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STUDIES ON ULTRASTRUCTURAL PATTERN OF OSTEOGENIC CELLS DURING BONE REPAIR

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Studies on the sequence of events in fracture healing have been carried out by many workers such as Keith (1918), Galli & Robertson (1919), Urist & McLean (1941), and Ham (1961), but they were based mostly on the histological and histochemical investigations. In recent years more work has been carried out with regard to certain biochemical changes (Neuman & Neuman 1958) occurring during the organic phase of fracture healing (Udupa & Prasad 1963) and also during the mineral phase (Bauer 1954, Nilsonne 1959). However, very few studies have so far been undertaken to investigate the ultrastructural changes that occur in the osteogenic cells during different phases of fracture healing. In addition to the electron microscopic studies of the cellular components of the growing bone under normal conditions (Takuma 1960, Robinson 1952), a few more studies have been carried out on bone cells during induced calcification (Clarke & Anderson 1967) and also on certain pathological conditions (Dotty 1966, Robinson & Cameron 1956). In view of this lacuna in our knowledge, we have undertaken this study to observe the various morphological changes that take place in the osteogenic cells during the different phases of fracture healing by using the electron microscope.

METHODS AND MATERIALS

Twelve Swiss strain mice of the same age, sex and approximately the same weight were selected for the study. The right humerus of each mouse was fractured by putting pressure on the bone with both hands. Thereafter all the mice were kept in the cages without any support and were given the usual laboratory diet and water *ad libidum*. Three mice were sacrificed by the decapitation method on the

4th, 8th, 12th, and 16th day of fracture. After sacrificing the animals their fractured bones were dissected out and separated from all the adherent structures. The area of callus was isolated and fixed in glutaraldehyde solution. Tissues were embedded in Araldite solution. Sections were cut in LKB Ultra Microtome. All the copper grids were dried in the incubator and then stained with uranyle acetate and lead citrate solution. The stained grids were examined under Philips Electron Microscope 200 M.

RESULTS

These studies showed that in the immediate period after injury, polymorphonuclear cells, eosinophils and large mononuclear cells accumulated in the region around the fracture. The morphonuclear cells with two or more nuclei reach the site of fracture containing an abundance of lysosomes. Some of these lysosomes within the cell

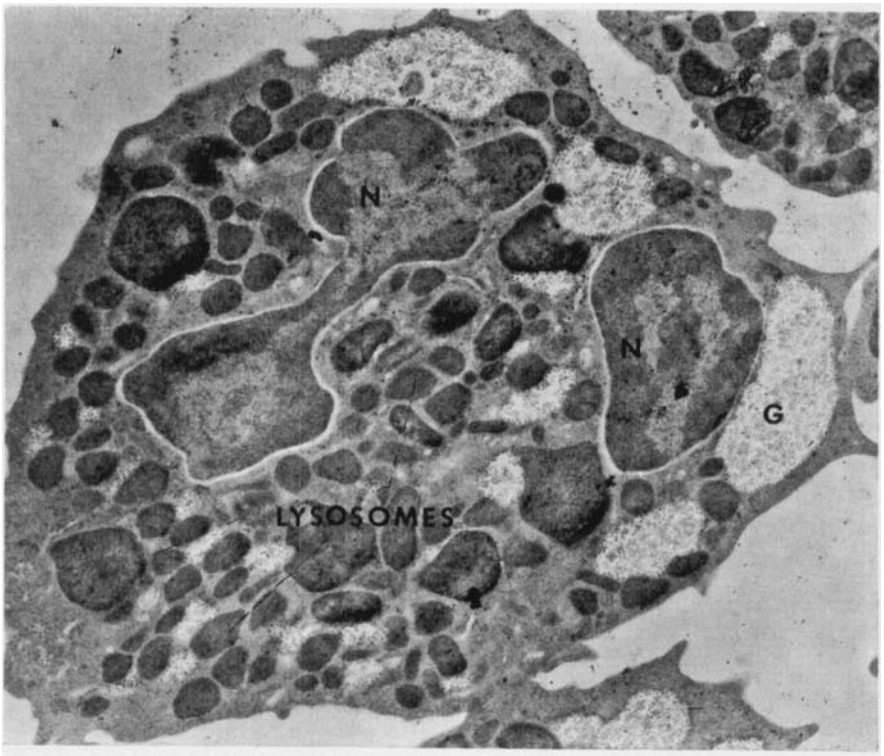

Figure 1. Shows polymorphonuclear leucocytes with three nuclei (N) and multiple lysosomal granules of different size. It also shows a few vacuolations inside the cytoplasm (G). × *5,500.*

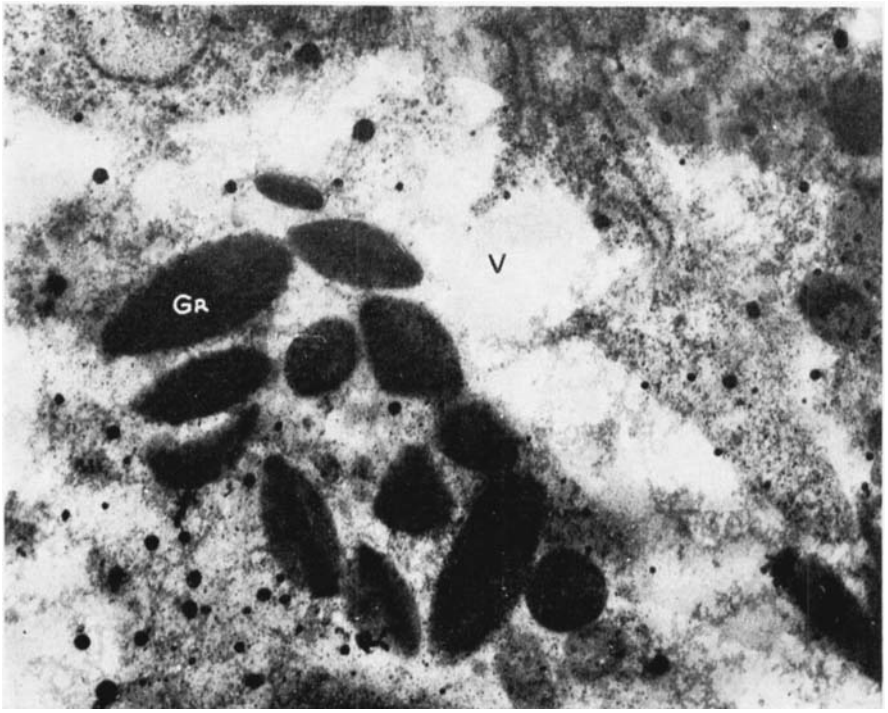

Figure 2. Eosinophilic cell with ill-defined cell membrane. Most of the cytoplasm is replaced by vacuolated material. The granules are of crystal type and multiple in number. × *8,600.*

are seen rupturing their membranous wall and causing vacuolations within the cytoplasm (Figure 1). This is done in order to phagocytose necrosed tissues and other small particles present at the site of injury. Once the cell cytoplasm becomes fully vacuolated, the membrane ruptures and the proteolytic enzymes present in the lysosomes are released into the extracellular space to produce liquification of organic materials. Similarly, the eosinophilic cells with their large granules in the cytoplasm can also be seen at the site of fracture (Figure 2).

On the eighth day many fibroblasts are present in the area with large nuclei and dilated rough endoplasmic reticulum (Figures 3 and 4). There are few collagen fibres seen floating on an amorphous substance known as mucopolysaccharides (Figure 5). From this finding it looks as though fibroblasts are responsible for the formation of both mucopolysaccharides and collagen fibres. As the days pass, more

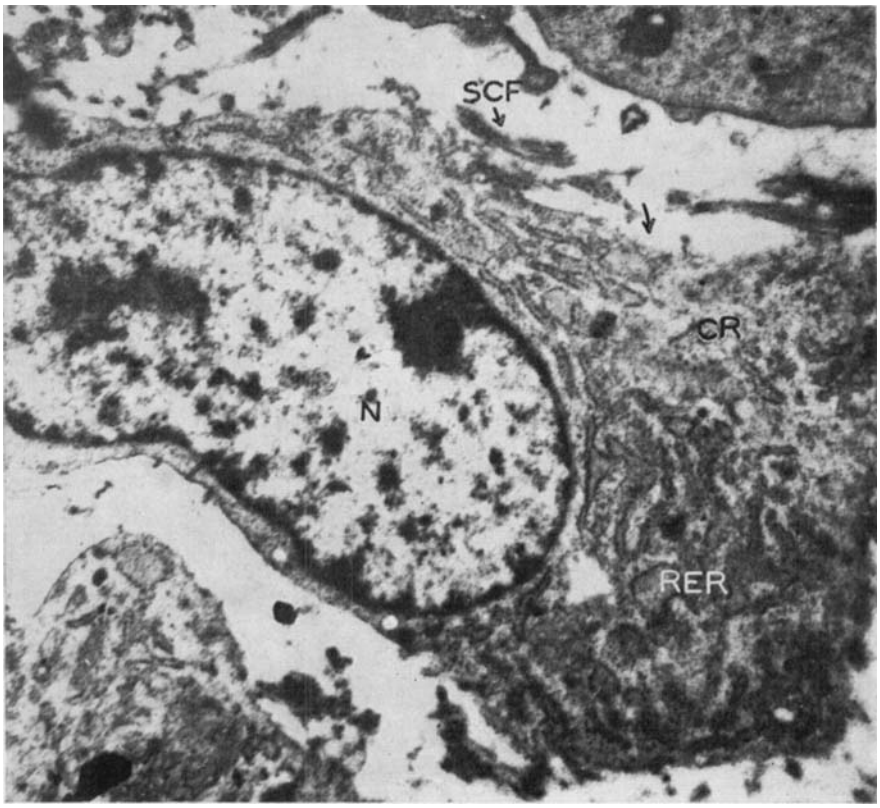

Figure 3. A fibroblast at the early stage of fracture healing showing a big oval nucleus (N) and dilated rough endoplasmic reticulum (RER). The secreting material (CR) is seen (SCF). × *5,500.*

and more collagen fibres become elongated and give an appearance of mature collagen fibres (Figure 6). At the same time, the amount of mucopolysaccharide present in the region gradually becomes less. The cell cytoplasm also gradually gets reduced, though the nucleus still remains elongated and prominent.

Thereafter, in different regions the areas of growth of cartillagenous cells known as chondroblasts can be seen. They have fairly big nuclei with an increased number of endoplasmic reticulum. These cells are surrounded by the newly formed organic matrix secreted by these cells (Figure 7). The cell membrane is not well defined and there is a spider-like projection into the matrix with a visible clear gap in between the two projections. This area is probably filled by the newly

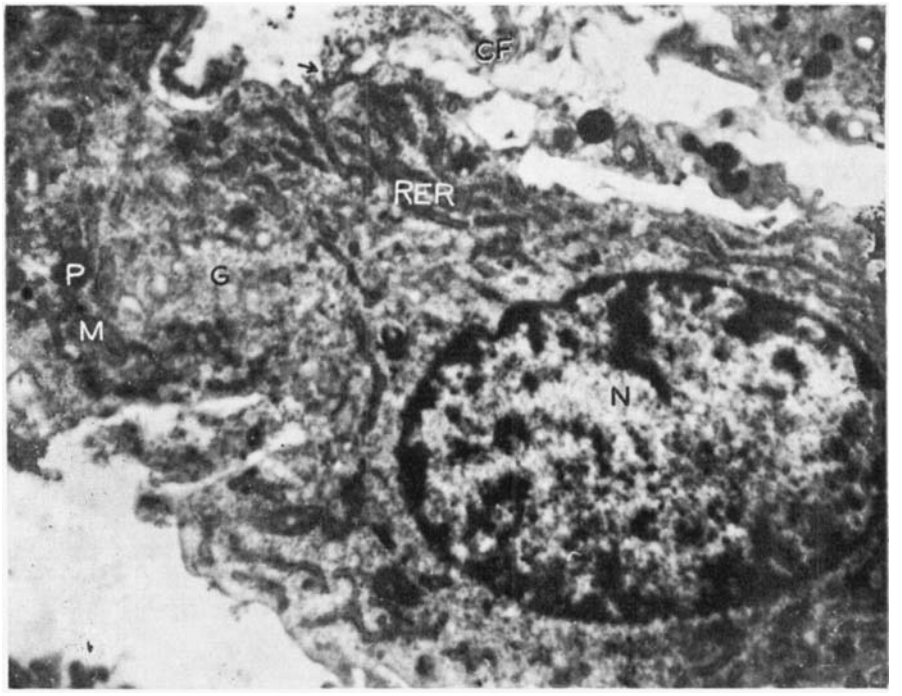

Figure 4. Fully developed fibroblast with oval nucleus and parallel dilated rough endoplasmic reticulum (RER). The mitochondria are well formed and seen in the cytoplasm. The Golgi-zone is hypertrophied (G). × *8,500.*

formed organic matrix which later on becomes polymerised. These cartilage cells gradually become hypertrophied and start degenerating. This will be followed by calcification of surrounding organic matrix. During this phase there is marked dilatation of the endoplasmic reticulum with decreased density of the cytoplasm (Figure 8). The nucleus also becomes gradually small and shrunken. This is followed by rupture of the cell membrane and dispersal of the cell fragments into the surrounding matrix.

The osteoblasts which appear at the fracture site at a much later stage usually have an oval shaped nucleus with plenty of endoplasmic reticulum (Figure 9). The golgi bodies are also well developed. There appears to be much activity in the cytoplasm followed by the synthesis of collagen fibres (Figure 10), which come out through the various pores present in the cell membrane.

The organic matrix formed at the site of fracture usually consists

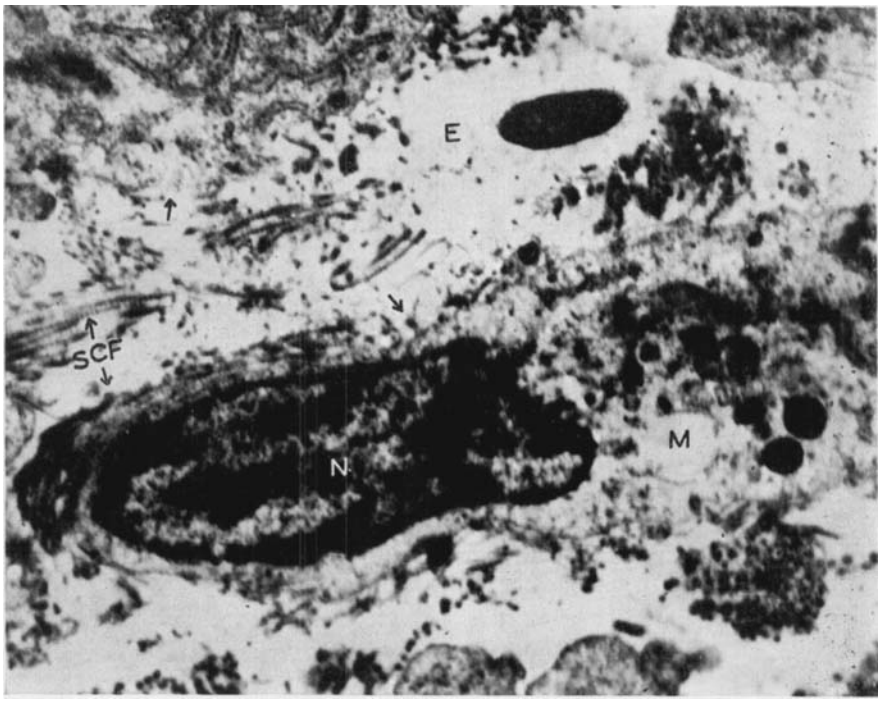

Figure 5. Shows amorphous proteinous materials accumulated between the two cells (E) after rupturing the cell membrane (arrowed). The cytoplasm becomes adherent to the collagen fibrils (SCF). × *5,500.*

of a network of irregularly arranged collagen fibrils in the initial period with a 600 to 800 °A axial period and a 250 °A diameter. These fibrils gradually join together with cross links to form full-fledged typical collagen fibres and are then arranged parallel to each other. Some of these collagen fibres are covered by some fusiform shaped opaque particles at different sites (Figure 11). This seems to be the early nucleus formation by the calcium mucopolysaccharide complex which later on extends to the whole of the fracture site for formation of the typical callus. Thus, we could see two types of callus formation, one through the calcification of chondroblastic cells and the other through the calcification of the collagen fibres directly. These are some of the sequences of events in fracture healing that we could see under the electron microscope.

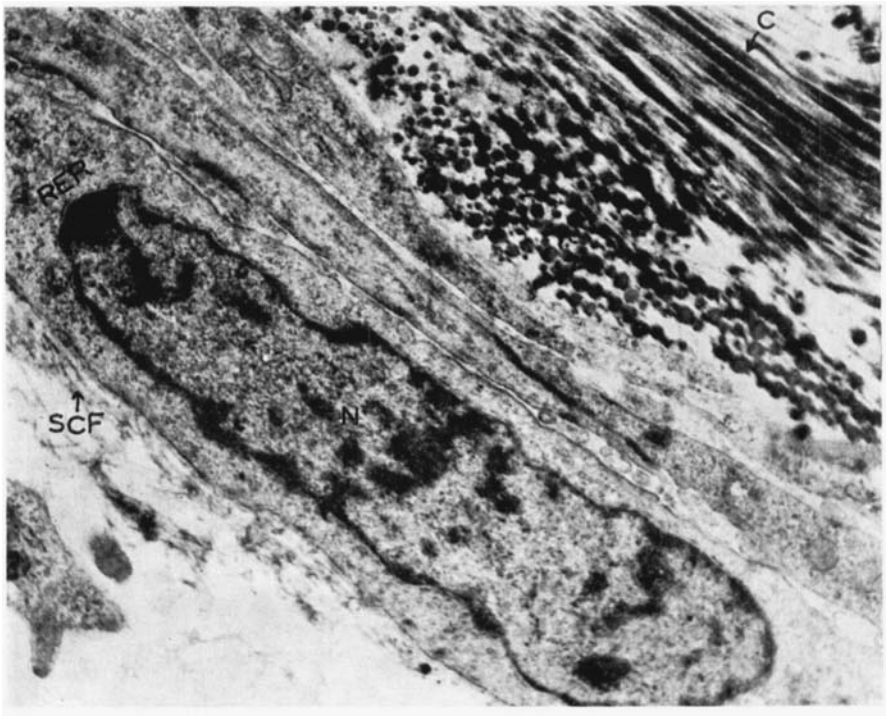

Figure 6. Shows mature collagen fibres on one side (C) and some of the newly formed small collagen fibrils on the other side (SCF) adjacent to the cell membrane (N). The cytoplasm is full of endoplasmic reticulum. × *15,000.*

DISCUSSION

As already mentioned earlier, a number of electron microscopic studies of the osteogenic cells had been carried out by Anderson (1967), Cameron (1963), Robinson (1956), Takuma (1960), and Fernandez & Engstrom (1956). Our studies on these cells during fracture healing showed that their behaviour appears to be somewhat different. Initially, after the occurrence of fracture, the inflammatory cells, like the polymorphs, large mononuclear and eosinophilic leucocytes, invade the surrounding tissues. These inflammatory responses are gradually replaced by the fibroblastic and chondroblastic cellular proliferations in the areas. The fibroblasts formed during this period showed large oval nucleus and elongated endoplasmic reticulum covered with ribosomes. Some of the rough endoplasmic reticulums were dilated; Golgi zone and mitochondria were hypertrophied. At this stage, the cell membrane

of the fibroblasts is seen to be broken at many places, and it is probably through these pores that they secrete their newly synthesised fibrous protein.

Besides fibroblasts and chondroblasts, osteoblasts were also seen at the fracture site. The chondroblasts found at the fracture site were similar to those found at the growing epiphysial cartilage, except that during the period of healing the cell membrane was not well defined. Spider-like projections were seen all around these cells, projecting into the surrounding zone of matrix. This well-defined space between the cell and matrix seems to be the zone of calcification. This process seems to be similar to the one that we see in the endochondral ossification of growing bones, in the same way as in some other areas where osteoblasts proliferate very quickly and engage themselves in the process of specific protein synthesis. They too synthesise mucopolysaccharide and collagen, which soon become calcified, whereas those

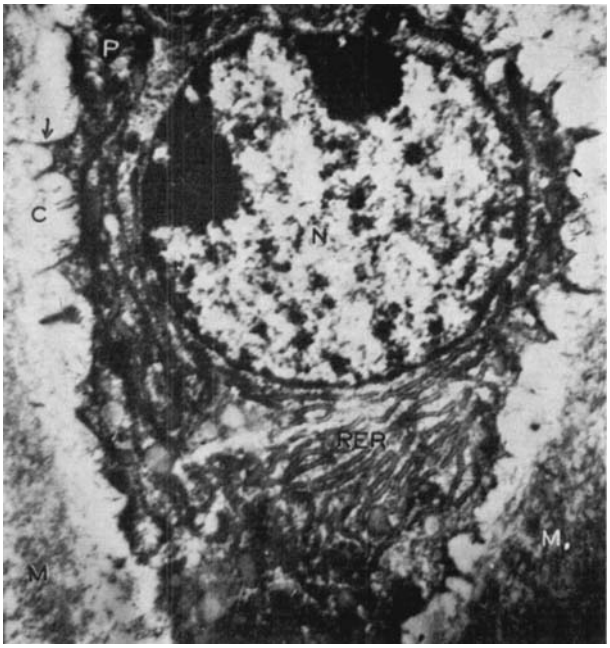

Figure 7. A chondroblast with degenerating nucleus (N) with dilated endoplasmic reticulum (RER) with some secretive materials in the cytoplasm (P). Cell membrane shows spider-like projections (arrowed) in the space (c) between the cell and matrix (M). × *10,500.*

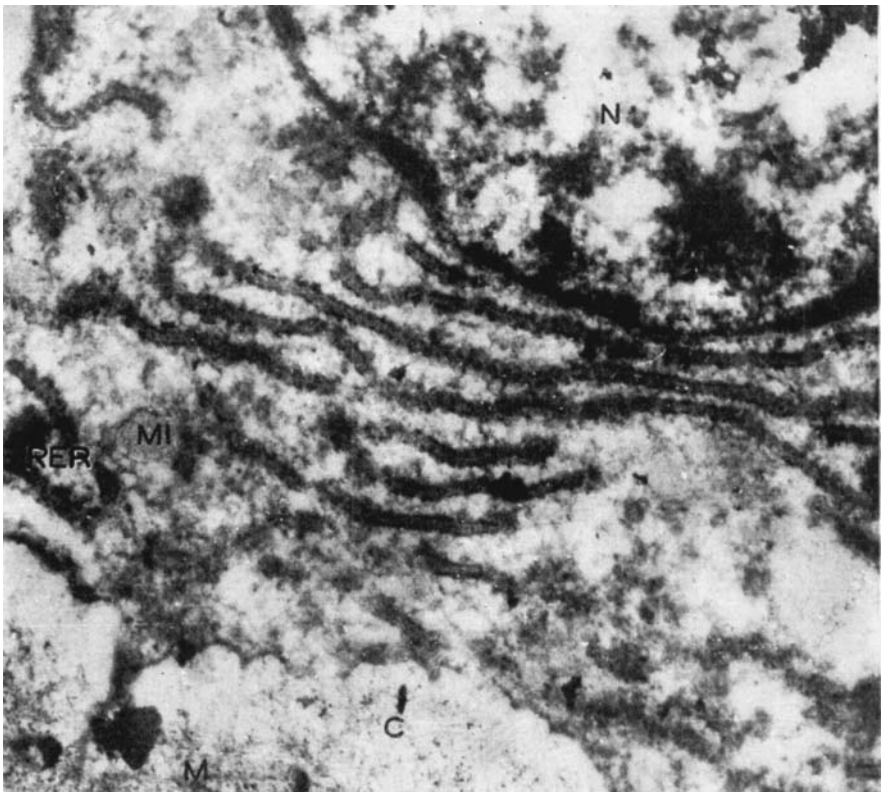

Figure 8. Another chondroblast at the time of calcification. Shows hypertrophy of the rough endoplasmic reticulum (RER). N = nucleus, M = mitochondria. × 60,000.

of other connective tissue cells are not calcified. This may be due to the fact that the mucopolysaccharides produced by these cells are of a specific type due to which their calcificability may be much more pronounced than others. However, this fact needs further investigation.

The matrix formed at the fracture site is composed of small and large bundles of collagen fibrils. The dense opaque material attached to the collagen fibrils at an early stage of healing could be composed of either protein polysaccharide complex or of proteinphosphokinase material, which may be the initial material required for the nucleation followed by the deposition of hydroxyapatite crystals, as reported by Glimcher (1966). It has been shown that most of the hydroxyapatite crystals were deposited over the leaf-like dense material. However,

the mechanism of the deposition of crystals and subsequent calcification is still not clear and needs further study.

SUMMARY

In this study the role of osteogenic cells in fracture healing was investigated with the help of the electron microscope. This was studied in the fractured bones of Swiss mice taken out on the 4th, 8th, 12th, and 16th day.

Initially after the fracture, the inflammatory response could be seen in the region, with the accumulation of polymorphs, eosinophils and large mononuclear cells, which continue to remain in the region till the regenerative cells of the bone invade the site.

Thereafter, the fibroblastic and chondroblastic cell proliferations in some regions and osteoblastic cell proliferation at other sites could

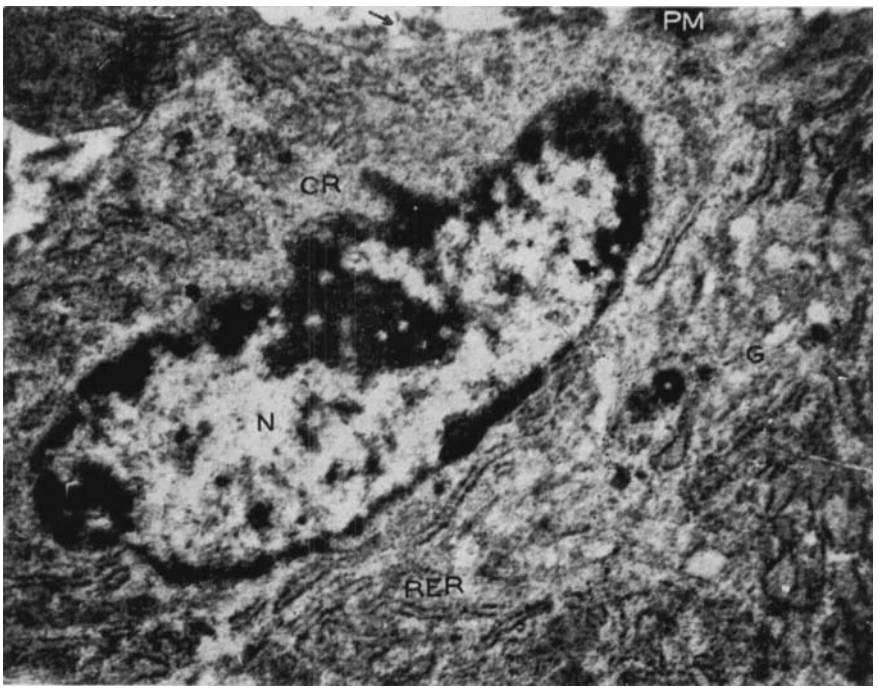

Figure 9. An osteoblast actively engaged in protein synthesis with a big nucleus (N) and granular (CR) endoplasmic reticulum at one side of the nucleus. Endoplasmic reticula (RER) are dilated and more in number. Golgi-zone (G) is also seen. × *15,000.*

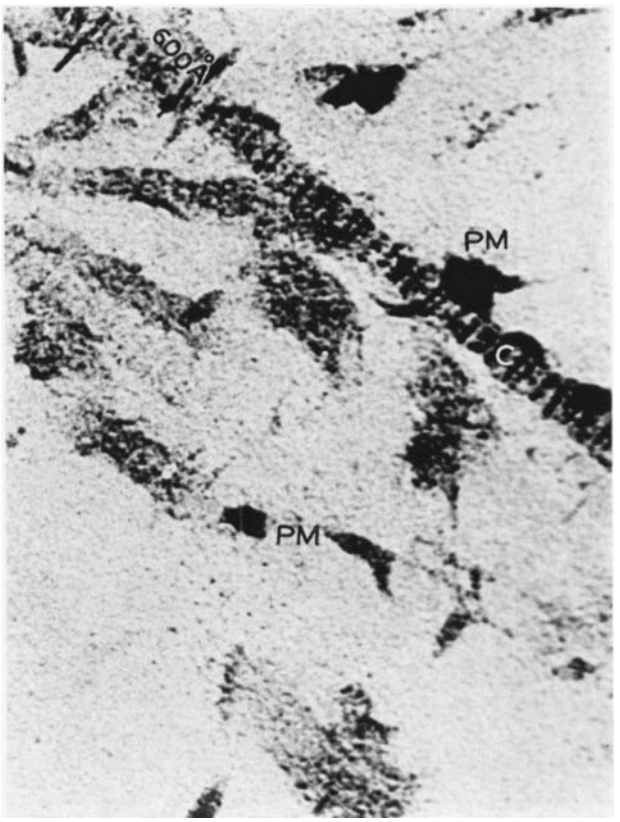

Figure 10. Collagen fibres showing (C) with 600 to 800 °A axial period with some opaque materials (PM) adherent to these fibre formed at fracture site. × 180,000.

be seen. All these cells proliferate and engage themselves in the synthesis of mucopolysaccharides and collagen fibres in varying degrees.

The collagen fibres produced by osteoblasts calcify much earlier, whereas those produced by chondroblasts calcify only when the osteoblasts invade the region. The fibres produced by fibroblasts become calcified only when the circumstances become favourable; the calcificable mucopolysaccharides accumulate in the region. These are some of the basic facts which we could observe in our study.

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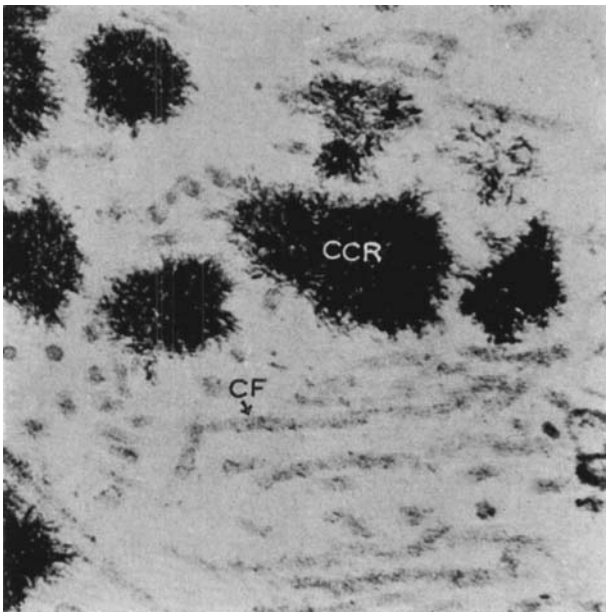

Figure 11. Showing the deposition of calcium crystals (CCR) over the collagen (CF) fibres formed at the site of calcification. × *65,000.*

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REFERENCES

Anderson, C. (1967) Electron microscopic studies of induced cartilage development and calcification. *J. Cell Biol.* **35**, 81.

Bauer, G. C. H. (1954) Rate of bone salt formation in a healing fracture determined in rats by means of radio calcium. *Acta orthop. scand.* **23**, 169.

Cameron, D. A. (1963) The fine structure of bone and calcified cartilage. A critical review of the contribution of electron microscopy to understanding of osteogenesis. *Clin. orthop.* **26**, 199.

Dotty, S. B. (1966) The electron microscopy of bone cells. *Berth. Defects.* Original article series. Vol. II, No. 1, 45.

Fernandez-Moran, H. & Engstrom, A. (1956) Ultrastructural organization of bone. *Nature* **178**, 494.

Gallie, W. E. & Robertson, D. E. (1919) The repair of bone. *Brit. J. Surg.* **7**, 211.

Glimcher, M. J. (1966) The ultrastructural organization of bone and the mechanism of calcification. *Berth. Defects.* Original article series. Vol. II, No. 1, 50.

Godman, G. C. & Porter, K. R. (1960) Chondrogenesis studied with the electron microscope. *J. biophys. biochem. Cytol.* **8**, 719.

Keith, A. (1918) Bone growth and repair. *Brit. J. Surg.* **5**, 685.

Keith, A. (1918) Bone growth and repair. *Brit. J. Surg.* **6**, 19, 160.

Neuman, W. F. & Neuman, M. W. (1958) *The chemical dynamics of bone minerals.* University of Chicago Press, Chicago, Ill.

Nilsonne, U. (1959) Biophysical investigations of the mineral phase in healing of fracture. *Acta orthop. scand.*, Suppl. 37.

Robinson, R. A. (1952) An electron microscopic study of the crystalline inorganic component of bone and its relationship to the organic matrix. *J. Bone Jt Surg.* **34**-A, 389.

Robinson, R. A. & Cameron, D. A. (1956) Electron microscopy of cartilage and bone matrix at the epiphyseal line of the femur in the new form infants. *J. biophys. biochem. Cytol.* **2**, Suppl., 253.

Takuma, A. (1960) Electron microscopy of the developing cartilaginous epiphysis. *Arch. oral Biol.* **2**, 111.

Udupa, K. N. & Prasad, G. C. (1963) Chemical and histochemical studies on the organic constituents in fracture repair in rats. *J. Bone Jt Surg.* **45**-B, 4.

Urist, M. R. & McLean, F. C. (1941) Calcification and ossification. Calcification in the callus in healing fractures in normal rats. *J. Bone Jt Surg.* **23**, 169.

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