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Human breast tumor slices: A model for identification of vitamin D regulated genes in the tumor microenvironment☆

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ABSTRACT

While many studies have addressed the direct effects of $1\alpha,25(OH)_2D_3$ on breast cancer (BC) cells, stromal–epithelial interactions, which are important for the tumor development, have been largely ignored. In addition, high concentrations of the hormone, which cannot be attained *in vivo*, have been used. Our aim was to establish a more physiological breast cancer model, represented by BC tissue slices, which maintain epithelial–mesenchymal interactions, cultured with a relatively low $1\alpha,25(OH)_2D_3$ concentration, in order to evaluate the vitamin D pathway. Freshly excised human BC samples were sliced and cultured in complete culture media containing vehicle, 0.5 nM or 100 nM $1\alpha,25(OH)_2D_3$ for 24 h. BC slices remained viable for at least 24 h, as evaluated by preserved tissue morphology in hematoxylin and eosin (HE) stained sections and bromodeoxyuridine (BrdU) incorporation by 10% of tumor cells. VDR mRNA expression was detected in all samples and CYP24A1 mRNA expression was induced by $1\alpha,25(OH)_2D_3$ in both concentrations (but mainly with 100 nM). Our results indicate that the vitamin D signaling pathway is functional in BC slices, a model which preserves stromal–epithelial interactions and mimics *in vivo* conditions.

1. Introduction

Epidemiological data suggest that a lower sun incidence, related with a vitamin D precursor lack, may be associated with a higher risk of many common cancers [1]. Other studies report a lower vitamin D serum concentration in breast cancer patients as compared with women without cancer [2,3]. Besides regulating calcium and phosphorus metabolism, the active form of vitamin D, $1\alpha,25(OH)_2D_3$, may induce growth arrest [4–6], differentiation and apoptosis [7] in cancer cell lines [8]. However, the majority of preclinical breast cancer research is based on established lineages, where antiproliferative effects were achieved through high concentrations of $1\alpha,25(OH)_2D_3$ or its analogs.

Stromal–epithelial cell interactions are key regulators of breast growth, development and differentiation [9], however our understanding of interactions between epithelium and stroma within the cancerous mammary gland is still rudimentary. An approach to the study of this interaction is the use of co-culture, which allows the investigation of soluble factor stimuli or the direct contact between two cell lines [10]. However, it does not consider the whole tumor microenvironment. Another model, organotypic culture preserves breast structural architecture with its complexity [11,12] and may be a model of greater physiological resemblance.

Another issue of interest is that $1\alpha,25(OH)_2D_3$ concentration that can be safely attained in humans, not causing hypercalcemia, has been evaluated in clinical studies [13] and is far below that used in cell culture experiments. $1\alpha,25(OH)_2D_3$ actions in lower concentrations using BC tissue slices would better mimic *in vivo* conditions. Hence, our aim was to establish a more physiological breast cancer model to study the vitamin D pathway.

2. Methods

2.1. Patients

Ten post-menopausal patients with invasive breast cancer (Table 1), in the absence of distant metastasis or previous treatment (chemotherapy, radiotherapy or endocrine therapy), were accrued at Instituto Brasileiro de Controle do Câncer (IBCC) and Hospital do Câncer A.C. Camargo, São Paulo, Brazil. This protocol

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Table 1
Characteristics of patients.

Patient	Age	HT	CS	LN	ER	PR	ErbB2
6[a]	70	IDC	IIIC	+	–	–	+
11[a]	74	IDC	IIIA	+	+	+	–
12[a]	56	IDC	I	–	–	+	–
13[a]	57	IDC	IIA	–	+	+	+
15[a]	76	IDC	IIIC	+	–	–	–
2[b]	56	IDC	IIIC	+	+	+	+
4[b]	54	IC	IIA	–	–	–	–
8[b]	69	ILC	IIIA	+	+	+	–
9[b]	56	ILC	IIA	–	+	+	–
10[b]	61	IDC	I	–	+	–	+

HT: Histological type; CS: clinical stage; LN: lymph node involvement; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma, IC: invasive carcinoma, ER estrogen receptor, PR progesterone receptor. –: Negative, +: positive and NA: non-available.

[a] Samples used in microarray studies.

[b] Samples used in qPCR experiments. (AJCC Cancer staging manual, 6th edition, 2002.)

was approved by the Institutional Ethics Committee and all patients signed the informed consent. Five patients had samples analyzed by microarray and another five patients had samples evaluated by qPCR. Mean age was 66 and 59 years, for patients with samples evaluated by microarray and qPCR, respectively. Patients characteristics are shown in Table 1.

2.2. Tissue slice preparation and treatment

Tumor fragments were obtained immediately after tumor resection by the pathologist, who selected an involved area, and placed them into culture medium (RPMI 1640 with antibiotics and fungicide). Tissue slicing was done using the Krumdieck tissue slicing system (Alabama Research and Development Corporation, Birmingham, AL, USA). Fragments thickness varied between 400 and 800 μm. Slices were cultured for 24 h in 6-well plates (1 slice/well) containing 2 ml of complete culture media (RPMI supplemented with 10%, v/v FBS, antibiotics and 0.001% ethanol (vehicle) or 1α,25(OH)$_2$D$_3$ (Calbiochem, Darmstadt, Germany) 0.5 nM or 100 nM, for 24 h.

2.3. Cell viability assay

Cell viability was evaluated using BrdU incorporation. Slices placed into wells were cultured with BrdU 10 μg/mL (Calbiochem) for 16 h and without BrdU for another 8 h. Slices were washed, fixed in 4% paraformaldehyde/PBS, incubated in 2N HCl for 30 s, followed by H_3BO_3 (0.1 M)–NaOH (0.15 M) inactivation. Sections were permeabilized in 0.3% Triton and blocked for 1 h with 0.2% Triton/1% horse serum/PBS. After 16 h incubation with mouse monoclonal anti-BrDU 1:150 (clone PRB1, Millipore, MA, USA) at 4 C, sections were exposed to secondary antibody 1:1000 (Alexa Fluor® 488 streptavidin conjugated, cat. S11223, Molecular Probes (Invitrogen, CA, USA)) for 1 h and counter-stained with DAPI (Invitrogen). Sections were sliced to 3 μm prior to glass slide preparation. BrdU positive nuclei were counted in 10–20 sections (1000 total cells) counter-stained with DAPI, using with *ImageJ* software package (http://rsb.info.nih.gov/ij).

2.4. Total RNA extraction from Tumor tissues

Total RNA was extracted using the RNeasy mini Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's protocols. Total RNA concentration was determined by NanoDrop ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purity and integrity (28S/18S ratio) were assessed by RNA 6000 pico assay chips using an Agilent Bioanalyzer Model 2100 (Agilent Technologies, CA, USA). Only samples with an RNA integrity number (RIN) higher than 6.5 (Agilent software) were included in the analysis.

2.5. cDNA microarray data analysis

One hundred nanograms of total RNA was processed for microarray analysis, in accordance with Affymetrix protocol, using Two Cycle Labeling Kit (Affymetrix, Santa Clara, CA, USA). Briefly, purified RNA was used to synthesize cDNA. Afterwards, Biotin-labeled cRNA was synthesized from double strand cDNA using IVT labeling kit (Affymetrix, Santa Clara, CA, USA). Quality and concentration were assessed using a NanoDrop and 2100 Bioanalyzer. Twenty micrograms of biotinylated cRNA were hybridized with Whole genome U133 plus 2.0 microarray chips (Affymetrix) and scanned with Affymetrixs GeneChip Scanner 3000. After image acquisition, raw fluorescent signal (.cel file) from Affymetrix GeneChip Operating Software (GCOS) was assessed with GeneSpring X software (Agilent Technologies, Santa Clara, CA, USA). After background correction, normalization and probe summarization, performed using Robust Multichip Analysis utilizing GeneSpring X, samples were categorized according to 1α,25(OH)$_2$D$_3$ treatment (ethanol, 0.5 nM and 100 nM) and repeated measures ANOVA, provided by GeneSpring GX software, was used to identify differentially expressed genes.

2.6. Quantitative Real time PCR

Reverse transcription reaction was performed with oligo(dT)$_{12–18}$ primers 0.5 μg/μL (Invitrogen), 10 nM dNTPs and SuperScript® III (Invitrogen, Santa Clara, CA, USA). cDNA samples (6 μL; 20 ng/μL) were subjected to qPCR assays in duplicates using SYBR Green methodology, followed by *Rotor-Gene 6 System* software analysis (Corbett Research, Mortlake, Australia). Gene-specific primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/) to generate a PCR product in the 3′ portion, spanning the translated region of the target mRNA (Table 2). Blast analysis was carried out to confirm specificity. Reactions conditions were: 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Results displaying a CT variation between them <1.0 were further used to calculate average values. GAPDH was used as an internal control. Relative gene expression of target genes was normalized to the mean of the control samples using the $2^{-\Delta\Delta Ct}$ method.

Table 2
Primer sets for quantitative real-time PCR.

Gene	GeneBank accession no.	Primer sequences	Product size (bp)
VDR	NM_002046	Sense 5′ AGATTGGAGAAGCTGGACGA 3′ Antisense 5′ GCCCACCATAAGACCTACGA 3′	203
CYP24A1	NM_000181	Sense 5′ TATTTGCGGACAATCCAACA 3′ Antisense 5′ GGCAACAGTTCTGGGTGAAT 3′	249
GAPDH	NM_002046	Sense 5′ ATTCCACCCATGGCAAATTC 3′ Antisense 5′ TGATGGGATTTCCATTGATGA 3′	72

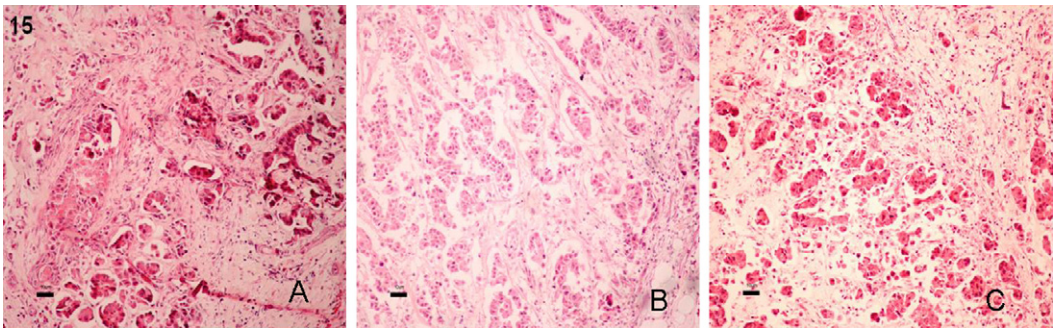

Fig. 1. Tissue architecture is preserved after tumor slices culture. Tumor slices maintained for 24 h in culture medium in the presence (100 nM) or absence (control) of $1\alpha,25(OH)_2D_3$ were stained with HE. Tumor slices (A) immediately after slicing; (B) after a 24 h period of culture without $1\alpha,25(OH)_2D_3$; (C) after a 24 h period of culture with 100 nM $1\alpha,25(OH)_2D_3$. Five independent assays were performed with similar results. Magnification ×20. Bars: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

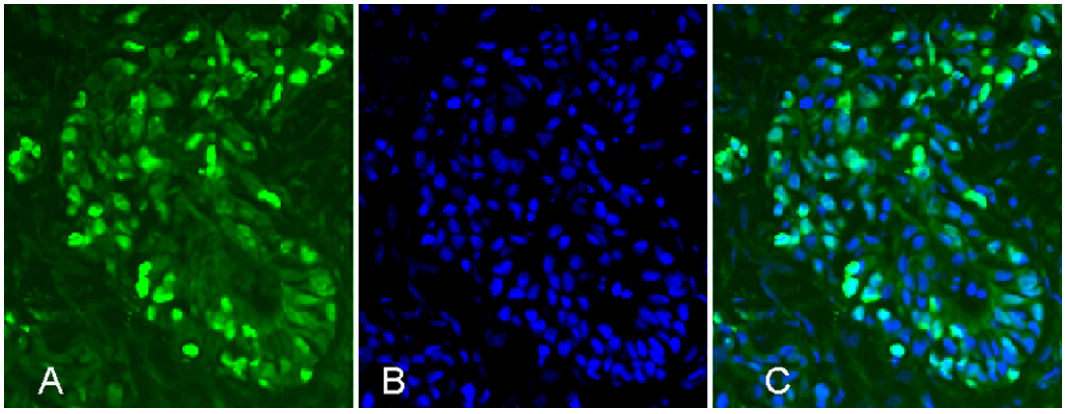

Fig. 2. Tumor viability in breast cancer organotypic culture, after a 24 h period. Proliferation rate evaluated by Bromodeoxyuridine (BrdU) incorporation was 10.44 ± 4.21%. (A) BrdU positive nuclei in green. (B) DAPI counterstaining of nuclei in blue. (C) Merged images. Magnification ×20 ($n = 5$, in duplicates). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

2.7. Statistical analysis

Repeated measures ANOVA, provided by GeneSpring GX software (Agilent, Santa Clara, CA, USA) was employed to analyze microarray data from samples treated or not (control) with 0.5 and 100 nM $1\alpha,25(OH)_2D_3$ or vehicle control. Differentially expressed genes were those satisfying a significance level <0.05 (two sided), expression fold change greater than 2.0 and agreement between treatment conditions with 0.5 and 100 nM $1\alpha,25(OH)_2D_3$ as compared to control. For qPCR experiments, data were analyzed by ANOVA provided by Instat (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Breast cancer tissue slices maintain cell viability for 24 h

One of our main objectives was to establish whether tissue manipulation would affect tissue viability. At first, tissue architecture was evaluated in sections of tumor slices, after a 24 h culture with and without $1\alpha,25(OH)_2D_3$, and histological analysis showed preserved tissue morphology (Fig. 1). Cell viability was then determined using BrdU incorporation by proliferating cells, which was positive in 10.44 ± 4.21% of tumor cells (Fig. 2). These results are consistent with preserved cell viability of tumor slices cultured for 24 h.

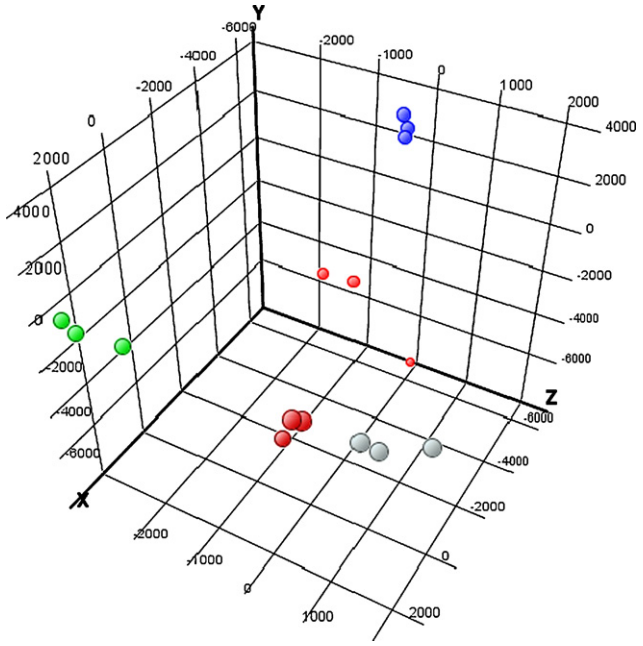

Fig. 3. Principal Component Analysis (PCA) plot representation of data quality. Samples from 5 patients (represented by a different color on the plot) were treated with either vehicle control, 0.5 nM or 100 nM $1\alpha,25(OH)_2D_3$. It is expected that replicates within a group should cluster together. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

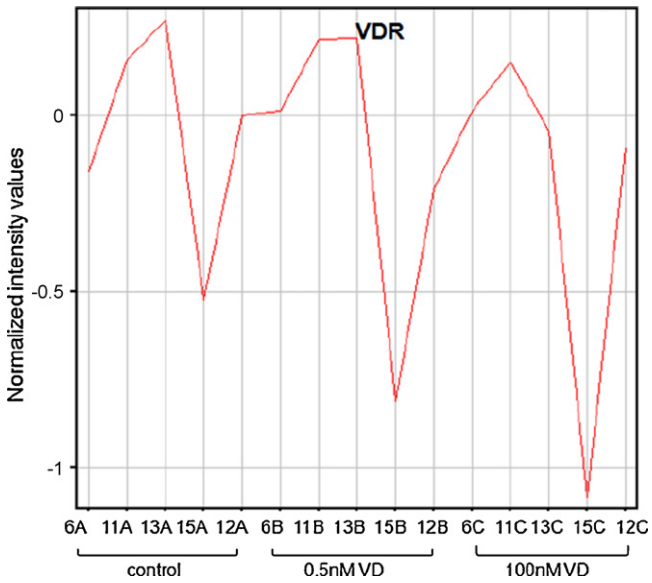

Fig. 4. VDR expression values (microarray data) in BC slices ($n = 5$) maintained without (control, left group) or with 1α,25(OH)$_2$D$_3$ (0.5 nM, center and 100 nM, right groups). *Y* axis: Relative expression values; *X* axis: tumor samples and treatment. Gray lines represent all the other differentially expressed genes.

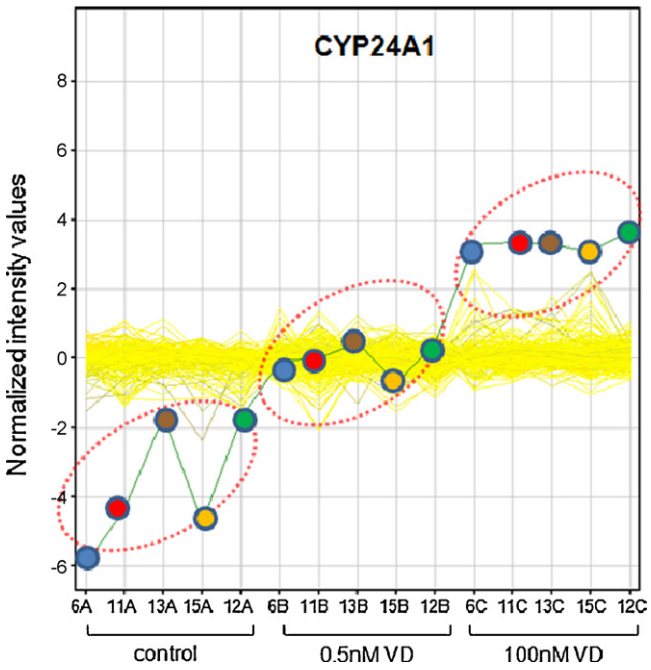

Fig. 5. CYP24A1 expression values (microarray data) in BC slices ($n = 5$) maintained without (control, left group) or with 1α,25(OH)$_2$D$_3$ (0.5, center and 100 nM, right groups). *Y* axis: Relative expression values; *X* axis: tumor samples and treatment.

3.2. Gene expression of breast cancer tissue slices is influenced by 0.5 and 100 nM 1α,25(OH)$_2$D$_3$

Tumor slices from five patients (invasive ductal carcinoma) were treated with 0.5 and 100 nM 1α,25(OH)$_2$D$_3$ or vehicle control for 24 h (Table 1) and gene expression was evaluated by microarray. Principal Component Analysis (PCA) showed that samples from the same patient tended to cluster together and samples each patient were clearly separated from the others, indicating good data quality (Fig. 3). In addition, a great homogeneity of gene expression among samples from the same patient indicates that even though 1α,25(OH)$_2$D$_3$ may regulate transcription of some genes, the individual expression profile predominates.

VDR expression was detected in all samples (Fig. 4) and VDR target gene, 24-hydroxylase (CYP24A1), was up regulated by 12- and 130-fold in 0.5 and 100 nM 1α,25(OH)$_2$D$_3$ BC samples, respectively (Fig. 5).

3.3. Expression of vitamin D receptor and CYP24A1 in another set of breast cancer samples – quantitative real time PCR

Expression of vitamin D receptor (VDR) and CYP24A1 was further evaluated in samples obtained from another five patients, cultured as tumor slices in the presence or absence of 1α,25(OH)$_2$D$_3$, using qPCR. Since genomic actions of 1 alpha,25 dihydroxivitamin D3 treatment are mediated by vitamin D receptor, we detected VDR mRNA expression in BC samples. VDR mRNA expression was similar in all conditions, in accordance with our microarray data (Fig. 6A). CYP24A1 mRNA was significantly up-regulated under near physiological (0.5 nM) and even more, under pharmacological (100 nM) concentrations of 1α,25(OH)$_2$D$_3$ (Fig. 6B).

4. Discussion

Tumor slices cultured *in vitro* may be an interesting model to evaluate hormone response, as it maintains some of the *in vivo* characteristics, as the epithelial mesenchymal relationship. An important issue is to guarantee a proper diffusion of oxygen and nutrients, which was indirectly evaluated in our organotypic culture system. As preserved tissue morphology and proliferation rate, were detected, we assume that tumor slices short term culture may be used to investigate tumor cell behavior *ex vivo*.

Our next step was to employ this tumor slice model to evaluate whether a relative low concentration of 1α,25(OH)$_2$D$_3$, 0.5 nM, which can be attained *in vivo* [13], would stimulate the vitamin D pathway, as determined by induction of the target gene CYP24A1. Up regulation of CYP24A1 expression was observed in a concentration dependent manner, but even though the lower 1α,25(OH)$_2$D$_3$ concentration (0.5 nM) induced the target gene in a less intense way, these results indicate that the vitamin D path-

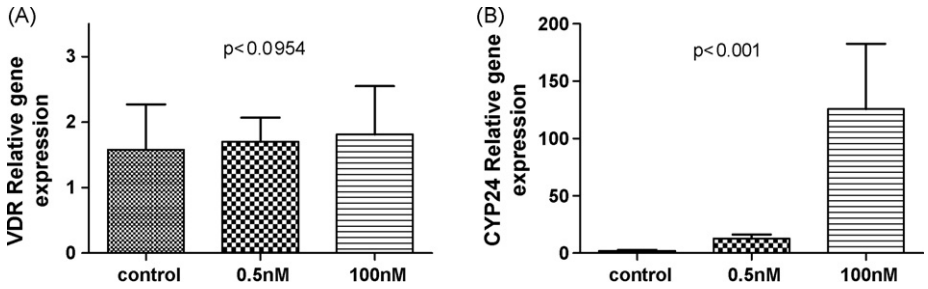

Fig. 6. VDR (A) and CYP24A1 (B) mRNA in BC samples from another set of 5 patients, evaluated by qPCR. Tissue slices were cultured either with vehicle control, 0.5 nM or 100 nM 1α,25(OH)$_2$D$_3$ for 24 h. Gene expression was evaluated by real-time RT-PCR. Error bars represent S.E.M. *p-values* one-way ANOVA ($p < 0.001$).

way is maintained in breast cancer. These data are in accordance with our preliminary results showing that tumor specimens from post-menopausal breast cancer patients, after calcitriol supplementation (dose to prevent osteoporosis), are growth inhibited, as evaluated by reduced Ki67 expression [14]. We are now comparing the relative effects of lower and higher concentrations of $1\alpha,25(OH)_2D_3$ on gene expression in this tissue culture model.

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