

Thymosin β_4 Secretion Due to Apoptotic Drugs in Macrophages and Fibroblasts

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Thymosin β_4 (t β_4), a 5 kDa protein, is a mediator of inflammation, with many functions in cellular motility, migration and damage repair. In spite of the fact that apoptotic cells exhibit pro-inflammatory signals, inflammation is absent during apoptosis. We believe that endogenous t β_4 is released during apoptosis from cells such as macrophages and fibroblasts, in turn suppressing inflammation. Our primary aims for this project were to develop and refine t β_4 detection assays/methods, mainly the ELISA. Competition fluorescence anisotropy was analyzed as a potential method for detecting t β_4 in cellular supernatant. The RAW 264.7 macrophage-like cell supernatant contained an unknown factor interfering with exogenous t β_4 added to cellular supernatant. We were unable to identify the interfering factor; however we were able to eliminate it through use of a protein concentrator, which likewise helped concentrate t β_4 in individual samples. These findings lay the groundwork for further investigation using apoptotic drugs as a t β_4 releasing agent in order to suppress inflammation.

Thymosin β_4 (t β_4) is a 43 amino acid, 5 kDa polypeptide with key roles in cell motility and organogenesis [1,2]. In addition, t β_4 has anti-inflammatory properties as seen in recent studies, where corneas treated with exogenous t β_4 exhibited higher recovery rates and decreased inflammation than those with a control of PBS [3]. During another study, exogenous t β_4 promoted survival and repair of postnatal cardiomyocytes after coronary ligation [4]. In these experiments, the effect of necrotic cells, which ordinarily cause inflammation to the surrounding living tissue, was diminished. Paradoxically, in spite of the release of pro-inflammatory mediators during apoptosis, apoptotic cells lack the inflammatory properties observed during cellular necrosis.

Based on these observations, we hypothesized that endogenous t β_4 released from cells during apoptosis suppresses the inflammatory response. A long-term research objective for this project is to determine drugs that trigger apoptosis, and subsequently cause cells to release t β_4 . These results could form the basis for development of new anti-inflammatory drugs. The FDA approved drug Paclitaxel, used against cancer, is one such an example of an apoptotic agent [5].

t β_4 is present in high concentrations in macrophages and fibroblasts [6,7]. Macrophage-like RAW 264.7 and fibroblast NIH-3T3 cell lines were used to study t β_4 release from apoptotic cells. Our hypothesis was that these cells treated with apoptotic drugs, such as Taxol or Etoposide, undergo apoptosis and release t β_4 in the extracellular media. Assays for detection of apoptosis, including measurements of Caspase-3 activation and

Annexin V exposure, were considered as a method to determine the percent of apoptotic cells.

Enzyme-Linked Immuno-Sorbant Assays (ELISA) were used to detect the concentration of t β_4 in cell supernatants. Based on our previous hypotheses, a positive correlation between the extent of apoptosis and the concentration of t β_4 in the media was anticipated. t β_4 exhibits extracellular activities at very low concentrations which indicates that t β_4 released into cellular media could be correspondingly small [8,9]. Therefore, quantitative and qualitative techniques for detection of t β_4 should be very sensitive. The possibility exists that ELISA assays are not sensitive enough to detect nanomolar concentrations at which t β_4 is observed to have activity.

METHODS

Cell cultures

RAW 264.7 cells were a generous gift from Dr. Shannon Holliday's Lab (University of Florida, Gainesville, FL). The cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 20 mg/L gentamycin and 4.5 g/L glucose, 90%; fetal bovine serum, 10%. Cells were cultured in 25 cm² plates and transferred once 80% confluency was reached.

Confluent 25 cm² flasks were scraped and frozen in a Styrofoam slow freeze contraption in DMEM/10% FBS supplemented with 5% DMSO at -80°C. Frozen cell vials were then transferred and stored in liquid nitrogen. Each freezer stock yielded enough for two plates when thawed.

Cell treatment with Paclitaxel

RAW 264.7 cells were treated with Paclitaxel (Taxol) 0-1000 nM diluted in 'no FBS' DMEM for a period of 10 hours at 37°C in 25cm² flasks. Controls were treated with 'no FBS' DMEM.

Standard ELISA

Immulon 4HBX ELISA plate was coated with 300 µl of recombinant tβ4 in 'No FBS' DMEM media containing Dulbecco's modified Eagle's medium (with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 20 mg/L gentamycin and 4.5 g/L glucose) and incubated at 22°C for 60 minutes. The plate was washed three times with PBS (0.43 mM Na₂PO₄, 0.27 mM KCl 13.7 mM NaCl), 0.02% Tween20 and blotted dry with a paper towel. Blocking buffer (5% BSA, 100 mM L-Lysine, pH 7.0) was added to each well and incubated at 22°C for 60 minutes. The plate was washed three times with PBS, 0.02% Tween20 and blotted dry with a paper towel. Primary antibody (monoclonal mouse anti-tβ4 produced in the University of Florida core facility) in PBS was added and incubated overnight at 4°C. The plate was washed three times with PBS, 0.02% Tween 20 and blotted dry with a paper towel. Secondary antibody (alkaline phosphatase mouse IgG, Jackson Labs, 115-055-003) in 0.01M Tris, 0.25 M NaCl was added and incubated at room temperature for 60 minutes. The plate was washed five times with 0.01 M Tris, 0.25 M NaCl and blotted dry with paper towel. Developing solution (5 ml DI H₂O, 2.5 µl 1M MgCl₂, 5 µl diethanolamine, 5 mg tablet of pNPP (Sigma S0942)) was added to each well. The plate was incubated at 22°C until color was visible. OD was read on a SpectraMax 5 spectrophotometer (Toronto, Canada) at 405 nm.

Fluorescence anisotropy

Data were collected using serial dilutions of samples in 0.3 ml in glass cuvettes with a Photon Technology International (South Brunswick, NJ) spectrofluorimeter. The Rhodamine labeled tβ4 (Rh-tβ4) was excited with a vertically polarized light at a wavelength of 546 nm, with horizontal (I_h) and vertical (I_v) components of the emitted light measured at 575 nm for ~30 s for each sample. Fluorescence anisotropy (r) was calculated using $r = (I_v - GI_h) / (I_v + 2GI_h)$. The G factor for each experiment was determined by exciting the labeled peptide in solution with a horizontally polarized light [10] and averaged over ~10 measurements. For the direct binding assay, anisotropy was measured as a function of mouse anti-tβ4 concentration with a Rh-tβ4 concentration of 10 nM. The observed anisotropy was a linear function of the concentration of Rh-tβ4 bound to the mouse anti-tβ4 antibody because total intensity of recombinant Rh-tβ4 fluorescence ($I_v + 2GI_h$) did not significantly change upon binding to monoclonal mouse anti-tβ4 [11].

Rhodamine-labeled tβ4 binding to plate

60 µl of Rhodamine labeled tβ4 was incubated in 'no FBS' DMEM or sample for 60 minutes at 22°C. Excitation at a wavelength of 530 nm and emission at 570 nm was measured on a spectrophotometer (SpectroMax 5) prior to incubation, after incubation, and after three washes of PBS.

Tubulin ELISA

Method described above under standard ELISA. Tubulin (Cytoskeleton, ML113) was used in place of the recombinant tβ4. Mouse Anti-Tubulin antibody (Sigma, T5168) was used in place of primary monoclonal mouse anti-tβ4 antibody.

Protein concentration

Cell-free supernatant and 'no FBS' DMEM were each adjusted to contain recombinant tβ4 (33.3 nM, 1 ml total volume). Each sample was loaded onto a Centricon-3 concentrator (Amicon, 42403) and spun for 45 minutes at 7500x g on a table-top centrifuge. Samples were washed by adding 1 ml of PBS to the concentrate. Samples were centrifuged again for 45 minutes at 7500x g. Initial flow-through and final concentrate were removed and analyzed using an ELISA assay.

RESULTS

A potential alternative method for tβ4 detection.

A fluorescence anisotropy assay was used to determine affinity of monoclonal mouse anti-tβ4 affinity to Rhodamine labeled tβ4 (Rh-tβ4). Increasing concentrations of monoclonal anti-mouse tβ4 were added to Rh-tβ4 (10 nM) until a maximal binding was achieved (Fig. 1). Direct binding data showed that tβ4 has a low affinity for monoclonal mouse anti-tβ4, with a K_d of 380 nM (Fig. 1). K_d is the equilibrium dissociation constant which has an inverse correlation to affinity. Using Osmolyte trimethylamine-N-oxide (TMAO), a molecular crowding agent, is a potential strategy to increase affinity and decrease K_d . The affinity of Rh-tβ4 to mouse anti-tβ4, while increased ($K_d = 120$ nM), was still low (Fig. 1).

Contents of RAW 264.7 cell-free supernatant interfere with the identification/quantification of endogenous tβ4 using an ELISA assay

In developing a standard ELISA for tβ4 using recombinant protein, a sensitivity of 5 pmol was determined (Fig. 2). Macrophage-like RAW 264.7 cells were treated with Paclitaxel and cellular supernatants were assayed for tβ4 using an ELISA assay (Fig. 2). No tβ4 was detected in the cellular supernatants with standard ELISA. The effect of cell-free supernatant on the sensitivity of ELISA detection was tested using modified tβ4 ELISA protocol (Fig. 3) as described below. Supernatant was collected from RAW 264.7 cells exposed

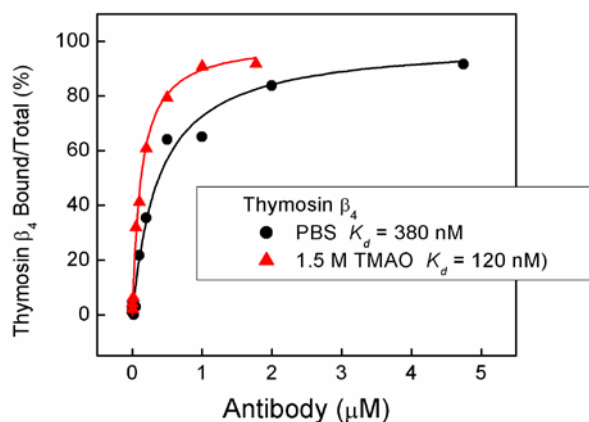


Fig. 1. Direct binding of monoclonal mouse anti-t β_4 to Rhodamine labeled (Rh-t β_4) using fluorescence anisotropy. Increasing concentrations of monoclonal anti-mouse t β_4 were added to Rh-t β_4 (10 nM) diluted in PBS buffer. Monoclonal mouse anti-t β_4 has a low affinity for t β_4 , with a K_d of 380 nM. The same assay was performed again in presence of at 1.5 M osmolyte trimethylamine-N-oxide (TMAO), a molecular crowding agent. TMAO was a potential strategy to increase affinity and decrease K_d , however the affinity of Rh-t β_4 to mouse anti-t β_4 , while increased ($K_d = 120$ nM), was still low. These data indicate that competition fluorescence anisotropy would not provide enough sensitivity with this monoclonal mouse anti-t β_4 antibody.

to 'no FBS' DMEM for 1 hour. The standard ELISA protocol described in Methods section was modified by including an extra 1 hour 'preincubation' prior to the normal 'incubation' with recombinant t β_4 or treated cell sample. The following were performed in duplicate with three PBS washes between each preincubation and incubation. (1) *Preincubation*: cell-free supernatant. *Incubation*: 66.6 nM t β_4 in 'no FBS' DMEM (2) *Preincubation*: 'no FBS' DMEM. *Incubation*: cell-free supernatants adjusted to 66.6 nM recombinant t β_4 (3) *Preincubation*: 66.6 nM t β_4 in 'no FBS' DMEM. *Incubation*: cell-free supernatant (4) *Preincubation*: 'no FBS' DMEM. *Incubation*: 0 or 66.6 nM recombinant t β_4 in 'no FBS' DMEM. Whenever the supernatant was incubated with the t β_4 , or prior to the t β_4 , an inhibition of OD signal at 405 nm occurred. The absorption dropped from 66.6 nM levels to 0 nM levels. In the samples where supernatant was added after t β_4 and allowed to attach to the plate, the OD signal was uninhibited. The observed inhibition of OD signal was confirmed by repeating the same experiment in triplicate (data not shown).

Contents of RAW 264.7 cell-free supernatant prevent the binding of Rh-t β_4 to ELISA plate

Rhodamine labeled t β_4 was used to confirm whether t β_4 bound to the ELISA plate in the presence of cell-free supernatant (Fig. 4). Fluorescence of Rh-t β_4 was a linear function of its concentration, as expected, when 60 μ l volume samples of various t β_4 concentrations

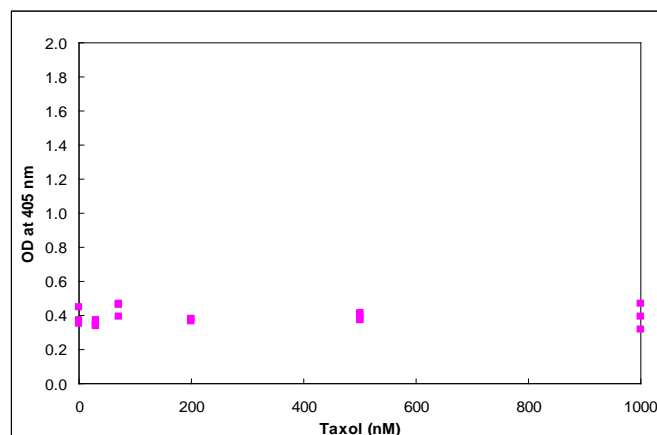
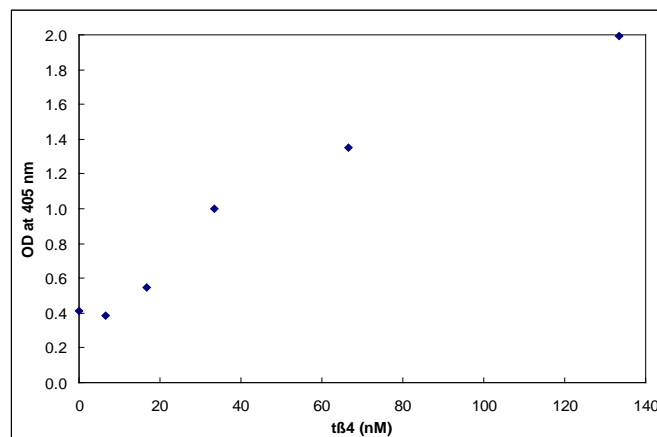


Fig. 2. ELISA of recombinant t β_4 in 'no FBS' DMEM and supernatant derived from Paclitaxel treated RAW 264.7 cells. Standard ELISA protocol described in Methods was followed for this assay. Paclitaxel treated (0-1000 nM) RAW 264.7 cells showed no visible t β_4 in their supernatant which could be due to an inhibitor in the supernatant or a concentration of t β_4 too low to detect with the ELISA.

were added to an ELISA plate and read on a spectrophotometer (Fig. 4A). A maximum of ~ 2 pmol Rh-t β_4 remained bound to the plate after three washes with PBS regardless of the initial concentration loaded. Rh-t β_4 was incubated on an ELISA plate in the presence or absence of cell-free supernatant (Fig. 4B). Cell-free supernatant and 'no FBS' DMEM were each adjusted to contain 1.6 μ M Rh-t β_4 and incubated on an ELISA plate for one hour. Emission from the samples was identical before and after the incubation. The ELISA plate was subsequently washed with PBS as per standard ELISA protocol and read for emission on the spectrophotometer. The cell-free supernatant/Rh-t β_4 samples had lower emission as compared to the 'no FBS' DMEM/Rh-t β_4 samples.

Interfering component of RAW 264.7 cell-free supernatant not specific for tβ4

In order to remove the inhibitor from the supernatant, an important step was to determine whether the inhibition was specific to t β_4 , or whether another ELISA would have

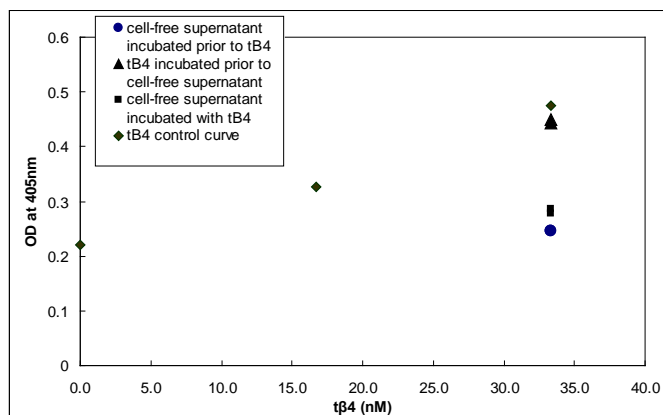


Fig. 3. Modified ELISA assay designed to reveal inhibition effects of cell-free supernatant. Standard ELISA protocol was modified to include a 1 hour 'preincubation' (P) before incubation (I) step. The samples were incubated as follows- (1) P: cell-free supernatant. I: 66.6 nM tβ4 in 'no FBS' DMEM, (2) P: 66.6 nM tβ4 in 'no FBS' DMEM. I: cell-free supernatant, (3) P: 'no FBS' DMEM. I: cell-free supernatants adjusted to 66.6 nM recombinant tβ4 (4) P: 'no FBS' DMEM. I: 0 or 66.6 nM recombinant tβ4 in 'no FBS' DMEM. After the plate was saturated with recombinant tβ4, cell-free supernatant could not inhibit ELISA absorbance and therefore, the inhibitor does not displace tβ4 bound to the plate or form a complex unrecognizable by primary mouse anti-tβ4.

inhibition as well. Using the developed standard ELISA protocol, an ELISA to an unrelated protein, Tubulin, was

developed (Fig. 5A). The ELISA plate was preincubated with 'no FBS' DMEM or cell-free supernatant. Tubulin was incubated in 1.6 μM concentration. When the cell-free supernatant was preincubated, the signal from Tubulin was inhibited by 50% as compared to the 'no FBS' DMEM preincubation.

Large cellular debris and lipids from RAW 264.7 cell-free supernatant are not responsible for tβ4 ELISA inhibition

In order to remove the possibility that large cellular debris or lipids were responsible for ELISA inhibition, RAW 267.4 cell-free supernatants were exposed to 80°C for 10 minutes, followed by centrifugation at 17,746x g for 10 minutes on a table-top centrifuge (Fig. 6). The treated supernatants were preincubated on an ELISA plate, followed by 33.3 nM recombinant tβ4 in 'no FBS' DMEM. Control wells were preincubated with 'no FBS' DMEM followed by 33.3 nM recombinant tβ4 in 'no FBS' DMEM. High temperature and centrifugation failed to remove the inhibitory component from the cell-free supernatant.

Inhibitor from RAW 264.7 cell-free supernatant separated using Centricon-3 concentrator

To eliminate small molecules in the cell-free supernatant, cell-free supernatant with recombinant tβ4 was concentrated and washed using a protein concentrator with upper cutoff of 3kDa (Fig. 7). The following

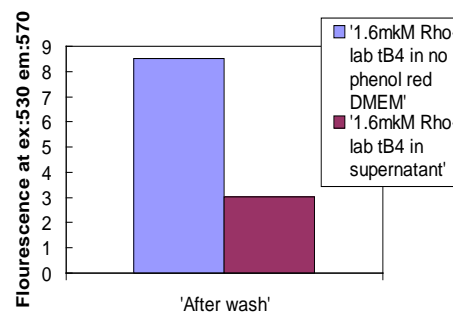
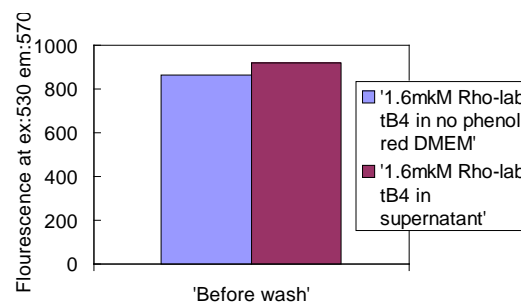
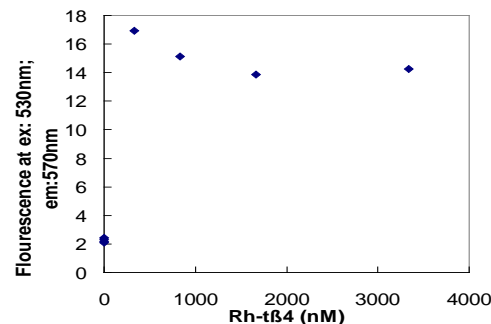
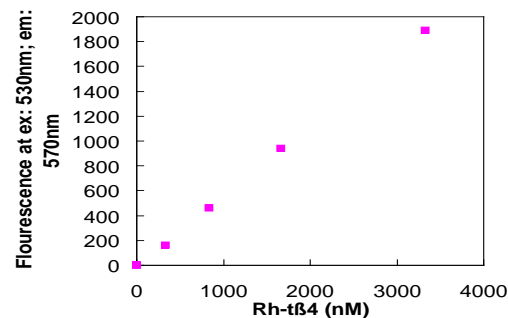


Fig. 4. Rhodamine labeled tβ4 (Rh-tβ4) binding to ELISA plate in the presence of cell-free supernatant. The Rh-tβ4 fluorescence was a linear function of Rh-tβ4 concentration when added to an ELISA plate and read on a spectrophotometer at Excitation: 530nm, Emission: 570nm (A). A maximum of 2 pmol of Rh-tβ4 remained bound to the plate after three washes with PBS regardless of the initial concentration loaded (B). Cell-free supernatant and 'no FBS' DMEM were each adjusted to contain 1.6 μM Rh-tβ4 and incubated on an ELISA plate (C). After wash, the cell-free supernatant/Rh-tβ4 samples had lower emission as compared to the 'no FBS' DMEM/Rh-tβ4 samples, indicating that Rh-tβ4 was unable to bind to the ELISA plate in the presence of cell-free supernatant (D).

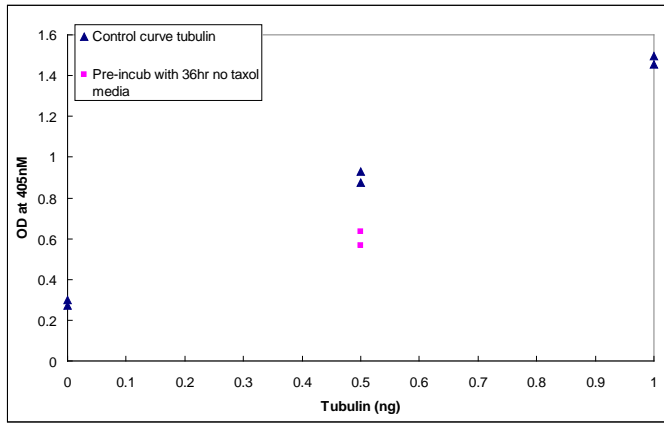


Fig. 5. Component in RAW 264.7 cell-free supernatant inhibits the standard ELISA assay for recombinant tubulin. The standard ELISA protocol was adapted for tubulin by using recombinant tubulin and monoclonal mouse anti- α -tubulin. Preincubation with cell-free supernatant as compared to 'no FBS' DMEM caused a 50% inhibition of 1.6 μ M tubulin signal, and demonstrated that the inhibitor was non-specific to t β 4.

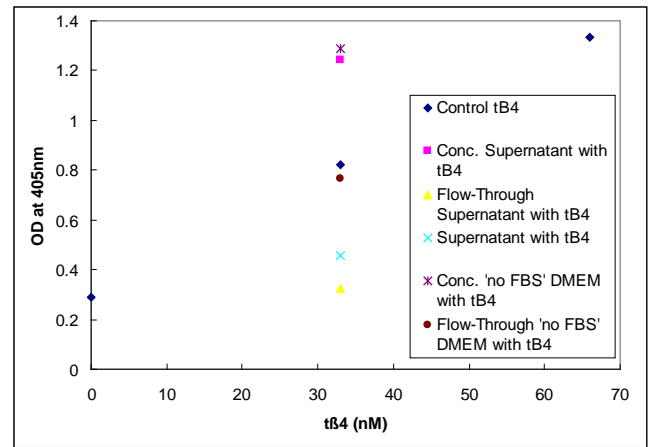


Fig. 7. ELISA of concentrates and flow-throughs from RAW 264.7 cell free supernatant and 'no FBS' DMEM adjusted to contain recombinant t β 4 (33.3 nM, 1 ml total volume). Each sample (1 ml of volume) was loaded onto a Centricon-3 concentrator (Amicon, 42403) and concentrated at 7500x g on a table-top centrifuge with 1 ml of PBS as a wash step. Control recombinant t β 4 (0, 33.3 nM and 66.7 nM) was diluted in 'no FBS' DMEM. All shown data is in duplicate. The Centricon-3 concentrator was efficient in removing the inhibitor from the cell-free supernatant however was not highly efficient for t β 4 concentration.

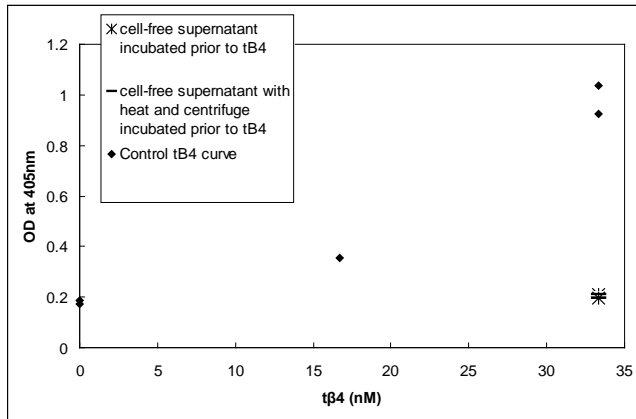


Fig. 6. ELISA of RAW 267.4 cell-free supernatants exposed to 80°C for 10 minutes, followed by centrifugation at 17,746 x g as compared to control recombinant t β 4 in 'no FBS' DMEM. Supernatant was preincubated followed by 33.3 nM recombinant t β 4 in 'no FBS' DMEM. Control t β 4 wells were preincubated with 'no FBS' DMEM. The centrifugation and heat samples still inhibited the signal from t β 4 in the ELISA, therefore indicating large cellular debris and lipids were not responsible for ELISA inhibition

experiment was performed in duplicate: RAW 264.7 cell free supernatant was adjusted to contain recombinant t β 4 (33.3 nM, 1 ml total volume). A control of 'no FBS' DMEM was adjusted to contain recombinant t β 4 (33.3 nM, 1 ml total volume). Each sample (1 ml of volume) was loaded onto a Centricon-3 concentrator (Amicon, 42403) and spun for 45 minutes at 7500x g on a table-top centrifuge. Initial flow-through was removed and saved for an ELISA assay. 1 ml of PBS was added to the concentrate as a wash step. Samples were spun again for 45 minutes at 7500x g (until 100 μ l were left of concentrate).

Using the concentrate and flow-through samples, an ELISA was performed. Control recombinant t β 4 (0, 33.3 nM and 66.7 nM) was diluted in 'no FBS' DMEM and showed a linear correlation as expected. Recombinant t β 4 with 'no FBS' DMEM and in cell-free supernatant in the Centricon-3 showed increase of t β 4 to 66.7 nM in the concentrate as compared to the control (33.3 nM). Recombinant t β 4 with 'no FBS' DMEM flow-through showed an equal amount of t β 4 as compared to the control. Uncentrifuged supernatant had a decreased signal of ~80% (down to 5 nM) as compared to the control (33.3 nM). Recombinant t β 4 with supernatant flow-through showed 100% (0 nM) inhibition of protein signal as compared to the control.

DISCUSSION

Working under the hypothesis that endogenous t β 4, a known anti-inflammatory agent, is released during apoptosis, therefore preventing inflammation, one of the primary goals of our project is to develop methods for detecting t β 4 in cell free supernatants of apoptotic cells.

The ELISA assay was used as a potential methods of detecting endogenous t β 4 and increasing sensitivity to t β 4 was the initial step. The lowest detectable recombinant t β 4 concentration was 5 pmol as seen in the ELISA. t β 4 has anti-inflammatory effects at low nM and even pM concentrations and therefore, our sensitivity of 20 nM for ELISA is possibly not high enough to detect physiologically relevant release of t β 4 [8,9].

Competition fluorescence anisotropy measures native t β 4 concentration in media or buffer by using fluorescent Rhodamine-labeled t β 4 (Rh-t β 4) to compete for the same binding site on an antibody. With increasing concentrations of unlabeled t β 4, the signal from Rh-t β 4/antibody complex diminishes until a saturation point with native t β 4 is reached, and all of the Rh-t β 4 is displaced. In order to effectively measure low t β 4 concentrations in a sample, an antibody with high affinity to an antigen is necessary. Direct binding fluorescence anisotropy assay was used to determine the affinity of monoclonal mouse anti-t β 4 antibody for t β 4, which turned out to be low (Fig. 1). With the addition of a molecular crowding agent, TMAO, the affinity increased 3 times, although this is not enough. The low affinity of monoclonal mouse anti-t β 4 suggests that competition fluorescence anisotropy using this particular antibody is not an effective method for identifying and/or quantifying t β 4 in cell-free supernatants.

Supernatant from RAW 264.7 cells treated with Paclitaxel, a known apoptotic agent, was assayed in the t β 4 ELISA mentioned above. Contrary to our expectations, no appreciable t β 4 signals in the supernatants could be detected as compared to the control, with recombinant t β 4 in 'no FBS' DMEM (Fig. 2B). A possible explanation is that t β 4 concentrations in cell-free supernatants with and without taxol treatment were lower than ELISA sensitivity. However, when t β 4 was added to the cell-free supernatants and analyzed in an ELISA for control purposes, an inhibitory factor was found (Fig. 3).

We stipulated the following explanations as to why t β 4 was not detected in supernatants by the ELISA assay. A component of the cell supernatant has some effect on the ELISA and could be acting to inhibit the assay by: (1) displacing t β 4 bound to the ELISA plate, (2) forming a complex with t β 4 which cannot bind to the plate surface, (3) binding to t β 4 and forming a complex which cannot interact with the primary mouse anti-t β 4 antibody, (4) binding to the plate, blocking all available t β 4 binding sites.

The ELISA protocol was modified to include a 1 hour preincubation step prior to the initial incubation. Incubating supernatant prior to, with, and after t β 4 revealed the affects of supernatant on the ELISA. After the plate was saturated with recombinant t β 4, cell-free supernatant could not inhibit ELISA absorbance. Therefore, the inhibitor neither displaces t β 4 bound to the plate nor forms with t β 4 a complex unrecognizable by primary mouse anti-t β 4. When supernatant was added to the plate to which t β 4 was already bound, it could neither displace t β 4 nor form a complex unrecognizable by primary mouse anti-t β 4. In the experiment when cell supernatant was incubated in the plate prior to addition of t β 4, it was washed out before t β 4 addition and therefore never contacted t β 4 directly in solution. Yet it could still inhibit ELISA. Therefore the

hypothesis for forming a complex with t β 4 which cannot bind to the plate surface also seems unlikely.

In order to determine whether the t β 4 ELISA was inhibited at the binding step, Rh-t β 4 was plated in the presence of cell-free supernatant. The cell-free supernatant/Rh-t β 4 samples had lower emission as compared to the 'no FBS' DMEM/Rh-t β 4 samples, indicating that Rh-t β 4 was unable to bind to the ELISA plate in the presence of cell-free supernatant (Fig. 4). Determination of whether the ELISA inhibition was specific for t β 4 was accomplished through an ELISA of a non-related protein using supernatant to preincubate the wells. The developed Tubulin ELISA demonstrated an inhibition of signal from a component of the cell-free supernatant, and therefore, that the interaction with t β 4 was non-specific (Fig. 5). Large cellular debris and lipids were still present in supernatant and were possibly responsible for ELISA inhibition. Heating and centrifugation of supernatant did not remove the inhibitory affects from the supernatant, therefore indicating large cellular debris and lipids were not the causative agent in decreased signal (Fig. 6).

The Centricon-3 concentrator was efficient in removing the inhibitor from the cell-free supernatant; however, it was not highly efficient for t β 4 concentration (Fig. 7). The concentration of t β 4 increased only twice, whereas the volume was decreased ten times, indicating a large amount (80%) of t β 4 (5kDa) passed through the 3kDa pores. Since the interfering was washed away through 3kDa pores of the cellulose acetate membrane, most likely the inhibitor was a small molecule which interacted with the ELISA plate to prevent t β 4 binding to the plate.

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