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Histological and biological changes in the epiphyseal plate during fracture healing

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Abstract The alterations that the epiphyseal plate undergoes during fracture healing are well documented microscopically, yet there are no reports in the literature which discuss the cellular and molecular changes that accompany this process. We studied fracture healing in 49 Wistar rats (5 weeks old) in which we inflicted a fracture to the distal third of the femur of the right hind leg (experimental side). The rats were killed 2 weeks later, and we dissected both hind legs from the hip joint to the knee joint, detaching all the surrounding soft tissues. We manually detached the distal epiphyses and the epiphyseal plates from both femurs. A piece of the epiphyseal plate was removed from the epiphyseal side of the femurs. In 25 animals, we analyzed the DNA content. In 8 animals, the specimen was studied under an electron microscope, and in the remaining 16 animals, the control and experimental sides were studied histologically. We found that healing was accompanied by an increase in DNA content, by a change in cellular activity, and by greatly accelerated apoptosis.

Key words Fracture healing · Epiphyseal plate

Introduction

Since the time of Hippocrates it has been realized that in children, reduction of a fracture of the femur should not be perfect, because this would result in a longer limb on the affected side. More than a century ago, Bryant[2] described the method for femoral fracture treatment, stressing that overlapping on the side of the fracture (and thus an initial shortening of the leg) is very important to avoid elongation of the affected side. This method of inducing healing of the fracture mainly restores axial deformities, as opposed to rotational ones. The restoration is more obvious when the fracture is near the epiphyseal plate.

During the past 50 years, many authors have reported that the overlapping of femoral fracture ends in children is a helpful means of restoring final leg lengthening.[1,3,5] The success of this measure was ascribed to the effects of the epiphyseal plates but the exact mechanism by which fracture healing close to the growth plate influences chondrocytes is unknown. To our knowledge, no one has studied the molecular and cellular basis of the healing-induced stimulation ascribed to the epiphyseal plate. Only a handful of experimental studies dealt with changes in the different cellular layers (zones) of the epiphyseal plate.[10] In all those studies, the authors agreed that there is a thickening of the zone of maturity, probably resulting from ischemia.[9,11] There is no mention of changes in cellularity or of molecular changes in the proliferating zone. The purpose of our study was to clarify the function of the epiphyseal cartilage cells (including the epiphyseal plate cartilage cells) in the proliferation zone by studying such cells microscopically and at the DNA level.

Materials and methods

Five-week-old male Wistar rats ($n = 49$) were housed at a constant temperature (22°C) with a 12-h light-dark cycle, and allowed water and food ad libitum. The experiments outlined below were conducted in accordance with the Guidelines for Animal Experimentation at the Aristotelian University of Thessaloniki.

With the animals under general anesthesia, we used mosquito forceps to trap and twist the bone and thus create a comminuted fracture of the right hind femur. We used a lateral approach to create the fracture in the distal third of the femur (not including the growth plate), where the quadriceps femoris and hamstring muscles are attached by tendons, because exposure of

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the bone in this region is easy and the incision is nearly bloodless.

The wound was closed with one absorbable stitch, and the animal was allowed to recover and to move freely about the cage. All animals began using the operated leg on the sixth day after surgery. Two weeks postoperatively, the animals were killed, and both hind legs were dissected at the hip level. The femoral bones from the hip joint to the knee joint were prepared after removal of the soft tissues, at which point we noted richly woven bone on the fractured side. The distal epiphysis of each femur was then visually identified and manually detached from the metaphysis via the epiphyseal plate. Our specimens therefore included the proliferating zone, with part of the maturing zone, as the other zones remained with the metaphyseal part of the plate.

In 33 of the 49 rats, a cartilagenous specimen of the epiphyseal side of the right (experimental) and left (control) epiphysis was removed. In 8 animals, the specimens were prepared for study under an electron microscope. In 25 animals, the DNA content of these cartilagenous specimens was studied in two ways: in 6 animals, pure DNA was extracted by the phenol-chloroform method and measured as described by Teixeira et al.,[8] and in the remaining 19 animals, the amount of DNA was assessed indirectly by fluorimetric methods.[7] Briefly, the specimen as a whole was ground in a solution containing Hoechst 33258 dye (which binds only DNA by intercalating in the double helix), and the fluorescence of the mix was then determined in a fluorimeter. As the fluorescence emission value is proportional to the amount of DNA in each sample, the DNA amount (in micrograms) of each sample was determined by comparing its fluorescence emission with that of samples containing known amounts of DNA.

In 16 of the 49 rats, the right (experimental) and left (control) femurs were demineralized, and slides were prepared which included the epiphysis, epiphyseal plate, and metaphysis. The slides were stained with hematoxylin and eosin and prepared for histological examination under an optical microscope.

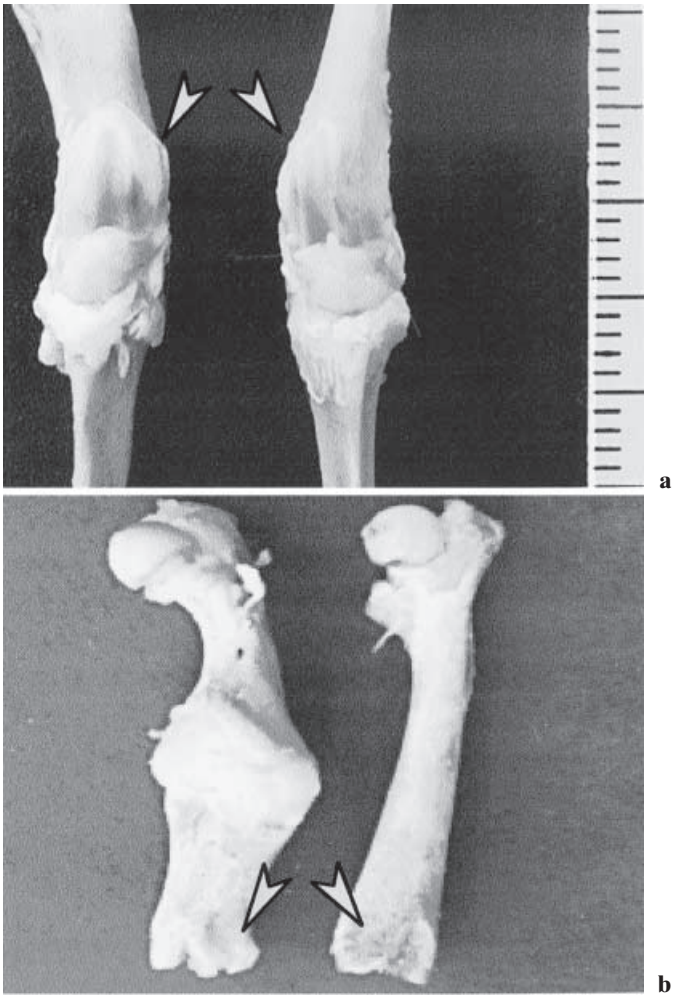

Fig. 1. a Gross specimen of the distal part of the femur and the proximal part of the tibia. The femur on the *right* (metaphysis and condyles) is larger (indicated by *arrowheads*). **b** The metaphysis on the right is broader and its ridges are shallower (*arrow heads*). The photograph was taken 2 weeks postoperatively; the distal part of the femur has become elongated and thus the fracture has migrated from the distal third toward the middle portion of the bone

Results

Histology

Macroscopically, the thickness of the right femur distal to the fracture was greater than that of the control distal femur (Fig. 1a,b compare right-side [5-mm] and left-side [3-mm] images). This difference was readily apparent when the bones shown in Fig. 1 were examined histologically (Fig. 2a,b corresponds to the left and right bones shown in Fig. 1a,b). After the epiphysis was, detached the remaining epiphyseal plate on the metaphyseal side showed shallow notches on the experimental side and deep notches on the control side (Fig. 1b). With the camera at 5× magnification, we observed that the diameter of the epiphysis of the right femur was much larger than that on the control (left) side. This was expected, because one result of a fracture is an increase in blood supply which in turn increases the activity of the ossification center.[9] Because of this enlargement of the epiphysis of the right femur, the folds of the epiphyseal plate on the right side were smooth, but on the left they were jagged (Fig. 2a,b). At higher magnification (25×), the above finding was more pronounced. We also observed that the right (experimental) epiphyseal

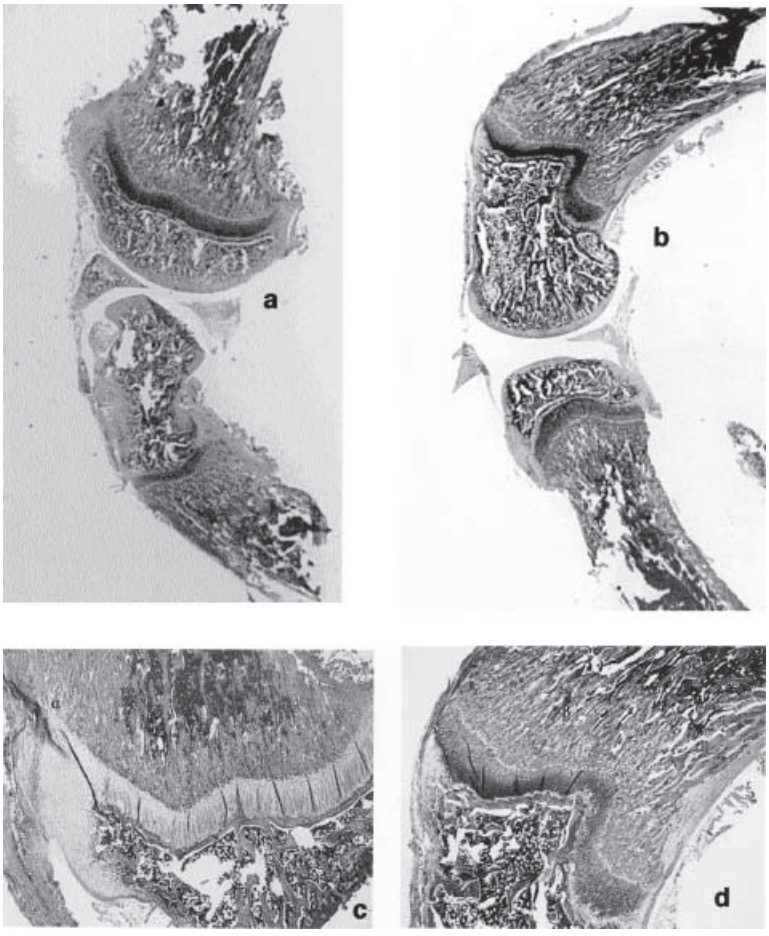

Fig. 2. **a** Under low magnification, the right distal femoral epiphysis (experimental side) appears thicker than the left (control), and the folds of the epiphyseal plate are smoother compared with the jagged appearance of the folds in the control epiphyseal plate. **b** The same findings are seen at a higher magnification. **a** and **b** ×5; **c** and **d** ×25

plate was thicker than the control plate (Fig. 2c,d). At a yet higher (45×) magnification, the proliferating zone of the epiphyseal plate on the experimental side was thicker and denser than that on the control side (Fig. 3a), while the hypertrophic zone appeared to be normal. Toward the metaphyseal area, the vascularization zone in samples from the right femur was thicker and contained greater numbers of giant osteoblasts and osteoclasts than the control. On additional (7×) magnification, it was clear that the interface between the resting and proliferation zones showed significant differences, as the chondrocytes of the resting zone clearly showed mitotic activity in the experimental, but not in the contol samples (Fig. 3b, left and right panels, respectively).

Electron microscopy

Under normal circumstances, in the epiphyseal plate, as one moves from the epiphysis to the metaphysis, one can distinguish the following four zones: resting cartilage, the proliferating zone, the maturing zone, and the calcifying cartilage zone. The cartilagenous cells move from the resting to the proliferating phase while pushing the cells of the maturing zone into the calcifying zone, where they eventually die by apoptosis. Therefore, an electron microscopic study of the cells in the proliferating zone of an epiphyseal plate shows changes from cell to neighboring cell, eventually resulting in the appearance of the apoptotic population.

We examined 230 sections per side from epiphyseal plate specimens by electron microscopy. As expected, in specimens from the control side, cells moving from the proliferating zone to the maturing zone showed gradual changes in the nucleus and the surrounding cytoplasm, consistent with apoptosis (data not shown). However, when we examined specimens from the right (experimental) side, we immediately noted that the change from normal to apoptotic cells was acute and occurred very rapidly from one cell to the next (as evidenced by the appearance of pyknotic nuclei, the size and shape of the nucleus, and the degeneration of the cytoplasm, as well as by the larger number of glucagon pools and the appearance of filopodia) (Fig. 4).

Cell death was also observed in sections from the control specimens. The reason that no data are pre-

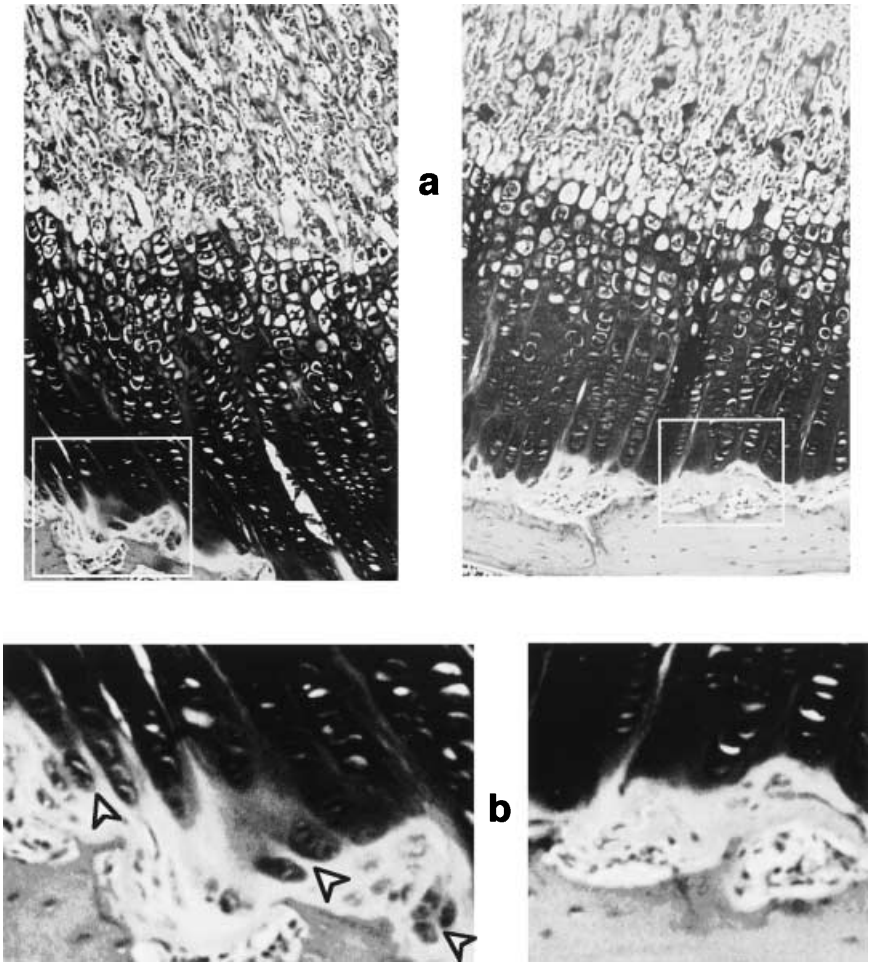

Fig. 3. **a** At 45× magnification , the vascularization and proliferation zones are thicker and the proliferation zone is denser in the fractured side than on the control side. **b** When we focused on the cells in the proliferation zone, significant cell division was taking place in the resting zone of the experimental side, while no cell division was taking place in the resting zone of the control side (*arrowheads* point at groups of dividing cells). **a** ×45; **b** ×52

sented here from the controls is that, as apoptosis in the controls was gradual, it would be impossible to show it in a single image (rather, a series of 20–30 images would have to be presented). In contrast, apoptosis in the experimental sections was dramatic, occurring from cell to neighboring cell (Fig. 4).

DNA content

We wanted to determine the molecular mechanism underlying the observed changes in the femur during fracture healing (Figs. 1 through 4) and others. We used DNA content as a measure of the activity of the epiphyseal plate. We measured the DNA in each specimen by fluorimetric methods. After accounting for animal size and normalizing for the control side (i.e., taking the ratio between experimental and control results), we observed that the DNA content in the cells of the epiphysis on the experimental side was up to eight times higher than that in the control side (Fig. 5). The median value of DNA recovered from the control side was half that recovered from the experimental side (0.9 and 2.2 μg DNA/mg of tissue, respectively; $P < 0.02$).

Discussion

The effects of fracture healing on the epiphyseal plate have been studied by many.[6,9,11] We studied the effects of fracture healing on the epiphyseal plate both macroscopically and microscopically. We found that, in gross specimens, the epiphysis and the metaphysis on the fracture side, were larger than those in the controls. When we examined the epiphysis, metaphysis, and the epiphyseal plate under low magnification, we found them to be almost twice as large as the control ones. In addition, we found that the folds of the epiphyseal plate (which appeared ribbon-like) were much shallower on the experimental side than on the control side. Higher magnification showed that the macroscopic differences were caused by a much larger and thicker proliferation zone on the right (experimental) side, with the vascu-

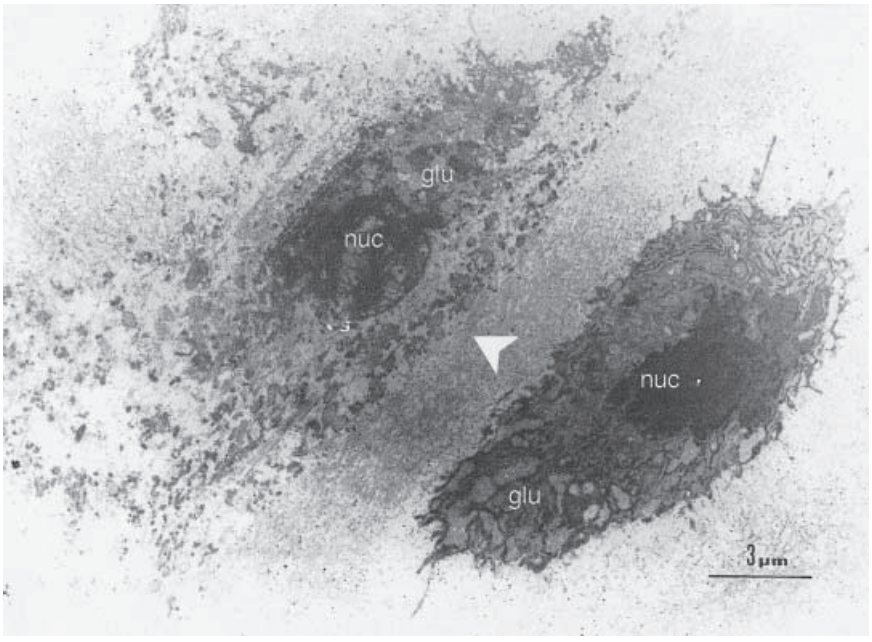

Fig. 4. Electron micrograph of two neighboring cells (specimen taken from the experimental side). A healthy-looking cell is next to a cell that is actively undergoing apoptosis, as evidenced by the pyknotic nucleus and the fragmented membrane. *nuc*, Nucleus; *glu*, pools of glucagon. *Arrowhead* indicates the transition from one cell to the next

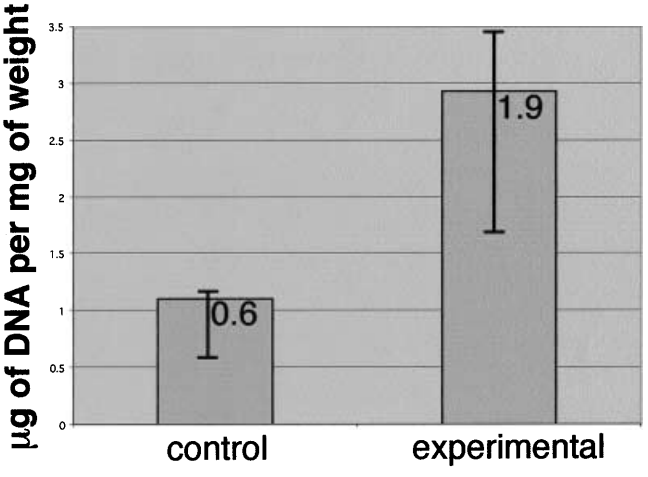

Fig. 5. Plots of the amount of DNA (in micrograms per milligrans of weight of tissue) recovered from each epiphyseal plate against the number of control and experimental sides in the animals, after normalizing for the weight of recovered tissue. *Bars* SDs in each set

larization zone being broader on the experimental side.

Our observations that the epiphyseal plate becomes thicker after femoral fracture, agree with those of previous authors.[6,10] Earlier reports have ascribed this difference in size to a tendency for the cells in the proliferation zone to have higher activity. Our electron microscope studies showed that generally after fracture, the cells in the epiphyseal plate of the right femur were changing from normal to apoptotic much more rapidly than those on the control side. In addition, the amounts of DNA recovered from the epiphyseal plate on the experimental side were much higher the amounts recovered from the controls. Therefore, the reason for the macroscopic differences between the control and experimental sides observed during fracture healing appears to be that the number of cells in the proliferation zone is much higher during fracture healing, and that those cells are being pushed from the epiphysis to the metaphysis at a much faster rate than under normal circumstances. As a result of this process, new bone formation is faster on the metaphyseal side, and the elongation of the fractured tubular bone appears to occur more rapidly than on the normal side.

Why do the cells of the epiphyseal plate of the fractured femur grow faster than their control counterparts? We speculate that bone-specific chemokines, such as the recently discovered osteogenic protein-1,[4] may be specifically secreted at the point of fracture as a result of the fracture damage, and may be specifically involved in triggering the healing responses we have documented here. It will be of interest to investigate the effect of such proteins on normal bones.

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References

1. Blount WP. Fractures in children, 2nd edn. Baltimore: Williams and Wilkins; 1964.
2. Bryant T. The practice of surgery: a manual, vol. 2. London: Churchill; 1872.
3. Burton VW, Fordyce AJW. Immobilization of femoral shaft fractures in children aged 2–10. Injury 1973;4:47–50.
4. Cook SD, Baffes G, Wolfe MW, et al. The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. J Bone Joint Surg Am 1994;76:827–8.
5. Griffin PP, Anderson M, Green WT. Fractures of the shaft of the femur in children. Orthop Clin North Am 1972;3:213–24.
6. James B, Wray MD. Acute changes in femoral arterial blood flow after closed tibial fracture in dogs. J Bone Joint Surg Br 1964: 1262–8.
7. Labarca C, Paiger K. Ultrasensitive method for measurement of DNA. Anal Biochem 1980;102:344–52.
8. Teixeira CC, Hatori M, Leboy RS, et al. A rapid and ultrasensitive method for measurement of DNA, calcium and protein content and alkaline phosphatase activity of chondrocyte cultures. Calcif Tissue Int 1995;56:252–6.
9. Trueta J. Studies of the development and decay of the human frame. London: Heiman Medbooks; 1968.
10. Trueta J, Amato VP. The vascular contribution to osteogenesis. J Bone Joint Surg Br 1960;42:571–9.
11. Yabsley RH, Harris WR. The effect of shaft fractures and periosteal stripping on the vascular supply to epiphyseal plates. J Bone Joint Surg Br 1965;45:551–6.