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Comparative analysis of innate immune system function in metastatic breast, colorectal, and prostate cancer patients with circulating tumor cells

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ABSTRACT

In recent years, circulating tumor cells (CTCs) in metastatic cancer patients have been found to be a promising biomarker to predict overall survival and tumor progression in these patients. A relatively high number of CTCs has been correlated with disease progression and poorer prognosis. This study was designed to assess innate immune system function, known to be responsible for the immune defense against developing neoplasms, in metastatic cancer patients with CTCs. Our aim is to provide a link between indication of poorer prognosis, represented by the number of CTCs to the cytotoxic activity of natural killer cells, an important component of the innate immune system, and to represent a promising expanded approach to management of metastatic cancer patients with CTCs. Seventy-four patients, with metastatic breast, colorectal, or prostate cancer, were recruited for this study. Using a flow cytometric assay, we measured natural killer (NK) cell cytotoxicity against K562 target cells; and CTCs were enumerated using the CellSearch System. Toll-like receptors 2 and 4 expression was also determined by flow cytometry.

We found that within each of our three metastatic cancer patient groups, NK cell cytotoxic activity was decreased in patients with a relatively high number of CTCs in peripheral blood compared to patients with a relatively low number of CTCs. In the breast and prostate cancer group, patients with CTCs greater than 5 had decreased NK cell cytotoxicity when compared to patients with less than 5 CTCs. In the colorectal cancer group, we found that 3 or more CTCs in the blood was the level at which NK cell cytotoxicity is diminished.

Additionally, we found that the toll-like receptors 2 and 4 expression was decreased in intensity in all the metastatic cancer patients when compared to the healthy controls. Furthermore, within each cancer group, the expression of both toll-like receptors was decreased in the patients with relatively high number of CTCs, i.e. greater than 5 for the breast and prostate cancer group and greater than 3 for the colorectal cancer group, compared to the patients with relatively low number, i.e. less than 5 or 3, respectively. Treatment options to increase NK cell cytotoxic activity should be considered in patients with relatively high numbers of CTCs.

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Introduction

The present investigation was designed to evaluate innate immune system function, particularly the cytotoxicity of natural killer (NK) cells against tumor cells. Metastatic breast, prostate, and colorectal cancer patients were recruited for the study and each was evaluated based on the number of circulating tumor cells (CTCs) to provide a link between indications of poorer prognosis to the cytotoxic activity of NK cells. CTCs are believed to be indicators of residual disease and to portend an increased risk of metastasis and poorer outcomes for those patients with CTCs (Plaks et al., 2013). Since the major cause of cancer-associated mortality is tumor metastasis, it is important to monitor the numbers of CTCs in metastatic cancer patients. CTCs are formed from tumor cells that separated from the primary tumor and intravasate into blood vessels or lymphatics. These cells are then free to migrate to different sites in the body, extravasate out of the blood vessels, and adapt to the new microenvironment. Eventually, they will seed, proliferate, and colonize to form metastases.

According to the National Cancer Institute, based on recent data, the number of new cases of colon and rectum cancer was 45.0 per 100,000 men and women per year, with the number of deaths at 16.4 per 100,000 men and women per year. For breast cancer, the number of new cases was 123.8 per 100,000 women per year and the number of deaths was 22.6 per 100,000 men and women per year. Lastly, the number of new cases of prostate cancer was 152.0 per 100,000 men per year.

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The number of deaths was 23.0 per 100,000 men and women per year. These present a multitude of medical and financial issues. With such large numbers, it is important to elucidate more effective ways to treat cancer.

This study focuses on the function of the innate immune system, which is believed to be the primary response against tumor growth and development. Natural killer (NK) cells are an important component of this system and play a vital role in eliminating cancer cells. NK cells recognize any cell lacking the Major Histocompatibility Complex (MHC) class I marker and release proteins that mediate the killing of the targeted cells (Bellora et al., 2013). When functioning properly, NK cells protect the host against a vast repertoire of potentially harmful agents, including cells having undergone cellular transformation in the process of forming a tumor. Other components of the innate immune system are the Toll-like receptors (TLRs), which are found in a number of cells including NK cells. TLRs recognize antigens that are not normally expressed within the host, including transformed cells (i.e. cancer cells). Upon recognition, TLRs promote the release of cytokines, chemokines, and other agents required in antitumor immunity (Erridge, 2010). In this study, TLRs were characterized on immune cell subsets using monoclonal antibodies by flow cytometric methods. In addition, patient lymphocytes were isolated from the peripheral blood and NK cell cytotoxic activity was measured using the flow cytometry assay (Bottley et al., 2007; Bryceson et al., 2010). This study aims to assess the effectiveness of the immune system against cancer in the presence of CTCs in the blood and to represent a promising expanded approach to management of metastatic cancer patients with CTCs.

Materials and methods

Subjects

Seventy-four patients visiting the Multispecialty Care Cancer Clinic at the Jackson Medical Mall in Jackson, MS were recruited for this study. Each patient had been previously diagnosed with metastatic breast, colorectal, or prostate cancer. A co-investigator from the study discussed the risks and benefits, privacy issues, and other pertinent information with potential participants before obtaining informed consent. After obtaining consent, peripheral blood was drawn via venipuncture into a 6.0 mL sodium-heparin tube for flow cytometry analysis and a 10 mL CellSave tube (purchased from Janssen Diagnostics, LLC) for circulating tumor cell enumeration. At least four milliliters and nine milliliters of blood samples are needed for the flow cytometry analysis and CTC enumeration, respectively. In addition, a total of ten healthy control were recruited for this study. Blood sample analysis was also conducted using flow cytometry.

Circulating tumor cell enumeration

The CellSearch System (Janssen Diagnostics, LLC) was used to identify the number of CTCs per 7.5 mL of peripheral blood collected in CellSave tubes. A 5 or greater CTCs per 7.5 mL whole blood is considered the threshold at which long term prognosis and survival of breast and prostate cancer patients is significantly diminished (Cristofanilli et al., 2005; Hayes et al., 2006; De Bono et al., 2008). In comparison, a 3 or greater CTCs per 7.5 mL whole blood is the threshold for colorectal cancer (Cohen et al., 2008). The CellSearch CTC test and control kits contain immunofluorescent reagents to capture and identify CTCs of epithelial origins. Before adding reagents, the CellSave tubes were inverted at least 5 times to ensure thorough mixing of the preservatives contained in the tubes with the blood samples. 7.5 mL of blood are taken and transferred into a 15-mL conical tube included in the test kit. 6.5 mL of dilution buffer is added and the tubes were capped and inverted at least 5 times. The tubes were then centrifuged for 10 min at 1800 rpm with no brake. The control was prepared following the manufacturer's guidelines. After centrifugation, the samples were loaded onto the CELLTRACKS® AUTOPREP® System as well as the reagents in the kit. Ferrofluid consisting of nanoparticles with a magnetic core captures cells expressing the epithelial cell adhesion molecule (EpCAM) for further examination. After immunomagnetic capture and enrichment, fluorescent staining reagents were added to visualize CTCs. Fluorescent staining reagents of anti-cytokeratin (CK) conjugated to PE, 4′-6-Diamidino-2-phenylindole (DAPI), and anti-CD45 conjugated with APC were used to label the intracellular protein cytokeratin (specific for epithelial cells), cell nucleus, and leukocytes, respectively (all reagents were obtained from Janssen Diagnostics, LLC). After processing, the samples were loaded onto the CELLTRACKS ANALYZER II® for analysis of the stained cells. Positive CTCs expressed EpCAM, CK, and DAPI and did not express CD45.

Peripheral blood lymphocyte isolation and activation

Peripheral blood lymphocytes (PBLs) were isolated from whole blood samples using Ficoll-Hypaque centrifugation. After inverting the sodium-heparin tube gently for 2–3 times to ensure thorough mixing of anticoagulant to whole blood, blood was centrifuged for 5 minutes at 3000 rpm. The resulting buffy coat was extracted using a transfer pipette and placed on a fresh tube. Approximately 2–5 mL PBS (purchased from Sigma-Aldrich) was added to the buffy coat and mixed well. A 9 inch pipette was placed on the tube. Using a syringe, 5 mL of Ficoll (purchased from GE Healthcare) were measured and added into the tube through the 9 inch pipette. This resulted in the Ficoll settling to the bottom of the tube. The tube was then centrifuged for 25 minutes at 2000 rpm with no brake at room temperature. The resulting mononuclear cell layer, interfaced between the two layers of blood plasma and Ficoll reagent, was removed with a transfer pipette and placed into a fresh, clean tube. PBS was added to wash and remove remaining traces of the Ficoll reagent. The tube was then centrifuged for 6 minutes at 1800 rpm. The supernatant was discarded and the cell button was resuspended in RPMI 1640 supplemented with 10% FCS (purchased from Gibco). Cells were concentrated at 10^6 cells/mL and distributed into 2 wells of a 12-well round bottom plate. Both wells contained 100 μL of 10 μg/mL LPS (purchased from Invivogen) to activate the toll-like receptors 2 and 4. The cells were then incubated at 37 °C in 5% CO_2 for 16 hours.

Immunofluorescent staining of peripheral blood lymphocytes

After the 16-hour incubation with LPS, the PBLs were prepared for flow cytometry by immunofluorescent staining of selected CD surface antigens and toll-like receptors 2 and 4. Two clean tubes were labeled 1 and 2, and to each, the incubated PBLs were transferred. Tube 1 was for analysis of toll-like receptor expression. Tube 2 was used for co-culturing with the target cells (K562) to measure the cytotoxic activity of the effector cells (NK cells). Antibodies specific to selected CD markers conjugated to different fluorochromes were purchased from Beckman Coulter. Antibodies directed against the toll-like receptors 2 and 4 were obtained from Biolegend.

To tube 1, antibodies directed against the CD surface antigens and the toll-like receptors were added in the following amount: 10 μL of CD45 conjugated with AlexaFluor 700, 10 μL of CD56 conjugated with APC, 10 μL of CD3 conjugated with ECD, 10 μL of CD14 conjugated with PC5.5, 5 μL of anti-human TLR2 antibody conjugated with FITC, and 5 μL of anti-human TLR4 antibody conjugated with PE. To tube 2, the following antibodies were added: 10 μL of CD45 conjugated with AlexaFluor 700, 10 μL of CD56 conjugated with APC, and 20 μL of CD3 conjugated with PE. Tubes were briefly vortexed and incubated in the dark for 30 minutes at room temperature.

After incubation, 2 mL of PBS were added to each tube. The tubes were then centrifuged for 5 minutes at 1800 rpm and the supernatant discarded. PBS was again added and the process was repeated twice more. After the final wash, 1.5 mL of PBS was added to tube 1. The