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Alteration of neutrophil function following exposure to conditioned medium derived from breast adenocarcinoma cells

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Abstract

Breast cancer, the most prevalent malignancy in women, is influenced by the tumor microenvironment (TME), where interactions between cancer cells and immune cells, including neutrophils, play a significant role in tumor progression. Neutrophils, which can have anti-tumor (N1) and pro-tumor (N2) functions, are involved in immune responses such as reactive oxygen species (ROS) production. However, the effects of tumor-derived conditioned media (CM) on neutrophil function remain poorly understood. This study investigates the impact of 4T1-derived CM (4T1-CM) on neutrophil activity, focusing on ROS production and antimicrobial function. We isolated neutrophils from healthy mice and exposed them to 4T1-CM. Our results show that 4T1-CM significantly enhances ROS production, as demonstrated by increased nitroblue tetrazolium (NBT) reduction, and improves neutrophil-mediated yeast killing. Importantly, 4T1-CM did not affect neutrophil viability, as assessed by MTT assay. These findings suggest that 4T1-CM enhances neutrophil function, specifically their ROS production and antimicrobial activity, without compromising cell health. This study highlights the potential role of CM in modulating immune cell responses in the TME, with implications for breast cancer immunotherapy strategies.

Keywords: Neutrophil, conditioned media, reactive oxygen species (ROS), yeast killing, NBT assay, 4T1 cells.

1. Introduction

Breast cancer refers to a condition in which the cells in the breast grow abnormally beyond their usual boundaries. Breast cancer is the most prevalent cancer among women globally. It accounts for up to 36% of all cancer cases. In 2018, there were 2.089 million cases of breast cancer diagnosed in women (1).

Tumors are made up of more than just neoplastic cells. The tumor microenvironment (TME) is now understood to play a vital role in tumor development and progression, in addition to serving as a key indicator of how the tumor responds to treatment (2). The breast cancer microenvironment is composed of various cell types, such as adipocytes, fibroblasts, leukocytes, endothelial, and myoepithelial cells, along with the proteins they produce and the extracellular matrix (ECM) in which they are located (2, 3). Continuous communication and interaction occur between the immune system, tumor cells, and stromal cells in the TME (4).

T cells and macrophages have been the most extensively studied immune cells in breast cancer. However, growing evidence indicates that neutrophils also play a crucial role in the oncogenesis and metastasis of breast cancer (5). Mediators such as cytokines, growth factors, lipids, and chemokines secreted by stromal and cancer cells within the TME can attract neutrophils, influencing tumor growth and metastasis (6). Tumor-associated neutrophils (TANs) are categorized into two types: those with anti-tumor activity (N1) and those with pro-tumor activity (N2). N1 neutrophils demonstrate strong anti-tumor activity primarily by releasing pro-inflammatory and immunostimulatory cytokines, including CCL3, CXCL9, CXCL10, tumor necrosis factor-alpha (TNF-α), and interleukin (IL)-12. On the other hand, N2 neutrophils exhibit significant tumor-promoting and immunosuppressive activities. They contribute to tumor angiogenesis, invasion, and metastasis by production of various factors, such as hepatocyte growth factor (HGF), reactive oxygen species (ROS), oncostatin M, reactive nitrogen species (RNS), neutrophil elastase, and matrix metalloproteinases (7). Understanding how neutrophils can either support or slow tumor growth, depending on the situation, is key to creating targeted therapies.

Conditioned media (CM) refers to the supernatant derived from cells and it contains various proteins and growth factors (8). Studies have shown that tumors-derived CM affects neutrophils (9, 10). For example, SenGupta et al. (2021) found that tumor-derived CM collected from highly aggressive, metastatic triple-negative breast cancer cells induced a polarized morphology and strong neutrophil movement, whereas CM from less aggressive breast cancer cells showed no such effect (9). However, there is limited understanding of how breast cancer-derived CM affects neutrophil function. Consequently, the objective of this study is to investigate the impact of CM from the 4T1 mouse breast tumor model (4T1-CM) on neutrophil function, with a particular focus on ROS production and antimicrobial activity.

2. Material and methods

2.1. Cell Culture

4T1 cells were obtained from GenIran Company (Iran) and grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Capricorn, Germany), supplemented with 10% fetal bovine serum (FBS; Anacell, Iran), 1% penicillin-streptomycin (Sigma-Aldrich, USA), 1% L-glutamine (Sigma-Aldrich, USA). The cells were maintained in a humidified incubator (Memmert, Germany) set at 37°C with 5% carbon dioxide. Cells at passage 3 were employed for the experiments.

2.2. Preparation of 4T1-CM

Cells at passage 3 were seeded in a T-75 flask and incubated under standard conditions (37°C with 5% CO_2). Upon reaching approximately 80% confluence, the DMEM medium was aspirated, and the cells were washed several times with phosphate-buffered saline (PBS).

Fresh FBS-free DMEM was then added, and the flask was incubated for a further 48 hours. Following this incubation, the CM was harvested, centrifuged to remove debris, and stored at −80°C for subsequent experiments.

2.3. Neutrophil isolation

Neutrophils were isolated from heparinized blood samples collected from healthy mice following the protocol outlined in (11). Each 5 mL blood sample was mixed with 3% dextran in a 1:1 ratio and incubated for 45 minutes at room temperature. After incubation, distinct layers formed, with the upper layer containing leukocytes or neutrophils. This layer was carefully extracted using a pipette and then centrifuged at 400 × g for 30 minutes using a Ficoll-Hypaque density gradient. The resulting pellet was resuspended in DMEM medium.

2.4.Neutrophil purity and viability

Isolated neutrophils were stained with Giemsa and trypan blue to assess their purity and viability, respectively. For Giemsa staining, a small drop of the neutrophil suspension was placed onto a glass slide and spread to create a thin blood smear. Once the smear dried, it was fixed by dipping the slide in methanol for 10 minutes. The slide was then stained with diluted Giemsa stain for 20 minutes. After staining, the slide was gently rinsed with distilled water and left to air dry at room temperature. For viability assessment using trypan blue, 50 µL of the neutrophil suspension was combined with 50 µL of trypan blue solution. The cells were then examined under an inverted microscope, and cell viability was recorded.

2.5. Pretreatment of neutrophils

Following neutrophil isolation and counting, the cells were transferred to microtubes and treated with CM at a 1:1 ratio. The control groups consisted of microtubes containing only the base medium (DMEM). All microtubes were incubated at 37°C for two hours to allow for treatment.

2.6. MTT assay

MTT is a tetrazolium salt commonly used in colorimetric assays to assess cell growth (12). Viable cells take up MTT and reduce it to formazan, which forms a purple color. For this assay, 100 µL of pretreated neutrophil suspension, adjusted to a concentration of 1×10^6 cells/mL, was added to each well of a 96-well microplate. Next, 20 µL of MTT solution (5 mg/mL final concentration) was added to each well, and the microplate was placed in an incubator at 37°C for 2 hours. After incubation, the microplate was centrifuged for 10 minutes at 3000 RPM. Following centrifugation, the media was carefully aspirated, and 100 µL of dimethyl sulfoxide (DMSO, Merck, Germany) was added to the wells. Finally, the absorbance was then measured at 570 nm.

2.7. Nitroblue Tetrazolium (NBT) test

In the NBT assay, which measures ROS generated by leukocytes (13), 500 µL of 0.01% NBT solution, 500 µL of pretreated neutrophils with 4T1-CM, and an opsonized yeast suspension were transferred to microtubes and mixed. The microtubes were incubated at 37°C for 30 minutes, followed by centrifugation at 400 × g for 10 minutes. A mixture of DMSO and 1 M potassium hydroxide (KOH) was then added to each microtube, and the microtubes were vortexed vigorously. Finally, 100 µL of each mixture was transferred to a 96-well plate, and the optical density (OD) was measured at 540 nm.

2.8. Yeast Killing Assay

The yeast-killing activity of neutrophils was evaluated in both test and control groups, with a final volume of 300 µL for each group. The test group contained a mixture of blood serum, opsonized yeast, and neutrophils treated with 4T1-CM.

The control groups were structured as follows:

a) A mixture of blood serum, opsonized yeast, and untreated neutrophils in DMEM to establish baseline neutrophil activity without any treatment.

b) Blood serum and DMEM with untreated neutrophils to assess background neutrophil activity.

c) A combination of blood serum, yeast, and DMEM to determine the baseline yeast viability.

For each group, 100 µL of each component was added to the sterile microtubes. For groups containing neutrophils or yeasts, the suspension was added at a density of 5 × 10^6 cells/mL. The microtubes were incubated at 37°C in a shaker incubator for 90 minutes to allow interactions between the neutrophils and yeast. Samples were collected at the start of the assay (0 minutes) and after the 90-minute incubation period. At each time point, neutrophils were lysed by adding sterile distilled water, followed by incubation at room temperature for 10 minutes. A 100 µL aliquot of the lysed sample was transferred to a 96-well microplate, followed by the addition of 20 µL of MTT solution (5 mg/mL) to each well. The microplate was incubated for two hours, after which 150 µL of DMSO was added. The OD was measured at 490 nm using an ELIZA reader.

2.9. Statistical analysis

The results are expressed as means ± standard deviation (SD) to reflect variability within the data. Statistical analyses were done using GraphPad Prism version 10 (GraphPad Software, USA). To assess the normality of the data distribution, the Kolmogorov–Smirnov test was applied. Group comparisons were conducted using a one-way analysis of variance (ANOVA). Statistical significance was defined as a p-value of < 0.05.

3. Results

3.1. Neutrophil purity and viability

The purity of isolated neutrophils was confirmed to be >95%, as shown in Figure 1, ensuring that the cells used in subsequent assays were predominantly neutrophils. Additionally, cell viability was assessed to be >98% using trypan blue exclusion, indicating that the neutrophils were in optimal condition for functional assays.

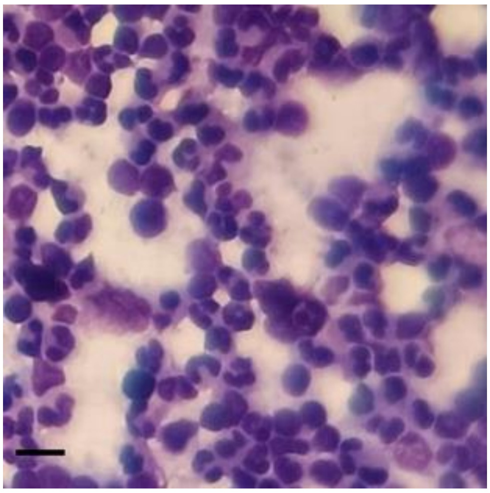


Figure 1. The neutrophil purity exceeded 95%, as determined by Giemsa staining.

3.2. MTT assay

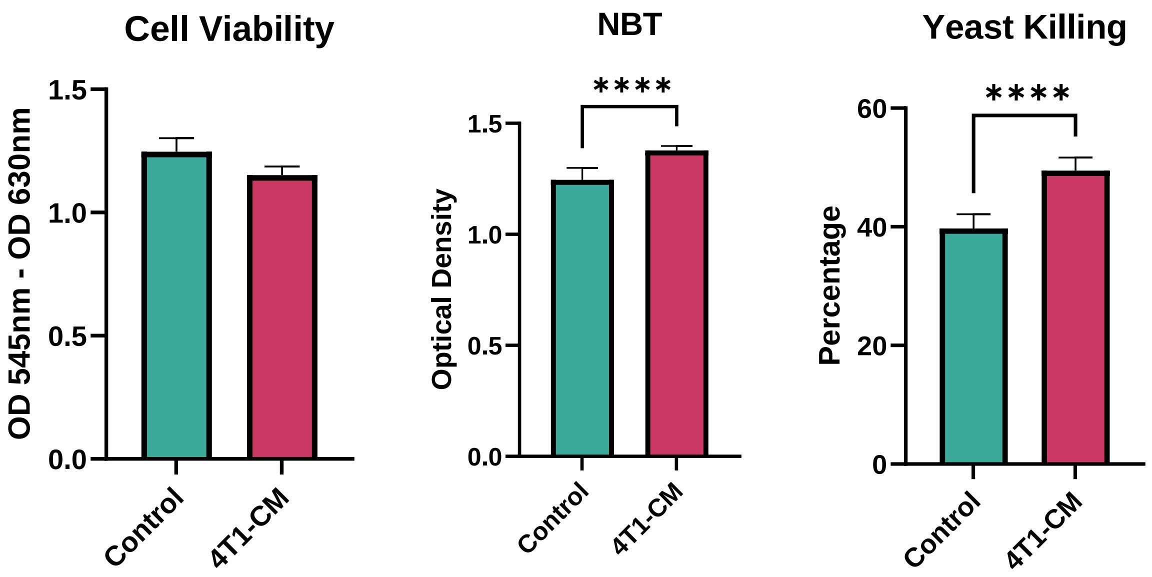
The MTT assay, which evaluates cell viability by measuring mitochondrial activity, relies on reducing MTT to form a purple formazan product. In this study, treatment with 4T1-CM had no discernible impact on neutrophil viability, as the viability of the treated group was comparable to that of the control group (Figure 1A).

3.3. NBT test

4T1-CM significantly enhanced NBT reduction ($P < 0.0001$), as shown in Figure 1B. This finding suggests that 4T1-CM promotes increased ROS production or heightened metabolic activity in neutrophils, as demonstrated by the elevated NBT reduction compared to the control group.

3.4. Yeast Killing Assay

Figure 2C demonstrates that 4T1-CM significantly enhanced yeast killing by neutrophils compared to the control group.



A **B** **C**

Figure 2. A) MTT assay showing neutrophil viability. B) NBT reduction of isolated neutrophils. C) Yeast killing assay by neutrophils. Data are the mean ± SD of three independent experiments. Statistical significance is indicated as ****P < 0.0001. Abbreviations: 4T1-CM, 4T1-derived conditioned media; ns, not significant.

4. Discussion

Breast cancer, the most prevalent cancer in women, highlights the critical need for a deeper understanding of its microenvironment to enhance diagnostic and therapeutic approaches. Neutrophils, as a key component of this microenvironment, have garnered considerable attention in breast cancer research. Tumor-associated neutrophils are categorized into two types: those with anti-tumor activity (N1) and those with pro-tumor activity (N2) (7). A key characteristic of neutrophils' actions is the activation of a potent oxidative burst (14). ROS are byproducts of aerobic metabolism, with the main types being superoxide anion, hydroxyl radical, and hydrogen peroxide (15, 16). ROS are made by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and can damage proteins, lipids, and DNA, leading to various pathological conditions (16, 17). The NADPH components are inactive in resting cells and become activated when triggered by pro-inflammatory mediators, phagocytosis, the presence of microbes, or the activation of PRRs (17). ROS also act as signaling molecules, regulating physiological and biological processes (16). In tumors, low concentrations of ROS can serve as signaling molecules that promote tumorigenesis and contribute to tumor heterogeneity. In contrast, at high concentrations, ROS may act as cancer modulators, exerting harmful, genotoxic, or even pro-apoptotic effects on cancer cells (18).

Previous studies have shown that cells-derived conditioned media affects ROS production. It can modulate ROS production by either enhancing or inhibiting its generation, highlighting its dual role in the regulation of oxidative stress (11, 19-22). In this study, we investigated the impact of 4T1-conditioned media (4T1-CM) on ROS production in neutrophils. Our findings show that 4T1-CM significantly increased ROS production in neutrophils, as evidenced by enhanced NBT reduction (Figure 2B). The increased NBT reduction suggests that 4T1-CM may stimulate neutrophils, enhancing their ability to generate ROS.

This result aligns with studies using CM from other cell types, such as mesenchymal stromal/stem cells (MSCs), which have similarly been shown to enhance neutrophil ROS production (11, 19, 20). However, they also showed that the CM they derived influenced neutrophil phagocytosis and lifespan. Similarly, another study found that stimulated CM from macrophages also increased ROS production in neutrophils (23). Additionally, CM from various cells has been demonstrated to impact neutrophil chemotaxis, adherence, apoptosis, and cytokine secretion (24-26). Given that CM contains a variety of secreted factors from the originating cells, these components are likely responsible for the observed changes in neutrophil activity. For example, Zhang et al. demonstrated that exosomal miR-146a derived from oxidized low-density lipoprotein-treated macrophages induced oxidative stress and promoted neutrophil extracellular trap formation (27).

However, contrary to these findings, some studies have shown that CM can also decrease ROS production. For instance, Bezerra et al. reported that CM from Wharton’s jelly-derived MSCs led to a reduction in ROS generation in isolated secondary follicles, suggesting a potentially inhibitory effect on oxidative activity (21). Another study by Widowati et al. observed a similar reduction in ROS levels when they used CM from human adipose tissue-derived MSCs (22).

The activity of CM depends on its composition, which varies based on the cell source and the conditions under which it is produced. The composition of CM is influenced by several factors in the cell culture microenvironment, including, cell aggregation, cell contact inhibition, cell growth and differentiation potential, as well as chemical and physical conditions (28). Moreover, the effects of CM can differ based on the cell type being studied and their response to the media. These conflicting results emphasize that the effect of CM on ROS production can vary based on the source of the CM, the conditions under which it was prepared, and the type of cells being targeted.

Additionally, our study showed that 4T1-CM significantly improved neutrophil-mediated yeast killing (Figure 2B). Given the enhanced ROS production observed, it is likely that ROS played a crucial role in this increased antimicrobial activity. Importantly, the MTT assay revealed that despite the observed increase in ROS production and enhanced antimicrobial activity, 4T1-CM did not affect neutrophil viability (Figure 2A). This indicates that 4T1-CM enhances neutrophil function without compromising their viability.

5. Conclusion

In this study, we investigated the effects of 4T1-CM on neutrophil function, specifically focusing on ROS production and yeast killing capacity. Our findings demonstrate that 4T1-CM significantly enhanced ROS production in neutrophils, and improved neutrophil-mediated yeast killing. Importantly, despite these enhanced functional responses, the MTT assay revealed that 4T1-CM did not affect neutrophil viability, indicating that it enhances neutrophil function without compromising cell health. These findings suggest that tumor-derived CM in the breast cancer microenvironment can modulate neutrophil activity, potentially influencing immune responses within the tumor. Further research is needed to better understand the specific factors involved and their implications for therapeutic strategies aimed at modulating immune cell functions in cancer.

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