoto S, Takahashi K, Amiel D, Coutts RD, Lotz M. Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. *Arthritis Rheum* 1998;41:1266-74.
9. Aizawa T, Kokubun S, Tanaka Y. Apoptosis and proliferation of growth plate chondrocytes in rabbits. *J Bone Joint Surg [Br]* 1997;79-B:483-6.
10. Vu TH, Shipley JM, Bergers G, et al. MMP-9/Gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;93:411-22.
11. Wyllie AH, Morris RG. Hormone-induced cell death: purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *Am J Pathol* 1982;109:78-87.
12. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.
13. Bronckers AL, Goei W, Luo G, et al. DNA fragmentation during bone formation in neonatal rodents assessed by transferase-mediated end labeling. *J Bone Miner Res* 1996;11:1281-91.

14. Fujita I, Hirata S, Ishikawa H, Mizuno K, Itoh H. Apoptosis of hypertrophic chondrocytes in rat cartilaginous growth plate. *J Orthop Sci* 1997;2:328-33.
15. Haughland RP. *Handbook of fluorescent probes and research chemicals*. 6th ed. Eugene, OR: Molecular Probes Inc, 1996.
16. Poole CA, Brookes NH, Gilbert RT, et al. Detection of viable and non-viable cells in connective tissue explants using the fixable fluoroprobes 5-chloromethylfluorescein diacetate and ethidium homodimer-1. *Connect Tissue Res* 1996;33:233-41.
17. Roach HI. Association of matrix acid and alkaline phosphatases with mineralization of cartilage and endochondral bone. *Histochem J* 1999;31:53-61.
18. Roach HI, Clarke NMP. 'Cell paralysis' as an intermediate stage in the programmed cell death of epiphyseal chondrocytes during development. *J Bone Miner Res* 1999;14:1367-78.
19. Wilsman NJ, Farnum CE, Hilley HD, Carlson CS. Ultrastructural evidence of a functional heterogeneity among physeal chondrocytes in growth swine. *Am J Vet Res* 1981;42:1547-53.
20. Bowen ID, Mullarkey K, Morgan SM. Programmed cell death during metamorphosis in the blow-fly, *Calliphora vomitoria*. *Microsc Res Tech* 1996;34:202-17.
21. Ansari B, Coates PJ, Greenstein BD, Hall PA. In situ end-labelling detects DNA strand breaks in apoptosis and other physiological and pathological states. *J Pathol* 1993;170:1-8.
22. Bott PJ, Roach HI, Clarke NMP. The mechanism of programmed cell death in chondrocytes of rabbit chondroepiphyses after induction of apoptosis with etoposide or prednisolone. *J Bone Joint Surg [Br]* 1999;81-B:Suppl, 1, 83.
23. Roach HI, Baker JE, Clarke NMP. Initiation of the bony epiphysis in long bones: chronology of interactions between the vascular system and the chondrocytes. *J Bone Miner Res* 1998;13:950-61.
24. Kass GEN, Eriksson JE, Weis M, Orrenius S, Chow SC. Chromatin condensation during apoptosis requires ATP. *Biochem J* 1996;318:749-52.
25. Hwang WS. Ultrastructure of human foetal and neonatal hyaline cartilage. *J Pathol* 1978;126:209-14.
26. Silbermann M, Frommer J. Heterogeneity among chondrocytes of the mandibular condyle in foetal and postnatal mice. *Arch Oral Biol* 1973;18:1549-54.
27. Zenmyo M, Komiya S, Kawabata R, et al. Morphological and biochemical evidence for apoptosis in the terminal hypertrophic chondrocytes of the growth plate. *J Pathol* 1996;180:430-3.
28. Kouri JB, Rosales-Encina JL, Chaudhuri PP, Luna J, Mena R. Apoptosis in human osteoarthritic cartilage: a microscopy report. *Med Sci Res* 1997;25:245-8.
29. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. *Am J Pathol* 1995;146:75-85.
30. Gibson G, Lin DL, Roque M. Apoptosis of terminally differentiated chondrocytes in culture. *Exp Cell Res* 1997;233:372-82.
31. Adams CS, Horton WE. Chondrocyte apoptosis increases with age in the articular cartilage of adult animals. *Anat Rec* 1998;250:418-25.
32. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl)* 1990;181:195-213.
33. Schwartz LM, Smith SW, Jones ME, Osborne BA. Do all programmed cell deaths occur via apoptosis? *Proc Natl Acad Sci USA* 1993;90:980-4.
34. Bowen ID. Apoptosis or programmed cell death? *Cell Biol Int* 1993;17:365-80.
35. Zakeri ZF, Bursch W, Tenniswood M, Lockshin RA. Cell death: programmed, apoptosis, necrosis, or other? *Cell Death Diff* 1995;2:87-96.
36. Lockshin RA, Zakeri Z. Programmed cell death: early changes in metamorphosing cells. *Biochem Cell Biol* 1994;72:589-96.
37. D'Herde K, De Prest B, Roels F. Subtypes of active cell death in the granulosa of ovarian atretic follicles in the quail. *Reprod Nutr Dev* 1996;36:175-89.
38. Evan GI, Brown L, Whyte M, Harrington E. Apoptosis and the cell cycle. *Curr Opin Cell Biol* 1995;7:825-34.
39. Aizawa T, Kokubun S, Kawamata T, Tanaka Y, Roach HI. c-Myc protein in the rabbit growth plate: changes in immunolocalisation with age and possible roles from proliferation to apoptosis. *J Bone Joint Surg [Br]* 1999;81-B:921-5.
40. Farnum CE, Wilsman NJ. Condensation of hypertrophic chondrocytes at the chondro-osseous junction of growth plate cartilage in Yacatan swine: relationship to long bone growth. *Am J Anat* 1989;186:346-58.