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The Hormonal Milieu in Early Stages of Bone Cell Differentiation Modifies the Subsequent Sex-Specific Responsiveness of the Developing Bone to Gonadal Steroids

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ABSTRACT

We have established previously that rat bone tissue, as well as rat and human-derived bone cells in culture, show a sex-specific response to gonadal steroids in stimulation of the specific activity of the BB isozyme of creatine kinase (CK) and DNA synthesis. This response could be modified by manipulation of the endocrine environment during early stages in rat development. To further examine the influence of changing hormonal steroid milieu and vitamin D status on the action of gonadal steroids in developing bone tissue, we used two models of ectopic bone formation: demineralized tooth matrix (DTM) implanted under the skin, and femoral bone marrow (BM) transplanted under the kidney capsule of a syngeneic recipient mouse. The response to gonadal steroids in ossicles developed from implanted DTM depended on the recipient's gender; injection of estradiol 17β (E_2; 5 μg) into young female mice 21 days after DTM implantation increased, 24 h later, CK activity in the newly formed ossicles by ~60%, whereas injection of dihydrotestosterone (DHT; 50 μg) had no effect on CK activity. In contrast, in male mice, DHT but not E_2 increased CK activity in the ossicles by ~50%. This sex-specific response was abolished in gonadectomized mice resulting in a similar response of the ossicles to both E_2 and DHT. When DTM was implanted into vitamin D–deficient female mice, there was a lower basal CK activity and a significantly diminished response to E_2 in the newly formed bone tissues. When BM, which contains mesenchymal and stromal cells and committed osteoprogenitor cells, was transplanted into 6-week-old intact or gonadectomized female or male mice, the response of the newly formed bone ossicles, 21 days after transplantation, to E_2 or to DHT was according to the gender of the donor. Bone formed from BM obtained from female mice responded to E_2 only and those formed from male BM responded to DHT only. Ossicles developed from BM obtained from gonadectomized mice showed lack of response to either gonadal steroid. Furthermore, only ~25% of the BM transplants obtained from castrated (CAST) male donors developed into ossicles. Ossicles formed from BM obtained from vitamin D–deficient female donors showed lack of response to gonadal steroids. These findings suggest that the manipulation of the hormonal milieu in early stages of the differentiation sequence of bone cells modifies the subsequent selective responsiveness of the developing bone tissue to gonadal steroids. (J Bone Miner Res 2001;16:823–831)

Key words: bone marrow, bone, cartilage, implants, estrogen, androgens, creatine kinase, vitamin D

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INTRODUCTION

WE HAVE established previously that rat bone in vivo and cultured rat-embryo calvaria cells,[1,2] human osteoblast-like cells,[3] and human untransformed bone cells[4] show sex-specific response to gonadal steroids; estradiol 17β (E_2) stimulated creatine kinase (CK) activity and DNA synthesis only in female bone cells, whereas testosterone or dihydrotestosterone (DHT) stimulated CK activity only in male bone cells. This sex-specific response of bone cells to gonadal steroids can be modified by manipulation of the endocrine environment in early development as we showed in young rats after gonadectomy,[5] in prenatally or neonatally androgenized female rats,[6] and in androgen-receptor–deficient (Tfm) male rats.[6] It also was shown that in vitamin D–deficient rats, E_2 or DHT failed to stimulate DNA synthesis in diaphyseal (Di) bone and the CK response was markedly lower.[7–9] To further examine the role of gender and/or the hormonal milieu on the action of gonadal steroids in developing bone tissues, we used two experimental models of ectopic bone formation in mice: (1) implantation of demineralized bone or tooth matrix (DTM) particles under the skin[10,11] (DTM particles contain collagens and bone morphogenic proteins (BMPs), which induce bone formation by pluripotent mesenchymal cells of the recipient)[12-18]; (2) transplantation of mouse femoral bone marrow (BM) under the renal capsule of syngeneic recipient, resulting in the formation of osteogenic tissue originated from stromal and/or osteoprogenitor cells from the donor mouse.[19–23] These experimental approaches enabled us to dissociate between the influence of the animals' gender from that of the hormonal milieu on the response of the newly formed ossicles to gonadal steroids.

In the present study we tested the hormonal regulation of the development of the two ectopic bone tissues, because it is well established that hormones such as gonadal steroids and vitamin D[7,24,25] regulate bone formation. We examined the effects of these hormones on the specific activity of the brain isozyme of CK in the two ectopic tissues. This enzyme has been used as a general genomic response marker for gonadal steroids. This enzyme is involved in cellular energy buffering and is related closely to changes in cell replication rate in various cell types.[24–26]

MATERIALS AND METHODS

Animals

C57Bl/6J mice were obtained from the colony at Tel-Aviv University, Sackler School of Medicine, and used at the age of 6 weeks. Intact male or female (immature) or gonadectomized mice (2 weeks postsurgery starting at the age of 6 weeks) as well as vitamin D–deficient mice were obtained as described previously for rats, namely, at the age of 4 weeks, the mice were switched to grow in the dark on a vitamin D–deficient diet supplemented with Ca (0.75%) and phosphate (0.55%).[7]

Preparation and implantation of DTM

DTM particles were prepared by washing mice teeth with distilled water and then dehydrating by ethanol and ether. The particles were then pulverized in a mortar in the presence of liquid nitrogen and sieved to select particles of about 75–300 μ. The particles were washed with 0.5 M HCl and dehydrated. All steps were carried out at 4°C.[27]

Mice at the age of 6 weeks were anesthetized and two samples of 2 mg of DTM each were implanted under the skin (subcutaneously) over the pectoralis muscle.

Transplantation of femoral BM

Mice were killed by ether and BM was prepared by pushing the femoral marrow out with a needle and keeping it in the cold. The BM was obtained from either 6-week-old intact female mice or 6-week-old intact male mice, ovariectomized (OVX) female mice, castrated (CAST) male mice, and vitamin D–deficient mice.

Two samples of femoral BM were transplanted under the renal capsules (one sample per kidney) of a syngeneic recipient mouse, separating it physically from other possible migrating cells, either intact female mice or intact male mice, OVX mice or CAST mice, and vitamin D–deficient mice.

Morphology

Three weeks after implantation, mice were killed and implants were collected, fixed in 10% neutral buffered formalin, decalcified, dehydrated, and paraffin embedded. Sections (3 μm thick) were stained for hematoxylin and eosin. Pictures were taken at $\times$100 enlargement.

Hormonal treatment

Three weeks (or other times if indicated) after implantation of DTM or transplantation of BM, female mice, male mice, OVX female mice, or CAST male mice were injected intraperitoneally (ip) with 5 μg of E_2 or 50 μg of DHT. Twenty-four hours later the mice were killed; the implants or transplants as well as bone, cartilage, or BM from the host mice were collected for enzyme measurements.

To examine the effect of vitamin D deficiency and vitamin D treatment on the response of the newly formed ossicles to the gonadal steroids, vehicle (0.01% ethanol), 3 ng/g body weight (BW) 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], or 30 ng/g BW 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] were injected daily for 3 weeks into vitamin D–depleted donors before obtaining the BM for transplantation followed, in some experiments, by gonadal steroids injections as described previously.

Enzyme extraction and assay

At the end of the hormonal treatment, bones formed from the implants, the mice bone, and cartilage were collected and homogenized in a cold homogenizing buffer using an Ultra Torax (Janke and Kunkel, Staufer Brisgov, Germany) and the CK was obtained by centrifugation at 14,000*g* as

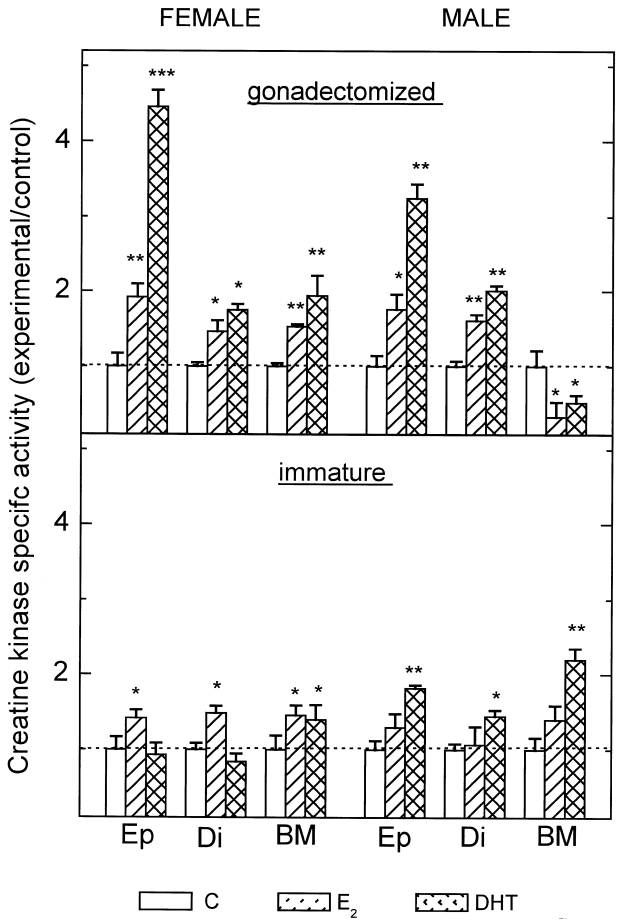

FIG. 1. The effect of E_2 and DHT on CK-specific activity in immature (6 weeks old) and gonadectomized male and female mouse organs. Animals were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. Mice organs: Ep cartilage, Di bone, and BM were collected and homogenized, and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for n = 5–10. *$p < 0.05$; **$p < 0.01$. The following were the basal levels (in μmol/minute per milligram proteins) of CK in immature female mice: Ep = 5.9 ± 1.0, Di = 12.6 ± 1.1, and BM = 0.67 ± 0.13; in immature males: Ep = 5±0.6, Di = 16.4 ± 1.25, and BM = 0.5 ± 0.08; in gonadectomized females: Ep = 2.6 ± 0.45, Di = 9.3 ± 0.45, and BM = 1.7 ± 0.07; in gonadectomized males: Ep = 5.1 ± 0.7, Di = 11.9 ± 0.8, and BM = 2.4 ± 0.5.

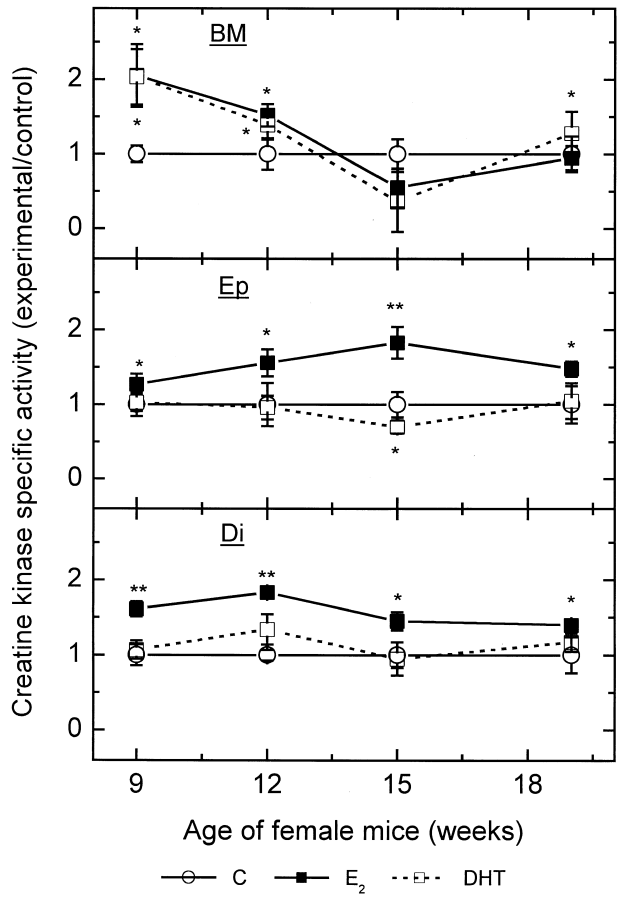

FIG. 2. Age-dependent stimulation by gonadal steroids of CK-specific activity in different organs in female mice. Female mice at the ages of 9, 12, 15, and 19 weeks were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. Mouse organs Ep cartilage, Di bone, and BM were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for n = 5–10. *$p < 0.05$; **$p < 0.01$. The basal levels (in μmol/minute per milligrams protein) of CK at 9 weeks were Ep = 7.9 ± 1.3, Di = 11 ± 1.5, and BM = 0.94 ± 0.1; at 12 weeks were Ep = 5.6 ± 1.7, Di = 8.6 ± 0.6, BM = 0.62 ± 0.13; at 15 weeks were Ep = 2.3 ± 0.4, Di = 8.6 ± 1.5, BM = 1.38 ± 0.28; at 19 weeks were Ep = 2.0 ± 0. 5, Di = 6.3 ± 1.5, and BM = 0. 78 ± 0.19.

described previously.[1,6] Enzyme activity was measured using the CK kit (Sigma-Aldrich, Rehovot, Israel) in a computerized automatic spectrophotometer (Kontron AG, Milan, Italy). A unit of enzyme activity was defined as the amount yielding 1 μmol of adenosine triphosphate (ATP)/minute at 30°C and the specific activity was defined as units per milligram of protein.[1]

Protein was assayed by the Coomassie brilliant blue dye binding assay[28] using bovine serum albumin (BSA) as the standard.

Statistical analysis

The significance of differences between experimental and control means was evaluated by Student's *t*-test for at least three different experiments with at least five implants or organs in each.

Reagents

All reagents were of analytical grades and were obtained from Sigma Co., Ltd.

RESULTS

The effect of gonadal steroids on CK activity in mouse bone and cartilage

Mouse bone responded to E_2 or DHT sex specifically as previously shown in rats.[1] E_2 stimulated CK-specific ac-

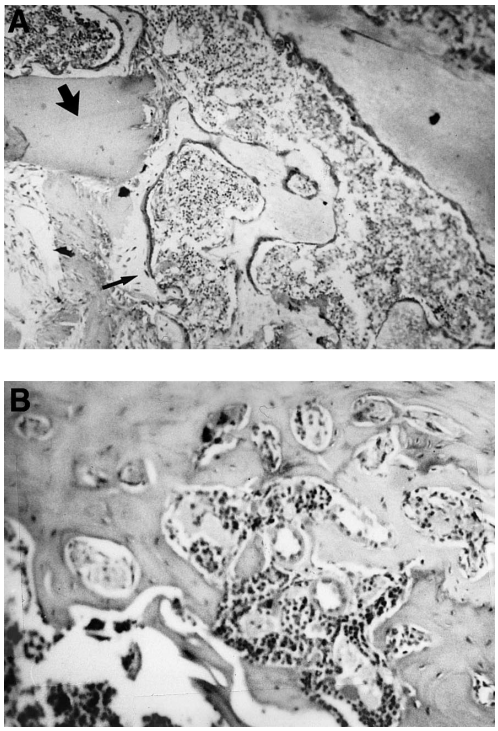

FIG. 3. Morphology of ossicles formed by either (A) DTM originated from females and transplanted in females or (B) BM obtained from females and implanted into females. The details are given in the Materials and Methods section.

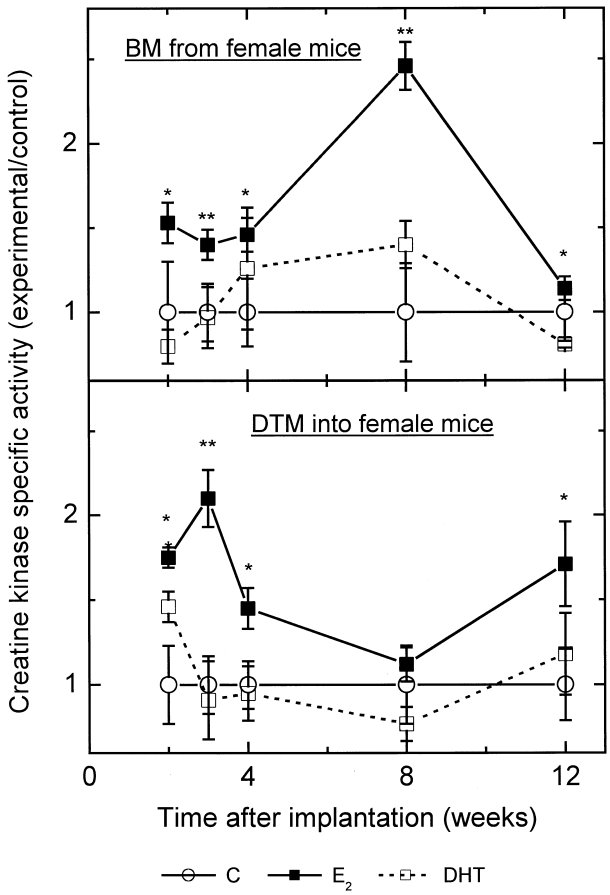

FIG. 4. Time-dependent stimulation by gonadal steroids of CK-specific activity in ossicles originated from DTM implantation and from BM transplantation. BM and DTM were implanted in intact female mice for different time periods. At the different times mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (BM and DTM) were collected homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control $\pm$ SEM for n = 5–10. *$p < 0.05$; **$p < 0.01$. The basal levels (in μmol/minute per milligram protein) were BM = 0.74 $\pm$ 0.06 and DTM = 4.1 $\pm$ 0.3.

tivity only in female Di bone by 40–50%, whereas DHT stimulated CK-specific activity only in male Di bone by 80–120% (Fig. 1). In contrast to rat epiphyseal (Ep) cartilage, mouse Ep cartilage also responded in a sex-specific pattern (Fig. 1), which means Ep cartilage from female mice responded to E_2 while Ep cartilage from male mice to DHT only. BM from female mice responded to both gonadal steroids, whereas from male mice there was no significant response to E_2. OVX female mice or CAST male mice responded to both gonadal steroids that is, loosing the sex specificity of the response in either Ep or Di, or male BM (Fig. 1). When female mice were used at different ages, the basal activity of CK in Ep cartilage, Di bone, and BM declined with age (Fig. 2), but their sex-specific response remained similar while in BM the response declined with age (Fig. 2).

The features of the ossicles formed by the implanted DTM or BM

Ossicles formed by implanted DTM show new bone tissues rich in osteocytes surrounded by a layer of osteoblasts (Fig. 3A, thin arrow). We also can see hematopoietic tissue between bone trabeculae. The DTM particle not covered by osteoblasts is seen in the upper left corner of Fig. 3 (thick arrow). Ossicles formed by implantation of BM under the renal capsule show bone tissue rich in osteocytes and covered by osteoblasts (Fig. 3B). New hematopoietic tissue is observed in the ossicle.

Gonadal steroids stimulation of CK activity in ossicles induced by DTM implants

When DTM from female mice was implanted in female mice, their constitutive CK-specific activity was changed with time after implantation. They responded only to E_2 and its magnitude was changed with time after implantation (Fig. 4). There was a significant response 21 days after implantation, and, therefore, this time point was chosen for analysis of their hormonal responsiveness. The response to gonadal steroids in ossicles developed from implanted DTM depended on the recipient gender; injection of E_2 into immature female mice 21 days after DTM implantation increased, 24 h later, CK-specific activity in the newly formed ossicles by 60% (Fig. 5), whereas DHT had no effect on CK activity. In contrast, in male mice, DHT but not E_2 increased CK activity in the newly formed ossicles by 50% (Fig. 5).

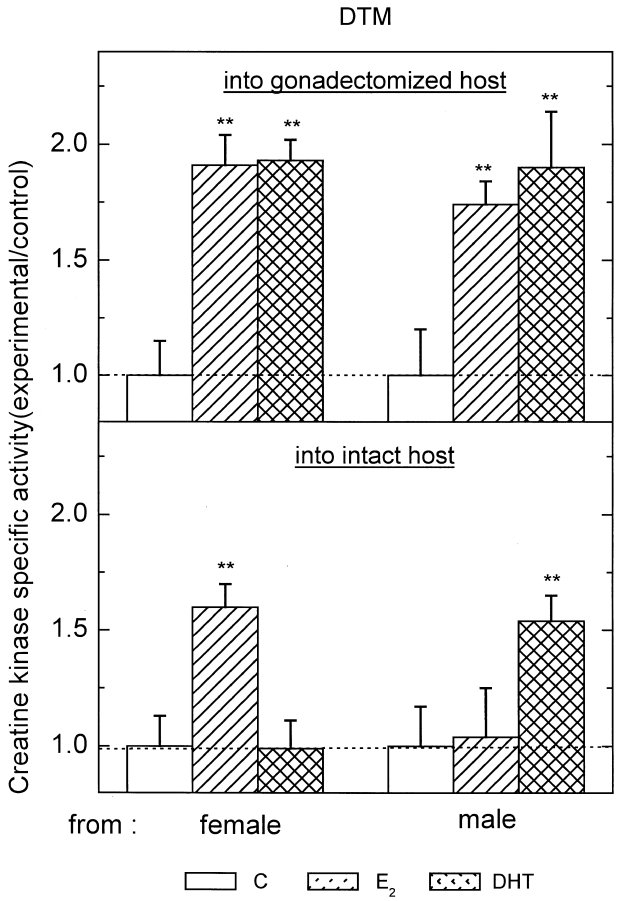

FIG. 5. Gonadal steroid stimulation of CK-specific activity in ossicles formed from DTM implanted into either intact male or female mice or gonadectomized male and female mice. DTM was implanted into intact male or female mice or gonadectomized male and female mice for 3 weeks and mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (DTM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control $\pm$ SEM for $n = 5$–10. *$p < 0.05$; **$p < 0.01$. The basal levels (in μmol/minute per milligrams protein) were DTM into intact male = 4.53 $\pm$ 0.77, DTM into intact female = 4.15 $\pm$ 0.52, DTM into gonadectomized male = 6.47 $\pm$ 1.29, and into DTM gonadectomized female = 5.32 $\pm$ 0.8.

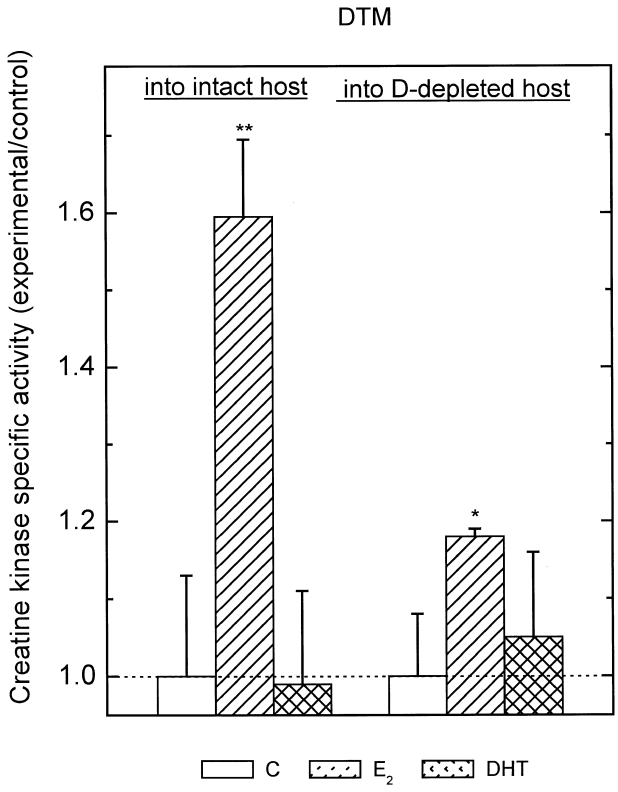

FIG. 6. Gonadal steroid stimulation of CK-specific activity in ossicles formed from DTM implanted into intact or vitamin D–depleted female mice. DTM from intact mice was implanted into intact or vitamin D–depleted female mice for 3 weeks, and mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (DTM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control $\pm$ SEM for $n = 5$–10. *$p < 0.05$; **$p < 0.01$. The following were the basal levels (in μmol/minute per milligrams protein): into intact female = 4.15 $\pm$ 0.52 and into vitamin D–depleted female = 2.23 $\pm$ 0.17.

This sex-specific response of ossicles induced by DTM was abolished in gonadectomized male and female mice, resulting in a similar response (70–90%) of the ossicles to both E_2 and DHT (Fig. 5). When DTM was implanted into vitamin D–depleted female mice, there was a significant lower CK basal activity compared with intact DTM implanted into intact mice and a significantly diminished sex-specific response to E_2 in the newly formed ossicles (20% compared with 60% stimulation; Fig. 6).

Gonadal steroids stimulation of CK activity in ossicles formed by BM transplantation

When BM from mice was transplanted under the kidney capsule of 6-week-old intact or gonadectomizes female or male mice, the response of the newly formed ossicles to E_2 or DHT, 3 weeks after transplantation, was determined by the gender of the donor; ossicles formed from BM obtained from female mice responded to E_2 by a 30–100% increase in CK activity (Fig. 7), whereas in those formed from transplanted male BM, the enzymatic activity was induced only by DHT by 40–70% (Fig. 7). When BM from intact female mice was transplanted into OVX mice, the ossicles formed had high CK-specific activity and responded to E_2 but not to DHT by a 300% increase in CK activity. When BM was transplanted into CAST mice, the CK activity was low but still responded only to E_2 by 40% (Fig. 8). When BM from intact male mice was transplanted into OVX mice, the basal CK activity in the ossicles was low but was stimulated significantly by DHT and not by E_2 (Fig. 8). BM obtained from OVX or CAST mice and transplanted either into intact male or intact female mice resulted in decreased basal CK activity with no response to either gonadal steroids (data not shown). Furthermore, only ~25% of BM transplanted obtained from CAST male donors developed into bone ossicles.

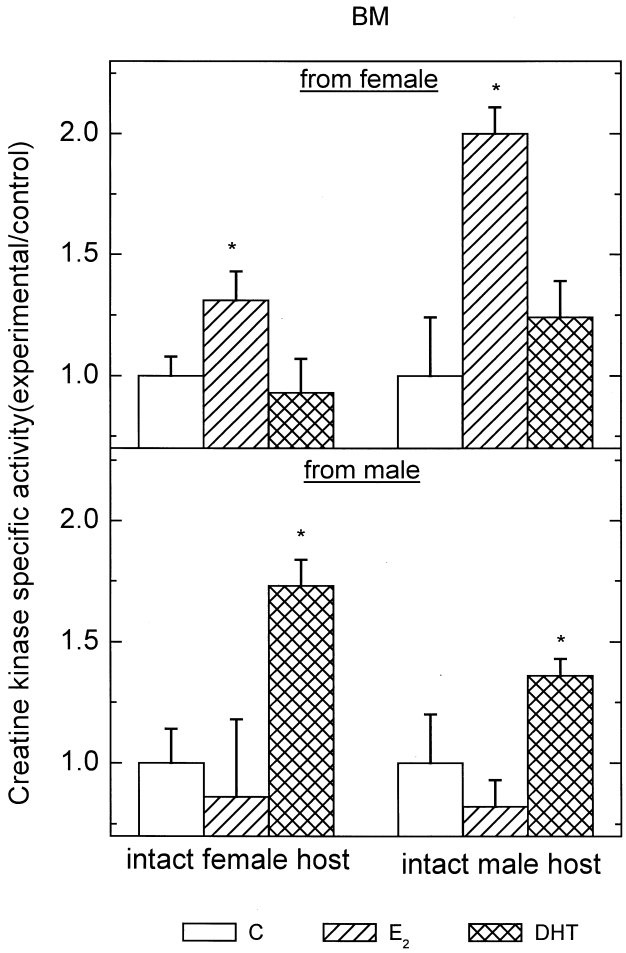

FIG. 7. Gonadal steroid stimulation of CK-specific activity in ossicles formed from BM transplanted into different mice. BM from male or female mice was implanted into intact male or female mice for 3 weeks and mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (BM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for $n = 5$–10. $*p < 0.05$; $**p < 0.01$. The basal levels (in μmol/minute per milligrams protein) were from female into intact female = 0.74 ± 0.06, from male into intact female = 0.22 ± 0.03, from female into intact male = 0.45 ± 0.11, and from male into intact male = 0.44 ± 0.09.

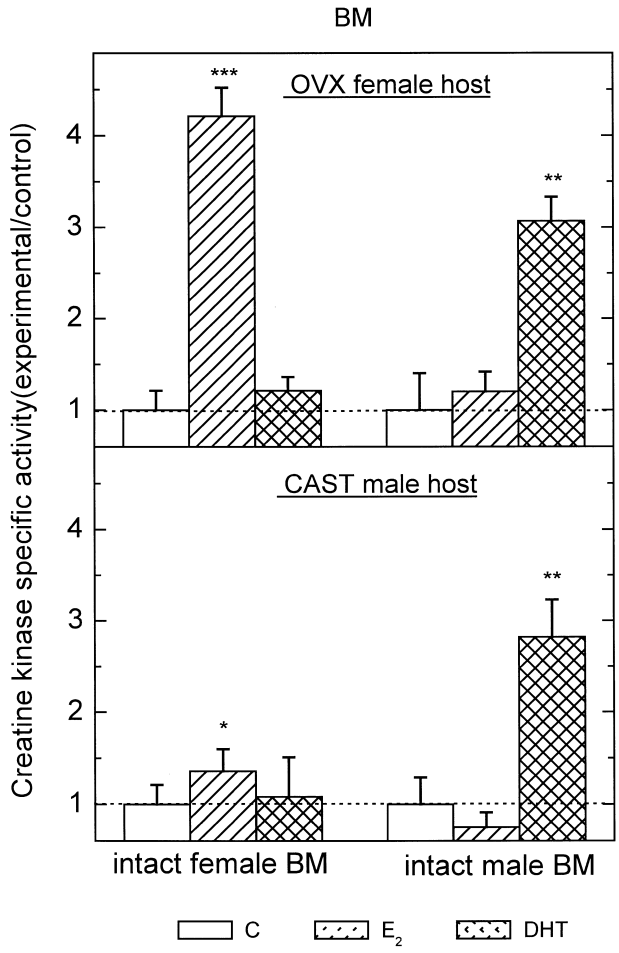

FIG. 8. Gonadal steroid stimulation of CK-specific activity in ossicles formed from BM implanted into gonadectomized animals. BM from male or female mice was implanted into gonadectomized male (CAST) or female (OVX) mice for 3 weeks and mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (BM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for $n = 5$–10. $*p < 0.05$; $**p < 0.01$. The basal levels (in μmol/minute per milligram protein) were from intact female into OVX female = 0.38 ± 0.08, from intact male into OVX female = 0.15 ± 0.06, from intact female into CAST male = 0.061 ± 0.013, and from intact male into CAST male = 0.068 ± 0.020.

BM obtained from intact female mice and transplanted into vitamin D–depleted female mice developed into bone tissue, which had low basal CK activity with no response to gonadal steroids (Fig. 9). Ossicles formed from BM obtained from vitamin D–deficient female donors transplanted into either intact female mice or into D-depleted female mice showed both low basal CK activity and lack of response to gonadal steroids (Fig. 9). BM from vitamin D–depleted female mice that had been replenished by daily injections of either $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ and then transplanted into intact female mice developed into bone tissues with higher basal CK activity compared with BM with no vitamin D treatment, and the newly formed ossicles showed a higher response to E_2 compared with BM with no vitamin D treatment, up to a 2-fold increase in CK activity (Fig. 10).

DISCUSSION

The investigation of the effects of gonadal steroids on bone cells was stimulated in the last decade by the finding of low concentrations of receptors for estrogen[29] and androgens[30] in bone cells and demonstration by several laboratories of direct effects of these hormones on the stimulation of bone cell proliferation and function. We have used markers of cell proliferation ([^{3}H]thymidine incorporation

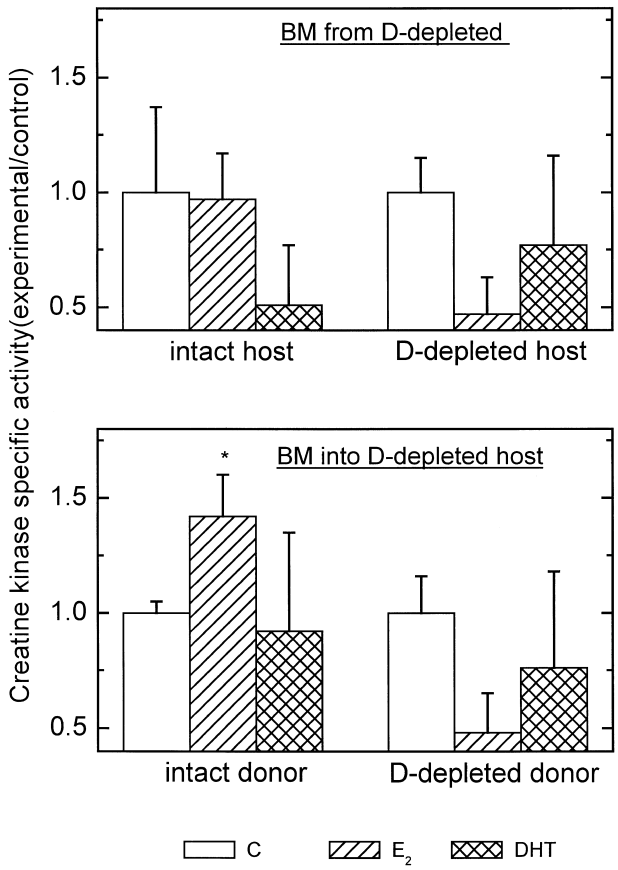

FIG. 9. Gonadal steroid stimulation of CK-specific activity in ossicles formed from BM obtained from intact or vitamin D–depleted female mice implanted into intact or vitamin D–depleted female mice. BM from intact female or vitamin D–depleted female mice was implanted into intact or vitamin D–depleted female mice for 3 weeks and mice were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (BM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for n = 5–10. *$p < 0.05$; **$p < 0.01$. The basal levels (in μmol/minute per milligram protein) were from intact into D-depleted = 0.120 ± 0.006, from D-depleted into D-depleted = 0.25 ± 0.04, from D-depleted into intact = 0.218 ± 0.080, and from D-depleted into D-depleted = 0.249 ± 0.037.

into DNA) and energy metabolism associated with cell division (the specific activity of CK[26]) to show that prepubertal rat bone in vivo, as well as cultured rat-embryo calvaria cells,[1,2] human osteoblast-like cells,[3] and human untransformed bone cells,[4] show a sex-specific response to gonadal steroids. Estrogens are active only in female-derived cells and androgens are active only in male cells. This sex-specific response of bone cells to gonadal steroids can be modified and broadened by manipulation of the hormonal milieu as we showed in prepubertal rats after gonadectomy,[5] in prenatally or neonatally androgenized female rats,[6] and in androgen-receptor deficient (Tfm) male rats.[6] The broadening of sex hormone specificity of rat bone responsiveness by in vivo changes in the hormonal milieu can be maintained in vitro, as shown in calvaria cell cultures obtained from female fetuses androgenized in utero.[6] However, in vitro treatment of female rat-embryo calvaria cells in culture with either testosterone or E_2 did not change their response to E_2.[6] These observations raised the question whether exposure to a certain hormonal steroid environment in early critical stages of bone cell lineage differentiation determines the subsequent responsiveness of rat bone cells to gonadal steroids. The results presented in this article show that in mice, as in rat bone, there was a sex-specific response of bone to gonadal steroids. However, in contrast to rat Ep cartilage,[2] which responds to both E_2 and DHT, mouse cartilage responded to gonadal steroids sex specifically (Fig. 1).[2] This may reflect a different pattern of differentiation, growth, and/or receptor development in the skeletal tissues of rats and mice. BM shows a peculiar behavior; BM originated from females responds to both hormones, whereas BM from males responds only to androgens and not to estrogens. It might be because of different modulation of the receptors to these hormones, which has to be analyzed further. Gonadectomy of mice abolished the sex-specific response of bone and cartilage in both sexes and male BM to gonadal steroids. It also acquired a response to the less dominant steroid, probably by counteracting the effects of each sex steroid on the action by the other, by an unknown mechanism that might involve modulation of receptors or their coactivators and/or corepressors (Fig. 1) similar to what was described for the rats. The results of this study, in which we used experimental models of ectopic bone formation, indicate that the hormonal milieu of the donors' BM mesenchymal stem cells or osteoprogenitor cells determines the development and the subsequent response. In contrast, ossicles originated from DTM implants responded sex specifically to E_2 or DHT, depending on the gender of the host, with no dependence on the gender of the DTM itself. Gonadectomy, resulted in loss of sex specificity of the response of the ossicles and they responded to both hormones, similar to rat skeletal tissues[5] (Fig. 5[8]). The results obtained by these models suggest that the critical time point for the appearance of active receptors to gonadal steroids in the skeletal tissues is in the early stages of bone development (i.e., on stem cells differentiation into osteoprogenitor cells). If the hormonal levels in the milieu are normal at this stage, even if implanted into a gonadectomized animal, there will be a normal sex-specific response. On the other hand, BM taken from gonadectomized mice, implanted into intact male or female mice, will lose the sex specificity of the response to gonadal steroids, which now respond to both. When gonadectomized mice were replenished before the experiments, the results were similar to those obtained using intact mice (data not shown).

Previously, we have shown that there was a mutual interaction between vitamin D analogs and gonadal steroids in affecting the development and responsiveness of the skeletal system.[7–9] Vitamin D depletion resulted in a decreased response of the CK activity and DNA synthesis of rat bones to gonadal steroids, but the sex specificity of the reduced response was retained.[7] Replenishment with $1,25(OH)_2D_3$ but not $24,25(OH)_2D_3$ restored the gonadal steroid responsiveness. In addition, both in vivo[7] and in vitro,[8] pretreatment with vitamin D analogs increased the responsiveness

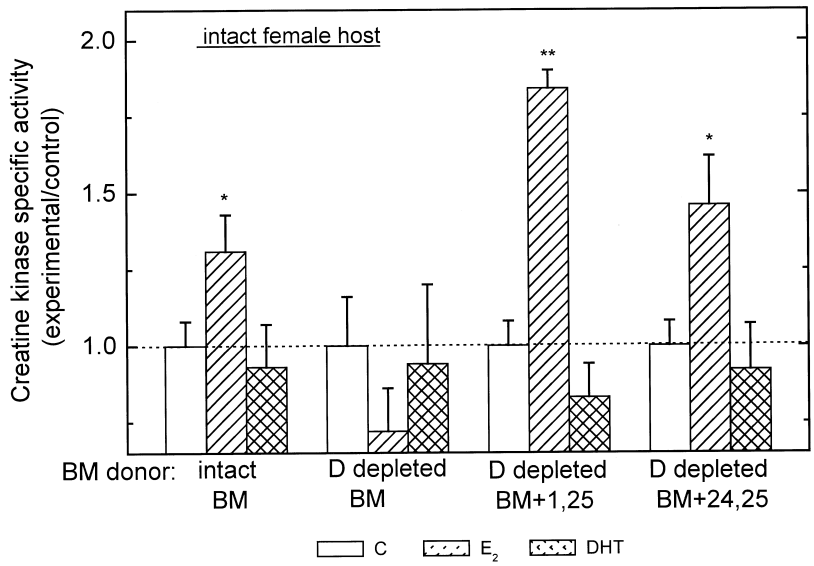

FIG. 10. Gonadal steroid stimulation of CK-specific activity in ossicles formed from BM obtained from intact or vitamin D–depleted female mice or vitamin D–depleted after replenishment female mice and transplanted into intact female mice. BM from females either intact, from vitamin D–depleted, vitamin D–depleted and injected for 3 weeks with 1,25 (3 ng/g), or vitamin D–depleted and injected for 3 weeks with 24,25 (30 ng/g) was implanted into intact female mice and then the hosts were injected with 5 μg of E_2 or 50 μg of DHT and killed 24 h later. The ossicles (BM) were collected and homogenized and enzyme was extracted and assayed as described in the Materials and Methods section. Results are expressed as experimental/control ± SEM for $n = 5$–10. *$p < 0.05$; **$p < 0.01$. The basal levels (in μmol/minute per milligram protein) were from intact = 0.74 ± 0.06, from vitamin D–depleted = 0.184 ± 0.029, from vitamin D–depleted + 1,25 = 0.397 ± 0.032, from vitamin D–depleted + 24,25 = 0.342 ± 0.027.

and the sensitivity of bone cells to gonadal steroids because of an increased number of the nuclear receptors for E_2.[8,9] Reciprocally, estrogen increases the concentration of $1,25(OH)_2D_3$ receptors in rat uterus.[31] Therefore, it is possible that in responsive tissues each of these hormones augment and/or modulate the receptors for the other hormones. Thus, the relationship between vitamin D and gonadal steroids also was studied in the ectopic bone formation systems presented in this article. Normal development of ossicles induced by DTM required normal levels of vitamin D in the host, because DTM transplanted into D-depleted mice had lower basal CK activity and a much reduced sex-specific response to gonadal steroids (Fig. 6). Moreover, BM obtained from intact mice, when transplanted into D-depleted mice responded to gonadal steroids as when transplanted into intact mice, but BM obtained from D-depleted mice, when transplanted into intact mice, had a lower basal enzyme activity and a significantly reduced sex-specific response to gonadal steroids (Fig. 9). Replenishment of mice donors of the BM with $1,25(OH)_2D_3$ restored the normal CK basal activity and response to gonadal steroids in the ossicles (Fig. 10). The ossicles formed had even significantly enhanced responsiveness to E_2. These observations suggest that the vitamin D status of the hormonal milieu, in which mesenchymal stem cells and osteoprogenitor cells differentiate, had a significant effect on the subsequent responsiveness to gonadal steroids of the newly formed bone. At present, it is not clear which mechanism determines the sex-specific responses of bone to gonadal steroids. Possible mechanisms involved may include : (1) the control of differentiation or selective proliferation of particular cell types, probably mesenchymal stem cells or osteoprogenitor cells, which preferentially respond sex specifically to a gonadal steroid, or (2) the regulation by a steroid hormone of the genes for the synthesis of receptors for the same or the other hormones.[9]

In summary, this study shows that gonadal steroids stimulate sex specifically CK specific-activity in bone and in cartilage of mice as well as in two ectopic bones formed as the result of DTM implantation or BM transplantation in these mice. The response of ossicles formed from DTM depended on the hormonal milieu of the host mice, whereas the response of ossicles formed from BM transplantation was dependent on the hormonal milieu of the donor mice. In all systems, the response to gonadal steroids depends on normal levels of both gonadal steroids and vitamin D in the early stages of skeletal development.

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