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THE INDUCTION OF BONE IN ORGANIZING HAEMATOMATA IN RATS

by

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The induction of ectopic bone formation has been described in a wide range of experimental systems. This has emphasized the fact that undifferentiated osteogenic precursor cells persist in post foetal life after embryonic differentiation has ceased. A key observation in the studies on the transformation of these cells into osteoblasts was made by Urist [1965] when he discovered that the decalcified organic matrices of bone and dentine had osteoinductive properties. This phenomenon is attributed to the physicochemical effect of an inductor present in these matrices which induces competent mesenchymal cells to differentiate into osteoblasts [Urist *et al.*, 1967]. The osteogenetic competence of mesenchymal cell populations was shown to be restricted in post-foetal life to specific areas of the body and in these areas mesenchymal cells showed different levels of readiness to differentiate into bone in response to implants of decalcified allogenic bone matrix (D.A.B.M.) [Urist *et al.*, 1967].

The use of an induction system to assist refractory skeletal regeneration is dependent upon it being able to initiate osteogenesis and maintain it until bone continuity is restored. Extraskeletal implants of D.A.B.M. however become encapsulated in fibrous connective tissue soon after implantation and this forms a barrier which confines the yield of induced bone to the limits of the enclosed matrix [Urist, 1965].

The rapid unrestricted ingrowth of woven bone into organizing fracture haematomata suggested that the cell population in granulation tissue may provide a favourable environment for bone induction.

This study was undertaken to determine whether implants of D.A.B.M. would induce osteogenesis in the granulation tissue of organizing haematomata held in perforated silicone rubber chambers implanted subcutaneously in rats.

MATERIAL AND METHODS

Preparation of silicone rubber chambers

Fifteen cylindrical silicone rubber chambers 12 mm long by 4 mm in diameter were prepared in order to hold blood clots and D.A.B.M. implants under standardized conditions in a soft tissue environment in rats (Fig. 1). The chambers were prepared using 250 μm thick silicone polymer sheeting (Silastic—Dow Corning, Michigan, U.S.A.). This material is biologically inert and has no inhibitory effects on cellular growth or differentiation [Basset, 1962]. The transparent sheeting was laid over a sheet of graph paper ruled with 1 mm squares and points situated 2 mm apart marked on to it in a square pattern. Using a dental rubber dam punch, holes 0.75 mm in diameter were punched through the rubber sheeting. The sheeting was then cut into 12 mm squares, rolled into tubes and the apposing edges held together by means of a thin strip of adhesive tape applied internally along the seam. A bead of self curing silicone polymer liquid (Medical Adhesive Type A—Dow Corning) was applied externally over the apposed edges of each tube and left to cure for 24 hours at room temperature. The adhesive tape on the inner surfaces was then removed and the open ends of the tubes closed with discs of silicone rubber 5 mm in diameter, applied with

the adhesive over the tube ends. After the adhesive had cured the chambers were washed in distilled water and autoclaved.

Preparation of D.A.B.M. implants

Four Long Evans rats weighing 150 g were killed with an overdose of pentobarbitone sodium administered intraperitoneally and their metatarsal bones were removed under aseptic conditions. The bones were scraped to remove all adhering soft tissues and the epiphyses were cut off. The remaining diaphyseal portions were decalcified in 250 ml of 0.6 N HCl for 24 hours at 4°C. After decalcification the bones were cut into 6 mm lengths, rinsed in several changes of physiological saline and then stored in 70% ethanol. Just prior to their use the D.A.B.M. implants were rinsed in physiological saline to remove the ethanol.

Surgical procedure

Fifteen 260 g male Long Evans rats were anaesthetized in turn with a neuroleptanalgesic preparation (Thalamonal-Janssens Pharmaceutica, Belgium) containing 0.05 mg/ml fentanyl and 2.5 mg/ml droperidol at a dosage rate of 2.5 ml/Kg administered intramuscularly. Each rat was positioned lying on its right side and the skin over the right inner thigh and left flank shaved and cleansed with an alcoholic solution of chlorhexidine (Hibitane I.C.I.).

A skin incision was made in the hind leg overlying the right femoral vein which was exposed by blunt dissection. 0.25 ml of blood was withdrawn from the vein in a syringe. A sterile silicone rubber chamber was filled with the blood and a D.A.B.M. implant placed in the chamber through a flapped open end of the chamber.

The filled chamber was placed in a petri dish and whilst the blood clotted, the leg wound was sutured. The left external oblique abdominal muscles were exposed through a flank incision and the chamber was sutured at each end to the muscle surface. The wound was then flooded with the residual blood held in the syringe filling the space surrounding the implanted chamber. The subcutaneous fat and skin was reapposed and sutured.

Retrieval of specimens

Groups of 3 rats were killed at intervals of 1, 2, 3, 4 and 6 weeks after operation and the implanted chambers were carefully dissected out enclosed in a small amount of surrounding soft tissue.

Tissue preparation

The specimens were fixed in buffered formol saline, dehydrated through serial alcohols, cleared in methyl salicylate and embedded in Paraplast (Sherwood Medical Industries Inc.). Serial sections, 7 μm thick, were cut transversely through the embedded chambers and stained with haematoxylin and eosin. In specimens in which newly calcified tissue was encountered, surface decalcification of the exposed end of the wax embedded tissue was performed in 5% nitric acid to enable a few decalcified sections to be prepared.

RESULTS

The operative wounds all healed by primary intention. At one week granulation tissue consisting of thin walled capillaries and young fibroblasts infiltrated with wandering macrophages and leucocytes had surrounded the chamber (Fig. 2). Granulation tissue had developed through the openings in the chamber walls and was proliferating into the blood clot within, along the inner surface of the chamber (Fig. 3). No changes had occurred in the D.A.B.M. implant at this stage.

At two weeks the granulation tissue had completely filled the chambers and enveloped the implants and filled their medullary cavities. Erosion chambers produced

by the proteolytic activities of wandering macrophages had appeared in the matrices of the implants and these were occupied by capillaries and mesenchymal cells (Fig. 4). At this stage the granulation tissue surrounding the implants had become less vascular and more fibrous, enclosing the implant in loose fibrous connective tissue.

At 3 weeks deposits of calcified tissue appeared in the implant matrices (Fig. 5). In partially decalcified sections induced osteoblasts were seen lying in contact with the newly formed matrix on the walls of excavation chambers situated around centrally placed capillaries (Fig. 6).

Deposits of new bone occurred in increasing amounts in the four and six weeks specimens. This only formed within the inner areas of the implanted matrix (Figs. 7 and 8).

DISCUSSION

The induction of bone within organizing extraskeletal haematomata has indicated that osteogenetically competent cells are present in the cell population of granulation tissue.

The presence of clotted blood in the implanted chambers invited an ingrowth of granulation tissue which initially is highly vascular but soon becomes more fibrous. The D.A.B.M. implants become surrounded in a thick fibrous connective tissue capsules before deposits of new bone appeared. Urist *et al.* [1967] showed that intramuscular implants of D.A.B.M. were enclosed in a thin fibrous membrane within three to four days of implantation. This restricted any subsequent bone formation to the enclosed area.

For bone induction to occur directly in granulation tissue, an inductive substrate would have to act on mesenchymal cells present before they differentiate into connective tissue cells. Encapsulation of the inductive substrate occurred long before osteoblast differentiation began, effectively isolating the substrate from any surrounding cells with osteogenetic potential.

The implantation of allogenic human dentin matrix in gingival papillae to assist the regeneration of osseous defects in periodontal disease [Register *et al.*, 1972] resulted in the formation of isolated ossicles of bone surrounded by connective tissue which did not attach to the alveolar bone.

For D.A.B.M. grafts to be used effectively direct contact between the inductive substrate and host bone is advisable to avoid encapsulation of the graft.

The restoration of experimental periodontal defects in dogs with D.A.B.M. by Narang and Wells [1972] showed that contact resulted in bony union between the graft and the host bone and stimulated the formation of new bone attached to host bone at the site of implantation.

The presence of clotted blood around decalcified allogenic bone matrix substrates creates conditions which are favourable for the isolation of induced bone in fibrous tissue.

SUMMARY

1. The induction of bone in extraskeletal haematomata with implants of decalcified allogenic matrix has been studied in rats.

2. The formation of new bone within the implants has indicated a source of osteogenetically competent mesenchymal cells in granulation tissue.

3. The implants were enclosed within a capsule of fibrous connective tissue within 14 days of implantation.

4. This surrounding connective tissue capsule confined new bone formation to the area of the implanted matrix.

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PLATE 1

Fig. 1. A silicone rubber implant chamber. Note the perforated walls to allow for granulation tissue ingrowth. (× 3)

Fig. 2. Photomicrograph showing a section through an implanted chamber at 1 week. Lying in the centre of a film meshwork (F) is the D.A.B.M. implant (I). Granulation tissue (G) is seen growing through sectioned holes in the chamber walls (arrowed). The clear areas between the granulation tissue ingrowth (S) are occupied by the chamber walls (× 100)

Fig. 3. Photomicrograph showing the granulation tissue ingrowth at 1 week, composed of thin walled capillaries (arrowed) and young fibroblasts, macrophages and lymphocytes growing into the film meshwork (F). (× 100)

Fig. 4. Photomicrograph of a section through the D.A.B.M. implant (I) at 2 weeks. Excavation chambers (arrowed) within it are occupied by capillaries and mesenchymal cells. (× 230)

PLATE 2

Fig. 5. Photomicrograph of a section through the implanted matrix at 3 weeks showing deposits of calcified bone (N) within the implant. (× 290)

Fig. 6. Photomicrograph of a section through a partially decalcified specimen at 3 weeks showing young osteoblasts (O) lying on newly deposited bone on the walls of excavation chambers in the implanted matrix. Chondroblasts (C) are also present in excavation chambers. (× 275)

Fig. 7. Photomicrograph of a section through an undecalcified specimen at 6 weeks showing a large deposit of new bone (N) lying with the transplanted matrix (I) which is enclosed in a loose fibrous connective tissue. (× 70)

Fig. 8. Photomicrograph of a section through a partially decalcified specimen at 6 weeks. A cement line marks the interface between the new bone (N) and implanted D.A.B.M. matrix (I). A line of osteoblasts (O) differentiated from mesenchymal cells can be seen on the surface of newly formed bone. (× 165)