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Physiological death of hypertrophic chondrocytes[1]

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Summary

Objective: Post-proliferative chondrocytes in growth cartilage are present in two forms, light and dark cells. These cells undergo hypertrophy and die by a mechanism that is morphologically distinct from apoptosis, but has not been characterized. The aims of the current study were to document the ultrastructural appearance of dying hypertrophic chondrocytes, and to establish a culture system in which the mechanism of their death can be examined.

Design: Growth cartilage from fetal and growing postnatal horses was examined by electron microscopy. Chondrocytes were isolated from epiphyseal cartilage from fetal horses and grown in pellet culture, then examined by light and electron microscopy, and quantitative polymerase chain reaction.

Results: In tissue specimens, it was observed that dying dark chondrocytes underwent progressive extrusion of cytoplasm into the extracellular space, whereas light chondrocytes appeared to disintegrate within the cellular membrane. Pellets cultured in 0.1% fetal calf serum (FCS) contained dying light and dark chondrocytes similar to those seen *in vivo*. Transforming growth factor-β1 or 10% FCS increased the proportion of dark cells and induced cell death. Triiodothyronine increased the differentiation of dark and light cells and induced their death. Dark cells were associated with higher levels of matrix metalloproteinase-13 expression than light cells, and light cells were associated with higher levels of type II collagen expression.

Conclusions: Light and dark hypertrophic chondrocytes each undergo a distinctive series of non-apoptotic morphological changes as they die. Pellet culture can be used as a model of the two forms of physiological death of hypertrophic chondrocytes.

Key words: Chondrocyte hypertrophy, Physiological cell death, Endochondral ossification, Ultrastructure, Transforming growth factor-β, Matrix metalloproteinase-13.

Introduction

During development and growth of the skeleton, the cartilage models of long bones are gradually replaced by bone tissue through the process of endochondral ossification. The formation of primary and then later secondary centers of ossification leads to the existence of growth cartilage in two locations: the physeal growth cartilage (PGC) or growth plate, which gives rise to metaphyseal bone, and the articular–epiphyseal growth cartilage (AEGC), which gives rise to epiphyseal bone. Chondrocytes in both of these locations undergo an orderly series of functional changes that allow the growth cartilage to drive longitudinal bone growth while producing a transient matrix that provides an appropriate level of structural support. Following proliferation, chondrocytes in growth cartilage undergo hypertrophy and then die, before the cartilage matrix that they have synthesized is invaded by osteoclasts and blood vessels, which prepare the tissue for ossification.

Physiological (or programmed) cell death *in vivo* can occur in many different ways, which have been subjected to a variety of classification systems by different authors[1–3]. The most studied forms of physiological cell death are apoptosis and autophagic cell death[4,5]. The typical features of these two forms of death and of the pathological form of death known as necrosis are summarized in Table I, part A. A dominant feature of apoptosis is intense chromatin condensation, whereas autophagic cell death is characterized by the presence of numerous autophagosomes. In the classification system developed by Schweichel and Merker[1] in 1973, dying hypertrophic chondrocytes were presented as an example of a third mode of cell death distinct from apoptosis and autophagic cell death; this paper continues to be cited in reviews of physiological cell death, referring to a mode of death ('non-lysosomal degradation') that remains uncharacterized[6]. Despite this, chondrocytes in growth cartilage are often described as dying by apoptosis, largely on the basis of detection of DNA strand breaks using the TUNEL method, or other molecular features known to be associated with apoptosis[7,8]. The definitive identification of apoptosis is, however, morphological[9]. In a series of recent papers including both presentation of primary data and critical reviews of the relevant literature, Roach and colleagues observed that ultrastructural studies of dying hypertrophic chondrocytes fail to identify cells with the typical features

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Table I
Summary of morphological characteristics of different forms of cell death

	Part A*			Part B†	
	Apoptosis	Autophagic cell death	Necrosis	Dark cell death	Light cell death
Nuclear chromatin	Condensed into sharply circumscribed round or crescent masses at margin of nuclear membrane	Condensed into single pyknotic mass in center of nucleus	Moderately condensed in irregular aggregates	Condensed into irregular patches throughout nucleus	Chromatin condensation inconsistent. Nuclear appearance varies from pale to uniformly condensed
Nuclear membrane	Progressively convoluted; initially well preserved, later discontinuous	Well preserved	Ruptured	Well preserved; often convoluted	Well preserved
Cytoplasm	Condensed; well preserved organelles compacted together	Contains numerous autophagocytic vacuoles	Cytoplasm and organelles grossly swollen and vacuolated	Cytoplasm condensed; often contains distended RER; gradually extruded into extracellular space	Cytoplasm and organelles progressively disintegrate
Cell membrane	Well preserved; often convoluted	Well preserved	Ruptured early	Extruded with cytoplasm	Appears well preserved
Final stage	Nucleus and cytoplasm fragmented into membrane-bound apoptotic bodies	Organelles degraded (amorphous cytoplasm); compacted nucleus	Cytoplasm and organelles disintegrated; chromatin degraded (nuclear ghost)	Cytoplasm and nucleus fragmented	Cytoplasm and nucleus fragmented
Timing of nuclear and cytoplasmic changes	Nuclear changes precede cytoplasmic alterations	Cytoplasmic changes precede nuclear alteration	Cytoplasmic changes precede nuclear alterations	Cytoplasmic changes precede nuclear alterations	Cytoplasmic changes precede nuclear alterations

*Reviewed in Refs. 4 and 35.
†New observations presented in the current manuscript.

of apoptosis: condensation of chromatin into geometric patterns, nuclear fragmentation and cell shrinkage[10–12]. Shapiro *et al.*[13] have contributed to the debate in a review putting forward the hypothesis that hypertrophic chondrocytes die by autophagic cell death.

Previous ultrastructural studies of growth cartilage have provided a variety of morphological descriptions of dying hypertrophic chondrocytes[1,10–12,14,15]. These studies have been conducted on samples obtained from different species, locations and developmental stages, and a variety of terminology has been used to describe the morphology of these cells as they die. Thus, although it is now clear that these cells do not undergo apoptosis *in vivo*, it is difficult to obtain from published literature a systematic spatiotemporal picture of the morphology of dying hypertrophic chondrocytes, and without this it is not possible to address the mechanistic question of how these cells die.

A fact which has received little attention in recent studies of growth cartilage, and which may complicate the understanding of how hypertrophic chondrocytes die, is the co-existence of two morphologically distinct types of chondrocytes ('light' and 'dark') in zones of proliferation and hypertrophy[14,16,17]. Light chondrocytes are usually oval or round cells with a few thin cytoplasmic processes extending into the surrounding matrix; the cytoplasm contains sparse endoplasmic reticulum and an inconspicuous Golgi region. Dark chondrocytes are of an irregular shape with numerous dense cytoplasmic processes and vesicles budding from the cell surface; the cytoplasm contains well developed endoplasmic reticulum and a prominent Golgi zone consisting of numerous vacuoles filled with fibrillar material. No information is available about molecular differences between light and dark chondrocytes. Distinct light and dark chondrocytes have not been described in mature cartilage.

The current study had three aims: to obtain a systematic overview of the morphology of dying hypertrophic chondrocytes by examining these cells in PGC and AEGC from different bones throughout the development and growth in a single large mammalian species; to develop a culture system in which the mechanism of physiological death of hypertrophic chondrocytes could be studied; and to investigate factors regulating physiological death of these cells.

Materials and methods

REAGENTS

DMEM, gentamicin, amphotericin B, L-glutamine and fetal calf serum (FCS) were obtained from Invitrogen. Recombinant human TGF-β1 was from R&D Systems, collagenase A from Roche and staurosporine from Alexis Biochemicals. Triiodothyronine (T3) and L-ascorbic acid were from Sigma–Aldrich.

SPECIMEN COLLECTION AND PROCESSING FOR LIGHT AND ELECTRON MICROSCOPY

Tissue specimens from fetal horses were obtained from a local abattoir; stage of gestation was estimated on the basis of crown:rump length[18]. Postnatal equine specimens were obtained from the abattoir or from animals that died from disease unrelated to the skeleton at the University of Melbourne Veterinary Clinic and Hospital. Fetal specimens were obtained from animals at the third, sixth, ninth and tenth month of gestation (referred to as F3, etc.), and postnatal specimens were obtained from animals of 6 months of age. Specimens were collected from the AEGC and PGC of the proximal end of the humerus and tibia and distal end of the femur. Cartilage with some adjacent bone was excised and fixed in 5% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate at 4°C. The specimens were decalcified in 0.33 M ethylene-diamine-tetra-acetic acid (EDTA). Specimens prepared by the standard method were postfixed in 1% osmium tetroxide/1.5% potassium-ferrocyanide[19] and embedded in Spurr's resin. For proteoglycan staining[20], after fixation and decalcification some specimens were stained *en bloc* with 0.1% safranin-O and postfixed in 1% osmium tetroxide/0.25% safranin-O before being embedded in Spurr's resin. Semi-thin sections for light microscopy were stained with methylene blue. Ultrathin sections of all specimens were stained with uranyl acetate and Reynold's stain and were examined under a transmission electron microscope (Philips 300).

CHONDROCYTE ISOLATION AND CULTURE

Metacarpophalangeal joints of prenatal foals (of various ages) were opened under aseptic conditions and the cartilage of the epiphyses excised. In some specimens the secondary center of ossification had not yet formed, and in others it was present. In each case the excised cartilage included the articular surface, and excluded any bone tissue and the zone of hypertrophy; it contained primarily resting, pre-proliferative chondrocytes. The specimens were dissected into small pieces, and chondrocytes were obtained by overnight digestion of the cartilage in 0.5% collagenase diluted in DMEM containing 1% FCS at 37°C, then cultured in pellets using a modification of the method of Kato *et al.*[21]. Briefly, 5×10^5 cells were placed into 15 ml polypropylene tubes in 1 ml aliquots of DMEM containing gentamicin (50 μg/ml), amphotericin B (2.5 μg/ml), L-glutamine (300 μg/ml), L-ascorbic acid (50 μg/ml) and 0.1% or 10% FCS. The cells were centrifuged to form pellets and then maintained at 37°C with the caps of the tubes closed. After 48 h, the cells formed disc-like pellets that could be manipulated with forceps. Pellets were harvested and fixed at 0, 7, 14, 21 and 28 days. In some experiments, TGF-β1 (10 ng/ml) or T3 (100 ng/ml) was included in the medium containing FCS (10% or 0.1%) from day 7 until day 28. In another experiment, pellets were cultured for 20 days in 0.1% FCS, then staurosporine (1 μM) was added to the medium of some pellets prior to harvest 24 h later. The pellets were fixed in cold 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate and processed for light and electron microscopy as described for tissue specimens.

Some cells were cultured in monolayer in 25 cm^2 flasks instead of pellet culture. These cells were grown to confluence in DMEM (containing10% FCS and all additives as for pellet culture except L-ascorbic acid), then treated with 0.1% FCS for 24 h. Cultures were then treated with or without staurosporine (1 μM) in DMEM containing 0.1% FCS for 24 h. The cells were trypsinised and centrifuged; the pellets were fixed and processed for light and electron microscopy as described for tissue specimens.

CELL COUNTS

Counting of light and dark chondrocytes and chondrocytes undergoing different forms of physiological death was undertaken using semi-thin sections of pellets stained with methylene blue. Digital images were captured and total cells counted manually. Following extensive comparison of light and electron micrographs of the same specimens, it was possible to identify light and dark cells, and cells dying with the typical features of dying light or dark cells *in vivo*, or undergoing apoptosis, on the basis of cell morphology in semi-thin sections (see criteria listed in Table I). These cell types were counted by eye and expressed as a percentage of total cells. Results are presented as mean ± S.E.M. ($n \geq 3$). Statistical differences between groups were evaluated using a one-way ANOVA with a Bonferroni *post-hoc* test; P values <0.05 were considered significant.

QUANTITATIVE POLYMERASE CHAIN REACTION

Isolated chondrocytes were cultured as pellets in 0.1% FCS until day 7, when they were treated with either T3 (100 ng/ml in 0.1% FCS) or 10% FCS. At day 21 or day 25, the pellets were collected and frozen in liquid nitrogen. Some pellets were processed for histology to confirm the presence of hypertrophic dark and light chondrocytes. For each treatment, three pools of at least 10 pellets were used for RNA extraction. RNA was extracted using TRI reagent (Sigma, St Louis, MO, USA) and further purified using the SV RNA Isolation Kit (Promega, Madison, WI, USA). Reverse transcription was carried out using MMLV Reverse Transcriptase RNase H-minus (Promega). Primers for quantitative polymerase chain reaction (qPCR) were designed as follows (numbers indicate exonic location of primer): Ribosomal protein S23 (RP-S23) 1-Fwd: CAGAGT GGCACGATAAGCAG, 2-Rev: CACACTTCCTGATGGCA GAA; Type II Collagen 20-Fwd: CAGACGGGTGAACCTGG TAT, 22-Rev: TCTCCACGAGCACCTCTTTT; Aggrecan 6-Fwd: CTACGACGCCATCTGCTACA, 7-Rev: ACCGTCTG GATGGTGATGTC; Runx2 4-Fwd: TCCCTGAACTCTGCA CCAAG, 5-Rev: GCCAGGTAGGAGGGGTAAGA; Sox9 1-Fwd: CCGAGCTCAGCAAGACTCTG, 2-Rev: CGCGGCTG GTACTTGTAATC; Connective tissue growth factor (CTGF) 4-Fwd: GCAGGCTGGAAAGCAGAGC, 5-Rev: ATGTCTTC ATGCTGGTGCAG (modified from published primers for human CTGF[22]); Vascular endothelial growth factor (VEGF) 1-Fwd: CTTGCCTTGCTGCTCTACCT, 3-Rev: GACGTCC AGAACTTCACCA; Fibroblast growth factor receptor-3 (FGFR3) 7-Fwd: TCTATCGGGTTTTCCCATCA, 8-Rev AC CACCAGGATGAAGAGGAG. Published primers for human matrix metalloproteinase-13 (MMP-13) were used[23]. RP-S23 was used as a housekeeping gene, as its expression has been shown to be consistent in both the proliferating and hypertrophic zones of the growth plate[24]. Automated sequencing was carried out on all PCR products to confirm their identity (AGRF, Melbourne, Australia). Real-time qPCR was performed using a Stratagene MX3000, using Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Groningen, The Netherlands). Results for qPCR were analyzed using the Pair-wise Fixed Reallocation Randomization Test from the Relative Expression Software Tool − 384, version 1[25]. The results for each gene of interest normalized to results for RP-S23 expression are presented as

level of expression in 10% FCS-treated pellets relative to levels in T3-treated pellets, or *vice versa*, depending on the treatment in which specific genes were more highly expressed (mean ± S.E.M.; $n = 3$).

Results

DEATH OF HYPERTROPHIC CHONDROCYTES *IN VIVO*

Ultrastructural studies were conducted on specimens of growth cartilage (PGC and AEGC, where relevant) from the humerus of fetal and postnatal horses ranging in age from F3 to 6 months. In all specimens of growth cartilage examined, light and dark chondrocytes (as described by Hwang[16]), could be identified from the zone of cells that had just undergone proliferation to the end of the zone of hypertrophic chondrocytes (Fig. 1). Light and dark chondrocytes could not be distinguished in the resting zone (not shown). The dark chondrocytes were characterized by electron dense cytoplasm containing abundant, often dilated, rough endoplasmic reticulum [RER; Fig. 2(A–D)]. Light chondrocytes, in contrast, were characterized by electronlucent cytoplasm that contained less developed RER [Fig. 2(E–H)].

Both types of chondrocytes could be identified, each undergoing a distinctive series of morphological changes, from the zone of proliferation, through the zone of hypertrophy to the bone–cartilage interface (Fig. 1). The dark chondrocytes gradually underwent condensation, but contained RER and secretory vacuoles until the last few lacunae before the bone–cartilage interface [Fig. 2(A–D)]. The secretory material contained abundant proteoglycan, as demonstrated by safranin-O staining [Fig. 2(D)]. For these cells, the process of dying involved progressive extrusion of their cytoplasm with secretory contents into the extracellular space. The nucleus underwent patchy chromatin condensation, without forming the intense geometric accumulations of chromatin typical of apoptosis [Fig. 2(B, C)]. In contrast, light chondrocytes appeared to disintegrate initially within the cellular membrane [Fig. 2(E–H)]. Their nuclei were pale and apparently functional, and the cytoplasm contained RER and proteoglycan granules until the cytoplasm had almost completely disintegrated [Fig. 2(E–H)]. Within the last row of lacunae before the bone–cartilage interface, most lacunae only contained cell remnants [e.g., Figs. 1(A, E) and 2(C, G)]. The morphological features observed for dying hypertrophic light and dark chondrocytes are summarized in Table I, part B.

The chondrocytes presented here are all from the proximal humeral PGC of fetal specimens. Although the depth of growth cartilage varied with age and anatomical location, the description of morphological changes of light and dark chondrocytes during hypertrophy and death presented above is generally relevant to all specimens examined.

A METHOD FOR INVESTIGATION OF DEATH OF HYPERTROPHIC CHONDROCYTES *IN VITRO*

Chondrocytes isolated from fetal equine growth cartilage were cultured for up to 28 days in pellet culture under a variety of culture conditions, and examined by light and electron microscopy. At day 0, pelleted cells appeared rounded and closely packed, with no evidence of extracellular matrix. By about 7 days, the cells had formed a semi-transparent cartilage-like tissue, which continued to expand with time in culture. Sections of pellets showed chondrocytes within lacunae surrounded by a metachromatic extracellular matrix. Most cells had the appearance of chondrocytes in the resting zone of growth cartilage, and could not be identified as light or dark cells. Dark and light chondrocytes could be distinguished by day 14, by which time some cells appeared morphologically hypertrophic. At the later stages of culture, cells showing the typical features of dying dark and light chondrocytes, as seen *in vivo*, were present [Fig. 3(A, D, E)]. In some cases a light and a dark chondrocyte were observed in the same lacuna [Fig. 3(C)]. Some cells undergoing apoptosis were also seen, usually toward the edges of the pellets, where the cells were flattened like cells in perichondrium; in some cases they were being phagocytosed by adjacent cells [Fig. 3(B)].

REGULATION OF CHONDROCYTE DIFFERENTIATION AND DEATH IN PELLET CULTURE

Chondrocyte pellet cultures were exposed to a variety of conditions to determine whether the proportion of light and dark cells could be regulated, and to investigate the regulation of different forms of death. In pellets cultured in 10% FCS, the majority (70–80%) of cells was dark chondrocytes and very few light chondrocytes were detected [Fig. 4(A)]. In pellets cultured in 0.1% FCS, there were more light than dark chondrocytes identifiable, but a high proportion of chondrocytes remained as resting cells, not distinguishable as either light or dark [Fig. 4(A)]. An analysis of cell death demonstrated that in pellets cultured in 10% FCS, at 21 days 30% of cells were dark cells undergoing the form of death seen for these cells *in vivo*, no dying light cells were detectable, and about 10% of cells were undergoing apoptosis; by 28 days, some dying light cells (similar to those seen *in vivo*) were detectable, but the vast majority of dying cells was dark chondrocytes [Fig. 4(B)]. In pellets cultured in 0.1% FCS, very few dying cells of any type (dying light cells, dying dark cells or apoptotic cells) were observed [Fig. 4(B)] at any time point.

In pellets cultured in the presence of T3 in 0.1% FCS, there were more light cells than dark cells. More light and dark cells were seen at 21 days and 28 days than in pellets cultured in 0.1% FCS alone [Fig. 4(C)], but at 28 days the ratio of light:dark cells was similar for the two treatment conditions (1.48 for T3-treated pellets and 1.69 for 0.1% FCS-treated pellets). Moreover, treatment with T3 led to the presence of increased percentages of dying light and dark cells by day 28 [Fig. 4(D)]. In pellets treated with TGF-β1 in the presence of 0.1% FCS, most cells were identifiable as dark cells by day 21 [Fig. 4(E)], and as dying dark cells by day 28 [Fig. 4(F)].

In order to investigate molecular differences between light and dark hypertrophic chondrocytes, expression of a number of genes involved in regulation of chondrocyte behavior was compared in pellets enriched in one or the other type of cell, using qPCR. Pellets cultured in 10% FCS until day 21 (which contained approximately 72% dark chondrocytes and 8% light chondrocytes in an early stage of hypertrophy; 'dark cell-enriched cultures') were compared with pellets cultured in the presence of T3 (which contained approximately 32% dark chondrocytes and 49% light chondrocytes in an early stage of hypertrophy; 'light cell-enriched cultures'). MMP-13, VEGF, CTGF and Sox9 mRNA were significantly more highly expressed in dark cell-enriched than in light cell-enriched cultures [Fig. 5(A)]. The highest level of relative expression was shown by MMP-13, which was 14-fold more highly expressed in dark cell-enriched than light cell-enriched cultures. Type II collagen was 7-fold more highly expressed in light cell-enriched cultures than in dark

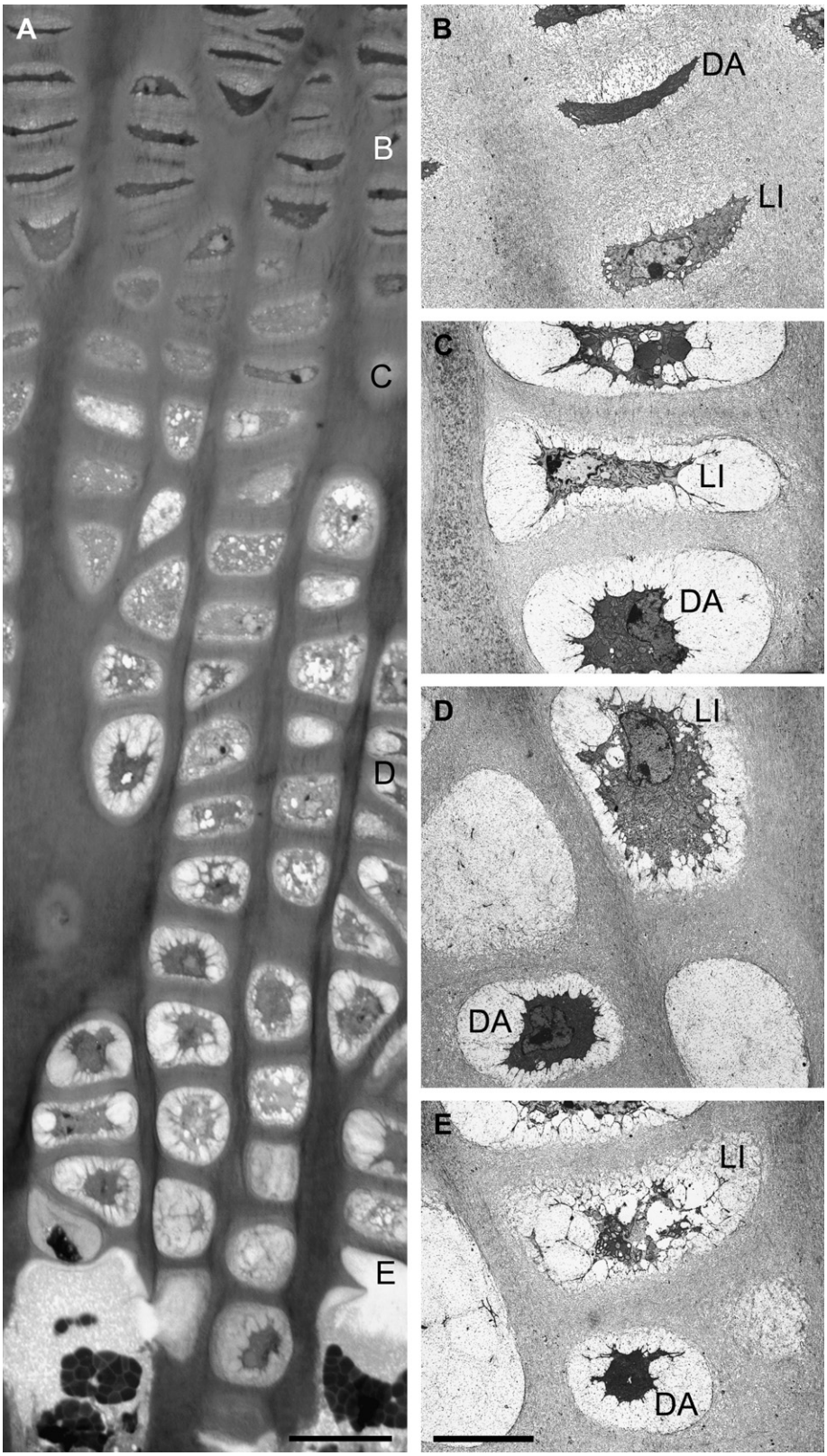

Fig. 1. Morphology of chondrocytes in growth cartilage from the proximal physis of the humerus of a 10-month equine fetus. A: Light micrograph; labels B–E indicate the regions corresponding to micrographs shown in parts B–E. B–E: Electron micrographs showing light (LI) and dark (DA) chondrocytes in the zone of proliferation (B), the beginning of the zone of hypertrophy (C), the middle of the zone of hypertrophy (D), and the last few lacunae before the ossification front (E). Bar in A = 25 μm. The magnification for parts B–E is the same; bar = 10 μm.

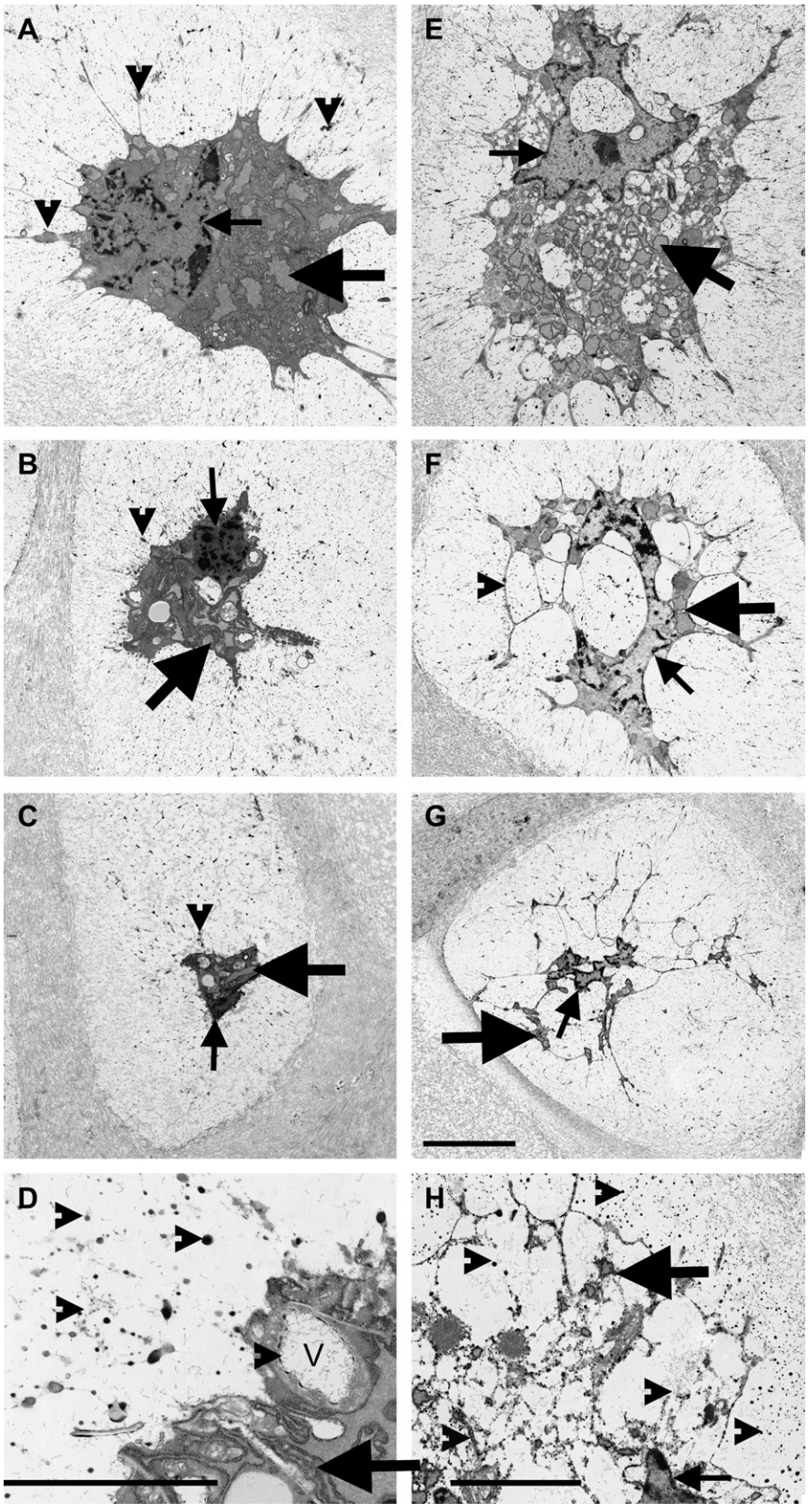

Fig. 2. Morphology of dying hypertrophic chondrocytes. Electron micrographs of the proximal physis of the humerus of a 3-month (A–C, E–G) or an 8-month (D, H) equine fetus. Sections in A–C and E–G were stained by the standard method, and sections in D, H were from tissues stained *en bloc* with safranin-O. A–D: Hypertrophic dark chondrocytes in the early (A), middle (B) and late (C, D) stages of degeneration. The nucleus (small arrows) shows progressive patchy chromatin condensation. Cytoplasm is progressively extruded into the extracellular space

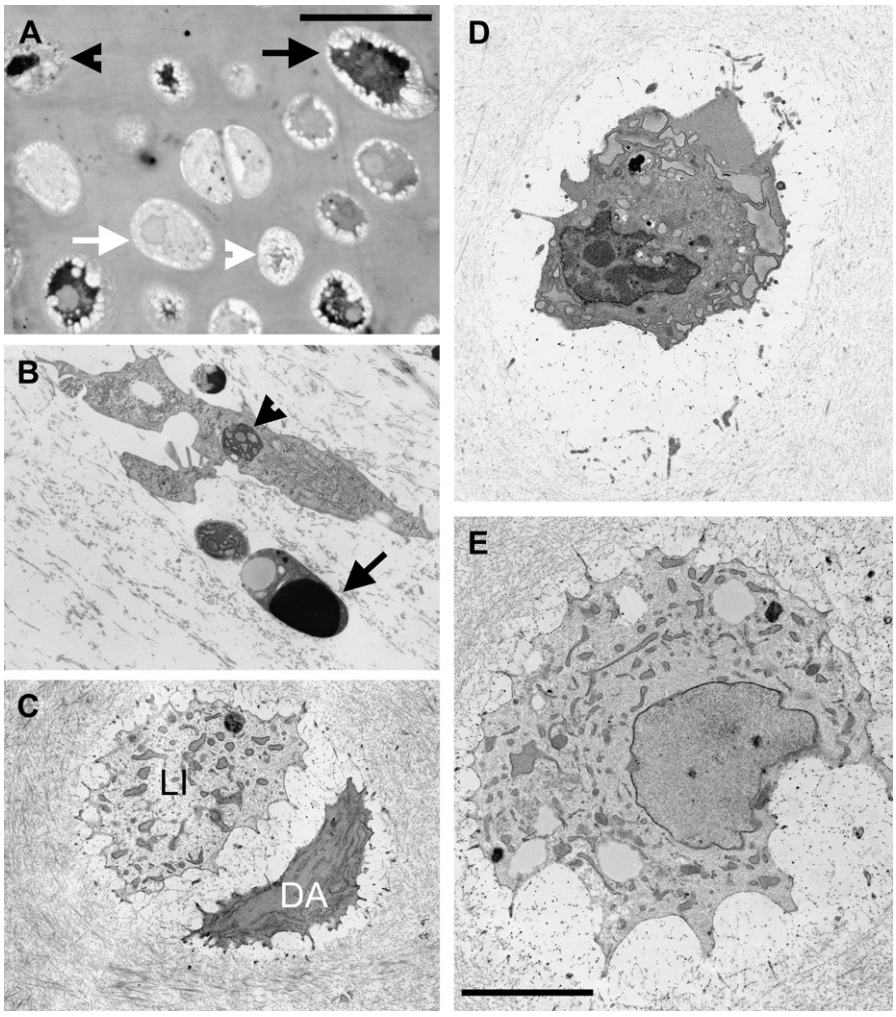

Fig. 3. Chondrocyte hypertrophy and death in pellet culture. Light micrograph (A) and electron micrographs (B–E) of chondrocytes cultured as pellets for 28 days in the presence of T3 in 0.1% FCS. A: Early (white arrow) and dying (white arrowhead) hypertrophic light chondrocytes, and early (black arrow) and dying (black arrowhead) hypertrophic dark chondrocytes can be seen. B: An apoptotic cell from the periphery of the pellet contains intensely condensed chromatin typical of apoptosis (arrow), and an adjacent cell has phagocytosed remnants of an apoptotic cell (arrowhead). C: Recently proliferated light (LI) and dark (DA) chondrocytes within the same lacuna. D: A dying hypertrophic dark chondrocyte. E: A dying hypertrophic light chondrocyte. Bar in A = 25 μm. The magnification in parts B–E is the same; bar = 2 μm.

cell-enriched cultures [Fig. 5(A)]. Pellets cultured in 10% FCS until day 25 (which contained approximately 67% dying dark chondrocytes and 2% dying light chondrocytes; 'dying dark cell-enriched cultures') were compared with pellets cultured in the presence of T3 for 25 days (which contained approximately 20% dying dark chondrocytes and 53% dying light chondrocytes; 'dying light cell-enriched cultures'). In these cultures, the difference in CTGF (15-fold), VEGF (8-fold) and MMP-13 (25-fold) expression between dying dark cell-enriched and dying light cell-enriched cultures was greater than for the early hypertrophic cultures, but Sox9 was no longer differentially expressed [Fig. 5(B)]. Type II collagen and Runx2 were more highly expressed in dying light cell-enriched cultures than in dying dark cell-enriched cultures [Fig. 5(B)]. Aggrecan and FGFR3 were not differentially expressed at either time point.

INDUCTION OF DEATH BY STAUROSPORINE IN CHONDROCYTES IN MONOLAYER AND PELLET CULTURE

Chondrocytes grown in monolayer culture were exposed to staurosporine for 24 h, and then examined by electron microscopy. Staurosporine was found to induce apoptosis in 43% of cells [Fig. 6(A–C)]. In contrast, in pellet culture, staurosporine did not induce apoptosis, but rather death of dark cells morphologically indistinguishable from that seen *in vivo* [Fig. 6(D–F)].

(arrowheads in A–D) until there is almost none left (C). RER (large arrows) is prominent at all stages, and a higher magnification (D) shows the presence of vacuoles (V) containing proteoglycans. E–H: Hypertrophic light chondrocytes in the early (E), middle (F) and late (G, H) stages of degeneration. The nucleus (small arrows) remains intact and apparently functional while the cytoplasm disintegrates within the cell membrane (arrowhead in F). RER (large arrows) is visible at all stages, and safranin-O-positive proteoglycan particles (arrowheads) are seen within the cell and in the surrounding matrix. The magnification in parts A–C and E–G is the same; all bars = 2 μm.

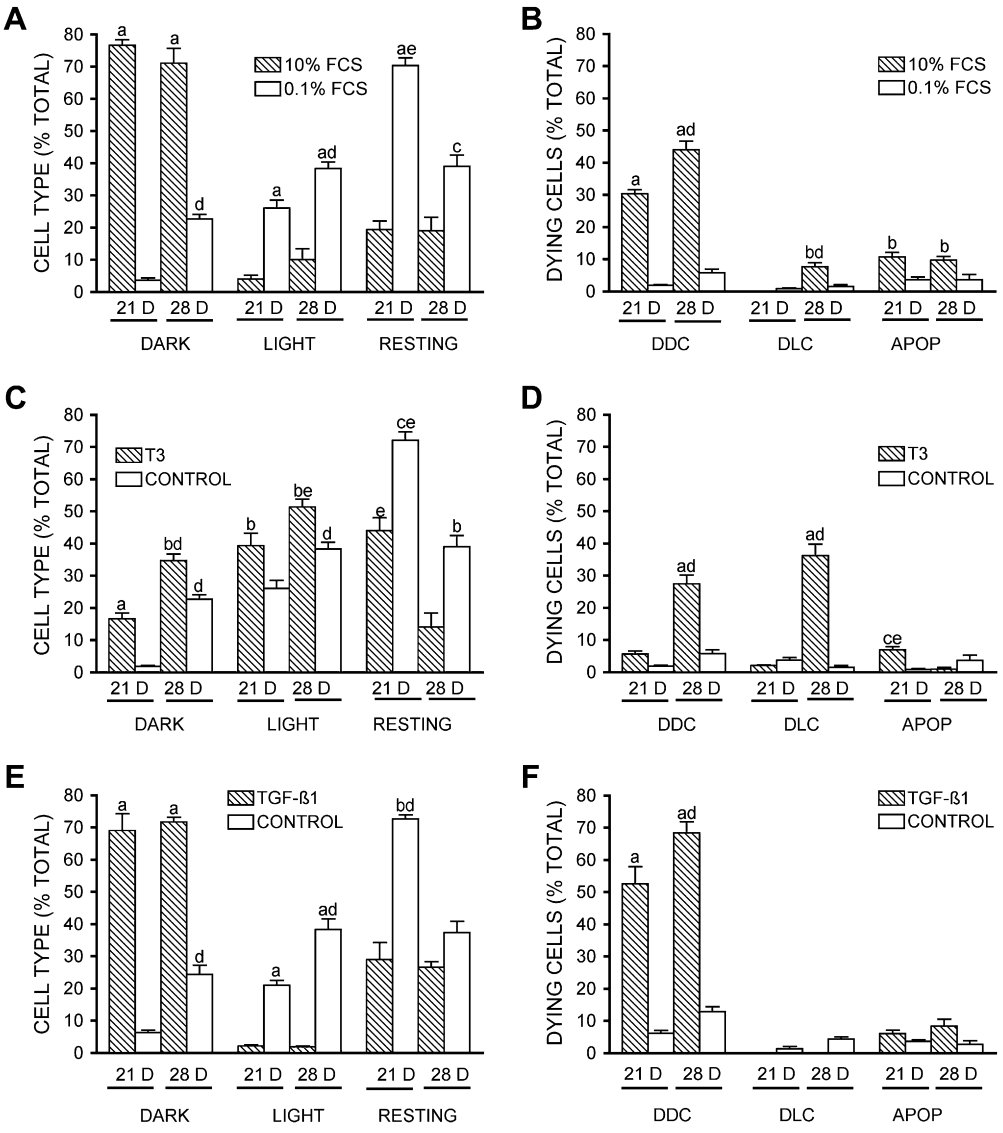

Fig. 4. Regulation of chondrocyte differentiation and death in pellet culture. Pellets were cultured in the presence of 10% or 0.1% FCS (A, B), T3 or 0.1% FCS (control; C, D), or TGF-β1 or 0.1% FCS (control; E, F). A, C, E: after 21 (21 D) or 28 days (28 D), dark, light and resting chondrocytes were counted and results expressed as a percentage of total cells. B, D, F: at the same time points, dying dark chondrocytes (DDC), dying light chondrocytes (DLC) or apoptotic chondrocytes (APOP) were counted and results expressed as a percentage of total cells. Data represent mean ± S.E.M. Significant differences between values are expressed as follows: a — $P < 0.001$, b — $P < 0.01$, c — $P < 0.05$ for comparison between treatment and control; d — $P < 0.001$, e — $P < 0.01$ for comparison between 21 D and 28 D.

Discussion

The first aim of the current study was to obtain a systematic overview of the morphology of dying hypertrophic chondrocytes across the spatiotemporal range of situations in which they are found. This aim was achieved through the demonstration that the two types of chondrocytes present in growth cartilage undergo distinctive, non-apoptotic forms of physiological death, as documented in Table I, part B. The fact that chondrocytes in growth cartilage do not undergo apoptosis has been clearly argued by Roach and Clarke[11] and does not need to be discussed further here. The idea that light and dark chondrocytes represent different cell types from the time of proliferation, each with its own distinctive form of death, has however not previously been proposed. Early ultrastructural studies that recognized the existence of light and dark chondrocytes did not specifically address the question of how these cells died[14,16]. Wilsman *et al.*[14] provided an elegant description of 'disintegrating' light and dark cells, but suggested that these may be different states of differentiation of one cell type. Roach and Clarke[11] provided a thorough characterization of the process of death undergone by dark chondrocytes corresponding to that observed in the current study, but paid no attention to the process of death being undergone in parallel by adjacent light chondrocytes (described by these authors as 'hydrated' cells).

As demonstrated here in Fig. 1, both light and dark cells were observed from the zone of proliferation to the last lacunae before the ossification front, each undergoing its own series of progressive changes during hypertrophy and

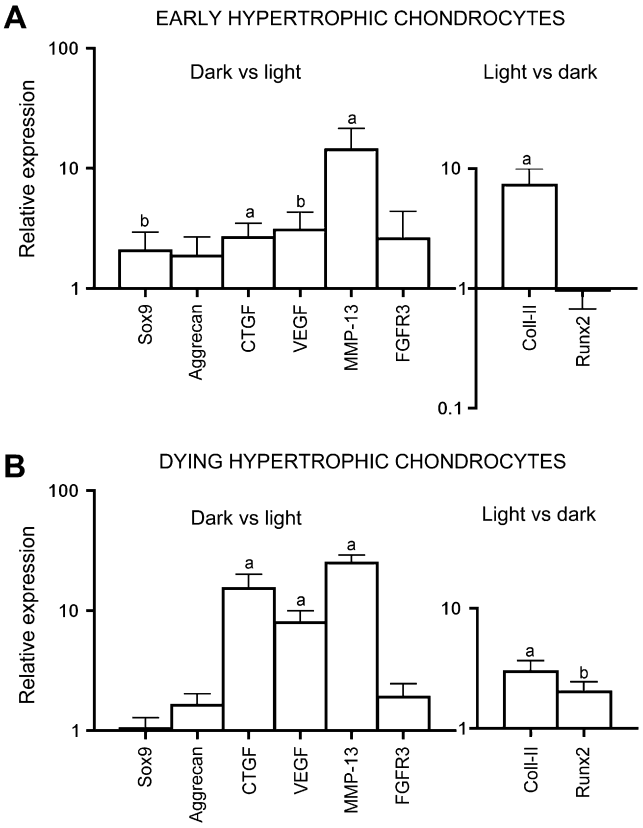

Fig. 5. Quantitative PCR analysis of mRNA expression in early (A) and dying (B) hypertrophic chondrocyte cultures. Cultures were enriched for dark or light cells by treatment with 10% FCS or T3, respectively, until day 21 (A) or day 25 (B). Results for Sox9, aggrecan, CTGF, VEGF, MMP-13 and FGFR3 expression in dark cell-enriched cultures are expressed relative to expression in light cell-enriched cultures. Results for type II collagen (Coll-II) and Runx2 expression in light cell-enriched cultures are expressed relative to expression in dark cell-enriched cultures. Data represent mean $\pm$ S.E.M.; $n = 3$. Significant differences between values for light and dark cells are expressed as follows: a $-$ $P < 0.001$; b $-$ $P < 0.01$.

death. The modes of death undergone by the two cell types were mutually exclusive, ruling out the possibility that they represent stages of a single continuum: light chondrocytes disintegrated by dismantling their cytoplasmic contents inside an intact plasma membrane, whereas dark chondrocytes disintegrated by breaking off packets of cytoplasmic contents and plasma membrane.

In 2004, Roach *et al.*[12] introduced the term 'chondroptosis' to the literature, describing the form of death that we document here as typical of dark chondrocytes. We do not consider this to be an appropriate name, partly because of the implication that it is a form of apoptosis, and partly because as demonstrated here it represents only one of the ways in which chondrocytes die. The mechanisms by which light and dark hypertrophic chondrocytes die remain to be determined. In their recent review, Shapiro *et al.*[13] suggest that hypertrophic chondrocytes die by autophagic cell death, but we see no evidence in dying light or dark chondrocytes for the increased number of autophagosomes or the nuclear changes typical of this form of cell death. The disintegration of the cytoplasmic contents of light cells is suggestive of non-lysosomal degradation[1,2], but further studies will be required to confirm such a conclusion. It is interesting to note that the example of this form of death shown by Schweichel and Merker[1] in their 1973 paper is a typical light chondrocyte similar to those presented here. Dark cells, in contrast, disintegrate by extrusion of their cytoplasmic contents (including secretory material) together with plasma membrane, until there is nothing but nucleus left; this process appears to be morphologically unique amongst modes of physiological cell death. Both light and dark cells appeared to maintain a high level of protein synthesis and secretion almost until they had completely disintegrated, as judged by the presence of RER and proteoglycan particles. In addition to proteoglycan, the secretory material presumably also included proteolytic enzymes such as MMP-13, which is expressed by hypertrophic chondrocytes and is essential for normal degradation of the cartilage matrix at the ossification front[26,27]. These modes of death seem to be particularly well suited to cells that need to remodel their surrounding matrix so as to assist in the invasion of osteogenic cells (including blood vessels, osteoclasts and osteoblasts), at the same time as they are killing themselves.

Cells undergoing apoptosis are normally phagocytosed by neighboring cells. The process of cellular condensation and formation of apoptotic bodies followed by phagocytosis is considered to be a mechanism for prevention of the inflammation that would ensue if cytoplasmic contents were released from dying cells. It seems that dark cells actually do release their cytoplasmic contents as they die, which as argued above is likely to assist in the necessary remodeling of the lacuna to allow invasion by osteogenic cells, but may also act as a chemotactic stimulus for such cells.

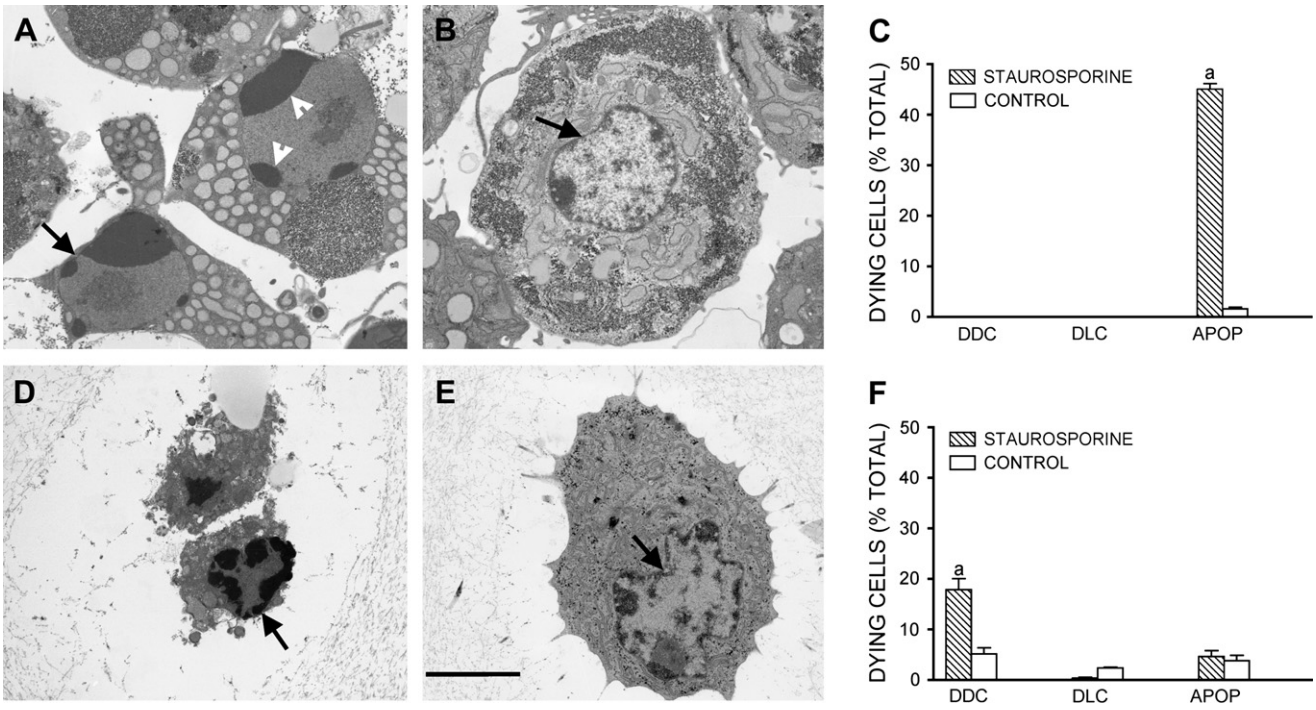

Fig. 6. Effect of staurosporine on chondrocytes isolated from growth cartilage. Chondrocytes were cultured in monolayer (A–C) or pellet (D–F) culture. Electron micrographs of cultures treated for 24 h in the presence (A, D) or absence (B, E) of staurosporine. Arrows indicate the nuclear membrane, and arrowheads in A indicate aggregations of condensed chromatin typical of apoptosis. All micrographs have the same magnification; bar = 2 μm. C, F: Dying dark chondrocytes (DDC), dying light chondrocytes (DLC) and apoptotic chondrocytes (APOP) were counted and results expressed as a percentage of total cells. Data represent mean ± S.E.M.; a – significant difference between treatment and control ($P < 0.001$).

Indeed, the invasion of these cells could perhaps be viewed as a controlled inflammatory response. Phagocytosis is clearly not possible for individual chondrocytes enclosed in lacunae. There is evidence for phagocytosis by osteoclasts of chondrocyte remnants in open lacunae at the ossification front[28]; our observations do not contradict this idea, but suggest that the chondrocytes have largely disintegrated by the time such phagocytosis occurs. Moreover, Farnum and Wilsman[29] have shown using serial sections that a small proportion of terminal lacunae in porcine growth cartilage are empty, indicating that some hypertrophic chondrocytes are able to digest themselves completely without the need for phagocytosis. We occasionally saw phagocytosed remnants of apoptotic cells in chondrocyte pellet cultures, but the phagocytic cells were elongated cells at the periphery of the pellets, where cells were not separated by large amounts of matrix, thus allowing contact between cells that does not occur for more differentiated chondrocytes.

The second aim of the current study was to develop a culture system in which the mechanism of physiological death of hypertrophic chondrocytes could be studied. The pellet culture system initially developed by Kato *et al.*[21] was selected for this purpose, since it allows chondrocytes to undergo hypertrophy. In the current study, it has been demonstrated that hypertrophic chondrocytes in pellet culture undergo the same forms of death as seen in growth cartilage *in vivo*. Furthermore, it has been demonstrated here that both the ratio of light:dark chondrocytes and the proportion of dying cells can be manipulated by altering the culture conditions. The effects of serum concentration and factors previously shown to modulate chondrocyte hypertrophy were investigated. TGF-β1 has been described as exerting a variety of effects on chondrocytes, depending on their state of differentiation. In monolayer culture where chondrocytes undergo de-differentiation, TGF-β1 has been reported to induce cell proliferation[30]. However, in pellet culture where chondrocytes maintain their differentiated state, TGF-β1 has been reported to prevent hypertrophy[31]. T3 has been shown to stimulate chondrocyte hypertrophy in organ culture[32]. In the presence of a low serum concentration (0.1%), more cells were identifiable as light chondrocytes than dark chondrocytes, and a similar ratio was seen with T3. In contrast, TGF-β1 or 10% FCS induced most cells to become dark chondrocytes. The low serum concentration was found to maintain survival up to 28 days in culture, whereas TGF-β1, T3 or 10% FCS accelerated chondrocyte death. For each of these inducers of chondrocyte death, the forms of death observed reflected the ratio of light:dark chondrocytes present in the cultures. Under all of these culture conditions, low proportions of cells (less than 10%) underwent apoptosis.

Culture conditions found to induce either light or dark chondrocyte differentiation were then used to compare gene expression patterns in the two cell types, both early and late in hypertrophy. Treatment with 10% FCS was used to obtain an almost pure dark cell population, whereas T3 treatment was used to obtain a light cell-enriched population. The results of the qPCR analyses indicate that dark cells express substantially higher levels of CTGF, VEGF and MMP-13 than do light cells, and that the difference increases as the cells begin to die. All of these gene products are known to be expressed by hypertrophic chondrocytes, and to play a variety of roles in endochondral

ossification. CTGF stimulates chondrocyte hypertrophy[33], VEGF stimulates vascularization of the cartilage matrix[34], and MMP-13 is required for the degradation of collagen within cartilage matrix[26,27]. In contrast, it appears that light cells express significantly higher levels of type II collagen than do dark cells, both during early hypertrophy and death. The cartilage-specific proteoglycan, aggrecan, appears to be expressed at similar levels by both cell types. This is the first documentation of differences in gene expression between light and dark hypertrophic chondrocytes.

Staurosporine induces apoptosis in a variety of cell types. The experiment with staurosporine presented here was conducted with the aim of inducing apoptosis in chondrocyte pellet culture; the intention was to establish culture conditions in which the molecular mechanism of the modes of death undergone by hypertrophic chondrocytes *in vivo* could be compared with that of apoptosis. Surprisingly, staurosporine did not induce apoptosis in chondrocytes in pellet culture, but rather induced the death of dark chondrocytes by the mode of death typically seen in dark chondrocytes *in vivo*. Staurosporine did, however, induce apoptosis in chondrocytes in monolayer culture, indicating that the mode of death undergone by these cells is determined by their three-dimensional environment and/or state of differentiation, rather than by the nature of the death stimulus. The fact that staurosporine accelerated the death of dark but not light cells in pellet culture may suggest that dark cell death shares a pathway with apoptosis that is not utilized during light cell death.

In conclusion, the results presented here demonstrate that light and dark hypertrophic chondrocytes undergo morphologically distinct non-apoptotic modes of death *in vivo*. These modes of death can be replicated in chondrocyte pellet cultures, and can be manipulated by varying serum concentration or treatment with TGF-β1, T3 or staurosporine. This culture system will be useful for the further study of factors regulating death of hypertrophic chondrocytes, as well as the molecular mechanisms by which light and dark chondrocytes die.

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