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Collagen Formation and Endochondral Ossification in Estrogen Treated Mice.* (30996)

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The ability of estrogen to stimulate endosteal bone formation in the skeleton of the mouse has been demonstrated many times since the original report of Gardner and Pfeiffer(1). It is not known why this response to estrogen occurs, nor why it appears to be specific (among mammals) to mice(2). The reaction begins in the metaphyses of growing long bones and eventually extends along the entire shaft. Ultimately, if treatment is prolonged, the marrow elements may be completely displaced by the centripetal growth of bone trabeculae. Vaes and Nichols (4) demonstrated *in vitro* that bone collagen formation, as measured by uptake of radioglycine, was enhanced in estrogen-primed mice. Autoradiographic studies with tritiated thymidine(4) in mice have shown that estrogen treatment increases the rate at which metaphyseal and endosteal osteoblasts are formed from undifferentiated reticular cells in the marrow. While, after estrogen treatment, the increase in osteoblast numbers may be adequate to explain the enhanced collagen synthesis observed *in vitro,* it is not known whether the hormone increases the ability of the individual osteoblasts to form collagen. To resolve this question, further studies of bone formation in mice have been pursued by high resolution autoradiography following administration of H^3-proline and H^3-glycine at different times after estrogen treatment.

Materials and methods. Male, CF_1 albino mice, 30 days of age, were injected with one subcutaneous dose of 1 mg estradiol valerate (Delestrogen)† to stimulate endosteal bone formation. The controls were not treated with estrogen. Tritiated-proline (1 μc/g body weight)‡ was administered intraperitoneally to 15 control and 15 estrogen-treated mice 24 hours after hormone treatment, and 3 animals from each series were sacrificed by decapitation thereafter at 1, 4, 8, 16 and 24 hours.

A second series of mice was injected subcutaneously with H^3-glycine (0.5 μc/g body weight)§ on the seventh day after estrogen treatment when the endosteal bone reaction had been well established. Four untreated control mice and 4 estrogen-treated mice were sacrificed by decapitation 1, 2, 4, 12, and 24 hours after isotope administration. The times at which the labeled amino acids were administered were based on data(3) which indicated (a) that the differentiation of osteoblasts from the precursor cell population was increased as early as 6 hours after estrogen treatment, and (b) that the population of

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‡ L-Proline-3,4,-H^3 Hydrochloride (Lot 64-191-3), New England Nuclear Corp., Specific Activity = 371 mC/mmole.

§ Glycine-1-C^{14}, New England Nuclear Corp., Specific Activity = 0.66 mC/mmole.

TABLE I. Number of Grains Counted per Metaphyseal Osteoblast* at Different Time Periods Following H^3-Proline Administration to Mice.

Time (hr)	Femur		Tibia	
	Control	Estrogen-treated	Control	Estrogen-treated
1	13.1 ± 3.4	15.3 ± 4.2	14.0 ± 4.0	16.1 ± 7.2
4	8.2 ± 4.0	9.7 ± 5.9	9.7 ± 3.2	9.9 ± 7.2
8	8.8 ± 3.7	6.2 ± 3.8	6.3 ± 2.7	6.9 ± 3.8
16	5.1 ± 1.9	4.9 ± 1.8	6.1 ± 2.9	5.4 ± 1.1
24	3.9 ± 1.1	5.4 ± 2.4	5.7 ± 2.1	4.6 ± 1.4

* Mean ± S.D. of 50 to 75 cells counted.

metaphyseal osteoblasts capable of being flash-labeled with tritiated thymidine (DNA synthesis) was nearly doubled after 24 hours.

At autopsy, the femurs and tibias were removed, fixed in 10% neutral formalin, decalcified in 10% EDTA (pH 7.4) and embedded in paraffin. Serial longitudinal sections were cut on a rotary microtome at 7 μ. Following deparaffinization, the slides were dipped in Kodak liquid nuclear emulsions NTB-2 (H^3-proline study) or NTB-3 (H^3-glycine study) according to the technique of Messier and Leblond(5). The slides were exposed in a freezer, developed and routinely stained with hematoxylin and phloxine. Sections were also stained with PAS prior to autoradiography, and these were counterstained with hematoxylin following exposure and development.

Results. In the controls, most of the metaphyseal (distal femur, proximal tibia) and endosteal cells labeled within the first hour after either radioproline or radioglycine administration were osteoblasts. Although proline is a more specific tracer for collagen formation than glycine, the deposition and relative intensity of the autoradiographic label from either amino acid at the same time after injection were identical. The grain counts over osteoblasts from 1-24 hours after injection of radioproline are presented in Table I. Since Tonna(6) reported peak labeling by H^3-glycine within 30 minutes after injection, the availability time of injected radioglycine and radioproline is probably of the order of minutes.

A greater proportion of the undifferentiated reticular cells were labeled by glycine than by proline, probably because glycine is utilized by every cell as a substrate for intracellular protein and nucleic acid synthesis as well as for collagen synthesis by osteoblasts. Silver grains were also observed over a few young osteocytes which were being buried in the bone, but mature osteocytes were rarely labeled. Osteoclasts were only infrequently and lightly labeled. The presence of extracellular silver grains between the osteoblasts and the surfaces of bone trabeculae as early as 1 hour after injection indicates that these cells were utilizing the labeled amino acids for collagen formation. The reactive bands were about 2 μ thick and probably represented a salt-soluble collagen fraction. The secretion of labeled amino acids from the cells was virtually complete by 16 hours after injection (Table I) when autoradiographs showed reduced silver grains lying over the recently deposited surfaces of trabeculae. On some trabeculae at 24 hours, the bands of silver grains appeared to have moved further away from surfaces and were sealed off by younger unlabeled bone lamellae. These reactive bands of mature collagen were somewhat thicker at 24 hours (4 μ) than the original surface deposits at 1 hour, no doubt because the osteoblasts had begun to utilize and secrete a greater proportion of unlabeled amino acids for collagen formation and the radioactive product had been progressively diluted.

It was also usual to find trabeculae delineated by surface grains and lined by labeled non-osteogenic spindle-shaped cells 16 to 24 hours after isotope administration. These are apparently resting surfaces which were growing at the time glycine and proline were administered. The lack of further growth and burial of the label can be attributed to a periodic modulation of the osteoblasts to un-

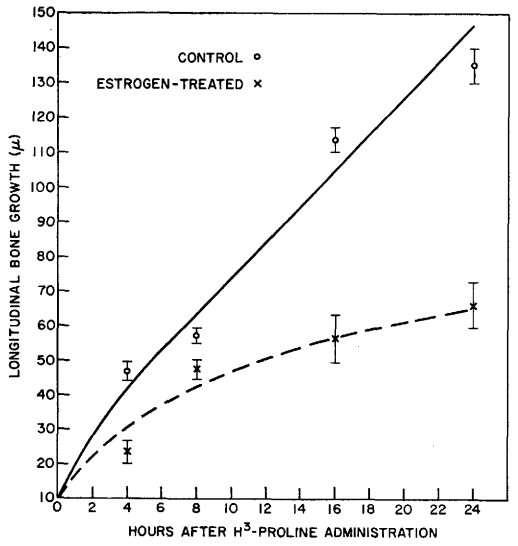

FIG. 1. Movement of the ossification front, away from the proximal epiphyseal cartilage of the tibia at times after labeling with H^3-proline in control and estrogen-treated mice.

differentiated reticular cells(7); osteoblastic activity along any trabeculum is not continuous.

In estrogen-treated mice the endosteal reaction, expressed by large numbers of newly formed trabeculae in the distal metaphyses of the femurs, was established a week after estrogen treatment. However, while estrogen is known to stimulate osteoblast formation within 6 hours(3) the serial autoradiographs of the bones exposed to radioproline (24 hours after injection) and radioglycine (7 days after injection) did not provide evidence that estrogen similarly altered the normal pattern of amino acid uptake or collagen formation by these newly formed osteogenic cells. Table I shows, for example, that the rate at which the cellular proline label was depleted and incorporated into collagen was identical in the control and estrogen-primed mice. Approximately 60% of the initial activity had been lost from the cells after 24 hours.

Because the labeled amino acids are rapidly fixed in the collagen of newly forming trabecular bone at the ossification front, the silver grains mark the position of the front at time of injection. The distance the label moves away from the epiphyseal growth cartilages with time after injection reflects the rate of *endochondral ossification* which is, in part, a measure of the proliferative activity of the chondrocytes in the cartilages. The comparative displacement of the band of silver grains from radioproline in the bones from the control and estrogen-primed mice during the 2nd day after hormone treatment has been plotted in Fig. 1. The overall growth in length of the bones due to cell divisions in the cartilages of the knee joint was 135 μ/day in the control and 66 μ/day after estrogen treatment.

Discussion. The choice of glycine and proline as tracers for collagen formation in autoradiographic and biochemical studies has been predicated on the knowledge that these amino acids are the largest contributors to the dry, ash-free collagen of bone(8). While molecular glycine and proline may be incorporated intact into the peptide linkage of collagen, a certain fraction of the available proline is first hydroxylated to yield collagen hydroxyproline(9). Although proline is the more specific tracer for collagen formation, the initial pattern of uptake and secretion of both amino acids in osteoblasts is quite similar.

These studies have again demonstrated that estrogen has the capacity to alter the micro-environmental conditions in bone and can promote endosteal bone formation. Quantitative studies with tritiated thymidine previously suggested that the hormone could stimulate the modulation of undifferentiated precursor cells to the osteoblast class(3). However, the hormone seemed not to have affected collagen formation by the individual osteogenic cells. The pattern of uptake of the labeled amino acids and the subsequent rate at which the label was moved out of the osteoblasts to bone surfaces was not altered by estrogen treatment. Moreover, the results obtained from the grain counts (H^3-proline) were in good agreement with those described previously for normal mice injected with H^3-glycine(10). Maximum labeling of cells was observed at 1 hour, and the label was found over the mature collagen lamellae of trabeculae 16-24 hours later. Estrogen, then, appears to stimulate only processes which influence cell differentiation, and has no effect on col-

lagen synthesis by mature osteoblasts. This difference in the response of mouse bone tissue to female sex hormone might have been predicted, for estrogen treatment had failed to reduce the time required for the self-burial of H^3-thymidine labeled osteoblasts in bone (3). It is evident that the amount of new bone formed in the shafts of mice treated with estrogen simply reflects a large population of competent osteoblasts. The effect of estrogen on bone formation in mice has variously been ascribed to some unspecified local insult to the primitive connective tissue cells of the marrow(11), or, alternatively, to malnutrition (hypoxia) owing to the constriction of the arterial terminals in the metaphyses by connective tissue hyperplasia(12). Because Brooks and Lloyd(13) failed to find any altered bone vascular supply in mice following estrogen treatment, it is probable that the endosteal reaction does not reflect a change in the gross nutritional status of the bone.

Because it is believed that estrogen treatment impedes both cartilage cell mitosis and mucopolysaccharide formation(14,15), it would be reasonable to assume that the hormone exerts its primary action on some regulatory cell organelle. Recently, the electromicrographic studies of Silberberg *et al*(16) demonstrated hypertrophy of the Golgi zone and accelerated organelle development in articular cartilage cells of mice as early as 2 hours after hormone treatment. These changes, which were maximum after 16 hours, were related to a disturbance of carbohydrate synthesis by these chondrocytes, *i.e.*, decreased production of mucopolysaccharides, and delayed endochondral ossification. In the present study, the radioproline marker demonstrated that estrogen had suppressed endochondral ossification (longitudinal bone growth) 24 hours after treatment.

Summary. The effect of estrogen on rate of collagen synthesis and new bone formation in the mouse was investigated by high resolution autoradiographic methods. H^3-proline and H^3-glycine were administered on the first and seventh days, respectively, following estrogen treatment, and the mice were sacrificed at various intervals during the 24 hours following each tracer injection. The position of the radioproline label on metaphyseal trabeculae indicated that the control bones grew in length 135 μ/day, whereas the bones from mice injected with estrogen 24 hours previously grew only 66 μ/day. Grain counts indicated that (a) uptake of the labeled amino acids by osteoblasts, and (b) subsequent transfer of the label with time after injection to surfaces of newly forming metaphyseal and endosteal bone were not affected by prior estrogen treatment. Silver grains were observed over the bone 16-24 hours after tracer administration. It was concluded that estrogen stimulates the processes which promote the differentiation of osteoblasts in the bones of mice, and that the hormone has no effect on the functional capacity of individual osteoblasts to synthesize and form collagen.

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