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THE EFFECT OF LONG-TERM OVARIAN HORMONE DEFICIENCY ON TRANSFORMING GROWTH FACTOR-β AND BONE MATRIX PROTEIN mRNA EXPRESSION IN RAT FEMORA

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Estrogen deficiency results in increased bone turnover and osteopenia. Decreased transforming growth factor beta (TGF-β) has been implicated as having a role in ovariectomy (OVX)-induced bone loss; however the decrease in TGF-β mRNA may simply be due to the reduction in cancellous bone volume that occurs following OVX. This investigation characterized the effect of long-term OVX (11.5 months) on mRNA levels for TGF-β, osteocalcin (OC), and type I collagen (TYPE I) and expressed these data relative to the amount of cancellous bone surface. Additionally, the short-term effect of estrogen administration on TGF-β, OC, and TYPE I mRNA was evaluated. OVX resulted in a 56% loss of bone surface. At the tissue level, TGF-β was reduced, OC was increased, and TYPE I was unchanged. Expressed relative to bone surface, TGF-β was unchanged and OC and TYPE I were significantly elevated. Four hours after estrogen administration, TGF-β mRNA was unchanged compared to vehicle-treated controls. The data suggest that bone turnover is elevated after long-term OVX, but do not suggest a direct role of TGF-β in OVX-induced bone loss.

Ovarian hormone deficiency in laboratory animals or humans results in an increase in bone turnover and a loss of bone matrix (1-3). Although the phenomenon of bone loss and the protective effect of estrogen treatment are well documented (4-6), the underlying mechanism of estrogen-deficient bone loss is not clear. Recently, Ikeda and colleagues (7) provided data indicating that mRNA levels for transforming growth factor beta (TGF-β) were reduced with relatively short-term (3-12 weeks) ovariectomy (OVX) and concluded that TGF-β plays a role in the estrogen-dependent maintenance of normal bone density. Although this hypothesis is attractive, there are alternative interpretations of the authors findings. TGF-β levels were not normalized to cancellous bone surface in the study by Ikeda, et al. (7). Since cancellous bone volume and surface decrease with time with OVX (8), the observed decreases in TGF-β mRNA in OVX'd rats may simply be due to a decrease in cancellous bone surface and bone cell number. Thus, it may be premature to implicate decreased TGF-β expression as having a role in mediating OVX-induced bone loss.

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The primary purpose of the present investigation was to characterize the effect of long-term ovarian hormone deficiency on steady-state mRNA levels for TGF-β and the bone matrix proteins [Type 1 collagen (TYPE I) and osteocalcin (OC)] and to evaluate these changes relative to the alterations in cancellous bone surface that occur with ovariectomy. A secondary purpose was to determine the short-term effects of estrogen on steady-state mRNA levels for TGF-β in cancellous bone of OVX rats.

MATERIALS AND METHODS

Animals

Experiment 1.--Twenty female Sprague Dawley rats (N=10/group) were ovariectomized (OVX) or sham-operated (CON) at three months of age. The animals were sacrificed 11.5 months later and the femora excised and immediately frozen in liquid nitrogen for future RNA isolation and histology. OVX was confirmed by the presence of atrophic uterine horns and the lack of ovarian tissue at the time of sacrifice.

Experiment 2.--Four female Sprague Dawley rats (N=2/group) were OVX'd at 2 months of age and were injected with either 200 µl of diethylstilbestrol (DES) (dose 625 µg/kg dissolved in 50% ETOH) or 200 µl of the vehicle (VEH) (50% ETOH) 4 hours prior to sacrifice. All animals were sacrificed 8 days after surgery. All animal protocols were approved by the appropriate animal care committee at the University of Florida or at the Mayo Clinic.

Total RNA Isolation.--The frozen left femora from 4 animals/group in Experiment 1 were cut at the distal end of the third trochanter and the distal portion was individually homogenized in guanidine isothiocyanate using a Spex Freezer Mill (Edison, NJ). From Experiment 2, cancellous bone from the tibial metaphysis was extracted as described (9) and individual samples were powdered using the Freezer Mill. Total cellular RNA was extracted and isolated using a modified organic solvent method (10) and the yields (200-400 µg/femur; 25-100 µg/cancellous bone) were determined spectrophotometrically at 260 nm. Ten µg of each sample were denatured by incubation at 52°C in a solution of 1M glyoxal, 50% dimethylsulfoxide in 0.1M NaH_2PO_4 solution, then separated electrophoretically in a 1% agarose gel. The amounts of RNA loaded and transferred were assessed by ethidium bromide staining of the gels and hybridization of the filters with a ^{32}P labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAP).

Northern Blot Analysis.--The RNA was transferred overnight via capillary action in 20X sodium saline citrate buffer to an Amersham Hybond nylon membrane (Arlington Heights, IL) and cross linked with a Stratagene UV Stratalinker 1800 before hybridization. The filters were prehybridized for 6 hours at 45°C in a buffer containing 50% deionized formamide, 10% Dextran Sulphate, 5X SSC (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 600 µg/ml heat denatured single strand salmon sperm DNA, and 2X Denhardts solution. Hybridization was carried out for 18-24 hours in a buffer containing the above ingredients in addition to a minimum of 10^6 cpm/ml ^{32}P labeled cDNA for TGF-β, TYPE I, OC, or GAP. GAP was unaffected by long-term OVX or a single injection of DES and was used to correct for unequal loading of RNA in the agarose gel. The message levels for TGF-β, TYPE I, or OC were normalized by dividing by the corresponding level of GAP and data were expressed as a percent of control values. cDNA probes were labeled by random sequence hexanucleotide primer extension using the Megaprime DNA labeling kit from Amersham (Arlington Heights, IL). The filters were washed for 30 minutes at 45°C in 2X SSC and for 15 to 60 minutes in 0.1X SSC at 45°C. The mRNA bands on the Northern blots were quantitated by densitometric scanning using a Molecular Dynamics Phosphor Imager (Sunnyvale, CA).

The cDNA probes used were: 1) rat TGF-β1 provided by Dr. MJ Sporn at the National Cancer Institute, National Institutes of Health; 2) rat OC, a gift from Dr. S. Rossi-Langer, Genetics Institute, Cambridge, MA (11); 3) rat Type I collagen obtained from Dr. C. Genovese, University of Connecticut, Farmington, CT (12); and 4) rat GAP, a gift from Dr. P. Fort, Laboratorie de Biologie Moleculaire, Montpellier France (13).

Histomorphometry

The frozen left femora from the remaining animals in Experiment 1 (N=6/group) were fixed in cold 70% ETOH for a minimum of 2 days, dehydrated in an ascending series of increasing concentrations of ETOH, embedded undemineralized in a mixture of methyl methacrylate: 2-hydroxyethyl-methacrylate (12.5:1) and sectioned at an indicated thickness of 5 μm.

Histomorphometric procedures on the left femur were carried out using a SMI-Microcomp semiautomatic image analysis system (Southern Micro Instruments, Inc., Atlanta, GA) consisting of a Compaq computer with Microcomp software interphased with a microscope and image analysis system. In this system, a high resolution video camera mounted on an Olympus BH-2 microscope displays the image of the specimen on a color monitor. The movement of a pen on a graphics tablet superimposes a tracing of the specimen on the video screen. By this method, the region of interest is traced, and the line length and area bounded by the tracing are calculated.

The sampling site in the distal femur consisted of the epiphysis and the area immediately distal to the growth plate extending 5 mm to encompass the entire metaphysis. Cancellous bone area and perimeter were measured in an area of approximately 45 mm^2. To normalize for OVX-induced cancellous bone loss, densitometric values for the mRNA messages were adjusted per mm bone surface.

Statistical Analysis

Differences in mRNA levels and histomorphometric indices between OVX and CON animals were assessed by unpaired Student t-tests.

RESULTS

Experiment 1.--As anticipated, OVX resulted in a significant loss of cancellous bone area (-46%) and bone surface (-56%) relative to age-matched sham-operated controls.

At the tissue level, TGF-β mRNA levels were significantly depressed to 60% of CON (Fig. 1). TYPE I was not different than CON (102%) (Fig. 2) and OC was significantly elevated to 352% of CON (Fig. 3).

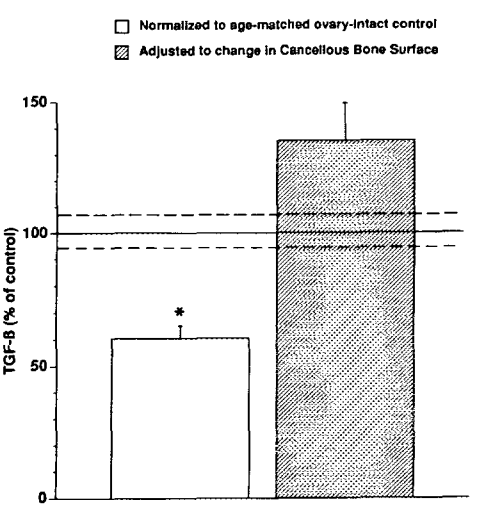

Figure 1. The effect of long-term ovarian hormone deficiency on steady-state mRNA levels for TGF-β. The data [mean ± standard error (SE)] are expressed as a percent of the sham-operated controls. Mean ± SE values for control animals are represented by the solid and dashed lines, respectively. Total RNA was obtained from femora from individual animals (N=4/group). *P≤p.05 vs control, by unpaired Student's t-test.

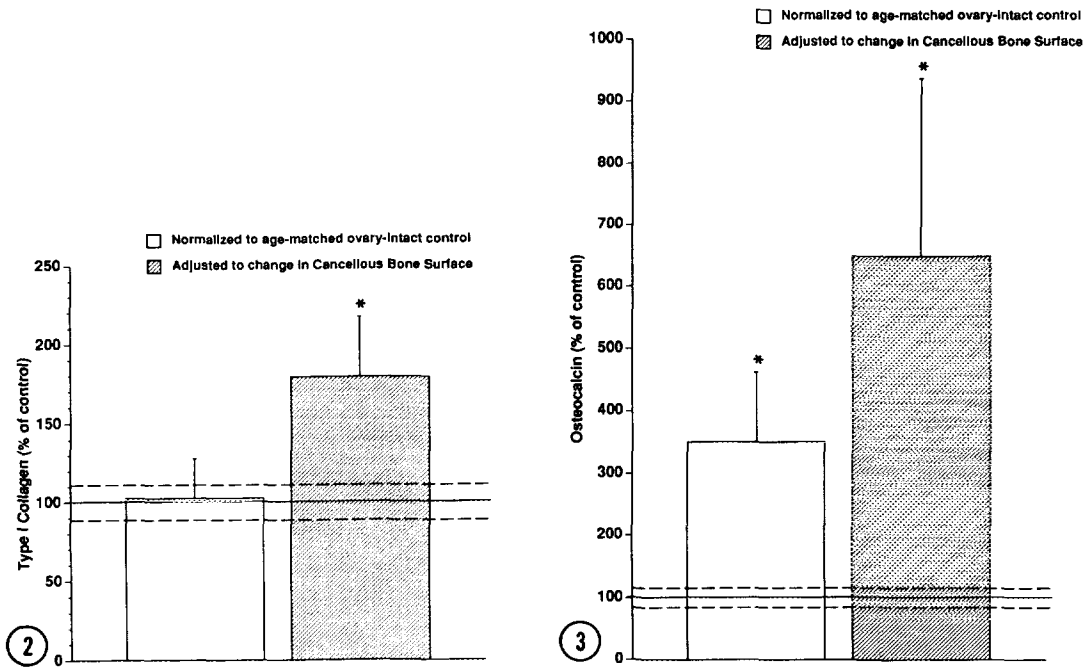

Figure 2. The effect of long-term ovarian hormone deficiency on steady-state mRNA levels for Type I collagen. The data [mean ± SE] are expressed as a percent of the sham-operated controls. Mean ± SE values for control animals are represented by the solid and dashed lines, respectively. Total RNA was obtained from femora from individual animals (N=4/group). *P≤p.05 vs control, by unpaired Student's t-test.

Figure 3. The effect of long-term ovarian hormone deficiency on steady-state mRNA levels for osteocalcin. The data [mean ± SE] are expressed as a percent of the sham-operated controls. Mean ± SE values for control animals are represented by the solid and dashed lines, respectively. Total RNA was obtained from femora from individual animals (N=4/group). *P≤p.05 vs control, by unpaired Student's t-test.

When the message levels were adjusted to account for the significant loss in cancellous bone surface, TGF-β was not depressed but rather was not significantly different from CON (135%) (Fig. 1). Further, both TYPE I and OC were now significantly elevated [179% (Fig. 2) and 649% (Fig. 3), respectively] relative to CON.

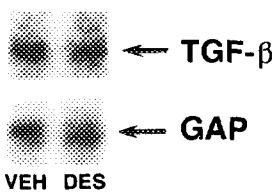

Figure 4. Representative Northern blot illustrating the hybridization of TGF-β and GAP cDNA to total cellular RNA from the cancellous bone of OVX'd rat tibiae. DES- or VEH-treated animals were injected with either DES or vehicle, respectively, 4 hours prior to sacrifice. Transcript sizes for TGF-β and GAP are 2.4 and 1.4 kb, respectively. Densitometric quantitation was performed with a Molecular Dynamics Phosphor-Imager. No difference was observed between DES and VEH treated rats.

Experiment 2.--Administration of DES 4 hours prior to sacrifice did not result in a change (101%) in mRNA levels for TGF-β when compared to the vehicle-treated animals. Figure 4 provides a representative Northern blot depicting the hybridization of TGF-β and GAP cDNAs to total RNA extracted from the cancellous bone of DES and vehicle-treated animals.

DISCUSSION

Ovarian hormone deficiency results in cancellous bone loss (1-3). This finding has repeatedly been observed in growing rats (1,9) and again, is supported by the findings in the present long-term investigation. However, the mechanism for this loss, or conversely the mechanism for the protective effect of the ovarian hormones, has not yet been elucidated.

Ikeda and colleagues present data indicating that estrogen deficiency caused a marked decrease and increase in mRNA levels of TGF-β and osteocalcin, respectively, in osteopenic OVX'd rats (7). In many ways, our results are similar to these authors. A statistically significant 40% decrease in TGF-β mRNA was observed one year after OVX and OC mRNA levels were concomitantly elevated. These findings would also suggest elevated bone turnover after long-term ovariectomy and a potential role of TGF-β in estrogen-deficient bone loss. Ikeda, et al. (7) did not measure TYPE-I message levels which were unchanged at the tissue level in our study. At the tissue level these findings may be valid; however if one were to express the "activity" (i.e., mRNA) based upon the amount of bone present, an entirely different explanation may be proposed.

The majority, if not all, of the osteocalcin synthesized in the body, is by osteoblasts (14) and, likewise, Type 1 procollagen synthesis is confined, in bone, to osteoblasts and preosteoblasts (9). In general, enhanced expression of these bone matrix protein has been interpreted to indicate increased osteoblast number or activity.

Using immunohistochemistry, TGF-β has also been localized to regions of cell differentiation and proliferation, in particular to osteoblasts and osteoclasts lining the bone surface (15,16). Although blood platelets and other tissues also appear to contain TGF-β, bone appears to be the major storage site (17). Thus, if a decrease in bone occurs, as with ovarian-hormone deficiency, decreases in TGF-β mRNA may result purely because of a reduction in the number of cells in which it is expressed as opposed to a reduction per cell. Similarly, changes in bone formation markers in whole bone extracts may underestimate the amount of activity occurring in the much smaller area of active bone surface.

Since the authors (7) see a 10% decrease in bone mineral density in the rat tibia within two weeks of ovariectomy, this suggestion seems to merit consideration. It should be noted that bone mineral density measures primarily cortical bone which turns over very slowly in adult rats. Thus, the actual loss of cancellous bone at that time probably exceeded 10%. In contrast, bone histomorphometry distinguishes the metabolically more active cancellous bone from the inactive cortical bone.

In the present study when message levels are expressed relative to the amount of cancellous bone surface present, mRNA levels for TGF-β were slightly, but non-significantly increased (135%), TYPE-I was elevated (179%) and osteocalcin was dramatically elevated (649%) relative to the sham-operated animals. These mRNA data continue to suggest elevated bone cell activity in agreement with dynamic bone histomorphometry but, in contrast, do not support decreased TGF-β levels one year post-OVX. It is unknown which, if either, method of normalizing data is valid. The extensive histological (1,18) and biochemical (19) data supporting increased bone turnover in OVX'd rats suggests that normalization to bone surface is more accurate because Type I collagen message is elevated. In any case, the potential disparate interpretations indicate the need to consider accompanying changes in bone area and/or surface when evaluating changes in gene expression over prolonged time intervals in complex tissues.

An alternative approach to evaluate the role of TGF-β in mediating estrogen-deficient bone loss is to study acute changes. Estrogen replacement therapy prevents cancellous bone loss in OVX rats (5,6). Thus, if decreased TGF-β expression mediates bone loss in OVX rats as hypothesized, then one would reasonably anticipate that estrogen treatment would rapidly upregulate steady-state mRNA for that growth factor. Furthermore, the upregulation should occur over a time frame that is very short compared to that required to alter changes in bone matrix protein expression or bone surface. In this regard, we have shown that estrogen regulates expression of another important growth factor, insulin-like growth factor-1 in a tissue specific manner within 3 hours of hormone treatment (20). In preliminary studies, 4 hours of treatment with diethylstilbestrol had no effect on steady-state TGF-β mRNA levels in cancellous bone from rat long bones. Although this result is inconsistent with a direct regulatory role for TGF-β in mediating the high bone turnover which follows ovariectomy, much more detailed time-course studies are necessary. Furthermore, as there is evidence that TGF-β protein levels are reduced in bone matrix of ovariectomized rats (21) it is possible that the growth factor has an indirect action on bone turnover.

We also wish to express concern in regards to the misuse of certain statistical analyses. Ikeda, et al. (7) indicate that all 6 tibiae per treatment were minced and homogenized <u>together</u>, and total RNA was extracted. By pooling the tissue, an N=1 per group is obtained and statistical comparisons between groups are not legitimate. However means and standard deviations from three Northern Blot analyses are presented in the results section. We interpret this to indicate that two additional gels or additional lanes were run with aliquots of total RNA from the same initial pools. These separate gels or lanes represent replicates and the results should be averaged to provide a reliable estimate of that individual sample. The authors do not indicate that the experiment was performed in triplicate and that the findings from one individual experiment confirms the results from another (i.e., a separate set of animals or bones). It is not surprising that the standard deviations were so small given that the samples came from <u>within</u> the same pool. We routinely are not so fortunate and see standard errors for TGF-β mRNA levels of 10-15%; however, these data represent separate samples of RNA from separate animals. Thus, we feel it is necessary to interpret the authors' findings

even more cautiously since the data, although pooled from six animals per group, still represent an N=1/group.

The present results indicate an increase in bone turnover one year following OVX whether expressed at the tissue or total bone surface level. However, our data do not necessarily implicate TGF-β as having a role in estrogen-deficient bone loss. The fact that our interpretation varies from that of Ikeda et al. (7) illustrates the importance of considering the "whole picture" when trying to understand cellular mechanisms. Although TGF-β mRNA may be depressed when viewed from the whole organ level following long-term ovarian hormone deficiency, when considered from another vantage point (i.e., the remaining perimeter lined by bone cells) it may be unchanged. Thus, TGF-β may not have a role in estrogen-deficient bone loss or in the protective effect of estrogen on the skeleton.

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REFERENCES

1. Wronski, T.J., Lowry, P.L., Walsh, C.C., Ignaszewski, L.A. (1985) Calcif. Tiss. Int. 37, 324-328.
2. Heaney, R.P., Becker, R.R., Saville, P.D. (1978) J. Lab Clin. Med. 92, 964-970.
3. Richelson, L.S., Wahner, H.W., Melton, L.J., Riggs, B.L. (1984) N. Engl. J. Med. 31, 1273-1275.
4. Christiansen, C., Christensen, M.S., Larsen, N.E., Transbol, I. (1982) J. Clin. Endocrinol. Metab. 55, 1124-1130.
5. Wronski, T.J., Cintron, M., Doherty, A.L., Dann, L.M. (1988) Endocrinology 123, 681-686.
6. Turner, R.T., Evans, G.L., Wakley, G.K. (1993) J. Bone Miner. Res. 8, 359-366.
7. Ikeda, T., Shigeno, C., Kasai, R., Kohno, H., Ohta, S., Okumura, H., Konishi, J., Yamamuro, T. (1993 Biochem. Biophys. Res. Comm. 194, 1228-1233.
8. Wronski, T.J., Cintron, M., Dann, L.M. (1988) Calcif. Tiss. Int. 43, 179-183.
9. Westerlind, K.C., Wakley, G.K., Evans, G.L., Turner, R.T. (1993) Endocrinology 133, 2924-2934.
10. Chomczynski, P., Sacchi, M. (1987) Anal. Biochem. 162, 156-159.
11. Celeste, A.J., Rosen, V., Buecker, J.L., Kriz, R., Wang, E.A., Wozney, J.M. (1986) Eur. Mol. Biol. Organ. J. 5, 1885-1890.
12. Genovese, C., Rowe, D., Kream, B. (1984) Biochem. 2, 6210-6216.
13. Forti, P., Marty, L., Piechaczyk, M., ElSabovty, S., Dane, C., Jeanteur, P. Blanchard, J.M. (1985) Nucleic Acid Res. 13, 1431-1442.
14. McCarthy, T.L., Centrella, M., Canalis, E. (1988) J. Bone Min. Res. 3, 401-408.
15. Joyce, M.E., Terek, R.M., Jingushi, S., Bolander, M.E. (1990) Annal. N.Y. Acad. Sci. 593, 107-123.
16. Andrew, J.G., Hoyland, J., Andrew, S.M., Freemont, A.J., Marsh, D. (1993) Calcif. Tiss. Int. 52, 74-78.
17. Centrella, M., McCarthy, T.L., Canalis, E. (1988) FASEB J. 2, 3066-3073.
18. Wronski, T.J., Walsh, C.C., Ignaszewski, L.A. (1986) Bone 7, 119-123.
19. Ismail, F., Epsteen, S., Fallon, M.D., Thomas, S.B., Reinhardt, T.A. (1988) Endocrinology 122, 624-630.
20. Turner, R.T., Backup, P., Sherman, P.J., Hill, E., Evans, G.L., Spelsberg, T.C. (1992) Endocrinology 131, 883-889.
21. Finkleman, R.D., Bell, N.H., Strong, D.D., Demers, L.M., Baylink, D.J. (1992) Proc. Natl. Acad. Sci. 89, 12190-12193.