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CHANGES IN ORNITHINE DECARBOXYLASE ACTIVITY DURING MATRIX-INDUCED CARTILAGE, BONE AND BONE MARROW DIFFERENTIATION

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SUMMARY

Subcutaneous transplantation of coarse powders of demineralized rat diaphyseal bone matrix into allogeneic recipients results in new bone formation. The changes in ornithine decarboxylase activity during such bone matrix-induced sequential differentiation of cartilage, bone and bone marrow were investigated. There was a peak in ornithine decarboxylase activity on day 3 corresponding to the appearance of fibroblasts in close contiguity to the bone matrix. This was followed by another peak of enzyme activity on day 8 which was correlated with the onset of proliferation of presumptive osteoblasts and vascular endothelial cells. The peak of ornithine decarboxylase activity on day 3 appears to be a demineralized bone matrix-specific event. Induction of ornithine decarboxylase activity represents one of the early responses to implanted bone matrix.

The occurrence of aliphatic polyamines during the growth and differentiation of many animal tissues is well known (1-3). Ornithine decarboxylase (EC 4.1.1.17; ODC) catalyzes the decarboxylation of ornithine to putrescine (1, 4 Diaminobutane) and has been shown to be involved in cell proliferation (4-6). Despite a recent report (7) of the occurrence of ornithine decarboxylase in cartilage, the possible roles of polyamines in connective tissue differentiation are not known. The differentiation and growth of endochondral bone is preceeded by the proliferation and hypertrophy of chondrocytes and the subsequent vascular invasion of the calcified cartilage matrix. Anatomically, these multitudinous events occur simultaneously in the epiphyseal growth plate which poses a technical problem for any biochemical

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approach. However, bone matrix-induced endochondral bone formation (8-10) circumvents these difficulties and offers a suitable system for biochemical studies. The present paper describes changes in ornithine decarboxylase activity during the sequential development of cartilage, bone and bone marrow.

MATERIALS AND METHODS

Demineralized bone matrix prepared from rat diaphyses was transplanted subcutaneously in the thoracic region of 28-35 day old male rats of Long-Evans strain in bilateral sites as described previously (8). The day of transplantation was designated as day 0, and all operations and autopsies were performed between 10:00 and 11:00 a.m. On designated days after transplantation the plaques were dissected free of adhering muscle and collected in ice cold solution containing 0.15 M NaCl, 0.003 M $NaHCO_3$, 0.01 M dithiothreitol (DTT) and 0.001 M EDTA at pH 7.4.

Plaques from at least 4 rats were pooled and homogenized three times for 10 seconds with a Polytron homogenizer set at maximum speed. The homogenates were centrifuged at 4° in a JA-20 rotor at 30,000 x g for 15 minutes in a Beckman refrigerated centrifuge. The supernatant was recentrifuged at the same speed for another 30 minutes and the resulting supernatant used for determination of ODC activity according to the method of Jänne and Williams-Ashman (11). The incubation mixture consisted of 0.1 M glycylglycine buffer, pH 7.2 containing 10 mM dithiothreitol, 0.2 mM pyridoxal phosphate (Sigma), 2 mM L-ornithine HCl (Sigma) and 0.2 µCi of [^{14}C] L-ornithine (New England Nuclear) in a final volume of 0.5 ml. The incubation was carried out at 37° for one hour in rubber stoppered glass tubes with polyethylene center wells (Kontes Glass) containing 0.1 ml of hyamine hydroxide. The reaction was stopped by injecting 0.5 ml of 40% trichloroacetic acid into each tube through the rubber septum and the tubes were further incubated for another 30 minutes to trap all the liberated $^{14}CO_2$. At the end of the incubation the center wells were removed and placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear) and counted in a Beckman Scintillation Counter. Appropriate control tubes containing no enzyme were incubated and processed as above. All of the determinations were made in triplicate and the control values were then subtracted from the experimental values. The results were expressed as picomoles of $^{14}CO_2$ released per hour per mg of protein; the protein in enzyme aliquots were determined according to Lowry et al. (12) after precipitation with 10% trichloroacetic acid to avoid interference due to DTT.

Ornithine decarboxylase was partially purified from 10% homogenates of day 3 plaques by ammonium sulfate fractionation and DEAE cellulose chromatography according to the methods of Jänne and Williams-Ashman (11). The characteristic properties of the ammonium sulfate fractionated enzyme were determined after dialysis against the homogenizing buffer in the absence of DTT.

Specificity of the ODC induction by demineralized bone matrix on days 3 and 8 was determined by transplanting mineralized

bone matrix and acid extracted rat tail tendon subcutaneously as described above.

The developmental stages of the plaques were determined through histological methods in all cases.

RESULTS AND DISCUSSION

Transplantation of demineralized bone matrix from the rat diaphysis to subcutaneous sites in allogeneic recipients resulted in new endochondral bone formation accompanied by hematopoietic bone marrow differentiation in the newly formed ossicle as described previously (8-10). In brief, on transplantation of demineralized bone matrix a transient inflammation-like response ensued. On day 3, responding fibroblasts (mesenchymal cells) appeared in the vicinity of the matrix particles, interacted with the matrix, proliferated and then emerged as chondroblasts on days 5 and 6. The differentiated chondrocytes then underwent hypertrophy, and on day 9 the onset of calcification of the matrix was evident. On day 10, vascular invasion into the calcified cartilage matrix occurred; this resulted in chondrolysis and osteogenesis. The newly formed bone was remodeled and hematopoietic bone marrow differentiation was evident in the ossicle.

The changes in ornithine decarboxylase activity during matrix-induced endochondral bone formation are depicted in Figure 1. Major peaks of enzyme activity were observed on days 3 and 8. The observed peak on day 3 could be correlated with the earliest stages in the proliferation of mesenchymal cells before their emergence as chondroblasts. Other experiments revealed that this peak was followed by a peak in DNA synthesis (A.H. Reddi, unpublished observations). The precipitous fall in ODC activity on day 4 may be due to the very short half-life of this enzyme (1). A second peak of ODC activity on day 8 was most probably due to proliferation of presumptive osteoblasts and vascular

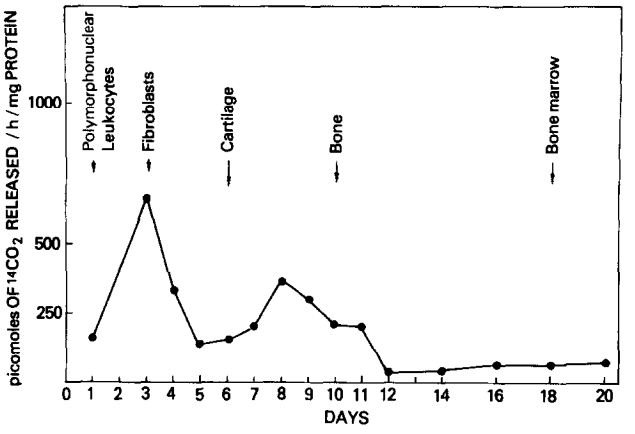

Figure 1: Changes in ornithine decarboxylase activity during matrix-induced endochondral bone differentiation.

Table 1. Specificity of the Induction of Ornithine Decarboxylase Activity.

Matrix	Ornithine Decarboxylase Activity (p moles $^{14}CO_2$ released/mg protein/h)	
	Day 3	Day 8
Demineralized bone powder	567	212
Mineralized bone powder	28	194
Acid-extracted rat tail tendon	159	143

The matrices were implanted subcutaneously and the activity of ODC in the implants were determined on days 3 and 8 as described in the text.

endothelial cells. Earlier investigations have documented amply the increase in ODC in rapidly proliferating cells (4-6). However, our inability to detect appreciable ODC in differentiating bone marrow (days 18-21) cannot be explained. It is worthy of note that in femoral bone marrow the enzyme activity was rather low, despite the abundance of proliferating cells in this tissue

Table 2. Characteristics of Ornithine Decarboxylase from day 3 Plaques

System	Ornithine Decarboxylase Activity (p moles of $^{14}CO_2$ released/mg protein/h)
No additions	*
Plus Pyridoxal Phosphate, 0.2mM	*
Plus Dithiothreitol, 10mM	*
Pyridoxal Phosphate, 0.2mM and Dithiothreitol, 10mM (complete)	419
Complete + Putrescine, 5mM	288
Complete + Spermine 5mM	300

*, not detectable

The enzyme source was 0.2-0.5 saturation ammonium sulfate fraction dialyzed against 0.15M NaCl, 3mM $NaHCO_3$, 1mM EDTA, pH 7.4.

(see Table 3). There was no change in enzyme activity when dialyzed bone marrow extracts were used.

The specificity of the ODC response, was examined by comparing the demineralized matrix with mineralized bone matrix and acid-extracted rat tail tendon matrix. It should be recalled that the latter two matrices are devoid of bone-inducing competence (13). On day 3, a maximal increase in ODC activity was elicited by demineralized bone matrix (Table 1). However, the increase in ODC activity on days 3 and 8 in response to rat tail tendon is due to granulomatous-tissue formation; similar findings have been reported earlier (5) in granulomas elicited by sponge implants. The increase in ODC activity in response to mineralized bone matrix on day 8 is due to proliferation and formation of multinucleated giant cells as observed histologically. Thus, the peak of ODC activity on day 3 is a

Table 3. Distribution of Ornithine Decarboxylase Activity in Various Rat Tissues

Tissue	Ornithine Decarboxylase Activity (p moles $^{14}CO_2$ released/mg protein/h)
Epiphysis, femur	56
Diaphysis, femur	N.D.*
Femoral head cartilage	219
Bone marrow, femur	51
Xipho-sternum	N.D.*
Rat tail tendon	86
Muscle, thoracic	122
Tooth pulp, incisor	131
Tooth, incisor	102
Ventral prostate	3424

N.D.* not detectable.

demineralized bone matrix-specific phenomenon.

A partial purification of the ODC in day 3 plaques was undertaken using the published procedure (11). Greater than 80% of the ODC activity in high speed supernatants was precipitated between 0.2-0.5 saturation with ammonium sulfate. Further purification on DEAE-cellulose column yielded about 30 fold purification. However, the purified enzyme was rather unstable and lost the activity in less than 24 h. In view of this, certain characteristics of the enzyme were determined from the partially purified 0.2-0.5 saturation ammonium sulfate fraction. The day 3 plaque enzyme had a pH optimum between 7 and 8. This enzyme activity is completely dependent on pyridoxal phosphate and

dithiothreitol (Table 2). Putrescine and spermine at 5 mM concentrations were slightly inhibitory. These properties are in general similar to those of the enzyme purified from rat ventral prostate gland (11).

Table 3 summarizes a survey of ODC activity in a variety of skeletal and dental tissues in comparison to ventral prostate, the richest source of this enzyme in growing (6-7 wk old) rats. In bone, most of the activity was confined to epiphyseal regions; the diaphyseal bone was devoid of activity. The latter finding confirms an earlier observation (7). Although bone marrow is a rapidly proliferating tissue, ODC activity was low. Moderate activity was detected in tail tendon and muscle. It is interesting that tooth pulp exhibited ODC activity. The activity in pulp-free tooth (incisor) could well be due to remnants of cell-processes and any attached ameloblasts and cementoblasts.

In summary, these results demonstrate the changes in ornithine decarboxylase activity in matrix-induced plaques and its possible relationship to cellular proliferation during endochondral bone differentiation.

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