

Effect of cathepsin D and prostate specific antigen on latent transforming growth factor-beta in breast cancer cell lines

*Shew Fung WONG, MSc, **Heng Fong SEOW, PhD and *Leslie C. LAI, FRCP, FRCPath

* International Medical University, Kuala Lumpur, Malaysia

** Faculty of Medicine and Health Sciences, University Putra Malaysia

Abstract

Transforming growth factor-beta (TGF β) is present, predominantly in latent forms, in normal and malignant breast tissue. The mechanisms by which latent TGF β is activated physiologically remain largely an enigma. The objective of this study was to assess whether the proteases, cathepsin D and prostate specific antigen (PSA) could activate latent TGF β_1 and TGF β_2 in conditioned media of the hormone-dependent MCF-7 and hormone-independent MDA-MB-231 human breast cancer cell lines, newly purchased from ATCC. Both of the cell lines were seeded in 6-well plates 2 days prior to treatment with varying concentrations of cathepsin D and PSA. Active TGF β_1 and TGF β_2 in the media were then measured by ELISA after 4, 8, 24 and 72 hours of treatment. TGF β_1 and TGF β_2 mRNA expression of both cell lines were measured by RT-PCR to determine whether any increase in level of active TGF β_1 and TGF β_2 was due to increased production. There was a significant increase in only active TGF β_2 levels in the MDA-MB-231 cell line with both treatments. Cathepsin D and PSA did not have any effect on TGF β_1 and TGF β_2 mRNA expression. Cathepsin D and PSA were unable to activate latent TGF β_1 and TGF β_2 in these two breast cancer cell lines. A constant level of TGF β_2 mRNA in the control and treated MDA-MB-231 cells suggests that the increase in level of active TGF β_2 was not a result of increased production but was likely to be due to activation by a mechanism independent of cathepsin D and PSA.

Key words: Transforming growth factor-beta; cathepsin D; prostate specific antigen.

INTRODUCTION

Transforming growth factor-beta (TGF β) is a dimeric protein, which plays a crucial and diverse role in tumour biology. It is involved in tumour development, progression, invasion, angiogenesis, immune functions and extracellular matrix formation. Both TGF β_1 and TGF β_2 have been shown to be potent growth inhibitors of many human breast cancer cell lines *in vitro*.

TGF β was first purified from platelets¹ and was found to bind covalently to a specific serum binding protein, α_2 -macroglobulin in the serum.² It is also secreted by virtually all cell types as a biologically inactive molecule. Latent TGF β is either secreted as a small latent complex consisting of mature TGF β and latency-associated protein (LAP) or as a large latent complex in which latent transforming growth factor binding protein (LTBP) is disulphide-linked to LAP.³ LAP may prevent latent TGF β from binding directly to ubiquitously expressed receptors and permits the maintenance of a large extracellular reservoir of latent TGF β , which can be activated

rapidly when active TGF β is needed. As a result, changes in synthesis and secretion of latent TGF β may have little biological consequences, whereas changes in the activation of latent TGF β are likely to influence its diverse cellular and biological effects.

A significant amount of active TGF β has been shown to be secreted by MCF-7 cells into the conditioned serum-free media without any acid activation.⁴ The amount of active TGF β secreted increased significantly in the presence of growth inhibitory hormones (antioestrogen LY 117018, 4-OH-tamoxifen, tamoxifen, dexamethasone) but decreased on treatment with growth stimulatory hormones (insulin and oestrogen). A measurable level of TGF β_1 was secreted constantly over 42 hours by the MDA-MB-231 breast cancer cell line in conditioned media.⁵

Several proteases such as cathepsin D⁶ and prostate specific antigen (PSA)⁷ have been shown to activate latent TGF β *in vitro*. Cathepsin D is present as 52 kDa and 27 kDa forms in the sera

of breast cancer patients and normal women.⁸ Malignant breast tissue contained the two forms of cathepsin D found in sera and an additional 31 kDa form. The 31 kDa form was found in significantly higher amounts in breast cancer tissues compared with benign and normal breast tissues.⁸

PSA has been detected in normal, hyperplastic and cancerous breast tissues.⁹ PSA is a favourable prognostic indicator in breast cancer and its presence has been found to be significantly associated with smaller tumours, tumours with low S-phase fraction, diploid tumours, younger patient age, tumours with lower cellularity and steroid hormone receptors as well as patients with earlier stage of disease.⁹ PSA levels in breast cyst fluid or nipple aspirate fluid can vary from non-detectable up to ~ 5000 µg/L.¹⁰ The average intracystic concentration of PSA in type I breast cysts which are lined by apocrine epithelium was significantly lower than in type II breast cysts which are lined by flattened epithelium.¹⁰ PSA is also found in the milk of lactating women ranging from less than 0.01 µg/L to 350 µg/L.¹¹ PSA has been shown to activate latent human TGFβ in bone-derived human prostate adenocarcinoma cell line PC-3 conditioned medium.⁷

The aim of the present study was to assess whether the proteases, cathepsin D and PSA were capable of activating latent TGFβ in the MCF-7 hormone responsive and MDA-MB-231 hormone unresponsive human breast cancer cell lines.

MATERIALS AND METHODS

Cell Culture

The hormone-responsive MCF-7 and the hormone-unresponsive MDA-MB-231 breast cancer cell lines were newly purchased from ATCC. Both of the cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, BRL Life Technologies Inc) supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine in 5% CO₂ at 37 °C in a humidified atmosphere.

For the experiments, 6-well plates were seeded with approximately 1 x 10⁶ cells in the above-mentioned media (2 ml). All the experiments were done in triplicate. Cells were grown at 37 °C in 5% CO₂. The next day, the media was discarded and the cells were treated with 50 ng/ml, 100 ng/ml and 200 ng/ml of cathepsin D or 50 ng/ml, 200 ng/ml and 400 ng/ml of PSA in RPMI 1640 (without phenol red) containing 5%

charcoal-dextran-stripped FBS and 2 mM L-glutamine. A similar volume of media, without any cathepsin D or PSA, was added to the control wells. The cell-culture media was collected and used directly for the determination of active TGFβ₁ and TGFβ₂ without any acid or alkali activation after 4, 8, 24 and 72 hours of treatment.

Enzyme-Linked Immunosorbent Assay (ELISA) TGFβ₁ and TGFβ₂ immunoreactivity were measured using ELISA kits purchased from R & D Systems (Minneapolis, USA) without any acid or alkali activation. These assays measure mainly active TGFβ. Thus, any increase in TGFβ₁ and TGFβ₂ levels in the cell-culture media would be a result of activation. We ascertained whether any increase in active TGFβ₁ and TGFβ₂ levels was due to activation or increased production by measuring the amount of TGFβ₁ and TGFβ₂ mRNA present in the cells using RT-PCR.

The sensitivities of the Quantikine human TGFβ₁ and TGFβ₂ ELISA were determined by adding two standard deviations to the mean optical density value of 20 zero standard replicates and reading the corresponding concentration from the standard curve. The sensitivity of the TGFβ₁ assay was 13 pg/ml and that of the TGFβ₂ assay was 20 pg/ml.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from both MCF-7 and MDA-MB-231 cell lines were extracted with Trizol Reagent (Gibco, BRL Life Technologies Inc) according to the manufacturer's protocol. The RNA obtained (5 µl) was reverse-transcribed with M-MLV RT (200 U/µl) (Promega Corp.). The cDNA obtained was amplified with *Taq* DNA polymerase (in storage buffer B, Promega Corp.) after undergoing a hot start at 95 °C for 5 minutes. The nucleotide sequences for human TGFβ₁ were 5' GCC CTG GAC ACC AAC TAT TGC T 3' (sense) and 5' AGG CTC CAA ATG TAG GGG CAG G 3' (anti-sense).¹² PCR conditions were as follows: 1 minute at 95 °C for denaturation, then 1 minute at 63 °C for primer annealing, followed by 1 minute at 72 °C for elongation (30 cycles) and, finally, 7 minutes at 72 °C for final extension. The predicted size for TGFβ₁ was 161 bp.

The nucleotide sequence of the forward primer for human TGFβ₂ was 5' GCA GAA CCC AAA AGC CAG AG 3' and the reverse primer was 5' GGA CAC GCA GCA AGG AGA AG 3'.¹³ The PCR mixture was cycled at 95 °C for 1 minute

(denaturation), 57 °C for 1 minute (primer annealing), 72 °C for 1 minute (elongation) for 30 cycles and 72 °C for 7 minutes (final extension). The expected size for TGF β_2 was 685 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal housekeeping gene and was amplified at 94 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 1 minute (25 cycles) and 72 °C for 10 minutes without undergoing a hot start. The primer sequences were 5' ACC ACA GTC CAT GCC ATC AC 3' and 5' TCC ACC ACC CTG TTG CTG TA 3'. The predicted size for GAPDH was 451 bp.

The PCR products obtained were analysed by size fractionation using 1.5% agarose gels stained with ethidium bromide and was visualised by ultraviolet illumination, photographed and semi-quantified (by comparing the intensity of the marker with the intensity of each treatment) using GeneGenius Gel Documentation and analysis system Genesnap (Syngene, Cambridge, UK).

The amount of DNA (ng) determined by SynGene Gene Tool analysis for each treatment was compared with control, different treatment concentrations and different time intervals of each treatment. The ratio between the individual TGF β_1 and TGF β_2 DNA amounts and GAPDH DNA amount was calculated (GADPH normalisation).

Statistical analysis

The effects of cathepsin D and PSA on TGF β_1 and TGF β_2 levels were compared using the unpaired Student's *t*-test. Results were considered to be statistically significant when $p < 0.05$.

RESULTS

Effect of cathepsin D and PSA on TGF β_1 level
TGF β_1 was not detectable in the MCF-7 and MDA-MB-231 breast cancer cell culture media up to 72 hours of treatment.

Effect of cathepsin D and PSA on TGF β_2 level
TGF β_2 was not detectable in cathepsin D or PSA treated and control MCF-7 culture media up to 72 hours.

In cathepsin D or PSA treated and control MDA-MB-231 breast cancer cell culture media, TGF β_2 was not detectable at 4 hours of treatment (Fig. 1). TGF β_2 was detectable in cathepsin D or PSA treated and control culture media at 8 hours and 24 hours at concentrations ranging between

10 and 30 pg/ml. The TGF β_2 level increased significantly at 72 hours in both cathepsin D or PSA treated and control cell culture media. However, both cathepsin D and PSA were unable to activate the latent forms of TGF β_2 in this cell line since a similar level of TGF β_2 was found in the controls.

Effect of cathepsin D and PSA on TGF β_1 and TGF β_2 mRNA expressions

Both cathepsin D and PSA did not have any significant effect on TGF β_1 and TGF β_2 mRNA expression in the MCF-7 and MDA-MB-231 breast cancer cell lines over the incubation periods studied.

DISCUSSION

TGF β_1 was not detectable in the cathepsin D or PSA treated and control MCF-7 and MDA-MB-231 cell culture media up to 72 hours. It is possible that both cathepsin D and PSA are able to activate TGF β_1 in the culture media but this is then rapidly cleared or rapidly bound to the cell surface receptors.

The inability to detect TGF β_1 in the experiment may also indicate that the levels of latent TGF β_1 produced by the MCF-7 and MDA-MB-231 cell lines were very low, thus any activation by cathepsin D or PSA may not result in detectable levels of active TGF β_1 . The situation is further complicated by the fact that the proteolytic activities of cathepsin D and PSA may decrease with time as both of these proteases tend to bind to other substances that are present in the culture media.

It is also plausible that α_2 -macroglobulin may prevent the activation of latent TGF β by proteases by masking the active site of the protease or by binding directly to the protease.^{14,15} α_2 -Macroglobulin may form complexes with proteolytic enzymes at the 'bait' region of α_2 -macroglobulin which is then cleaved, causing a conformational change in α_2 -macroglobulin which sterically traps the proteases resulting in the removal of the proteases.¹⁶ Two forms of α_2 -macroglobulin exist: an electrophoretically slow form which is capable of binding proteases and an electrophoretically fast form resulting from protease binding.¹⁷ The slow form circulates in plasma while the fast form is reversibly produced after binding to proteases. The fast form can bind to cell receptors, be internalised and, thereby, cleared from the circulation. Hence, α_2 -macroglobulin may trap cathepsin D or PSA in the culture media and prevent them from

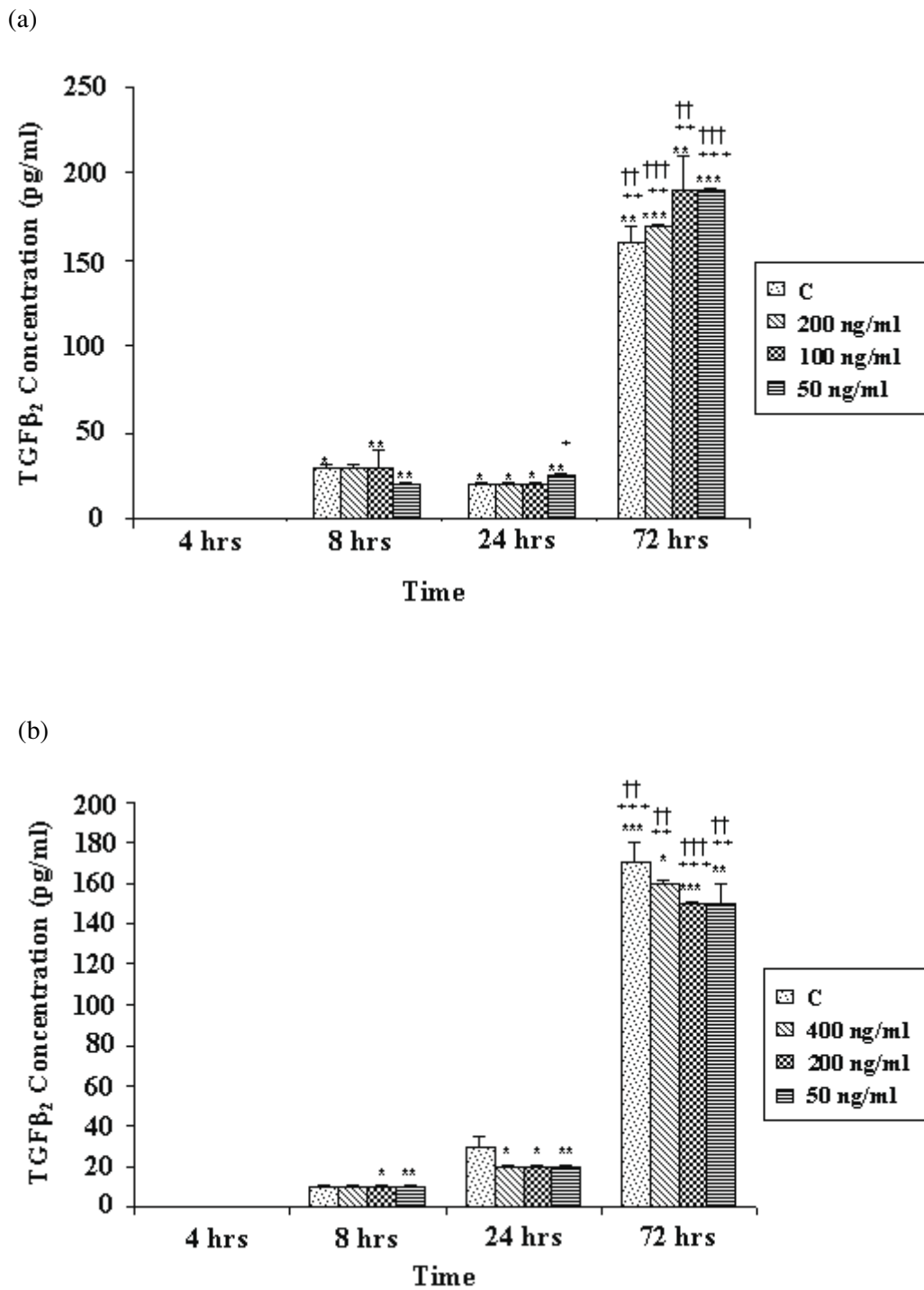


FIG. 1: Effect of (a) Cathepsin D and (b) PSA on conditioned media concentrations of TGFβ₂ in the MDA-MB-231 breast cancer cell line. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus results obtained for the corresponding treatment concentrations at 4 hours; *****P* < 0.001, ***P* < 0.01, **P* < 0.05 versus results obtained for the corresponding treatment concentrations at 8 hours; ††† *P* < 0.001, †† *P* < 0.01 versus results obtained for the corresponding treatment concentrations at 24 hours.

activating latent TGF β . In addition, α_2 -macroglobulin may also rapidly trap TGF β_1 or TGF β_2 that is present in the culture media. Crookston *et al.*¹⁸ have shown that TGF β_1 and TGF β_2 can bind to the fast form of α_2 -macroglobulin with the same affinity and can bind the slow form noncovalently.

TGF β_2 was not detectable at 4 hours in the treated and control MDA-MB-231 breast cancer cell culture media. Active TGF β_2 was detectable at 8 and 24 hours in low concentrations while significantly higher concentrations were found at 72 hours in both cathepsin D or PSA-treated and control MDA-MB-231 breast cancer cell culture media. Nevertheless, both cathepsin D and PSA were unable to activate latent TGF β_2 since the differences between treated and control groups were not statistically significant.

Lyons *et al.*⁶ were able to detect a faint 25 kDa band corresponding to the active form of TGF β by immunoprecipitation analysis of radiolabelled cell conditioned media after cathepsin D and plasmin treatment. They incubated confluent NRK-49F cells in 75 cm² tissue culture flask with [³⁵S]cysteine in cysteine-free MEM for 48 hours. The conditioned medium was treated with plasmin (0.4 U/ml) for 2 hours at 22 °C and cathepsin D. The number of MCF-7 and MDA-MB-231 cells in the 6-well plates which we used may have been too few compared with the NRK-49F cells in the 75 cm² tissue culture flasks. Hence, the amount of latent TGF β in the cell culture media may have been too low to detect even if the latent forms were activated by cathepsin D or PSA, with the exception of TGF β_2 in the MDA-MB-231 cell line.

In conclusion, both cathepsin D and PSA were unable to activate latent TGF β_1 and TGF β_2 in the MCF-7 and MDA-MB-231 breast cancer cell lines. A constant level of TGF β_2 mRNA in the control and treated MDA-MB-231 cells suggests that the increase in level of active TGF β_2 in the MDA-MB-231 cell line is likely to be due to activation by a mechanism independent of cathepsin D and PSA and was not due to increased production.

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