RESEARCH ARTICLE

Estudy the Effect of Breast Cancer on Tlr2 Expression in Nb4 Cell

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Abstract

Background: Breast cancer is the most common neoplasm in women and the most frequent cause of death in those between 35 and 55 years of age. All multicellular organisms have an innate immune system, whereas the adaptive or 'acquired' immune system is restricted to vertebrates. This study focused on the effect of conditioned medium isolated from cultured breast cancer cells on NB4 neutrophil-like cells. Materials and Methods: In the current study neutrophil-like NB4 cells were incubated with MCF-7 cell-conditioned medium. After 6 h incubation the intracellular receptor TLR2, was analyzed. Results: The results revealed that MCF-7 cell-conditioned medium elicited expression of TLR2 in NB4 cells. Conclusions: This treatment would result in the production of particular stimulants (i.e. soluble cytokines), eliciting the expression of immune system receptors. Furthermore, the flow cytometry results demonstrated that MCF-7 cell-conditioned medium elicited an effect on TLR2 intracellular receptors.

Keywords: Breast neoplasms - serum - antigens - CD14 - gene expression

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Introduction

Breast cancer is the most common neoplasm in women and the most frequent cause of death in women between 35 and 55 years of age. Various studies estimate the 5-year overall survival (OS) to be 65 %. Prognostic indicators include tumor size, axillary lymph node status, histological grade, tumor type, vascular invasion, and estrogen receptor status (Lee et al., 2002; Olewniczak et al., 2002). Several immunological markers of the disease have been reported, and recent developments in molecular and cytogenetic analysis have provided new means to evaluate prognosis (Giatromanolaki et al., 2002).

All multicellular organisms have an innate immune system, whereas the adaptive or 'acquired' immune system is restricted to vertebrates (Fearon and Locksley, 1996). The innate immunity system is comprised of physical barriers such as skin and mucosal linings of soluble factors such as complement components, chemokines and cytokines, and cells such as monocytes, macrophages, dendritic cells and polymorphonuclear blood cells (PMNs). Breaks in physical barriers such as a skin cut or severe burn can increase susceptibility to infection.

Pathogen associated molecular patterns (PAMPs) are conserved microbe-specific molecules that are associated with groups of pathogens. More precisely, PAMPs are small molecular motifs conserved within a class of microbes. Pathogen associated molecular patterns are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) that are proteins expressed by cells of the innate immune system. Cluster of differentiation 14 (CD14) (Fearon and Locksley, 1996; Medzhitov et al., 1997) and mannan-binding lectin (MBL) are the examples of these receptors.

TLR-bound PAMPs activate signal transduction pathways that result in the expression of specific genes that code for cytokines involved in immunological responses. To date, 13 TLRs have been identified, all possessing an intracellular domain similar to interleukin-1 (IL-1), that so-called TIR (toll-IL-1 receptor) domain too (Medzhitov et al., 1997).

It has been demonstrated that PAMP-mediated activation of phagocytic cells, such as monocyte-derived macrophages involves a cell-surface complex of CD14 and TLRs (specifically TLR2). TLR2 recognize highly conserved structural motifs known as PAMPs, which are exclusively expressed by microbial pathogens (Underhill et al., 1999).

Neutrophils and Their Functions: Neutrophil is a particularly abundant PML type, comprising 50-60% of circulating leukocytes. They are the first line of defense against infection and injury. The life span of a terminally differentiated leukocyte in the blood stream is about 6-8 hours (Cassatella et al., 1997). However, neutrophils exposed to certain external stimuli such as interleukin-1β (IL-1β), IL-6, IL-8, TNF-α, GM-CSF, and oncostatin M (OSM), can survive for longer periods of time (Cross et al., 2003). The presence of neutrophils, found typically at sites of inflammation, infection, trauma and ischemia, is mostly beneficial. However, they have been implicated in cancer development and progression (Baggiolini et al., 1989). Since neutrophils are terminally

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differentiated, they have little capacity for biosynthesis, but they can produce considerable amounts of cytokines (Cassatella, 1995) and chemokines (Scapini et al., 2000). Both are responsible for chemotaxis of effector cells, their activation and proper functioning. Neutrophils are among the first response elements to infiltrate an injured tissue where they produce TNFα (Feiken et al., 1995) and interleukins (IL-1α and IL-1β) (Hubner et al., 1996). These protein inflammatory messengers recruit leukocytes to the site of inflammation and mediate tissue repair by stimulating fibroblasts (Chedid et al., 1994) to produce matrix metalloproteinases (MMPs) and keratinocyte growth factor (KGF/FGF-7). Another important function of neutrophils is the production of oxidative radicals in the infected tissue and phagocytosis of infective agents (Kohen and Nyska, 2002).

Toll-like Receptors 2 (TLR2): Pattern recognition receptors (PRRs), comprise a diverse, evolutionary conserved set of proteins that recognize specific PAMPs. Thereby, PRRs allow the innate immune system to distinguish self-molecules from pathogen associated non-self-structures and to initiate the host defense response (Medzhitov and Janeway, 1998; Janeway and Medzhitov, 2002). PAMPs represent the molecular signatures of potentially noxious substances and may be perceived as a 'danger signal' (Medzhitov and Janeway, 1998) by the innate immune system (Janeway, 1989c; Janeway, 1989b; Janeway, 1989a; Fearon and Locksley, 1996).

TLRs are broadly expressed on macrophages, dendritic cells, epithelial cells, B-cells (TLR4 and 9) and T-cells (TLR2). TLRs are transmembrane proteins with an extracellular domain containing leucine-rich repeats that recognize conserved motifs on pathogens, and a cytoplasmic domain similar to the corresponding domain of the IL-1 receptor involved in signal transduction (Aderem and Ulevitch, 2000; Hallman et al., 2001). The capacity of TLRs to alter the phenotype of the cell on which they are expressed, makes them attractive candidates for the initiators of the entire program of host defense, be it innate or acquired.

The role of TLRs in the recognition of pathogens and the initiation of adaptive immune responses against them is well known. However, TLRs have been identified on several tumor cell types, including tumor cells of human malignancies (Zeromski et al., 2008). Their expression in cancer results either in promoting or inhibiting tumor progression. It has been demonstrated that several TLR agonists (natural and synthetic) may have a beneficial effect on tumor-mediated disease, leading to potentiation of the immune response to tumor-associated antigens.

TLR proteins can be used by the host to recognize and conserve PAMPs (So and Ouchi, 2010). However, some TLRs are able to detect specific host molecules, such as high-mobility group box protein 1 (HMGB1) and heat shock proteins (HSP), leading to inflammatory responses. Thus, it has been suggested that TLRs are involved in the development of many pathogenic conditions. Recent advances in TLR-related molecules have highlighted the therapeutic potential of these proteins against diseases, such as autoimmune disease and cancer.

TLR2 is found on the cell surface, but after activation it enters phagosomes (Janeway and Medzhitov, 2002). Self-antigens have been reported that can stimulate TLR2 including heat shock protein 60 (HSP60) (Ohashi et al., 2000), heat shock protein 70 (HSP70) and endoplasmin (Vabulas et al., 2002), which are components of the extracellular matrix (fibronectin) (Liu-Bryan et al., 2005).

Xie et al. (2009) reported invasion behavior by MDA-MB-231 cells, mediated by TLR2 through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ϰB) pathway. TLR2 is expressed in MDA-MB-231 cells at high levels and mediates aggressive cell invasion. As compared to other breast cancer cell lines, the MDA-MB-231 cell line produces 10 fold greater amounts of TLR2. One consequence is that, TLR2 activation leads to the secretion of growth and attachment factors including, IL-6, transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) (Xie et al., 2009). TLR2 and TLR9 have been reported to promote tumor invasive behavior via metalloproteases (Chen et al., 2007) and integrins (Coussens and Werb, 2002).

TLR stimulation in vitro of several types of tumor cell lines leads to their enhanced survival and increased cell proliferation. Cells isolated from patients suffering from multiple myeloma expressed increased levels of TLR2 receptors. TLR ligands that are encoded by pathogens have been divided into three categories based on their chemical structure. TLR4 receptors recognize lipopolysaccharides (LPS) from Gram-negative bacteria, belong to the lipoprotein and lipid category (Akira et al., 2006). There is evidence to indicate the over expression of TLRs in mouse (Huang et al., 2005) and in human prostate cancer, lung cancer, breast cancer and neuroblastoma. Different types of tumors have been reported to express a wide array of TLRs, but this has been correlated with increased cell survival, progression, tumor mass growth, metastasis and invasion of the cancer cells (Droemann et al., 2005; Hassan et al., 2006; Ilvesaro et al., 2007). TLR4 expression in human breast cancer cells leads to immune escape of breast tumor cells and their metastasis and invasion to other parts of the body (Ilvesaro et al., 2007).

Materials and Methods

Cell line and cell culture

NB4 Cell Line: Acute promyelocytic leukemia (APL) is a well-defined sub-type of acute myelogenous leukemia, cytogenetically characterized by t (15; 17) (q22; q11-12) translocation. The cell line NB4 is the only permanent cell line with t (15; 17), and was established from the leukemia cells of a patient with APL. APL cells have a strikingly low proliferation potential in vitro. Mitotic cells are often scarcely detected after 3 to 4 days in culture. However, APL cells do survive in culture, while many leukemia cell types die. This suggests that they have no requirement for survival factors, unlike normal promyelocytes or factor-dependent cell lines.

The NB4 cell line was used as a model for neutrophils in this project because NB4 cells have a myeloblastic morphology and lack typical APL granules. This finding does not necessarily contradict the promyelocytic nature of the leukemia, because hypogranular forms of APL have

been described (Lubbert and Koeffler, 1988).

Methods

Cell Culture: NB4 cells and MCF-7 cells were cultured in RPMI-1640 medium (PAA Laboratories Gmbh, Austria) containing 10 % FCS (PAA Laboratories Gmbh, Austria). Cells were cultured at 37°C in a humidified 5% CO_2 incubator. Staining immune system receptor in NB4 cells for flow cytometry analysis.

NB4 cells were stimulated with FCS, MCF-7 cell-conditioned medium (prepared by culturing MCF-7 cells in RPMI-1640 medium). Positive controls comprised treating NB4 cells with 100 IU/ml of recombinant human interferon-gamma (rh IFN-γ) (Immuno Tools; CAT#11343536) and 1 μg/ml LPS 055:B5 from Escherichia coli (SIGMA; CAT#L4524). Cells were stained using fluorescein isothiocyanate (FITC), TLR2 (Biolegend; CAT#325604). IgG isotype FITC mouse antibody (Biolegend; CAT#400212) was used as the negative control. Samples were then analyzed using a Aria generation 1 (BD Biosciences) system.

Western blots: The NB4 cells were stimulated with FCS, MCF-7 cell-conditioned medium, 100 IU/ml IFN-γ and 1 μg/ml LPS. To prepare whole cell lysate, Proteo JET™ Mammalian cell Lysis (Fernataz; CAT#K0301) and protease inhibitor (Sigma-Aldrich) were mixed at a 100:1 ratio and added to NB4 cells. THP-1 cell lysate, used for analyzing TLR2 protein expression, served as positive controls.

For each cell line, 5×10^6 cells were lysed by incubation on ice with 400 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Lysates were centrifuged for 20,000 g at 4°C for 10 min, and 20 μg of each protein samples were run on SDS-PAGE under reducing conditions. Proteins were electrotransferred to a nitrocellulose membrane and detected with a primary antibodies mouse anti-human TLR2. secondary antibody goat anti-mouse IgG IRDye 800CW (ODYSSEY; CAT#926-32210). The membrane was read using a Li-Cor scanner using Odessy software. PageRuler™ Plus Prestained Protein Ladder from Fermentas (Thermo Fisher Scientific; CAT#26616) was used to estimate the molecular weight of the proteins.

Results

Intracellular Expression

A summary of the data obtained are shown in the bar charts of the expression of intracellular receptor in NB4 cells cultured with FCS (A), MCF-7 cell-conditioned medium (B), IFN-γ and LPS (C) stained with FITC-conjugated mouse-monoclonal antibodies specific for human TLR2. IgG isotype antibody used as negative control (Figure 1). These flow cytometer results show that TLR2 expression was up-regulated in NB4 cells incubated with MCF-7 cell-conditioned medium (B).

A) Expression of TLR2 receptor in NB4 cell cultured with FCS. B) Expression of TLR2 receptor in NB4 cell cultured with MCF-7 cell-conditioned medium. C) Expression of TLR2 receptor in NB4 cell cultured with IFN-γ + LPS after 6 hours incubation. C is the positive control. Part A is the negative cells and part b is the positive cells.

Western Blotting

In order to detect the expression of TLR2 in NB4 cells by western blot, cell lysates were prepared from NB4 cells incubated with FCS, MCF-7 cell-conditioned medium, IFN-γ and LPS, and then separated on 12% SDS-PAGE (Figure 2).

1) Protein ladder, 2) NB4 cells stimulated with MCF-7 cell-conditioned medium, 3) NB4 cells stimulated with 10 % concentration of FCS, 4) NB4 cells stimulated with 100 IU/ml IFN-γ +1 μg/ml LPS, 5) THP-1 cell lysate as positive control. All stimulations were done for 6 hours. TLR2 detected at its expected molecular weight 90-100 kDa.

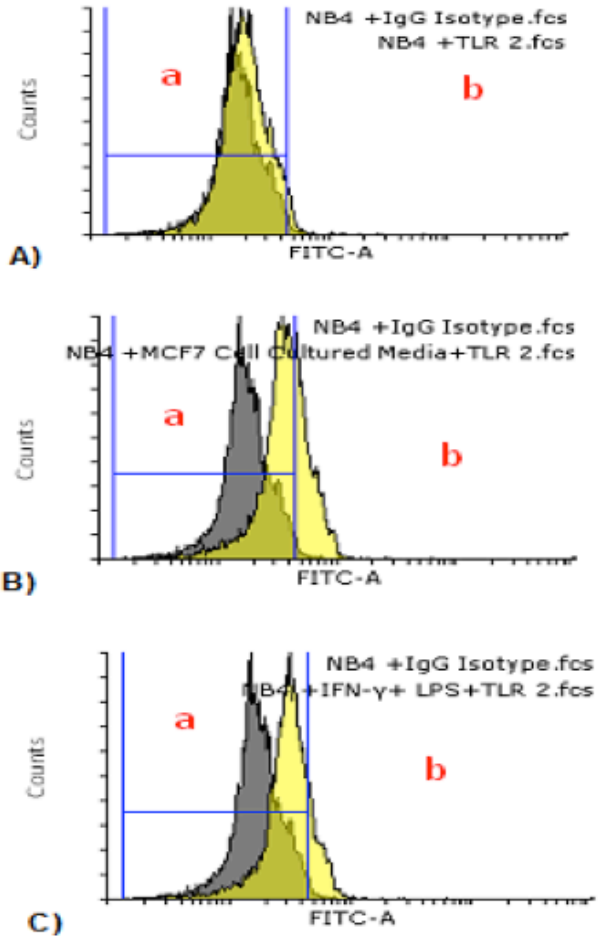

Figure 1. Intracellular expression of TLR2 receptor in NB4 cell cultured with FCS (untreated negative control), MCF-7 cell-conditioned medium or 100 IU/ml IFN-γ+1 μg/ml LPS (treated positive control) confirmed by flow cytometry. Black filled histograms show Isotype Control, and yellow filled histograms represent mean IMF intensity of NB4 cells labelled with FITC antibodies

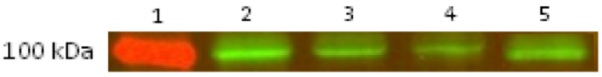

Figure 2. Western Blotting of TLR2 Receptor in NB4 Cells Lysate

Discussion

This study has focused on the effect of conditioned medium isolated from cultured breast cancer cells on NB4 neutrophil-like cells. Intracellular immune receptor was examined. The human promyelocytic cell line NB4 was used as a model system as it is an easily accessible cell type that propagates *in vitro*. This cell line has been previously used as it closely resembles the phenotype of real neutrophils from normal adults. NB4 cells exhibit the chromosomal translocation t (15; 17), specific for acute promyelocytic leukemia (APL) that results in the fusion of the retinoic acid receptor alpha (RARA) gene to the promyelocytic leukemia (PML) gene. The characterization of PML is a putative zinc finger protein and potential transcription factor, with at least three transcription products (Goddard et al., 1991). In the current study presented here it was hypothesized that patients suffering from breast cancer have a stimulated immune system and hence trigger the release of soluble proteins that alters the phenotype of cells including neutrophils and granulocytes. If so, it is likely that some of those soluble proteins would up-regulate or down-modulate immune receptors. This effect can be local at the site of a tumor or at systemic level. In previous studies it has been shown that secretion of cytokines released into the circulation and present in the plasma could lead to systemic activation of immune receptors and hence affects host tumor interactions (Silverman et al., 2005).

Conditioned medium is the general term used to describe complete medium fluid in which cells have been cultured for a period of time. Conditioned media are obtained from short-term or long-term cultures of the cancer cells, which are likely to contain different soluble mediators or secreted factors. These mediators include metabolites such as glucose, amino acids, and nucleosides; growth factors such as interleukins, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF); and matrix proteins such as collagen, fibronectin and cytokines. Collectively, these products are likely to have a strong influence on the cancer cells and may promote either the growth or inhibition of neoplasma cells and metastasis.

In normal healthy individuals neutrophils are able to attach and eliminate microorganisms with the release of cytotoxic substances (Smith, 1994). Activated neutrophils also release proteinases into the extracellular environment, directing to damage of surrounding host tissue (Pham, 2006). Moreover, neutrophils produce cytokines and chemokines, which contribute to inflammation and cell recruitment and thus modifies the immune response (Nathan, 2006). This process of PMN recruitment and activation, found in during infection, is enhanced within the tumor microenvironment. In this context, PMNs could act to the damage of the host cells.

Previous studies have shown that a factor present in the conditioned media obtained from hepatocellular carcinoma cells induced the production of hepatocyte growth factor (HGF) from neutrophils, which promoted increased invasiveness by the tumor cells (Imai et al., 2005). These authors suggest that, although neutrophils release a paucity of cytokine in contrast to macrophages and other cells within the tumor microenvironment, the temporal-spatial contribution of these substances by PMN performs unique roles in tumor progression.

In the current study neutrophil-like NB4 cells were incubated with MCF-7 cell-conditioned medium. After 6 h incubation the receptor TLR2, was analyzed as intracellular on NB4 cells. These molecules were investigated in NB4 cells. The results revealed that MCF-7 cell-conditioned medium elicited the expression of TLR2 in NB4 cells. This observation is of utmost significance as it is known that TLRs are considered important innate recognition molecules of the immune system. Their role in the recognition of pathogens and the initiation of adaptive immune responses well established. TLRs have been previously reported on several tumor cells (Sato et al., 2009). Activation of TLRs with their ligands directly or with aid of accessory proteins such as CD14 and MD2 (in the case of TLR2 and TLR4), cells evoke strong inflammatory responses. This mechanism is capable of coordinating the immune system in the whole organism, protecting the host from pathogen spreading after infection (Takeda and Akira, 2005). In this context it was reported that TLR expression in many tumors or cell lines is inducible and hence likely to play an anti-tumor effect (Yu and Chen, 2008). Also, in some tumor models, polymorphism of TLR2 and TLR4 is known to be a contributory factor to the risk of cancer. This implies that genetic variation or alleclic forms of TLR may be connected with specific tumor progression (El-Omar et al., 2008). In the current study it is shown that TLR2 is affected by MCF-7 cell-conditioned medium in the cytoplasm of NB4 cells.

TLR2 is the receptor for several microbial ligands, including gram-positive bacteria, peptidoglycan, yeast zymosan, and mycobacterial lipoarabinomannan (LAM) (Means et al., 2000; Zhang and Ghosh, 2001). On the other hand TLR4 is the receptor for gram-negative bacteria, LPS, and some viruses (Means et al., 2000; Zhang and Ghosh, 2001; Kurt-Jones et al., 2002). The receptors TLR2 and TLR4, like other TLR group members, have a conserved intracellular signaling motif also found in the intracellular domain of the IL-1 receptor. This motif is responsible for NF-ϰB activation/translocation after TLR or IL-1 receptor engagement. It is also a crucial signaling pathway for IL-1β and TNF-α secretion (O'Neill, 2000).

Intracellular Receptor Expression in NB4 Cells: The experiments for this study were carried out by incubating NB4 cells at 37 °C for a maximum period of 6 h with 10 % FCS, the breast cancer cell line MCF-7. It was anticipated that this treatment would result in the production of particular stimulants (i.e. soluble cytokines), eliciting the expression of immune system receptors. However, the flow cytometry results demonstrated that MCF-7 cell-conditioned medium elicited an effect on TLR2 receptors as intracellular receptor. A reasonable hypothesis is to as same that the patient with cancer have a stimulated immune system if so it is likely but immunological receptors as well as cytokines are expressional locally at the site of cancer systemically. There for the reasonably

as submission is to expect the present function of soluble cytokine which stimulate the biological receptors of immune system.

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