PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 445–448 (1979)

Inhibition of Mineralization by Experimental Lathyrism during Matrix-Induced Endochondral Bone Differentiation (40701)

A. H. REDDI AND N. E. SULLIVAN

Laboratory of Biological Structure, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

The various types of collagens have recently been implicated in cell attachment, cell differentiation, and morphogenesis (1). There is a growing realization of the importance of cross-linked collagenous matrix for mineralization of bone (2–4). The availability of specific agents that inhibit collagen cross-linking leading to experimental lathyrism (5) enables an experimental scrutiny of the influence of this condition on bone differentiation and mineralization. The use of the matrix-induced endochondral bone differentiation system characterized in our laboratory (6–8) permits the study of discrete stages of endochondral bone differentiation, such as mesenchymal cell proliferation, chondrogenesis, cartilage calcification, osteogenesis, and bone mineralization. This system avoids many of the difficulties inherent in the use of the epiphyseal growth plate. The present study describes the influence of β-aminopropionitrile (BAPN)-induced experimental lathyrism on the differentiation of endochondral bone.

Materials and methods. Animals. Thirty-six 23- to 25-day-old (50–60 g) male rats of Long–Evans strain were divided into two groups. The control group was pair fed with a powdered rat chow while the experimental group was fed the same diet supplemented with 0.3% (w/w) of β-aminopropionitrile fumarate (BAPN; Aldrich Chemical Co.). At the end of 2 weeks the rats were implanted subcutaneously with demineralized diaphyseal bone matrix powder in the thoracic region as described earlier (6). The rats in each group were pair fed the designated diet until the termination of the experiment. The day of implantation was designated as Day 0 and the rats were utilized for different experiments on designated days as described in the tables. The present investigation was performed during the following distinct stages of matrix-induced endochondral bone differentiation: (i) proliferation of mesenchymal cells on Day 3, monitored by the activity of ornithine decarboxylase (ODC); (ii) chondrogenesis on Day 7 as evidenced by $^{35}SO_4$ incorporation into proteoglycans; (iii) cartilage calcification on Day 9 as indicated by alkaline phosphatase and ^{45}Ca incorporation; and (iv) osteogenesis and bone mineralization on Days 14 and 21 as quantitated by alkaline phosphatase activity and ^{45}Ca incorporation. The proximal tibial epiphyses and tibial metaphyses were utilized as internal controls for assessing the influence of BAPN on chondrogenesis and osteogenesis, respectively.

Ornithine decarboxylase (ODC) assay. Matrix-induced plaques (implants) were dissected out on Day 3, cleaned of adherent tissue, and homogenized in ice-cold 50 m*M* Tris–HCl buffer containing 5 m*M* dithiothreitol and 1 m*M* EDTA at pH 7.4. The samples were centrifuged at 30,000 *g* in a Beckman JA-21 centrifuge for 30 min and the supernatant fractions were used for the ODC assay. The assay was performed as described (9) and the results expressed as pmoles of $^{14}CO_2$ released from L-[1-^{14}C]ornithine per hour per milligram protein. Protein was determined as described (8, 9) using bovine serum albumin as standard.

$^{35}SO_4$ incorporation. Sodium (^{35}S) sulfate in saline was injected intravenously at a dose of 1 μCi/g body wt. The rats were killed 2 hr after injection; the plaques were dissected out and cleaned, and a portion of each was weighed and homogenized in ice-cold 10% (w/v) trichloroacetic acid (TCA) containing 10 m*M* nonradioactive sodium sulfate. The samples were kept cold for 30 min and centrifuged at 20,000 *g* for 15 min. Aliquots of the acid-soluble supernatant fraction were used for determination of ^{35}S radioactivity. The acid-insoluble precipitate was washed twice with 5 ml of ice-cold 10% TCA and dissolved in 0.5 ml 23 *M* formic acid at 90° for 10 min and transferred to scintillation

0037-9727/79/110445-04$01.00/0

vials, and the radioactivity was determined in a liquid scintillation counter. The results were expressed as cpm/mg of tissue and statistically evaluated by means of the Student's *t* test. A portion of each plaque was also used for determination of alkaline phosphatase activity as described earlier (6).

^{45}Ca incorporation and alkaline phosphatase activity. $^{45}CaCl_2$ was injected intravenously via the caudal vein in saline at a dose of 1 μCi/g body wt 2 hr prior to the termination of the experiment. The plaques were dissected out and homogenized in ice-cold 0.15 *M* NaCl containing 3 m*M* $NaHCO_3$ and centrifuged at 20,000 *g* for 15 min at 4°. Alkaline phosphatase activity in the supernatant was determined as described previously (6). The saline-insoluble sediment representing the mineral fraction was suspended in 10 ml 0.1 *M* $CaCl_2$ and stirred for 30 min. After centrifugation at 3000 *g* the supernatant fractions were discarded. The sediment fractions were washed twice in 10 ml of deionized glass distilled water and the pellets were stirred for 2 hr in 10 ml 0.5 *M* HCl and centrifuged at 3000 *g* for 5 min. Radioactivity in the aliquots of the supernatant acid extract were determined in a Beckman liquid scintillation counter. The ^{45}Ca incorporation was expressed as cpm/mg tissue and represented the rate of mineralization during the 2-hr pulse of $^{45}CaCl_2$. Alkaline phosphatase activity was expressed as units/mg protein. The statistical significance was evaluated by means of the Student's *t* test.

Calcium determination. Aliquots of the acid-soluble extracts of the sediment were diluted in 0.5% HCl containing 0.1% lanthanum oxide for determination of calcium content by atomic absorption spectrophotometry. The results were expressed as μg calcium/mg tissue and represented the total extent of mineralization throughout the experimental duration.

Preparation and assay of bone and tooth matrices from lathyritic rats. Thirty-six, 21-day-old (45–50 g), Long–Evans rats were divided into two groups. The control group was fed Purina rat chow while the lathyritic group was fed the same diet supplemented with 0.4% (w/w) of BAPN. Both groups were fed their respective diets *ad libitum* for 4 weeks. On termination of the experiment demineralized bone matrix was prepared as described earlier (6) from femora and tibiae of control and BAPN-fed rats. Tooth matrix was similarly prepared from mandibular and maxillary incisors. Demineralized bone and tooth matrices, with a particle size of 74–420 μm, were implanted subcutaneously during ether anesthesia into 28-day-old, male, Long–Evans rats (6). The day of implantation was day 0. On Day 11, the alkaline phosphatase activity and ^{45}Ca incorporation in the implants were determined in order to quantitate the osteogenic response as described above.

Histology. Portions of the plaques were fixed in Bouin's fluid and 5-μm sections were cut and stained with toluidine blue.

Materials. L-[1-^{14}C]Ornithine (56 mCi/mmole), (^{35}S) sodium sulfate (960 mCi/mmole), $^{45}CaCl_2$ (18 Ci/g), Aquasol, and Hyamine hydroxide were purchased from New England Nuclear; L-ornithine HCl, EDTA, *p*-nitrophenylphosphate, pyridoxal phosphate, and bovine serum albumin were purchased from Sigma Chemical Company. Dithiothreitol and folin phenol reagents were obtained from Fisher Scientific Company.

Results and discussion. Implantation of demineralized diaphyseal bone matrix to subcutaneous sites in allogeneic rats resulted in sequential differentiation of endochondral bone as described earlier (6–8). A transient inflammation-like response ensued on Day 1 and was followed on Day 3 by mesenchymal cell proliferation in the vicinity of the implanted bone matrix; this was quantitated by ODC activity and revealed a peak on Day 3 (9). On Days 6–8 there was extensive chondrogenesis as indicated by $^{35}SO_4$ incorporation into proteoglycans (10) and the appearance of type II collagen (11). The hypertrophy of the chondrocytes was followed by calcification of the cartilage matrix on Day 9 (6–8). With vascular invasion beginning on Day 9 there was chondrolysis and Days 10–11 were marked by initial osteogenesis as indicated by induction of alkaline phosphatase activity and ^{45}Ca incorporation (8). The BAPN-fed rats exhibited an identical sequence of development as the control rats. However, histologically the amounts of new bone formed was less in the BAPN-fed rats. This was confirmed by the ^{45}Ca incorporation experiments described later. The newly

formed bone was remodeled (Days 14–18) and hematopoietic bone marrow differentiation occurred within the ossicle and was maximal by Day 21 (7, 8).

Mesenchymal cell proliferation. In our previous study we demonstrated a peak of ODC activity on Day 3, concomitant with extensive proliferation of mesenchymal cells (9). In the present experiments BAPN-fed rats exhibited ODC activity (303 ± 19 pmole/mg protein/hr) that was not statistically different from that in the controls (278 ± 27 pmole/mg protein/hr).

Chondrogenesis. The influence of experimental lathyrism on cartilage differentiation was assessed by $^{35}SO_4$ incorporation into proteoglycans. As seen in Table I, in Day-7 plaques there is an inhibition of $^{35}SO_4$ incorporation into the acid-precipitable fraction of proteoglycans. Also noteworthy is that the alkaline phosphatase activity is decreased. These inhibitory effects were also seen in the proximal tibial epiphyses. The inhibitory effect on $^{35}SO_4$ uptake was confined to the acid-precipitable fraction in both the plaques and tibial epiphyses. This inhibition was probably not related to pool sizes as $^{35}SO_4$ in the acid-soluble fraction in BAPN-fed rats was not different from controls. The present observations on the inhibitory effects of BAPN on chondrogenesis and $^{35}SO_4$ incorporation corroborate the earlier work on chick embryos (12, 13).

Mineralization of cartilage and bone. The influence of BAPN-diet on cartilage calcification and bone mineralization as indicated by alkaline phosphatase activity and ^{45}Ca incorporation is summarized in Table II. On Day 9 during cartilage mineralization lathyrism significantly inhibited both alkaline phosphatase activity and ^{45}Ca incorporation and calcium content. It would appear that there was no delay in the onset of mineralization but the rate of mineralization was impaired. During bone formation and remodeling on Day 14 there was a significant ($P < 0.01$) inhibition of ^{45}Ca incorporation into bone mineral in BAPN-fed rats. In contrast, the alkaline phosphatase levels were maximal in both controls and lathyritic rats on Day 14. The inhibitory effect on ^{45}Ca incorporation in BAPN rats was sustained through Day 21. Alkaline phosphatase and ^{45}Ca incorporation in Day-21 plaques declined in both controls

TABLE I. ALKALINE PHOSPHATASE ACTIVITY AND $^{35}SO_4$ INCORPORATION INTO PROTEOGLYCANS IN DAY 7 PLAQUES

Tissue	Group	Mean body weight (g)	Alkaline phosphatase (U/mg protein)	$^{35}SO_4$ incorporation (cpm/mg)	
				Acid-soluble supernatant	Acid-insoluble precipitate
Plaques	Control	138	0.76 ± 0.13[a]	1309 ± 142	470 ± 87
	BAPN-Fed rats	106	0.31 ± 0.06[b]	1723 ± 150	251 ± 52[b]
Tibial epiphysis	Control		3.0 ± 0.2	1264 ± 61	1207 ± 72
	BAPN-Fed rats		2.3 ± 0.2	1105 ± 49	550 ± 90[b]

[a] Mean ± SEM of eight observations from four rats.
[b] Significant difference ($P < 0.05$) from control.

TABLE II. ALKALINE PHOSPHATASE ACTIVITY AND ^{45}Ca INCORPORATION IN MATRIX-INDUCED PLAQUES

Tissue	Day	Mean body weight (g)		Alkaline phosphatase (U/mg protein)		^{45}Ca incorporation (cpm/mg tissue)		Calcium content (μg/mg tissue)	
		Control	BAPN	Control	BAPN	Control	BAPN	Control	BAPN
Plaques	9	148	114	1.4 ± 0.2[a]	0.5 ± 0.1[b]	910 ± 230	261 ± 96[b]	1.97 ± 0.65	0.22 ± 0.09[b]
	14	153	124	2.2 ± 0.3	2.8 ± 0.4	1410 ± 180	565 ± 75[b]	17.7 ± 3.8	10.3 ± 1.8
	21	152	124	1.5 ± 0.1	1.6 ± 0.3	550 ± 70	304 ± 62[b]	47.2 ± 8.6	19.6 ± 2.7[b]
Tibial metaphysis				6.6 ± 0.6	5.1 ± 0.6	6705 ± 588	3654 ± 462[b]	63.6 ± 2.9	57.8 ± 1.4

[a] Mean ± SE of eight observations from four rats.
[b] Significant difference ($P < 0.05$) from control.

and BAPN-fed rats. It is noteworthy that the inhibitory effect of BAPN on ^{45}Ca incorporation was also seen in the tibial metaphysis (Table II).

The present results demonstrate that experimental lathyrism was inhibitory to the rate of mineralization in both cartilage and bone. While there was no discernible influence on the onset of mineralization the rate (^{45}Ca incorporation) and extent (calcium content) of this process were profoundly inhibited. While it is attractive to postulate that the deficiency in collagen cross-linking may be responsible for impaired mineralization, it is possible that this effect could in part be due to some unknown metabolic action.

Our results confirm the earlier work (2, 3) on the inhibitory effects of BAPN on mineralization and extend it to discrete stages of endochondral bone mineralization. The present study enabled us to assess the influence of BAPN on *de novo* mineralization of cartilage and bone during development.

Osteoinductive potency of lathyritic bone and tooth matrices. The foregoing results described the influence of BAPN on matrix-induced endochondral bone differentiation. It was of interest to investigate the osteoinductive capacity of normal and lathyritic bone and tooth matrices. Previous studies (14) have demonstrated that bone and tooth matrices from adult rats (1 year old) are equipotent in bone induction. The results are summarized in Table III. The lathyritic bone and tooth matrices are not as active as their normal counterparts in induction of new bone. It is noteworthy that comparison of bone and tooth matrices from 7-week-old rats revealed the latter to be more potent in osteoinduction as measured by alkaline phosphatase activity and ^{45}Ca incorporation. The lathyritic bone matrix was previously demonstrated to be devoid of the inductive signal for bone formation (15). The present observations demonstrate that the lathyritic tooth matrix was as active as the control bone matrix.

TABLE III. COMPARISON OF THE OSTEOINDUCTIVE POTENCY OF BONE AND TOOTH MATRICES OBTAINED FROM CONTROL AND LATHYRITIC RATS IN NORMAL RECIPIENTS

Matrix	Group	Alkaline phosphatase (U/mg protein)	^{45}Ca incorporation (cpm/mg tissue)
Bone	Control	2.2 ± 0.3^a	2570 ± 356
	Lathyritic	0.2 ± 0.03^b	45 ± 6^b
Tooth	Control	5.4 ± 0.5	6330 ± 770
	Lathyritic	4.3 ± 0.5	3375 ± 420^b

[a] Mean ± SEM of eight observations.
[b] Significant difference ($P < 0.05$) from control.

Summary. The influence of experimental lathyrism generated by β-aminopropionitrile (BAPN) treatment on matrix-induced endochondral ossification was investigated. Although the mesenchymal cell proliferation was not affected, BAPN was inhibitory to chondrogenesis as quantitated by $^{35}SO_4$ incorporation. Both cartilage and bone mineralization, as indicated by ^{45}Ca incorporation, were profoundly inhibited by BAPN treatment. The initial rate and the final extent of mineralization was impaired by the lathyrism. Matrices prepared from lathyritic bone and tooth were not as active as controls in induction of new bone as quantitated by alkaline phosphatase activity and ^{45}Ca incorporation.

1. Reddi, A. H., *in* "Biochemistry of Collagen" (G. N. Ramachandran and A. H. Reddi, eds.), p. 449. Plenum Press, New York (1976).
2. Glimcher, M. J., *in* "Handbook of Physiology—Endocrinology" (G. D. Aurbach ed.) Vol. 7, p. 25. American Physiological Society, Washington, DC (1976).
3. Rosenquist, J. B., Baylink, D., and Spengler, D. M., Proc. Soc. Exp. Biol. Med. **154,** 310 (1977).
4. Goren, A. D., Singh, I. J., and Pentel, L., Amer. J. Anat. **150,** 193 (1977).
5. Tanzer, M. L., *in* "Biochemistry of Collagen" (G. N. Ramachandran and A. H. Reddi, eds.), p. 137. Plenum Press, New York (1976).
6. Reddi, A. H., and Huggins, C. B., Proc. Nat. Acad. Sci. USA **69,** 1601 (1972).
7. Reddi, A. H., and Huggins, C. B., Proc. Nat. Acad. Sci. USA **72,** 2212 (1975).
8. Reddi, A. H., and Anderson, W. A., J. Cell Biol. **69,** 557 (1976).
9. Rath, N. C., and Reddi, A. H., Biochem. Biophys. Res. Commun. **81,** 106 (1978).
10. Reddi, A. H., Hascall, V. C., and Hascall, G. K., J. Biol. Chem. **253,** 2429 (1978).
11. Reddi, A. H., Gay, R., Gay, S., and Miller, E. J., Proc. Nat. Acad. Sci. USA **74,** 5589 (1977).
12. Hall, B. K., Calc. Tiss. Res. **8,** 276 (1972).
13. Elders, M. J., Smith, J. D., Smith, W. G., and Hughes, E. R., Biochem. J. **136,** 985 (1973).
14. Reddi, A. H., Advan. Biol. Med. Phys. **15,** 1 (1974).
15. Strates, B. S., and Urist, M. R., Clin. Orthop. Rel. Res. **66,** 226 (1969).

Received May 17, 1979. P.S.E.B.M. 1979, Vol. 162.