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THE SURVIVAL OF TRANSPLANTED EMBRYO BONE GRAFTED TO CHORIOALLANTOIC MEMBRANE, AND SUBSEQUENT OSTEOGENESIS

BY N. M. HANCOX

From the Histological Laboratory, Department of Histology and Physiology, University of Liverpool

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The extent to which bone transplants survive and the source of any new bone subsequently formed are still matters of controversy. Three main hypotheses have been put forward.

Barth (1893, 1895) came to the conclusion that all cells, layers, and tissues of transplanted bone perish, and that subsequent osteogenesis was brought about through the agency of host tissue. Since that time, the 'death and resurrection' theory has received the support of innumerable investigators. Leriche & Policard (1928) state: 'The question of the death of the transplant should be considered solved. It is 32 years since Barth conclusively shewed it. It is truly a waste of time still to seek to verify facts so well demonstrated.' Watson-Jones (1943) states: 'It is undoubted that the graft never participates in the circulation of the host.' Proponents of the 'death and resurrection' theory believe that boiled bone, on grafting, is entirely replaced, but the process is complicated by the relative impermeability of the graft to invasion by host tissue.

Ollier (1867, 1891) thought that periosteum, bone and marrow can under certain circumstances not only survive transplantation but that the newly formed bone which arises is formed by graft and not by host cells. Hey Groves (1939) inclines to this view. Macewen (1912), Matti (1936) and Mowlem (1944) employed small chip grafts, and interpret their excellent clinical results as suggestive of survival of the transplanted fragments.

In 1918 Nageotte demonstrated ossification in the region of some transplants fixed in alcohol. Polletini (1923) also observed occasional osteogenesis following transplantation of alcohol-fixed bone; he concluded that a substance with osteogenic properties diffused from the fragment. Levander (1938) transplanted small fragments of bone subcutaneously and examined the host area histologically from the second day onwards. He was of the opinion that

all grafted tissue perished, and that the new bone which formed in close association with the graft was produced as a result of the permeation of the surrounding host vascular connective tissue by a specific osteogenic substance originating in the graft. He then extracted callus with alcohol; on injection of the extract, osteogenesis sometimes occurred. In 1940 he repeated the experiments along similar lines, employing bone-marrow, freed of bone spicules as far as possible, with similar results. Annersten (1940, 1943) continued the experiments and improved the extraction technique; but several positive results occurred among his control experiments. He suggested the existence of an osteogenic hormone. Bertelsen (1944) repeated the work, and compared the potency of extracts obtained from corticalis, total bone, marrow, periosteum and epiphysis. In his experiments, marrow extract proved most potent. He also had positive results among his control experiments (liver). Attempts at concentration and purification were unsuccessful. Lèvander (1945) later extended the specific osteogenic hormone theory to cover many similar cases of tissue induction. Lacroix (1945) also has produced an alcoholic extract of cartilage capable of inducing osteogenesis on injection.

So far as experimental evidence of the fate of grafts is concerned, our knowledge depends upon subjective interpretation of histological and radiological appearances. The degree of radiopacity of a transplanted fragment is held to be a measure of its vascularization. Histologically, the criteria appear to have been the presence in sections of recognizable blood vessels containing blood cells; or the gross appearance of the bone fragment (a somewhat difficult tissue to preserve histologically) has been studied. The disadvantage in all previous experiments is that the grafts have been available for microscopical examination only in the fixed and dead condition.

The present account deals with the results of experiments in which the transplanted fragments could be examined microscopically in the living condition at any time.

In the past, a series of observations has been made on bone anlagen transplanted to the chorioallantoic membrane (Murray, 1926; Murray & Selby, 1932; Studitsky, 1936). These experiments, however, were directed towards a different end, and the technique employed did not allow of direct examination.

METHODS

Aseptic precautions were observed throughout. Fragments of os frontale of the embryo chick (14 days' incubation) were prepared. They measured approx. $2 \times 2 \times 0{\cdot}5$ mm. and were placed either in buffered saline directly for use as live fragments, or were first transferred to boiling Ringer's solution for 30 sec. Samples of each batch of boiled bone were explanted as hanging-drop tissue cultures, and since no cell outgrowth occurred, it was assumed that no viable cells remained after this treatment.

A fragment of bone was placed on a sterile cover-slip in a small drop of cock plasma-chick embryo mixture; the ensuing clot served temporarily to hold the bone in position. The chorioallantoic membrane of another embryo (10 days' incubation) having been exposed by the customary

technique, the cover-slip bearing the fragment was inverted over the hole in the shell and sealed down with a beeswax mixture. The chorioallantois was then brought into apposition with the under surface of the cover-slip by inflation (Hoffstadt & Omundsen, 1940). The egg was then returned to the incubator in the vertical position in which it was maintained throughout.

For low-power microscopical examination the specimens were kept warm in a simple type of stage incubator and illuminated from one side by the focused image of a Pointolite lamp; for more critical work a Leitz Ultropak was employed with the same light source and incubator.

For histological purposes the bone and bone-bearing area of the membrane were excised and fixed in Carnoy's fluid after preliminary fixation with the same fluid in situ. Decalcification was carried out in Formol-nitric solution. Paraffin sections were generally stained according to Masson's Trichrome or Heidenhein's Azan technique. In some preparations filtered India ink was injected into the embryo circulation via a large membrane vessel. After fixation the bone-bearing area was excised, cleared in cedarwood oil, mounted and studied in toto.

Some technical difficulties were encountered. Critical examination showed that exposure of the membrane frequently led to capillary thrombosis in the exposed zone. Although this generally resolved within 24 hr., its presence was undesirable since survival of the bone fragments must depend upon re-establishment of a circulation within the briefest period. It was found that a few drops of 1/5000 heparin solution added to the shell membrane before exposure of the chorioallantois usually prevented capillary thrombosis.

In some eggs the membrane tended to sag away from the cover-slip, generally within the first 24 hr. of incubation. Under these circumstances, presumably according to the extent of clot liquefaction, the bone sometimes remained on the cover-slip (i.e. not in contact with the membrane) and inevitably dried up; sometimes it remained attached to the membrane and sagged away with it. The former preparations were discarded while the latter were 'pumped up' again as before until bone and membrane re-established contact with the cover-slip. The fragment generally remained in situ thereafter.

In spite of every precaution, some eggs became infected and were discarded.

RESULTS

The most striking observation concerned the remarkable rapidity with which a complete circulation became established within the live fragments. Of forty successfully transplanted live fragments, thirty-two were extensively vascularized within 18 hr. and the remainder within 48 hr. Of twenty-five boiled fragments studied, none were vascularized within 48 hr. A negligible circulation was present in one at 72 hr., but the remainder did not vascularize until a considerably longer period elapsed and, as will be described below, the appearance of the vascular patterns was quite different.

Observations on the living preparations

Live fragments. At the start of the experiment, primitive Haversian systems, often containing vessels full of stationary blood cells, could be seen in the bone fragment; the trabeculae of matrix and the bone-cell lacunae could also be seen. The richly vascularized membrane was naturally obscured in the region of the bone, where it was indented slightly, but elsewhere was clearly visible, and the circulation of blood cells within the vessels could easily be followed (Pl. 1, fig. 1).

After 5 hr. the indentation was less clear, and in many cases haemorrhage obscured the bone fragment. In one fragment, however, a slow but definite

movement of blood corpuscles along a vessel within a Haversian canal was already apparent.

At 18 hr. a marked change had occurred. Much of the extravasated blood had been removed and the entire fragments were penetrated by a complete system of vessels filled with circulating blood. Following the primitive Haversian canals, the vessels seemed to permeate the entire substance of the fragment. The vascular pattern of the surrounding membrane had also become augmented to some extent (Pl. 1, figs. 2, 3).

The fragment itself seemed to have sunk somewhat into the substance of the membrane so that the indentation had disappeared. Two main types of vessel were distinguishable. There were large tortuous sinusoidal channels through which the flow was relatively placid, and in which the lumen might carry perhaps 6–10 red corpuscles abreast, and there were also smaller and straighter capillaries, passing perhaps 1–3 corpuscles together at considerable speed. The size and course of any particular vessel seemed to be conditioned by the size of its canal; the vessels could easily be followed on their tortuous course through the matrix. The fact that leucocytes could be seen adhering to and rolling along the sticky endothelium showed that some local abnormality was present for the first few days.

Bone fragments were studied for as long as 10 days. Both resorption and deposition of matrix occurred. The precise details of the laying down and resorption of bone, particularly in relationship to the developing vascular pattern, are now being studied in more detail. In general terms it can be stated that growth in length or in width did not occur in the case of single grafts, but that the thickness of the fragment continued to increase. In many cases some peripheral resorption took place, which, together with central deposition, led to the formation of a sphere. The fragments remained fully viable until the host embryo hatched.

Boiled fragments. From the outset these appeared quite different. The matrix of the fragments seemed more refractile and granular and the Haversian contents were more opaque.

No vascularization occurred within 48 hr., although the vascular pattern of the membrane was somewhat augmented (Pl. 1, fig. 4). When vessels finally appeared within the bone, they were seen to consist of short and fine capillary leashes, which generally entered the bone vertically. The impression was gained that they simply penetrated the fragment by the shortest possible route. Little anastomosis within the bone was present until a late stage, and the final pattern was never so profuse as in the live fragments.

Large numbers of wandering phagocytes were present around and within these fragments.

Observations on fixed and stained material

Scrutiny of sections confirmed and amplified these findings. The unboiled fragments seemed perfectly normal. No cell degeneration was present and mitoses were seen among the osteoblasts. As in vivo, some of the cells seemed (on morphological grounds) to be actively osteogenic and others in a resting condition. In some places osteoclasts were seen. The vessels were in no way remarkable. The impression gained from sections of fragments after varying periods of incubation was, simply, that the fragment of bone had become incorporated in the host circulation and that modelling of the fragment was continuing (Pl. 1, figs. 5–7).

It was apparent that the fragments had shifted from their original situation, i.e. resting against the ectoderm, and had sunk within the substance of the membrane so as to become surrounded by mesoderm. Islands or 'pearls' of ectodermal cells were sometimes found within the marrow spaces of the fragments. In one case, such a pearl (Pl. 1, fig. 7) was traced in serial section to the ectoderm. Just beneath the fragments, but generally separated from them by one or more fusiform connective tissue cells, were syncytial masses, also of ectodermal origin. Danchakoff (1918) reported similar findings in spleen grafts. Goodpasture & Anderson (1937) evidently saw similar cells but described them as foreign body giant cells.

Sections of boiled fragments showed a different state of affairs. The matrix was shrunken and distorted with remains of cells and connective tissue present. Some cellular infiltration was apparent at 48 hr. (Pl. 1, fig. 8), and later a richly cellular and highly vascular connective tissue surrounded the grafts. There was little histological evidence of resorption and the boiled fragments seemed to be well tolerated. The reaction of the host tissue was rather slight, consisting chiefly of mononuclear cell infiltration; occasional osteoclasts were seen.

DISCUSSION

There are two possibilities regarding the manner in which the newly developed circulation arose from the membrane. First, it may have arisen completely de novo as capillary sprouts which pushed their way through the Haversian systems; secondly, newly formed and relatively short vascular buds may have grown up to, and fused with the cut ends of the vessels already present in the bone. The latter explanation seems the more likely; the time factor is in its favour, and the phenomena seem to parallel closely those observed in the junction of lymphatic vessels within transparent ear chambers (Clark & Clark, 1937). The early haemorrhages seen in the region of the graft thus probably arose as a result of the junction of a host capillary with one end of a graft vessel; a preliminary 'flushing' of the fragment would thus take place before

the establishment of a complete and closed vascular channel. This process was actually witnessed on one occasion, at the 5th hour.

Thus, under the present experimental conditions, which, essentially, predicate a highly reactive and vascular graft bed, it is safe to assume that transplanted fragments of live bone not only survived, but that, through the agency of their surviving cells they deposited fresh bone substance. The necessity for postulating a specific osteogenic inductor does not, therefore, arise in these experiments.

In the light of the foregoing it seems justifiable to suggest an alternative explanation for the occurrence of fresh bone in the grafting experiments of Levander (1938); namely, that some osteogenic cells within the fragment survived operation, and, having wandered out in their characteristic fashion from the fragment, brought about deposition of bone in the surrounding host tissue.

Fell (1932) demonstrated osteogenesis in tissue cultures 6 days after explanation of endosteal osteoblasts; Levander (1938) also observed it within 6 days of transplantation. In the present experiments trabecular thickening was often found after 4 days, a result which agrees well with the former observations. Osteogenesis following extract injection seems to develop only after a prolonged latent period and the results reported here shed no light upon the developmental history of such bone, nor upon bone formation following transplantation of dead tissue. The extent to which the results can be applied to the more general problem of osteogenesis is difficult to estimate. They constitute perhaps, evidence in support of the view that chip grafts survive and form osteogenic foci; they lay emphasis also on the need for delicate handling of transplant fragments.

SUMMARY

1. Grafts of living embryo bone, transplanted to chick chorioallantois, survive and rapidly become vascularized.

2. Boiled fragments do not become vascularized in a comparable manner.

3. Osteogenesis occurs in the former, but not in the latter.

4. It seems likely that short capillary buds developing from the host join up with vessels pre-existing in the transplanted fragment.

5. The application of these results to other work is discussed.

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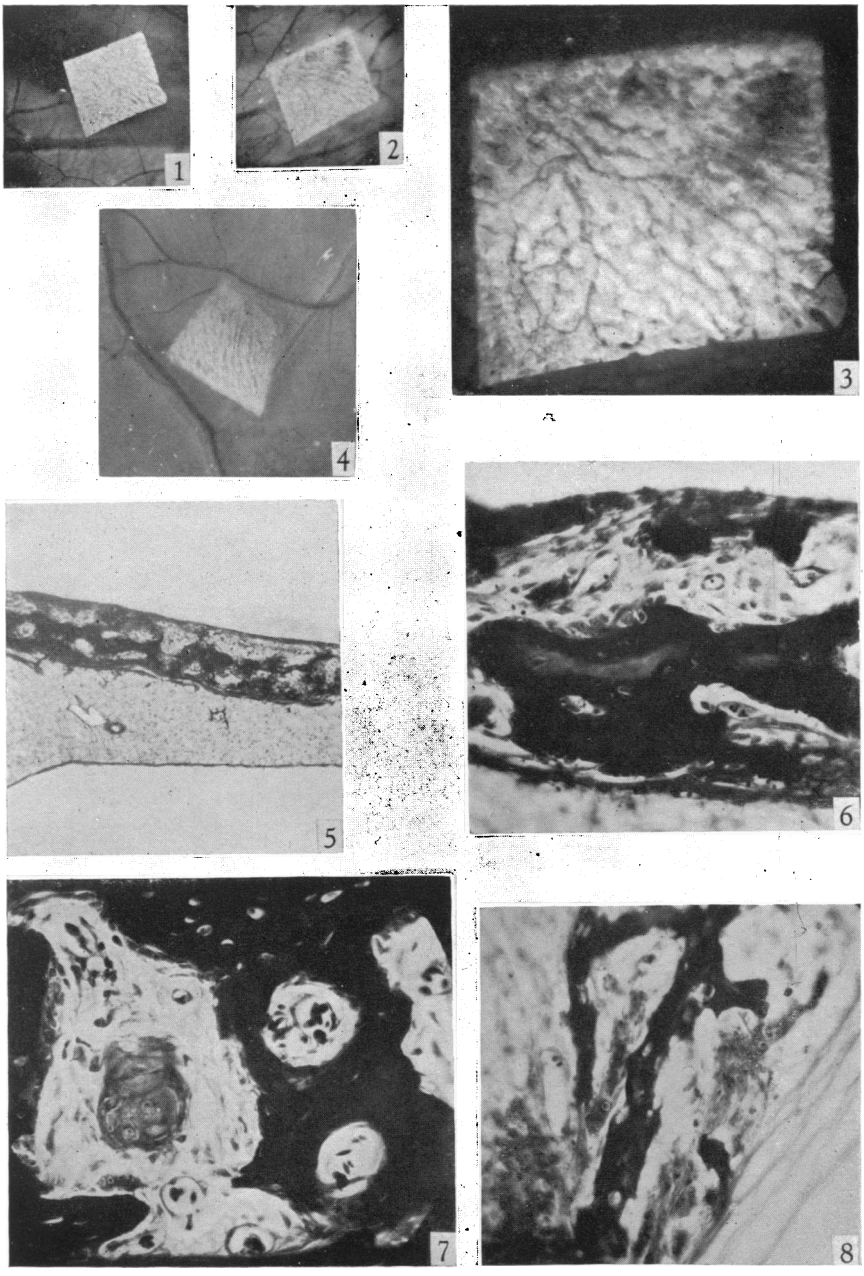

Figs. 1–8.

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EXPLANATION OF PLATE I

Fig. 1. Fragment of normal living bone at start of experiment. Outlines of primitive Haversian canals are visible. The chorioallantoic membrane can be seen around the fragment. ×6.

Fig. 2. Same fragment, 48 hr. incubation. The top right-hand corner is to some extent obscured by haemorrhage. Vessels can be distinguished within the fragment. ×6.

Fig. 3. Higher magnification of fig. 2. The course of vessels is easy to distinguish. Note ramification near lower left-hand corner. ×30.

Fig. 4. Fragment of boiled bone, 48 hr. incubation. Outlines of primitive Haversian canals can be seen but there is no vascularization. ×6.

Fig. 5. Vertical section of normal bone on membrane, 24 hr. incubation. Masson Trichrome stain. ×50.

Fig. 6. Similar, 3 days' incubation. The bone appears normal. ×400.

Fig. 7. Similar, 8 days' incubation. A primitive Haversian canal is seen. An 'ectodermal pearl' occupies a central position. Above and to its left, active osteoblasts are present: below, an osteoclast. Two small canals containing capillaries are present to the right. ×400.

Fig. 8. Boiled bone, 48 hr. incubation. Shrunken and distorted lamellae are visible. Commencing cellular infiltration of bone. ×400.