The Effect of Fibronectin Extra Domain A on the Proliferation of Triple Negative Breast Cancer Cell Lines

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Abstract

Breast Cancer affects many women around the world, as it is one of the most common cancers in women. One of the types of breast cancers is triple-negative breast cancer (TNBC). Triple-negative breast cancer is negative to three things: the human epidermal growth factor receptor 2, estrogen receptor, and progesterone receptor. My study aims to see if Fibronectin EDA will decrease the proliferation of three types of TNBC: MCF7, MDA-MB-468, and MDA-MB-231. In this study we tested each cell line with different dosages of FnEDA to see the response. We also tested to see if this response was by the activation of TLR5. We tested this by using an anti TLR5 neutralizing antibody to see if it would rescue the proliferation of the different cell types. It was confirmed that MCF7 and MDA-MB-468 cells are suppressed by FnEDA and that the proliferation was rescued by the anti TLR5 neutralizing antibody.

Introduction

In recent years, breast cancer (BC) has confidently taken a leading position in the structure of morbidity and mortality from cancer in most countries of Eastern and Western Europe, Asia, and America. According to a study by the global project GLOBOCAN, it is the most common cancer in women, accounting for 25.1% of all cancers. BC is a very heterogeneous disease with a different course, prognosis, sensitivity to therapy, and other characteristics, due to the variety of genetic aberrations against this disease. Most often, an immunohistochemical (IHC) study is used to determine the subtype of the disease, which allows determining the level of expression of receptors on the surface of tumor cells – estrogen receptors (ER), progesterone receptors (PR), and oncoprotein HER-2/neu (human epidermal growth factor receptor 2 – the second epidermal growth factor receptor). (Baranova, 2022)

Gene expression studies have identified several major subtypes of breast cancer: the luminal subtypes, which typically express hormone receptor-related genes, and two hormone receptor-negative subtypes – the human epidermal growth factor receptor 2 (HER2) positive/estrogen receptor (ER) negative subtype and the basal-like subtype. (Irvin, 2008)

In arrays, BBCs are characterized by low expression of ER-related genes and HER2-related genes; for this reason in clinical specimens they are usually ER-negative, progesterone receptor (PR) PR-negative and lack HER2 overexpression. This is called the ‘triple-negative’ phenotype. (Irvin, 2008) The ‘triple-negative’ phenotype is resistant to many types of treatments and therapies.

In a study that analyzed the frequency of local and locoregional metastases in 335 patients who underwent organ-sparing surgery and neoadjuvant therapy for stage 2–3 breast cancer, TNBC was detected in 61 patients (18.2%). At TNBC, the 5-year survival rates without locoregional and local recurrences were the lowest compared to other subtypes (79.6% and 84.6%, respectively). Locoregional recurrences were detected in 21.3% (most often in subclavian and intramammary lymph nodes), local – in 14.8%, distant metastases – in 29.5% of patients with this subtype of breast cancer. According to the multivariate analysis, the triple-negative subtype had the highest risk of local recurrence (Baranova, 2022). The cell lines used in this study were MDA-MB-468, MDA-MB-231, and MCF7.

Increasing evidence showed that Toll-like receptors (TLR), key receptors in innate immunity, play a role in cancer progression and development but activation of different TLRs might exhibit the exact opposite outcome, antitumor or protumor effects. TLR function has been extensively studied in innate immune cells, so we investigated the role of TLR signaling in breast cancer epithelial cells. We found that TLR5 was highly expressed in breast carcinomas and that TLR5 signaling pathway is overly responsive in

breast cancer cells. Interestingly, flagellin/TLR5 signaling in breast cancer cells inhibits cell proliferation and anchorage-independent growth, a hallmark of tumorigenic transformation. In addition, the secretion of soluble factors induced by flagellin contributed to the growth-inhibitory activity in an autocrine fashion. The inhibitory activity was further confirmed in mouse xenografts of human breast cancer cells. These findings indicate that TLR5 activation by flagellin mediates innate immune response to elicit potent antitumor activity in breast cancer cells themselves, which may serve as a novel therapeutic target for human breast cancer therapy. (Cai, 2011)

Within this study the glycoprotein of the extracellular matrix, Fibronectin is used. The microenvironment of tumors is characterized by structural changes in the fibronectin matrix, which include increased deposition of the EDA isoform of fibronectin and the unfolding of the fibronectin Type III domains. The impact of these structural changes on tumor progression is not well understood. The fibronectin EDA (FnEDA) domain and the partially unfolded first Type III domain of fibronectin (FnIII-1c) have been identified as endogenous damage-associated molecular pattern molecules (DAMPs), which induce innate immune responses by serving as agonists for Toll-Like Receptors (TLRs).

In response to paracrine signals from the tumor cells, stromal fibroblasts differentiate into highly contractile myofibroblasts. These cancer-associated fibroblasts (CAFs) assemble an extracellular matrix (ECM) enriched for the alternatively spliced isoform of fibronectin which contains an extra Type III Domain, Extra Domain A (EDA). EDA fibronectin has been identified as a damage-associated molecular pattern molecule (DAMP). DAMPs are endogenous molecules, derived from the extracellular matrix or released from damaged cells, which mediate sterile inflammation in a variety of cell types through the activation of Pattern Recognition Receptors (PRRs). (Ambesi, 2022)

Statement of Purpose and Hypotheses

There were two purposes to my study:

1. To examine if in vitro treatment of breast cancer cells with recombinant Fibronectin EDA (FnEDA) suppresses cell proliferation
2. To determine if the suppression result in response to FnEDA was Toll-like receptor 5 (TLR5) dependent

We hypothesized that the Fibronectin EDA domain will suppress breast cancer cell proliferation. We will test this by performing experiments that count cells at different time intervals in the presences of increasing concentrations of cell treatments. We also hypothesized that the suppression in response to the FnEDA will be TLR5 dependent. We will test this secondary hypothesis by performing cell counts in the presence of cell treatments and a TLR5 neutralizing antibody.

Methods and Materials

Cell Culture - Human breast adenocarcinoma cell lines, MDA-MB-468 (ATCC; HBT-132) and MDA-MB-231, and MCF7 (ATCC; CRM-HTB-26), were maintained in a complete medium [Dulbecco's modified eagle medium (DMEM), Invitrogen/Life Technologies, Corp., Grand Island, NY, USA) supplemented with 1% Pen-Strep (Gibco), 1% GlutaMAX (Gibco) and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA)] in a humidified chamber at 37 °C/8% CO_2. (Ambesi, 2022)

Cell Treatments - Unless otherwise stated, cells were plated onto 48-well culture plates (3×10^4 cells per well) in complete medium, cultured overnight, then rinsed once with 0.1% BSA/DMEM [serum-free medium; DMEM containing 0.1% bovine serum albumin (BSA; Roche Applied Science, Indianapolis, IN, USA), 1% Pen-Strep, 1% GlutaMAX, 1X non-essential amino acids (NEAA; Gibco) and 10 mM HEPES (Gibco)] prior to all treatments. Treatment with inhibitors or blocking antibodies was typically carried out in serum-free medium for 1 h prior to the addition of fibronectin DAMPs (FnEDA and FnIII-1c) and TLR PAMPs (flagellin and Pam3CSK4). Specific treatment times are described in the Figure Legends. Unless otherwise stated, experiments where conditioned medium was collected and used for IL-8 expression analysis, cells were treated for 4 h. IL-8 was measured using a human enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's recommended procedure. To capture phosphorylated intermediates of the NFκB signaling pathway, cells were treated with DAMPs and PAMPs for 1 h prior to lysis (Ambesi, 2022)

Antibodies and Reagents - TLR5 neutralizing antibody and TLR agonists (flagellin) were obtained from InvivoGen (San Diego, CA, USA). Recombinant FnEDA… prepared and purified as previously described. (Ambesi, 2022)

Invitrogen Countess 3 Automated Cell Counter - Used for determining cell density (cells/ml) after lifting and resuspending.

EVOS Digital Cell Imaging System - Used to image cells that were fixed and stained with Crystal Violet over 4 days.

BioTek Synergy 2 Plate Reader and BioTek Gen 5 - For data collection (absorbance values) of eluted dye. After fixation cells were stained with toluidine blue and rinsed, stain was eluted to a 96-well plate.

Proliferation Assay - Breast cancer cells (MDA-MB-468, MCF7, and MDA-MB-231) were maintained in T75 tissue culture flask. To perform the proliferation assay all of these steps were done under a tissue culture hood. Media was aspirated from the cells and the cells were rinsed with 10 ml of

37°C PBS EDTA, which was aspirated after a rinse. Then 3 ml of 0.25% trypsin (37°C) was added to each flask until the cells detached. The trypsin was neutralized with 10 ml of complete media (37°C). The cells were then transferred to a 15 ml conical tube and spun in a centrifuge for 1000 rpm for 5 minutes at room temperature. The cells were then counted and diluted to 20,000 cells/ml in complete media in 14 ml total volume. Then 3 ml of cell suspension was placed into wells of four 6-well tissue culture plates and incubated at 37°C and 8% CO2. The cells were fixed for the next 4 consecutive days and imaged.

Dose Dependent Response - Breast cancer cells (MDA-MB-468, MCF7, MDA-MB-231) were maintained in T75 tissue culture flasks and all steps were performed in a tissue culture hood under sterile conditions. Media was aspirated from the cells and the cells were rinsed with 10 ml of 37°C PBS EDTA, which was aspirated after a rinse. Then 3 ml of 0.25% trypsin (37°C) was added to each flask until the cells detached. The trypsin was neutralized with 10 ml of complete media (37°C). The cells were then transferred to a 15 ml conical tube and spun in a centrifuge for 1000 rpm for 5 minutes at room temperature. The cells were then counted and diluted to 250,000 cells in 15 ml. Then 0.3 ml of cell suspension was placed into wells of a 48-well tissue culture plate and 3 wells of a second 48-well plate. Place 0.1 ml of flagellin or EDA of each concentration, to each respective well. Then place 0.4 ml of complete media in 3 empty wells of each of the plates as a negative control. Culture the cells at 37°C and 8% CO2 for 1 day (for plate #1) and 4 days (remainder). After 24 hours aspirate media from plate #1 and fix cells with paraformaldehyde. Store sealed with parafilm at 4°C until remaining plates are fixed over the next 4 days.

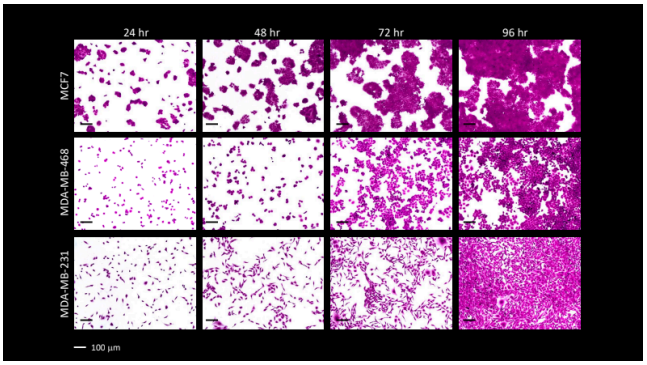
Effects of TLR5 Neutralizing Antibody - Breast cancer cells (MDA-MB-468, MCF7, MDA-MB-231) were maintained in T75 tissue culture flasks and all steps were performed in a tissue culture hood under sterile conditions. Media was aspirated from the cells and the cells were rinsed with 10 ml of 37°C PBS EDTA, which was aspirated after a rinse. Then 3 ml of 0.25% trypsin (37°C) was added to each flask until the cells detached. The trypsin was neutralized with 10 ml of complete media (37°C). The cells were then transferred to a 15 ml conical tube and spun in a centrifuge for 1000 rpm for 5 minutes at room temperature. The cells were then counted and diluted to 200,000 cells in 12 ml. Then 0.3 ml of cell suspension was placed into wells of a 48-well tissue culture plate. Add 0.05 ml of anti-TLRr5 neutralizing antibody or 0.05 ml of control IgG to appropriate wells. Incubate at 37°C and 8% CO2 for 60 minutes. Then add either flagellin or FnEDA to designated wells and use complete medium as no-treatment control. Place 0.4 ml of complete media in 3 empty wells of the 48-well plates to act as a negative control. After 24 hours, aspirate media from plate #1 and fix cells with paraformaldehyde; store sealed with parafilm at 4°C until remaining plates are fixed. Repeat this step after 4 days on treated cells.

Toluidine Blue Assay - Warm fixed cell tissue culture plates to room temperature. Aspirate off fixative, paraformaldehyde. Then wash the cells 3 times with 0.5 ml distilled H2O. Then add 0.25 ml

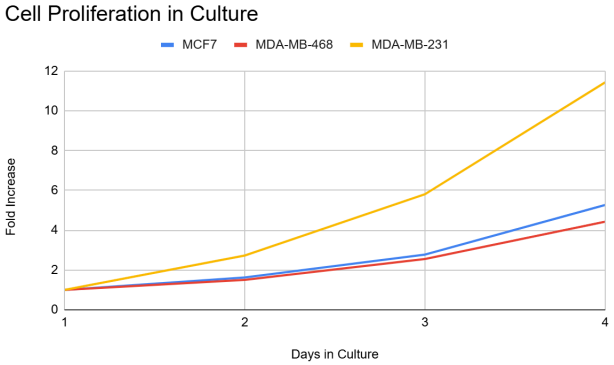
0.05% toluidine blue dissolved in distilled H2O. Allow the toluidine blue to sit on the cells for at least an hour and then aspirate the toluidine blue off. After that wash the cell layers 4 times with 0.5 ml distilled H2O. Then elute toluidine blue with 250 µl of 10% acetic acid and swirl plates for 5 minutes. Lastly transfer 200 µl to a 96-well plate and read OD650-OD405 using a cell-free well as a blank.

Results

Crystal Violet Staining and Toluidine Blue Assay for Cell proliferation - Figure 1.1 shows images of the crystal violet stained cells. The crystal violet stains the proteins and nucleic acids of each cell. This pictures all 3 cell types in culture over the course of 4 days and shows that each cell line increases exponentially. Figure 1.2 shows the average proliferation of the three different cell lines used in this study over a 4 day period. The cell proliferation graph shows that the MCF7 cell line increased (on average) by 5.2 fold over the period. MDA-MB-468 cells were shown to have increased slightly less with a 4.4 fold increase. The MDA-MB-231 cells increased the most dramatically with an 11.4 fold increase.

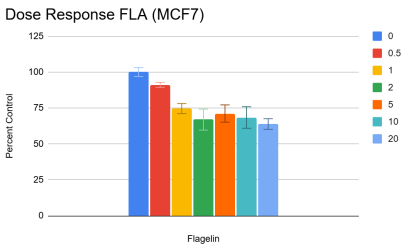


(Figure 1.1) *Crystal violet staining of each cell line*

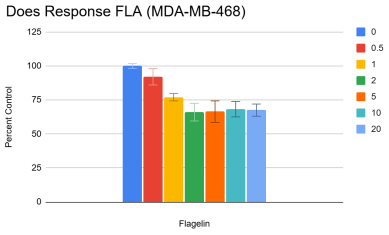


(Figure 1.2) *Graph of Proliferation fold increases of each cell line (all error bars are based on SD)*

Dose Dependent Responses (FLA) - Figures 2.1, 2.2, and 2.3 show the average dose responses for MCF7, MDA-MB-468, and MDA-MB-231 cell lines to Flagellin over the course of 4 days. In Figure 2.1 it shows that the MCF7 proliferation was suppressed by the flagellin and responded the most to the 20 nanogram dose. In Figure 2.2 it shows that the MDA-MB-468 cell proliferation was also suppressed by the flagellin and responded the most by the 2 nanogram dose. In Figure 2.3 it shows that the MDA-MB-231 cell proliferation was not affected by the flagellin doses.



(Figure 2.1) (*All error bars are based on SD)*



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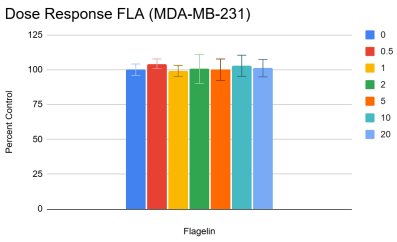
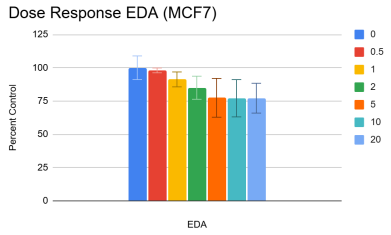
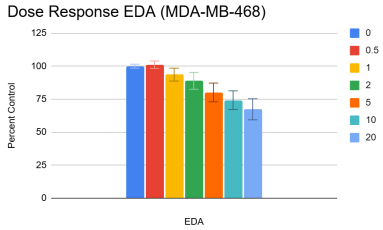


(Figure 2.3) (*All error bars are based on SD)*

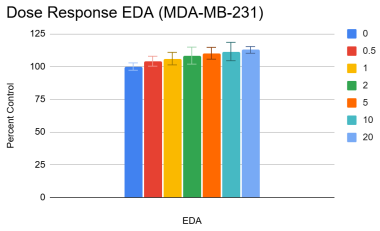
Dose Dependent Response (EDA) - Figures 3.1, 3.2, and 3.3 show the average dose responses for the 3 cell lines to Fibronectin EDA over a 4 day period. Figure 3.1 shows that the MCF7 cell proliferation was suppressed by the Fibronectin EDA and responded the most to the 10µl dose. In Figure 3.2 it shows that the MDA-MB-468 cell proliferation was also suppressed by the Fibronectin EDA and responded the most to the 20µl dose. In Figure 3.3 it shows that the MDA-MB-231 cell proliferation was not affected by the Fibronectin EDA.



(Figure 3.1) *(All error bars are based on SD)*

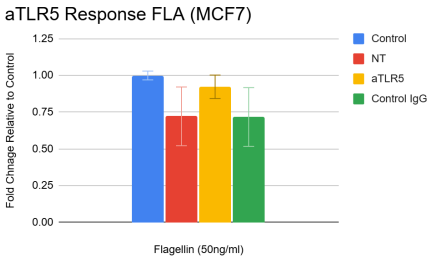


(Figure 3.2) *(All error bars are based on SD)*

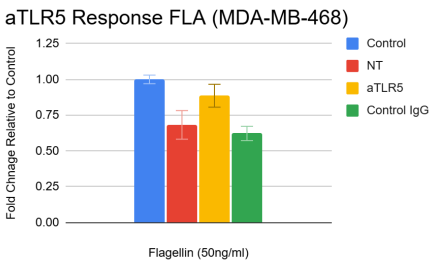


(Figure 3.3) *(All error bars are based on SD)*

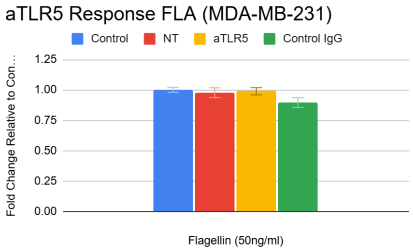
Anti TLR5 Neutralizing Antibody (FLA) - Figures 4.1, 4.2, and 4.3 show all 3 cell types exposed to an anti TLR5 neutralizing antibody, and a control IgG, along with control and no treatment wells, all treated with flagellin except the control well. Figure 4.1 shows that the MCF7 cell proliferation was rescued by the neutralizing antibody. Figure 4.2 shows that the MDA-MB-468 cell proliferation was also rescued by the neutralizing antibody. Figure 4.3 shows that the MDA-MB-231 cell proliferation was not affected by the neutralizing antibody.



(Figure 4.1) *(All error bars are based on SD)*

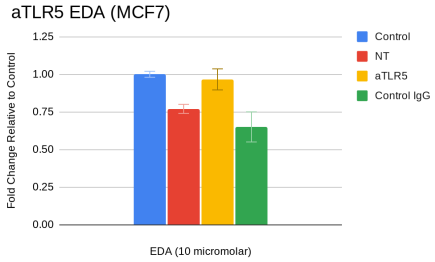


(Figure 4.2) *(All error bars are based on SD)*

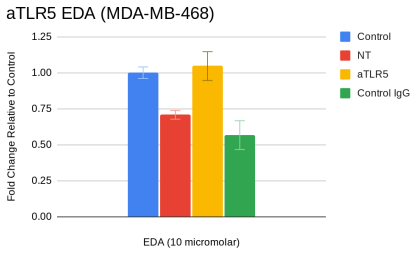


(Figure 4.3) *(All error bars are based on SD)*

Anti TLR5 Neutralizing Antibody (EDA) - Figures 5.1, 5.2, and 5.3 show all 3 cell types exposed to an anti TLR5 neutralizing antibody, and a control IgG, along with control and no treatment wells, all treated with Fibronectin EDA except the control well. Figure 5.1 shows that the MCF7 cell proliferation was rescued by the neutralizing antibody. Figure 5.2 shows that the MDA-MB-468 cell proliferation was also rescued by the neutralizing antibody. Figure 5.3 shows that the MDA-MB-231 cell proliferation was not affected by the neutralizing antibody.



(Figure 5.1) *(All error bars are based on SD)*



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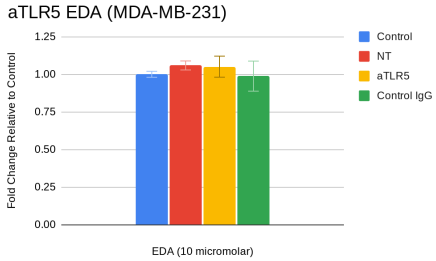

(Figure 5.3) *(All error bars are based on SD)*

Discussion and Conclusion

Our results have given us varied conclusions, and many more questions to be further investigated in the future. Something that could be looked at in the future would be determining what TLR5 signaling drives cancer cell progression. Possible errors in this study would be from the eluted stain from the toluidine blue assays. Since it is a protein stain, decreased stain may be decreased protein synthesis. Another possible, but unlikely error, would be that the TLR5 neutralizing antibody may be causing off-target effects, blocking something else other than TLR5.

In this study we hypothesized that the FnEDA would suppress breast cancer cell proliferation in a dose-dependent manner. Our secondary hypothesis was that FnEDA was an activator of TLR5. This study supported our hypothesis. From my first experiment it was concluded that the MCF7 and MDA-MB-468 cells were both suppressed by dosages of FnEDA. From the second experiment it was proven that the FnEDA was an activator of TLR5 as the anti-TLR5 neutralizing antibody rescued the proliferation of the cells. If I were to continue further research I would have tested more cell lines and tested different treatments.

References

1. Baranova, A., Krasnoselskyi, M., Starikov, V., Kartashov, S., Zhulkevych, I., Vlasenko, V., ... & Vinnyk, Y. (2022). Triple-negative breast cancer: current treatment strategies and factors of negative prognosis. *Journal of medicine and life*, *15*(2), 153.
2. Irvin Jr, W. J., & Carey, L. A. (2008). What is triple-negative breast cancer?. *European journal of cancer*, *44*(18), 2799-2805.
3. Cai, Z., Sanchez, A., Shi, Z., Zhang, T., Liu, M., & Zhang, D. (2011). Activation of Toll-like receptor 5 on breast cancer cells by flagellin suppresses cell proliferation and tumor growth. *Cancer research*, *71*(7), 2466-2475.
4. Ambesi, A., Maddali, P., & McKeown-Longo, P. J. (2022). Fibronectin functions as a selective agonist for distinct toll-like receptors in triple-negative breast cancer. *Cells*, *11*(13), 2074.