Formation of marrow cavity and ossification in mouse limb buds grown \textit{in vitro}

By D. R. JOHNSON$^1$

From the Department of Anatomy, Medical and Dental Building, The University of Leeds

\textbf{SUMMARY}

Explanted mouse limb buds aged 12, 13 or 14 days cultured for 9 days \textit{in vitro} failed to develop a marrow cavity or show endochondral ossification.

Explants aged 15 days developed a marrow cavity and showed signs of endochondral ossification. The periosteum in these limbs was perforated and laminated and cells appeared to be passing between the laminae to populate the marrow cavity. This suggests that there is a periosteal contribution to the bone marrow even in the absence of a blood supply.

\textbf{INTRODUCTION}

It is now more than 50 years since Fell's (Fell \& Robinson, 1929) pioneer work on the organ culture of limb rudiments. In all that time there seems to have been only one report (Crelin \& Koch, 1965) of the occurrence of endochondral ossification in a 'long bone' \textit{in vitro} (Crelin \& Koch in fact described ossification in the mouse pubis). In the hands of most workers (e.g. Fell \& Robinson, 1929; Holder, 1978) the more usual situation has been development proceeding as far as a solid collar of periosteal bone surrounding the cartilaginous centre of a long bone rudiment, and no further.

Slightly later explants of bone rudiments already containing a marrow cavity grow well in culture (Jones \& Keeler, 1971) and it has been suggested by a variety of workers that progress towards ossification beyond the collar stage \textit{in vivo} depends upon the invasion of the developing marrow cavity by blood vessels. In their absence \textit{in vitro} the osteoblasts and preosteoblasts in the developing periosteum are regarded as being excluded from the marrow cavity by the layer of osteoid which they (or their fellows) have secreted. The blood vessels have been given four distinct roles: (1) as the bringers of osteoclasts which penetrate the periosteum; (2) as 'carriers' transporting osteoblasts to the future bone marrow; (3) as bringers of increased oxygen tension (Shaw \& Basset, 1967); (4) as bearers of haemopoietic precursor cells.

Crelin \& Koch (1965, 1967) explanted the mesenchyme which was to form

\footnotesize{$^1$ Author's address: Department of Anatomy, Medical and Dental Building, The University of Leeds, Leeds LS2 9JT, U.K.}
the pelvis from 13-day mouse embryos, cultured it, and demonstrated endochondral ossification. Labelling experiments showed that the chondroclasts and osteoblasts formed in vitro arose from released chondrocytes. Crelin & Koch point out specifically that the periosteum was completely separated from calcified cartilage by a continuous sheath of periosteal bone (my italics) precluding any contribution to the cells in the marrow cavity from the periosteum.

Streeter (1949) described the process of the establishment of a blood supply in man a little differently. He saw ‘osteoblasts and other cells from the inner layer of the periosteum streaming through the primary osseous shell by way of a small pore’. These invaders became osteoblasts, the forerunners of marrow cells, or angioblasts which tended to branch out as elongated endothelial cells, some becoming capillaries. At this early stage he did not see fully formed blood vessels or haemoglobin-carrying red cells.

In the light of these findings, it was decided to re-investigate the development of ossification of mouse limb buds in culture. Could they be induced to develop beyond the classic ‘periosteal ring’ stage, and, if so, was there any sign of Streeter’s invading mesenchymal cells in the absence of a blood supply?

MATERIALS AND METHODS

Tuck A strain mice were mated and the day of the vaginal plug taken as day 0 of gestation. Pregnant females were sacrificed by cervical dislocation on the mornings of days 12–17, their uteri removed under sterile conditions and the embryos dissected out. Forelimb buds were removed under a dissecting microscope and stored temporarily in Hanks B.S.S. at 37 °C. Half were fixed in 10 % formol saline as controls. The contralateral limbs were transferred via a polythene pipette to Millipore filters (pore size 1·2 μm) which were placed upon triangular sterile stainless steel grids in 5 cm diameter sterile plastic petri dishes. Four limbs were usually placed on each filter. Three ml of culture medium (Beggars B. G. J. – Beggars Gwatkin & Heyner, 1961), supplemented with fetal calf serum (25 % by volume), glutamine (29·2 μg/ml), penicillin and streptomycin (5000 i.u./ml of each), mycostatin (100 units/ml) and ascorbic acid (15 mg/ml), were added and the dishes transferred to a 37 °C incubator saturated with water vapour and gassed with 95 % air 5 % CO₂ mixture. The culture medium, freshly supplemented with ascorbic acid (Harrison, 1979), was changed at 3-day intervals and the cultures harvested after 9 days of incubation.

After fixation in 10 % formol saline, limbs were dehydrated in alcohol, stained with 1 % methylene blue and cleared for preliminary examination and/or photography. They were then wax embedded, serially sectioned at 8 μm, stained with alcian blue, haematoxylin and chlorantine fast red (Lison, 1954) and examined under the light microscope. A minimum of eight cultured limbs and eight controls were examined from each age group.
Fig. 1. Longitudinal section through the radius of a 14-day-old forelimb grown in culture for 9 days. Note the well-developed periosteal collar (p) and the fact that no marrow cavity is present.

Fig. 2. Longitudinal section through the radius of a 15-day-old forelimb grown in culture for 9 days. Note the line of the original periosteal collar (arrowed), the laminar appearance of newly deposited osteoid outside this collar and the presence of mesenchymal cells (mcs) in the developing marrow cavity. Towards the proximal end of the rudiment endochondral bone (b) is being laid down.
RESULTS

All the limb buds explanted were in a healthy condition after 9 days' incubation, although some had developed further than others. Because of the proximo-distal gradient in the developing limb, which results in the humerus, for example, being much better developed at a given time than the phalanges, only findings from the radius and ulna will be discussed.

Limb buds explanted at 12, 13 or 14 days developed a periosteal collar after 9 days in culture (Fig. 1), but proceeded no further. The collar was made up of a solid band of osteoid of varying thickness. The cartilage cells within this collar were rounded and a little swollen but showed no signs of degeneration.

Fifteen-day-old explants presented quite a different picture (Fig. 2). The line of the periosteal collar could still be traced as a darkly staining band (arrowed), somewhat compressed towards the centre of the rudiment by a series of laminations of osteoid which had been laid down on its outer surface. These were thickest at the midshaft region and thinner towards the cartilaginous ends of the rudiment. These laminations of osteoid are well separated from each other, giving a latticed appearance in longitudinal section: in the interstices of the lattice are many small cells. The original darkly staining periosteum had clearly been breached at several places and these small cells were in contact with the marrow cavity via the gaps.

In the centre of the rudiment cartilage had broken down, only a few strands of (calcified?) matrix indicating the site of the original cartilage cells. Endochondral ossification was proceeding near the ends of the newly developed marrow cavity (b in Fig. 2) which was populated by at least three cell types: small, rounded cells, elongated fibroblasts, and small numbers of large, multinucleate cells. Fig. 3 shows details of the multilayered periosteum developed in culture with clear communication between the marrow cavity and the exterior.

Explants at 16 and 17 days resemble those made at 15 days.

In the control series perforations of the radius and ulna were first seen in three out of nine 16-day control limbs (Fig. 4). In the example illustrated, a blood vessel (bv) running between radius and ulna has perforated the periosteum of the ulna. It seems, however, that this is a recent perforation as only one cell can be seen invading the putative marrow cavity.

DISCUSSION

The experiment reported above demonstrates that mouse limbs explanted at 12, 13 or 14 days and cultured for 9 days in the system chosen do not progress beyond the solid periosteal collar stage of development, and do not develop a marrow cavity. Those explanted at 15 days or later do develop a marrow cavity.
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Fig. 3. A further example of the laminar periosteum (p). Mesenchymal cells seem to be invading the developing marrow cavity at two points (arrowed). 15-day-old limb cultured for 9 days.

Fig. 4. Control limb (16-day). This was one of the earliest examples seen of an invading blood vessel (bv) which has perforated the single-layered periosteum (p) of the radius at the point indicated by the arrow. Note the non-hypertrophied cartilage of the radius and compare with Fig. 1.

which becomes populated by cells, some of which at least appear to reach the cavity via spaces between the laminations of the developing collar of osteoid. As the first perforations in controls were not seen until 16 days and were then not universally present, it seems reasonable to suggest that explants made 24 h earlier at 15 days had an intact periosteum at explant, as indeed did all the 15-day-old control material. If this is so, then perforations can develop and the marrow cavity can become populated, at least partially, in the total absence of a blood supply.
These results seem to favour the view expressed by Streeter (1949) that the initial invasion of the developing marrow cavity is by mesenchymal buds, which then develop into a number of structures including capillaries, rather than by formed blood vessels. Presumably, whatever factors predispose these cells to form blood vessels are not present \textit{in vitro} in the absence of the rest of the blood system.

According to the views of Crelin & Koch (1965, 1967), what we have seen is simply the transformation of chondrocytes into other skeletogenic elements within a sealed tube. Their labelling experiments (1967) are convincing and it seems likely that, as they suggested, some marrow cavity elements arise from transformed chondrocytes. However, their view that this is the sole source of marrow cavity cells in the absence of a blood supply seems not to be valid. The present experiments differ from theirs in the nature of the periosteum. Crelin & Koch stress its intact, impermeable nature while in this series the periosteum was made up of a series of well-separated layers of osteoid with ample spaces between, providing communication between the marrow cavity and the mesenchyme surrounding the bone rudiment.

Multinucleate cells were observed in the marrow cavity both in this material and in that of Crelin & Koch. Current evidence seems to favour the derivation of osteoclasts from macrophages (Jotereau & Le Dourain, 1978; Owen, 1978). If the multinucleate cells seen by the author and by Crelin & Koch were osteoclasts, then it seems possible that they may have an additional origin.

The sharp demarcation between 14- and 15-day explants (for radius and ulna) is interesting. It seems that the timing is a function of the invaded rather than of the invader. Cartilage resists invasion, both vascular and nonvascular. Eisenstein \textit{et al.} (1973) grafted various tissues on Millipore filters to the chorioallantoic membrane of host chick eggs. Hyaline cartilage, live or devitalised, resisted vascularisation whilst calcified cartilage and bone were rapidly vascularised. Extraction of the cartilage with 1 M-GaHCl (and, therefore, extraction of glucoseaminoglycans) reduced this resistance considerably. Kuettner \textit{et al.} (1977) noted that human osteosarcoma or metastasized mammary carcinoma cells cultured with human growth plate invaded bone but not the unmineralized cartilage. When cartilage is (rarely) the host of a tumour, the latter tends to encapsulate cartilaginous nodules rather than invade the tissue.

It seems that the process of cartilage hypertrophy (whatever that may entail) has not been initiated by 14 days in the radius and ulna of the strain of mice used in this study, but that by 15 days it has. Once the cartilage is committed to hypertrophy, it is open to invasion by mesenchymal cells/blood vessels. In culture, of course, blood vessels do not invade as there are none, but mesenchymal cells do. \textit{In vivo} both types of invasion may take place. It is perhaps more than coincidental that alkaline phosphatase activity (implicated in cartilage hypertrophy by Fell & Robinson, 1929) should rise sharply in normal mouse femur, tibia and fibula after 14 days (Krotoski & Elmer, 1973). It may be that
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the presence of a high level of alkaline phosphatase activity in a rudiment removed for culture indicates that it will go on to ossify, whereas a low level signals the fact that it will remain a cartilaginous entity.

REFERENCES


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