Journal of Cellular Biochemistry 44:81–91 (1990)

Gene Expression During Endochondral Bone Development: Evidence for Coordinate Expression of Transforming Growth Factor β_1 and Collagen Type I

Rita Bortell, Leesa M. Barone, Melissa S. Tassinari, Jane B. Lian, and Gary S. Stein

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Subcutaneous implantation of demineralized bone particles (DBP) into rats induces the formation of a bone ossicle by a tightly controlled sequence of chondro- and osteo-inductive events which are directly comparable to those which occur in normal endochondral bone development. Although the morphological and biochemical sequence associated with endochondral bone formation in this model has been well characterized, to date little information is available as to the gene regulation by which these events occur. To examine the expression of genes in this system, RNA was isolated from implants every 2 days over a time course spanning 3 to 19 days after implantation of DBP into rats. Cellular levels of mRNA transcripts of cell-growth-regulated and tissue-specific genes were examined by slot blot analysis and compared to the morphological changes occurring during formation of the ossicle. Analysis of the mRNA levels of histone H4 and c-myc, markers of proliferative activity, revealed several periods of actively proliferating cells, corresponding to 1) production of fibroprogenitor cells (day 3), 2) onset of bone formation (day 9), and 3) formation of bone marrow (day 19). The mRNA levels of collagen type II, a phenotypic marker of cartilage, peaked between days 7 and 9 post-implantation, corresponding to the appearance of chondrocytes in the implant, and rapidly declined on day 11 (to 5% of maximum value) when bone formation was observed. The peak mRNA levels of collagen type I, found in fibroblasts and osteoblasts, occurred first with the onset of bone formation (days 7–10) and again during formation of bone marrow (day 19). This study has demonstrated that the temporal patterns of mRNA expression of cartilage type II and bone type I collagens coincide with the morphological sequence in this model of endochondral bone formation. Further, the mRNA levels of transforming growth factor β_1 (TGFβ) were compared to those of collagen types I and II; a direct temporal correlation of TGFβ mRNA levels with that of collagen type I was found throughout the developmental time course. This observation of a tightly coupled relationship between TGFβ and type I collagen mRNA levels is consistent with a functional role for TGFβ in extracellular matrix production during in vivo bone formation.

Key words: TGFβ, extracellular matrix, slot blot analysis, DBP, RNA

Received April 25, 1990; accepted June 20, 1990.

The developmental sequence of endochondral bone formation has been well characterized both morphologically and at the biochemical level [1–3]. The series of developmental events which occur during endochondral bone formation in the skeleton include the appearance of chondrocytes, the production of a cartilage matrix, subsequent mineralization of the cartilage matrix, with coincident vascular invasion of this primary ossification site. This is followed by the appearance of osteoblasts which produce an osteoid matrix that mineralizes, leading to the formation of bone.

A convenient in vivo model to study endochondral bone formation is the DBP-induced bone implant system. In this system the subcutaneous implantation of demineralized bone particles (DBP) into rats induces a series of events which result in the production of an ectopic bone ossicle at the site of injection. Further, the sequence of chondro- and osteo-inductive events occurring in this bone particle implant is directly comparable to that which takes place in the skeleton [4].

Because bone is one of the major sources of TGFβ in the body and osteoblasts both synthesize TGFβ and possess high-affinity receptors for it, this polypeptide growth factor has been implicated as an important regulator of skeletal formation [5–8]. Indeed, numerous in vitro models of cartilage or bone suggest that TGFβ may play a regulatory role in these tissues in vivo. In cultured mesenchymal cells, for example, TGFβ has been shown to induce chondrogenesis [9], and the protein originally named cartilage-inducing factor A was found to be identical to TGFβ [10]. In other studies, osteoblasts either increased or decreased proliferation or alkaline phosphatase activity (a marker for the osteoblastic phenotype) in response to TGFβ [8,11–14]. These experiments were done in vitro, however, and the response observed was dependent on the concentration of TGFβ, the density and stage of differentiation of the cells, and the composition of the media.

In the present study we examined, in vivo, the expression of selected cell-growth-regulated and tissue-specific genes during cartilage and bone formation in the DBP implant model system. Based upon the cellular levels of growth-regulated mRNA's, we have identified three periods of increased proliferative activity during formation of the bone ossicle. Also, our results indicate that cellular levels of mRNA for essential extracellular matrix proteins (collagen type II in cartilage and collagen type I in bone) correlated with the morphological changes we observed, as well as with the known developmental sequence of the synthesis of these proteins in DBP implants [4,15,16]. Further, we examined the level of TGFβ mRNA in the DBP implants and related it to the expression of extracellular matrix genes. Our data showed a coupled temporal relationship between the mRNA levels of TGFβ and collagen type I, but not type II. This supports a functional role for TGFβ in extracellular matrix production during normal bone development.

METHODS

Preparation and Implantation of DBP

Bone powder was prepared from long bones of adult rats (8–10 weeks). The diaphyses of the long bones were cleaned, pulverized in liquid nitrogen in a Spex Mill (Metuchen, NJ), sieved to particle size of 75–250 μm, and then acid demineralized following the procedure described by Glowacki and Mulliken [17]. Fifty milligrams of DBP was implanted in bilateral subcutaneous pockets on the thoracic region in 28-day-old male Sprague Dawley CD strain rats (Charles River Breeding Lab,

Kingston, RI) as described previously [15]. Within 24 h the DBP becomes encapsulated, forming a discrete implant. To demonstrate specific stages of the endochondral bone sequence, the implants were removed from the rats over a time course spanning 3 to 19 days after implantation.

Histology

At the time of harvest, one of the implants from each rat was fixed in 2% paraformaldehyde and prepared for JB-4 plastic embedding (Polysciences, Warrington, PA). Serial sections (3 μm) were cut from each implant and stained with toluidine blue for matrix and cells or with 3% silver nitrate (Von Kossa) for mineral [18], and counterstained with hematoxylin and eosin.

RNA Isolation

Total cellular RNA was isolated from the bone implant material according to the method of Plumb et al. [19]. The implant was excised from the rat and immediately immersed in liquid nitrogen. The specimen was then pulverized under liquid nitrogen into a powder which was digested with 15 μg/ml proteinase K in 2 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2.5% sodium dodecyl sulfate (SDS) and 10 μg/ml polyvinyl sulfate. The lysate was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with an equal volume of chloroform, and the DNA and RNA were precipitated in ethanol.

DNA was removed by incubation with 100 μg/ml of proteinase K–treated DNase I in 10 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 10 mM $MgCl_2$, and 12 μg/ml polyvinyl sulfate for 30 min at 37°C. The lysate was re-extracted as above and the RNA was precipitated in ethanol. The RNA was resuspended in diethyl-pyrocarbonate-treated water and quantitated by absorbance at 260 nm, and its intactness was confirmed by electrophoretic fractionation on 6.6% formaldehyde-1% agarose gel and staining with ethidium bromide.

Slot Blot Hybridization Assay

RNA was incubated in 13.5% formaldehyde/7.5× SSC (0.65 M NaCl, 0.06 M trisodium citrate, pH 7.0) at 65°C for 15 min, adjusted with 15× SSC to a final volume of 300 μl, and vacuum blotted on Zetaprobe blotting membrane (BioRad, Richmond, CA) by using a slot-blot apparatus (Schleicher & Schuell, Keene, NH). The filters were baked in vacuo at 80°C for 1 h and prehybridized a minimum of 4 h at 42°C in 50% formamide, 5× SSC, 10× Denhardt's solution, 1% SDS, 50 mM sodium phosphate (pH 6.8), and 250 μg/ml of heat-denatured *E. coli* DNA. The cDNA or genomic probes were labelled with [^{32}P]-dCTP by the random primer method [20] and included: $TGF\beta_1$ (from R. Derynck, Genetech, San Francisco, CA), H4 histone [21], c-myc [22], and collagen types I and II (from D. Rowe, Univ. of Conn., Farmington, CN). Hybridizations were carried out for 18 h at 42°C in the same solution supplemented with 10% dextran sulfate and 1×10^6 cpm/ml of radioactive probe. The blots were washed for 30 min at room temperature with two changes of 2× SSC/0.1% SDS, followed by 30 min at 60°C with 2× SSC/0.1% SDS and 30 min at 60°C with 1× SSC/0.1% SDS. Blots probed with homologous probes were washed with a final wash of 0.2× SSC/0.1% SDS for 30 min at 60°C. The blots were exposed on pre-flashed Kodak X-AR5 film, and the resulting autoradiograms were quantitated by scanning densitometry.

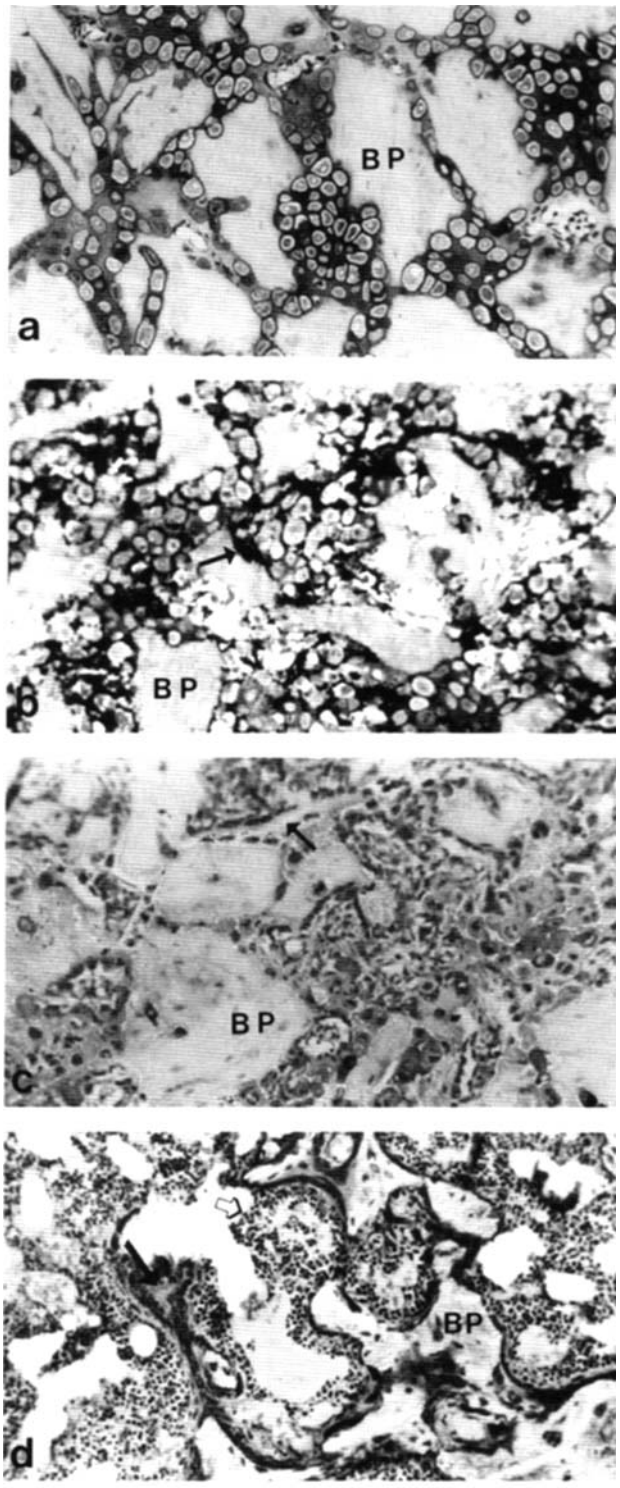

Figure 1.

RESULTS

Morphological Development in the DBP Implants

The time points in the present study were selected to illustrate the progressive stages of endochondral bone formation in the DBP implants. By day 3 the early stage of recruitment of fibroprogenitor cells around the particles of the implant was apparent. By day 7 chondroblasts were observed in the areas adjacent to the bone particles (Fig. 1a). On day 9, more of the implant had undergone chondrogenesis and the cartilage matrix had mineralized, as determined by Von Kossa staining (Fig. 1b). Bone formation, noted by the presence of osteoblasts and newly formed osteoid, was observed on day 11 (Fig. 1c). Completion of bone formation and the onset of the bone remodeling stage were observed by day 15, and marrow formation and hematopoeisis were evident at day 18 (Fig. 1d). Thus, the DBP implants exhibited the chondro- and osteogenic events which normally occur during endochondral bone formation.

mRNA Levels of Cell-Growth-Regulated Genes in the DBP Implants

Cellular levels of mRNAs transcribed from a cell-growth-regulated H4 histone gene were examined over the entire 19-day time course and showed a characteristic pattern (Fig. 2) in relation to the sequential periods of endochondral bone development as shown by this and other studies [4,16]. The variations in histone H4 mRNA levels, which are tightly coupled with DNA synthesis [19], suggested that there were three periods of enhanced proliferative activity initially occurring at day 3, the time when fibroprogenitor cells populate the implant. The second proliferative period peaked on day 9, the period of calcified cartilage formation and onset of osteogenesis. A third burst of proliferation occurred with the formation of bone marrow in the ossicles at day 19.

In a second independent experiment (n = 4 animals/time point), three periods of elevated histone H4 mRNA levels were again observed (Fig. 2). These periods coincided with those of the first experiment except that in the first period there is an apparent 2-day lag before histone H4 mRNA levels drop. This is probably a reflection of the more heterogeneous population of cells present during the earliest stages of endochondral bone formation in this system.

The pattern of expression of the c-myc proto-oncogene, which is characteristic of proliferating cells [23], closely paralleled that of histone H4 for the time points examined (Fig. 3). As with histone H4, c-myc mRNA levels were highest during the period of calcified cartilage formation and onset of osteogenesis (day 9) and lowest during the period of bone remodeling (day 15). Subsequent hybridization of the same blots with a 28S ribosomal gene probe indicated less than 15% variation in RNA levels throughout the time course, indicating that the hybridization signals observed on the autoradiograms accurately reflected cellular levels of RNA transcribed from the genes.

Fig. 1. Histologic sections of DBP implants at selected time points. All sections were stained with toluidine blue. × 100. **a:** Day 7—chondrogenesis: bone particles (BP) surrounded by newly formed chondrocytes. **b:** Day 9—calcified cartilage: black areas (arrow) show areas of mineralization after staining silver nitrate. **c:** Day 11—bone formation: arrow indicates area of osteoblasts and newly formed osteoid. **d:** Day 18—new bone (black arrow) is surrounded by marrow cells (open arrow).

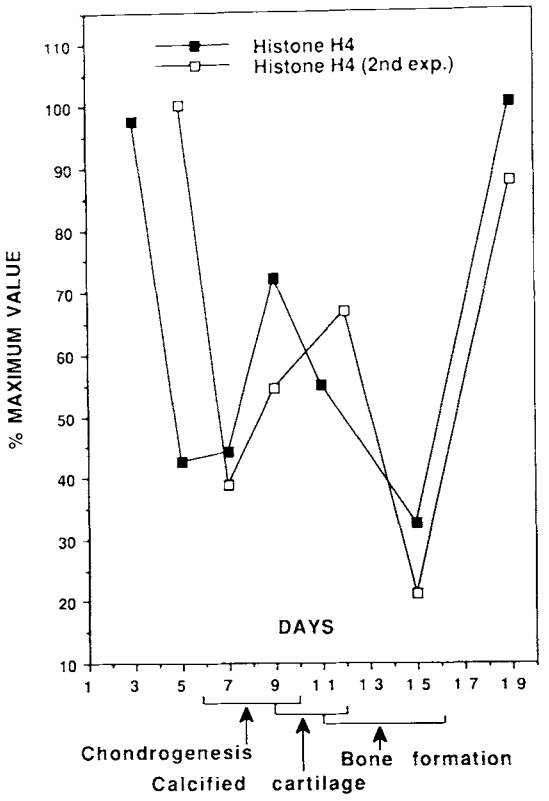

Fig. 2. Time course of the expression of histone H4 mRNA in the DBP implants. Day 0 is the time at which DBP was implanted subcutaneously into rats. Results are the mean of the data from 3 animals/time point. A second independent experiment is also shown in which each point represents the mean of data from 4 animals/time point. The time of appearance of various cell types as determined histologically is shown.

mRNA Levels of Extracellular Matrix Genes in the DBP Implants and Correlation to Morphology

The mRNA levels of cartilage-specific type II collagen (Fig. 4) were elevated at days 7–9 post-implantation, corresponding to the chondrogenic period defined by morphology (Fig. 1a,b) and biochemistry [4]. By day 11, however, the mRNA levels had decreased to $<5\%$ of the peak value and thereafter remained low. In contrast, the mRNA levels of type I collagen (Figs. 4, 5), in fibroblasts and osteoblasts, were high in the early stages of the implant and peaked at day 9 during the onset of the osteogenic period. The mRNA levels then decreased through day 15, the period of bone remodeling, and subsequently increased $\sim$2-fold at day 19 during marrow development.

mRNA Levels of TGFβ in the DBP Implants and Correlation to Extracellular Matrix Genes

When TGFβ mRNA levels were correlated with those of the extracellular matrix genes, the striking observation was that TGFβ mRNA levels closely paralleled

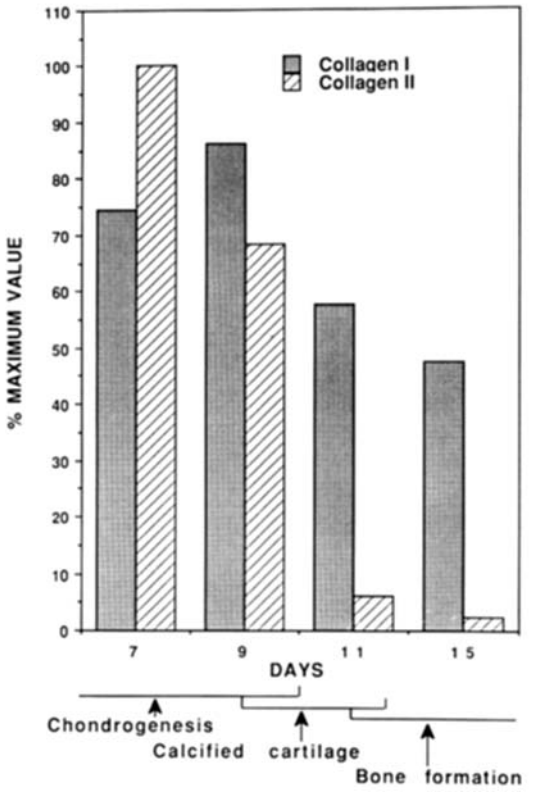

Fig. 3. Time course of the expression of histone H4 and c-myc mRNA's in the DBP implants. Day 0 is the time at which DBP was implanted subcutaneously into rats. Results are the mean of the data from 3 animals/time point.

those for collagen type I throughout the 3–19-day time course (Fig. 5). Although elevated levels of TGFβ mRNA were observed corresponding to the maximum level (day 7) of collagen type II mRNA (Fig. 4), the collagen type II mRNA declined rapidly by the onset of osteogenesis (day 11), whereas TGFβ mRNA remained elevated. Thus, mRNA levels of TGFβ correlated more closely with that of collagen type I, which was elevated initially during late chondrogenesis as calcified cartilage formation occurred, and remained elevated during osteogenesis (day 10). Then, TGFβ and collagen type I mRNA levels decreased together during the period of ossicle remodeling (day 15), and a subsequent increase of both mRNA levels was observed with the selective enrichment of type I collagen gene expression during marrow development (day 19).

DISCUSSION

The DBP implant system used for this study has the advantage that it is an in vivo post-natal model of endochondral bone development in which the implants are able to respond to the physiology of the rat. In addition, the implants provide samples of specific stages of bone formation, from undifferentiated cells to cartilage induction

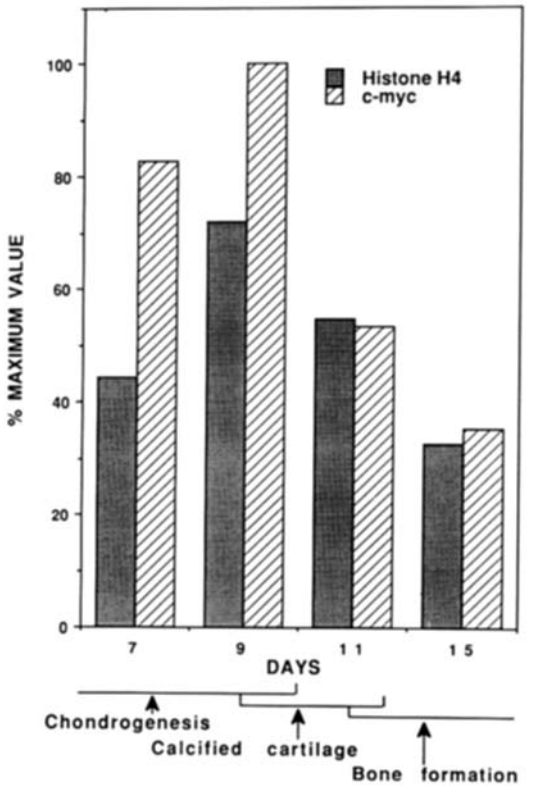

Fig. 4. Time course of the expression of collagen types I and II mRNA's in the DBP implants. Day 0 is the time at which DBP was implanted subcutaneously into rats. Results are the mean of the data from 3 animals/time point. The time points were selected to encompass the histologically determined transition from cartilage to bone.

and the subsequent calcification and bone formation and remodeling stages. In this study, we examined endochondral bone development at the molecular level by characterizing the expression of various cell-growth-regulated and tissue-specific genes in the DBP implants.

The levels of cell-growth-regulated histone H4 mRNAs suggested that there were three periods of enhanced proliferative activity in endochondral bone development. These periods corresponded to 1) the recruitment and proliferation of fibroprogenitor cells, 2) the induction and proliferation of chondroblasts, followed by osteoblast induction and proliferation, and 3) an additional proliferative period during marrow development and hematopoeisis.

In this study, we also determined the expression of tissue-specific collagen types I and II during endochondral bone formation. Collagens comprise the major extracellular matrix proteins of cartilage and bone (40 and 90%, respectively), and the extracellular matrix provides the functional integrity of the skeleton [24]. Our data showed that mRNA levels for the cartilage-specific collagen type II were elevated coincident with the active period of chondrogenesis. Type II mRNA levels subsequently dropped to less than 5% of the peak value when chondrogenesis had ended and bone formation

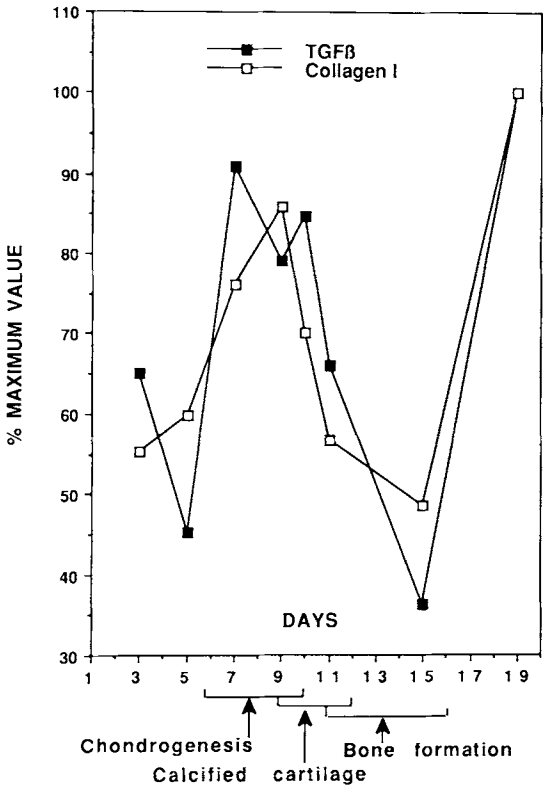

Fig. 5. Time course of the expression of TGFβ and collagen type I mRNA's in the DBP implants. Day 0 is the time at which DBP was implanted subcutaneously into rats. Results are the mean of the data from 3 animals/time point. The time of appearance of various cell types as determined histologically is shown.

had begun. In contrast, mRNA levels for collagen type I, the major protein produced by the osteoblast, were elevated during the transition from calcified cartilage to the formation of bone matrix by osteoblasts. A subsequent increase in collagen type I mRNA during later stages reflects active bone remodeling, where new bone formation by osteoblasts is replacing the bone resorbed by osteoclasts. Thus, the expression of collagen type I and II mRNAs showed distinct temporal patterns which correlated with the morphological events we observed, as well as those described previously for the endochondral bone sequence in the DBP implant model [16].

Because TGFβ is known to stimulate synthesis of extracellular matrix components in a number of phenotypically diverse systems [25–28], we examined the expression of TGFβ mRNA in the implants and compared it with that of the collagens. Our data revealed a direct temporal correlation of relative type I collagen mRNA levels, but not type II, with that of TGFβ. This observation that TGFβ expression paralleled collagen type I expression suggests that TGFβ may be involved in the regulation of production of a specific tissue extracellular matrix during DBP-induced endochondral bone formation. In support of this, numerous in vitro studies have shown increased collagen type I production in response to TGFβ. For example, in fibroblast and myoblast cell lines, the addition of exogenous TGFβ resulted in both

an increased mRNA and protein level of collagen type I [26,29,30]. In fetal rat calvarial bone cells, TGFβ has been shown to affect the production of matrix by stimulating the production of collagen (primarily type I) [31].

TGFβ may directly influence the level of collagen synthesis by mediating transcription of the gene. This has been observed in fibroblastic cells treated with TGFβ which exhibit an increase in steady-state levels of type I collagen that appears to occur at the transcriptional level by stimulation of the alpha 2(I) collagen promoter. This activation is mediated by a specific sequence in the promoter that is a binding site for nuclear factor 1 (NF1) [32]. Indeed, one of the earliest genomic responses to TGFβ appears to involve the enhanced gene expression of the transcription factors junB and c-jun [33]. Alternatively, TGFβ and collagen type I gene expression may be coordinately regulated by another, as yet unknown factor.

In summary, our results show that endochondral bone formation in the DBP implants was associated with three periods of proliferative activity. Further, the expression of the collagen genes for types I and II correlated well with the morphological changes we observed in the implants. Our finding that endogenous TGFβ mRNA levels were elevated at times corresponding to the onset of osteogenesis is supported by the recent work of Carrington et al. [34], who examined TGFβ mRNA and protein levels in this same model system. Their data showed a large increase in TGFβ protein during the onset of osteogenesis, but this observation was not correlated with the expression of other components of bone formation. Our finding that TGFβ mRNA expression shows a direct temporal correlation with type I collagen suggests a tightly controlled functional coupling of these two genes at the mRNA level. Because type I collagen is the major organic component of bone, it may be that TGFβ regulates the formation of the extracellular matrix by regulating the production or deposition of type I collagen. This suggests a role for TGFβ in the osteogenic phase of endochondral bone development in vivo.

ACKNOWLEDGMENTS

The authors would like to thank Drs. Steven Dworetzky and Vicki Shalhoub for critically reviewing the manuscript.

These studies have been supported by grants from the National Institutes of Health (GM32010, AR33920, AR35166, AR39588), The March of Dimes Birth Defects Foundation, The Northeast Osteogenesis Imperfecta Society, and The International Life Sciences Institute.

REFERENCES

1. Fell H: J Morphol Physiol 40:417–459, 1925.
2. Urist M: Science 150:893–899, 1967.
3. Holder N: J Embryol Exp Morphol 44:15–29, 1978.
4. Reddi A: Coll Res 1:20, 1981.
5. Centrella M, Canalis E: Proc Natl Acad Sci USA 82:7335–7339, 1985.
6. Hauschka P, Mavrakos A, Iafrati M, Doleman S, Klagsbrun M: J Biol Chem 261:12665–12674, 1986.
7. Pfeilschifter J, Mundy G: Proc Natl Acad Sci USA 84:2024–2028, 1987.
8. Robey P, Young M, Flanders K, Roche N, Kondaiah P, Reddi A, Termine J, Sporn M, Roberts A: J Cell Biol 105:457–463, 1987.
9. Seyedin S, Thomas T, Thompson A, Rosen D, Piez K: Proc Natl Acad Sci USA 82:2267–2271, 1985.

10. Seyedin S, Thompson A, Bentz H, Rosen D, McPherson J, Conti A, Siegel N, Galluppi G, Piez K: J Biol Chem 261:5693–5695, 1986.
11. Ibbotson K, D'Souza S, Mundy G: J Cell Biochem [Suppl] 10B:30, 1986.
12. Noda M, Rodan G: Biochem Biophys Res Commun 140:56–65, 1986.
13. Centrella M, McCarthy T, Canalis E: J Biol Chem 262:2869–2874, 1987.
14. Rosen D, Stempien S, Thompson A, Seyedin S: J Cell Physiol 134:337–346, 1988.
15. Reddi A, Huggins C: Proc Natl Acad Sci USA 69:1601–1605, 1972.
16. Reddi A, Gay R, Gay S, Miller E: Proc Natl Acad Sci USA 74:5589–5592, 1977.
17. Glowacki J, Mulliken J: Clin Plast Surg 12:233–241, 1985.
18. Clark G: In Clark G (ed): "Staining Procedures, 4th Edition." Baltimore, MD: Williams and Wilkins, 1981, p 187.
19. Plumb M, Stein J, Stein G: Nucleic Acids Res 11:2391–2410, 1983.
20. Feinberg A, Vogelstein B: Anal Biochem 132:6–13, 1983.
21. Grimes S, Weisz-Carrington P, Daum H III, Smith J, Green L, Wright K, Stein G, Stein J: Exp Cell Res 173:534–545, 1987.
22. Toscani A, Soprano D, Soprano K: Oncogene Res 3:223–238, 1988.
23. Muller R, Bravo R, Burckhardt J, Curran T: Nature 312:716–720, 1984.
24. Glimcher M: In Greep RO, Astwood EB (eds): "Handbook of Physiology: Endocrinology," Vol. VII. Washington, DC: American Physiological Society, 1976, pp 25–116.
25. Fine A, Goldstein R: J Biol Chem 262:3897–3902, 1987.
26. Ignotz R, Massague J: J Biol Chem 261:4337–4345, 1986.
27. Roberts A, Sporn M, Assoian R, Smith J, Roche N, Wakefield L, Heine U, Liotta L, Falanga V, Kehrl J, Fauci A: Proc Natl Acad Sci USA 83:4167–4171, 1986.
28. Thompson K, Assoian R, Rosner M: J Biol Chem 263:19519–19524, 1988.
29. Ignotz R, Endo T, Massague J: J Biol Chem 262:6443–6446, 1987.
30. Keski-Oja J, Raghow R, Sawdey M, Loskutoff D, Postlethwaite A, Kang A, Moses H: J Biol Chem 263:3111–3115, 1988.
31. Wrana J, Maeno M, Hawrylyshyn B, Yao K, Domenicucci C, Sodek J: J Cell Biol 106:915–924, 1988.
32. Rossi P, Karsenty G, Roberts A, Roche N, Sporn M, de Crombrugghe B: Cell 52:405–414, 1988.
33. Pertovaara L, Sistonen L, Bos T, Vogt P, Keski-oja J, Alitalo K: Mol Cell Biol 9:1255–1262, 1989.
34. Carrington J, Roberts A, Flanders K, Roche N, Reddi A: J Cell Biol 107:1969–1975, 1988.