Vol. 94, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1980 Pages 373-380

GLUCOCORTICOID RECEPTOR IN CLONAL OSTEOSARCOMA CELL LINES: A NOVEL SYSTEM FOR INVESTIGATING BONE ACTIVE HORMONES

M.R. Haussler, S.C. Manolagas and L.J. Deftos

Endocrine Section, San Diego Veterans Administration Medical Center and University of California, La Jolla, California 92161

Received April 2,1980

Summary: Cytosol from cultured osteogenic sarcoma cells contains a high affinity(K_d=1.4x10^{-9}M) triamcinolone(TRM) binding component which adsorbs to DNA-cellulose. The TRM binding molecule sediments at 4S in 0.3M KCl-sucrose gradients, but appears as a 6-7S species when sodium molybdate is present. The concentration of TRM binder is 100fmol/mg cytosol protein (~60,000 receptors/cell) and it displays the classical hierarchy of glucocorticoid potency in competition experiments. Thus, osteogenic sarcoma possesses a typical glucocorticoid receptor. This osteoblast-like malignant bone cell should serve as a model for studying the actions and interactions of glucocorticoids, 1,25-dihydroxyvitamin D and other bone active hormones.

Osteosarcomas are highly malignant tumors that rapidly erode bone but are locally restricted by epiphyseal and articular cartilage. The growth and differentiation of these neoplasms are modified by certain hormones and glucocorticoids have been shown to elicit ultrastructural changes in cultured osteogenic sarcoma (OS) cells (1). These changes include a marked increase in cell size as well as augmented nuclear and mitochondrial volumes, thus resembling the effects of glucocorticoids on thymus cells (2). To date, specific glucocorticoid receptors have not been reported in OS tumor cells, although such information would be significant not only to characterizing the actions of glucocorticoids in these tumor cells, but because of the potential therapeutic implications of understanding glucocorticoid mediation of osteosarcoma growth and/or differentiation.

Moreover, osteogenic sarcoma cell lines recently have been proposed as a useful model for the study of hormonal influences on bone cell metabolism (1,3). Established OS cell lines retain their differentiated osteoblast-like functions such

---

Abbreviations: OS, osteogenic sarcoma; TRM, triamcinolone acetonide; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; hypotonic buffer, 0.01M Tris-HCl, pH 7.4, 0.001M EDTA, 0.012M thioglycerol; isotonic buffer, 0.25M sucrose, 0.025M KCl, 0.005M $MgCl_2$, 0.001M EDTA, 0.012M thioglycerol, 0.05M Tris-HCl, pH 7.4; hypertonic buffer, 0.3M KCl, 0.0005M dithiothreitol, 0.0015M EDTA, 0.01M Tris-HCl, pH 7.4.

0006-291X/80/090373-08$01.00/0

as the production of osteoid and alkaline phosphatase and the ability to mineralize (4). Like normal bone cells, intact OS cells and cell membrane preparations respond to parathyroid hormone, prostaglandins and calcitonin by enhancing cyclic AMP production (5). Finally, Manolagas, et al. (3) have observed a specific, high affinity receptor for 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in OS cell cytosol. Because of the current interest in the relationship between glucocorticoids and the functioning of the calcitropic hormones in bone, as well as documented biological effects of glucocorticoids in OS cells, we evaluated several OS lines for the presence of glucocorticoid receptors.

MATERIALS AND METHODS

Materials. [1,2,4(n)-^{3}H] Triamcinolone acetonide (20Ci/mmol) was obtained from the Amersham Corp. Unlabelled steroids were purchased from Sigma, except that 1,25-dihydroxyvitamin D_3 was a gift from Dr. M. Uskokovic. DNA-cellulose is a product of Worthington Biochemicals.

Cell Culture and Cytosol Preparation. Frozen OS cells derived from rat tumors were generously provided by Dr. T.J. Martin, University of Melbourne (OS-12) and Dr. G. Rodan, University of Connecticut (ROS 17/2). The cells were cultured in our laboratory on the surface of plastic vessels at 37°C in 5% CO_2 in air with either minimal essential medium (MEM) or Coon's F12 medium, 10% fetal calf serum and penicillin-streptomycin (10 mg/ml). The cells reach confluence in 4 to 5 days and are readily subcultured in new vessels to establish continuous cell lines. The cells were freed via trypsinization (0.025% trypsin) and then washed three times at 4°C with isotonic buffer (0.25M sucrose, 0.025M KCl, 0.005M $MgCl_2$, 0.001M EDTA, 0.012M thioglycerol, 0.05M Tris-HCl, pH 7.4) in the presence of trypsin inhibitor (Type IIL, Sigma, 1 mg/ml). Washed cells were then suspended in hypertonic buffer (0.3M KCl, 0.0005M dithiothreitol, 0.0015M EDTA, 0.01M Tris-HCl, pH 7.4) and sonicated for 15 sec/10^6 cells. The sonicate was centrifuged at 2°C in a Beckman L5-65 Ultracentrifuge for 1 hour at 105,000 x g to obtain a supernatant (cytosol) fraction.

DNA-Cellulose Chromatography. After labelling the cytosol from $125x10^6$ cells with [^{3}H]TRM (4.6nM) for 30 minutes at 25°C, binding macromolecules were precipitated with 40% saturated ammonium sulfate. Resolubilization of receptor was done with 10 ml hypotonic buffer (0.01M Tris-HCl, pH 7.4, 0.001M EDTA, 0.012M thioglycerol) and this solution applied to a 12 ml DNA-cellulose column (6). After elution with 60 mls hypotonic buffer a 90 ml linear gradient of 0 to 0.6M KCl in the same buffer was run. Salt concentrations were monitored via conductivity and 1 ml aliquots were counted for tritium in Aquasol (34% efficiency).

Charcoal Assay for Specific Binding. 100 µl portions of OS cytosol were monitored for specific [^{3}H]TRM binding by incubating at 4°C for 24 hours with increasing concentration of [^{3}H]TRM alone, or along with a 200-fold excess of nonradioactive TRM. Bound steroid was determined by adding 1.5ml of dextran-coated charcoal (0.625% Norit A and 0.0625% dextran T-70 in 0.1% gelatin, 0.15M NaCl, 0.015M sodium azide, 0.1M Na_2HPO_4, 0.039M Na_2HPO_4, pH 7.03). After 10 minutes at 4°C, the solution was centrifuged at 5,000 x g for 10 minutes and 1 ml of supernatant counted. Competition experiments with various other steroids were carried out similarily, except that 5- and 25-fold excesses of nonradioactive steroids were included. Protein concentration in cytosol was assayed by the method of Bradford (7).

RESULTS AND DISCUSSION

To detect putative glucocorticoid receptors in osteogenic sarcoma cells grown in tissue culture, the macromolecular binding of triamcinolone acetonide (a potent synthetic glucocorticoid) was first assessed by DNA-cellulose chromatography. Glucocorticoid receptors (8,9), as well as other steroid hormone receptors (10,11) associate with high affinity and selectivity to DNA-cellulose, possibly as a reflection of the proposed activity of the steroid-receptor complex in modulating gene expression. In the present experiment (Figure 1), OS-12 cytosol was precipitated with 40% saturated ammonium sulfate after labelling at 25°C with [^{3}H]TRM. When applied to DNA-cellulose under low-salt conditions, a significant peak of bound [^{3}H]TRM adsorbs to the column and subsequently elutes at 0.15M KCl (Figure 1). The fact that only about 1/3 of the bound [^{3}H]TRM adsorbed to DNA-cellulose is consistent with multiple forms of the receptor, and LeFevre, et al. (8) obtained only a 40% efficiency for DNA-cellulose binding of the hepatic glucocorticoid receptor. Thus, the results in Figure 1 provide initial evidence for the existence of a glucocorticoid receptor in OS-12 cytosol.

Sucrose gradient centrifugation was used to further identify macromolecular [^{3}H]TRM binding in OS cytosol (Figure 2). OS-12 cytosol contains a TRM binding

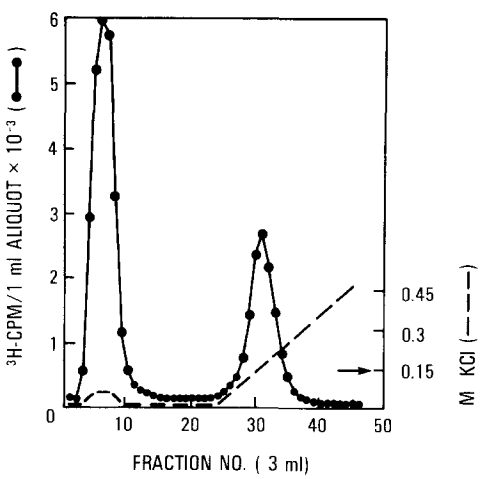

Figure 1. DNA cellulose chromatography of osteogenic sarcoma cell cytosol labelled with [^{3}H]triamcinolone. Results were obtained from 2.5 ml of OS-12 cytosol (125×10^6 cells) incubated with 4.6nM [^{3}H]TRM as detailed in Methods. The arrow indicates the salt concentration at which the adsorbed peak elutes.

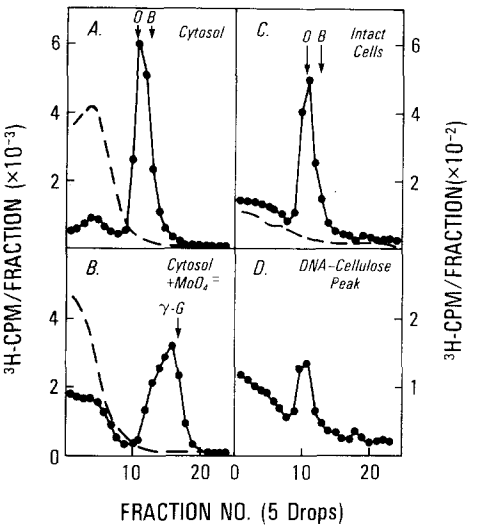

Figure 2. Sucrose gradient sedimentation of glucocorticoid receptor from rat osteogenic sarcoma cells. 4-24% sucrose gradients were run in hypertonic buffer as described elsewhere (6). Arrows indicate the sedimentation positions of protein markers: O = ovalbumin (3.7S), B = bovine serum albumin (4.4S), and γ-G = human γ-globulin (7S). A.) OS-12 cytosol ($15x10^6$ cells/0.3 ml; protein conc. = 60 mg/ml) was incubated 3 hours at 4°C with 4.6nM [^{3}H]TRM alone (—●—) or 4.6nM [^{3}H]TRM plus 920nM nonradioactive TRM (---). B.) Cytosol from OS-12 cells was prepared in hypertonic buffer supplemented with 10mM sodium molybdate and 4-24% sucrose gradients also were performed in sodium molybdate supplemented hypertonic buffer. Otherwise, conditions were identical to Panel A. C.) $20x10^6$ intact OS-12 cells were incubated 30 minutes at 37°C in isotonic buffer plus bovine serum albumin (1 ml/ml) with 4.6nM [^{3}H]TRM alone (—●—) or 4.6nM [^{3}H]TRM along with 920nM unlabelled TRM (---). After extensive washing with isotonic buffer, the cells were sonicated in 0.55 mls hypertonic buffer and 0.3 ml of resulting cytosol sedimented in sucrose gradients. D.) 300 µl of fraction no. 31 of the DNA-cellulose peak (see Figure 1) was anlayzed via sucrose density centrifugation.

component which sediments in 0.3M KCl-sucrose gradients between ovalbumin and BSA at approximately 4S (Figure 2A). Binding to this 4S species is abolished by a 200-fold excess of unlabelled TRM, indicating a finite number of high affinity sites. The TRM receptor-like molecule sediments as a larger complex (~6-7S) when sodium molybdate is included in the homogenizing medium and the gradient (Figure 2B) This observation is in concert with the recently discovered role of molybdate ion in preventing "activation" or "transformation" of the glucocorticoid (9) and progesterone (12) receptors. In the case of the progesterone receptor, molybdate prevents the 6-8S inactive form of the receptor from dissociating to the active 4S macromolecule (12). Thus molybdate interacts with the glucocorticoid receptor in OS cytosol to stabilize an aggregated form of the macromolecule, although partial dissociation in 0.3M KCl is evident from Figure 2B. In data not shown

here, results identical to those of Figures 2A and 2B were obtained with cytosol from another OS cell line (ROS 17/2), suggesting that glucocorticoid receptors are not unique to the OS-12 line.

To insure that the 4S binding component occurs intracellularly, [^{3}H]TRM was incubated with intact OS-12 cells to establish binding to receptor inside the cells prior to sonication. Cytosol prepared after extensively washing these cells also displays the 4S TRM binder which is inhibited by incubating cells with excess nonradioactive TRM (Figure 2C). Since the cytosol was prepared in 0.3M KCl, receptors expected to be translocated into the nucleus during the 37°C incubation would also be extracted in the cytosol fraction during sonication. Finally, an aliquot of the DNA-cellulose adsorbed peak (Figure 1) was run on a sucrose gradient and migrated to the 4S position (Figure 2D), proving the identity of the "activated" 4S TRM binder and the species adsorbing to DNA-cellulose. Because the glucocorticoid receptors from mouse liver cytosol (13) and AtT-20 mouse pituitary tumor cells (14) both display a sedimentation coefficient of 4S in high-salt sucrose gradients, it is clear that a very similar or identical receptor is present in OS cells.

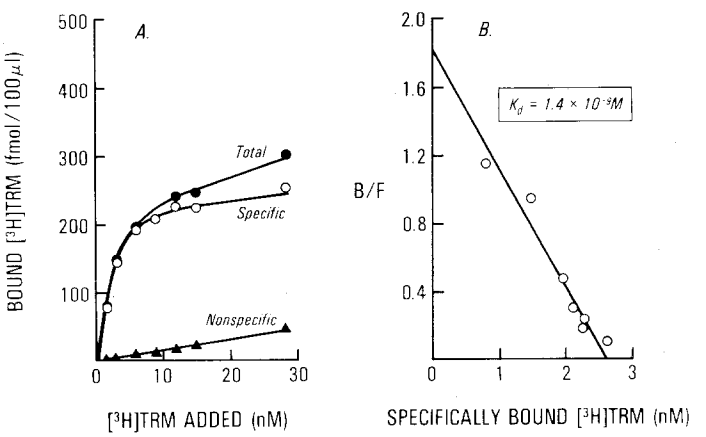

Figure 3. Saturation analysis of triamcinolone binding in osteogenic sarcoma cell cytosol. A.) OS-12 cytosol (2.5×10^6 cells or 2.6 mg protein per 100 µl) was incubated to equilibrium with increasing concentrations of [^{3}H]TRM (—●—) or [^{3}H]TRM plus a 200-fold excess of radioinert TRM (—▲—). Bound hormone was then separated from free using dextran-coated charcoal. Nonspecific binding (that unaffected by a 200-fold excess of unlabelled hormone) was subtracted from total binding to yield specific binding (—○—). B.) Scatchard analysis of specific binding. Each point represents the mean of duplicate determinations.

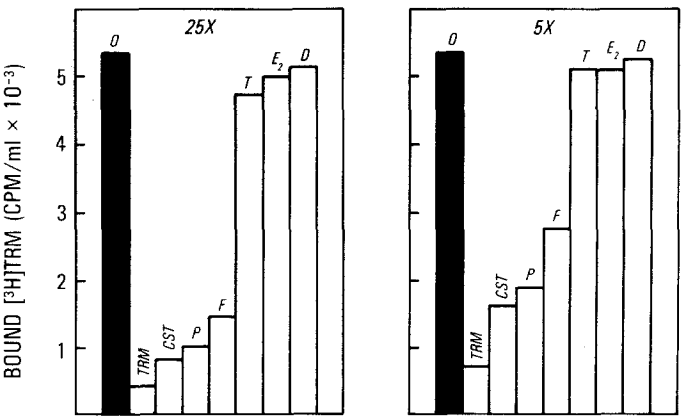

Figure 4. Competition of unlabelled steroids for [^{3}H]triamcinolone binding in OS-12 cytosol. OS-12 cytosol ($3.3x10^6$ cells or 3.0 mg protein/100 µl) was incubated 24 hours at 4°C with 11.75nM [^{3}H]TRM alone or in the presence of 25- or 5-fold excess nonradioactive steroids. Bound [^{3}H]TRM was determined using charcoal separation. Steroid code: O = no competitor, TRM = triamcinolone acetonide, CST = corticosterone, P = progesterone, F = cortisol, T = testosterone, E_2 = estradiol-17β, and D = 1,25(OH)$_2$D$_3$. Each value represents the mean of duplicate determinations.

To further characterize this receptor, saturation analysis and specificity studies were carried out. Figure 3A shows that saturation of specific binding occur at TRM concentrations of ~10nM. Scatchard analysis of specific binding (Figure 3B) indicates a single class of independent binding sites with a K_d of $1.4x10^{-9}$M. From these data we calculate a receptor concentration in OS-12 cell cytosol of 100 fmol/mg protein or approximately 60,000 receptors per cell. In competition studies (Figure 4), the binding of [^{3}H]TRM to the cytosol receptor was largely inhibited by unlabelled corticosterone, progesterone, and cortisol, but unaffected by testosterone, estradiol, and 1,25(OH)$_2$D$_3$. Because the relative competitive potency of the series of steroids in Figure 4 is strikingly similar to that observed for competition of [^{3}H]TRM binding to receptor in normal rat bone (15) and in mouse pituitary tumor cells (14), we conclude that OS cytosol is endowed with a classical glucocorticoid receptor which most likely mediates the biologic action of glucocorticoids in these bone tumor cells. This receptor is distinct from the 3.3S 1,25(OH)$_2$D$_3$ receptor in OS cytosol (3), and the fact that OS cells possess both glucocorticoid and 1,25(OH)$_2$D$_3$ receptors but lack estradiol or testosterone receptors (3), further illustrates the similarities between these tumor cells and normal bone cells.

Since glucocorticoid receptors are almost ubiquitous (16), the significance of the present results lies not in their existence in OS cells, per se, but in the utility of employing OS cells in culture to study hormone action on bone. Glucocorticoid therapy in humans causes osteopenia (bone rarefaction), primarily by decreasing osteoblastic activity at the cell level (17,18). Although glucocorticoid induced osteopenia is primarily the result of decreased bone cell replication and increased osteoblast differentiation (19), it has recently been found that glucocorticoids maintain or stabilize bone receptors for $1,25(OH)_2D_3$, the potent bone-resorbing sterol (20). Therefore, OS cells comprise a unique established cell line in which the interrelationship between glucocorticoids and $1,25(OH)_2D_3$ in bone can be probed at the molecular level. Moreover, a complete definition of the hormonal requirements for OS cell growth could offer new insights into the pathogenesis and management of osteosarcoma.

ACKNOWLEDGEMENTS

The authors wish to thank M. Larsen, M. LaFrance and D. Meler for excellent technical assistance. This research was supported by the National Institutes of Health, the American Cancer Society, the Veterans Administration and the Foundation Alexander Onassis. MRH is a faculty scholar of the Josiah Macy, Jr. Foundation.

REFERENCES

1. Martin, T.J., Ingleton, P.M., Coulton, L.A., and Melick, R.A. (1979). Clin. Orth. Rel. Res. 140, 247-254.
2. Kodama, T., and Kodama, M. (1972). Cancer Res. 32, 208-214.
3. Manolagas, S.C., Haussler, M.R., and Deftos, L.J. (1980) J. Biol. Chem., in press.
4. Martin, T.J., Ingleton, P.M., Underwood, J.C.E., Michelangeli, V.P., and Hunt, N.H. (1976) Nature 260, 436-438.
5. Underwood, J.C.E., Melick, R.A., Loomes, R.S., Dangerfield, V.M., Crawford, A., Coulton, L., Ingleton, P.M., and Martin, T.J. (1979) Europ. J. Cancer 15, 1151-1158.
6. Haussler, M.R., Manolagas, S.C., and Deftos, L.J. (1980) J. Biol. Chem., in press.
7. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
8. Le Fevre, B., Bailly, A., Sallas, N., and Milgrom, E. (1979) Biochim. Biophys. Acta 585, 266-272.
9. Leach, K.L., Dahmer, M.K., Hammond, N.D., Sando, J.J., and Pratt, W.B. (1979) J. Biol. Chem. 254, 11884-11890.
10. Gschwendt, M. (1980) Biochim. Biophys. Acta 627, 281-289.
11. Pike, J.W., and Haussler, M.R. (1979) Proc. Natl. Acad. Sci., USA, 76, 5485-5489.
12. Nishigori, H., and Toft, D. (1980) Biochemistry 19, 77-83.
13. Miras, M.E., and Harrison, R.W. (1979) J. Steroid Biochem. 11, 1129-1134.
14. Watanabe, H., Orth, D.N., and Toft, D.O. (1973) J. Biol. Chem. 243, 7625-7630.
15. Manolagas, S.C., and Anderson, D.C. (1978) J. Endocrinol. 76, 379-380.

16. Baxter, J.D., and Funder, J.W. (1979) New Engl. J. Med. 301, 1149-1161.
17. Hahn, T.J., Halstead, L.R., Teitelbaum, S.L., and Hahn, B.H. (1979) J. Clin. Invest. 64, 655-665.
18. Bressot, C., Meunier, P.J., Chapuy, M.C., Lejeune, E., Edouard, C., and Darby, A.J. (1979) Metab. Bone Dis. Rel. Res. 1, 303-311.
19. Raisz, L.G., Kream, B.E., Rowe, D.C., and Canalis, E.M. (1980) Proceedings of the Sixth International Congress of Endocrinology, Abstract S-92.
20. Manolagas, S.C., Anderson, D.C., and Lumb, G.A. (1979) Nature 277, 314-315.