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REGULAR ARTICLE

Chondrocyte apoptosis enhanced at the growth plate: a physeal response to a diaphyseal fracture

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Abstract Post-traumatic overgrowth of growing long bones is a common clinical phenomenon in paediatric traumatology and is the result of an enhanced stimulation of the nearby growth plate after fracture. To date, the exact post-fractural reactions of the growth plate are poorly understood. The aim of this study has been to determine the impact of fracture on the frequency of chondrocyte apoptosis of the growth plate. Rats sustained a mid-diaphyseal closed fracture of the left tibia or were left untreated. All animals were killed 3, 10, 14 or 29 days after trauma. The left and right tibiae were harvested and apoptotic chondrocytes of the proximal tibial growth plate were detected by TUNEL staining. The apoptosis percentage of physeal chondrocytes was statistically compared among fractured bones, intact contra-lateral bones and control bones. The physeal apoptosis rate of the fractured bone was significantly higher than that of the contra-lateral intact bone (valid for all evaluated days) and the control bone (valid from day 10 onwards). Contra-lateral intact tibiae never showed significantly higher apoptosis rates compared with control tibiae. Thus, mid-diaphyseal fracture influences the nearby growth plate by stimulating chondrocyte programmed cell death, which is associated with cartilage resorption and bone replacement. The lack of a significant difference between the intact contra-lateral and the intact control bone suggests that fracture only has a local effect that contributes to the greater apoptosis rate of the adjacent physis.

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Introduction

Bone fractures of the growing skeleton are unique in their potential to cause growth disturbances. Bone overgrowth represents a clinical complication of a fractured growing bone and can only occur if the corresponding epiphyseal plates of the fractured long bone have not yet closed. Stimulating growth phenomena are associated with an increased activity of the growth plate, which can be affected either in its entirety or partially. The former results in limb length discrepancy, whereas the latter leads to angular and joint deformities and is an established risk factor in the development of early arthrosis (Hasler and von Laer 2000; Linhart and von Laer 2005)

Overgrowth occurring after femoral shaft fracture has been well documented in a variety of retrospective trials (Clement and Colton 1986; Edvardsen and Syversen 1976; Reynolds 1981; Stephens et al. 1989; Zimmermann et al. 1999). Indeed, post-fractural overgrowth is to be obligatorily expected in every fractured growing bone (Hasler and von Laer 2000; Linhart and von Laer 2005). The responsible factors for physeal stimulation may be, on the one hand, an increased blood flow to the adjacent growth plates and, on the other hand, the influence of hormones and growth factors (Breitfuss et al. 2006; Hasler and von Laer 2000). Although the underlying molecular mechanisms that occur at the fracture site and that are responsible for the formation of callus and fracture healing have been elucidated in detail (Einhorn 1998; Einhorn et al. 1995; Gerstenfeld et al. 2003; Gerstenfeld and Einhorn 2003; Landry et al. 1996; Tsiridis et al. 2007), little is known about the molecular influence of

a fracture to the adjacent growth plate and the growth process itself. Recently, growth plate reactions to a femoral fracture have been investigated in a global gene expression study by Ashraf and colleagues in a rat model, giving evidence that mitotic genes are initially up-regulated at the physis after fracture (Ashraf et al. 2007). Furthermore, increased bone growth after fracture in rats has previously been reported in literature (Ashraf et al. 2007; Garces et al. 1997; Kaya Alpar 1986).

In order to acquire more information about post-fractural reactions of physeal cells, we have investigated to what extent the fracture of a growing bone influences chondrocyte apoptosis in the growth plate. Physeal chondrocytes undergo a life cycle of proliferation and differentiation (columnar zone of the epiphyseal plate, Fig. 1a) and hypertrophy (hypertrophic zone, Fig. 1a) before they are finally eliminated through programmed cell death (Ballock and O'Keefe 2003; Forriol and Shapiro 2005; Shum and Nuckolls 2002). Unlike necrosis, apoptosis is an active ATP-requiring process that does not cause inflammation. Apoptotic cell death is characterised by typical morphological changes in the cell, such as chromatin condensation, DNA cleavage, cell shrinkage, nuclear and cell fragmentation and disassembly into membrane-enclosed vesicles called apoptotic bodies (Baynes and Dominiczak 2005; Hockenbery 1995; Kressel and Groscurth 1994; Lemasters 2005; Lodish et al. 2008). The key molecules of the apoptotic process are intracellular enzymes termed caspases; they cleave important cell components such as proteins of the nuclear lamina and cytoskeleton and activate specific DNases resulting in the fragmentation of genomic DNA (Baynes and Dominiczak 2005; Lemasters 2005; Lodish et al. 2008).

This study aims to determine the impact of fracture on cellular apoptosis of the growth plate in rats, an indication of physeal cell turnover hypothesised to be stimulated by fracture. Our work should improve the understanding of the effect of post-fractural reactions on the growth plate.

Animals in the experimental group sustained a mid-diaphyseal closed fracture of the left tibia and were extensively compared with control animals. Apoptosis of physeal chondrocytes was determined by using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL). The difference in the apoptosis rate of the proximal tibial growth plate between the fractured bone, the intact contralateral bone and the control bone was statistically evaluated.

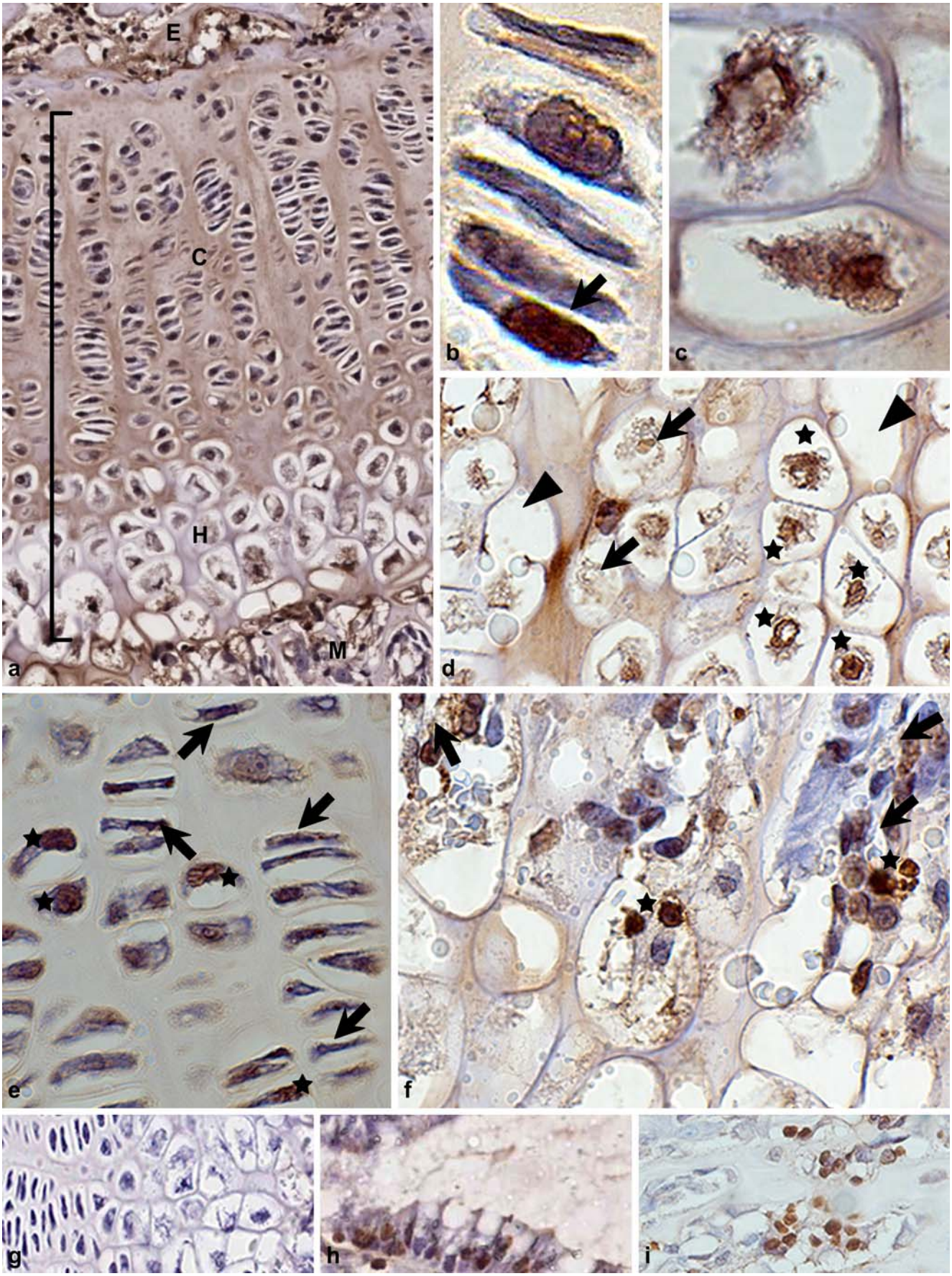
TUNEL is based on the specific TdT-catalysed binding of nucleotides to 3′-OH ends of nuclear DNA breaks (Gavrieli et al. 1992). Several previous studies have successfully used this assay to identify apoptosis in chondrocytes (Chrysis et al. 2002; Ploumis et al. 2004; Silvestrini et al. 1998; Zenmyo et al. 1996). Gavrieli and collaborators (1992) have established this method as a specific, simple and reproducible tool for the in situ detection of programmed cell death at a single-cell level, whilst also preserving tissue architecture and enabling the additional visualisation of apoptotic morphological changes.

Materials and methods

Animals

Bone samples were obtained from 56 male Sprague-Dawley rats (Division for Laboratory Animal Science and Genetics in Himberg, Medical University Vienna, Austria) aged 4 weeks and weighing 100–120 grams. All rats were caged in pairs with a light:dark cycle of 12:12 h. They received food and purified water treated with 37% hydrogen chloride (8.6 ml HCl per 20 l water) ad libitum. The rats were randomly distributed into two main groups, viz. the experimental and the control group, which were further divided into several subgroups, depending on the day post-fracture on which they were killed. Each subgroup consisted of 7 rodents. Anaesthetised experimental animals were subjected to a closed transverse mid-diaphyseal fracture of the left tibia by using a guillotine according to Bonnarens and Einhorn (1984). Postoperatively, rats were returned to their litters for nursing and activity at discretion. Painkiller (Rimadyl, Pfizer Animal Health, Louvain-La-Neuve, Belgium; 4-5 mg/kg body weight) was injected subcutaneously every 24 h to ensure analgesia. After 7 days, Rimadyl was discontinued and, subsequently, Novalgin (Aventis Pharma Deutschland, Bad Soden, Germany) was added to the drinking water (20 drops per 400 ml).

Fig. 1 Light microscopy of several growth plate sections stained by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) with haemalum counterstain. **a** Tibial growth plate in its total length. The columnar zone (*C*) can be easily distinguished from the hypertrophic zone (*H*). The full length of the growth plate, which separates the epiphysis (*E*) and the metaphysis (*M*) is marked (*bracket*). **b** A TUNEL-positive *brown* cell nucleus (*arrow*) lies in the columnar zone of the tibial growth plate. **c** Apoptotic nuclei of hypertrophic chondrocytes become condensed and fragmented. **d** Hypertrophic chondrocytes. Note the empty lacunae (*arrowheads*), lacunae with extracellular matrix and debris (*arrows*) and TUNEL-positive chondrocytes (*stars*). **e** Proliferative chondrocytes arranged in columns (*stars* apoptotic cells). In non-apoptotic cells, the nucleus is dyed *blue* (*arrows*). **f** Lowermost hypertrophic zone, adjacent to the metaphysis. Ingrowth of vessels (*arrows*) is accompanied by cells of mesenchymal origin with some of them being apoptotic (*stars*). Many lacunae that are devoid of a cell can be observed. **g** Negative control specimen: no TUNEL-positive cells. **h** Positive control: section of rat small intestine. **i** Internal positive control: bone marrow cells. The *brown* colouring of cells (**h**, **i**) indicates that they are apoptotic. Original magnification ×60)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Concomitantly, rats of the control group also received painkillers. Depending on the subgroup, the experimental animals were killed 3, 10, 14 and 29 days after fracture, together with control rodents, in order to allow comparisons of the age and weight between the animals of both main groups. The animals were initially narcotised with volatile anaesthetic (Forane, Abbott Laboratories, Kent, UK) and then killed by an intracardial Thiopental Sandoz (Sandoz, Kundl, Austria) injection. Subsequently, the left and right tibiae were harvested and cautiously dissected from soft tissue. An extension of the fracture callus into the growth plate never occurred.

All animal experiments were approved by the Austrian Federal Ministry of Science and Research (file number: BMBWK-66.010/0054-BrGT/2006).

Tissue preparation

Bones were fixed in 10% formaldehyde solution for up to 24 h, decalcified in EDTA, pH 7.0, for 2 weeks and embedded in paraffin after dehydration through ethanol and acetone. Longitudinal sections (4 μm thick) were cut, mounted on coated glass slides (Micro Slides x-tra adhesive, Surgipath Medical Industries, Richmond, Ill., USA), stored at 37°C for at least 10 h and finally incubated at 60°C for 1 h. Before proceeding with staining, the sections were routinely deparaffinised and hydrated through xylene and a graded alcohol series.

TUNEL assay

We detected apoptotic DNA fragmentation by using the TUNEL method, established by Gavrieli and collaborators (1992). For permeabilisation, tissue sections had to be incubated with 20 μg/ml proteinase K (Roche Diagnostics, Mannheim, Germany), for 15 min at room temperature and slides were then washed in phosphate-buffered saline (PBS) twice for 4 min each. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 30 min at room temperature. TUNEL staining was performed according to the manufacturer's protocol (TUNEL kit: In Situ Cell Death Detection Kit, POD; Roche Diagnostics). In detail, sections were covered with a mixture of TdT and fluorescein-labelled nucleotides and incubated in a humid atmosphere at 37°C for 1 h. The reaction was terminated by transferring the slides to PBS. Sections were then treated with the peroxidase-conjugated anti-fluorescein antibody solution (supplied with the TUNEL kit) at 37°C for 30 min and subsequently rinsed with PBS. Diaminobenzidine (DAB; 50 μl; Dako, Glostrup, Denmark) was used to produce a dark brown precipitate in the presence of peroxidase and thus to highlight nuclei with DNA fragmentation; it was applied directly onto tissue sections and left thereon at room temperature for about 1 min. The reaction had to be followed under a light microscope in order to ascertain the exact time required to wash out the substrate in PBS. The treated sections were counterstained with filtrated Mayer's haemalum solution for 10 s and then rinsed well with warm running tap water for 2 min. After dehydration in 50%, 70% and absolute ethanol for 1 min each and clearing in xylene twice for 2 min, tissue sections were coverslipped with GCL mounting medium (Sakura Finetek Europe, Zoeterwoude, The Netherlands).

The accuracy of the TUNEL method was evaluated by means of control specimens. Positive controls were sections of rat small intestine submitted to the same treatment as the tibial tissue sections. In addition, bone marrow cells were used as an internal positive control. In negative control sections, the enzyme TdT was omitted from the reaction mixture.

Quantification of apoptotic cells

For further tissue analysis, TUNEL-stained tissue sections were scanned with an Aperio ScanScope scanner (Aperio Technologies, Vista, Calif., USA) in order to create digital slide images. TUNEL-positive cells of the proximal tibial growth plate were counted manually by using an ImageJ Cell Counter (Kurt de Vos, University of Sheffield/ Academic Neurology, England), with part of the public domain Java image processing program ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda, Md., USA). Simultaneously, each nucleus was additionally evaluated with an optical microscope under 60-fold magnification by a second person. Cells were considered as TUNEL-positive when the nucleus of a chondrocytic cell was a brown, dark brown or black colour. Additionally, this nuclear staining had to show a crescent, annular or fragmented shape, or the staining pattern had to affect the whole nucleus, depending on the temporal phase of apoptosis that the cell was passing through at the time of anaesthesia (Gavrieli et al. 1992). The frequency of TUNEL-positive cells was measured separately in the columnar zone and the hypertrophic zone of the proximal tibial growth plate (Fig. 1).

Each zone was further subdivided into one central and two lateral regions. In each area (a total of six for each growth plate), 100 cells, regardless of colour and morphology, were manually and arbitrarily enumerated. Out of these 100 cells, TUNEL-positive cells were counted. The number of apoptotic chondrocytes per 100 cells was defined as the apoptosis rate in percent. The apoptosis rates of the three different regions (one central and two lateral regions) were finally averaged; hence, the mean apoptosis rate of the hypertrophic zone, the columnar zone and the entire growth plate was obtained.

Statistical analyses

SPSS version 15.0.1 (SPSS, Chicago, Ill., USA) was used for statistical data analysis and data presentation. All data are presented as medians (p25–p75 percentiles). Normality of data was checked by using the Shapiro-Wilk test.

Mann-Whitney *U* tests were used for two-group comparison (experimental group versus control group) because of non-normally distributed data. The Wilcoxon signed-rank test was performed to evaluate the discrepancy in the apoptosis rate between the fractured and the contra-lateral intact bone of each experimental animal. A potential disparity of the apoptosis percentage between the four subgroups (group day 3, day 10, day 14 and day 29) within the experimental group and the control group was tested by using the Kruskal-Wallis test with subsequently performed pairwise comparisons with Bonferroni corrections. Additionally, the Friedman test was performed for confirmation. The difference in the apoptosis rate between the hypertrophic zone and the columnar zone of the epiphyseal plate in one animal was evaluated by using the Wilcoxon signed-rank test.

A *P*-value of less than 0.05 was considered as being statistically significant.

Results

Morphology of growth plate

The growth plate can be found between the epiphysis and the metaphysis of a long bone. Several zones within the growth plate represent the life cycle of chondrocytes: proliferation and maturation take place in the columnar zone, whereas cell hypertrophy occurs in the hypertrophic zone. Ingrowth of vessels can be observed in the lowermost hypertrophic zone (Fig. 1).

Columnar zone versus hypertrophic zone

The amount of apoptotic cell bodies in the columnar zone of the growth plate was always higher than that in the hypertrophic zone, regardless of the group to which the bone belonged. The hypertrophic zone contained many empty lacunae of degenerated hypertrophic chondrocytes and lacunae with only debris or extracellular matrix (Fig. 1d,f). Such lacunae, which were devoid of nuclei, were not counted as apoptotic cell bodies as only those cells including a visible black or brown apoptotic nucleus were considered as TUNEL-positive.

Generally, apoptosis was spread uniformly over the full physeal width. An increase of apoptosis in a more lateral or central region was not observed.

Positive controls showed staining as expected. Negative controls lacked any TUNEL-positive cells (Fig. 1g–i).

Fractured bone versus contra-lateral bone

The percentages of apoptotic cells within the growth plates of the fractured and the contra-lateral bones of the experimental group are shown in Table 1. As seen in Fig. 2, the epiphyseal plate of the left fractured tibia showed a higher physeal apoptosis rate compared with the contra-lateral intact tibia. This was found to be statistically significant on all evaluated days.

The apoptosis rate of the chondrocytes at the growth plate of the fractured tibia increased continuously over time within the measured time interval, however, without statistical significance. We observed a maximum level of physeal apoptotic cells on day 29 (Fig. 2). The trend of a continuous increase of the apoptosis incidence over time could be described neither for the contra-lateral intact bone of the experimental group, nor for the control bone. The apoptosis percentage of both intact tibiae consisted more of an irregular rising and falling over time, without a significant difference in the apoptosis rate between individual days, with only one exception: the control bone showed a significant increase in chondrocyte apoptosis from day 14 to day 29. This was valid for the columnar zone (P=0.006), the hypertrophic zone (P=0.002) and the entire growth plate (P=0.003).

Fractured bone versus control bone

The evaluation of the difference between the growth plates of the left tibiae of the control and experimental group revealed a

Table 1 Summary of physeal apoptosis rate (in %) of the left and right tibiae of the experimental group; n=7, each. The median, the first and third quartile (in *parentheses*) and the *P*-value are shown

Day after fracture	Experimental group		*P*-value
	Left tibia (fractured)	Right tibia (not fractured)	
Day 3	21.00 (16.00–25.17)	14.33 (13.33–19.83)	0.018*
Day 10	22.00 (16.33–27.50)	16.50 (12.83–19.33)	0.043*
Day 14	22.17 (19.83–24.50)	16.50 (15.50–19.00)	0.018*
Day 29	25.00 (21.83–26.17)	15.33 (15.17–18.50)	0.018*

*P<0.05 with the Wilcoxon test

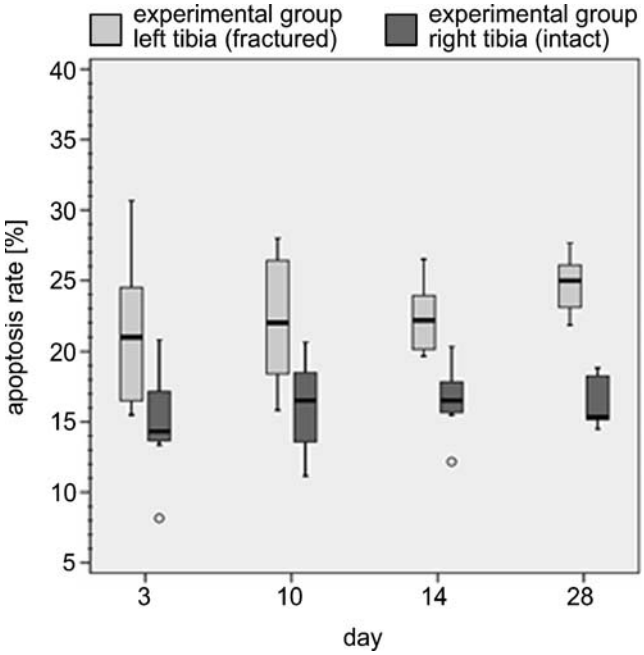

Fig. 2 Apoptosis rates (in %) of the fractured left (*light grey*) and unfractured right (*dark grey*) tibiae of the experimental group; n=7, each. The *x-axis* presents the number of days post-trauma on which the rats were killed, whereas the *y-axis* specifies the apoptosis rate in %. Significance (P<0.05) was reached on all days

significantly higher apoptosis rate in the fractured bone on days 10, 14 and 29, but not on day 3 (Fig. 3). Table 2 shows the incidence of apoptosis for both groups and statistically important differences between them.

When examining the apoptosis rate of the columnar and hypertrophic zones separately from each other (Fig. 4), the columnar zone of the left fractured tibia showed significantly higher (P<0.01) apoptosis percentages compared with the columnar zone of the control bone from day 10 onwards. This significance was only reached on day 14 for the hypertrophic zone.

Contra-lateral bone versus control bone

Interestingly, the right intact tibia of the experimental group never demonstrated a significantly higher physeal apoptosis rate compared with the unfractured left tibia of the control group. No significant difference was seen in the incidence of apoptotic death between both intact bones on day 3 (P=0.123), day 10 (P=0.522) and day 14 (P=0.055). However, day 29 revealed a significantly higher physeal apoptosis rate of the control bone (P=0.010).

Discussion

Bone overgrowth presents a clinically well-known paediatric phenomenon that occurs after bone fracture and whose underlying pathomechanism has not yet been clarified. The present study provides evidence that a mid-diaphyseal fracture in an animal model enhances chondrocyte apoptosis at the nearby growth plate.

In order to gain better insights into post-fractural reactions and alterations of the growth plate after the occurrence of a long-bone shaft fracture, this study has focused on the determination of physeal chondrocyte apoptosis in a rat model.

Rat model

Much of the knowledge regarding the cellular and molecular biology of fracture healing has been enlightened by investigations conducted on rats (Einhorn 1998; Tsiridis et al. 2007). Indeed, their bone development, with the formation of growth plates and primary and secondary ossification centres, resembles that of humans. The TUNEL-stained histological sections from our study have revealed that the cellular architecture of the growth plate is almost identical to that of human growth plates. In contrast to human bones for which the closure of the growth plate upon reaching skeletal maturity is responsible for the cessation of longitudinal bone growth, the growth plates of rats remain open, even in aged rats (Kilborn et al. 2002; Roach et al. 2003). Roach and collaborators have described morphological changes that occur in the growth plates of older rats and that would lead to deceleration and finally to

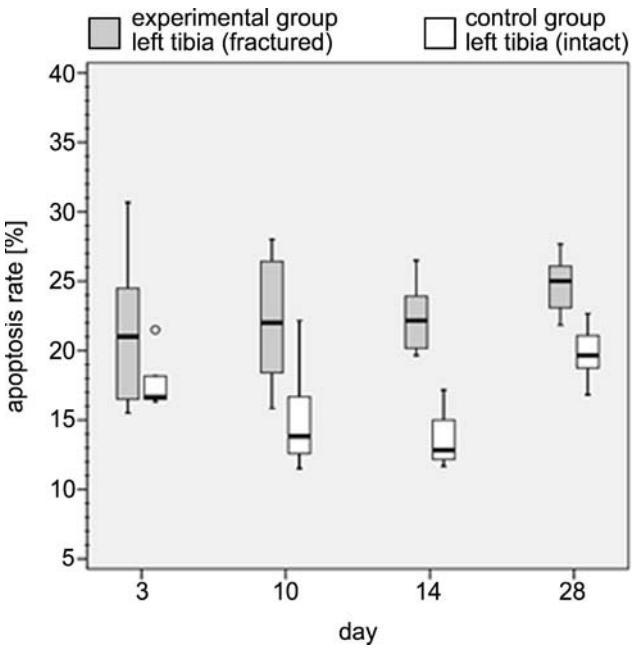

Fig. 3 Apoptosis rates (in %) of the fractured left tibia of the experimental group (*grey*) and the left tibia of the control group (*white*); n=7, each. Significance was reached on day 10 (P<0.05), day 14 and day 29 (both P<0.01)

Table 2 Summary of the physeal apoptosis rate (in %) of the left tibia of the experimental group and the left tibia of the control group; n=7, each. The median, the first and third quartile (in *parentheses*) and the P-value are shown

Day after fracture	Experimental group Left tibia (fractured)	Control group Left tibia (unfractured)	P-value
Day 3	21.00 (16.00–25.17)	21.00 (17.00–21.33)	0.465 .
Day 10	22.00 (16.33–27.50)	16.00 (13.67–20.33)	0.018* .
Day 14	22.17 (19.83–24.50)	14.67 (13.00–17.67)	0.002**
Day 29	25.00 (21.83–26.17)	24.00 (20.33–24.67)	0.004**

*P<0.05, **P<0.01 with the Mann-Whitney U test

the cessation of bone growth, despite the continued presence of the physis (Roach et al. 2003). In rats, the growth rate increases continuously during the first 5 weeks of life, followed by a decline until skeletal maternity is reached at the age of 11.5–13 weeks. After a period of highly reduced growth rate, rat bones stop growing at around the age of 26 weeks (Roach et al. 2003). During our experiments, all rats were aged 4–8 weeks and therefore demonstrated consistent and rapid bone growth throughout the whole experimental period.

Bonnarens and Einhorn (1984) developed a rat model for the production of a standard closed transverse experimental mid-shaft fracture by the use of a guillotine driven by a dropped weight. Investigations on 40 male Sprague-Dawley rats established that fractures were highly reproducible in terms of configuration and location and were accompanied by minimal soft tissue damage. Radiographic evaluation revealed that, in all 40 cases, a bicortical mid-diaphyseal femoral fracture had been produced and that, in 38 cases, the fracture was transverse. The authors reported uncomplicated fracture healing taking place in all rats (Bonnarens and Einhorn 1984). In our study, radiographic documentation of the fracture was abandoned because of the apparentness of the transverse closed fracture by confirmation in the operative finding.

To summarise, the rat seems to serve as an appropriate model for the study of bone fractures. However, the present study methods differ from clinical routine methods in the sense that, in human patients, fractures are stabilised by conservative or operative treatment, whereas the fractures

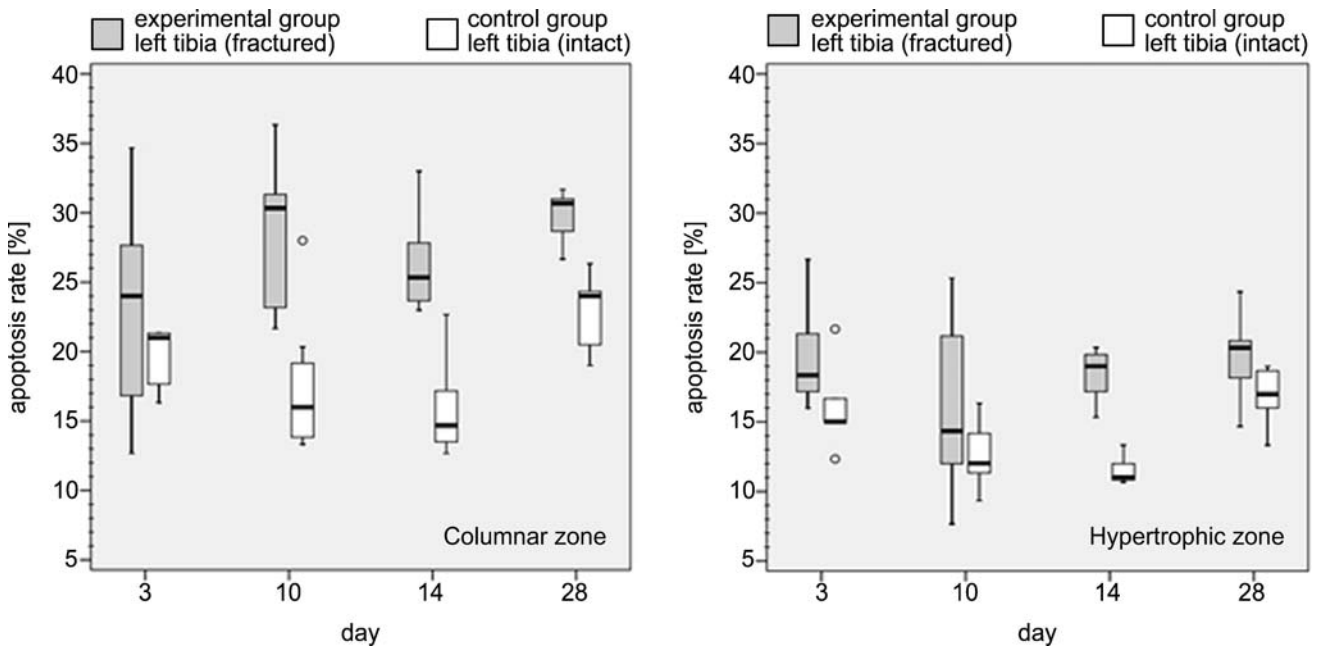

Fig. 4 Apoptosis rates (in %) of the fractured left tibia of the experimental group (*grey*) and the left tibia of the control group (*white*); n=7, each. Rates were analysed separately for the columnar zone (*left*) and the hypertrophic zone (*right*) of the growth plate. Concerning the columnar zone, significance was reached on day 10 (P=0.006), day 14 (P=0.002) and day 29 (P=0.004), whereas the apoptosis rate of the two hypertrophic zones (of the fractured bone and of the control bone) showed significant difference only on day 14 (P=0.002)

of the rats of this study were not immobilised. Hence, the remodelling capacity of growing bones was utilised and maximum growth plate stimulation was achieved in order to demonstrate the stimulative effect of a fracture on the adjacent growth plate.

Fate of hypertrophic chondrocytes

Chondrocytes of the human growth plate experience a life cycle that ranges from resting to proliferating before becoming hypertrophic cells and finally undergoing programmed cell death (Ballock and O'Keefe 2003; Forriol and Shapiro 2005; Shum and Nuckolls 2002), with their apoptotic bodies supposedly being phagocytosed by osteoclasts (Bronckers et al. 2000). Until now, opinions regarding the ultimate fate of terminally differentiated hypertrophic chondrocytes have been conflicting. Several studies have confirmed the occurrence of chondrocyte apoptosis both in vitro (Cheung et al. 2003; Gibson et al. 1997) and in vivo (Chrysis et al. 2002; Ploumis et al. 2004; Zenmyo et al. 1996) and some review articles refer to apoptosis as being the most likely mechanism of hypertrophic chondrocyte deletion (Ballock and O'Keefe 2003; Forriol and Shapiro 2005; Shum and Nuckolls 2002). However, other authors refer to variants of physiological cell death showing an ultrastructural morphological appearance different from the hallmark features of classical apoptosis (Roach et al. 2004; Roach and Clarke 1999, 2000; Shapiro et al. 2005). Roach and collaborators coined the term chondroptosis for the description of non-apoptotic physiological cell death found in chondrocytes (Roach et al. 2004), whereas Shapiro and colleagues preferred to use the term autophagy (Shapiro et al. 2005). Unlike apoptosis, chondroptosis/autophagy is not mediated by caspases but involves lysosomal proteinases. Finally, the complete self-destruction of the chondrocyte and the appearance of an empty lacuna can be observed. As chondroptosis is not dependent on phagocytosis, it may serve as an alternative mechanism of cell deletion without inflammation for situations in which phagocytosis of apoptotic bodies would be difficult (Roach et al. 2004; Shapiro et al. 2005)

With regard to our study, which as aimed at determining the incidence of post-fractural physeal apoptosis by means of the TUNEL method, the disagreement regarding the ultimate fate of physeal hypertrophic chondrocytes (apoptosis versus chondroptosis) has no influence, since TUNEL is not capable of distinguishing apoptosis from chondroptosis and shows positive reactions during both processes (Roach et al. 2004).

Moreover, the hypothesis of chondroptosis could feasibly explain the appearance of intact, empty and only debris-containing lacunae that we could constantly observe at the lowermost hypertrophic zone of the growth plate (Fig. 1d,f). In TUNEL-stained sections of growth plate, Ploumis and co-workers (2004) also discerned a large number of terminal lacunae that were devoid of cells. They provided another possible explanation for this by suggesting that these lacunae appear because of the loss of degenerating hypertrophic chondrocytes that are discarded during the staining process.

Apoptosis detection by the TUNEL method: a controversial subject

Apoptosis can be detected by a variety of techniques such as annexin V labelling, immunohistochemistry for caspases and apoptosis-related proteins, detection of DNA fragmentation by either the in situ nick-end labelling method (TUNEL) or DNA agarose gel electrophoresis with the occurrence of a DNA ladder pattern and the identification of characteristic ultrastructural morphological changes under electron microscopy, which remains the most secure way to verify apoptotic death. In our study, we have used TUNEL, established by Gavrieli and colleagues (1992) and well known as being an easily applicable (Ploumis et al. 2004) and specific method (Gavrieli et al. 1992).

Some researchers have questioned the accuracy of TUNEL and postulate that false-negative and false-positive results lead to underestimation and overestimation, and to ambiguous and widely varying results (Aigner 2003; Roach et al. 2004). For example, Chrysis and collaborators (2002) have evaluated the apoptosis rate in growth plates of rats and found that one quarter of the hypertrophic chondrocytes show morphological characteristics of apoptosis, although the number of TUNEL-positive cells is much lower. Consequently, the data concerning the extent of apoptotic cells in the growth plate are controversial and vary widely; additionally, investigations have been carried on different animal species at different ages (Ploumis et al. 2004). Furthermore, growth rates and physeal activities differ depending on the origin of the epiphyseal plate. For instance, the human growth plates that are closer to the knee (distal femur, proximal tibia) show a higher level of activity than their opposite plates and contribute the most to the longitudinal growth of the lower extremities (Forriol and Shapiro 2005). Some studies estimate that the number of physeal apoptotic cells may be low at a one-digit percentage level (Chrysis et al. 2002; Ploumis et al. 2004; Zenmyo et al. 1996). Another study has found that up to a quarter of physeal cells undergo apoptosis, dependent on the age of the animal (Aizawa et al. 1997).

Our work has revealed the large amount of physeal chondrocytes in apoptotic stages, reaching up to 25%, even in the control group. Although this result disagrees with several of the above-mentioned studies, the comparison of the apoptosis rate between the experimental and control group remains possible, as all growth plates have been

investigated by using the same intra-experimental technique. Hence, the conclusion that the fractured bone demonstrates a significantly higher incidence of physeal apoptosis from day 3 onwards when compared with the contra-lateral bone, and from day 10 onwards when compared with the control bone, is still valid. The aim of our study has been to compare the fractured, contra-lateral and control bones and not to determine the exact apoptosis rate in the growth plate. This apoptosis analysis should be seen more as a qualitative than a quantitative study. Therefore, the TUNEL apoptosis rate has not been verified by additional methods, such as immunohistochemistry for caspases and apoptosis-related proteins, DNA electrophoresis or the evaluation of ultrastructural changes by the use of electron microscopy, as some other authors demand (Aigner 2003; Roach et al. 2004) and have carried out in their own apoptotic studies (Chrysis et al. 2002; Ploumis et al. 2004; Zenmyo et al. 1996).

Furthermore, we have not used additional staining of specific chondrocyte markers for the identification of cell type or the biochemical distinction between proliferating and hypertrophic chondrocytes, as TUNEL staining perfectly preserves the histology of the growth plate. Chondrocytes can thus be easily identified. Only growth plate cells that form columns have been selected and assigned to the columnar proliferating zone. The resting zone of the epiphyseal plate has not been evaluated. Enlarged cells surrounded by a lacuna are considered to be hypertrophic cells. Cells in the so-called transition zone that have not been definitely classified as columnar or hypertrophic cells have not been counted. In the lowermost hypertrophic zone with vascular ingrowth (Fig. 1f), cases of uncertainty arose as to whether an apoptotic cell in an open lacuna belonged to the hypertrophic cells or was of mesenchymal origin; such cells were skipped and not evaluated.

The manual counting of the cells turned out to be extremely time-consuming. It also involved the risk of bias. An automated counting by means of appropriate software was not possible, since the colour and morphological changes had to be considered and the intensity of counterstaining varied in each staining run.

Increased growth plate apoptosis after fracture

Our study reveals that the number of programmed cell deaths in chondrocytes is significantly higher in the growth plate of fractured bones compared with both the contra-lateral intact bone and the control bone. This result is consistent with an electron microscope study of Papavasiliou (2002) who showed that, in a rat model of femur-shaft fracture, physeal chondrocytes differentiate faster and sooner become apoptotic in the fractured bone compared with the contra-lateral bone; thus, bone formation is enhanced.

Furthermore, our work has demonstrated the lack of a significant increase of apoptotic cells in the intact contra-lateral bone when compared with the control bone. Therefore, we conclude that the fracture influences chondrocyte apoptosis of the adjacent growth plate via a local effect. A systemic effect of the fracture would have additionally influenced the contra-lateral epiphyseal plate.

As described in the literature, the death and removal of terminally differentiated hypertrophic chondrocytes provides space for the ingrowth of new blood vessels that bring bone cell precursors, osteoblasts, osteoclasts and chondroclasts (Ballock and O'Keefe 2003; Forriol and Shapiro 2005; Rauch 2005; Shum and Nuckolls 2002). Thus, cartilage resorption and bone replacement, which are essential for longitudinal bone growth, take place and are associated with the programmed cell death of hypertrophic chondrocytes. These findings are in agreement with our study as we report a stimulative effect of the fracture on apoptosis of physeal chondrocytes.

Age-dependency of apoptosis of physeal chondrocytes

This work is consistent with previous data stating that, under normal growth conditions, the number of physeal apoptotic cells increases with age (Aizawa et al. 1997; Chrysis et al. 2002; Ploumis et al. 2004), whereas the number of proliferating chondrocytes decreases (Aizawa et al. 1997). Regarding control animals, this study shows a statistically significant increase of chondrocyte apoptosis from day 14 to 29, whereas the apoptosis rate of the fractured bones tends to rise over time without statistical significance.

Columnar versus hypertrophic chondrocytes

Another finding of our work is the constantly higher number of TUNEL-positive cells in the proliferating zone when compared with the hypertrophic zone. This leads to the assumption that the overall increase of apoptotic cells in the fractured bone is primarily driven by the rise of apoptosis in the columnar zone. Some studies have previously demonstrated TUNEL-positive cells in the hypertrophic zone and also in the resting and proliferating zones (Aizawa et al. 1997; Chrysis et al. 2002; Ploumis et al. 2004), but with the highest rate of apoptosis in the hypertrophic zone. Given that empty lacunae and lacunae containing only debris or extracellular matrix have not been counted as apoptotic cell bodies in our study, the higher incidence of apoptosis in the columnar zone might be partly caused by this kind of cell counting. Moreover, some apoptotic hypertrophic cells might have been lost during the staining process. The conclusion that the rate of physeal cellular deletion must be the same as the rate at which the

cells proliferate (Shapiro et al. 2005), combined with the assumption that bone fracture stimulates the proliferation of growth plate chondrocytes, serves as a comprehensible explanation for the higher apoptosis rate, even among proliferative chondrocytes. Ploumis and colleagues (2004) have also assumed that DNA fragmentation starts early in the process of chondrocyte differentiation.

This innovative work has successfully demonstrated that bone fracture during growth affects the growth plate. Thus, the physeal apoptosis rate of the fractured bone is significantly higher in comparison with the contra-lateral intact bone (valid for all evaluated days) and with the control bone (valid from day 10 onwards). The lack of a significant difference between the intact contra-lateral and the intact control bone suggests that the local effect on the adjacent physis exceeds the systemic effect that would additionally influence the contra-lateral epiphyseal plate.

The type of local effect that leads to post-traumatic elevated programmed cell death remains unclear. The influence of certain cytokines and hormones, such as tumor necrosis factor-α and fibroblast growth factors, the down regulation of parathyroid hormone-related peptide (a known potent inhibitor of chondrocyte apoptosis; Ballock and O'Keefe 2003) or a higher chondrocyte proliferation rate may be considered as possible stimuli for programmed cell death occurring more often in physeal chondrocytes after fracture. The last-mentioned supports the idea that an increased number of hypertrophic chondrocytes might lead to the release of more matrix vesicles containing alkaline phosphatase, an enzyme that increases the concentration of phosphate ions necessary for the calcification process. Phosphate is suggested to trigger apoptosis by promoting mitochondrial membrane permeability transition, leading to the mitochondrial release of cytochrome c (Ballock and O'Keefe 2003). Nevertheless, the above possibilites for the exact stimulus leading to the increased apoptosis rate observed after fracture remain hypotheses that require additional research.

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