TRANSFORMATION OF FIBROBLASTS BY ALLOGENEIC AND XENOGENEIC TRANSPLANTS OF DEMINERALIZED TOOTH AND BONE*

By CHARLES HUGGINS, M.D., SOL WISEMAN, and A. H. REDDI, Ph.D.

(From The Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Illinois 60637)

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In postfetal life after embryonic differentiation has ceased, some cells of the connective tissue still remain in an undifferentiated state which persists lifelong. These primitive dormant cells have a remarkable attribute—they can be readily induced to differentiate by experimental means.

Under a certain set of conditions, grafts of demineralized matrix of dentin or bone in adult animals invariably and rapidly changed the phenotype of fibroblasts adjacent to the transplant. The visible and biochemical characters of the altered cells were changed so profoundly under these artificial conditions that we shall refer to the phenomenon as transformation. Under other circumstances cells of the graft survived but were not transformed.

The change from normal into cancer cell can be brought about by many different experimental procedures. In animals the change of normal cell into a normal cell of different sort has been accomplished only in a single cell type, the fibroblast (mesenchyma, stem cell of connective tissue). After transformation, the responding fibroblasts emerge as chondroblasts or osteoblasts and their fate is altered permanently.

In the present experiments useful and simple techniques to study transformation in vivo were developed. The results of allogeneic and xenogeneic transplantation are presented in this paper.

There are two distinctive experimental methods to alter the phenotype of competent fibroblasts; these consist of bringing them in contact with, respectively, (*a*) osteogenic epithelium or (*b*) demineralized matrix of tooth and bone.

The remarkable and unique mutability of fibroblasts which permits this radical change of their phenotype was found out in the dog (1). The surgical approximation of bladder epithelium with fascias of the trunk or limb evoked large amounts of bone which was evident in 10–12 days; it is noteworthy that

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cartilage was not observed. Three sorts of epithelium are osteogenic: urinary tract, biliary tract (2), seminal vesicle (3). Epithelial osteogenesis can be induced invariably in the dog and guinea pig (3), but bone is induced in the fibroblasts of the rat and rabbit rather infrequently. Bone does not occur in the normal bladder or gall bladder despite the fact that the submucosal fibroblasts lie in close proximity to repressed but potentially powerful osteogenic epithelium. The fibroblasts are of two physiologic types (1), respectively competent or incompetent to undergo the transformation.

Autologous grafts of tooth without demineralization to the fascias of puppies led to osseous creeping substitution (4) of dentin and enamel of the graft and bone formation in the pulp space.

A key observation in the transformation of animal cells in postfetal life was made by Urist (5) who discovered that decalcified lyophilized matrix of bone and tooth is an "inductive substrate" since it evokes differentiation of mesenchyma into cartilage and bone. The transformation occurs in vivo and in tissue culture (6). The transforming factor is heat-stable (7) and resistant to strong acids (8); it is destroyed by dilute solutions of sodium hydroxide and formaldehyde and by UV irradiation.

Xenogeneic transplants (7) of bone matrix of calf or man to rat or rabbit produced extensive purulent exudates; scanty amounts of cartilage or bone were reported to occur in a few instances after several months.

Methods

Tooth and bone of several species were processed rapidly after removal from the donor. A surgical specimen of human bone was obtained from the operating room; it was comminuted by hammer blows before demineralization. Incisor teeth of animals were excised following decapitation and the dental pulp was removed with a wire. All of the experiments were performed with dehydrated powder (200–400 μ) of tooth or bone.

Mineralized dentin contained its natural minerals and was not treated with acid or phenol. The tooth was morsellated to a fine powder with hammer blows, extracted repeatedly with ethanol for 24 hr, and dried at 37°C. Bacteriologic cultures were made on eosin-methylene blue agar.

Demineralization refers to the preparation now to be described. Tooth and bone were extracted with 0.5 N hydrochloric acid (circa 1 cc/mg) at room temperature (27–30°C) with magnetic stirring. After 1 hr the extremities of the bone were amputated and discarded; the tooth and bone were cleaned, shredded, and again extracted with fresh 0.5 N hydrochloric acid (several changes) for an additional 2 hr. The shreds were extracted with ethanol, with water-saturated phenol for 0.5 hr, with 70% ethanol, and with water. Then the specimens were frozen with liquid N_2 and powdered with mortar and pestle. The powders were dehydrated with ethanol followed by ether. The time of processing was less than 8 hr after excision. The powders were dried overnight at 37°C and sieved. A sample of dry powder was hydrolyzed for 18 hr at 105°C; the content of Ca^{2+} was determined in an atomic absorption spectrophotometer.

The recipients were not inbred. They were: male or female guinea pigs, circa 200–500 g in weight, purchased from a dealer; female mice of CF-1 stock, age 7–10 wks; male or female rats of Long-Evans strain, age 25–35 days.

The recipients were anesthetized with ether. Under sterile conditions an incision, circa 1 cm in length, was made in the skin of abdomen or thigh and a subcutaneous pocket was prepared by blunt dissection. A knife point full (Messerspitze) of powder (10–15 mg) was deposited in the surgically prepared pocket and the incision was closed with a metallic skin clip. Usually transplants were made in five locations at one sitting in each recipient. The day of transplantation is denoted day 0.

At harvest the grafts were weighed and alkaline phosphomonoesterase (3.1.3.1) was determined by chemical (9) and histochemical (10) methods. A portion of the specimen was excised for histologic study and the remainder homogenized. After centrifugation the phosphatase and protein (11) concentrations of the supernatant were determined. One unit was defined as the enzyme activity which liberated 1 μmole of *p*-nitrophenol/0.5 hr under the stated conditions (9). The results are given as specific activity: enzyme units/mg of protein.

RESULTS

Events Following Allogeneic Transplantation (Rat).—The rate of extraction of Ca^{2+} from rat tooth was determined (Fig. 1). The matrix was calcium-free at 60 min.

The transforming factor was present in all parts of the demineralized pulpless tooth. These comprise: enamel and dentin, crown and root. Age and sex of the donor were not factors in the transformation; transformability is approximately equipotent in tooth of rats age 30 and 180 days both in the males and in the females.

The transforming factor was stable in pooled, dehydrated powders of demineralized dentin. These retained their transforming potency when kept on the laboratory table at room temperature for 6 + months. Many of our studies were carried out on two samples, each circa 25 g, of coarse powder (200–400 μ) of demineralized rat tooth and bone. The material was devoid of Ca^{2+} and alkaline phosphatase. Transformation was accomplished in more than 500 consecutive allogeneic transplants of each of these large pools.

10 consecutive samples of coarse bone powder were uniform in the transformations that they evoked. On day 0 the coarsely powdered, dehydrated, demineralized rat bone or tooth, 10–15 mg, was transplanted to rats; on days 9–21 hard, calcified, radio-opaque conglomerates, 60–150 mg, were harvested. The content of alkaline phosphatase increased progressively for 2 wk (Fig. 2); high levels of activity were found on days 9–14 after which the values declined. Our samples of bone and tooth were rather similar in the quantitative effects which they called forth.

The following observations were made after allogeneic transplantation of our sample of coarse powder of dehydrated demineralized dentin. There was an early and intense attraction of the dentin for fibroblasts of the subcutaneous tissue. Within 24 hr fibroblasts surrounded the graft and fused with its surface; there was induction of alkaline phosphatase not only in the enveloping fibroblasts but

in their neighbors at some distance from the transplant. Cartilage was first found on day 6 and its presence was transitory; on day 14 many of the chondrocytes had succumbed. We did not observe cartilage after day 18. Bone was detected first on days 10–11 and hemopoietic bone marrow on day 15. On day

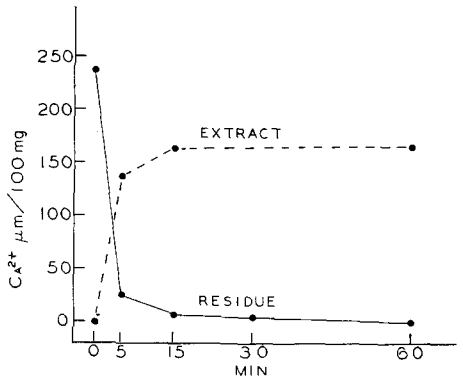

FIG. 1. The rate of removal of calcium from rat dentin by 0.5 N hydrochloric acid (designated "extract") at 30°C. The demineralized dentin residue was calcium-free at 60 min.

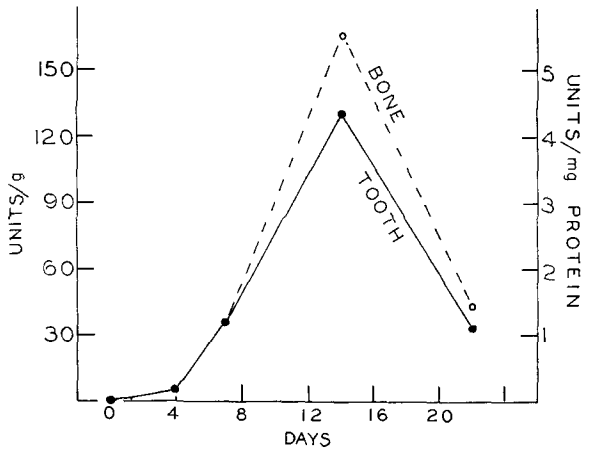

FIG. 2. Alkaline phosphatase concentration (unit/gram) and specific activity (units/milligram protein) of allografts of demineralized rat bone and tooth in the subcutaneous tissue.

365 the dentin grafts were composed of living bone which in the gross appeared like ivory pegs.

Many substances failed to induce transformation of fibroblasts after allogeneic transplantation in the rat. They were inserted in surgically prepared subcutaneous pockets and harvested on days 11–14.

Among the Inactive Materials were.—(*a*) Reconstituted collagen[1] extracted from rat skin or tendon with cold acetic acid (3%). These materials calcified in vivo in the test period but there was no induction of alkaline phosphatase, bone, or cartilage. (*b*) Residue of rat tendon after demineralization with 0.5 N hydrochloric acid and phenol. (*c*) Demineralized intervertebral disc. (*d*) Mineralized rat dentin. Powdered rat dentin containing its original minerals was sterilized and extracted with ethanol for 24 hr and dried before transplantation. This mineralized dentin did not induce alkaline phosphate, cartilage, or bone. A control demineralized sample of the same pool was very active in transforming fibroblasts. Expressed as specific activity, mean values of alkaline phosphatase were: mineralized graft 0.03, demineralized graft 2.6, and control fibroblasts, 0.03.

Xenogeneic Transplantation of Dentin.—Our sample of mineralized dentin of rat was bacteria-free; it evoked purulent exudates in mouse and guinea pig and cartilage or bone were not detected. Demineralized dentin did not elicit abscesses in the foreign species. It was evident that the process of acid extraction had diminished the histoincompatability of the graft.

Demineralized coarse powder of dentin of guinea pig, mouse, and rat was transplanted systematically to recipients of the same three species; in addition demineralized unpowdered mouse teeth were transplanted similarly. Each recipient received simultaneously grafts of its own and the other two species. The transplants were harvested on days 9–14.

There was no dichotomy in the induction of cartilage and bone, both tissues or neither were observed. Day 11 was particularly favorable for harvest of the transplants since, in the positive cases, both chondrocytes and osteoblasts are present at this time. Histochemical determination of alkaline phosphatase was advantageous in enabling the recognition of small nests of transformed cells. The presence of cartilage and bone in allogeneic and xenogeneic grafts on day 11 in three species is summarized (Fig. 3).

Recipient Guinea Pig.—15 guinea pigs were studied. Cartilage and bone were not observed in connection with any transplant in this species. Regardless of the species of the donor of the graft, the reaction of histoincompatibility was not apparent but the graft underwent dissolution in the guinea pig. In five guinea pigs, grafts of guinea pig origin vanished in four cases within 14 days; the transplant became fibrillar, edematous, and dispersed in small pieces. A similar dissolution (Table I) of mouse dentin occurred in guinea pig. Demineralized rat dentin was more hardy and it induced small amounts of alkaline phosphatase.

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Recipient Mouse.—Demineralized mouse dentin evoked large amounts of cartilage and bone and rat dentin induced small amounts (Fig. 3). Guinea pig dentin was inactive as a transformer. The rejection response characteristic of histoincompatability, which is particularly severe in grafts of unmodified foreign tissue to mouse, was not evident in the mouse recipients of demineralized dentin.

Recipient Rat.—Demineralized dentin, whose origin was guinea pig, mouse, or rat, evoked large quantities of cartilage (Fig. 4) and bone in the rat.

Donor	Recipient of Graft		
	Guinea pig	Mouse	Rat
Guinea pig	0	0	++++
Mouse	0	++++	++++
Rat	0	+	++++

FIG. 3. Cartilage and bone in allografts and xenografts of dentin from three species on day 11: +, slight; ++++, large amounts; 0, no cartilage or bone.

TABLE I

Alkaline Phosphatase Values in Allogeneic and Xenogeneic Transplants of Demineralized Dentin

Species of donor	Species of recipient		
	Guinea pig	Mouse	Rat
Guinea Pig	0.07*	0.05 (0.01–0.09)	0.80 (0.31–1.69)
Mouse	0.10*	0.52 (0.39–1.02)	0.80 (0.54–0.91)
Rat	0.08 (0.04–0.14)	0.20 (0.02–0.36)	4.28 (1.09–5.93)

Specific activity of alkaline phosphatase in transplants on days 9–14. The mean and range of values are given. There were five grafts in each category.

* Four of five grafts disappeared.

Alkaline Phosphatase.—In rat, large concentrations of alkaline phosphatase were induced by demineralized dentin of the three species, guinea pig, mouse, and rat. In mouse, alkaline phosphatase was induced by mouse dentin and, to a smaller extent, rat dentin. The highest concentration of alkaline phosphatase was observed (Table I) when dentin of rat was transplanted to rat as recipient.

Transplantation of Human Bone to Rat.—Our specimen consisted of a coarse powder of dehydrated, demineralized bone (occiput) of a child age 3; it was transplanted to rat. On day 14 the transplant was pearly, translucent, and resembled cartilage. Alkaline phosphatase (specific activity) values were: mean 0.13, range 0.02–0.50. In histological sections on day 14 we found (*a*) induction of alkaline phosphatase in fibroblasts adjacent to the graft, and (*b*) small islands of cartilage (Fig. 5).

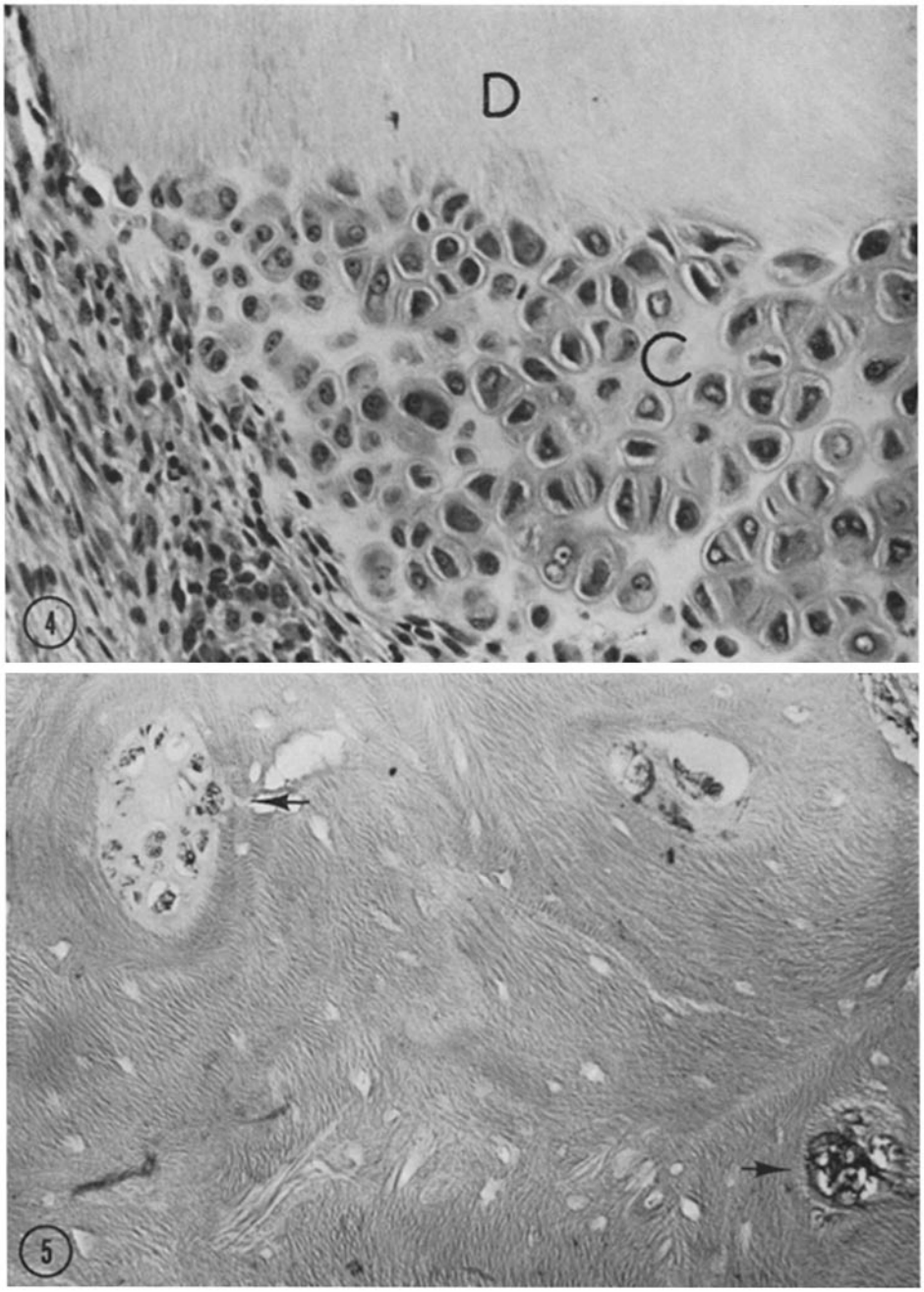

FIG. 4. Xenogeneic transplant of demineralized mouse dentin (*D*) to the subcutaneous tissue of the rat on day 7. × 350. A large mass of cartilage (*C*) is apparent.

FIG. 5. Xenogeneic transplant of demineralized human bone to subcutaneous tissue of rat on day 13. Arrows point to islands of cartilage. × 100.

DISCUSSION

The histochemical demonstration of alkaline phosphatase in transplants was of special value in detecting minute amounts of cartilage or bone.

Most of our experiments were performed with pooled samples of coarse powders of dehydrated, demineralized dentin of the rodent species. These preparations were uniform, stable for many months, easy to manipulate, and yielded reproducible results.

Sterile mineralized rat dentin, untreated with acid, failed to elicit transformation and it caused abscesses around allogeneic transplants. The extraction of rat dentin by strong acids and water-saturated phenol sterilized and demineralized. Proteins were denatured so that transplants into foreign species were not rejected. Demineralization makes the transforming factor available and transplant becomes tolerable in foreign species.

Systematic xenogeneic transplantation of demineralized dental matrix into three species of rodents permitted an analysis of (*a*) competence of cells to respond by transformation and (*b*) potency and availability of the transformer.

We found differences in competence of fibroblasts to be transformed by demineralized dentin. Rat fibroblasts were most susceptible to transformation of this sort; they were transformed by demineralized dentin of guinea pig, mouse, and rat. Mouse fibroblasts were transformed by dentin transplants from mouse and rat, but not by dentin from guinea pig. Fibroblasts of guinea pig were not transformed by dentin.

As a recipient, guinea pig had a singular reaction to grafts of dentin. Most of the transplants of demineralized dentin of guinea pig or mouse into guinea pig underwent dissolution and vanished in a few weeks. Rat dentin persisted for 2 wk but did not induce alkaline phosphatase or transformation.

Whereas fibroblasts of guinea pig did not undergo the dentin transformation, it is remarkable that they are highly susceptible to transformation by the osteogenic epithelia. Conversely, rat fibroblasts, which are most prone to dentin-transformation, are rather insusceptible to the osteogenic transformation induced by epithelium.

Transformation was effected by all species of demineralized dentin and bone which we examined. Rat dentin and rat bone were the most potent. Human bone was least potent in the induction of alkaline phosphatase and cartilage, but it is certain that the power to transform fibroblasts resided in the specimen which we studied.

Transformation of fibroblast into cartilage cell is a lethal effect and the chondroblasts produced in this way succumbed within 14 days. But the newly formed osteoblasts and their descendants persisted without obvious change for more than 1 yr.

SUMMARY

Xenogeneic transplants of powdered, dehydrated, demineralized matrix of bone and tooth were well tolerated in three species of rodents. Differences between the species were found in competence of fibroblasts to be transformed into cartilage and bone in vivo by these preparations. Rat fibroblasts were most susceptible to transformation of this sort; they were transformed by demineralized dentin of guinea pig, mouse, and rat, and to a limited extent, by a specimen of decalcified human bone.

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