ROLE OF DEXAMETHASONE IN THE TREATMENT OF HUMORAL HYPERCALCEMIA OF MALIGNANCY (HHM) USING JAR HUMAN CHORIOCARCINOMA CANCER CELL LINE AS A MODEL.

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Summary: 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 commonest site of metastatic 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 remedy for the ailing HHM. Here JAR human choriocarcinoma cells was used with PTH-rP with and without Dexamethasone also, 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 JAR human choriocarcinoma cells. Thus, the experiment may act as a spring board for alleviating the sufferings and possible treatment of patients with Humoral Hypercalcaemia of Malignancy (HHM)

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. 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 commonest site of metastatic disease in tumors of all types and the second most common in breast and prostate cancer.

It occurs frequently in patients with breast Hennipman, et al., (1989) and lung cancer Burton and Knight (1992) and very characteristic of the unique form of the bone disease associated with myeloma Boyde 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, hypercalcemia and compression syndrome, the most serious of which is spinal cord compression Munday and Guise (1998).

Osteoblastic bone disease is much less common than osteolytic bone disease and occurs most frequently in patients with carcinoma of the prostate Chybowsky, 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 cause hypercalcaemia of malignancy Robert, et al., (1992) and Saaticioglu et al.(1993).

Dexamethasone is a synthetic glucocorticoid. All natural and synthetic

glucocorticoid bind to glucocorticoid receptor. The 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 contain nine cysteine residues. This region folds into two finger-like structure stabilized by zinc ions constrain by cysteine to form two tetrahydrons. This part of the molecule bind to specific sites of the DNA, the glucocorticoid response element (GRE) Danielson et al., (1998). The interaction can result in increase or decrease gene expression. Ultimately, *c-fos* and *c-jun* may be implicated as early components of gene regulation. Angel and Karen (1991). Since many other factors including PTH-rP and PTH interact at the level of *c-fos* and *c-jun*, it is very possible that PTH-rP interact with dexamethazone 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, dexamethazone inhibits growth and enhances cAMP production with PTH-rP and PTH(1-34) with equal potency in rats Rodan et al., (1988), a welcome development supporting my hypothesis.

Thus, PTH-rP produced by tumor cells of various forms is a killer in at least 15% of the 1,000, 000 cases reported by Munday and Guise (1998) 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 inhibits the action of PTH-rP in-vitro and therefore providing a spring board for the treatment of HHM. Here, JAR cell line, a human choriocarcinoma was with PTH-rP and PTH-rP+ Dexamethasone and proliferation assays were carried out such as DNA and thymidine incorporation proliferation.

Materials and methods

A. Effect of pth-rp and dexamethasone on proliferation of jar cells

JAR cells were grown to 80% confluence in defined media (RPMI) supplemented with 10% fetal calf serum, 2mM L-glutamine, 1% HB (1320mg oxaloacetic acid, 80mg insulin/100ml.)

and 1% PSA. They were growth arrested for 24 hrs prior to any experiment by replacing with serum free medium. Cells were trypsinised [10% trypsin EDTA in calcium/magnesium, free Hank's Balanced Salt (HBBS)] from the culture flasks and set up into two flasks one control and the other treated with dexamethasone 10^{-7} M in 2.5% SFCS at an initial density of 0.5µg DNA/flask (equivalent to approximately 0.5 x 10^6 cells). The cells were incubated for 72 hours at 37°C in 5% CO_2 in air. They were growth arrested for 24 hrs by replacing with serum free medium, the treated flask boosted with the same dose of dexamethasone. Cells were trypsinised with 10% T.E., in Hanks Balanced Salt (HBS) from the culture flasks and set up control and treated separately in 24 well plates at an initial density of 0.5µg DNA/well with PTH-rP(1-34) with or without dexamethasone 10^{-7} 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^{-6} M. The cells were incubated for 72 hours at 37°C in 5% CO_2 in air. DNA and Thymidine incorporation assays were carried out.

B. 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 10ng DNA. 0.05M Na_2 HPO4 (3.549g.) were dissolved in 500ml analar water, and titrated against 0.05M Na_2 HPO4 (3.001g.) also dissolved in 500ml analar water and adjusted to pH 7.4 and a total volume of ~800ml. 2M NaCl molecular biology grade 116.88g per litre was added, and dissolved. The pH was adjusted to 7.4 using NaOH or HCl, and volume made up to 1l with analar water, and stored at 4°C. Bis-benzamide was prepared from stock (2mg in 10ml 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 8.0µg/ml concentration were prepared in DNA assay buffer. Three tubes each containing 100µl of cells from cell culture were sonicated with 900µl DNA assay buffer for 5min. and 1ml diluted BIS was added to make the total 2ml in all the eight tubes. Each tube's contents were transferred into cuvettes. The fluorescence was read at 342nm and 458nm excitation and emission.

C. 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, ^{3}H-thymidine assay may be toxic to S-phase cells (Maurer, 1981). Most normal cells have the ability to take up exogenously 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/ml. 50µl/well were added and plates incubated at 37°C in 5% CO_2 in air for three hours. At the end of the incubation period medium was discarded and cells washed twice with 250µl phenol red free Hank's 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%.

Results

Proliferation was assessed using the DNA and thymidine incorporation assays. Results were expressed as % control for each experiment. Data are M +/- SEM of four experiments. Data were analyzed by Student's T-Test.

A. Effect of PTH-RP (1-34) with or without Dexamethasone on the Proliferation of Jar cells.

PTH-rP (1-34) increased cell proliferation in JAR control cells with the maximum at 2.5 nM to 150% above the control (P<0.001), while dexamethasone 10^{-7} M decreased the stimulated proliferation by PTH-rP(1-34) in JAR cells over a dose range of 1.25-5nM to 55% of control values (P<0.001) (Table 1)

Likewise, PTH-rP(1-3) increased cell proliferation in JAR cells pretreated with 10^{-7} M dexamethasone with the maximum at 2.5 nM to 130% above the control (P<0.001). Also PTH-rP(1-34) stimulated cell proliferation in JAR was inhibited by dexamethasone 10^{-7} M to 80% of control value (P<0.001) (Table 1)

Table 1: DNA Assay of Jar cells.

CONTROL	PTH-rP (1-34)	SIGNIFICANCE	PTH-rP (1-34) + DEXAMETHASONE (10^{-7} M)	SIGNIFICANCE
100%	150%	P<0.001	55%	P<0.001 *
100%	130%	P<0.001	80%	P<0.001 **

* NOT TREATED WITH DEXAMETHAZONE (10^{-7}M) ** PRETREATED WITH DEXAMETHAZONE (10^{-7}M)

The thymidine incorporation assay showed 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 JAR to a maximum at 2.5nM of 157% above the control (P<0.001) in control cells, while in the dexamethasone pretreated cells the peptide increased the proliferation to 130% (P<0.001) above the control at 2.5nM. Also PTH-rP(1-34) stimulated cell proliferation was inhibited by

dexamethasone (10^{-7}) to a minimum of 85% (P<0.001) of control values in control cells, while dexamethasone(10^{-7} M), inhibited cell

proliferation to 80% of control values (P<0.001) in pretreated cells (Table 2).

Table 2: Thymidine incorporation assay of Jar cells.

CONTROL	PTH-rP (1-34)	SIGNIFICANCE	PTH-rP (1-34) + DEXAMETHASONE (10^{-7}M)	SIGNIFICANCE
100%	157%	P<0.001	80%	P<0.001 *
100%	130%	P<0.001	85%	P<0.001**

* NOT TREATED WITH DEXAMETHAZONE (10^{-7}M) ** PRETREATED WITH DEXAMETHAZONE (10^{-7}M)

Discussion

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 metastatic disease in tumors of all types and the second most common in breast prostate cancer.

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Karen (1991). Since many other factors including PTH-rP and PTH interact at the level of c-fos and c-jun. The possibility that PTH-rP interact with dexamethazone to decrease or stimulate its action was exploited with the intention of finding a way by which patients with HHM are treated.

Thus, PTH-rP produced by tumor cells of various forms is a killer in at least 15% of the 1,000, 000 cases reported by Munday and Guise (1998) in U.S. and Western Europe a significant number that is hard to ignore.

The result of the experiments is as expected. While PTH-rP increased very significantly cell proliferation in JAR human choriocarcinoma (P<0.001), to cause HHM dexamethasone on the other hand inhibited same very significantly, in both the control hard and pre-treated (P<0.001). This experiment may be used as a spring-board for further experiment which may pave way to the treatment of Hypercalcemia of Malignancy (HHM).

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