

## **Bcl-2, tissue transglutaminase and p53 protein expression in the apoptotic cascade in ribs of premature infants**

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### ABSTRACT

Apoptotic cells of the human growth plate have not previously been demonstrated *in situ*. We have investigated the distribution of apoptotic cells in costosternal growth plates and bone of premature infants aged 4–11 d with a gestational age of ~ 26 wk. In addition, we investigated the immunolocalisation of apoptosis-related proteins within the growth plates and associated bone. A proportion of late hypertrophic chondrocytes and osteocytes within newly formed primary spongiosa showed evidence of highly fragmented DNA. The incidence of osteocyte apoptosis decreased as the distance from the chondroosseous junction increased. Tissue transglutaminase (tTG) expression was associated with apoptosis of osteocytes and hypertrophic chondrocytes. In contrast the presence of tTG was demonstrated in osteoblasts and bone lining cells but it did not colocalise with evidence of apoptosis. The anti-apoptotic gene product Bcl-2 was absent from the growth plate but was present in osteocytes. Visual assessment indicated a greater occurrence of the protein in cells occupying regions of low apoptosis. P53 was not demonstrated in the growth plate or bone. These findings would indicate that human growth plate chondrocytes appear to show little provision for ensuring cell longevity. In contrast osteocyte apoptosis appears negatively correlated with the skeletal distribution of Bcl-2 protein in the human infant, implying a potential selective vulnerability in individual cells. Lack of Bcl-2 and the high incidence of osteocyte apoptosis in the more rapidly remodelling bone of the human infant suggest a potential role of osteocyte apoptosis in the remodelling process.

*Key words:* Bone; growth plate; apoptosis; DNA fragmentation; tissue transglutaminase; Bcl-2; p53 protein.

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### INTRODUCTION

The purpose of the present paper is to provide a description of the morphological and biochemical changes affecting chondrocytes and cells of the osteocyte/osteoblast lineage in the vicinity of the human epiphyseal growth plate and to correlate these changes with the expression of certain proteins believed to play key regulatory roles in the control of apoptosis. Whilst some data exist regarding these changes in animal models of bone growth, to date we have no equivalent data obtained for growing human bone.

Apoptosis may play an important role in growth plate function and longitudinal bone growth. Endochondral ossification is characterised by the ordered progression of chondrocytes from the resting through the proliferating to the hypertrophic state and

terminal differentiation. In the vicinity of hypertrophic chondrocytes, the cartilage is invaded by capillary loops and endochondral ossification is initiated (Shapiro, 1992). What prompts this vascular invasion is unclear. From evidence gained in animal models it has been suggested that chondrocyte apoptosis may either initiate tissue resorption and vascularisation in the chick (Gibson *et al.* 1995) or conversely be the product of these two processes (Ganey *et al.* 1995).

Evidence of chondrocyte death was first described in 1872 (Clarke & Clarke, 1996) and much later it was found that apoptosis was occurring in hypertrophic chondrocytes in the rat paw (Aeschlimann *et al.* 1993), and porcine and rat (Noble *et al.* 1995), neonatal rodent (Bronckers *et al.* 1996) and chick (Roach *et al.* 1995) long bones. Studies by our group on rat epiphyseal bone showed a decrease in osteocyte apoptosis with increasing distance from the growth

plate and the newly-formed endochondral bone (Tomkinson et al. 1998).

Of the key regulatory proteins believed to be important in apoptosis, tissue transglutaminase (tTG) is perhaps one of the most intriguing. It is associated with cells undergoing apoptosis and considered to be a key regulator in skeletal tissues, associated with chondrocyte apoptosis, calcification and crosslinking of the matrix (Aeschlimann et al. 1993). In addition recent findings point to a signal transducing role (Melino & Piacentini, 1998). Furthermore, a transforming growth factor beta1/bone morphogenetic protein 4 (TGF $\beta$ 1/BMP4) response element has been located within the mouse tTG promoter (Ritter & Davies, 1998) which could act within bone forming osteoblasts. Osteocalcin (a known bone growth inhibitor) has been identified as the first known protein inhibitor of tTG-mediated crosslinking of the bone matrix protein osteopontin (Kaartinen et al. 1997). It is therefore important to investigate where tTG protein is being expressed within the human growth plate to help understand its role in regulating cell function.

Bcl-2 is known to be important in bone. Bcl-2 mRNA is upregulated in Paget's disease of bone (Mee et al. 1997) and enforced expression of Bcl-2 in monocytes partially reverses osteopetrosis in op/op mice (Lagasse & Weissman, 1997) which suffer from premature death of osteoclasts. The ratio of Bcl-2 to Bax (i.e. the balance of Bcl-2/Bcl-2 homodimers to Bcl-2/Bax heterodimers) determines in part the susceptibility of many cells to undergo apoptosis. In the mouse, parathyroid hormone-related peptide (PTHrP) appears to inhibit the apoptosis of growth plate chondrocytes specifically by stimulating their production of Bcl-2 protein (Amling et al. 1997). It has been suggested that, in this species, PTHrP thereby performs an important regulatory role in limb development in association with indian hedgehog protein (Ihh) and other gene products (Vortkamp et al. 1996). These important aspects of growth plate regulation might be relevant to clinical disorders of growth plate function.

P53 is a proto-oncogene with an important role in the apoptotic pathway induced by DNA damage. P53 knockout mice have shorter and thinner bones than the wild type and reveal an absence of apoptotic chondrocytes during development. However, apoptotic cells are present in the perichondrium (Ohyama et al. 1997). These mice do not develop a calvarium, suggesting p53 is also critical in fetal developmental patterning. The distribution of this proto-oncogene is also investigated here.

Earlier we reported the association of higher proportions of osteocytes undergoing apoptosis in bone with increased rates of turnover, as found in growing bone and in certain types of pathological bone (Noble et al. 1997). We put forward the hypothesis that the apoptotic process in osteocytes acts as a homing signal for the targeted removal of bone. Here we report the distribution of apoptotic cells in the costal growth plate and associated bone in relation to tTG, Bcl-2 and p53 protein. In situ studies of this kind should help identify certain of the control mechanisms regulating the growth plate and enable us to understand further the complex pattern of bone development set by the terminal differentiation of chondrocytes.

## MATERIALS AND METHODS

### *Source of human bone samples*

Five samples of ribs were obtained from postmortem examinations of infants born prematurely. One was excluded from the study on the basis of having abnormal regions in the growth plate and another was excluded due to its unavailability for processing within 24 h of the postmortem. Details of the samples ( $n = 3$ ) used in this study are outlined in Table 1. Samples were coated in 5% polyvinyl alcohol (PVA, Sigma, UK) and snap frozen in a hexane chilling bath before being stored at  $-70^{\circ}\text{C}$  before sectioning in a cryostat.

### *Immunohistochemical staining for bcl -2, tTG and p53*

Frozen longitudinal costal tissue sections (10  $\mu\text{m}$ ) (see Fig. 1) were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, washed thoroughly with PBS and air-dried before demineralisation in 0.25 M ethylenediaminetetraacetic acid (EDTA) in 50 mM Tris-HCl pH 7.4 for 10 min. The sections were then treated with 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in PBS for 15 min to block any endogenous peroxidase activity before blocking any nonspecific binding with horse serum for 20 min (1:40, Vector Labs, UK). Sections were incubated with mouse monoclonal antibodies raised to human Bcl-2 (0.2  $\mu\text{g}/\text{ml}$ , Santa Cruz, USA), or human p53 amino terminal (0.5  $\mu\text{g}/\text{ml}$ , Santa Cruz) or human tTG (1:75 dilution of supernatant, Neomarkers, USA) for 1 h at room temperature (RT) in a humidified chamber. Controls were incubated with serum only or a noncross-reactive antibody. Subsequently, the sections were incubated with a biotinylated horse

Table. Infant mortality information for the postmortem material ( $n = 3$ ) used in this study

Case number	Sex	Gestational age at birth (wk)	Development in utero	Age at death (d)	Cause of death	Comorbidities
1	M	25	N	11	Respiratory distress syndrome	Anaemia
2	F	28	N	4	Respiratory distress syndrome, suppurative meningitis	Septicaemia
3	M	26	N	5	Massive pulmonary haemorrhage, respiratory distress syndrome	

N = normal.

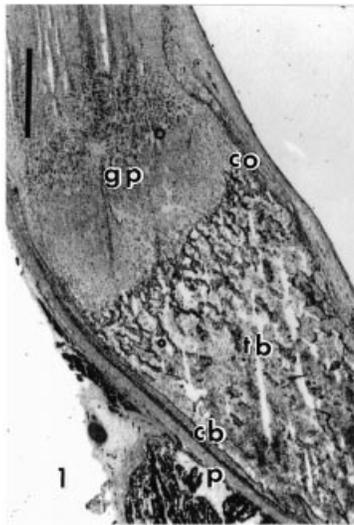


Fig. 1. Regions of rib examined for evidence of apoptosis and related gene expression: gp, growth plate; co, chondro-osseous junction; tb, trabecular bone; cb, cortical bone; p, periosteum;  $\times 50$ , bar 240  $\mu\text{m}$ .

antimouse antibody (Vector Labs, UK), amplified with ABC and developed with diaminobenzidine (DAB). Sections were rinsed in PBS before mounting in CITIFLUOR (Agar Scientific). Immunostaining for tTG was also performed using a fluorescein isothiocyanate (FITC)-labelled secondary goat antimouse antibody (1:50 Sigma). Unmasking of certain growth plate proteins was attempted by treatment with chondroitinase ABC for 1 h at 40 mU/ml in Tris buffered saline (TBS), pH 7.4. Protein localisation was determined by use of light microscopy to identify dark brown staining positive cells and in the case of tTG also by fluorescence microscopy to identify FITC-green positive cells against a population of propidium iodide (PI) (Sigma) counterstained cells.

All monoclonal antibodies were commercially available and therefore tested for specificity in human tissues by their respective suppliers. In addition, sections of marrow cells from human osteophyte and bone from growing calvaria were used as positive

control tissue for Bcl-2 (Crescenzi et al. 1988) and p53 (Lynch et al. 1998) immunostaining respectively.

#### DNA nick translation

Fragmented DNA was detected in cell nuclei in situ using an adaptation of previously described methods (Fehsel et al. 1994; Midgley et al. 1995) in which the sensitivity of the system has been reduced to increase the specificity for just those cells containing very large numbers of DNA breaks (Noble et al. 1997). Briefly, cryostat sections (10  $\mu\text{m}$ ) were fixed in formaldehyde for 10 min and air dried before demineralisation as before. Sections were then treated with nick translation mixture: 3  $\mu\text{M}$  DIG-11-dUTP, 3  $\mu\text{M}$  each of dGTP, dATP, dCTP, 50 mM Tris-HCl, pH 7.5, 5 mM  $\text{MgCl}_2$  and 0.1 mM dithiothreitol, either with or without (negative control) 0.5 U/100  $\mu\text{l}$  Kornberg polymerase for 1 h at 37  $^\circ\text{C}$  (all reagents obtained from Boehringer Mannheim). Sections were washed in PBS before incubation with FITC-labelled anti-DIG antibody, 5% normal sheep serum in PBS for 1 h at room temperature. After washing, sections were counterstained for nuclear DNA with PI, mounted in CITIFLUOR and examined by fluorescence photomicrography.

#### Morphology using high resolution light microscopy

PI counterstained sections from the in situ DNA nick translation assay were investigated for nuclear morphology, i.e. ordered disintegration of the nucleus to produce PI positive fragments, visible at high power, using oil immersion.

#### Assessment of cell viability in situ by LDH activity

Cells that were alive until the time of sampling were identified in undecalcified cryostat sections by means of their lactate dehydrogenase (LDH) activity. Histo-

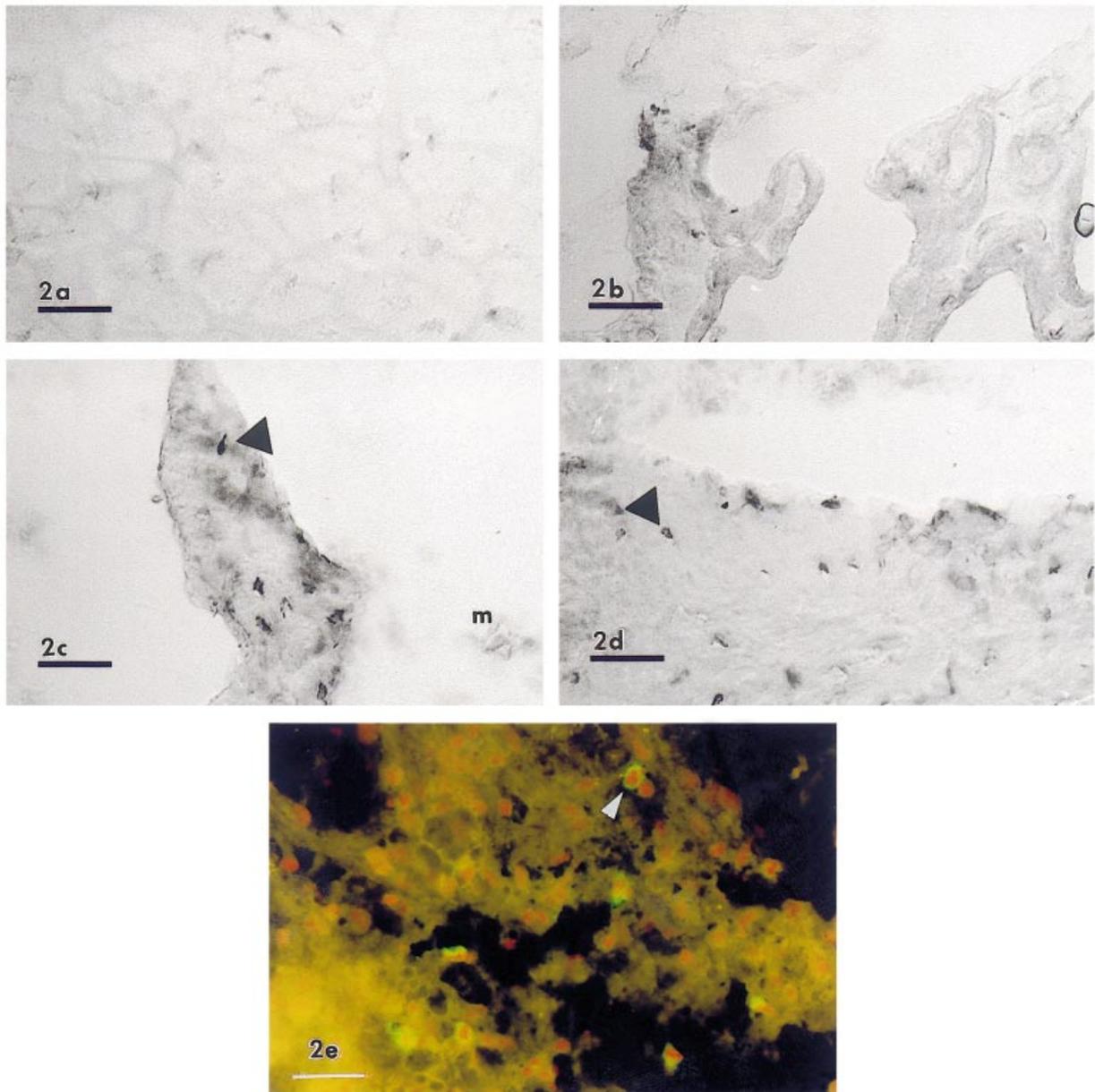


Fig. 2. (a) Bcl-2 (greyscale) immunostaining of rib. No staining in growth plate hypertrophic chondrocytes. (b) Primary spongiosa and recently formed trabecular bone showing low expression of Bcl-2 protein. Less recently formed bone with Bcl-2 positive osteocytes (arrowhead) in trabecular bone (c) and cortical bone (d); m, marrow:  $\times 200$ , bar  $60 \mu\text{m}$ . (e) Positive control tissue: human osteophyte marrow cells showing cytoplasmic FITC immunolocalisation of Bcl-2 protein (arrowhead), PI counterstained;  $\times 200$ , bar  $60 \mu\text{m}$ .

chemical staining was undertaken according to published methods (Farquharson et al. 1992) with minor modifications. Briefly  $10 \mu\text{m}$  tissue sections were reacted in  $1.75 \text{ mg/ml}$  nicotinamide adenine dinucleotide (NAD),  $60 \text{ mM}$  lactic acid,  $3 \text{ mg/ml}$  nitroblue tetrazolium (NBT) and  $40\%$  POLYPEP (Sigma) in  $0.05 \text{ M}$  glycyglycine buffer (pH 8.0), for 3 h at  $37^\circ\text{C}$  in a humidified chamber. After reaction, sections were rinsed in warm water, fixed in  $4\%$  formaldehyde in PBS for 10 min and then mounted in Citifluor. Dark blue/purple stained cells were regarded as viable.

#### *NADPH diaphorase activity*

To identify cells with high levels of NADPH diaphorase activity, as a means of labelling osteoclasts (Brandt et al. 1995) in situ we used the method of Spessert et al. (1994). Fresh cryostat sections ( $10 \mu\text{m}$ ) were incubated in  $50 \text{ mM}$  Tris-HCl, pH 7.6,  $1 \text{ mM}$  nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) (Melford Laboratories, Ipswich, UK) with or without (negative control)  $0.8 \text{ mM}$  nitroblue tetrazolium (NBT) (Sigma) for 1 h

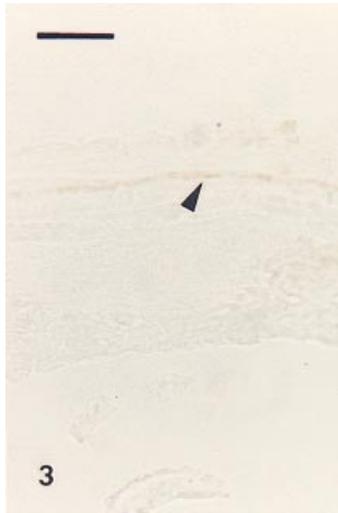


Fig. 3. P53 immunostaining of rib. P53 protein present in surrounding fibrous tissue (arrowhead) but not associated with growth plate or bone.  $\times 200$ , bar  $60 \mu\text{m}$ .

at  $37^\circ\text{C}$ . After rinsing in acetone for 1 min to remove soluble pink formazan, the sections were washed in PBS and mounted in Citifluor. Dark blue/purple stained cells were positive for diaphorase activity.

*Osteoblast and osteoclast activity*

Osteoblastic activity was assessed using an in situ assay for alkaline phosphatase (ALP). Formaldehyde-fixed sections were reacted in 2 mM  $\alpha$ -naphthyl acid phosphate, 2 mM  $\text{MgCl}_2$  and 1 mg/ml Fast Blue RR in 0.1 M barbitone buffer pH 9.2 at  $37^\circ\text{C}$  for 10 min. Osteoclastic activity was assessed by tartrate resistant acid phosphatase (TRAP) by reacting formaldehyde-fixed sections in 0.1 M acetate buffer pH 4.5, containing 1 mM naphthol ASBI phosphate and 10 mM sodium tartrate for 10 min at  $37^\circ\text{C}$ . The sections were

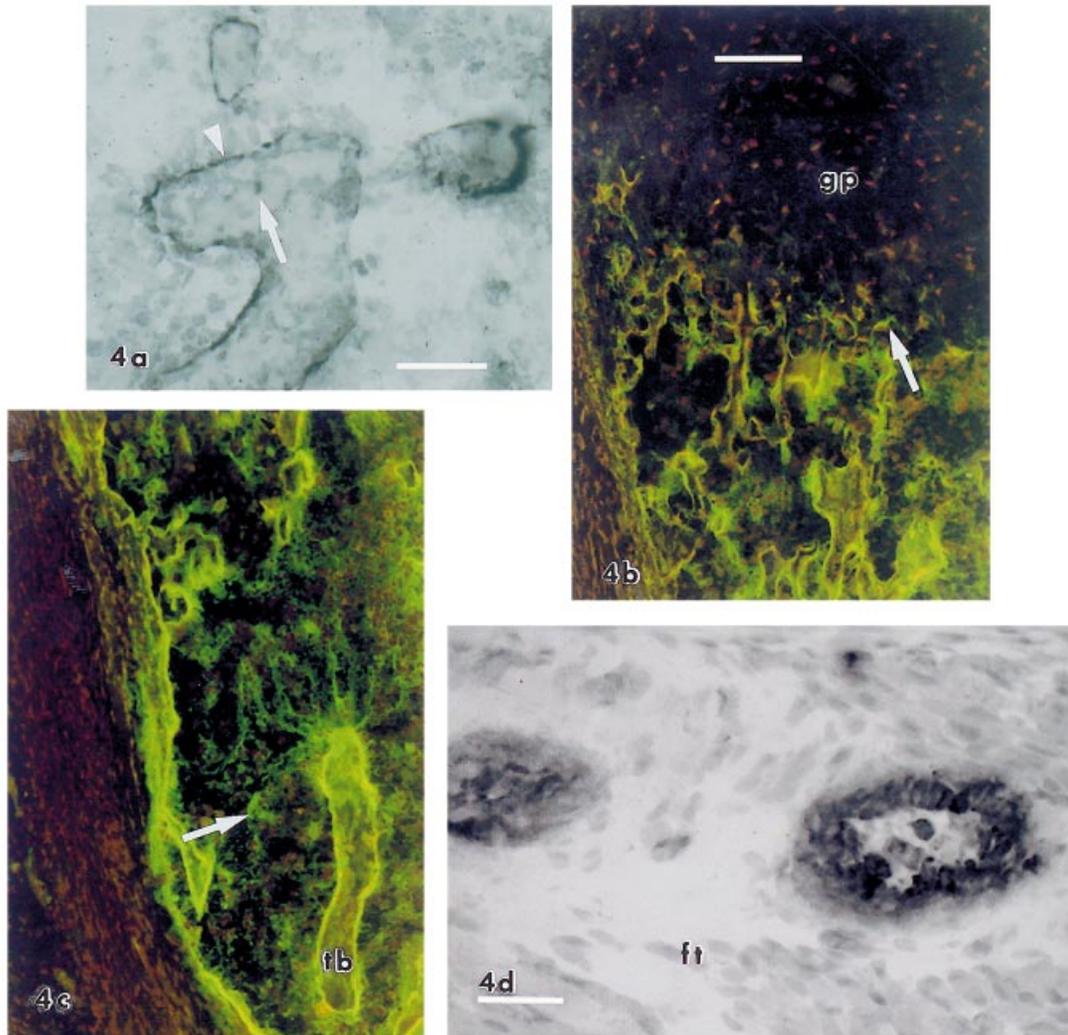


Fig. 4. (a) Tissue transglutaminase (tTG) immunostaining in rib. Greyscale immunolocalisation of tTG protein in bone lining cells (arrowhead) and osteocytes (arrow);  $\times 200$ , bar  $60 \mu\text{m}$ . (b,c) Hypertrophic chondrocytes and marrow cells (FITC, arrows); gp, growth plate; tb, trabecular bone;  $\times 100$ , bar  $110 \mu\text{m}$ . (d) Vascular channels in the outer fibrous tissue (FITC, grey scale); ft, fibrous tissue;  $\times 400$ , bar  $25 \mu\text{m}$ .

washed in 50 mM sodium fluoride and then post-coupled in 0.1 M acetate buffer pH 6.2 containing 2.2 mM Fast Garnet GBC at RT for 6 min.

## RESULTS

### *Immunohistochemical staining for Bcl-2, tTG and p53 proteins*

The growth plate region, including the primary spongiosa of the costal bone was negative for Bcl-2 immunostaining (Fig. 2a), whereas the recently formed trabecular bone showed low levels of Bcl-2 protein, with the occasional osteocyte being positive (Fig. 2b). With increasing distance from the growth plate more bone osteocytes showed expression of Bcl-2 (Fig. 2c, d). Marrow from human osteophyte tissue showed positive cytoplasmic staining for Bcl-2 protein in the cytoplasm of some cells (positive control, Fig. 2e).

P53 protein was absent from the growth plate and bone but appeared to be present in the outer fibrous tissue in discrete patches (Fig. 3).

Expression of tTG protein was found only in the hypertrophic chondrocytes of the growth plate and in bone lining cells, cortical and trabecular bone osteocytes (Fig. 4a) and cells of the marrow (Fig. 4b, c). Its association with haematopoietic cells was not restricted to the lumen of the shaft. Many tTG positive cells were found associated with marrow channels within the growth plate and specifically locating to vascular channels within the peripheral tissue (Fig. 4d). Pretreatment of samples with chondroitinase ABC did not affect staining for tTG, other than revealing some protein in late hypertrophic chondrocytes.

### *Nick translation (NT) in growth plate sections*

Sections of infant growth plate stained positive for fragmented DNA in the late hypertrophic chondrocytes which are known to undergo apoptosis in other species (Fig. 5a). Chondrocytes of the proliferative zone and early hypertrophic zone remained negative (Fig. 5b). Cells labelled for DNA breaks were found at the chondro-osseous junction (Fig. 5c) and in surrounding periosteal tissue (Fig. 5d), where expansion of the growth plate unit is thought to be initiated. Some cortical bone osteocytes at the base of the growth plate (Fig. 5e), bone marrow cells and osteocytes of recently formed trabecular bone were labelled positive for DNA breaks (Fig. 5f). Bone lining cells were generally negative for NT-labelling.

### *Morphology using high resolution light microscopy*

PI counterstained sections showed ordered disintegration of the nuclei to produce PI positive fragments, particularly in the late hypertrophic chondrocyte region (Fig. 6). Many PI positive fragments were also labelled by FITC for DNA breaks.

### *Cell viability*

Cells from the costal bone sections were positive for LDH activity (Fig. 7) and it was possible to eliminate from the study samples which had growth plates with zones of dead tissue.

### *NADPH diaphorase/TRAP and ALP activity*

Cells which stained positive for NADPH diaphorase activity included chondrocytes, some osteocytes and bone lining cells. Multinuclear osteoclasts, however, showed the most consistent and highest intensity staining and were clearly seen invading cortical regions at the base of the growth plate (Fig. 8a) at the chondro-osseous junction. The latter area was also the site of a considerable number of osteocytes undergoing DNA fragmentation in the bone.

Osteocytes in trabecular bone stained positive for NADPH diaphorase (Fig. 8b). ALP staining was widespread; trabecular bone surfaces and the region of the chondro-osseous junction showed particularly high levels of activity.

## DISCUSSION

Whilst previous animal studies have sought to clarify the importance of apoptotic cell death in the function of the epiphyseal growth plate, it should be noted that there are distinct differences in growth plate morphology between species. In the chick, there are specialised regions across the growth plate such that it is nonuniform, chondrocytes have a longer life span and resorption and bone formation are temporally and spatially much more separated than in mammalian systems. The porcine growth plate is bidirectional and the rat does not fully close the growth plate on reaching sexual maturity. To assess the clinical relevance of the previous animal findings it was desirable to examine human material.

In the present study, we have for the first time described the distribution of apoptotic cells in the human growth plate. Observed morphologically and by nick translation, the distribution was similar to

that seen in avian, porcine and rat systems (Aeschlimann et al. 1993; Hatori et al. 1995; Noble et al. 1995, 1997; Roach et al. 1995; Roach & Erenpreisa, 1996; Fujita et al. 1997). The majority of apoptotic chondrocytes reside in the hypertrophic region adjacent to the primary spongiosa.

The expression of tTG mirrored the distribution of cells with fragmented DNA, with the notable exceptions of bone lining cells and marrow cells, where tTG was present in much greater abundance than was expected from the numbers of visibly apoptotic cells. The role of tissue transglutaminase in the mammalian growth plate is known to be complex. It has been debated whether apoptosis is triggered by tTG crosslinking (Melino et al. 1994) or controlled and slowed by organisation of proteins (Furuya & Isaacs, 1994). It is postulated that tTG reduces leakage of soluble proteins from dying cells by crosslinking (Fesus et al. 1989), thereby encouraging the ordered formation of apoptotic bodies.

An interesting finding in the present study was that osteoblasts in the rapidly growing human nearly all expressed tTG. This raises the possibility that the expression of tTG in osteoblasts might indicate bone forming activity. Future experiments should investigate this possibility.

The lack of staining for Bcl-2 protein in the human growth plate was unexpected, since it is clearly expressed in the growth plate of other species (Amling et al. 1997; Wang et al. 1997). PTHrP-mediated elevation of Bcl-2 protein in proliferating chondrocytes is thought to delay their apoptosis (Amling et al. 1997). A second feedback mechanism exists such that indian hedgehog (Ihh) expression by differentiating chondrocytes is thought to activate parathyroid hormone related peptide (PTHrP) signalling molecule in the perichondrium, which in turn prevents more chondrocytes from differentiating (Vortkamp et al. 1996). Constitutively activated PTH-PTHrP receptors are thought to be responsible for the short-limbed dwarfism seen in patients with Jansen's metaphyseal chondroplasia (Schipani et al. 1996), indicating the physiological importance of PTHrP and its receptor in the human growth plate. Bcl-2 has been demonstrated in human articular chondrocytes grown in culture (Feng et al. 1998), suggesting that these related cells are capable of expressing Bcl-2. It is possible therefore that the human growth plate relies on other or additional feedback mechanisms to control the rate of differentiation of chondrocytes. It is also possible that we failed to detect Bcl-2 protein due to low levels of expression at this stage in development or inaccessibility of the growth plate cells to our antibody

incubation procedure. However its expression in osteocytes distant from the growth plate and in the marrow cells (in which it was first described), suggests that our procedures were capable of Bcl-2 detection in other cell types.

Whilst we found Bcl-2 to be present in osteocytes, clearly not all of the cells were expressing it. In regions of bone with few apoptotic cells, osteocytes containing Bcl-2 protein were more common and vice versa. These findings are observational and have not been quantified in any way. They are however consistent with the presumed function of Bcl-2: to prevent apoptosis in cells exposed to a range of inducers of apoptosis. One possible explanation of these findings is that Bcl-2 might protect 'older bone' osteocytes from apoptosis as they become distanced from the growth plate so preventing cell death in this relatively quiescent and less rapidly remodelling bone. In support of the possibility that some osteocytes could be more vulnerable to death induction than others, it has been shown that a small proportion of osteocytes show evidence of the death associated caspase CPP32 (Krajewska et al. 1997). Thus there may be a distribution of vulnerability so that common death inducers could have a regional influence. Similarly, Bcl-2 was absent from most but not all bone lining cells/osteoblasts.

This study is limited in its interpretation by the paucity of the rarely available samples used and the observational nature of the study. However our results have served to highlight some potentially important differences between human growth plate function and that of other species commonly used as models for human bone regulation in research.

The biochemical inducers of apoptosis in the growth plate are unknown but of interest is the ability of osteocytes to produce nitric oxide upon loading. The role of Bcl-2 as an antioxidant molecule (Hockenbery et al. 1993) may be of relevance to the signalling function of nitric oxide and related molecules. We were unable to show that p53 featured in the growth plate or associated bone at this stage in development. On the other hand, it was seen in peripheral tissues, indicating the validity of the staining technique. P53 induction is associated in particular with ionising radiation damage and in many cases its presence facilitates apoptosis, by bringing about growth arrest. Our data would suggest that p53 does not play a dominant role in the function of normally growing human bone in the vicinity of the growth plate.

The theory that chondrocyte apoptosis may initiate tissue resorption and vascular invasion (Gibson et al. 1995) could not be tested here but the visual

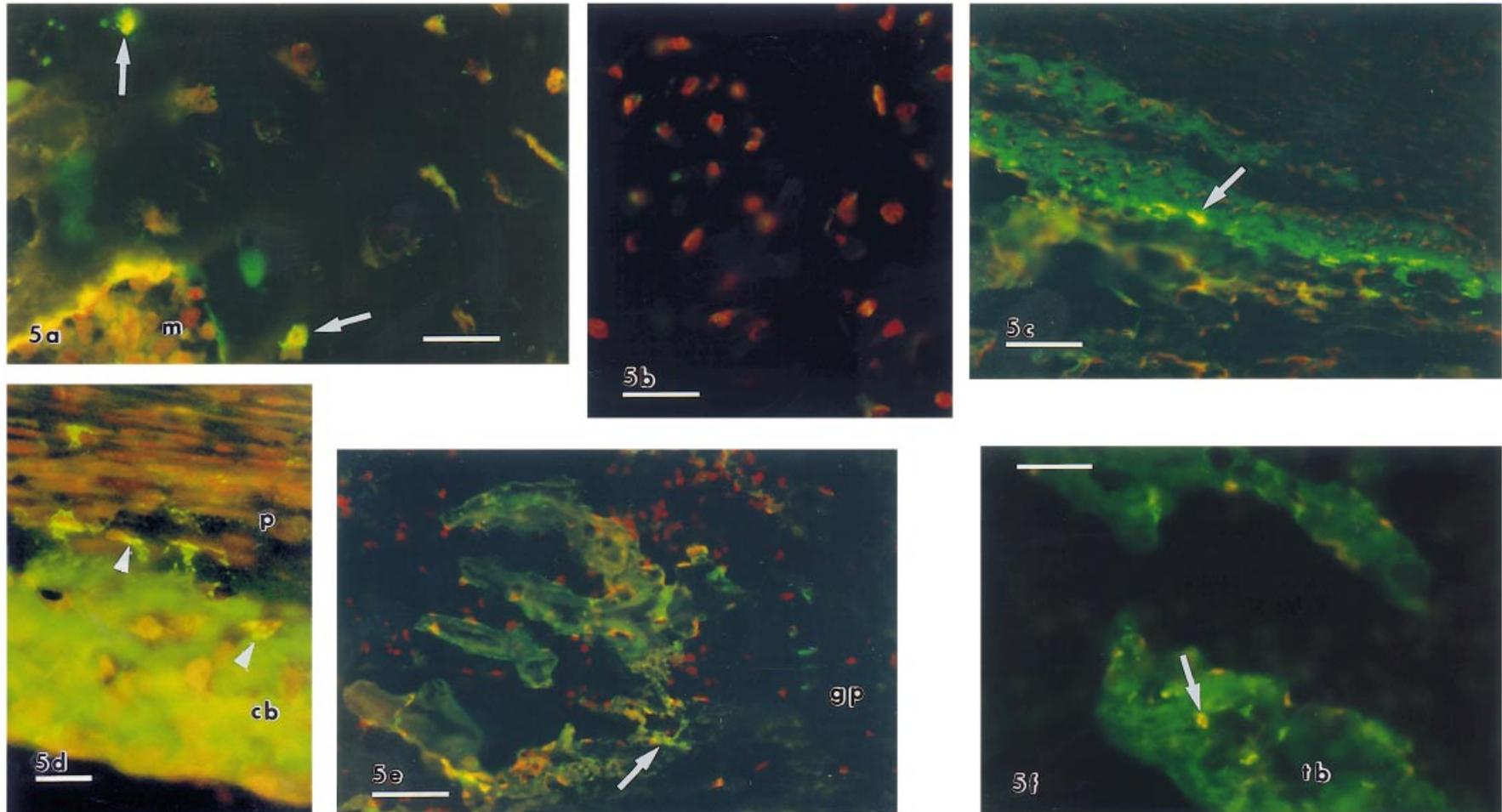


Fig. 5. (a) Evidence of DNA fragmentation in cells from rib (FITC). Late hypertrophic chondrocytes positive for nick translation (NT) (arrows); m, marrow;  $\times 400$ , bar  $25\ \mu\text{m}$ . (b) Proliferative and early hypertrophic chondrocytes are negative for NT;  $\times 400$ , bar  $25\ \mu\text{m}$ . (c) Positive cells at chondro-osseous junction (arrow) and (d) in surrounding periosteal tissue (arrowheads); cb, cortical bone; p, periosteum;  $c \times 200$ , bar  $60\ \mu\text{m}$ ;  $d \times 400$ , bar  $25\ \mu\text{m}$ . (e) Cortical bone osteocytes at the base of the growth plate (arrow); gp, growth plate;  $\times 200$ , bar  $60\ \mu\text{m}$ . (f) Marrow cells and osteocytes (arrow) in newly formed bone positive for DNA breaks; tb, trabecular bone;  $\times 400$ , bar  $25\ \mu\text{m}$ .

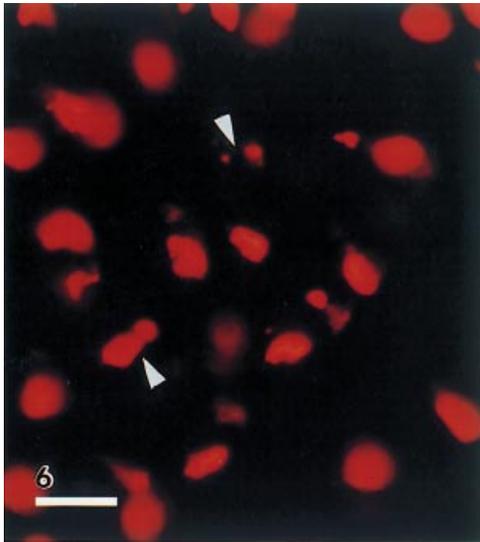


Fig. 6. Evidence of apoptotic morphology in cells from rib. PI staining of hypertrophic chondrocytes showing regions of DNA fragmentation (arrowheads);  $\times 400$ , bar 25  $\mu\text{m}$ .

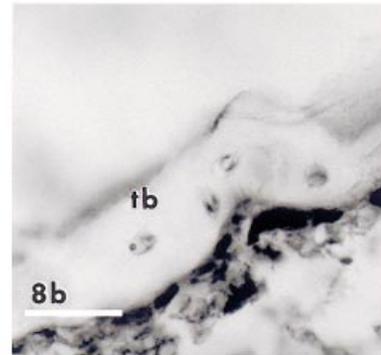
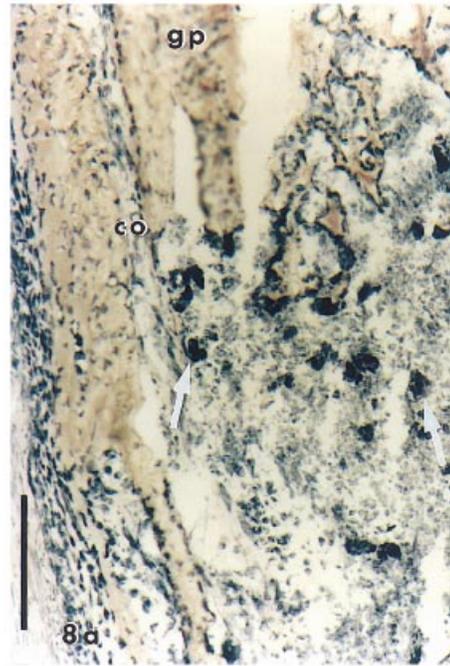


Fig. 8. (a) Location of osteoclastic cells determined using NADPH diaphorase. NADPH diaphorase activity (purple staining) in osteoclasts (arrows), osteocytes, bone lining cells and marrow; gp, growth plate; co, chondro-osseous junction ;  $\times 200$ , bar 60  $\mu\text{m}$ . (b) Diaphorase activity (grey scale) in bone osteocytes at high power; tb, trabecular bone;  $\times 400$ , bar 25  $\mu\text{m}$ .

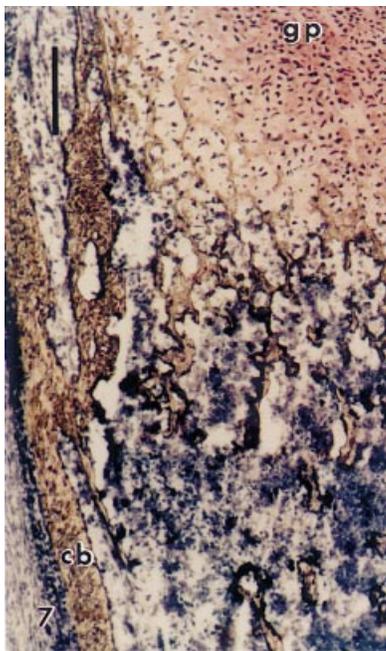


Fig. 7. Lactate dehydrogenase enzyme activity in cells, used as a marker of cell viability. LDH staining of one side of the growth plate showing viable (purple) chondrocytes, osteocytes and marrow cells; gp, growth plate; cb, cortical bone;  $\times 100$ , bar 110  $\mu\text{m}$ .

association between osteoclasts and regions of bone containing osteocytes with fragmented DNA supports other evidence suggesting a possible means for osteocyte-directed bone resorption (Bronckers et al. 1996, Noble et al. 1997).

In this study, we have identified in human bone, cell behaviours associated with apoptotic death which might play a role in human bone growth and growth

plate function. We have detailed the anatomical distribution of apoptotic cells within the human epiphyseal growth plate and associated bone and found that in general the pattern of chondrocyte apoptosis is comparable to that of animal models. An exception to this similarity concerns our failure to demonstrate Bcl-2 protein in the chondrocyte population in human bone using immunocytochemical techniques. This finding is surprising and brings into question the applicability of the PTHrP/Bcl-2 feedback loop seen in animal models to the control of human longitudinal bone growth. However, the current study cannot definitively rule out Bcl-2 production in the growth plate and future studies using molecular biological techniques should clarify the situation.

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