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The Effect of Sympathetic Neurotransmitters on the Hepatic Metastatic Niche to Regulate Dormancy and the Reactivation of Disseminated Breast Cancer Cells

Introduction

Breast cancer is recognized as one of the most frequently diagnosed malignancies and is associated with a high metastatic rate of 30% [1][2]. Disseminated breast cancer cells are able to migrate to other organs and become dormant even before the primary tumor starts growing; they settle in this dormant state for a period of time before reactivating to form metastases [3]. Among various metastatic areas, the liver is a frequent target due to the nature of its microenvironment. The metastatic niche of an organ can be influenced by many different factors including signaling from neurotransmitters.

Scope: This study will focus on Triple-negative breast cancer (TNBC), a type of invasive breast cancer. TNBCs lack receptors for estrogen, progesterone, and HER2 which enables this study to focus on targeting the Tumor Microenvironment (TME) instead of the receptors itself [4]. Moreover, this study will focus on norepinephrine (NE), epinephrine (Epi), and acetylcholine (ACh) as the sympathetic neurotransmitters.

Aims: This research will investigate how sympathetic neurotransmitters alter the Hepatic metastatic microenvironment, particularly on the regulation of disseminated breast cancer cells dormancy and reactivation. This study plan to focus on three key elements:

1. The effect of sympathetic neurotransmitters on the Hepatic metastatic niche, specifically in the regulation of dormant disseminated breast cancer cells.
2. The biological mechanistic interactions between sympathetic neurotransmitters and the Hepatic metastatic microenvironment.
3. The therapeutic possibilities in exploring whether targeting neurotransmitter-mediated pathways can reduce the risk of metastatic relapse.

Importance: Since the TME plays a central role in cancer progression, findings from this study allow scientists to utilize the dormant period of cancer cells as an opportunity to prevent the residual cancer cells from reactivating. Currently, breast cancer survivors still undergo conventional treatments like chemotherapy and radiotherapy after surgery due to the potential presence of disseminated cancer cells (DCCs) in other organs. However, these therapies have side effects which often reduce patient quality of life. For that reason, the ability to prevent relapse in breast cancer by manipulating and remodelling the TME through neurotransmitter-mediated pathways may change breast cancer treatment plans for the better.

Research Question: How do sympathetic neurotransmitters affect the hepatic metastatic niche to regulate dormancy and the reactivation of disseminated breast cancer cells?

Literature Review

The Mechanism of Breast Cancer Metastasis and Dormancy

The metastatic cascade is a sequence of steps a tumor has to undergo in order to metastasize to other organs. It begins with the invasion of cancer cells into the surrounding healthy tissue by altering cell-to-cell and cell-to-extracellular matrix adhesion. This is done through the

downregulation of Epithelial-cadherin, a transmembrane protein that maintains cellular adhesion. The DCCs then intravasate into circulatory organs like the blood stream and lymphatic vessels. The cells stop their cell cycle and adhere to the capillaries within the target organ before extravasating onto the parenchyma tissue [5]. The cancer cells are now in a cellular dormancy state, where the small cluster of DCCs simply exists in the target organ without undergoing their cell cycle. DCCs can remain in this dormant state through therapies and natural biological mechanisms such as antiproliferative therapies, niche-derived signaling, primary-site signaling, and immune-mediated dormancy. However, DCCs may escape dormancy and re-enter cell cycle through mechanisms through mutation-induced genetic loss of signaling receptors and inflammation [6]. Regardless, the cancer cells are able to secrete both dormancy and reactivation markers depending on the state they are in.

The Hepatic Metastatic Niche (Hepatic TME)

Existing studies have proven that different organs have different components in their metastatic niches. In the context of the liver, the components can be divided into two categories: cellular and acellular. In many cases, changes in the cellular components will lead to changes in the acellular components. According to a study done by T. Williamson, cellular components of the liver microenvironment include tumor-associated macrophages (TAMs), Kupffer cells (KCs), Liver sinusoidal endothelial cells (LSECs), tumor-associated neutrophils (TANs), fibroblasts, hepatocytes, etc. On the contrary, the acellular liver microenvironment is made up of molecules such as collagen proteins, cell-adhesion molecule carcinoembryonic antigen (CEA) and other cell adhesion molecules (CAMs), CXC motif-chemokines (CXCLs), VEGF, MAPK, etc. Further information regarding the function of involvement of these components in metastasis can be found in the journal article published by Clinical and Translational Medicine [7].

The Role of the TME and Cancer Progression

The idea of a Tumor Microenvironment (TME) dates back to the 1980s when Stephen Paget came forward with the 'Seed and Soil' theory. The theory proposes that the seed (tumor) can only grow in a suitable soil (TME). Now, it is known that the TME is composed of the Extracellular Matrix (EMC), fibroblasts, endothelial cells, immune cells, mural cells of blood and lymph vessels, and many more specialized cells. The DCCs in the targeted site consistently interact with the TME, or in this case, the metastatic niche of the targeted organ. Acknowledging the various hallmarks of cancer, it is evident that the cancer cells can utilize the cells and biological compounds found in the TME to promote its own growth [5]. The existing studies done on the TME emphasizes its relevance to cancer growth and proliferation.

Research Gaps

While existing studies in this area of oncology provide essential information regarding the role of the TME in tumor progression, there remains little to no papers exploring the role of the TME in DCCs reactivation and dormancy. Specifically, there is evident lack of research done on the role

of Hepatic metastatic niche in the reactivation and dormancy of disseminated breast cancer cells. Furthermore, there is yet to be a study done with the aim of investigating the interaction between the sympathetic neurotransmitters and the components of the liver metastatic niche. For that reason, this research gap exemplifies the need for a specific study that delves into the effect of sympathetic neurotransmitters on the hepatic metastatic niche to regulate dormancy and the reactivation of disseminated breast cancer cells. Amid a plethora of generalized studies done on cancer metastasis and the TME, this research paper will serve as a specialized study that focuses on one of the most prevalent cancers with a high metastatic rate.

Methodology

This study will utilize laboratory-based research. The independent variable in this experiment is the neurotransmitter exposure and the dependent variables that will be measured are dormancy and reactivation markers. The experiment will require co-cell-culture systems since it involves the combination of cells in one media: the TNBC cell line and the cellular components of the liver metastatic niche.

Experimental Setup: Due to economic reasons, this study will use 2D co-culture to imitate the liver microenvironment.

1. TNBC cells will be grown in culture flasks until a sufficient amount is obtained to conduct the experiment. The media used will have a 1:1 ratio of DMEM and hepatocyte medium along with FBS and antibiotics [8].
2. The TNBC cells will then be transferred to well-plates with seeding density of 100,000 cells and 1mL of media per well.
3. The cellular components of the liver metastatic niche will then be transferred to the well-plates with seeding density of 100,000 cells per well.
4. After a 24 hour incubation period, the sympathetic neurotransmitters will then be added to each well. The variation in treatments will be 10μM NE; 5μM NE; 2.5μM NE; 10μM Epi; 5μM Epi; 2.5μM Epi; 10μM ACh; 5μM ACh; 2.5μM ACh; control.

Data Collection: In this experiment, the data collected will be the identification and the quantification of the protein biomarkers of TNBC cells' dormancy and reactivation.

1. After a 48 hour treatment period, the hepatocytes and the media will be removed, leaving the adherent TNBC cells on the base of the well plates.
2. A detergent is then going to be added to each well to lyse the TNBC cells, allowing the protein biomarkers to be obtained. A western blot will be done to identify the proteins and an image analysis software will be used to quantify the proteins.
3. This will be done five times to reduce random error.

Data Analysis: The data from the Western blot will be analysed by separately comparing the presence of dormancy markers and reactivation markers across the different treatment groups.

The software ImageJ will be used to quantify the band intensities. A one-way ANOVA will test for significant differences between the treatment groups. If the test shows significant difference, individual t-tests will be done for each treatment against the control to pinpoint the conditions which show this difference. The statistical significance will be set at $p < 0.05$, meaning that there is a 95% confidence of a significant difference (not due to chance) that there is an elevated expression of dormancy/reactivation biomarkers in that specific treatment condition. It is important to note that the data analysis should be done twice: one for the dormancy biomarkers and another for the reactivation biomarkers.

Timeline

Week 1	***Preparation:*** Purchase cell lines, media, neurotransmitters, and other equipments
Week 2-3	***Cell Culture:*** Thaw and grow TNBC cells and liver TME cells in the media
Week 4	***Hepatic Niche:*** Mimic the hepatic niche by co-culturing the liver TME cells (hepatocytes, LSECs, KCs, etc.)
Week 5-7	***Main Experiment:*** Seed the cells into the well-plates and treat them with their respective neurotransmitters (NE, Epi, ACh). Incubation period: 48 hours.
Week 8	***Biomarker Collection:*** Remove the media; lyse the cells; run a western blot to detect dormancy and reactivation biomarkers; and use ImageJ for quantification of the Western blot.
Week 9	***Data Analysis:*** Perform a one-way ANOVA and individual t-tests. Compare significant differences in both dormancy and reactivation markers.
Week 10	***Data Interpretation:*** Interpret findings and data and relate the results back to the hypothesis and research question.

Conclusion

In summary, this research seeks to address the ambiguity in breast cancer metastasis to the liver, particularly in understanding the effect of sympathetic neurotransmitters on the hepatic TME. Acknowledging the importance of the TME in cancer growth, this research will serve as a tool to determine whether neurotransmitters alter the hepatic TME in ways that either maintain cell dormancy or trigger activation. The findings from this study have potential to build scientific

understanding on the underlying biological mechanisms driving liver metastasis in TNBC. This knowledge can be used to develop treatments that involve the neurotransmitter-mediated pathways to prevent the activation of the disseminated breast cancer cells which may result in improvements in long-term outcomes for breast cancer patients.

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