

## Rapid new bone tissue remodeling during distraction osteogenesis is associated with apoptosis

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### Abstract

During the process of distraction osteogenesis new bone forms and undergoes rapid remodeling. Apoptosis may be one of the regulatory mechanisms governing the removal of the redundant callus during distraction osteogenesis. A rabbit tibial lengthening model was used and lengthened at 0.7 mm/day for 3 weeks. The regenerating tissues from the distraction gap were examined for apoptotic changes by transmission electron microscopy (TEM) and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method. Osteoclastic bone resorption activities were demonstrated by tartrate resistant acid phosphatase (TRAP) staining. The apoptotic cells were mainly present in the transitional regions between the fibrous tissue and the new bone in the mineralization front, and close to or on the new bone surfaces near the center of the regenerate. The TUNEL labeling was greatly reduced in the mature bone near the osteotomied bone ends. TEM examination confirmed the presence of cells with apoptotic changes at various regions of the regenerate. TRAP staining revealed that osteoclastic bone resorption activities in the regenerate were in a similar pattern of distribution to those of the TUNEL labeling. The localization of apoptotic cells at the different regions of the regenerate, accompanied by the osteoclast activities, suggest that apoptosis is closely related to bone formation and remodeling during distraction osteogenesis.

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### Introduction

During the process of distraction osteogenesis, when distraction is applied at an optimal rate with no surrounding soft tissue complications, new bone tissue forms and undergoes rapid remodeling [1,7,10,11,18,19,21]. However, little is known about the regulatory mechanisms which govern the removal of the redundant callus during this process.

Apoptosis or programmed cell death is a fundamental property of cells, allowing unwanted cells to be eliminated quickly and neatly [12,38,39]. Tissue homeostasis is achieved by a delicate balance between the processes of cell death and cell proliferation [29]. There are in-

creasing reports that apoptosis is involved in a variety of hard tissue cell types in various physiological and pathological conditions [2,9,32]. Osteoblasts, chondrocytes and osteoclasts have been demonstrated to undergo apoptosis in vivo [14,16,17,22,25,30,33] and in vitro [13,23]. Apoptotic changes were also found in osteocytes in normal and pathological human bone, suggesting a functional relationship between bone turnover and controlled cell death of osteocytes [24,28]. Furthermore, apoptosis of osteocytes within bone could impair the proposed mechano- and/or trauma-receptor function of these cells, and thus delay the removal of damaged bone and so increase the risk of fracture [2]. The role of apoptosis in the regulation of osteoblast activity is much less clear. However, it may be hypothesized that increased osteoblast apoptosis as a consequence of ageing could lead to a reduced matrix deposition and thinner bone trabeculae, which are characteristic of age-related bone loss [24].

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Previous studies on cell proliferation during distraction osteogenesis have indicated that cell proliferation was activated and affected by the rate of distraction, and the population of cells within the regenerate was consistently changing [18]. In such a cell population, the proportion of proliferating to dying cells may be varying, and to understand this process it is important to define the populations of cells undergoing apoptosis, the time course of cell death and the factors influencing this process. This study tests the hypothesis that apoptosis may be of major importance during bone regeneration, and that rapid bone tissue remodeling is triggered by the cells in the regenerating tissues undergoing apoptosis during distraction osteogenesis.

## Materials and methods

### *Animal model of distraction osteogenesis*

All animal experimental procedures were approved and performed under the control of the guidelines for Animals (Scientific Procedures) Act 1986, British Home Office. Mid-tibial osteotomies were performed in six adult male NZW rabbits (age 24 weeks, body weight 3.0–3.5 kg), and the tibiae stabilized with external fixators as previously described [18–20]. After a 7 days latency period, twice daily distraction was initiated at rate of 0.7 mm/day for 3 weeks, until the tibiae were lengthened by 20% (approximately 1.5 cm). At this time, the animals were killed. During the distraction period, the animals were free to weight-bear on the operated leg, and weekly radiographs were taken to confirm the extent of lengthening.

### *Sample preparation and histological examination*

The rabbits were killed by anesthetic overdose at the end of the experiment. The central portion of the regenerated tissue in the distraction gap, including 2–5 mm of the neighboring cortical bone, was excised and divided midsagittally. One half of the specimen was prepared for electron microscopic examinations as described below. The other half of the specimen was fixed in 4% paraformaldehyde (pH 7.4) for 24 h before decalcification in buffered 14.5% EDTA (pH 7.2) for 3–4 weeks at 4 °C. The decalcification was confirmed by radiography and the decalcified specimens embedded in paraffin wax. 5 µm thick microtome sections were placed on poly-L-lysine (Sigma, Poole, UK) coated slides and stored for future use. For histological examination, the sections were stained routinely with haematoxylin and eosin (H&E).

### *Preparation for transmission electron microscopy examination*

The central portion of the regenerated tissue in the distraction gap was excised and divided in the midsagittal plane. One half of the specimen was fixed at 4 °C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 h. Specimens were then given 4 × 5 min washes in 0.1 M sodium cacodylate buffer and left in buffer overnight at 4 °C. Post-fixation was for 1 h in 1% osmium tetroxide (O<sub>8</sub>O<sub>4</sub>) in 0.1 M sodium cacodylate buffer. The tissues were washed with buffer to remove O<sub>8</sub>O<sub>4</sub> and dehydrated through an ascending alcohol series into propylene oxide prior to gradual infiltration and embedding in TAAB resin. Polymerization was for 48 h at 60 °C. Ultrathin sections (60–90 nm) were cut on a Reichert Ultracut microtome (Reichert-Jung Optische Werke, Austria), and placed on Formvar<sup>TM</sup>-coated 200 mesh copper grids (Agar Scientific Ltd., UK). Sections were stained with saturated uranyl acetate solution in 50% ethanol for 15 min, washed briefly in ethanol and distilled water before additional staining in Reynolds lead citrate for 4 min, washing in

buffered water 2 × 5 min, examined in a Jeol 100 CXII transmission electron microscope.

### *Tartrate-resistant acid phosphatase staining*

To detect bone-resolving cells, tartrate-resistant acid phosphatase (TRAP) staining was performed. In brief, paraffin sections were de-waxed, rehydrated through ethanol, washed in tris-HCl buffer (pH 9.0) and incubated with acetate buffer (0.5% w/w sodium acetate, 0.83% v/v acetic acid, pH 5.0) for 1 h before incubation in substrate solutions consisting of Naphthol AS BI phosphate (15 mg, Sigma, Poole, Dorset, UK), sodium tartrate (75 mg), pararosaniline (1 mg), sodium nitrite (0.8 mg) in 26 ml acetate buffer (pH 5.0) at 37 °C for 1 h. Sections were rinsed in running water for 5 min, with or without counterstaining with haematoxylin, and mounted in aquamount (BDH, Poole, England).

### *Detection of DNA fragmentation by TUNEL method*

The morphological features of apoptosis are easily recognized ultrastructurally, but are only detected with difficulty by light microscopy. Detection of apoptosis on a DNA level requires extraction of DNA and analysis by agarose gel electrophoresis. However, assay systems of this type are difficult to quantify and fail to evaluate apoptosis on a cell-by-cell basis. Since extensive DNA fragmentation is an important characteristic of the apoptotic process, visualization of DNA breaks could greatly facilitate the identification of apoptotic cells. Modak and Bollum [27] discovered DNA breaks during nuclear degeneration in 1970. Gavrieli et al. [4] further established the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP-biotin nick end-labeling (TUNEL) method that allows detection of apoptosis at the single-cell level. This method uses TDT to label 3'OH ends of DNA fragments generated during the process of apoptosis. TUNEL staining has been used in the present study to detect DNA fragments of apoptotic cells in histological sections.

The reliability and the quality of the TUNEL staining is dependent upon the methods used for tissue preparation, non-specific background staining is minimal in paraformaldehyde-fixed tissue [35]. The conditions for TUNEL staining used in the present study were optimized to ensure minimum non-specific staining. The method used for permeabilization prior to TUNEL staining also affects the quality of TUNEL staining. Both proteinase K and Triton X-100 provided similar results when the proteinase K was used at a low concentration for a short period of time at room temperature. However, at higher concentrations, proteinase K increased non-specific staining [36]. In contrast, permeabilization treatment with Triton X-100 provides more reliable results and this was confirmed in preliminary experiments of this study. Sections permeabilized for 10 min using 0.5% (w/v) Triton X-100 in PBS at room temperature were found to provide the most reliable and reproducible results, with best preservation of morphology of the sections.

A cell death detection kit (Boehringer Mannheim, East, Sussex, UK) was used to detect and quantify apoptosis at the single-cell level by TUNEL staining. In brief, slides were de-paraffinized, rehydrated and washed in tris-buffered saline (TBS). The sections were permeabilized for 10 min using 0.5% (w/v) Triton X-100-PBS at room temperature. Following 2 × 5 min TBS washes, TUNEL reaction mixture (40 µl/slide) was added, the section covered with a glass cover slip and incubated for 60 min at 37 °C. To make 500 µl of the TUNEL reaction mixture, the following solutions, provided with the kit, were added: 50 µl TDT from calf thymus in storage buffer and 450 µl label solution containing fluorescein-labeled dUTP and nucleotide mixture in reaction buffer. The reaction was terminated by immersing the slides in tris-EDTA buffer (pH 8.) for 15 min. The slides were rinsed with distilled water followed by phosphate-buffered saline (PBS), and incubated with a sheep anti-fluorescein antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Poole, UK) at a dilution of 1:5 in TBS for 30 min at room temperature. Following washes with TBS, the slides were developed with substrate solution containing 10 ml TBS (pH 7.4), 10 mg Fast red TR (BDH, Poole, UK), and 400 µl Naphthol AS MX buffer (Sigma, Poole, UK) for 15 min in the dark at room temperature. The slides were then rinsed with distilled water, counterstained with Mayer's haematoxylin for 5 min, mounted with water-soluble mountant (Aquamount, BDH, Poole, UK), and

analyzed under light microscopy. The TUNEL-positive staining cell was pink/red in color.

For a methodological positive control, prior to the TUNEL staining, tissue sections were treated with DNase I (Sigma, Poole, UK, 20 µg/ml) for 30 min at room temperature, to generate DNA strand breaks. For a negative control, slides were incubated only with a solution containing fluorescein-labeled dUTPs, but without TDT, or with TBS buffer alone. In addition, tissues known to have low cell turnover, including brain and peripheral nerve, were processed by TUNEL staining as real negative controls.

## Results

### *Radiographic and histological evaluation of the distraction regenerate*

In all the animals, the distraction gap was seen to consist of three regions at the end of the lengthening period. These regions, assessed by histological and radiographic appearances, are shown in Fig. 1 and were defined as previously reported [18,19]: (1) The central fibrous zone (FZ), localized in the middle of the distraction gap as a radiolucent area. (2) The primary mineralization fronts (PMF), which yielded sclerotic bands on the radiographs lying on both sides adjacent to the central fibrous zone, containing longitudinally arranged, well vascularized collagen bundles which were undergoing mineralization. (3) The peripheral new bone zone (NBZ), lying between the PMFs and the osteotomy bone ends. This zone mainly consisted of mineralised woven and lamellar bone when examined by polarized light microscopy, and was relatively radiolucent in radiographs compared with the primary mineralization front.

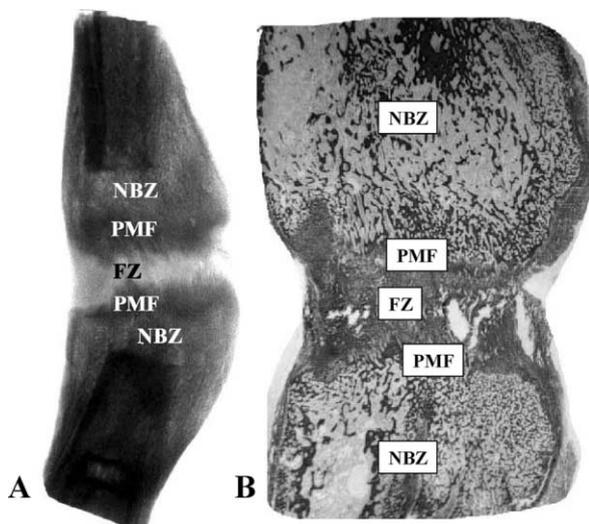


Fig. 1. The three zones of the distraction regenerate defined. A: Radiography of the regenerate. B: Histological appearance of the regenerate, H&E staining, original magnification  $\times 40$ . FZ: fibrous zone, PMF: primary mineralization fronts, NBZ: new bone zone.

### *Transmission electron microscopy examination*

Transmission electron microscopy (TEM) is a useful approach to prove apoptotic changes qualitatively. Apoptotic cells showed a loss of cell contact, shrinking away from neighboring cells, leading to the formation of halos around the cell bodies. Nuclear and cytoplasmic condensation caused apoptotic cells to obtain a more spheroid morphology and rounded appearance. The TEM features of cells undergoing apoptosis include especially segregation of condensed chromatin and cell breakdown into dense apoptotic bodies.

Apoptotic changes of various stages were observed in the osteoblastic cells in all of the three defined regions of the regenerate, with an impression that the number of apoptotic cells was particularly high in the PMF and the NBZ adjacent to PMF (Fig. 2B and C).

### *TUNEL labeling in the distraction regenerate*

No TUNEL labeling was found in any of the negative control sections, when the slides were incubated only with a solution containing fluorescein-labeled dUTPs, without TDT, or with TBS buffer alone. A very few TUNEL-positive cells were found in the tissue sections of rabbit brain and peripheral nerve. For methodological positive controls, extensive TUNEL-positive labeling was seen in all of the tissue sections treated with DNase I as expected.

TUNEL staining was performed in at least 3 sections from each specimen. All sections were examined by a light microscopy under  $\times 400$  magnifications. The staining intensity was graded semi-quantitatively in the three defined regions of the regenerate by two observers (the mean grading of the two observers was used). The intensity of grading was used as follow: +++: strong presence; ++: moderate presence; +: rare presence; -: absence.

TUNEL-positive cells were present in all of the three regions, the FZ, PMF and NBZ throughout the regenerate. Majority of the TUNEL-positive cells were present in the FZ (Fig. 3A) and the PMF (Fig. 3B). Some TUNEL-positive cells were also seen in the cartilage region of the regenerate, with dense labeling in chondrocytes in close proximity to invading blood vessels (not shown). In the NBZ near the PMF, the TUNEL-positive cells were seen close to or on bone surfaces, and some of the newly formed osteocytes in the new trabeculae were also positive (Fig. 3C). However, the TUNEL labeling was reduced in the NBZ close to the osteotomied bone ends, the number of the TUNEL-positive cells in this region was about one third of that in the region near the PMF, and most of the TUNEL-positive cells were osteocytes (Fig. 3D). TUNEL-positive labeling was not detected in the intact cortical bone of the osteotomied bone ends and in the adjacent

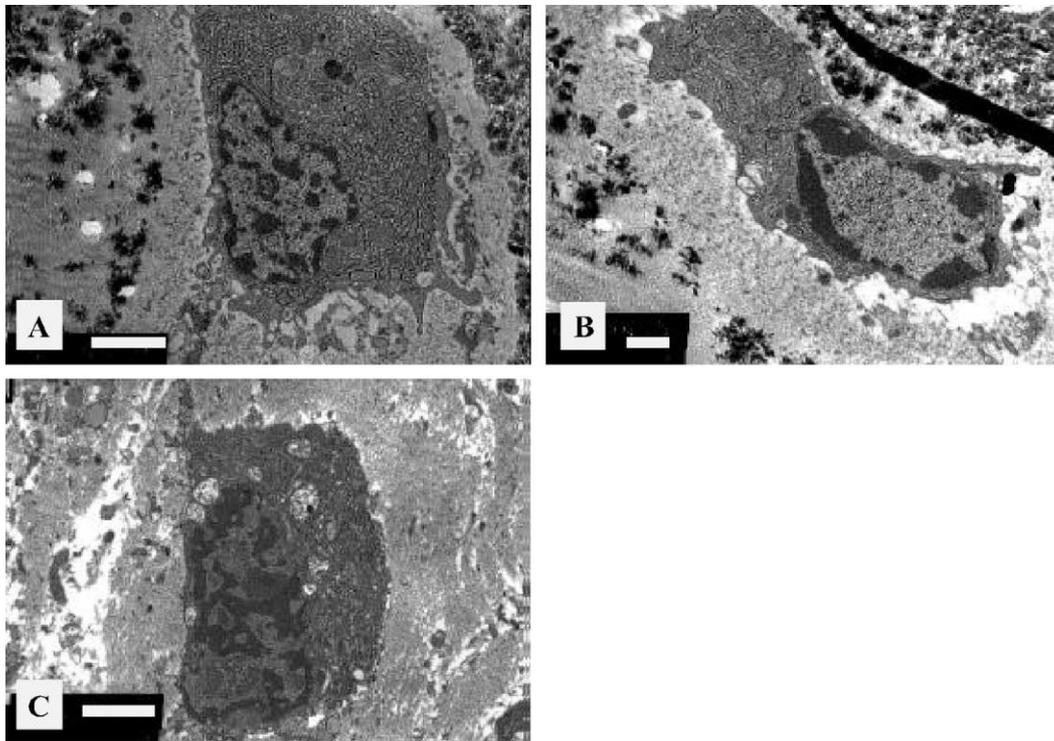


Fig. 2. TEM images of osteoblasts present in the NBZ displaying nuclei in various stages of normal and apoptotic features. A: Normal appearance of an osteoblast in the NBZ. Bar; 1  $\mu$ m. B: Apoptotic changes of an osteoblast in the NBZ, showing chromatin condensing and clumping around the periphery of the nucleus. Bar; 1  $\mu$ m. C: More advanced stage of apoptosis of an osteoblast in the NBZ, the chromatin has clumped together and the cell can be seen to begin to break down. Bar; 1  $\mu$ m.

surrounding periosteum. The extent and intensity of the TUNEL labeling was summarized in a semi-quantitative manner and shown in Fig. 4.

#### *Osteoclast activity in the distraction regenerate*

TRAP-staining in the regenerate revealed similar patterns of distribution to those of the TUNEL labeling as summarized in Fig. 4. TRAP-positive osteoclastic cells were seen in the PMF and the NBZ resolving the newly formed callus (Fig. 3E and F). Considerable numbers of TRAP-positive cells were also seen in the FZ. There were more TRAP-positive cells in the NBZ near the PMF than in that of the NBZ close to the osteotomied bone ends. The extent and intensity of the TRAP staining together with the TUNEL labeling were summarized in a similar way as the TUNEL labeling and shown in Fig. 4.

#### **Discussion**

Distraction osteogenesis is a unique process in which bone formation occurs in parallel with rapid bone remodeling [1,7,10,11,20]. This technique is widely used clinically for the treatment of many challenging orthopedic conditions, such as correction of congenital de-

formities and limb reconstruction following tumor resection [10,11]. The rabbit model of leg lengthening is a well-established model of distraction osteogenesis and has been used extensively to study various aspects of bone regeneration during distraction osteogenesis [3,7, 18–21]. The radiography and histology findings in the present study agree with previous reports using the similar model. The optimal rate of lengthening of 0.7 mm, twice daily [18,19] was used in this study to ensure a good quality of bone regeneration.

The TUNEL staining method, like most other methods involving in vitro transcription, does not necessarily distinguish between apoptosis and other causes of DNA fragmentation. In a study on cell death of chick hypertrophic chondrocytes using TUNEL staining, Gibson et al. [5] found that up to 30% of the cells in the developing chick sterna was TUNEL-positive. In another study, Hatori et al. [8] applied flow cytometry and estimated that only 8% of chondrocytes of the avian growth plate were apoptotic at one time. These reports suggest that TUNEL staining tends to over estimate the true number of apoptotic cells by up to two to three times. However, none of the negative control tissues (peripheral nerve and brain) known to be homeostatic showed significant labeling when examined by TUNEL staining and there was little TUNEL-positive staining seen in intact cortical bone tissues. This suggests that the

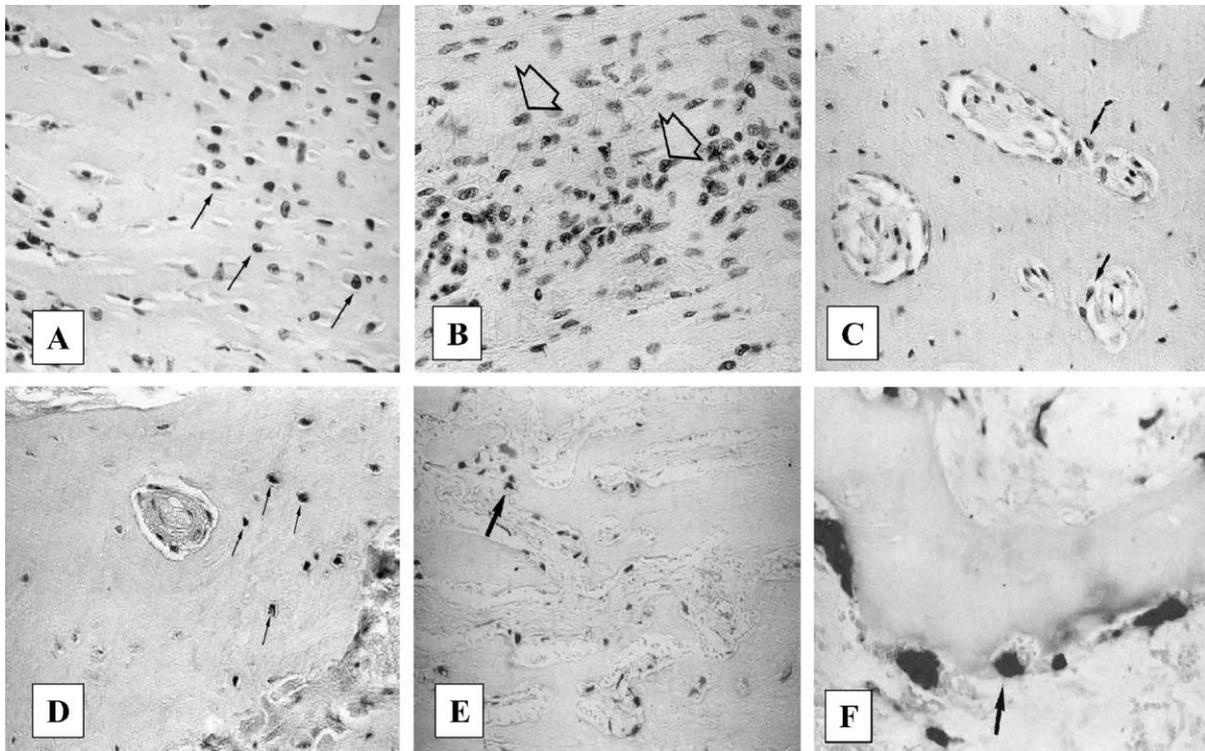


Fig. 3. Representative views of TUNEL and TRAP staining in the distraction regenerates. A: TUNEL positive labeling (arrows) in the FZ of the regenerate. B: TUNEL staining in the PMF, the majority of TUNEL-positive cells (arrows) were present in the transitional region between the PMF and the NBZ. C: In the NBZ near the PMF, TUNEL-positive cells were close to or on the bone surfaces (arrows). D: TUNEL positive osteocytes (arrows) were found in the new bone close to the osteotomy ends. E: TRAP staining in the new bone zone of a regenerate, showing that there were many TRAP-positive cells (in red, arrow) in the new bone zone near the PMF. F: Close up of TRAP staining showing active osteoclastic bone resorption in the NBZ. A–E, original magnification  $\times 100$ ; F,  $\times 400$ .

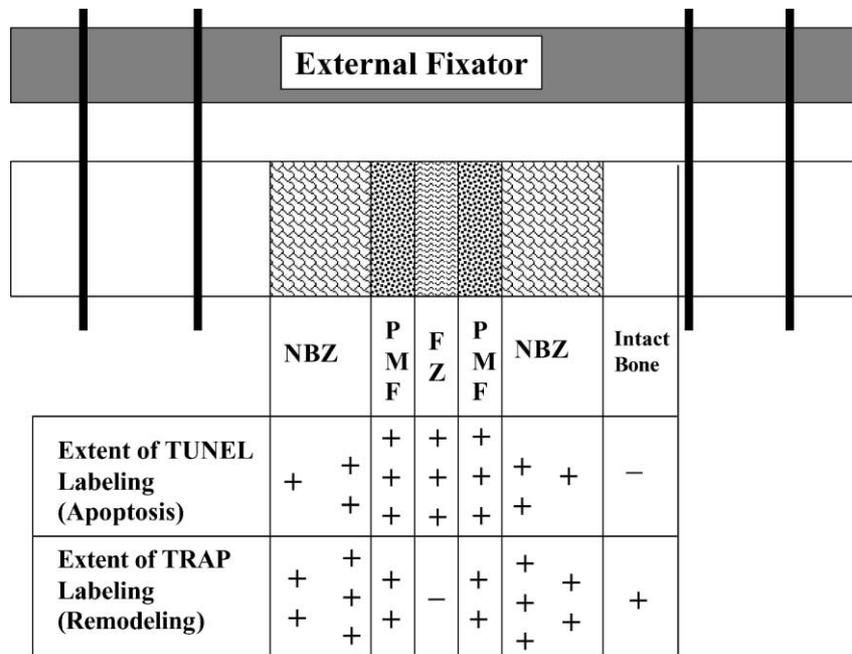


Fig. 4. Diagrams showing the morphological features of the distraction regenerate, and summarizing the extent and intensity of apoptosis by TUNEL labeling, osteoclastic activity by TRAP staining in the different regions of the regenerate. +++: strong presence; ++: moderate presence; +: rare presence; –: absence.

result of TUNEL staining in the present study reflects reliably the cell death situation in the regenerating tissues. Further consideration of the TUNEL labeling results is that some of the DNA fragmentation could be occurring *ex vivo* during the processes of sample preparation and decalcification. To clarify this issue, samples of rabbit thymus and liver were obtained and immediately snap frozen and frozen sections were examined using the TUNEL staining. It was found that the distribution of TUNEL-positive cells was similar to that of the paraformaldehyde-fixed paraffin-embedded thymus and liver samples. In addition, the potential effects of the decalcifying process on the tissue were examined. Paraformaldehyde-fixed tissues of the rabbit thymus, liver and skeletal muscle were treated with 14.5% EDTA (pH 7.4) for 2 weeks, paraffin-embedded and examined by TUNEL staining. The distribution of TUNEL-positive cells was found to be similar to that of the samples without EDTA treatment. Taken together, these data strongly suggest that the tissue preparation and processing methods used in this study do not lead to increased fragmentation of DNA. Since we have used the TUNEL labeling method with great caution in the present study, one would expect the artifacts (if any) would apply to all the tissues examined, creating a background level, which would not prevent comparisons. Consequently, DNA fragmentation identified by TUNEL staining in the present study in all probability reflects the situation *in situ* in the distraction regenerates. The TEM results have further confirmed the presence of apoptosis in the regenerates.

The most intense TUNEL labeling for apoptosis in the distraction regenerate was seen in the central fibrous zone, the mineralization front and the newly formed bone zone adjacent to the mineralization front. DNA fragmentation was detected readily in these regions by TUNEL labeling and it was also confirmed by TEM examination. Distraction osteogenesis is a process demanding rapid cell proliferation, differentiation and tissue turnover (bone remodeling). It is possible that during the development of new bone tissue in this process, the number of potentially osteogenic cells generated exceeds the number of cells required to make bone, and the superfluous cells are eliminated through programmed cell death. In general, there are three pathways that could lead to apoptosis [15,38,39]: (i) DNA and protein damage by inappropriate physical and/or chemical conditions; (ii) positive induction by ligand binding to a plasma membrane receptor such as Fas; and (iii) negative induction by loss of a suppressor signal, e.g. growth factors.

There are a number of possible explanations for the induction of apoptosis during distraction osteogenesis. First, it may be due to inadequate vascularization for tissue maintenance. Gottlieb et al. [6] reported that apoptosis could be induced in cardiac myocytes by

ischemia and reperfusion. A similar situation may exist in the central zone of the regenerating tissues during distraction osteogenesis, where rapid tissue formation and increase in cell number, may cause severe local hypoxia and acidosis. Subsequent neovascularization occurs in the center of the regenerate, and the alternating ischemia (caused by rapid tissue formation) and reperfusion (by neo- or revascularization) in the regenerating tissue, may lead to oxygen radical formation, which may induce DNA fragmentation and apoptosis in the cells of the newly formed tissues. The distribution and the changes in the number of TUNEL-positive cells in the different regions of the regenerate, concurs with the changes in angiogenesis in these regions, which were reported previously [19]. Furthermore, in areas of intact cortical bone where revascularization activity was low, there was little TUNEL-positive labeling.

Another alternative explanation for the induction of apoptosis during distraction osteogenesis is lack of surviving growth factors or increases of cytokines or growth factors that trigger apoptosis. The viability of many cells is dependent on a constant supply of growth factors and cytokines and, in the absence of these factors, cells undergo apoptosis. The cell population in the regenerate during distraction osteogenesis changes rapidly and some cells may be deprived of conditions favoring their survival. The expression and distribution of several proteins known to be mediators of the apoptotic process, such as bcl-2, Fas and p53 [26,37,40], in the distraction regenerate should be examined in future studies.

Apoptosis is closely involved in tissue remodeling during development and repair [12,13,16,17,22,25,30]. Gibson et al. [5] studied endochondral ossification of chick sterna, and suggested that apoptosis may be an initiating event in bone tissue remodeling and revascularization. Noble et al. [28] and Monolagas [24] suggested a functional relationship between bone turnover (remodeling) and the controlled cell death of osteocytes. Previous studies of osteogenesis *in vitro* have revealed the participation of cell death in the course of calcification [13,34]. These observations are confirmed in a recent study of ossification in foetal mouse calvariae [41], mineralization of the osteoid was accompanied by necrosis of the osteoblasts and the number of the latter increased markedly as the ossification of the long bone cortices proceeded [30]. The authors have suggested that calcium and phosphate being stored in cells are possibly liberated by cell death to supply mineral constituents at sites of rapid mineralization [41]. This hypothesis is supported by our findings that high levels of phosphorus, zinc and copper are found in the distraction regenerate in a similar model of distraction osteogenesis in the rabbit [31]. In addition, in our recent study of fracture healing, we have confirmed that cell proliferation and apoptosis are coupled events during fracture repair,

cell proliferation is active at the early stages and apoptosis is active during the phase of callus remodeling [22]. The reliance on certain signals emphasizes the existence of a high turnover state in which cell proliferation and cell death are likely to coexist, with their relative quantities being determined by the surrounding microenvironment. The critical choice for cells depends upon additional considerations such as the availability of growth factors and local mechanical environment.

In this study, the increased TUNEL positivity in the new bone zone near the mineralization front is accompanied by increased TRAP-positive staining in this region, indicating high osteoclastic bone resorption activity. This suggests that rapid new bone remodeling seen in distraction osteogenesis through attraction of bone-resolving cells may be initiated by osteoblasts undergoing apoptosis. Thus, the apoptosis seen during distraction osteogenesis may be an important cellular mechanism by which redundant callus tissue is removed, and local tissue turnover is controlled. Further knowledge on the availability of certain cell types at a line of bone regeneration and their coordinated removal by apoptosis could result in better prognosis of bone healing and provide a better insight to bone disorders and disease states.

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