Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 56-65

TYPE-β TRANSFORMING GROWTH FACTOR INHIBITS PROLIFERATION AND EXPRESSION OF ALKALINE PHOSPHATASE IN MURINE OSTEOBLAST-LIKE CELLS

Masaki Noda and Gideon A. Rodan

Merck Sharp & Dohme Research Laboratories
Department of Bone Biology and Osteoporosis
West Point, Pennsylvania 19486

Received August 18, 1986

Summary. TGF-β modulates growth and differentiation in many cell types. MC3T3E1 is a clonal non-transformed murine bone cell line which differentiates in culture. We tested the effect of porcine TGF-β on the proliferation and differentiation of MC3T3E1 cells in monolayer cultures by following cell number, and alkaline phosphatase activity. TGF-β treatment (2 ng/ml) altered the shape of MC3T3E1 cells from cuboidal to elongated/spindle-shape. TGF-β inhibited the growth of MC3T3E1 by up to 40% ($P<0.02$) in a dose-dependent manner with half maximal inhibition at 1 ng/ml. Growth inhibition depended on serum concentration, maximal inhibition occuring at 2% serum. Expression of alkaline phosphatase, which peaks in vitro when the cells reach confluence, was strongly inhibited by TGF-β, in a dose-dependent manner with half maximal inhibition at around 0.05 ng/ml and complete inhibition at 2 ng/ml. Alkaline phosphatase inhibition was irreversible after 24 hours exposure to TGF-β.

Transforming growth factor β (TGF-β) is a 25,000 dalton homodimer (1) which stimulates anchorage independent growth (2,3) and modulates growth and differentiation in various cell types (4,5,6,7). TGF-β is isolated from platelets (8), kidney (9) and placenta (10), is very abundant in bone (200 μg/kg tissue) (11,12) and is probably made by osteoblasts (13-15). TGF-β induces bone resorption in cultured mouse or rat calvaria (16,17) and increases the level of alkaline phosphatase (AP) in rat osteosarcoma cells (17). In cells from neonatal rat muscle, TGF-β stimulates chondrogenic differentiation in vitro, in the same manner as extracts from bone matrix (18). Indeed, partial amino acid sequence of the purified extract is identical to TGF-β (19). To further study the potential role of TGF-β as an autocrine regulator of bone metabolism we examined its effect on morphology, proliferation and alkaline phosphatase expression in the clonal murine

0006-291X/86 $1.50

calvaria-derived cell line MC3T3E1, which shows differentiation and mineralization in vitro (20).

Materials and Methods

TGF-β was purchased from R&D Systems, Inc. (Minneapolis, MN). It was obtained from procine platelets and is reported to be 96% pure. The sample of TGF-β used in this study stimulated anchorage independent growth in normal rat kidney cells NRK-49F. Alpha(α)-MEM and kanamycin sulfate solution are from GIBCO Grand Island, NY, fetal bovine serum (FBS) from KC Biological, Laneza, KS.

MC3T3E1 cells were kindly provided by Dr. Kodama and were maintained in 5% CO_2 at 37°C. Cells were subcultured every three days at 2,000 cells/cm^2 in a α-MEM medium with 10% FBS and 60 μg/ml kanamycin sulfate. For experiments, cells were plated at 2,000 cells/cm^2 and were grown for 24 hours in a α-MEM with 10% FBS. Cells were then cultured in fresh media with TGF-β. All control cells were cultured with vehicle (0.1% v/v, 4mM HCl). Unless indicated otherwise media contained 10% FBS. In experiments where serum concentration was varied, cells were rinsed three times with serum free α-MEM before the addition of media with TGF-β or vehicle.

At given time points, cells were trypsinized and counted by Coulter Counter (Coulter Electronics Inc., FL). Morphological observations were carried out with a phase contrast microscope (Nikon, Japan).

At indicated times, medium was removed and the cells were rinsed with Ca^{2+}-Mg^{2+}-free Hank's solution and then scraped into 10mM Tris HCl pH 7.5, 0.5 mM $MgCl_2$, 0.1% Triton X-100, and frozen. Alkaline phosphatase activity was measured in thawed and sonicated samples as described (21). Protein was measured in the same sample by Coumassie blue binding using bovine serum albumin as standards (22). Statistical significance was estimated by Student's t-test.

Results

At low cell density the osteogenic MC3T3E1 cells display a fibroblastic morphology, which is similar to that of lipogenic and myogenic cells of the same origin (23); near confluence, the osteogenic cells become cuboidal and have a cobblestone-like arrangement (20). Forty eight hours after TGF-β addition at (2 ng/ml) these cells became elongated, and spindle shaped (Figure 1) at all serum concentrations tested (0.2,2,10%). In serum free cultures, TGF-β caused cell rounding while control cells maintained their processes.

TGF-β, at 2 ng/ml, inhibited the growth of MC3T3E1 cells by 18% ($p<0.01$) at 3 days and 40% ($p<0.02$) at 6 days, relative to corresponding controls, in the presence of 10% FBS. This effect was dose dependent: the EC_{50} estimated 6 days after initiation of treatment was 1.0 ng/ml (Figure 2).

Since TGF-β stimulation of NRK cell growth in soft agar requires the presence of EGF we examined the effect of serum concentration on the action

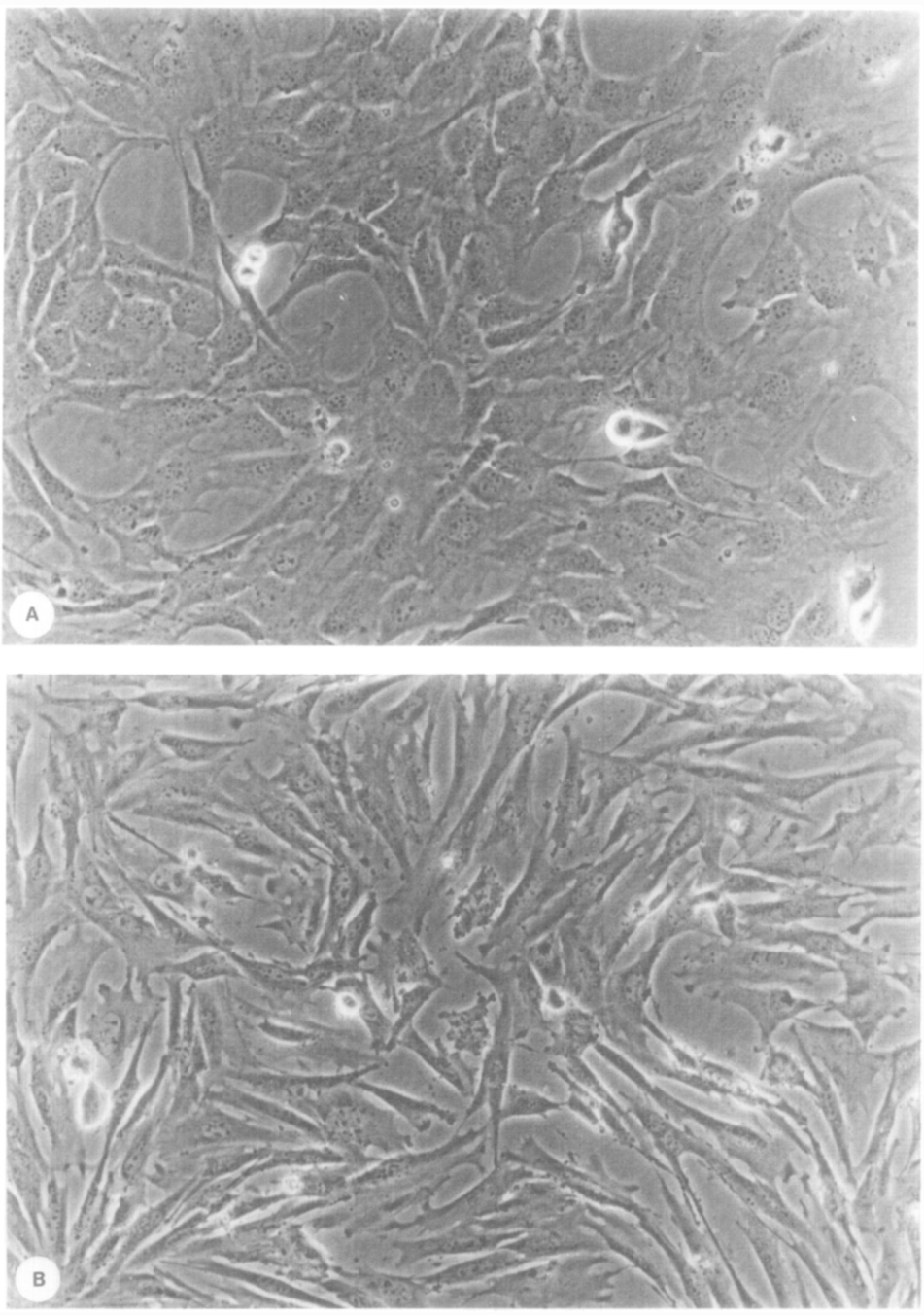

Figure 1. Effect of TGF-β on the morphology of MC3T3E1 cells. Cells were seeded at 2,000 cells/cm in 2 cm wells in α-MEM supplemented with 10% FBS. After 24 hours, media were replaced with fresh medium with or without TGF-β. The phase contrast micrographs were taken on day 4 of culture. (A) Control cells. (B) Cells treated with TGF-β 2 ng/ml.

of TGF-β (2 ng/ml). Cell growth and growth inhibition by TGF-β were dependent on serum concentration (Figure 3A). No inhibition was observed at

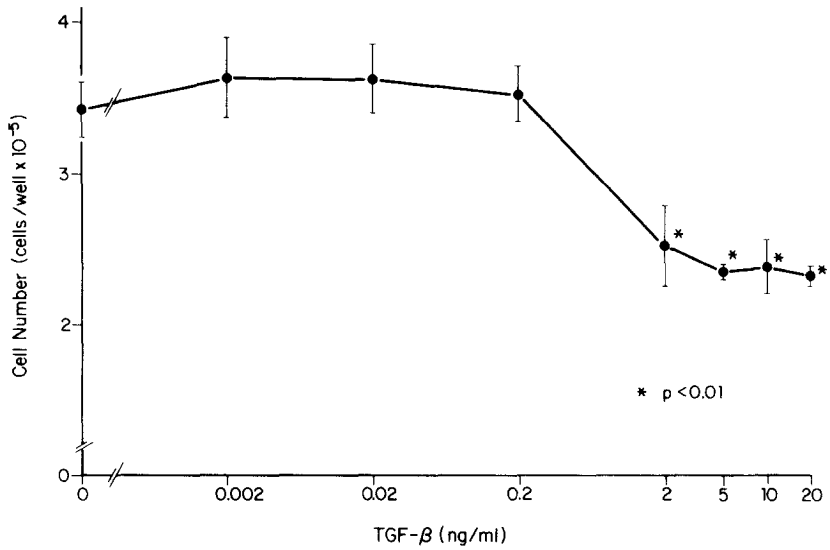

Figure 2. Dose-response relationship of the effect of TGF-β on growth of MC3T3E1 cells. Cells were cultured in media with 10% FBS as described in legend to Figure 1. Cells were trypsinized and were counted on day 7 by Coulter Counter. Data are mean values (●) from four samples ± standard deviations. This is one of two similar experiments.

0 and 0.2% serum, although the cell number increased three and ten fold respectively. Maximal inhibition, 47% ($p<0.01$), was observed at 2% serum, at 10% serum inhibition was 24% ($p<0.01$).

Alkaline phosphatase (AP) is one of the phenotypic markers of osteoblasts. AP levels rose in MC3T3E1 cells seeded at 2,000 cells/cm^2 from below 0.05 μmole/min/mg protein to 0.99±0.25 μmole/min/mg protein after 11 days in culture, when the cells reached confluence. TGF-β added one day after seeding (2ng/ml in 10% FBS) inhibited the expression of AP by 83% ($p<0.05$) (Figure 4), completely preventing the rise in AP observed in control cultures. The TGF-β effect was dose dependent (Figure 5) with half maximal inhibition around 0.05 ng/ml and complete inhibition at 2 ng/ml. The rise in AP in control cultures depended on serum concentration. TGF-β (2ng/ml) inhibition was only seen when AP levels rose considerably at 2% and 10% FBS (85% inhibition, $p<0.01$, and 73%, $p<0.05$, respectively, figure 3B). To test the time of exposure required for producing the TGF-β effect, cells were cultured with TGF-β (2ng/ml) for 24 or 72 hours and, after rinsing

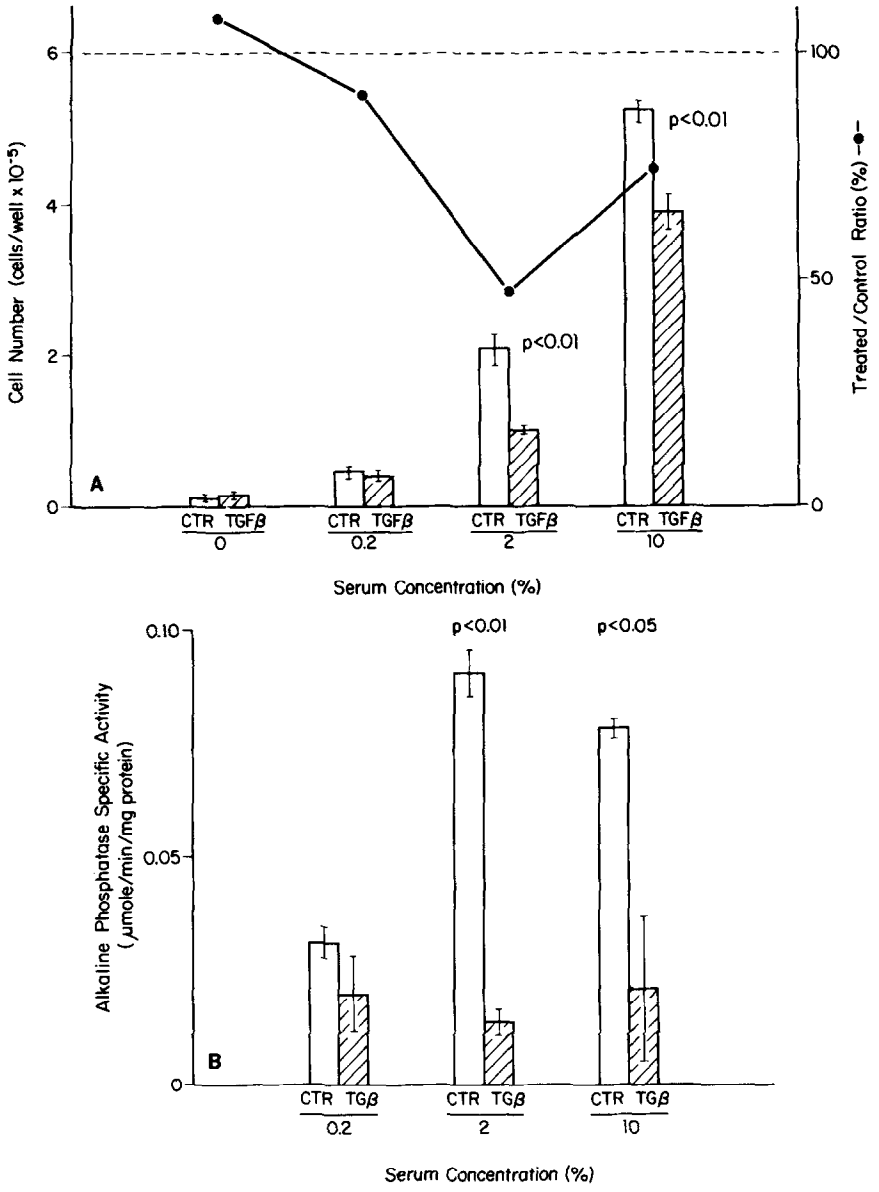

Figure 3. Effect of serum concentration on TGF-β inhibition of (A) growth and (B) AP in MC3T3E1 cells. Cells were cultured as described in legend to Figure 1 at the indicated serum concentrations. Cells were trypsinized and counted on day 7. AP was assayed on day 11. Open bars are mean values from two to four samples of cells cultured with vehicle and hatched bars are from cells treated with TGF-β (2ng/ml) ± standard deviations.

three times, the culture was continued to confluence in the absence of TGF-β. This treatment inhibited AP by about 50% ($p<0.01$) (Figure 6A) but had no effect on cell number (Figure 6B).

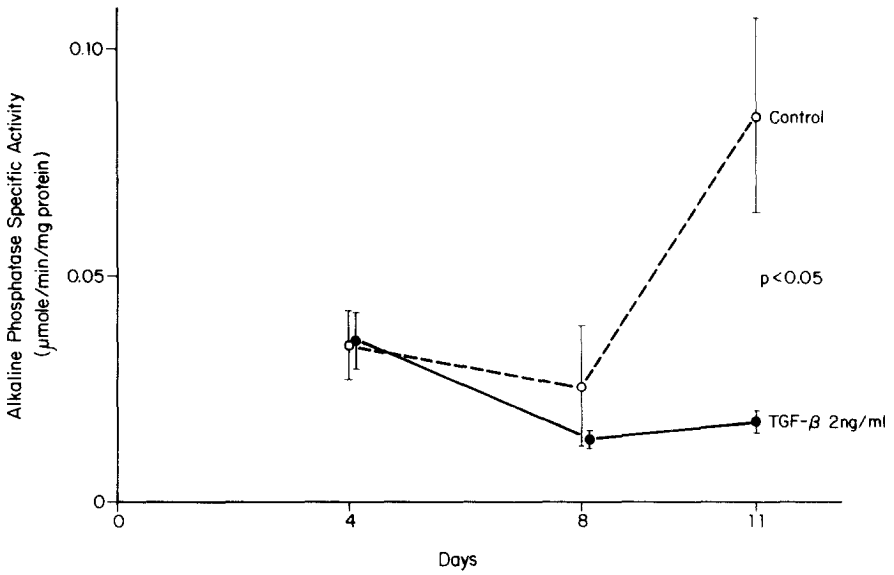

Figure 4. Time course of the TGF-β effect on the expression of alkaline phosphatase in MC3T3E1 cells. Cells were cultured in media with 10% FBS as described in legend to Figure 1. AP specific activity was measured as described in Methods. (O) Controls cultured with vehicle. (●) Cells treated with TGF-β (2ng/ml). Data are mean values from two to four samples ± standard deviations from one of two similar experiments.

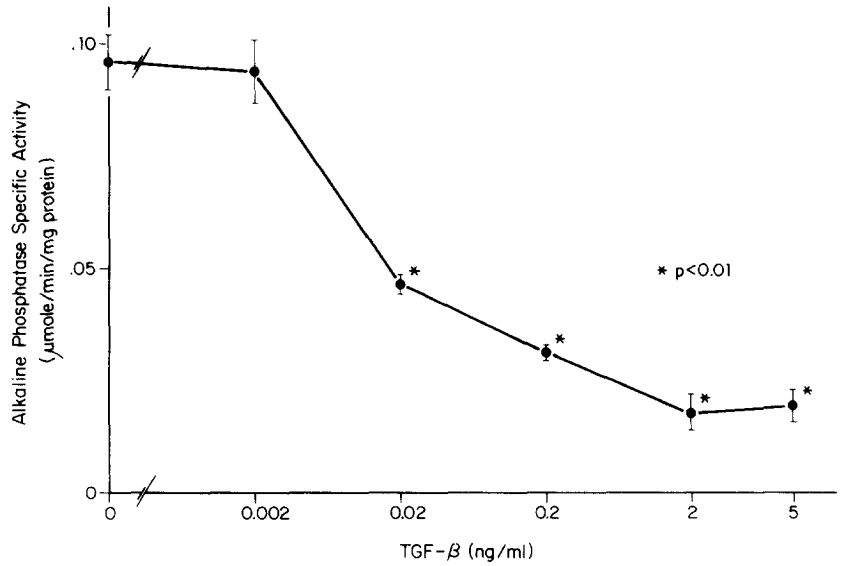

Figure 5. Dose response relationship of TGF-β effect on alkaline phosphatase in MC3T3E1 cells. Cells were cultured in media with 10% FBS as described in legend to Figure 1. AP specific activity (●) was measured on day 11. Data are mean values from two to four samples ± standard deviations from one of two similar experiments.

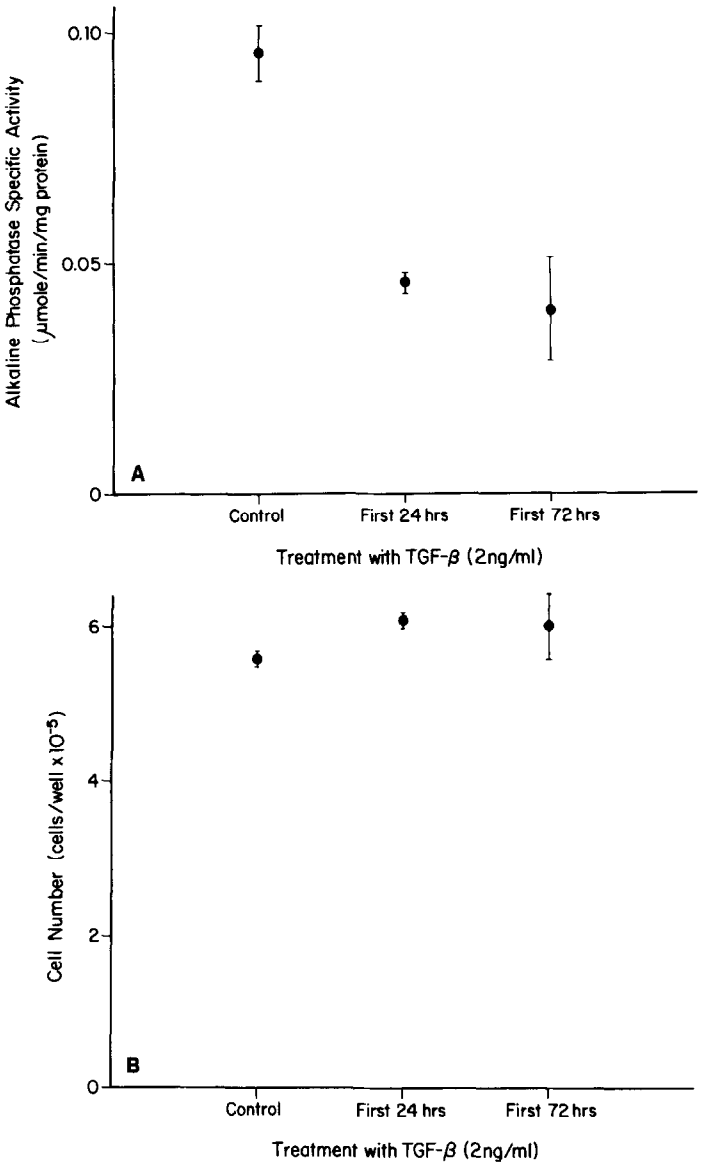

Figure 6. Effect of transient esposure to TGF-β on the expression of (A) alkaline phosphatase and (B) growth in MC3T3E1 cells. Cells were seeded as described in legend to Figure 1. After 24 or 72 hours treatment with TGF-β (2ng/ml) in the presence of 10% FBS, cells were cultured in fresh media without TGF-β. AP activity was measured at day 11 and cells were counted at day 7. Data are mean values from two to four samples ± standard deviations from one of two similar experiments. Experiments were repeated twice.

Discussion

TGF-β induces shape changes, inhibits growth and suppresses alkaline phosphatase expression in MC3T3E1 cells. Should this occur *in vivo* TGF-β may inhibit bone formation in addition to its stimulation of bone resorption

(16). However, the action of TGF-β in bone may be more complicated since it was shown to stimulate DNA synthesis in fetal rat calvaria organ cultures and in isolated calvaria cells, as well as in the osteoblastic osteosarcoma cells UMR-106, seeded at low density (24). On the other hand, TGF-β at higher concentrations (10-30 ng/ml) inhibited DNA synthesis in the same cells (24). We have found that TGF-β also inhibited growth in the ROS 17/2.8 osteoblastic rat osteosarcoma cells (data not shown) along with stimulation of alkaline phosphatase (13,17). TFG-β inhibition of growth and bifunctional effects on cell growth have been observed in many other cells (3,4,7,25). This may be a general feature of this factor possibly due to the dependence of TGF-β action on other growth factors, such as EGF, and on the state of the responsive cells (4). The dependence of the effects of TGF-β on serum concentration, reported here, is consistent with this notion, which dictates caution in extrapolating in vitro growth control effects to the in vivo situation.

TGF-β inhibition of alkaline phosphatase, a marker of osteoblastic differentiation in these cells, was very pronounced and is analogous to the suppression of adipocytic differentiation in 3T3-L1 cells (5). Interestingly, a 24-hour exposure to TGF-β during the early growth phase, was sufficient to completely prevent the rise in AP seen at confluence, but had no detectable effect on cell growth. These findings are consistent with the prevention of 3T3-L1 cell-differentiation by a short (4 hr) exposure to TGF-β prior to the commitment point (5) and with the reversible inhibition of proliferation observed in prokeratinocytes (26).

In contrast TGF-β increased alkaline phosphatase levels in the rat ROS 17/2.8 cells (13,17). It is not clear if the difference between TGF-β effects on MC3T3E1 cells and ROS cells is due to species differences (mouse vs. rat), to properties associated with the malignant nature of the ROS 17/2.8 cells, to the state of differentiation of the two cell populations when exposed to TFG-β or to the presence of other modulating factors. It should be noted that MC3T3E1 cells exhibit a much larger change in AP

expression between the growing phase, when AP levels are very low, and confluence, when they increase considerably (27).

In conclusion, TGF-β which is abundant in bone and promotes the differentiation of muscle derived mesenchymal cells into cartilage (18) inhibits the proliferation and suppresses the differentiation of osteogenic MC3T3E1 mouse cells. This factor may thus play a regulatory role in bone development, but its site and time of action and ultimate physiological effect remain to be determined.

References

1. Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., and Goeddel, D.V. (1985) Nature 316, 701-705.
2. Roberts, A.B., Frolik, C.A., Anzano, M.A., and Sporn, M.B. (1983) Fed. Proc. 42, 2621-2625.
3. Sporn, M.B., and Roberts, A.B. (1985) Nature 313, 745-747.
4. Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., and Stern, D.F. (1985) Proc. Natl. Acad. Sci. 82, 119-123.
5. Ignotz, R.A., and Massague, J. (1985) Proc. Natl. Acad. Sci. 82, 8530-8534.
6. Masui, T., Wakefield, L.M., Lechner, J.F., LaVeck, M.A., Sporn, M.B., and Harris, C.C. (1986) Proc. Natl. Acad. Sci. 83, 2438-2442.
7. Sporn, M.B., Roberts, A.B., Wakefield, L.M., and Assoian, R.K. (1986) Science 223, 532-534.
8. Assoian, R.K., Komoriya, A., Myers, C.A., Miller, D.M., Sporn, M.B. (1983) J. Biol. Chem. 258, 7155-7160.
9. Roberts, A.B., Anzano M.A., Meyers, C.A., Wideman, J., Blacher, R., Pan, Y-C.E., Stein, S., Lehrman, S.R., Smith, J.M., Lamb, L.C., and Sporn, M.B. (1983) Biochem. 22, 5692-5698.
10. Frolik, C.A., Dart, L.L., Meyers, C.A., Smith, J.M., and Sporn, M.B. (1983) Proc. Natl. Acad. Sci. 80, 3676-3680.
11. Seyedin, S.M., Thomas, T.C., Thompson, A.Y., Rosen, D.M., and Piez, K.A. (1985) Proc. Natl. Acad. Sci. 82, 2267-2271.
12. Seyedin, S.M., Thompson, A.Y., Bentz, H., Rosen, D.M., and Piez, K.A. (1986) J. Cell. Biochem. Suppl. 10B, 105.
13. Harrod, J., Hill, D.J., and Russel, R.G.G. (1985) Calcif. Tiss. Int. 38, Suppl. S16.
14. Centrella, M., and Canalis, E. (1985) Proc. Natl. Acad. Sci. 82, 7335-7339.
15. Young, M.F., Robey, P.G., Reddi, A.H., Roberts, A.B., Sporn, M.B., and Termine, J.D. (1986) J. Bone Mineral Res. 1, Supp. 1, Abstr. 8th Ann. Meet. Am. Soc. Bone Mineral Res.
16. Tashjian, A.H.Jr., Voelkel, E.F., Lazzaro, M., Singer, F.R., Roberts, A.B., Derynck, R., Winkler, M.E., and Levine, L. (1985) Proc. Natl. Acad. Sci. 82, 4535-4538.
17. Ibbotson, K.J., D'Souza, S.M., and Mundy, G.R. (1986) J. Cell Biochem. Suppl. 10B, 108.
18. Poser, J.W., Coppinger, W.J., and Lucas, D.S. (1985) Calcif. Tiss. Int. 38, Suppl. S21.
19. Seyedin, S.M., Thompson, A.Y., Bentz, H., Rosen, D.M., McPherson, J.M., Conti, A., Siegel, N.R., Galluppi, G.R., and Piez, K.A. (1986) J. Biol. Chem. 261, 5693-5695.

20. Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., and Kasai, S. (1983) J. Cell Biol. 96, 191-198.
21. Majeska, R.J., and Rodan, G. A. (1982) Calcif. Tissue Int. 34, 59-66.
22. Specter, T. (1978) Anal. Biochem. 86, 142-146.
23. Kodama, H., Amagai, Y., Koyama, H., and Kosai, S. (1982) J. Cell. Physiol. 112, 83-88.
24. Centrella, M., McCarthy, T., and Canalis, E. (1986) J. Bone Mineral Res. 1, Suppl. 1, Abstr. 9th Ann. Meet. Am. Soc. Bone Mineral Res.
25. Wang, J.L., and Hsu, Y-M. (1986) Trends Biochem. Sci. 11(1), 24-26.
26. Shipley, G.D., Pittelkow, M.R., Wille, J.J., Scott, R.E., and Moses, H.L. (1986) Canc. Res. 46, 2068-2071.
27. Kodama, H., Amagai, Y., Sudo, H., Kasai, S., and Kamamoto, S. (1981) Jpn. J. Oral. Biol. 23, 899-901.