Fracture healing of chick femurs in tissue culture

Katsuro Takahashi[1], Michael D. G. Shanahan[2], Leslie A. Coulton[2] and Thomas Duckworth[2]

Embryonic chick femurs were isolated, and a transverse osteotomy was performed at the midshaft. The femurs were transplanted individually onto the host chorioallantoic membrane and incubation continued. Cultured femurs were harvested at intervals from 1 to 9 days after the transplant. Histologic examinations showed that repair progressed rapidly: the fracture gap was invaded by blood vessels and fibrous ingrowth. Ossification followed, and the repair process was completed by Day 9.

Departments of Orthopedics, [1]Nagasaki University School of Medicine, Japan, and [2]University of Sheffield, Sheffield, England
Correspondence: Dr. L. A. Coulton, Dpt. of Orthopedics, Floor I, Royal Hallamshire Hospital, Sheffield, S10 2JF, England
Tel +44–742 766222 ext. 2933
Submitted 90-09-29. Accepted 91-03-03

The chorioallantoic membrane culture system has been used to investigate embryonic bone repair in rat tibia (Schmidt and Brighton 1983) and chick long bone (Monro and Shearer 1984). Although fracture repair of chick femur was shown to occur within 10 days, there have been no detailed publications of the changes in histology during this period.

We report our examination of the histology of fractures of embryonic chick femurs cultured on the chorioallantoic membrane of avian eggs.

Material and methods

Fertile chicken eggs (Mytholmroyd Hatcheries, W. Yorkshire) were maintained at 10 °C until required. The storage time before incubation was kept to a minimum. Eggs were incubated in two batches; the donor eggs were incubated for 14 days, and after a 5-day delay, the host eggs were incubated for 9 days; thus, both batches of eggs were ready on the same day. The eggs were kept in a purpose-built humidified incubator, which was maintained at 37 °C. The eggs were turned automatically every hour.

All the procedures were performed under aseptic conditions. Donor femurs were removed from 14-day-old embryos, and a stereomicroscope was used to aid the dissection of soft tissue from the bones. A transverse osteotomy was made with a scalpel at the midshaft of the isolated femur. Soft tissues on the medial and lateral sides of the femoral shaft were left intact to maintain bone alignment and contact.

The 9-day-old host eggs were prepared to receive the donor femurs. The air sac was pierced, thus allowing the chorioallantoic membrane to be displaced to create space for the graft. A 1-cm square window was cut with a dental burr in the side of the egg. The egg-shell membrane was moistened with sterile phosphate-buffered saline, which facilitated the removal of the shell membrane while leaving the chorioallantoic membrane intact. Donor femurs were placed on the chorioallantoic membrane parallel to the long axis of the eggs (Figure 1). The window and drill hole were sealed with clear tape. The eggs were incubated on their sides for 1 to 9 days. The donor femurs, which were harvested daily, were dissected along with the attached chorioallantoic membrane.

The isolated femurs were fixed in phosphate-buffered formalin and decalcified in 10 percent ethylenediaminetetraacetic acid in phosphate buffer. Decalcification was complete within 2 weeks. The femurs were processed and embedded in wax.

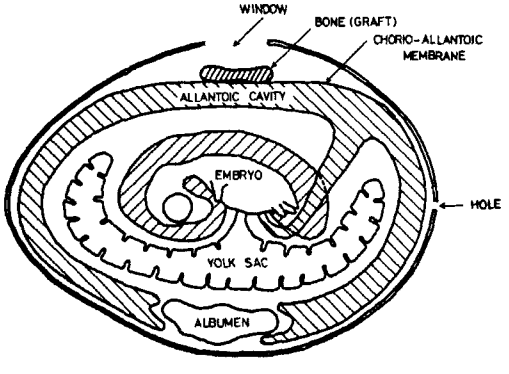
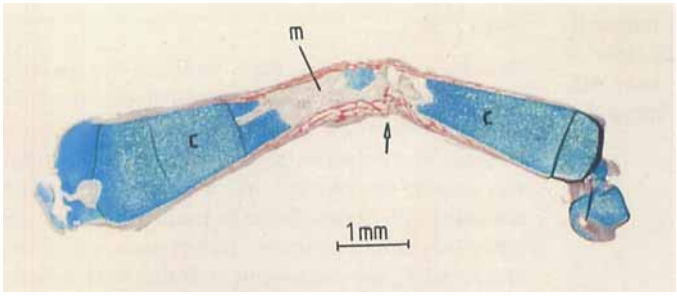
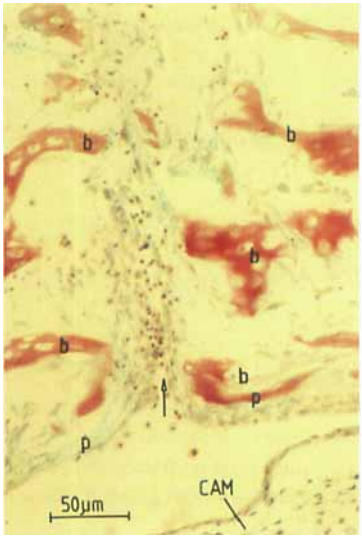


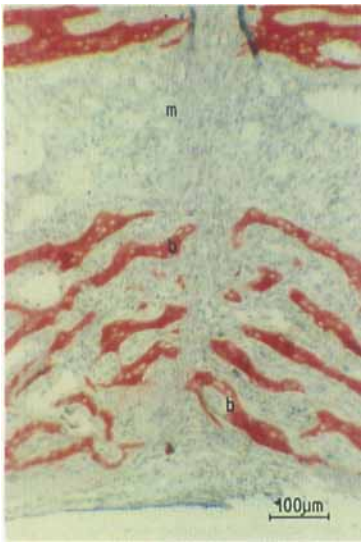
Figure 1. Cross section of the host egg with a transplanted femur. The air space has been punctured by a hole through the shell. The upper part of the chorioallantoic membrane has dropped and has allowed space for the transplanted bone.



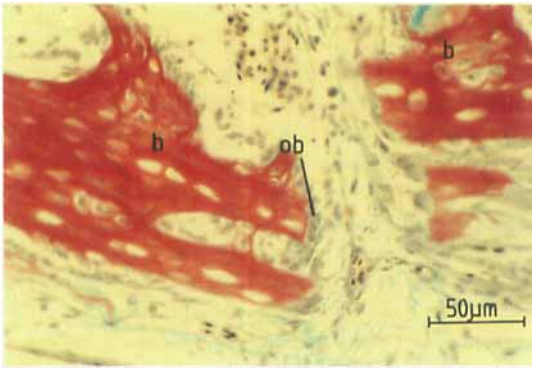
A. Day 0. The arrow marks the site of osteotomy.



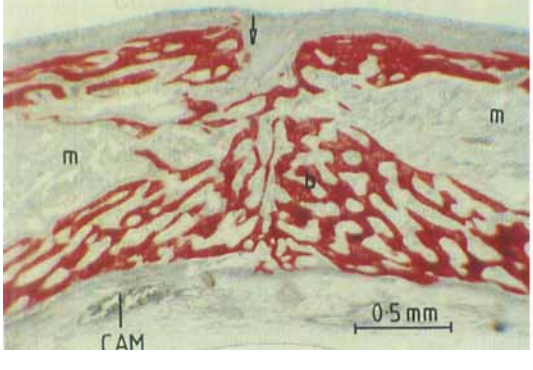
B. Day 2.



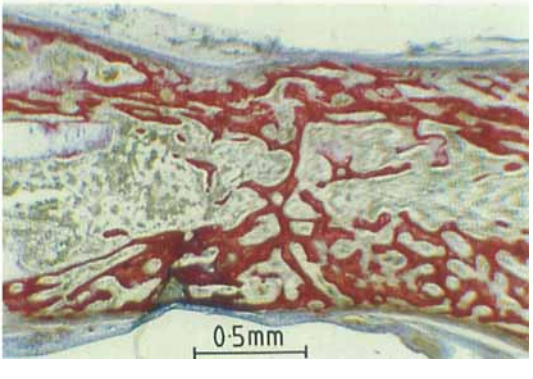
C. Day 3. Increase in the number of cells occupying the fracture site.



D. Day 4. Osteoblasts line the surface of the cut bone.



E. Day 7. The arrow marks a wider fracture gap where the repair is not so far advanced.



F. Day 8. Bone running transversely across the shaft.

Figure 2. Fracture healing after osteotomy of a 14-day-old chick femur transplanted onto the chorioallantoic membrane. Stains: alcian blue and chlorantine fast red (A–E) or polychrome (F, Herovici 1963).
b bone, c cartilage, m marrow cavity, ob osteoblasts, p periosteum.

Serial 7-μm sections of femurs were cut longitudinally, stained, and examined microscopically. One of the following stains was used: hematoxylin and eosin, safranin O and fast green, alcian blue and chlorantine fast red, or polychrome (Herovici 1963).

Results

Day 0

Histologic examination confirmed that good bone contact was maintained after osteotomy (Figure 2A). In these 14-day-old femurs, the marrow cavity occupied approximately one third of the total bone length and was rich in small blood vessels.

Days 1–3

Twenty-four hours after placement on the chorioallantoic membrane, there was no change from the controls. By Day 2, most specimens had become adherent to the chorioallantoic membrane, and there was fibrous ingrowth at the gap, with accompanying osteoblasts and erythroblasts (Figure 2B). The fibrous ingrowth was continuous with the periosteum, and periosteal involvement was obvious even at this early stage. The processes of ingrowth and chorioallantoic membrane adhesion were present in all the viable grafts by Day 3, by which time the chorioallantoic membrane had enveloped the femur.

The bone repair was carried out against a background of normal fetal bone development, in that the marrow cavity continued to extend towards the epiphyses, the bony cylinder around the shaft thickened, and the overall diameter steadily increased.

Although the marrow cavity was rich in blood vessels, very few were found at this stage in the mass of cells occupying the site of the osteotomy (Figure 2C).

Days 4–6

On Day 4, the site of the osteotomy was still apparent. There was an increase in the number of osteoblasts within the repair region. By Days 5 and 6, these osteoblasts had lined the bony margins of the osteotomy (Figure 2D). The fibrous tissue from the initial response to the trauma showed a progressive increase in vascularity and a concurrent decrease in fibrous content.

Days 7–9

Osseous bridging across the cortical gap was observed in most specimens by 7 days, although the site of the osteotomy remained discernible in many sections. In contrast, where a wider gap existed (Figure 2E), repair was slower; the gap was still lined with osteoblasts and filled with fibrous tissue. In the marrow cavity the repair was almost complete, in that there were many blood vessels and surrounding cells that were indistinguishable from the normal marrow contents. There was, however, some evidence in a few sections of bone running transversely across the shaft and projecting into the cavity (Figure 2F). No external callus or a cartilage transition stage from fibrous tissue to bone were seen.

By 8 or 9 days, complete bone union and extensive remodeling at the site of the osteotomy made identification difficult except where angulation had occurred. In the case of these bent specimens, the original site of the osteotomy could still be distinguished, but the extent of bone repair and remodeling in such a short time period could be appreciated.

Discussion

Fracture healing in the chorioallantoic membrane model is characterized by 1) rapid healing without fracture hematoma or external callus; 2) the presence of the periosteum, but no surrounding soft tissue; and 3) totally exogenous blood supply to the graft bone from the chorioallantoic membrane.

Bone union in the chorioallantoic membrane model took place without hematoma formation, suggesting that the hematoma is not a mandatory factor for fracture healing.

There have been two theories on the source of the osteogenic cells for fracture healing. One is that the repair tissues derive from the osteoprogenitor cells, which have a predetermined commitment to bone formation (Owen 1970). The other is that uncommitted fibroblasts in the surrounding tissues are stimulated or induced, by the fracture, to develop an osteogenic ability (Urist and McLean 1952).

In our experiments the source of the osteogenic cells in the chorioallantoic membrane model was not investigated. The repair tissue may have arisen from the periosteum, bone, and bone marrow of the fracture femur or may have had an exogenous origin from the chorioallantoic membrane and host embryo. However, the fact that complete bone healing did occur in the chorioallantoic membrane model suggests that bone

Table 1. Number of cultured femurs harvested on each day after the osteotomy

	Days of culture									
	1	2	3	4	5	6	7	8	9	Total
Successful	4	10	2	8	4	8	3	7	3	49
Failure	0	1	3	3	1	4	3	6	0	21
Total	4	11	5	11	5	12	6	13	3	70

union can take place without any influences from the soft tissue around the fracture.

The third major feature of our model was that the blood supply for the healing process was totally derived from the chorioallantoic membrane vessels of the host.

Cartilage forms in relatively avascular conditions, because it is able to grow under low oxygen tension (Bassett and Herrman 1961). There have also been suggestions that cartilage and callus form where massive movement of the fragments exists or where shearing stress is exerted (Anderson 1965). Mechanical stress to the site of the osteotomy is negligible in the chorioallantoic membrane model, although some movement of the host embryo in the egg exists. These features of the chorioallantoic membrane model might explain the reason for the absence of both the external callus and cartilage formation. Additionally, the absence of the soft tissue surrounding the fracture may have been an affecting factor in the lack of external callus formation.

We observed in a few sections bone running transversely across the shaft; a temporary orientation of new bone across the fracture gap has been seen in other fracture healing models (Olerud and Danckwardt-Lilliestrom 1968).

We had a rather high failure rate (Table 1). The most common cause of failure in our experiments was partial necrosis of the donor femur. Total necrosis was rare, as was death of the host. The reason for this partial necrosis was most likely nonadherence to the chorioallantoic membrane, although we found no explanation as to why this should occur at only one end of a graft. Necrotic specimens were excluded from the study.

Two other factors proved to be variable. The first factor was the number of sterile eggs. This varied between 1 and 5 eggs in any batch, and probably reflected a seasonal variation. The second factor was the stage of development of the donor femur as judged by length. However, within any batch of eggs the difference in lengths was small (± 0.25 mm).

The chorioallantoic membrane model does have some technical problems due to the size of the bone, causing difficulties in making and fixing the osteotomy. The model also has some other disadvantages: it is an embryonic system, and hence bone repair takes place against a background of normal bone growth and development; normal growth does not occur in cultured mammalian bone (Stephenson and Tomkins 1964); and the overall length of the bone is restricted to that which will fit inside an egg. However, this rapid and reproducible repair model has several major advantages over in vivo models of fracture healing.

In this initial study, we have used simple histologic techniques to compare chick embryonic bone repair on the chorioallantoic membrane with known patterns of mammalian fracture healing. We have shown that the underlying patterns of repair are similar. Further studies of the repair process would require a more sophisticated approach and necessitate the use of hard-tissue embedding techniques and probably histomorphometry to exploit the full potential of this model.

Acknowledgement

The authors wish to express their thanks to Professor Iwasaki of Nagasaki University School of Medicine for his advice and to Dr. Torigoshi of Rosai Hospital for his support during this study.

References

Anderson L D. Compression plate fixation and the effect of different types of internal fixation on fracture healing. *J Bone Joint Surg* (Am) 1965; 47: 191–208.

Bassett C A L, Herrmann I. Influence of oxygen concentration and mechanical factors on differentiation of connective tissues in vitro. *Nature* 1961; 190: 460–1.

Herovici C. A polychrome stain for differentiating precollagen from collagen. *Stain Techn* 1963; 38: 204–5.

Monro P P, Shearer J R. The use of the chorioallantoic membrane cultured 14-day-old chick femurs to investigate bone growth, differentiation and repair. *J Embryol Exptl Morph* (Suppl 259) 1984: 82.

Olerud S, Danckwardt-Lilliestrom G. Fracture healing in compression osteosynthesis in the dog. *J Bone Joint Surg* (Br) 1968; 50 (4): 844–51.

Owen M. The origin of bone cells. *Int Rev Cytol* 1970; 28: 213–38.

Schmidt R G, Brighton C T. In vitro mammalian fracture healing on the chick chorioallantoic membrane. *Orthop Trans* 1983; 7: 364.

Stephenson N G, Tomkins J K N. Transplantation of embryonic cartilage and bone on to the chorioallantois of the chick. *J Embryol Exptl Morph* 1964; 12: 825–39.

Urist M R, McLean F C. Osteogenetic potency and new bone formation by induction in transplants to the anterior chamber of the eye. *J Bone Joint Surg* (Am) 1952; 34: 443–76.