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Growth Hormone Dependent Matrix-Induced Heterotopic Bone Formation (40788)[1]

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Normal skeletal growth and repair is dependent upon adequate levels of circulating vitamins, minerals, and hormones plus a myraid of known and unknown serum growth factors. One of the most important agents supporting osseous tissue development is growth hormone (GH) whose action is mediated through various somatomedins (1, 2). Most previous studies have utilized fully differentiated intact or explanted skeletal tissue in examining effects of GH excesses and deficiencies (3) but information concerning *de novo* cartilage and bone formation under these conditions is lacking. The heterotopic production of cartilage and bone which occurs 10–20 days following intramuscular implantation of demineralized cortical bone (4) provides an experimental model for investigating hormonal influences on skeletal tissue induction (5–7), and is used in the present research report to study the effects of hypophysectomy on chondroosseous cytodifferentiation.

Materials and methods. Twenty-two 125- to 150-g (5–7 weeks old) hypophysectomized male Sprague–Dawley rats (Hormone Assay Lab; Chicago, Ill.) and their normal litter mates were kept under standard laboratory conditions and weighed at regular intervals for 60 days prior to beginning the experiment. At that time several hypophysectomized (HYPOX) and normal rats were sacrificed by ether overdose for collection of the long bones. The midshafts of the femur and tibia were removed, cleaned, and demineralized in 0.6 *N* HCl (2°C) for 24 hrs and then lyophilized for storage. When implanted intramuscularly into allogenic recipients this preparation of demineralized whole bone matrix consistently induces new cartilage by the 10th day and woven bone by the 15th day (4).

The above osteoinductive implants prepared from HYPOX and normal donors were each bioassayed in both HYPOX and normal rats of approximately 3.5–4 months of age. All group II recipients received daily s.c. injections of 200 μg bovine growth hormone (bGH) which was kindly supplied by Dr. C. H. Li. Calculated on the basis of 100 g of body weight, the dose given was 110 μg for the HYPOX recipients and 50 μg for the corresponding normal group. Because the response to exogenous GH is correlated more closely with age than body weight (8), the fixed daily injection of 200 μg bGH was selected to correspond with a dose previously demonstrated by Murphy *et al.* (9) to restore radiosulfate uptake in young HYPOX rat tibial epiphysis. Group III consisted of HYPOX rats given the above dose of bGH for 20 days prior to sacrifice and implantation of their long bones into normal untreated hosts.

De novo cartilage production was quantitated by $^{35}SO_4$ uptake. On the 10th postoperative day, a single 100-μCi dose of $Na_2{}^{35}SO_4$ (757 Ci/*M*) was injected ip. Twenty-four hours later the animals (three per group) were killed and the implants (three per animal) excised for analysis of ^{35}S incorporation. Two specimens were used for histological examination while the remaining samples (5–7) were counted for radioactivity. For the latter procedure, the grafts were minced and washed in 0.05 *M* Na phosphate buffer (ph 7.4) containing nonradioactive 0.1 *M* Na_2SO_4 for 2 hr followed by three washings in distilled water. Samples were then solubilized in 2 ml of

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90% formic acid at 100°C for 1 hr. Aliquots (0.5 ml) were dissolved in 15 ml of scintillation cocktail and counted in a Beckman LS-100 spectrometer. The results were expressed as counts per minute/10 mg dry preimplant weight and represented sulfation of cartilage chondromucoprotein (10).

New bone formation was estimated on the 15th day by ip injection of 100μCi of $^{45}CaCl_2$ (2200 Ci/*M*). The implants were removed the following day and rinsed in nonradioactive 0.1 *M* $CaCl_2$ followed by a thorough washing in distilled water. Overnight hydrolysis in 0.5 *N* HCl was carried out at room temperature (10). Aliquots (0.5 ml) were counted and results interpreted as uptake by newly calcified bone.

Results. During the 60 days prior to beginning the experiment the average body weights of HYPOX rats reached peak levels of 183 ± 12 g compared with 404 ± 30 g for the normal siblings. For the purposes of this study, hypophysectomy was judged complete by macroscopic examination of the sella turcica in combination with the body weight data (11). There was a significant ($P < 0.01$) increase in chondromucoprotein synthesis as measured by $^{35}SO_4$ levels following bGH injection into HYPOX recipients (group II) implanted with matrix from hypophysectomized and normal rats. No increase was observed when the same matrix implants were bioassayed in normal rats receiving bGH. Cortical bone prepared from hypophysectomized rats pretreated for 20 days with bGH stimulated a significant ($P < 0.05$) increase in $^{35}SO_4$ incorporation when compared with untreated controls (Table I).

Injections of the host with bGH significantly elevated ^{45}Ca uptake during bone morphogenesis in all group II donor–recipient combinations. Group III implants also showed greater uptake ($P < 0.01$) of ^{45}Ca indicating GH replacement can restore some osteoinductive activity to matrices of HYPOX animals (Table II). Although reduced in quantity, the quality of new cartilage and bone formed appeared normal in all the implants examined. Figure 1 shows the matrix-induced bone morphogenesis as it occurs in normal animals and Fig. 2 illustrates the same reaction in HYPOX recipients 15 days after implantation of matrix from normal donors.

Discussion. Bone morphogenesis refers to the differentiation of competent mesenchymal-type cells into cartilage and bone which forms not only as a tissue but as an organ unit consisting of a complete ossicle filled with hematopoietic marrow (4). This reaction occurs in response to intramuscular implants of demineralized bone (4), bone matrix gelatin (12), and most recently, a soluble collagenase resistant component of bone matrix (13). The inductive agent, termed bone morphogenetic protein (BMP) is a glycoprotein (13) closely associated with but separable from the intact collagen molecule. BMP prepared either by methods, without (14) or with enzymic digestion (13) induces mesenchymal-like cell differentiation into cartilage and bone. The present study demonstrates that the quanti-

TABLE I. $^{35}SO_4$ INCORPORATION INTO CHONDROMUCOPROTEIN ON DAYS 10–11 AFTER IMPLANTATION OF BONE MATRIX (cpm/10 mg preimplanted matrix wt)

Donor	Recipient	Group I	Group II	Group III
HYPOX	Normal	316 ± 122[a]	338 ± 92 (3)[b]	517 ± 45*
HYPOX	HYPOX	77 ± 22	411 ± 132 (8)**	
Normal	Normal	659 ± 165	757 ± 89 (4)	
Normal	HYPOX	412 ± 68	1071 ± 259 (10)**	

[a] Mean ± SD based on 5–7 samples.

[b] Percentage increase in body weight of recipients receiving 200 μg bGH daily for 10 days. Group I are untreated controls. Group II recipients received daily fixed injections of 200 μg bGH which corresponds to a dose of 110 μg/100 g body wt for HYPOX rats and 50 μg/100 g body wt for normal rats. Group III HYPOX donor rats (4) received 200 μg bGH daily for 20 days prior to sacrifice which resulted in a 21% increase in body weight. Their long bones were then removed for implantation into normal untreated hosts.

* $P < 0.05$. Groups II and III compared with Group I using Student's *t* test.

** $P < 0.01$.

TABLE II. ^{45}Ca INCORPORATION ON DAYS 15–16 AFTER IMPLANTATION OF BONE MATRIX (cpm/10 mg preimplanted matrix wt)

Donor	Recipient	Group I[a]	Group II	Group III
HYPOX	Normal	557 ± 73[b]	692 ± 95 (4)[c,*]	790 ± 100**
HYPOX	HYPOX	93 ± 32	468 ± 60 (18)**	
Normal	Normal	3096 ± 192	3573 ± 256 (5)*	
Normal	HYPOX	196 ± 45	2646 ± 512 (16)**	

[a] See Groups in Table 1.
[b] Mean ± SD based on 5–7 samples.
[c] Percentage increase in body weight of recipient receiving 200 μg bGH daily for 15 days.
* $P < 0.05$. Groups II and III compared with Group I using Student's t test.
** $P < 0.01$.

ty of new bone is deficient in the absence of hypophyseal hormones, and that synthesis of the BMP in the matrix of bone may be GH dependent.

Although the amount of cartilage and bone produced was diminished in HYPOX recipients implanted with normal demineralized bone, the morphogenetic process, once initiated, seemed to proceed as normal. This quantitative decrease in the product of differentiation may be related to the known inhibitory effects of GH deficiency on mitogenesis (15). Because bone morphogenesis is dependent upon initial mesenchymal cell proliferation (16), the lowered $^{35}SO_4$ and ^{45}Ca uptake in hypophysectomized recipients and the stimulation of these processes following replacement therapy could have been due to reduced progenitor cell division in the former case and subsequent recovery of this capacity in the latter. How BMP action is integrated with accessory metabolic and endocrine agents in modifying the pro-

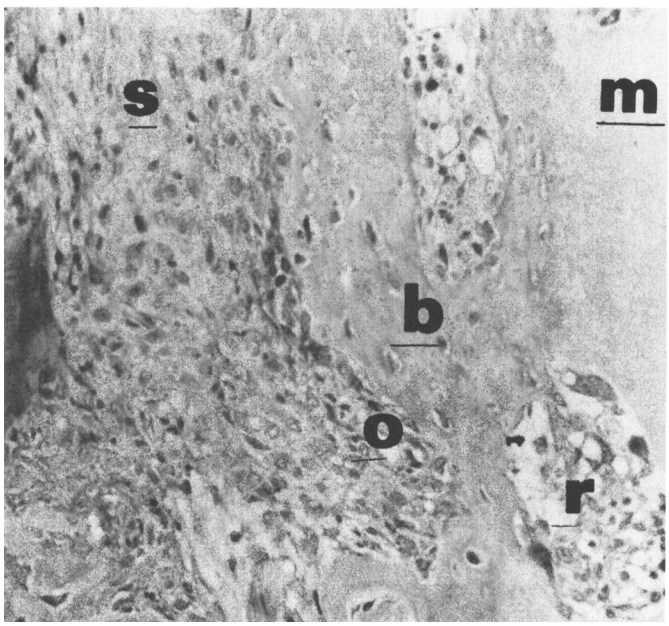

FIG. 1. Photomicrograph of deposits of new bone induced by bone matrix (right) prepared from a normal rat and implanted in a normal rat, 15 days after implantation. Note: unabsorbed matrix (*M*); osteoblasts (*O*); mesenchymal cells and preosteoblasts (*S*); new bone (*b*); new bone marrow (*r*). Hemotoxylin, eosin, and azure II stains ×64.

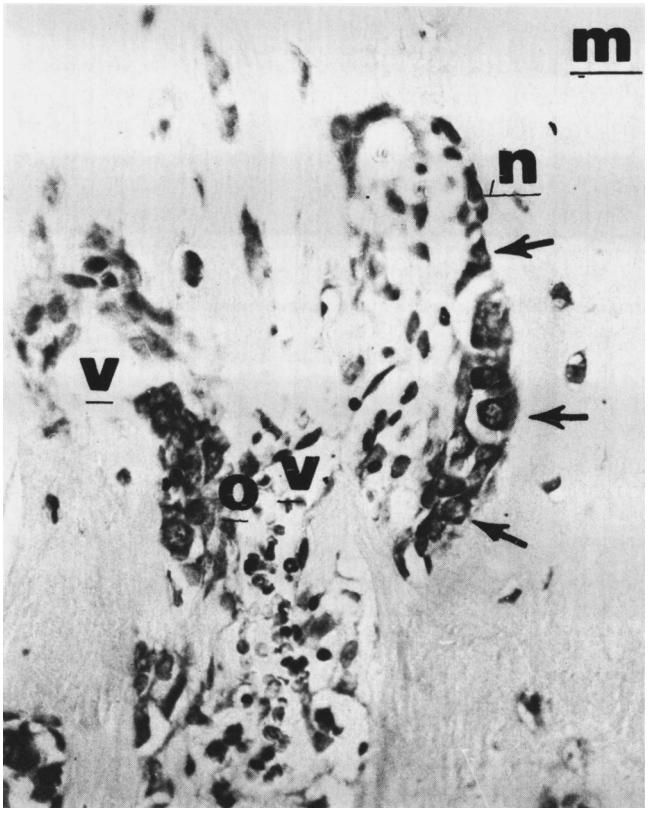

FIG. 2. Photomicrograph of new bone formation induced by demineralized bone matrix prepared from a normal rat and implanted in a muscle pouch in a hypophysectomized rat, 15 days after the operation. Note the nest (o) and layer (arrows) of osteoblasts but the paucity of extracellular matrix. The layer of cuboidal shape osteoblasts (arrows) is arranged in a palisades. The golgi vacuoles lie on the matrix side of the nuclei and also identify these cells as osteoblasts. Note: unabsorbed matrix (*m*); layer of new bone including osteocytes (*n*); capillaries (*v*); nest of osteoblasts (*o*).

genitor–genitor cell sequences during osteoinduction is unknown at present.

As documented by others in intact epiphysis (9) and as demonstrated here for heterotopic chondrogenesis, administration of GH to normal rats has little effect on chondromucoprotein $^{35}SO_4$ uptake compared to untreated controls. In contrast, bone formation as measured by ^{45}Ca levels was elevated in the presence of excess GH. This observation corroborates several previous reports of increased skeletal mass in orthotopic (17) and heterotopic (6, 7) sites in response to GH injection. The differential response of chondrogenesis and osteogenesis to GH excess is unclear at present and will require further investigation.

The present report significantly extends previous research (6, 7) dealing with GH influences on heterotopic bone formation. In a review article, Reddi includes an unpublished experiment demonstrating an attenuated response in matrix-induced osteogenesis in HYPOX rats (7). In a report by Koskinen *et al.* (6) the experiments deal with the enhancement of matrix-induced bone formation by bGH in normal and not hypophysectomized rats. Reddi described the response of HYPOX rats to normal bone matrix and did not test the response to bones of HYPOX rats. Our data demonstrate that HYPOX rat bone matrix induces a low response in normal and a still lower, hardly detectable response, in HYPOX recipients.

Recently, Canalis *et al.* (18) have shown

in vitro that somatomedin enhanced both collagen and noncollagen protein synthesis in fetal rat calvaria. The restoration of BMP activity following GH injections into HYPOX rats may have been due to a comparable GH stimulation of noncollagenous matrix protein synthesis including a BMP. Experimental lathyrism (18), rickets (18), and now GH deficiency are known to attenuate the osteoinductive property of bone matrix. It would appear that any agent or condition retarding bone regeneration might be expected to affect either matrix-induced bone formation or matrix storage of the BMP, or both.

Summary. The bone morphogenetic property in bone matrix, recently demonstrated to be a glycoprotein, and the mesenchymal cell response to it, are growth hormone dependent. Both can be partially restored by the administration of bGH to hypophysectomized rats.

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