



ROLE OF DEXAMETHASONE IN THE TREATMENT OF HUMORAL HYPERCALCEMIA OF MALIGNANCY (HHM) USING SAOS-2 CELLS, HUMAN OSTEOBLAST CANCER CELL LINE AS A MODEL

Dikko, A.A.U

Department of Human Physiology, Faculty of Medicine Bayero University, Kano.

ABSTRACT

Tumors cause multiple effects on the skeleton and on calcium homeostasis, but they do so in specific patterns which are becoming better defined as the mediators responsible become more fully characterized. Approximately 1,000,000 people die each year in Western Europe and the United States from these three malignancies bone, lung, and breast and the majority of these have bone metastases. Bone is the third common site of metastasis disease in tumors of all types and the second most common in breast and prostate cancers. PTH-rP produced by tumor cells of various forms is a killer in at least 15% of the 1,000, 000 cases reported in U.S. and Western Europe a significant number that is hard to ignore. The sole aim of this research is to establish whether dexamethazone inhibit the action of PTH-rP in-vitro and therefore providing a possible relief for the ailing HHM. Here SaOS-2 cells human osteoblast cancer cells was used with PTH-rP with and without Dexamethasone and DNA and thymidine incorporation proliferation assays were carried out to determine the extent of stimulation by PTH-rP or inhibition by dexamethasone. The stimulation aggravates HHM, while the inhibition is assumed to alleviate the sufferings of patients with HHM. Dexamethasone was found to inhibit the stimulated cell proliferation by PTH-rP in SaOS-2 cells human osteoblast cancer cells. It is therefore concluded that this experiment may act as a spring board for alleviating the sufferings and possible treatment of patients with Humoral Hyper Calcemia of Malignancy(HHM).

Keywords: Humoral Hyper Calcemia of Malignancy (HHM). PTH-rP Parathroid related peptide. SaOS-2 osteoblast cancer cell.

INTRODUCTION

Tumors cause multiple effects on the skeleton and on calcium homeostasis. But they do so in specific patterns which are becoming better defined as the mediators responsible become more fully characterized (Munde and Guise, 1998). Approximately 1,000,000 people die each year in Western Europe and the United States from these three malignancies bone, lung, and breast and the majority of these have bone metastases. Bone is the third common site of metastatic disease in tumors of all types and the second most common in breast and prostate cancers (Munday and Guise, 1998).

It occurs frequently in patients with breast cancer (Hennipman *et al.*, 1989) and lung cancer (Burton *et al.*, 1992) and very characteristic of the unique form of the bone disease associated with myeloma (Boyd *et al.*, 1986). The steps involved in the formation of an osteolytic bone metastasis are multiple but to date most attention has been focused on the final step, namely the increase in osteoclastic bone resorption. Osteoclastic bone disease, is responsible for catastrophic consequences in the patient with malignant disease- intractable bone pain susceptibility to fracture following trivial injury or even spontaneously. Amongst the most serious is compression syndrome leading to the compression of spinal cord (Munday and Guise, 1998).

Osteoblastic bone disease is much less common than osteolytic bone disease and occurs mostly frequently in patients with carcinoma of the

prostate (Chvbowskv *et al.*, 1991) and urinary tract (Burton, *et al.*,1990), but is also seen in patients with carcinoma of the breast and other tumors and particularly with Hodgkin's disease and with carcinoid syndrome. A number of important growth factors with powerful bone stimulatory activity have been identified and are probably responsible alone or in combination causing hypercalcaemia of malignancy (Robert and Sporn, 1992; Saaticioglu *et al.*, 1993).

PTHrP (1-34) was shown to activate osteoblastogenesis and inhibit adipogenesis in pluripotential mouse C3H10T1/2 cells (Chan *et al.*, 2003), while the C-terminal fragment of PTHrP containing the 107-111 epitope –named osteostatin- is a potent inhibitor of bone resorption in vitro (Cornish *et al.*, 1997). In addition, recent studies have shown that its intermittent administration induces various osteogenic effects through interaction with VEGF receptor 2 in these cells (Alonso *et al.*, 2008; de Gortazar *et al.*, 2006).

Dexamethasone is a synthetic glucocorticoid. All natural and synthetic glucocorticoids bind to glucocorticoid receptors. Glucocorticoid receptor is a cytoplasmic or nuclear receptor of about 800 amino-acids. Dexamethasone is hydrophobic and therefore diffuses through the cell membrane to reach the receptor. The receptor is divided into several domains (Evans,1988). The glucocorticoid/dexamethasone receptor binding domain is located at carboxyl-terminus of the molecule where hormone binds (Danielson *et al.*, 1998).

The DNA-binding domain is in the mid-portion of the protein and contains nine cysteine residues. This region folds into a two-finger-like structure stabilized by zinc ions coordinated by cysteine to form two zinc-fingers. This part of the molecule binds to specific sites of the DNA; the glucocorticoid response element (GRE) (Danielson et al., 1998). The interaction can result in an increase or decrease in gene expression. Ultimately, *c-fos* and *c-jun* may be implicated as early components of gene regulation (Angel and Karin, 1991). Since many other factors including PTH-rP and PTH interact at the level of *c-fos* and *c-jun*. Dexamethasone downregulates the expression of parathyroid hormone-related protein (PTHrP) in mesenchymal stem cells (Ahlgren et al., 2009). Thus, PTH-rP and Dexamethasone application on SaOS-2 cells may provide an outlet for the long desired method in the treatment of cancer.

It is very possible that PTH-rP interacts with dexamethasone to decrease or stimulate its action. Our attention is basically focused on the decrease as this may be a way by which cancer patients with HHM could have a reprieve. Furthermore, dexamethasone inhibits growth and enhances cAMP production when with PTH-rP and PTH(1-34) with equal potency in rats (Rodan et al., 1988), a welcome development supporting my hypothesis.

MATERIALS AND METHODS

Effect of PTH-rP and Dexamethasone on the Proliferation of SaOS-2 Cells

SaOS-2 cells were grown to 80% confluence in defined media (McCoy's) supplemented with 10% fetal calf serum, 2mM L-glutamine, 1% PSA. They were growth arrested for 24 hrs prior to any experiment by replacing with serum free medium. Cells were trypsinised (10% TE in HBS) from the culture flasks and set up into two flasks one control and the other treated with dexamethasone 10 M in 2.5% SFCs at an initial density of 0.75µg DNA/flask. The cells were incubated for 72 hours at 37°C in 5% CO₂ in air. They were growth arrested for 24hrs by replacing with serum free medium. The treated flasks were supplemented with the same dose of dexamethasone. Cells were trypsinised (10% TE in HBS) from the culture flasks and set up as control and treated in 24 well plates at an initial density of 0.75µg DNA/well with PTH-rP(1-34) with or without dexamethasone 10 M. Another set of plates were prepared from the control and the dexamethasone pretreated flasks with increasing dose of dexamethasone 10⁻⁸, 10⁻⁷ and 10⁻⁶M. The cells were incubated for 72 hours at 37°C in 5% CO₂ in air. DNA and thymidine incorporation assay carried out.

DNA Assay

This technique was first used in 1979 by Labarca and Paigen, and is based on the enhancement of fluorescence seen when bis-Benzamide binds to DNA. This assay could be used to detect as little as long DNA. 0.05M Na₂HPO₄ (3.549g.) were dissolved in 500ml analar water. and titrated against 0.05M Na₂HPO₄ (3.00 g.) also dissolved in 500ml analar water

and adjusted to pH 7.4 and a total volume of 800ml. 2M NaCl molecular biology grade I 16.88g per litre was added, and dissolved. The pH was adjusted to 7.4 using NaOH or Rd. and volume made up to 1 litre with analar water, and stored at 4°C. Bis-benzamide was prepared from stock (2mg in 1 Oml analar water) and stored at 4°C, and was diluted immediately before use with DNA assay buffer to 1:100. Standards were prepared, using 1ml volumes of calf thymus DNA. Standard solutions of 0.5, 1.0, 2.0, 4.0, and 8.0 µg/ml concentration were prepared in DNA assay buffer. Three tubes each containing 100 µl of cells from cell culture were sonicated with 900 µl DNA assay buffer for 5mm. and 1ml diluted BIS was added to make the total 2ml in all the eight tubes. Each tube contents were transferred into cuvettes. The fluorescence was read at 342nm and 458nm excitation and emission (Labarca and Paigen, 1979).

Thymidine Incorporation Assay

³H-Thymidine is a nucleotide that is incorporated into the cellular DNA. Therefore, this assay is a measure of the rate of DNA synthesis within a given time. However, ³H-thymidine assay may be toxic to S-phase cells (Maurer, 1981). Most normal cells have the ability to take up exogenous supplied thymidine. This phenomenon is referred to as the *Salvage pathway*, because it relies on exogenous rather than the endogenous thymidine. The rate-limiting step is the uptake and thymidine incorporation into the cellular DNA is the enzyme thymidine kinase. Any procedure affecting thymidine kinase production affects also thymidine uptake.

The thymidine incorporation assay was employed in several experiments with pretreated cultured cells as described above. At the end of the experiment the medium was tipped off from the plates, and replaced with 450µl thymidine free DMEM/F12 medium. Some of 1.0 mCi/ml from the stock ³H-Thymidine was diluted with this DMEM to 65 µCi/1ml. 50p1/well were added and plates incubated at 37°C in 5% CO₂ in air for three hours. At the end of the incubation period medium was discarded and cells washed twice with 250µl phenol red free Hanks balanced salt solution (HBS). The cells were trypsinised with 250µl/well (10% TE in HBS). Well contents were transferred into LP3 tubes and wells washed with an additional 500µl HBS. Cells were then lysed with ice cold 10% TCA, and left overnight 4°C. The lysates were filtered through GF/C filter Millipore filtration manifold and washed twice with 5ml of 5% TCA followed by 5ml methanol. The filters were placed in scintillation vials and left to dry. 3ml of scintillation fluid (Pharmacia/LKB Hisafe) were added and shaken and the vials were counted in a scintillation counter LKB Rak-beta with tritium efficiency of 30%. Proliferation was assessed using the DNA and thymidine incorporation assays. Results were expressed as % control for each experiment. Data are SEM of four experiments. Data were analyzed by Student's T-Test.

RESULTS

Effects of PTH-rP (1-34) with or without Dexamethasone on the Proliferation of SaOS-2 CELLS

PTH-rP(1-34) increased cell proliferation in SaOS-2 untreated cell with the maximum at 5 nM to 120% above the control (Table 1) (P<0.001), while dexamethasone 10⁻⁷M decreased the stimulated proliferation of PTH-rP(1-34) in SaOS-2 cells over a

dose range of 1.2-5nM to the minimum of 78%(P<0.001) (Table 1),

Like wise, PTH-rP (1-34) increase cell proliferation in SaOS-2 dexamethasone pretreated cells with maximum effect at 2.5nM reaching 122% (P<.001) above control, while dexamethason 10⁻⁷m decreased the PTH-rP (1-34) stimulated proliferation to maximum of 85% (P<.001) below the control (Table 1).

Table 1: DNA assay of SaOS-2 CELLS

CONTROL	PTH-rP (1-34)	PTH-rP (1-34) + DEXAMETHASONE (10 ⁻⁷ M)
100%	120% ^S	78% ^S
100%	122% ^S	85% ^S

Key: Not Significant = NS, Significant = S (P<0.001).

Again, the thymidine incorporation assay show a similar trend to the DNA assay (Table 2) in both the control and dexamethasone pretreated cells. PTH-rP(1-34) increased cell proliferation in SaOS-2 to a maximum of 128% (P<0.001) above the control in untreated cells (Table 2) at 5nM, while in the

pretreated cells the peptide increased the proliferation to 125% (P<0.001) above the control.

However, PTH-rP (1-34) stimulated cell proliferation was inhibited by dexamethasone 10⁻⁷M to the minimum of 45% (P<0.001). Also dexamethasone 10⁻⁷M. inhibited cell proliferation to minimum of 45% (P<0.001) in pretreated cells (Table 2).

Table 2: Thymidine Incorporation Assay of SaOS-2 CELLS

CONTROL	PTH-rP (1-34)	PTH-rP (1-34) + DEXAMETHASONE (10 ⁻⁷ M)
100%	128% ^S	45% ^S
100%	125% ^S	45% ^S

Key: Not Significant = NS, Significant = S (P<0.001).

DISCUSSION

The problem of cancer generated a lot of interest in the biological scientist research circles, being a disease that is yet to discover its treatment, most especially at advanced stage or metases stage. Approximately 1,000,000 people die each year in Western Europe and the United States from these three malignancies bone, lung, and breast and the majority of these have bone metastases (Munday and Guise,1998).

Dexamethasone and PTH-rP(1-34) were found to increase cell proliferation in SaOS-2 cells individually acting through the *c-jun* and *c-fos* corresponding to the findings of Angel and Karen (1991). While, Dexamethasone on the other hand

inhibited PTH-rP proliferation very significantly, in both the dexamethasone untreated and pre-treated cells (P< 0.001) corresponding to the finding of Ahlstrom, et. al., (2009) who found dexamethasone to down-regulate the expression of parathyroid hormone-related protein (PTHrP) in mesenchymal stem cells, supporting our assertion that; dexamethasone and PTH-rP could ultimately interact through the *c-fos* and *c-jun* to cause inhibition in the proliferation of SaOS-2 cells.

It could therefore be conclude that this experiment may be used as a spring-board for further experiments which may pave way to the management/treatment of Hyper Calcemia of Malignancy (HHM) resulting from cancer of the bone.

REFERENCES

Angel, P. and Karin, M. (1991). The role of Jun and Fos and the AP-1 complex in cell proliferation and transformation. *Biochem. Biophys. Acta* 1072: 129-157.

Alonso, V, de Gortazar AR, Ardura JA, Andrade-Zapata I, Alvarez-Arroyo MV, Esbrit P. (2009). Parathyroid hormone-related protein (107-139) increases human osteoblastic cell survival by activation of vascular endothelial growth factor receptor-2. *J Cell Physiol* 217:717-727

Ahlstrom M, Pekkinen M, Lamberg-Allardt, C. (2009). Dexamethasone downregulates the expression of parathyroid hormone-related protein (PTHrP) in mesenchymal stem cells. *Steroids*;74:277-82

Boyde, A., Machonnachie, E., Reid S.A., Delling, G., and Munday, G.R. (1986) Scanning electron microscopy in bone pathology: Review of methods. Potential and application. *Scanning Electron Microscopy* 4 1537-1554.

- Burton, P.B.J., Moniz. C., Quircke. P., Malik. A., Bui. T.D. and Juppner, H. (1992). Parathyroid hormone-related peptide: expression in fetal and neonatal development *J Pathol.* 167:291-296.
- Chan, G.K, Miao D, Deckelbaum R, Bolivar I, Karaplis A, Goltzman D (2003). Parathyroid hormone-related peptide interacts with bone morphogenetic protein 2 to increase osteoblastogenesis and decrease adipogenesis in pluripotent C3H10T 1/2 mesenchymal cells. *Endocrinology* 144:5511-5520.
- Chybowski, F.M. Keller. L. Bergstral. E.J., and Oestren. J.E. (1991) Predicting Radionuclide bone scan findings in patients with newly diagnosed. untreated prostate cancer: prostate specific antigen is superior to all clinical parameters. *Journal of Urology* 145 : 313-318.
- Cornish J, Callon K.E, Nicholson G.C, Reid IR (1997). Parathyroid hormone-related protein-(107-139) inhibits bone resorption in vivo. *Endocrinology*; 138:1299-304.
- Danielson, M., Hinck. L. and Ringold, G.M. (1998). Two aminoacids within the knuckle of the first zinc finger specify DNA response element activation by glucocorticoid. *Cell* 57:1131-1138.
- de Gortazar A.R, Alonso V, Alvarez-Arroyo M.V, Esbrit P (2006). Transient exposure to PTHrP (107-139) exerts anabolic effects through vascular endothelial growth factor receptor 2 in human osteoblastic cells in vitro. *Calcif Tissue Int.* ;79:360-9
- Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science.* 240:889-895.
- Hennipman, A., van Oirschort B. A. Smits J. Rijksen G. and Staal G.E. (1989). Tyrosine kinase activity in breast cancer, benign breast disease, and normal breast tissue, *Cancer Research* 49:516-521.
- Labarca, C. and Paigen K. (1979). A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem* 102: 344-352
- Maurer, H.R. (1981). Potential pitfalls of 3H-thymidine techniques to measure cell proliferation, *Cell Tissue Kinet.* 14:111-120.
- Mundy, G.R. and Guise, T.A. (1998). Role of Parathroid hormone-related peptide in hypercalcemia of malignancy: *Endocrine-Related Cancer: Journ. of Endocrinol.* 5:15-26.
- Rodan, S.B., Noda. M., Wesolowski. G., Rosenblatt, M. and Rodan G.A. (1988). Comparison of post receptor effects of 1-34 human hypercalcemia factor and 1-34 parathroid hormone in rat osteosarcoma cells. *Endocrinology*, 18: 199-203.
- Robertson, A.B. and Sporn M.B. (1992). Mechanistic interrelationship between two super-families: the steroid/retinoid receptors and transforming growth factor- β . In: *Cancer Survey*, 4: 205-220. Ed. MG Parker, Cold Spring Harbor Laboratory Press.
- Saaticioglu, F., Claret F-X, and Karin, M. (1994). Negative transcriptional regulation by nuclear receptors. In: *Seminars in Cancer Biology*, Vol.15. pp 347-359. Ed. M Parker. London: Academic Press.