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# Ultrastructural aspects of human nonunion

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**Summary.** A histological study on the tissue of nonunion of tibias of two young patients was performed to evaluate the ability of cells to start the mineralization of the matrix. The observations can be summarized as follows: 1) Tissue vessels often appear occluded by thrombotic material; 2) Fibroblasts and chondrocytes found in the nonunion tissue seemed normal, with a good secretion apparatus; 3) The cell membranes were able to produce matrix vesicles; 4) Matrix vesicles and cell membrane looked positive to ALPase reaction, 5) Hydroxyapatite crystals could be observed in the cell matrix or inside matrix vesicles.

It may be concluded that cells populating nonunion tissue are well equipped to induct the mineralization of the matrix, but the absence of a blood supply, enough to bring them a normal calcium amount, is the real reason for the nonunion.

**Key words:** Human nonunion, Human pseudoarthrosis, Ultrastructure

## Introduction

The spontaneous trend of bone fractures to repair themselves may be prevented by adverse circumstances that may retard or completely prevent the fracture healing. This last event is called «nonunion» or «pseudoarthrosis».

Factors inhibiting normal bone fracture healing are well known through several clinical and experimental studies; such as the micro or macro motion between the bone fragments (Yamagishi and Yoshimura, 1955; Ashhurst, 1986; Page et al., 1986); large periosteal loss (Connoly et al., 1977; Jacobs et al., 1981); muscle interposition (Altner et al., 1975; Alpar, 1984); pharmacological influences (Rohe et al., 1980a,b), etc.

Morphological studies on human aseptic nonunion, performed on bioptic tissue (Urist et al., 1954; Judet et al., 1958; Bohr, 1971), or on autoptical fragments (Sevitt, 1981b), showed that a fibro-cartilaginous tissue or osteoid tissue developed between the bone stumps instead of primary bone or definitive osteonic bone.

In order to evaluate if the cells of the nonunion tissue are able or not to start mineralization into the matrix they have produced, and which type of mineralization is present, light and electron microscopic pictures of the nonunion tissue of the long bone, removed from two young patients, are described.

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## Materials and methods

### *Clinical cases*

The fragments examined were withdrawn from two male patients (18 and 23 years old) affected by comminuted fracture of the tibia at the middle third, without involvement of the fibula, and treated by a uniaxial fixation device (Castaman, Italy) a week after injury.

Both patients obtained immediate passive and active motion of the hip, knee and ankle, while walking was possible in two weeks with one crutch.

At four-month follow up, radiographic findings did not demonstrate any periosteal calcification and at eight-month follow up both patients showed a typical radiographic pattern of nonunion and were operated on by osteotomy of the fibula and compression of the fracture by the shortening of the external fixation device.

During the operation the pseudoarthrosis tissue was removed by a 5 mm biopsy cannula under fluoroscopic control. Macroscopically this tissue appeared as a homogeneous connective tissue with elastic consistence.

The nonunion tissue was fragmented. No fragment was decalcified, because of the scarce mineralization.

*Light microscopy*

Some tissue was fixed in 10% formaldehyde for light microscopy. The fragments for light microscopy were dehydrated in ascending concentrations of cold ethanol and after a 20-minute treatment with benzene were embedded in paraffin at 60° C. Six μm sections were obtained and then stained with Eosin Hematoxilin or Giemsa stain.

The slides were then observed and photographed on a Leitz Orthoplan.

*Electron microscopy*

For ultrastructural morphology some other small fragments were fixed in cold 4% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2, for 6 hours at 4° C.

To evaluate alkaline phosphatase (ALPase) activity some other fragments were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, at 4° C for one hour. The fragments were then incubated in 0.03 M tris HCl buffer containing 20Mm beta-glycerophosphate 4mM $MgSO_4$ and 2mM lead citrate. The final pH was 9.2. The incubation was performed at room temperature for 15 minutes (Mayahara et al., 1967).

Control specimens were prepared by incubating 1) in medium deprived of beta-glycerophosphate (substrate), 2) in full medium deprived of MgSO4 (activator), 3) in full medium with 10mM levamisole (inhibitor of ALPase).

All fragments prepared for electron microscopy were postfixed in 1% $OsO_4$ solution in 0.1M cacodylate buffer at pH 7.2, at room temperature and then dehydrated in ascending concentrations of cold ethanol, passed through cold propylene-oxide for 20 minutes and embedded in epoxy resin.

The semi-thin sections (0.5μm) were stained with 1% toluidine blue in order to observe them with the light microscope.

The thin sections were stained with uranyl-acetate, sodium-bismuth (Riva, 1974) or uranyl-acetate, and lead-citrate and were examined with a Jeol 100 electron microscope.

## Results

Light and ultrastructural findings were similar in both patients and so can be described together.

On light microscopic study very different pictures were observed: there were some necrotic fragments of bone belonging to fractured bone, and connective and cartilage tissue.

**Fig. 1.** The nonunion tissue is composed of connective tissue (thin arrow), cartilage (large arrow), and fragmented osteoid-like trabeculae (arrowhead).ide of x 2.5

Inside the connective tissue fragmented osteoid-like trabeculae were frequent (Fig. 1).

A lot of blood vessels, principally capillary vessels, were present in the tissue: they often appeared free of blood cells (Fig. 2), and were occluded by thrombi at various organization stages.

Cartilage cells had a hypertrophic aspect and frequently presented degenerative aspects.

Pericellular matrix did not show a homogeneous reactivity to staining solutions: the pericellular halo was frequently evident and interterritorial matrix had a motly aspect (Fig. 3).

At ultrastructural examination, fibroblasts were elongated, their cytoplasm was very well stained and presented a well developed secretion apparatus. Some were more enlarged and presented a low stainability (Fig. 4).

The cell membrane of either fibroblasts or osteoblasts presented frequent evaginations and a lot of vesicles either outside the cell membranes or inside the interterritorial matrix.

The matrix vesicles were sometimes filled by strongly electronopaque needle-like particles of 80 Å in diameter and about 1500 Å long that are the morphological representation of hydroxyapatite crystals. The electronopaque needle-like particles frequently collected in packs either inside vesicles or in the pericellular matrix around collagen fibrils (Fig. 5).

The vesicles were mainly stored on the cell membrane face at the opposite side from capillary vessels. Only in this zone did they mineralize (Fig. 6). The capillaries inhabiting the nonunion tissue were completely transparent or filled with fibrillar material, without blood cells or platelets (Fig. 7). Some of them were completely occluded by various staged thrombotic occurrences.

Condrocytes presented a secretive aspect: they were provided with a large RER and often Golgi apparatus was detected. They were enlarged and very similar to hypertrophic metaphyseal ones (Fig. 8).

Also condrocyte pericellular matrix presented a lot of matrix vesicles accumulated on the opposite side of the capillary vessels.

Frequently, vesicles were filled with needle-like hydroxyapatite structures.

Fibroblasts, cartilage cell membranes and vesicles presented a clearly evident positiviti to ALPase reaction: the morphological evidence of ALPase activity was represented by electronopaque thin segments of about 300 Å in diameter and 500 to 1000 Å long, and these segments were also easily observable without any further staining procedure owing to the precipitation of lead citrate.


**Fig. 2.** Capillary vessels (arrowhed) are often free of blood cells. × 25

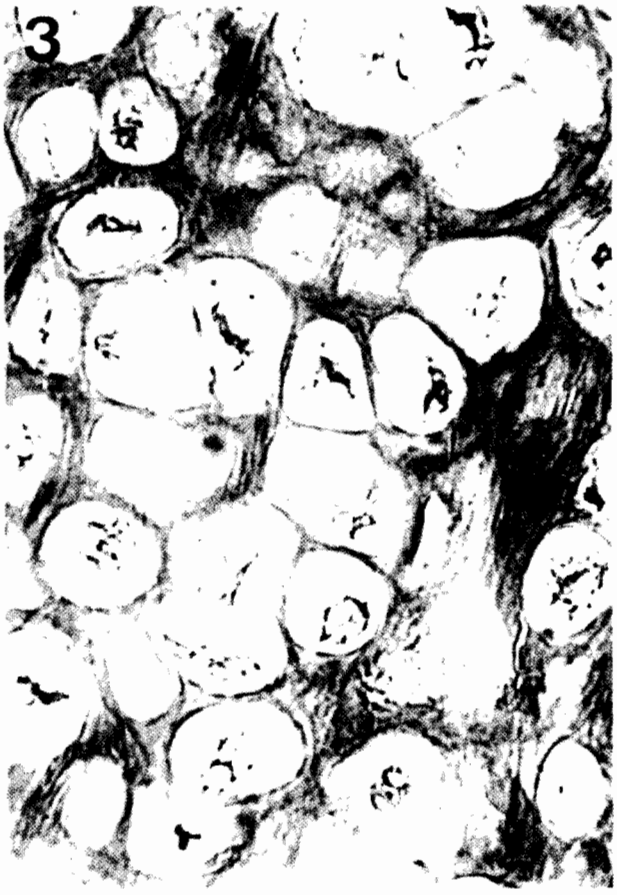


**Fig. 3.** Cartilage tissue condrocytes are hypertrophic or degenerative. Extracellular matrix has a motly aspect. × 25

The ALPase reaction was also present inside the cell but the enzyme was better evidenced in the pericellular area.

Some matrix vesicles presented ALPase activity inside them, but the main enzymatic activity was present outside and strictly connected to the vesicle membrane (Fig. 9).

All control reactions were completely negative.

Around the fibroblast-like cells the matrix was characterized by periodic collagen fibrils collected in bundles and having a regular diameter of 500 Å.

Around cartilage-like cells collagen periodic fibrils showed a more variable diameter and often random disposition. With great frequency matrix granules are observed in the pericellular and perifibrillar space. This corresponds to the pattern of proteoglycan aggregates and characterizes all cartilage tissues. Matrix granules were clearly evident particularly if stained with lead-citrate.

## Discussion

Two young patients were selected for our observations, because of the decrease of osteogenetic periosteal activity in old patients (Simmons, 1979). This event cannot be supposed in our young patients without systemic disease.

Light microscope aspects of nonunion tissue at eight months from fracture were very similar to the callus normally present at two or three weeks from fracture (White et al., 1977; McKibbin, 1978), in fact either osteoid tissue or cartilage and fibrous osteoblastic-like cells can be observed.

The presence of a lot of capillary vessels demonstrated that angiogenic stimuli, also normally required for callus formation, were present. This was not an unexpected finding; in fact the external fixation enhances vascularization of the callus, as demonstrated by Kiliuchevechii et al. (1983) and Court-Brown (1985), even if the middle third of the tibia has a scarce vascularization (Trias and Frey, 1979). But all capillary vessels were free of red cells and also presented various stages of thrombization. These findings gave value to the hypothesis of Mckibbin (1978) that for good fracture healing it is not only necessary good osteogenetic capacity but also a sufficient apportation of calcium and phosphate.

The cells populating nonunion tissue may be divided into fibroblast-like ones and cartilage-like ones.

Among the fibroblasts the largest and most electrontrasparent ones were very similar to young osteoblasts as described by Marks and Popoff in 1988.

Condrocytes mainly had a hypertrophic or degenera-

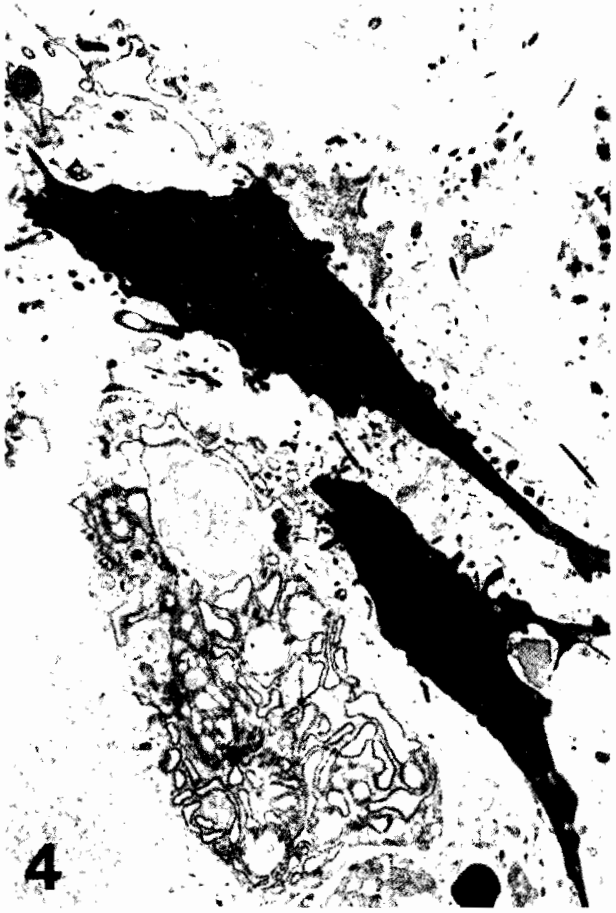


**Fig. 4.** Fibroblasts are elongated and present a well developed secretion apparatus. Some are enlarged and have a low stainability (different from osteoblasts). × 6,900

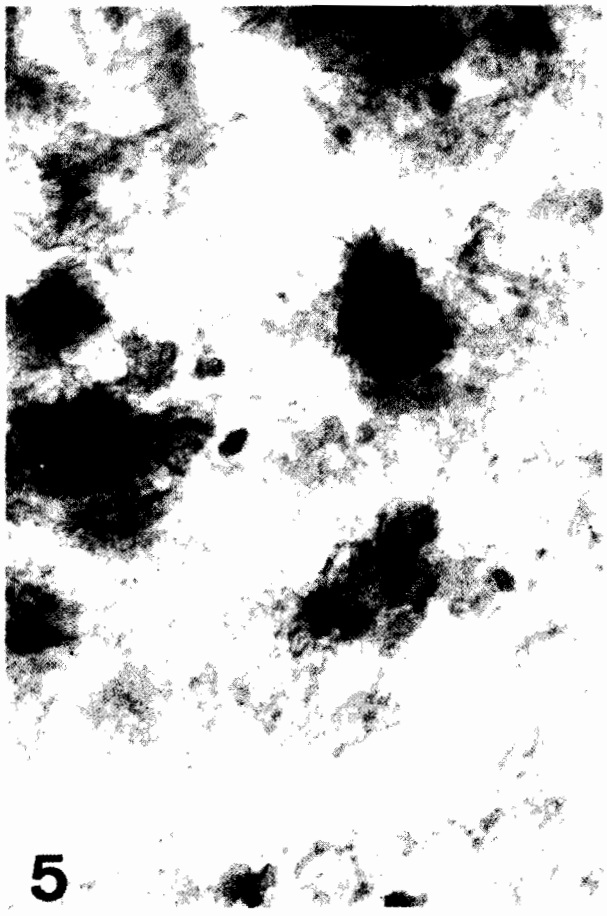


**Fig. 5.** Strongly electron opaque needle-like particles are present inside matrix vesicles and pack together. × 71,000

tive aspect: this evolution of cartilage cells is a prelude to mineralization of their matrix either in metaphysis (Hincliffe and Johnson, 1983) or in normal callus (McKibbin, 1978; Sevitt, 1981a).

Both cell types presented a well expanded secretion apparatus which certified an active proteic and glycoproteic secretion, thus the abundant matrix regularly organized in fibrils and fibril bundles can be explained.

The light microscopical motly aspect of the matrix is characteristic of ones that are calcifying (Hincliffe and Johnson, 1983).

It is necessary to emphasize that the cell membrane was able to produce matrix vesicles, as nearly all cells do, particularly calcifying ones, when $PO_2$ falls (Brighton, 1984).

Cells and matrix vesicle membranes presented an evident positivity to ALPase reaction. The presence of this enzyme usually characterizes the mineralization process (Lewinson et al., 1982; Quacci et al., 1990).

All these events are always involved in calcification processes (Wuthier, 1982).

In addition, the presence of hydroxiapatite crystals inside vesicles allow us to affirm that no deficiency inhibits the Ca-P definitive precipitation, as happens in normal callus (McKibbin, 1978).

So it is possible to suppose that morphologically and functionally the cells inhabiting the nonunion tissue eight months after injury had the osteogenetic properties of normal callus cells.

The presence of osteoid tissue trabeculae, even if incomplete, also allows us to affirm that the callus formation process was started (Simmons, 1985).

On the other hand, the mineralization process was too low and ineffective to allow a normal calcification because of the very scarce blood flow (empty or thrombized vessels), which determines a low Ca and P apportation.

The presence of calcified vesicles on the side opposite the capillary vessels is a common observation also in young self-making osteons (Rodan and Rodan, 1984), but the immediately subsequent phase is the calcification of the matrix on the capillary vessel face: in our findings no evidence of calcification was seen on the vascular side of the fibroblasts, osteoblasts or condrocyte membrane. This observation supports the hypothesis of the lack of Ca and P supply.

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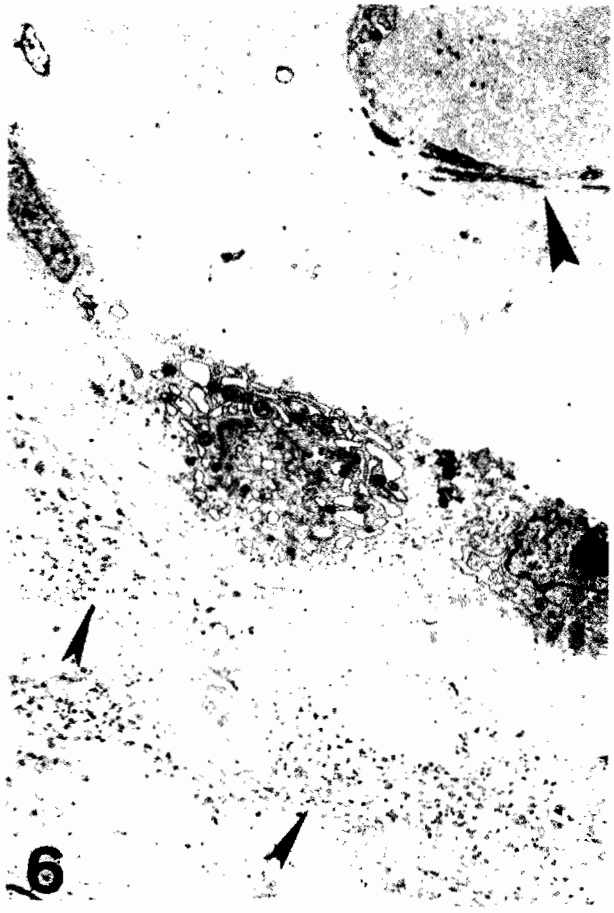


**Fig. 6.** Calcified matrix vesicles (thin arrowhead) are disposed on the opposite side of the void capillary vessels (large arrowhead). × 3,200.


**Fig. 7.** Capillary vessels always look devoid. × 3,200

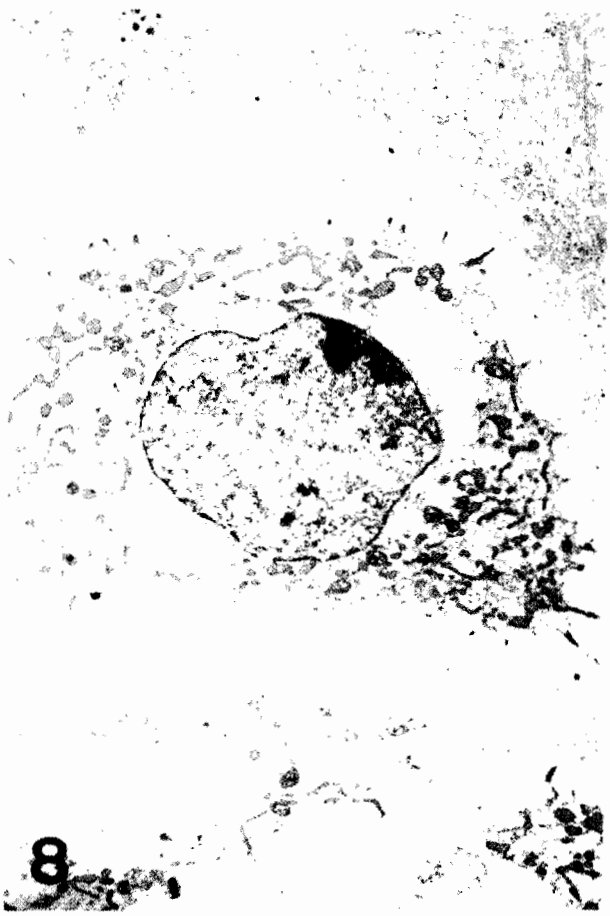

**Fig. 8.** In cartilage tissue condrocytes have a hypertrophic aspect and are provided with large RER. × 3,200

**Fig. 9.** The condrocyte cytoplasm is positive to ALPase reaction but the main stain is on the outer layer of cell membrane, which corresponds to matrix vesicles which accumulate in the interterritorial matrix. × 7,100

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