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Hormonal regulation of the cytokine microenvironment in the mammary gland

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ABSTRACT

The mammary gland is a unique organ that undergoes hormone-driven developmental changes over the course of the ovarian cycle during adult life. Macrophages play a role in regulating cellular turnover in the mammary gland and may affect cancer susceptibility. However, the immune microenvironment that regulates macrophage function has not been described. Hormonal regulation of the cytokine microenvironment across the ovarian cycle was explored using microbead multiplex assay for 15 cytokines in mammary glands from C57Bl/6 mice at different stages of the oestrous cycle, and in ovariectomised mice administered oestradiol and progesterone. The cytokines that were found to fluctuate over the course of the oestrous cycle were colony-stimulating factor (CSF)1, CSF2, interferon gamma (IFNG) and tumour necrosis factor alpha (TNFA), all of which were significantly elevated at oestrus compared with other phases. The concentration of serum progesterone during the oestrus phase negatively correlated with the abundance of cytokines CSF3, IL12p40, IFNG and leukaemia inhibitory factor (LIF). In ovariectomised mice, exogenous oestradiol administration increased mammary gland CSF1, CSF2, IFNG and LIF, compared with ovariectomised control mice. Progesterone administration together with oestradiol resulted in reduced CSF1, CSF3 and IFNG compared with oestradiol administration alone. This study suggests that the cytokine microenvironment in the mammary gland at the oestrus phase of the ovarian cycle is relatively pro-inflammatory compared with other stages of the cycle, and that the oestradiol-induced cytokine microenvironment is significantly attenuated by progesterone. A continuously fluctuating cytokine microenvironment in the mammary gland presumably regulates the phenotypes of resident leukocytes and may affect mammary gland cancer susceptibility.

1. Introduction

During the ovarian cycle, the mammary gland epithelium undergoes proliferation, differentiation and apoptosis under the direction of ovarian hormones oestrogen and progesterone (Fata et al., 2001; Ramakrishnan et al., 2002), and this cellular turnover has been linked to the high

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susceptibility of the mammary gland to tumour formation (Ramakrishnan et al., 2002). During the oestrus phase of the cycle, the mammary epithelium exhibits a basic ductal structure. As serum progesterone rises, secondary branching and alveolar bud development occur, with alveolar bud appearance predominantly during dioestrus (Fata et al., 2001). If pregnancy does not proceed, the fall in progesterone at proestrus induces apoptosis of the newly formed alveolar buds, and the tissue is remodelled back to the basic architecture.

Macrophages are immune system cells with multiple roles in epithelial cell turnover during the course of the ovarian cycle (Chua et al., 2010). Macrophages promote oestradiol- and progesterone-induced epithelial cell proliferation and alveolar development following ovulation, and when progesterone levels fall towards the end of the cycle, macrophages promote phagocytosis of apoptotic cells and remodel the tissue to its rudimentary structure. These differing macrophage functions are associated with altered phenotype, with fluctuation in expression of MHCII, CD204 and NKG2D by mammary gland macrophages across the cycle under hormonal control (Hodson et al., 2013). In order to recruit macrophages and regulate specific phenotypes and functions of these cells, an array of cytokines and chemokines are likely to be present in the mammary gland microenvironment.

Cytokines comprise the interleukins, lymphokines and related regulatory glycoproteins released by cells of the immune system or non-haematopoietic cells, to act as intercellular mediators in the generation of an immune response. Previous studies have shown that cytokines, including colony-stimulating factor 1 (CSF1), interleukin 4 (IL4) and interleukin 13 (IL13), play important roles in mammary gland development during puberty and pregnancy (Pollard and Hennighausen, 1994; Gouon-Evans et al., 2000; Ingman et al., 2006; Khaled et al., 2007). CSF1, for example, is known to be a regulator of macrophage survival. *Csf1* null mutant mice have significantly reduced macrophage abundance and display impaired mammary gland development during puberty and lactation (Gouon-Evans et al., 2000; Ingman et al., 2006). IL4 and IL13 play a role in the promotion of luminal mammary epithelial cell development during pregnancy through the Stat6/Gata3 signalling pathway. Mice with a double mutation in both the *Il4* and *Il13* genes show a significant decrease in the number of mammary gland side branches and alveolar buds (Khaled et al., 2007).

Increased breast cancer risk is associated with early menarche, late menopause and increased cumulative number of menstrual cycles (Chavez-Macgregor et al., 2008; Collaborative Group on Hormonal Factors in Breast Cancer, 2012). Circulating oestrogens are elevated in young women with early menarche (Shi et al., 2010) and this early exposure to oestrogen may be responsible for the increased risk of breast cancer in women with early menarche over and above the increased risk associated with menstrual cycling (Collaborative Group on Hormonal Factors in Breast Cancer, 2012). Fluctuating ovarian hormones presumably create a pro-tumourigenic environment in the mammary gland that, over time, increases the risk of cancer. The biological factors behind this pro-tumourigenic microenvironment have yet to be elucidated, but may include impaired immune surveillance and/or inflammation associated with altered cytokine regulation (Need et al., 2014).

The aim of this study was to define the cytokine microenvironment across the four phases of the mouse ovarian cycle and to investigate hormonal regulation of cytokines in the mammary gland. We demonstrate that the concentration of specific cytokines in the mammary gland fluctuates over the course of the ovarian cycle, with the oestrus phase being associated with a relatively pro-inflammatory cytokine microenvironment compared with other stages of the cycle. Furthermore, circulating progesterone negatively correlates with a number of these cytokines at the oestrus phase of the cycle, and in ovariectomised mice progesterone suppresses the oestradiol-induced cytokine profile. Fluctuations in the cytokine microenvironment in the mammary gland over the course of the ovarian cycle may affect susceptibility to mammary gland tumourigenesis.

2. Materials and methods

2.1. Mice

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004). Mice were maintained in specific pathogen-free conditions with controlled light (12 h light, 12 h dark cycle) and temperature at the Laboratory Animal Services Medical School facility.

The oestrous stages of naturally cycling C57Bl/6 mice were determined using cytological characteristics of vaginal smears (Bronson et al., 1968) performed daily. The mice were tracked for a minimum of two weeks through at least one normal four- to five-day oestrous cycle and were 12–15 weeks old at mammary gland dissection. Mice were euthanised at each of the four stages of the oestrous cycle (oestrus, $n = 16$; metoestrus, $n = 20$; dioestrus, $n = 18$; and proestrus, $n = 14$). At the time of euthanasia, blood was collected by cardiac puncture and the fourth abdominal pair of mammary glands was dissected, snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ before protein extraction.

To investigate hormonal regulation of the mammary gland cytokine microenvironment, 12-week-old mice were ovariectomised under 2% isoflurane anaesthesia and rested for one week. Subcutaneous 17β-oestradiol (1 μg) alone or in combination with progesterone (1 mg) was administered in 100 μl of sesame oil daily for three days, while control mice were given vehicle alone. On the fourth day, approximately 1 mL of blood was collected by cardiac puncture and the fourth pair of inguinal mammary glands was dissected. The section between the lymph node and the distal end of the gland was snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ for cytokine analysis. Blood was centrifuged for six minutes at 350 g and serum was collected and stored at $-80\,^{\circ}C$.