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Cyclic Electrochemical Inactivation and Restoration of Competence of Bone Matrix to Transform Fibroblasts

(bone matrix/Evans Blue/hexadimethrine/^{45}Ca/cartilage)

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ABSTRACT **Brief exposure of rat bone matrix to highly charged electrochemicals had profound but reversible effects on its competence to transform fibroblasts into chondroblasts and osteoblasts. Suppression and subsequent reactivation of this function were influenced critically by the charge of the electrochemical reagent and the pH of the reaction mixture. In dilute acids, Evans Blue, a long electronegative molecule, suppressed transforming competence, whereas hexadimethrine, a polycationic quaternary ammonium base, failed to do so. A cycle of inactivation-restoration of transforming competence was achieved by sequential treatment of bone matrix with Evans Blue followed by hexadimethrine; an electrochemical complex was demonstrated by histochemistry. In dilute alkalies, hexadimethrine inhibited, whereas, Evans Blue did not suppress the transforming function of the bone matrix. Electric charge characteristics on the surface of the bone matrix are of crucial significance for the fibroblast-transforming attribute.**

Fibroblasts possess the singular attribute of transformability, wherein their gene expression can be altered profoundly. The change in the phenotype of fibroblasts is brought about by rather simple experimental procedures, and the products comprise two general types of altered cells: (*i*) the fibroblasts are converted into neoplastic fibroblasts (1) which elicit fibrosarcoma on allogeneic transplantation, and (*ii*) the fibroblasts are changed into normal or neoplastic cartilage and bone cells. This paper is concerned with a physiologic transformation of fibroblasts. In the present experiments it was found that electrochemical properties of the surface of bone matrix have critical significance in determining its competence to transform fibroblasts into normal chondroblasts and osteoblasts.

The experimental methods to change fibroblasts into bone or cartilage are threefold: (*i*) a virus (2) can convert chicken fibroblasts into osteochondrosarcoma cells (*ii*) transitional epithelium (3) transforms fibroblasts into nonmalignant osteoblasts but cartilage cells are not observed and (*iii*) demineralized matrices of bone (4) or tooth (5) bring about the fibroblast–chondroblast–osteoblast transformation.

The change of the fibroblasts by physiologic methods in postnatal life is reminiscent of embryonic differentiation. The cell's new program is expressed according to a strict and reproducible timetable (6). In addition to dramatic cytologic alterations, the sequential alteration of phenotype is expressed by changes in lactic dehydrogenase (7) and organic acids (8), and incorporation of ^{35}S into chondroitin sulfate and of ^{32}P and ^{45}Ca into bone mineral (6). Moreover, there is a positive correlation between alkaline phosphatase activity and the emergence of chondroblasts and osteoblasts (9). The temporal sequence and the yield of transformation products are dependent on the geometry (10) of the matrix as transformant.

It has been observed (11) that contact of the transforming matrices of bone and tooth with heparinized blood plasma of guinea pig causes (*i*) loss of their transforming competence and (*ii*) coagulation of plasma. In the experiments to be described, bone matrix-transformant of fibroblasts (TF) was exposed briefly to Evans Blue or hexadimethrine, alone or sequentially, at two levels of hydrogen ion concentration (pH 3.6 and 8). We investigated the effects of these strongly charged electrochemicals on the competence of bone matrix to transform fibroblasts into normal cartilage and bone.

MATERIALS AND METHODS

Chemicals. Evans Blue (EB), from Eastman Kodak, Rochester, N.Y., was recrystallized from water-ethanol; hexadimethrine (Hex) (molecular weight about 6000) was donated by J. M. Price, Abbott Laboratories North Chicago, Ill.; sodium dextran sulfate (Dex) (molecular weight about 500,000) was purchased from Sigma Chemical Co., St. Louis, Mo.; and sodium polyvinylsulfonate (PVS) was synthesized by John Pataki, Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill.

Preparations of TF. TF was prepared at room temperature, about 25°. Sieved powders of cleansed, desiccated, diaphyseal rat bone were extracted (6) with 0.5 N HCl, water, ethanol, and diethyl ether. The particle size of the product was 74–420 μm; total phosphorus content was 12.9 $\pm$ 0.8 μmol/g. In this paper, bone matrix (TF) refers to demineralized preparations of this sort. In one of the experiments, TF was treated for 10 min with 0.15 M glycine–NaOH buffers (pH 10.8–12.8), washed four times with saline, and dehydrated with ethanol/ether.

Aliquots of our sample of TF were exposed to electrochemicals, singly or sequentially, in buffered solutions of 0.15 M acetate-acetic acid (pH 3.6) or 0.15 M Tris·HCl (pH 8). The sample was moistened with the appropriate buffer with magnetic stirring. At 0 min, a solution of the electrochemical (20 ml/g) was added; the final concentration of the reagent was

Abbreviations: Dex, sodium dextran sulfate; EB, 4,4′-*bis*-[7-(1-amino-8-hydroxy-2,4-disulpho)-naphthylazo]-3,3′-bitolyl tetrasodium; Hex, poly(*N,N,N′N′*-tetramethyl-*N*-trimethylene-hexamethylenediammonium dibromide); PVS, sodium polyvinylsulfonate; RTT, rat tail tendon; TF, bone matrix-transformant of fibroblasts; Gly, glycine.

TABLE 1. *Effect of treatment of bone matrix with buffers on its competence to transform fibroblasts*

		Plaque		
Buffer	pH	Alkaline phosphatase (units/g)	^{45}Ca (cpm/mg)	Calcified cartilage
Acetic acid-acetate	3.6	43.2 ± 4.3	8,496 ± 2,864	++++
Tris·HCl	7.4	35.2 ± 5.3	8,574 ± 2,074	++++
Gly-NaOH	10.8	34.0 ± 2.2	6,190 ± 1,456	++++
Gly-NaOH	11.4	26.7 ± 4.6	2,301 ± 633	++++
Gly-NaOH	12.0	7.5 ± 1.7	542 ± 144	++
Gly-NaOH	12.8	1.32 ± 0.33	90 ± 7	0

±, Standard error of mean of eight observations.

0.5% (w/v). At 5 min, the mixture was centrifuged; the precipitate was washed with solutions (40 ml) of 0.15 N NaCl and equilibrated again with buffer. In sequential exposure to electrochemicals, the foregoing procedure was repeated at this stage with a reagent bearing an electric charge of opposite sign and in ratio of 1:1 with its predecessor.* Finally the precipitates were washed thrice with 0.15 N NaCl (40 ml) dehydrated with ethanol/ether. The pH of each aqueous solution at every stage of the preparation was measured with a glass electrode.

Bioassay of Transformation. Male rats of Long–Evans strain, age 28–42 days, were anesthetized with ether. Under sterile precautions, an incision (1 cm) was made in the skin and a pocket in the subcutaneous tissue was prepared by blunt dissection. A knife-point-full (10–20 mg) of TF was inserted on the floor of the surgical pocket. The incision was closed with a metallic clip. The surgical procedure was repeated at eight sites in the trunk of the animal. Only one substance was assayed in an individual rat. The day of transplanation is denoted day 0.

On day 9 the grafts were harvested 4 hr after an intravenous injection of ^{45}Ca in saline at a dose of 1 μCi/g of body weight. At harvest, the transplantation plaque was weighed and portions were homogenized for alkaline phosphatase determination and ^{45}Ca incorporation as described (6) earlier. A unit of alkaline phosphatase [orthophosphoric-monoester phosphohydrolase(alkaline optimum), EC 3.1.3.1] is defined as enzyme activity that liberates 1 μmol of *p*-nitrophenol per 0.5 hr under stated conditions (12). Portions of the plaque were fixed in Bouin's fluid or in neutral formalin for histologic examination; staining with $AgNO_3$ was performed as a routine.

RESULTS

Control experiments consisted of allogeneic transplantation of acid-insoluble bone matrix which had not been exposed to electrochemical reagents. On day 1, discrete plaques were found; these consisted of masses of fibroblasts which encapsulated the TF and migrated between its particles to form a conglomerate.

* (EB; Hex) denotes an electrochemical sequence in which Evans Blue was the initial reagent.

TABLE 2. *Effects of electrochemical reagents and hydrogen ion concentration on transforming competence of bone matrix*

		Plaque		
Reagent	pH	Alkaline phosphatase (units/g)	^{45}Ca (cpm/mg)	Calcified cartilage*
None; control	—	39.9 ± 4.8	4,245 ± 932	++++
EB	3.6	1.8 ± 0.3	81 ± 16	0
EB	8.0	16.8 ± 2.2	1,168 ± 675	+++
Hex	3.6	50.1 ± 4.8	14,738 ± 2,643	++++
Hex	8.0	5.5 ± 1.9	1,904 ± 974	+

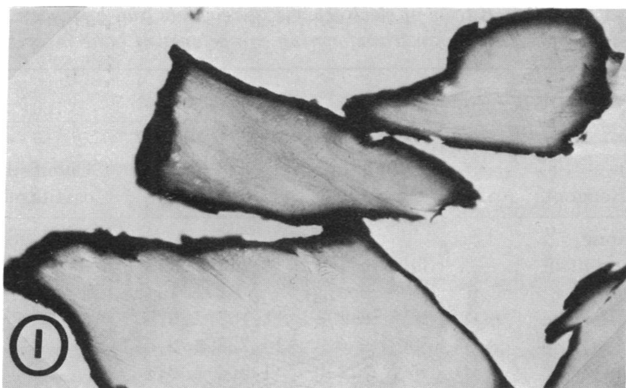
±, Standard error of mean of eight determinations.
* +, trace; ++++, abundance of cartilage.

In most of the experiments, the transplanted material was harvested on day 9. The transplantation plaques were firm, white, plano-convex, button-like structures; the activity of alkaline phosphatase and the incorporation of ^{45}Ca were high (Tables 1 and 2); staining with $AgNO_3$ revealed large masses of calcified cartilage; osteoblasts were present in parallel stacks; and early bone was evident. By our definition, when findings of this sort are present in every parameter, the fibroblasts have been transformed. The results were remarkably uniform in control experiments insofar as fibroblasts were transformed in each of 100 consecutive control experiments.

The effect of hydrogen ion concentration on the bone matrix used in control experiments was investigated; TF was exposed for 10 min to buffers of 0.15 M concentration and pH 3.6–12.8. Bone matrix which had been placed in buffers of pH 3.6–11.4 retained full transforming activity (Table 1). At pH 12, transforming activity was diminished, and at pH 12.8 it was abolished. TF was exposed to 0.1 N KOH for varying lengths of time and transforming competence was measured; after immersion for 7 min, competence was retained, whereas after exposure for 20 min it was abolished.

Electrochemical Inactivation of Transforming Competence of Bone Matrix. It was found that the ability of TF to transform fibroblasts *in vivo* was abolished by electrochemical reagents under special conditions; inactivation was a function of the electric charge of the reagent and the concentration of hydrogen ions in the reaction mixture. In acid solutions, EB inactivated, whereas Hex did not modify transforming competence of the bone matrix. In dilute alkali, Hex inactivated, whereas EB did not suppress transforming function.

TF was placed in 0.5% solution of EB dissolved in 0.15 M acetate (pH 3.6). After exposure for 5 min, the solution was centrifuged; the precipitate was washed rapidly with 0.15 N NaCl, dehydrated, and tested in bioassay of transformation. On day 9, discrete well formed plaques, light blue in color, were found. The activity of alkaline phosphatase and the incorporation of ^{45}Ca in the plaques were low (Table 2). Generally, cartilage was absent and calcification was not observed. In a few plaques, chondroblasts were found in small numbers; these were situated in deep crevices between fragments of TF. By contrast, companion samples treated with



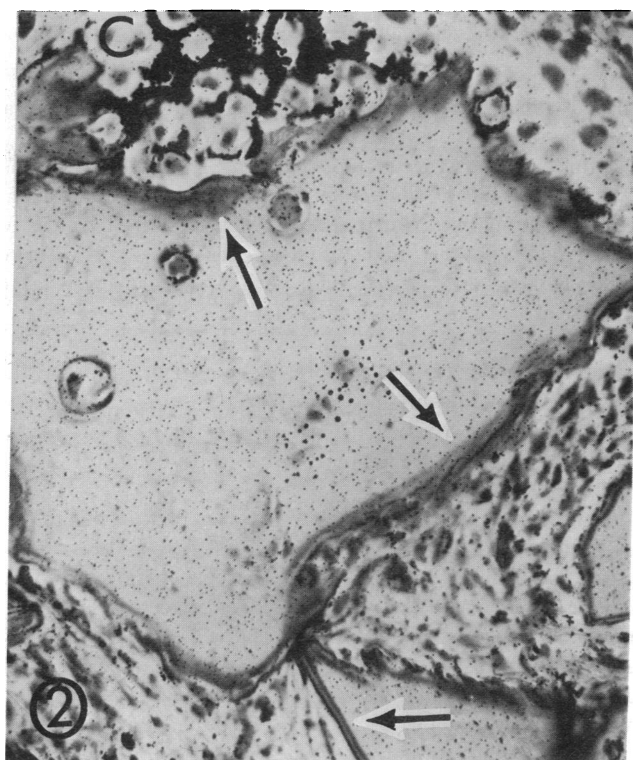


FIG. 1. Histological section of rat bone matrix treated at pH 3.6 *in vitro* for 5 min with (EB; Hex); the dense electrochemical band on the surface of TF is evident. Eosin stain. ×400.

FIG. 2. Transplantation plaque (day 9) containing calcified cartilage (C) created by subcutaneous transplant of bone matrix which had been exposed for 5 min sequentially to (EB; Hex) at pH 3.6. *Arrows* designate the coat of dye on the surface of fragments of transplanted TF. Hematoxylin, eosin, and silver nitrate stains. ×200.

EB dissolved in 0.15 M Tris·HCl buffer (pH 8) retained competence to transform fibroblasts (Table 2).

The experiment was repeated under similar conditions except that Hex replaced EB as the electrochemical reagent; the results were opposite to those of the preceeding experiment (Table 2). In acetate buffer (pH 3.6), TF exposed to Hex for 5 min retained full transforming competence, whereas in Tris·HCl buffer (pH 8), the transformability of the bone matrix was much reduced.

Cyclic Inactivation-Restoration of Transforming Competence. A series of solutions of Hex and the polyanions was prepared; each solution contained a single electrochemical reagent, 0.5% (w/v) dissolved in 0.15 M acetate buffer (pH 3.6). The solutions were mixed in pairs in 1:1 proportion. The solutions remained clear when members of any pair had electric charge of the same sign. A voluminous precipitate formed immediately when members of the pair had electric charge of opposite sign. The precipitate was stable for more than 1 month in solution containing the following pair: EB + Hex. The precipitates dissolved within 48 hr in solutions containing the following pairs: Dex + Hex or PVS + Hex.

TABLE 3. *Electrochemical inactivation and restoration of transforming competence of bone matrix at pH 3.6*

Electrochemical sequence	Plaque: Blue color	Plaque: Alkaline phosphatase (units/g)	Plaque: ^{45}Ca (cpm/mg)	Plaque: Calcified cartilage
None; control	None	45.5 ± 6.0	11,265 ± 3,037	++++
(EB; Hex)	Dark	31.7 ± 9.1	5,288 ± 586	++++
(Hex; EB)	Light	3.6 ± 0.6	517 ± 223	None

±, Standard error of mean of eight observations.

Samples of TF were exposed to solutions of EB or Hex at pH 3.6 in sequence. After the reaction had taken place for 5 min, the sample was washed with large quantities of 0.15 N NaCl followed by 0.15 M acetate buffer (pH 3.6) before exposing it to the following member of the reaction sequence. At the end of the sequential reactions the samples were desiccated and the dried powders transplanted for bioassay of transformation.

Transforming competence of the bone matrix was abolished in an electrochemical reaction with the following sequence: (Hex; EB). On day 9 each animal had a dark blue color due to staining of the deep fascia with EB. The transformation plaques were well formed, discrete, light blue in color, and soft; alkaline phosphatase level and ^{45}Ca incorporation were low (Table 3); cartilage and bone were absent.

Transforming competence of the bone matrix was restored in an electrochemical reaction with the following sequence: (EB; Hex). On day 9 each animal had a light blue color; the transformation plaques were dark blue in color and firm in consistency. The levels of alkaline phosphatase and ^{45}Ca incorporation were high (Table 3); cartilage was abundant.

Histochemistry of Bone Matrix Exposed to Electrochemical Sequences. It was found that treatment of bone matrix *in vitro* for periods of 5 min with solutions of EB, singly or in sequential combinations with Hex, resulted in blue staining of the surface of the solid phase; the interior of the granules of TF was colorless. The blue dye was manifest histochemically in paraffin sections of material fixed in neutral formalin; it was not visible when Bouin's solution was the fixative. The localized dye was found in test tube preparations of TF (Fig. 1) and in transplantation plaques (Fig. 2) after sojourn in animals for 9 days.

The blue staining on the surface of the matrix protein was of two sorts: (*i*) a diffuse coating of pale hue and (*ii*) a sharp, dense, dark blue electrochemical band. The nature of the localization was determined by the sequence of electrochemical reagents and by the pH of the reaction mixture.

The diffuse blue coating was found only in test tube preparations. It was observed on the surface of TF treated at different pH levels with the following electrochemical reagents: (*i*) EB singly at pH 3.6, (*ii*) EB singly at pH 8 and (*iii*) (Hex; EB) at pH 3.6. The diffuse coloration was unstable; samples of TF in plaques elicited in animals after the foregoing electrochemical treatments were colorless.

The sharp, dense, dark blue band was present in TF after the following electrochemical sequences: (*i*) (EB; Hex) at pH 3.6, (*ii*) (EB; Hex) at pH 8, and (*iii*) (Hex; EB) at pH 8. In addition, the dense electrochemical band was stable; it was found in test tube preparations and in transplantation plaques on day 9 (Fig. 2).

The transformation of the sort elicited by the rat bone matrix exhibits remarkable specificity; among other insoluble collagenous matrices from rat tested, only tooth matrix has been found to have competence as a transforming agent; rat tail tendon (RTT) and skin collagen are ineffective in this regard (13). However, the histochemical properties of RTT with respect to binding EB at pH 3.6 and the formation of tinctorial complexes by sequential treatment *in vitro* with (EB; Hex) were indistinguishable from similar properties of rat bone matrix. Additionally, it was found that the alteration of surface charge characteristics of RTT with EB and Hex, singly or in sequential combinations, did not confer transforming competence on tendon.

DISCUSSION

The coordinated triad of fibroblast transformation consists of the induction of alkaline phosphatase, the incorporation of ^{45}Ca, and the presence of calcified cartilage in cells of the connective tissue which, in control animals, are devoid of these components or possess them in trace amounts. In the present work, the coordinated triad was present in pronounced degree on day 9 in 100 consecutive assays of bone matrix in control experiments; hence, day 9 was selected for routine harvest of tissue for possible transformation.

It has been observed that electrochemicals influence the growth of virus-transformable hamster fibroblasts. A line of hamster fibroblasts, BHK 21/13, which is specially susceptible to fibrosarcoma transformation *in vitro* by polyoma virus, possesses the ability to form three-dimensional colonies in semisolid agar gels. Montagnier (14) found that colony formation of BHK 21/13 fibroblasts occurred in low frequency in crude agar due to impurities which were identified as sulfated polysaccharides. Sanders and Smith (15) showed that colony formation of BHK 21/13 cells was enhanced 4- to 5-fold in the presence of native collagen derived from skin. Nevo and Dorfman (16) found that many highly charged polyanionic substances stimulated the synthesis of chondromucoprotein by chondrocytes in cell culture.

The bone matrix-induced transformation proceeds in two phases. In *phase 1* the transplanted bone matrix is surrounded by fibroblasts to form a conglomerate; in control experiments button-like plaques in which the transformant was encapsulated were present on day 1. In *phase 2* the invading fibroblasts in the center of the plaque, but not in its rim, infiltrate the transforming matrix (9) blending with it by a creeping substitution. The inhibitory electrochemicals did not prevent the early formation of plaques constituting *phase 1*. Under stated conditions, treatment of bone matrix with EB or Hex blocked the infiltrative process of *phase 2* which requires the closest contact between transformant and fibroblast.

Quantitatively, the bone matrix consists of collagen (17) as its chief component. The finding that the inhibitory potency of EB, a long electronegative molecule, was greatest in acid solutions was not unexpected, as the protein constituent of the bone matrix would be more electropositive at high H^+ concentration. Conversely, the bone matrix would be less electropositive in alkali as more carboxyl groups of the protein are ionized. This assumption is in agreement with the finding that Hex, a polycationic quaternary ammonium base, inactivated the transforming competence of bone matrix in alkaline but not in acid solutions.

It has been found (11) that the demineralized bone matrix possesses the ability to coagulate heparinized blood plasma in the range of pH 5.3–7.2. The coagulant attributes of bone matrix are reminiscent in many respects of the antiheparin attributes of Hex (18). It would appear that an optimal number of electropositive centers on the insoluble demineralized bone matrix contribute to its plasma coagulant and transforming activities.

It is well known that most animal cells possess negative charge due to the presence on the cell surface of *N*-acetylneuraminic acid (19), chondroitin sulfate (20), and heparan sulfate (21). In the present experiments, EB was inhibitory to transformation and it is plausible that the electronegative dye inhibits by preventing surface–surface interaction between matrix and fibroblast. Under the conditions of the present experiments, brief exposure of TF in the sequence (EB; Hex) resulted in a stable electrochemical complex which was demonstrated histochemically as a blue coat on the surface of the bone matrix. This sort of electrochemical complex is not specific for the collagen of the bone matrix; it also occurred on the surface of tendon collagen exposed to the sequence (EB; Hex).

From the foregoing observations it is clear that perturbation of electric charge on the surface of the bone matrix inactivates but does not destroy its transformant attribute, since alteration of surface charge of the inactivated bone matrix with appropriate electrochemicals rapidly restored its transforming competence.

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