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Fetuin-A Modulates Tumor Growth and Invasion in a Basal-like Triple Negative Breast Cancer Cell line, MDA-MB-468

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Abstract

The present studies were undertaken to address the innovative role of fetuin-A in the growth and invasion potential in a triple negative breast cancer (TNBC) cell line, MDA-MB-468. Basal like TNBC that express high levels of ectopic fetuin-A have poorer prognosis for the patients compared to those that express low levels of the protein. We overexpressed fetuin-A in MDA-MB-468 and then determined the invasive potential of fetuin-A overexpressing cells vs controls transfected with empty vector. We also determined the adhesion and growth potential of the cells in the presence of only fetuin-A in serum free medium and also in complete medium. Our data suggest that fetuin-A overexpression significantly enhances the invasive potential of the cells and also the expression of Toll like receptor 4 (TLR4) on these cells. More importantly, the cells rely on fetuin-A-TLR4 signaling network for growth and invasion because the specific TLR4 inhibitor CLI-095 (resatorvid) abrogates fetuin-A mediated growth and invasion. Taken together, the data suggest that fetuin-A-TLR4 signaling network plays a significant role in the growth and invasion potential of TNBC.

Keywords: Fetuin-A, Triple Negative, Breast Cancer, TLR4, Invasion, Basal-like

Introduction

We previously demonstrated the significance of fetuin-A in the initiation and progression of mammary tumors using a transgenic mouse model for breast cancer. In this model system, we were able to demonstrate that lack of fetuin-A in the PyMT transgenic background significantly prolonged the latency period of mammary tumor development. Furthermore, tumors that formed in mice that lacked fetuin-A were much smaller in size with low number of proliferating cells [1]. However, this model was based on liver produced fetuin-A. In the prese nt studies we considered not only the role played by fetuin-A in the medium supplied to the tumor, but also fetuin-A produced by the tumor (ectopic fetuin-A).

Fetuin-A is presently considered a multifunctional protein with a potential to play several roles in tumorigenesis [2]. It is a serum glycoprotein primarily synthesized and secreted by liver parenchymal cells. It has a molecular weight of approximately 50 kDa and is heavily glycosylated [3]. Whereas its main physiological function is inhibition of ectopic calcification, reports show that ectopic fetuin-A plays pivotal roles in the progression of lung cancer

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Figure 3: Toll like receptor 4 (TLR4) expression in MDA-MB-468 sub-clones. Surface TLR4 expression levels were determined by flow cytometry in MDA-MB-468-EV (upper panels) and MDA-MB-468-FA (lower panels) in the absence and presence of added fetuin-A (FA)

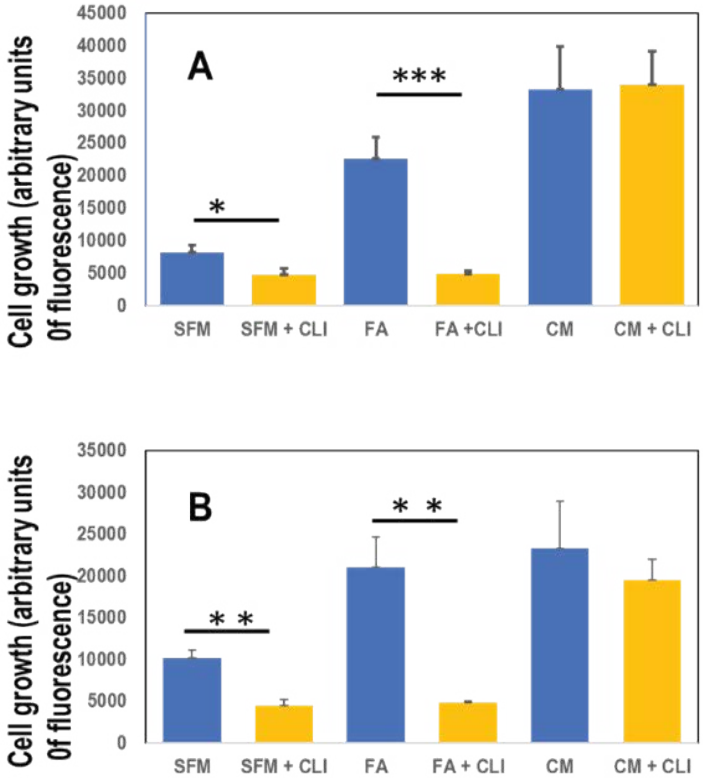

Figure 4: Fetuin-A mediates 2-D and 3-D growth of MDA-MB-468 via TLR4 signaling. The cells were seeded (2 x 10^4 cells/well) in attachment (2-D) (**panel A**) or ultra-low attachment (3-D) (**panel B**) 96-well plates in SFM; SFM + CLI-095 (CLI); Fetuin-A (FA); FA + CLI; complete medium (CM); and in CM + CLI. After 6 days of growth, Alamar blue was added to each well, incubated for 2 h and fluorescence measured as described. *P< 0.05; **P< 0.001; ***P<0.0001; N= 4

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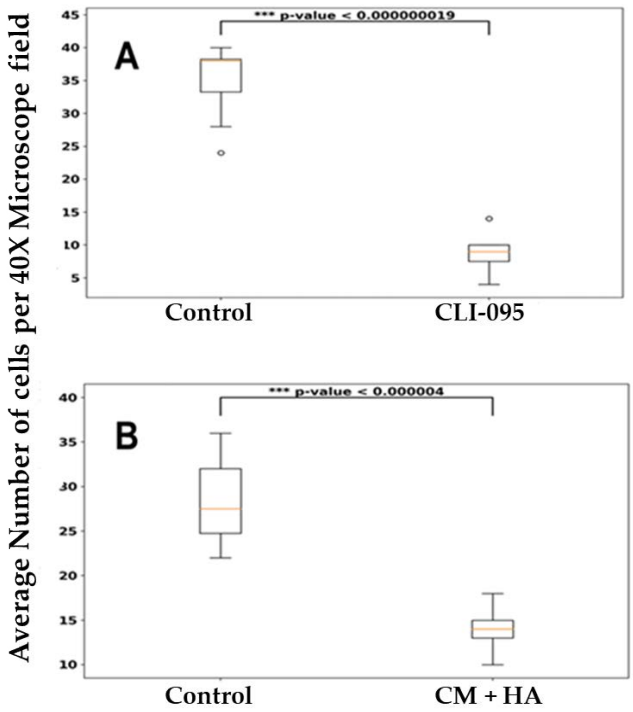

Figure 5: Attenuation of fetuin-A mediated invasion by inhibition of TLR4 signaling network. In **A**, control chambers had only cells in SFM, while experimental chambers had CLI-095 (28 µM) in SFM (upper chambers) of trans-well assay. The bottom chambers had complete medium (CM). In **B**, controls had CM only in the bottom chambers while experimental chambers had CM depleted of fetuin-A (CM + HA). Number of invading cells was determined as described in Materials and Methods.

Discussion

The role that fetuin-A synthesized by cancer cells plays in tumor cell growth has been rather difficult to explain from a mechanistic viewpoint, given that the concentration of fetuin-A in the blood is relatively high and likely to supply all the needs of the growing tumor mass. Indeed, in the present studies, the ectopically produced fetuin-A was not sufficient to drive the growth of the cells in serum free medium as had been shown for other tumor cells such as glioblastoma [5]. Interestingly, glioblastoma cells also express high levels of ectopic fetuin-A and are more motile and invasive, phenotypes that were attenuated when fetuin-A in these cells was knocked down [5]. The report by Mintz et al suggested that the ectopically produced fetuin-A drove the progression of prostate cancer culminating in the metastatic spread to the bones [6]. We hereby demonstrate that overexpression of fetuin-A by the basal like triple negative breast cancer cell line, MDA-MB-468, enhances its capacity for invasion. The basal like triple negative breast cancer cell line MDA-MB-468 is an excellent prototype of the cancer it represents in that it lacks estrogen, progesterone and epidermal growth factor 2 receptors [18, 22]. Triple negative breast cancer, a type of basal breast cancer that disproportionately affect women of color has poor prognosis with fewer treatment options [24]. A recent survey of TCGA survival data (Kaplan Meir Plotter) showed that basal type breast cancers including triple negative that express high levels of fetuin-A, have worse overall survival compared to those that express low levels of the protein [21], suggesting that fetuin-A upregulates metastatic processes in these cells. Based on these studies, we overexpressed fetuin-A in MDA-MB-468 to determine if this was sufficient to confer rapid growth, adherence and spreading in these cells, cellular phenotypes that we had previously attributed to fetuin-A [5]. Overexpression of fetuin-A in MDA-MB-468 did not confer in vitro growth advantage (2-D or 3-D) to these cells (data not shown), suggesting that overexpression of fetuin-A in these cells would likewise not confer growth advantage in vivo. Nevertheless, the increased invasive capacity as well as TLR4 expression in the TNBC cells resulting from overexpression of ectopic fetuin-A, suggested that other signaling pathways that promote invasion and metastasis are also upregulated.

The basal like triple negative breast cancer cell line, MDA-MB-468 typically express very low levels of TLR4 [22]. We believe this is the first report showing that fetuin-A overexpression can upregulate TLR4 expression in these tumor cells. Even more interesting was the fact that in the transfection controls where the expression of TLR4 was almost negligible, the presence of fetuin-A at approximately 2 mg/ml was sufficient to increase surface expression of TLR4 underscoring the role of fetuin-A in the tight regulation of surface expression of TLR4 on tumor cells. A number of studies have reported the role of TLR4 in tumor motility, invasion and metastasis (13). However, the ligand that initiates TLR4 signaling in tumor cells in particular has been a subject of intense debate. Whether it is the natural ligand for TLR4 lipopolysaccharide (LPS) or some other cellular protein such as high-mobility group box-1 protein (HMGB1) [13]. Fetuin-A was originally proposed to be a ligand for TLR4 by Pal et al [25]. The publicly available protein/protein binding data (STRING) shows that other than serum albumin, TLR4 has the strongest binding interaction with fetuin-A. The present studies suggest that cell surface TLR4 is the receptor through which fetuin-A interacts with MDA-MB-468 cells. It is not unusual for a ligand to modulate the expression of its receptor and vice-versa [26, 27].

Fetuin-A supplied to the tumor cells either from the medium or blood (in vivo) modulates cellular adhesion, motility, and growth in a very elaborate mechanism. To begin with, fetuin-A has to enter the cell as a prelude to cell adhesion and cell spreading. We have reported the ability of fetuin-A to prepare a unique population of extracellular vesicles/exosomes that then promote the cellular adhesion and

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spreading. We have demonstrated that only those exosomes that are isolated and secreted in the presence of fetuin-A have the ability to promote adhesion and cell spreading [9]. Those prepared in the absence of fetuin-A, lack this ability [9]. In the present studies we show that fetuin-A alone is sufficient to drive the adhesion and spreading and growth of MDA-MB-468 cells both in 2-D as well as 3-D, processes that were abrogated by the specific TLR4 inhibitor. These we believe are the cells that also have the propensity for invasion.

The present data suggest that even though fetuin-A is capable of signaling growth and adhesion in MDA-MB-468 cells, other adhesion and growth signals are at play in the presence of complete medium, so the cells will use these default pathways and bypass the fetuin-A/TLR4 signaling network when it is specifically targeted by CLI-095. The fact that CLI-095 only reduced the 2-D and 3-D growth in wells containing fetuin-A but not complete medium shows that the action of CLI-095 was specific for the fetuin-A mediated adhesion and growth. Lastly the data suggest that in addition to other known chemo-attractants such as EGF, fetuin-A is the dominant chemo-attractant in complete medium (CM) that is usually added to the lower chambers in trans-well invasion and motility assays [23]. The depletion of fetuin-A in complete medium significantly attenuated the ability of CM to attract the cells as they invaded through a bed of Matrigel. Likewise, in blood vessels next to a growing tumor mass and where fetuin-A is maintained at a concentration of approximately 0.3 mg/ml, it is likely to play a role in the initial attraction of invading tumor cells during the process of intravasation.

In summary we have demonstrated that elevated expression of fetuin-A in a triple negative breast cancer cell line promotes invasion capacity of the cells and upregulates TLR4 expression in these cells. These we believe are the novel aspects of this study because, the findings could explain at least in part why TNBC which express high levels of ectopic fetuin-A have poorer prognosis. The data also suggest that fetuin-A in the medium can modulate the surface expression of TLR4 on tumor cells (either upregulation or downregulation) to promote adhesion, invasion and growth. More importantly our data show that fetuin-A is the putative ligand that signals through TLR4 to promote the growth and invasive capacity of MDA-MB-468.

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