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Combined calcitriol and menadione reduces experimental murine triple negative breast tumor

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ABSTRACT

Background: Calcitriol (D) or 1,25$(OH)_2D_3$ inhibits the growth of several tumor cells including breast cancer cells, by activating cell death pathways. Menadione (MEN), a glutathione-depleting compound, may be used to potentiate the antiproliferative actions of D on cancer cells. We have previously shown *in vitro* that MEN improved D-induced growth arrest on breast cancer cell lines, inducing oxidative stress and DNA damage *via* ROS generation. Treatment with MEN + D resulted more effective than D or MEN alone.
Objective: : To study the *in vivo* effect of calcitriol, MEN or their combination on the development of murine transplantable triple negative breast tumor M-406 in its syngeneic host.
Methods: Tumor M-406 was inoculated s.c., and when tumors reached the desired size, animals were randomly assigned to one of four groups receiving daily i.p. injections of either sterile saline solution (controls, C), MEN, D, or both (MEN + D). Body weight and tumor volume were recorded three times a week. Serum calcium was determined before and at the end of the treatment, at which time tumor samples were obtained for histological examination.
Results: None of the drugs, alone or in combination, affected mice body weight in the period studied. The combined treatment reduced tumor growth rate (C *vs.* MEN + D, $P < 0.05$) and the corresponding histological sections exhibited small remaining areas of viable tumor only in the periphery. A concomitant DNA fragmentation was observed in all treated groups and MEN potentiated the calcitriol effect on tumor growth.
Conclusions: As previously observed *in vitro*, treatment with MEN and D delayed tumor growth *in vivo* more efficiently than the individual drugs, with evident signals of apoptosis induction. Our results propose an alternative protocol to treat triple negative breast cancer, using GSH depleting drugs together with calcitriol, which would allow lower doses of the steroid to maintain the antitumor effect while diminishing its adverse pharmacological effects.

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1. Introduction

Although calcitriol (D) or 1,25-dihydroxyvitamin D_3 is well known for its effects on calcium and phosphate metabolism, antiproliferative non-classical effects were recently described for this steroid hormone [1–4]. Moreover, calcitriol has been shown to regulate almost all cancer hallmarks such as cellular proliferation, differentiation, cell death, angiogenesis, metastasis, immunomodulation and inflammation [5,6]. On the other hand, the antitumor activity of menadione (MEN) or vitamin K_3, a chemically synthesized compound that appears to have anticancer effects [7,8], is still not completely understood. However, MEN is a glutathione (GSH)-depleting drug and tumor resistance is associated with high intracellular GSH levels. MEN or D,L-buthionine-S, R-sulfoximine (BSO), another GSH-depleting drug, are used in clinical practice and may act as sensitizers when administered in combination with conventional chemotherapeutic agents in various cancer types [9,10].

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Breast cancer (BC) cells have been shown to be targets of vitamin D compounds [4,11]. We have previously demonstrated that D in combination with MEN or BSO, elicited antiproliferative effects on MCF-7 and other breast cancer cell lines [1,2], and that the inhibitory action on cell growth *in vitro* was even more potent than using D or MEN alone. Moreover, we have shown a similar antineoplastic activity of this combined therapy on colon adenocarcinoma cells [12]. In these studies, the effects of MEN and D on tumor cell growth were associated with oxidative stress induction, cell cycle arrest and activation of cell death pathways.

BCs are often classified by the expression profile of three membrane receptor proteins: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). During the last 15 years, five intrinsic molecular subtypes of breast cancer have been identified through gene-expression profiling, (Luminal A, Luminal B, HER2-enriched, Basal-like, and Claudin-low), which have improved our knowledge of breast cancer biology [13]. Particularly, mRNA profiles have been used to define the prognostic value of these subtypes of breast cancer [14]. The presence or absence of these receptors confers distinctive biological characteristics to these BC subtypes that determine the response patterns to different therapeutic modalities and their clinical outcome. The triple-negative breast cancer (TNBC), a subtype that lacks the expression of ER, PR, and HER2, represents a very heterogeneous group of breast diseases, associated with a significantly higher probability of relapse and poorer overall survival in the first few years after diagnosis [15,16]. One of the BC cell lines studied in our previous work, the human triple-negative breast cancer (TNBC) HMLER cell line [17], was susceptible to calcitriol or GSH-depleting drugs and much more to the combined treatment. In the conditions of the experiment, the HMLER cells treated with GSH-depleting drugs, D or both showed a significant delay in their doubling time compared to the control [2]. Since cytotoxic chemotherapy is currently the only treatment option for TNBC [18], the aim of the present work was to analyze the effect of calcitriol, MEN or their combination in an *in vivo* setting, using the CBi-IGE murine model of a transplantable triple-negative breast tumor [19].

2. Materials and methods

2.1. Chemicals

MEN was obtained from Sigma (St. Louis, MO, USA) and was used at 2 mg/kg body weight (BW). $1,25(OH)_2D_3$ was a generous gift from Roche Diagnostic GmbH (Mannheim, Germany), and used at 0.15 μg/kg of BW. All other reagents were of analytical grade.

2.2. Experimental design

2.2.1. Animals

Twenty-seven CBi female mice (11–14 weeks old) of the CBi-IGE colony from the Animal Facilities of the Instituto de Genética Experimental, Facultad de Ciencias Médicas, Universidad Nacional de Rosario (from here on, CBi-IGE stock), were used. The CBi-IGE stock comprises five genetically distinct lines obtained from selection experiments, currently in their 140th generation of selective breeding. A description of the selection procedure is given elsewhere [20]. The average weight of the animals at the beginning of the experiment was 32 g. Mice were kept in the IGE animal facility, in a room with a constant temperature of 24 ± 2 °C, a relative humidity of 50 ± 10% and a 12-h-on/12-h-off light cycle. Animals were fed the same diet (Gepsa Feeds pelletized, Grupo Pilar S.A., Córdoba, Argentina) and water *ad libitum*. Experiments were performed during the first half of light cycle. Mice were treated following the institutional regulations (Facultad de Ciencias Médicas, Universidad Nacional de Rosario, permit # 081/2014) which comply with the guidelines issued by the Institute for Laboratory Animal Resources, National Research Council, USA (2011) and the Canadian Council on Animal Care (1998). All experiments were performed with prior approval from the Bioethics Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Rosario.

2.2.2. Tumor

M-406 is a type B semi-differentiated mammary adenocarcinoma [21,22] that arose spontaneously in an inbred CBi female mouse in 1998 and is maintained *in vivo* in syngeneic mice by serial intraperitoneal implantation every 14 days. When the tumor is inoculated subcutaneously (*s.c.*) in the line of origin, the lethality is 100% (100% take and 0% regression). M-406 was characterized as a triple negative tumor (ER-, PR-, HER2-) [19].

2.2.3. Experimental approach

Mice were challenged *s.c.* with M-406 by trocar in the right lateral flank. When tumors reached an approximate size of 150 mm^3 (5 days post-inoculum), the animals were randomly divided into four experimental groups which were treated daily, from Monday to Friday, with sterile *i.p.* injections of saline (Control, Group C, n = 6), 2 mg MEN/kg BW (Group MEN, n = 7), 0.15 μg 1,25 $(OH)_2D_3$/kg of BW (Group D, n = 7) or the combination of both drugs (Group MEN + D, n = 7). Body weight (g) and minor/major tumor diameters (mm) were measured, and the general health status monitored, three times a week throughout the experiment. Tumor volume (TV, mm^3) was estimated according to the formula TV = (minor diameter)^2 x major diameter x 0.4 [23,24]. Tumor volume was plotted as a function of time (days), and the graph was fitted with an exponential function. A parameter of this curve, the tumor doubling time (Tdt), is clinically relevant [25], and was used to compare the effect of the different treatments. Tumor growth rate was also calculated as the ratio between volumes measured at two consecutive times (rate of tumor volume increase, as depicted in Fig. 1). Mice were sacrificed by cervical dislocation when tumors from the control group had reached the maximum size permitted by ethical standards. At that time, the tumors were removed and processed for histological examination.

2.3. Histology

Tumors were removed after euthanasia, fixed in buffered 10% formalin solution for 24 h and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) for histopathological studies and for counting nuclei in mitotic cell division.

2.4. TUNEL technique

DNA fragmentation was detected by the terminal transferase mediated dUTP nick-end labeling assay (TUNEL) employing ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA), as previously described [26]. The detection of peroxidase activity was done using 3,3 − diaminobenzidine (DAB; Thermo Scientific, Rockford, USA), as a chromogen and the sections were counterstained with 0.5% (w/v) methyl green for 10 min at room temperature. The apoptotic cells were counted at 400× magnification in at least three sections from three animals for each treatment, which was accomplished by two independent researchers in a blinded fashion. Positive and negative controls were also performed. Positive controls were established using the slides contained in the same kit following the manufacturer's instructions. Sections processed without TdT

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