Biochemical Sequences in the Transformation of Normal Fibroblasts in Adolescent Rats

(bone matrix/alkaline phosphatase/ ^{32}P / ^{35}S / ^{45}Ca)

A. H. REDDI AND CHARLES HUGGINS

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

Contributed by Charles Huggins, March 25, 1972

ABSTRACT Coarse powders of acid-insoluble matrix of diaphysis and calvarial parietal bone rapidly and consistently transformed fibroblasts into masses of cartilage and bone containing hemopoietic marrow. The transformant was encapsulated by fibroblasts within 24 hr to form a plaque. Transformation was restricted to the central thicknesses of the plaque. Under the stated conditions the alteration of the phenotype, fibroblast to chondroblast, was an unstable transformation, whereas the phenotype change, fibroblast to osteoblast, was stable. The transformation occurred on a rigid timetable of sequences. Measurements of alkaline phosphatase activity and incorporation of radioactive sulfate, phosphate, and calcium were sensitive and quantitative assays for the appearance of the transformed products, cartilage and bone.

The aim of this work was to develop reproducible, rapid, and quantitative methods (a) to induce the fibroblast-chondroblast-osteoblast transformation, and (b) to differentiate the major links in the biological sequence.

Long after embryonic differentiation has ceased, fibroblasts still retain the singular potential of transformability (1) into cells of other sorts, an attribute that persists throughout the life of the animal. The visible and biochemical characters are altered so profoundly that we refer to the phenomenon as transformation (2). Approximation of transformant (TF) and competent responding fibroblasts (R) initiates a series of interconnected biological reactions that yield products which we shall designate: P_1 , cartilage; P_2 , bone; P_3 , hemopoietic bone marrow.

Urist discovered that intramuscular transplants of lyophilized segments of demineralized bone (3) or tooth (4) transform fibroblasts to form bone by endochondral ossification in 24-26 days. Chondrogenesis occurs in cell culture (5), as well as *in vivo*. There are two convenient enzyme assays to study the transformation of the fibroblasts of fascia into cartilage, followed by bone: a. activity of alkaline phosphatase (6); b. determination of the quotient of activity: lactate dehydrogenase/malate dehydrogenase (7).

The present experiments consisted of allogeneic transplantation of a weighed amount of sized desiccated powder of acid-insoluble bone matrix to the subcutaneous tissues of young rats. This technique provided a simple, quick, and standardized method to induce the transformation. The biochemical sequences of cartilage and bone in the chain reaction were analyzed by measurement of enzyme activities and incorporation of isotopes in the transformation products.

MATERIALS AND METHODS

Preparation of Transformant. Manufacture and final storage of all preparations were at room temperature (about 25°). When liquids of any sort were used, the biological materials were immersed in the fluids in a jar with a magnetic stirrer, where they were propelled around the vortex created by vigorous stirring.

Large adult rats of both sexes were used as donors throughout. The rats were decapitated. Parietal bones of the calvarium were removed and fragmented. Long bones were excised, their extremities were amputated, and the bone marrow was discarded. The diaphyses were scrubbed with a stiff brush and cut into chips with a bone cutter; the adherent soft tissues were removed meticulously. The bones were washed with copious amounts of water, 2 hr; absolute ethanol, 1 hr; and ethyl ether, 0.5 hr. They were dried at 37° overnight and stored. The dry preparations retained transforming potency for periods of storage as long as 2 years.

Demineralized Bone Powder. Dehydrated bone chips were crushed with hammer blows and sieved. All experiments were conducted with a pool of the dry powders having particle size 74-420 μm . The powders were demineralized as follows: 0.5 N HCl, 25 meq/g for 3 hr; repeated changes of water, 2 hr; absolute ethanol, 1 hr; and ethyl ether, 0.5 hr. They were dried overnight at 37°. The residue was devoid of Ca^{2+} and inorganic P. Organic phosphorus content of the demineralized bone powder was $12.9 \pm 0.8 \mu\text{mol/g}$ dry weight; total N content was $15.6 \pm 0.5\%$. Incineration of the residue for 12 hr at 900° in a platinum crucible yielded a trace of black ash.

Bioassay and Enzyme Assays. Rats of the Long-Evans strain, 25-35 days old, were anesthetized with ether. A 1-cm incision was made in the skin of thorax, abdomen, or loin under sterile conditions, and a pocket was prepared by blunt dissection. A weighed knife-point-full of bone powder (10-20 mg) of known size was inserted as a compact deposit on the muscle forming the floor of the surgically prepared pocket. The incision was closed with a metallic skin clip. The day of transplantation is designated day 0.

At harvest, the grafts were cleansed of adherent soft tissue and weighed. It was useful to relate the weight of the original transplant (in) to the weight of the product (out); transplant weight ratio is the quotient of weight out/in. Tissues for histological section were preserved in Bouin's fluid; paraffin sections were stained with hematoxylin-eosin. Tissues pre-

Abbreviation: TF, transformant fibroblasts.

served in neutral formaldehyde were stained additionally with silver nitrate.

Aliquots of the harvested tissues were homogenized in ice-cold 0.15 M NaCl–3 mM NaHCO₃ in a Polytron homogenizer for three 10-sec bursts at the maximum setting. Homogenates were centrifuged at $12,000 \times g$ for 15 min at 2°; the supernatant was removed for enzyme assays. One unit of alkaline phosphatase (EC 3.1.3.1) was defined as the enzyme activity that liberated 1 μ mol of *p*-nitrophenol per 0.5 hr under stated conditions (8).

Radioactivity: Materials. H₃³²PO₄ (carrier free), Na₂³⁵SO₄ (716 Ci/mol), and ⁴⁵CaCl₂ (2200 Ci/mol) were obtained from

New England Nuclear Corp. Papain (EC 3.4.4.10) was purchased from Worthington Biochemical Corp.

³⁵S Incorporation. 100 μ Ci of Na₂³⁵SO₄ in 0.2 ml of saline was injected intravenously *via* the caudal vein. 4 Hr later, the animals were killed and the grafts were dissected out. Half of the plaque was used for histology and enzyme assays, the other half was used in the determination of radioactivity. For the latter procedure, the grafts were cut into 1- to 2-mm cubes and washed with stirring in 0.1 M Na₂SO₄ (nonradioactive) in 0.05 M sodium phosphate buffer (pH 7.4) for at least 30 min. Tissues were rinsed three times in deionized water, blotted dry, and weighed. Tissue samples were solubilized in 2 ml of 88% (w/v) formic acid at 90° for 40 min. 0.5-ml Aliquots of formic acid were counted in duplicate, and the results, expressed as cpm/mg tissue, represented the total radioactivity in the chondromucoprotein.

Identification of Chondroitin Sulfate. Weighed quantities of the grafts were minced and digested with papain in 25-ml erlenmeyer flasks in a shaken water bath at 60° for 2 hr. The incubation mixture was in a final volume of 4 ml and consisted of: sodium phosphate buffer (pH 6.0), 400 μ mol; Na₂ EDTA, 8 μ mol; *L*-cysteine, 20 μ mol; papain, 8 mg. After incubation, the reaction was terminated by immersion of the flask in a boiling-water bath for 15 min. After cooling in an ice bath for 30 min, the contents of the flask were centrifuged at $8000 \times g$ for 10 min at 2°. The supernatant was dialyzed against 2 liters of deionized water for 8 hr. The nondialyzable material was centrifuged at $8000 \times g$ for 10 min at 2°. Aliquots were used to determine radioactivity before and after dialysis. Samples of the nondialyzable material were analyzed by paper electrophoresis on 3 $\times$ 30-cm strips of Whatman No. 1 paper in 0.1 M sodium phosphate buffer (pH 7.0) for 2 hr at 100 V. Strips were dried and sprayed with 1% acridine orange (w/v) aqueous solution; the paper strips were destained in running water and air dried. Authentic samples of chondroitin sulfate isolated from bovine nasal septum (a generous gift from Dr. M. B. Mathews) were run simultaneously as standards. 2-cm Pieces were cut and placed in scintillation vials, which were filled with 15 ml of scintillation fluid for the determination of radioactivity.

Radioactivity Was Determined in a Packard Tricarb liquid scintillation spectrometer. The background was 30–35 cpm. Counting fluid contained xylene-*p*-dioxane-ethanol 8:8:9, naphthalene (7.5% w/v), 2,5-diphenyloxazole (PPO) (0.45% w/v), and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP) (0.0045% w/v). Under the conditions used there was no appreciable quenching due to formic and hydrochloric acids.

³²P and ⁴⁵Ca Incorporation. 100 μ Ci of either H₃³²PO₄ or ⁴⁵CaCl₂ in 0.2 ml of saline was injected in the caudal vein; 4 hr later, at harvest, the plaques were obtained and cleansed of adherent tissues. Tissues from animals that received ³²P were cut into 1- to 2-mm cubes and placed in 40 ml of 0.05 M sodium phosphate buffer (pH 7.4) and stirred for at least 30 min; a similar procedure was used for plaques obtained from animals injected with ⁴⁵Ca, except a 0.1 M solution of CaCl₂ (nonradioactive) replaced the sodium phosphate buffer. After they were rinsed three times in deionized water, tissues were blotted dry, weighed, and transferred to tubes containing 50 ml of 0.5 M HCl. The contents of the tube were stirred

FIGS. 1 and 2. Plaques (day 7) containing calcified cartilage (C) created by subcutaneous transplantation of desiccated acid-insoluble residue of bone powder (B). Arrows designate calcification of cartilage matrix. Hematoxylin and eosin stains; Fig. 2 was stained additionally with silver nitrate. $\times 250$.

for 3 hr at room temperature (25–27°). After centrifugation, 0.5-ml aliquots were used to determine radioactivity. The results were expressed as cpm of ^{32}P or ^{45}Ca per mg of tissue incorporated into an acid-soluble fraction, representing *de novo* calcification and mineral formation.

RESULTS

Matrix of Calvarium and Femur as Transformants. Acid-insoluble powders of femur and parietal were transplanted subcutaneously, and the plaques that ensued were harvested on day 7. Alkaline phosphatase values of the plaques, expressed as units/g fresh weight, were: femoral plaques 15.4 ± 4.6 ; parietal plaques 4.2 ± 0.7 . On histologic examination, much cartilage was found in plaques derived from femoral matrix, whereas small amounts of cartilage were found in plaques induced by acid-insoluble residue of parietal bone.

Histologic Sequence. Acid-insoluble diaphyseal bone powder, 10–15 mg, was transplanted to each of 6 subcutaneous sites in groups of young rats. There were no inflammations in any of the experiments. The transplanted powder had an intense attraction for fibroblasts.

On day 1, the transplant had formed an encapsulated conglomerate of TF surrounded and invaded by fibroblasts comprising the transformation plaque, about 1 cm in diameter. On day 3, the plaques consisted of TF, fibroblasts with edema fluid containing a few leukocytes. On day 5, the plaque consisted of a compact mass of particles of TF with associated fibroblasts and immature cartilage progenitors; edema fluid

and leukocytes had disappeared. Many of the fibroblasts were small curvaceous basophilic cells associated in clusters. The absence of giant cells was noteworthy.

Cartilage (P_1) was evident (see Table 2) on day 5 in small amounts. The transformation plaque was a plano-convex disc consisting of a thick opaque center with a narrow, thin translucent periphery. The flat surface was in apposition to the underlying muscle. On days 7 and 8, cartilage was abundant (Fig. 1) and many of the chondrocytes were in cell division. A strongly metachromatic extracellular matrix was a characteristic feature. On day 7 the matrix was calcified (Fig. 2). Cartilage was found exclusively in the center (Fig. 3) of the plaque, whereas the rim, composed of TF and fibroblasts, was devoid of chondrocytes. On day 9, there was an incursion of capillaries into the plaques, soon followed by three great events: *a.* a crop of osteoblasts was present; *b.* chondrolysis began; and *c.* erythroblasts were observed, but only in the capillaries. Moreover there was a close association between the chondrolytic foci and the capillaries. Chondrolysis became extensive and cartilage had disappeared from most of the plaques before day 14, but a few cartilage cells remained in some of the discs. Cartilage had vanished from all plaques before day 18.

On day 10, bone (P_2) was evident for the first time, but only in sparse amounts. On day 12, there were large quantities of bone associated with a few cartilage cells. On day 14, the plaques were pearl gray in color. Ossification was present in pronounced degree, and a few cartilage cells were present. On days 18–21, the transformation plaques consisted of large

FIG. 3. Plaque (day 9) containing calcified cartilage created by subcutaneous transplantation of desiccated acid-insoluble residue of bone powder. The rim of the plaque contains particles of transformant, but is devoid of transformed cells. Hematoxylin, eosin, silver nitrate stains. $\times 30$.

TABLE 1. Alkaline phosphatase activity and incorporation of ^{35}S , ^{45}Ca , and ^{32}P in normal tissues of rat

Tissue	Alkaline phosphatase units/g	Incorporation cpm/mg		
		^{35}S	^{45}Ca	^{32}P
Skeletal muscle	1.63 ± 0.2	20 $\pm$ 5	7 $\pm$ 3	13 $\pm$ 8
Ensiform cartilage	1.5 ± 0.5	1559 ± 415	0	—
Femur	7.9 ± 1.2	—	15,720 ± 2527	6,157 $\pm 1,626$
Parietal bone	7.8 ± 2.8	—	7,589 ± 453	3,421 ± 900

($\pm$) Standard deviation of mean; $n = 16$ for all determinations.

masses of bone with red centers and white translucent rims. Ossification was limited to the center of the plaque; the rim was devoid of bone. On day 700, the plaques consisted of living bone with much hemopoietic bone marrow; TF had vanished.

On day 9, the first sign of hemopoiesis (P_3) had been observed when a few erythroblasts were found in close proximity to the capillaries. Thereafter, the amount of bone marrow increased. On days 18–700, there was vivid red coloration in the centers of the ossicles due to extensive erythropoiesis.

Enzyme Sequences. Alkaline phosphatase activity of the cytosol of normal skeletal muscle at the transplant sites was 1.63 ± 0.2 units/g (Table 1).

Acid-insoluble diaphyseal bone powder was transplanted in rats, and enzyme assays were performed at daily intervals from days 3 to 21 on the transformation plaques which ensued; 18 plaques from three rats were assayed each day. Alkaline phosphatase activity of the plaques rose slowly (Table 2) from days 3 to 6; there was an explosive rise on day 7. Maximum values were found on day 10. The values declined thereafter.

Sequential Incorporation of ^{35}S . Values for incorporation of ^{35}S in normal tissues (Table 1) were expressed as cpm/mg. These tissues included skeletal muscle, because the transformant had been inserted on muscle at the time of transplantation on day 0; ensiform cartilage was an internal control for incorporation of radioactive sulfate in cartilage. The incorporation (Table 1) of ^{35}S in muscle was minute; ensiform cartilage showed a considerable incorporation.

Acid-insoluble diaphyseal bone powder was transplanted in groups of rats and daily assays (9 samples from 3 rats) of incorporation of ^{35}S in the transformation plaques were made. On day 3, the incorporation of ^{35}S was considerable (Table 2) and there was a steady rise in incorporation from until day 8. There was a pronounced decline in the incorporation of ^{35}S values on day 9; still lower values were found on days 12 and 21.

To identify the nature of the product containing ^{35}S , the plaques obtained on day 7 were digested with papain, dialyzed, and subjected to paper electrophoretic analysis. A major proportion of the product (74–88%) was nondialyzable. On electrophoresis, the radioactivity migrated to a single spot (Fig. 4), corresponding to the mobility of chondroitin sulfate. Recovery of radioactivity ranged from 84–94%. On the basis of electrophoretic mobility and acridine orange staining, the ^{35}S -labeled material appears to be chondroitin sulfate.

Sequential Incorporation of ^{32}P and ^{45}Ca . The values for incorporation of acid-soluble ^{32}P and ^{45}Ca into skeletal muscle were very low (Table 1). In ensiform cartilage, ^{45}Ca was not detected. The incorporation of ^{45}Ca and ^{32}P was determined in acid-soluble fractions of femoral diaphysis and parietal bone; the isotope values in parietal were about 50% of those in the femur. Alkaline phosphatase activities were similar in both bones (Table 1).

Acid-insoluble diaphyseal bone powder was transplanted subcutaneously in groups of rats and daily assays (9–18 samples; 3 rats) of incorporation of acid-extracted ^{32}P in the transplantation plaques were made.

On day 7, the incorporation of acid-soluble ^{32}P was low (Table 2). There was a considerable increase in incorpora-

TABLE 2. Major transformation products, alkaline phosphatase activity, and incorporation of ^{35}S , ^{45}Ca , and ^{32}P in plaques elicited by acid-insoluble residue of diaphyseal bone

Day	Major transformation products	Transplant weight ratio	Alkaline phosphatase units/g	Incorporation cpm/mg		
				^{35}S	^{45}Ca $\times 10^{-3}$	^{32}P $\times 10^{-3}$
3	—	8.3 $\pm$ 2.5	2.8 $\pm$ 0.7	284 $\pm$ 63	—	—
5	P_1	7.5 $\pm$ 2.9	4.3 $\pm$ 1.1	577 $\pm$ 140	0.020 $\pm$ 0.01	—
6	P_1	6.5 $\pm$ 2.3	7.4 $\pm$ 3.1	651 $\pm$ 389	0.030 $\pm$ 0.01	—
7	P_1	6.6 $\pm$ 1.9	25.9 $\pm$ 17	753 $\pm$ 222	0.121 $\pm$ 0.14	0.204 $\pm$ 0.13
8	P_1	7.1 $\pm$ 2.1	27.6 $\pm$ 13	698 $\pm$ 217	3.217 $\pm$ 2.20	1.313 $\pm$ 0.84
9	P_1	6.2 $\pm$ 1.7	45.1 $\pm$ 21	334 $\pm$ 72	4.659 $\pm$ 3.10	1.498 $\pm$ 0.81
10	$\text{P}_1;\text{P}_2$	6.1 $\pm$ 1.3	79.8 $\pm$ 31	—	8.886 $\pm$ 2.50	4.296 $\pm$ 1.68
11	$\text{P}_1;\text{P}_2$	4.6 $\pm$ 1.1	52.2 $\pm$ 24	—	8.470 $\pm$ 2.40	5.129 $\pm$ 4.14
12	$\text{P}_1;\text{P}_2$	5.4 $\pm$ 1.6	61.4 $\pm$ 18	106 $\pm$ 15	8.932 $\pm$ 2.83	4.778 $\pm$ 2.40
14	$\text{P}_1;\text{P}_2$	5.0 $\pm$ 1.8	47.0 $\pm$ 16	—	7.582 $\pm$ 2.50	4.283 $\pm$ 2.10
18	$\text{P}_2;\text{P}_3$	5.0 $\pm$ 1.5	42.3 $\pm$ 10	—	9.603 $\pm$ 1.20	4.062 $\pm$ 0.92
21	$\text{P}_2;\text{P}_3$	4.3 $\pm$ 1	35.7 $\pm$ 9	257	7.293 $\pm$ 2.19	3.718 $\pm$ 0.86

($\pm$) Standard deviation of mean; $n = 18$ for all determinations.

tion of day 8–10. The values for ^{32}P remained on a high plateau from days 10 to 21.

The incorporation of ^{45}Ca in transformation plaques exceeded that of ^{32}P , but the rates of incorporation of both isotopes were rather similar, suggestive of a coordinated incorporation into bone mineral.

DISCUSSION

The transformation of fibroblasts in adult animals is reminiscent of the process of differentiation in embryonic life. Transformability is a regulated function that is inactive in bone in its native state; it becomes active when bone minerals are removed.

Powders of acid-insoluble bone matrix had an intense chemotactic attraction for fibroblasts, which aggregated around the transformant to encapsulate it within 24 hr to form the transformation plaque; it is noteworthy that giant cells and inflammations were absent. The plaque had a thick center and a thin periphery. Transformation occurred exclusively in the center of the plaque, never in its rim.

Techniques described in this paper provided a convenient method to produce *de novo* large masses of cartilage, bone, and erythropoietic bone marrow in adult rat. Rather coarse powders (particle size 74–420 μm) were highly efficient in creating large quantities of the transformation products. There was a strict timetable of transformation that was reproduced repeatedly in our experiments. It was noteworthy that transformation plaques formed in 486 consecutive transplantations in our experiments.

In the present experiments, chondrogenesis was an unstable transformation. Chondroblasts appeared on day 5 and large masses of cartilage were present on days 7 and 8. Chondrolysis began on day 9 and in most cases cartilage had vanished before day 18. Chondrolysis and the subsequent replacement of cartilage by bone began with the invasion of the transformation plaque by capillaries.

The change of phenotype of fibroblast to osteoblast was a stable transformation. Living bone, with hemopoietic bone marrow, was found on day 700, long after the transformant had disappeared.

The optimal time for harvest of newly created cartilage, devoid of bone, was day 7–8. To obtain bone without cartilage, days 18–21 were advantageous for harvest. Because of the large content of erythropoietic bone marrow, the newly formed ossicles were vivid red in color from days 18 to 700+.

It was noteworthy that the concentration of ^{35}S was ap-

FIG. 4. Paper electrophoresis of ^{35}S -labeled material in papain digests of plaques after dialysis. The inset shows the position of an authentic sample of chondroitin sulfate (CS) on the electropherogram.

preciable in the sulfur-containing macromolecules of the transformation plaques before cartilage cells were observed.

The parietal bone of the calvarium develops in the embryo by membranous ossification without an intervening stage of cartilage. But, transplants of acid-insoluble residue prepared from parietal bones yielded a typical endochondral ossification.

This work was supported by grants from the American Cancer Society, The Jane Coffin Childs Memorial Fund for Medical Research, and United States Public Health Service, National Institutes of Health (no. CA11603).

1. Huggins, C. B. (1930) *Proc. Soc. Exp. Biol. Med.* **27**, 349–351.
2. Huggins, C., Wiseman, S. & Reddi, A. H. (1970) *J. Exp. Med.* **132**, 1250–1258.
3. Urist, M. R. (1965) *Science* **150**, 893–899.
4. Bang, G. & Urist, M. R. (1967) *Arch. Surg.* **94**, 781–789.
5. Urist, M. R. & Nogami, H. (1970) *Nature* **225**, 1051–1052.
6. Huggins, C. B. & Urist, M. R. (1970) *Science* **167**, 896–897.
7. Reddi, A. H. & Huggins, C. B. (1971) *Proc. Soc. Exp. Biol. Med.* **137**, 127–129.
8. Huggins, C. & Morii, S. (1961) *J. Exp. Med.* **114**, 741–760.