SYNOPSIS
Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student: Rajshri Singh

2. Name of the Constituent Institution: BARC

3. Enrolment No.: LIFE 01200804013

4. Title of the Thesis: Role of macrophages in growth and progression of breast cancer.

5. Board of Studies: Life Sciences

SYNOPSIS

Introduction

The tumor microenvironment is composed of malignant and immune cells, cytokines, chemokines and stromal components including extracellular matrix (ECM) and plays an important role in facilitating cancer progression and metastasis. Interactions between tumor and immune cells through the soluble factors they secrete influence the tumor cell survival and proliferation, integrity of the ECM, invasion, angiogenesis and metastasis [1]. The importance of macrophages, one of the prominent infiltrating immune cells, in growth and metastasis of breast
cancer is well documented. Macrophages participate in a number of pathophysiological settings, due to high plasticity of their functional responses. Macrophages populate the microenvironment of most if not all tumors. They secrete a variety of growth factors, cytokines, chemokines and enzymes that regulate tumor growth, angiogenesis, invasion and metastasis [2]. The tumor associated macrophages (TAM)-derived conditioned medium can induce angiogenesis in various in vivo model systems [3].

Focal macrophage infiltration is an important prognostic factor in breast invasive carcinoma and reduced survival is associated with high infiltration rates [4]. A large number of studies indicate that many of the inflammatory components present in the tumor microenvironment actively support cancer development and progression [1, 2, 5, 6]. But how the inflammation link operates in breast cancer is still an open question. Breast cancer is no longer seen as a single disease but rather a multifaceted disease comprised of distinct biological subtypes with diverse natural history, presenting a varied spectrum of clinical, pathologic and molecular features with different prognostic and therapeutic implications. Consensus regarding the definitive prognostic/predictive analysis is yet to be reached, but significant progress continues to be made in the ongoing search for a specific, rigorous and reproducible method of identifying successful treatment algorithms utilizing biological markers. The hypothesis of this study is that macrophages influence the growth and progression of breast cancer. To test this hypothesis the effect of monocyte and macrophage conditioned media was tested on growth and migration of breast cancer cells in vitro. The importance of some of the prominent proteins identified during this in vitro study was also evaluated in archived fibroadenoma and invasive ductal carcinoma (IDC) samples. The specific aims of the studies included in this thesis are:
1. To study the role of macrophages and inflammatory mediators in growth and migration of breast cancer cell lines in vitro.

2. To identify the soluble factors secreted by macrophages responsible for tumor growth promotion.

3. To understand the relationship between inflammatory response, DNA damage and survival factor signaling pathways in breast cancer by immunohistochemical labeling of representative markers: iNOS (inflammation), CD68 (macrophage), pCREB (pro-survival transcription factor), γ-H2AX (DNA damage) and p53 (tumor suppressor protein) in benign fibroadenomas as well malignant invasive ductal carcinoma.

4. To identify if any of these proteins could serve as a biomarker of malignancy or metastasis.

The work embodied in this thesis is divided into four chapters: Chapter 1: General introduction and review of literature. Chapter 2: Materials and Methods. Chapter 3: Results. Chapter 4: General Discussion and conclusion. The 'Results' chapter is further sub divided into three sections (3.1): Role of macrophages in non invasive and invasive breast cancer cell lines. (3.2): Characterization of monocyte and macrophage conditioned media. (3.3): Evaluation of the expression of biomarkers pCREB, iNOS, CD68, γH2AX and p53 proteins in fibroadenoma and invasive ductal carcinoma by immunohistochemistry.

**Chapter 1.** This chapter describes the general information on breast cancer and review of literature on role of macrophages in cancer progression. TAMs promote cancer metastasis through several mechanisms, including promotion of angiogenesis, induction of tumor growth, and enhancement of tumor cell migration and invasion. A variety of cytokines and growth
factors, such as tumor necrosis factor (TNF-α), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), epidermal growth factor (EGF) etc., have been implicated in tumor-stroma cross-talk. The TGF-β-pathway is one of the major pathways altered in tumors, including breast cancer [7, 8].

Metastasis is a biological cascade of multiple steps: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue and eventual colonization at a distant site. Mechanisms that induce epithelial mesenchymal transition (EMT) involve multiple extracellular triggers and intracellular signaling pathways [9-11]. These include oncogenic signaling [12], Wnt3/β catenin signaling, increased reactive oxygen species (ROS) [11] as well as DNA damage [10]. This chapter describes the various signaling and survival pathways involved in macrophage mediated cancer progression. In response to DNA damage or extracellular signals, expression of several transcription factors like Cyclic AMP (cAMP) response element binding protein (CREB), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), c-fos and c-jun allows the cells to overcome stressful or deleterious environment. Previous studies have reported the tumor promoting function of CREB in breast cancer, melanoma, and hepatocellular carcinoma [13-15]. CREB also acts as a proto-oncogene to regulate hematopoiesis and to contribute to the leukemia phenotype [16, 17]. In melanoma, CREB activation regulates the expression of many genes important for invasion, inflammation, and survival including MCAM/MUC18, MMP2, IL-8, and BCL2 [18-20].

Macrophages are a key component of a chronic inflammatory response and constitute part of the heterogeneous population of cells in tumors. Macrophages and nitric oxide (NO) have been implicated in the activation of p53 [21] in inflammatory bowel disease (IBD) and the activation of the Akt pathway in breast cancer [22]. The literature related to different clinical
markers used for breast cancer and their regulatory effect on various cellular processes would be described in this chapter.

Chapter 2. This chapter describes the details of materials used along with their sources and common experimental methods used in this study. Detailed protocols of different techniques and approaches used in this study will be described under three different categories: (i) cell culture techniques including maintenance of cell lines, preparation of conditioned medium, colony forming assay, cell cycle analysis, estimation of ROS and RNS, flow cytometric analysis, immunofluorescence, wound healing, migration assay, si-RNA mediated transfection, and western blots, (ii) techniques used for characterization of CM like ELISA, 1D and 2D gel electrophoresis, MALDI analysis and preparation of exosomes and (iii) clinical techniques including archived breast cancer biopsy sample collection, tissue microarray (TMA) preparation, IHC labeling, scoring and statistical analysis.

Chapter 3. The results obtained from this study have been presented in three sections.

3.1. Role of macrophages in non invasive and invasive breast cancer cell lines.

In this study, the macrophage – tumor interaction was studied by employing monocyte conditioned medium (MCM) and macrophage conditioned medium (MφCM) treatment to epithelial breast cancer cell line, MCF7 and invasive breast cancer cell line, MDA-MB-231. Differential effects of MφCM were observed in these two cell lines differing in their invasive nature. MφCM treatment resulted in increased merging of colonies accompanied by EMT responses in MCF7 cells and larger sized, multinucleated cells resembling senescence type phenotype in MDA-MB-231 cells. The macrophage conditioned media contained various pro-inflammatory cytokines like TNF-α, IL-1β and IL-6. These cytokines in turn induced secretion of
TGF-β1 in MCF7 cells. As a multifunctional factor, TGF-β1 is involved in the regulation of many biological processes and induced concomitant apoptosis and EMT responses in hepatocytes [23, 24]. It has been referred to as a “double edged sword” because of its dual function as tumor suppressor and tumor promoter (reviewed in [25]). TGF-β1 caused apoptosis in some of the cells and significant increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation in the surviving cells. This oxidative and nitrosative stress resulted in DNA damage response signaling as observed by expression of phosphorylated ATM and H2AX proteins. In contrast, there was no increase in apoptosis, ROS, RNS or DNA damage in MDA-MB-231 cells. CREB, a 43 kDa-basic/leucine zipper (bZip) transcription factor plays important roles in cell differentiation [26], survival [27, 28], proliferation [17], development [29], cell cycle progression [30] and glucose metabolism [31]. A significant increase in pCREB and total CREB was observed in MφCM treated MCF7 cells in contrast to MDA-MB-231 cells where basal level expression of CREB was higher and was unaffected by the treatments. There was a marked increase in vimentin expression (a EMT marker) as well as increase in migration of MCF7 cells treated with MφCM. MφCM induced expression of pCREB and invasion in MCF7 cells was significantly decreased following neutralization of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6 in the conditioned media) or treatment with N-acetyl cysteine (NAC) or inhibitors of iNOS and ATM/ATR. Decreased phosphorylation of pCREB as well as decreased migration was observed in MCF7 cells with siRNA mediated downregulation of ATM.

3.2. Characterization of monocyte and macrophage conditioned media.

The tumor promoting activities of TAM are the result of the ability to express numerous mediators, such as growth factors, angiogenic molecules, ECM degrading enzymes, inflammatory cytokines and chemokines. In order to identify the constituents of the conditioned
media, two approaches were followed. The proteins were precipitated using ammonium sulfate or concentrated through centricon filtration with a 10 kDa cutoff. The conditioned media concentrated using centricon filters could not be absorbed onto isoelectric focusing (IEF) strips or move in the electric field indicating the presence of lipid components. Exosomes are small membrane vesicles that can be secreted *in vitro* by most cell types and the general idea is that they could play roles as “intercellular messengers”, transferring various kinds of informations or signals between cells. To determine if exosomes secreted by macrophages were involved in the induction of EMT signals in breast cancer cells, they were purified and the supernatant obtained following ultra centrifugation was collected and termed as exosomes free conditioned medium (EFCM). The protein profile of exosomes secreted by monocytes and macrophages were similar and the exosomes from both cell types was taken up by MCF7 cells indicating that it did not play a major role in the growth promoting effects exerted by MϕCM. On the other hand the effect of EFCM and EFMϕCM on the growth of MCF7 cells was identical to MCM and MϕCM. The EFMϕCM treatment resulted in decrease in the colony forming ability in MCF7 and MDA-MB-231 cells along with an increase in merging of colonies in MCF7 cells. These results thus confirmed that the soluble mediators present in the CM and not exosomes were responsible for the observed changes.

Presence of some of the cytokines was detected using ELISA. The cytokines IFN-γ and TGF-β1 were absent in MCM and MϕCM whereas the pro inflammatory cytokines like TNF-α, IL-1β and IL-6 were present only in MϕCM. In order to further characterize the proteins present in MϕCM, the conditioned media were subjected to 2D electrophoresis. The proteins were precipitated with ammonium sulfate for this purpose. Though the number of proteins observed in MϕCM was higher in 1D, this difference was not seen in 2D gel electrophoresis. This could
happen due to proteins with lower or higher pI or multicomplex proteins. Hence the proteins were separated in 1D gel electrophoresis followed by MALDI analysis of the differentially expressed proteins. The protein samples were subjected to peptide mass fingerprint (PMF) analysis by MALDI-TOF and protein IDs were generated. The proteins upregulated in MφCM were found to be isoforms of matrix metalloproteinase 1 (MMP1) pre proprotein variant, MMP 9 pre proprotein variant, chitinase-2, plasminogen activator inhibitor-2, myoferlin, L-plastin, dual specificity protein kinase CLK3, etc. These proteins are known to have their role in breast cancer growth and progression. TNF-α is an important mediator during the inflammatory phase of wound healing and TNFα stimulates secretion of active MMP-2. Confirmation of the identity of MMPs was also carried out by gelatin zymography.

3.3. Expression of pCREB, iNOS, CD68, γ-H2AX and p53 protein in fibroadenomas and invasive ductal carcinoma samples by IHC.

The relationship between inflammatory mediators, DNA damage, survival signaling and migration was studied in breast cancer cells in vitro. To confirm if these biomarkers have a practical relevance and can be used to predict malignancy or metastasis, CD68, a macrophage marker, iNOS, a marker for inflammation, pCREB, a pro-survival transcription factor, γ-H2AX, effector of the DNA damage response pathway and p53 as a representative tumor suppressor protein were assessed in fibroadenoma and invasive ductal carcinoma samples by IHC labeling. Three cores (2 mm each) from each of these donor samples representing different areas of tumor were placed on a recipient paraffin block in the form of tissue microarray (TMA). Tissue sections (5-7 µm thick) were cut from the TMA paraffin embedded blocks on a microtome and mounted on the slides. After appropriate deparaffinzation, rehydration and unmasking of the antigens, they were labeled with various antibodies. The images of stained
tissues were taken with Metasystems Imager. Z2 Zeiss microscope enabled with Metaviewer V2 software. The scoring for protein expression for each sample was given as the product of intensity of labeling and percentage positive cells. The labeling of 91 IDC samples and 23 fibroadenoma samples was carried out. There was a statistically significant increase in iNOS and CD68 expression (p<0.01) in IDC as compared to fibroadenoma. No such difference was observed with respect to pCREB as a marker.

**Chapter 4.** This chapter covers the general discussion and conclusions on the results obtained in the study. Macrophages play an ambiguous role in cancers. Macrophages and tumor cells mutually influence each other’s behaviour in majority of cancers, with the tumor cell attracting macrophages and sustaining their survival and they, in turn, producing a myriad of factors to promote or regulate tumor growth and angiogenesis. The main finding of this study is that the pro-inflammatory cytokines secreted by macrophages induce secretion of TGF-β1 in MCF7 cells. This results in apoptosis in a fraction of cells. In the remaining cells, there is increase in oxidative stress and DNA damage which trigger CREB mediated survival signaling inducing EMT responses. The data presented herein not only provide evidence that macrophage mediated release of soluble factors result in EMT responses in tumor cells but also point out that a differential effect on tumors depending on their invasive nature. There was a statistically significant increase in expression of markers CD68 and iNOS in IDC as compared to the fibroadenoma samples. The expression and correlation of iNOS, CD68, pCREB, γ-H2AX and p53 in breast cancer samples will be discussed in this chapter in light of their value as prognostic or predictive factors and in turn their potential for integration into clinical practice.
Figure 1: The schematic representation of the effect of MφCM on MCF7 cells

References:


Publications in Refereed Journal


Other Publications: Symposium presentations:
