Cytokine-macrophage regulatory network in mammary gland development and tumourigenesis

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Abstract

Development and function of the mammary gland involves complex and dynamic interactions between epithelial and stromal cells under the influence of hormones and cytokines. Macrophages are a major component of the mammary gland stroma and they are capable of many roles in mammary gland development; importantly, their functions are tightly regulated by signals within the local cytokine microenvironment. The mammary epithelium secrets a number of cytokines, including transforming growth factor beta 1 (TGFB1) and chemokine ligand 2 (CCL2), that might affect the phenotype and function of adjacent stromal macrophages. Furthermore, alterations in cytokine secretion, and macrophage abundance and phenotype have been observed throughout different stages of normal mammary gland development and in tumourigenesis. A number of studies have demonstrated the significance of TGFB1 and CCL2 in regulating macrophages in many other tissues; however, the importance of the function of this cytokine-macrophage regulatory network in mammary gland development and tumourigenesis is yet to be investigated. The studies described in this thesis aimed to investigate the significance of epithelial cell-derived TGFB1 and CCL2 in regulation of macrophages in mammary gland development and mammary cancer susceptibility in the mouse and human mammary gland.

Utilising a mouse mammary gland transplant model whereby the mammary gland tissue from *Tgfb1* null mutant and wild-type mice were transplanted into TGFB1 replete recipients, we have demonstrated that deficiency in epithelial cell-derived TGFB1 caused a 50% increase of F4/80-positive macrophages invaded into the mammary epithelium, moreover, the number of iNOS-positive ("M1") and CCR7-positive ("M1") macrophages was increased by 78% and 200% respectively in the absence of epithelial cell-derived TGFB1. Similarly, immunohistochemical analysis of human non-neoplastic breast tissue revealed that there was a significant inverse relationship between the abundance of latent TGFB1 protein and the abundance of CD68-positive macrophages. We also observed a significant positive relationship between the abundance of latent TGFB1 and the density of stromal-associated CD206-positive ("M2") macrophages.

Further investigation of the role of TGFB-regulated macrophages in mammary gland development and tumourigenesis was undertaken utilising a transgenic (*Cfms-rtTA* x *TetO-TgfbrII*) mouse model whereby a dominant negative TGFB receptor is activated in macrophages in the presence of doxycycline, which in turn attenuates TGFB signalling in macrophages in these mice. Whole mount and H&E analysis revealed that impaired TGFB signalling in macrophages caused a 15% and 7% increase in the number

of ductal branch points and the percentage of alveolar epithelium respectively in the mammary gland at diestrus. Immunohistochemical analysis using macrophage markers indicated that impaired TGFB signalling in macrophages resulted in a similar alteration in macrophage phenotypes observed in TGFB replete mice transplanted with *Tgfb1-/-* epithelium. There was a 50% increase in abundance of macrophages invaded into the mammary epithelium, and the number of iNOS-positive ("M1") macrophages and CCR7-positive ("M1") stromal macrophages was increased by 110% and 37% respectively. The effect of impaired TGFB signalling in macrophages on mammary gland cancer susceptibility in mice was investigated by challenging the mice with DMBA carcinogen; a significant decrease in mammary tumour incidence and prolonged tumour free survival was observed in mice with impaired TGFB signalling in macrophages compared to controls.

The role of epithelial cell-derived CCL2 in regulation of macrophages in mammary gland development and cancer susceptibility was explored in a transgenic mouse model, *Mmtv-Ccl2,* whereby CCL2 is constitutively expressed by the mammary epithelium under the control of the MMTV promoter. Whole mount and H&E analysis revealed that the number of ductal branch points and the area comprised by alveolar epithelium were increased by 26% and 22% respectively in the presence of abundant epithelial cell-derived CCL2 at proestrus. Immunohistochemical analysis revealed that CCL2 did not affect the proliferation or apoptosis of mammary epithelial cells; however, there was a 40% and 53% increase in macrophage density and collagen deposition respectively around the ductal epithelium of mammary glands of transgenic mice compared to non-transgenic controls. Moreover, quantitative PCR analysis showed that the expression of *Lox* and *Timp3* was increased by 160% and 170% respectively in the mammary glands with constitutive CCL2 expression. In addition, we investigated the effect of constitutive expression of epithelial cell-derived CCL2 on mammary gland cancer susceptibility by challenging the *Mmtv-Ccl2* mice with DMBA carcinogen. A significant increase in mammary gland tumour incidence and reduced tumour latency was seen in mice with overabundant CCL2 expression compared to controls. Non-neoplastic breast tissue exhibited variable expression of CCL2 in the epithelium, with protein abundance ranging from low, to moderate and high. However, immunohistochemical analysis of human non-neoplastic breast tissue did not show a significant correlation between the expression of CCL2 and the abundance of macrophages. Interestingly, it was demonstrated that a significant negative relationship was found between the expression of CCL2 and the abundance of stromal-associated iNOS-positive cells in our human breast tissue.

Together, these studies suggest that epithelial cell-derived TGFB and CCL2 exert effects on mammary gland development and tumourigenesis through regulation of macrophage functions and phenotypes.

This implies that the finely orchestrated cytokine-macrophage regulatory network may be a contributing factor in mammary gland cancer susceptibility. These studies also reveal the possibility of targeting both TGFB and CCL2 signalling as a novel therapeutic approach to breast cancer prevention and/or treatment. However, more research will first be required on the upstream signalling events and underlying mechanisms that affect epithelial cell-derived TGFB and CCL2 macrophage-mediated mammary cancer risk.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications arising from this thesis

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2. **Sun X**, Robertson SA, Ingman WV. *Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland*. Cytokine. 2013; 61(2):377–88.

3. **Sun X**, Robertson SA, Ingman WV. (In preparation) *TGFB-regulated macrophages constrain mammary gland development and promote tumourigenesis.*

4. **Sun X**, Robertson SA, Ingman WV. (In preparation) *The role of epithelial cell-derived CCL2 in regulation of macrophages in mammary gland development and tumourigenesis.*

Abstracts arising from this thesis

2014

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"The role of CCL2 in mammary gland development and cancer"*, Research Centre for Reproductive health (RCRH) conference, Adelaide, Australia. Poster Presentation, November 2014.

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Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"TGFB1 is a key regulator of mammary gland macrophages"*, Research Centre for Reproductive health (RCRH) conference, Adelaide, Australia. Poster Presentation, November 2013.

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Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"The effect of epithelial cell-derived TGFB1on macrophage abundance and phenotype in the mammary gland"*, The Queen Elizabeth Hospital Research Day conference, Adelaide, Australia, Oral Presentation, October 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Faculty of Health Science (FHS) Meeting, Adelaide, Australia, Poster Presentation, August 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. *"Regulation of mammary gland macrophages by epithelial cell-derived TGFB1"*, Australian Society for Medical Research (ASMR) Scientific Meeting, Adelaide, Australia, Oral Presentation, June 2011.

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Epithelial cell-derived TGFB1 regulates macrophages abundance and phenotypes in the mammary gland"*, Pacific Rim Breast and Prostate Cancer Conference, Tweed Coast, Australia, Poster Presentation, May 2011.

2010

Xuan Sun, Sarah A Robertson, Wendy V Ingman. "*Location of active TGFB1 in the mammary gland during different stages of development*", Society of Reproductive Biology (SRB) Annual Scientific Meeting, Sydney, Australia, Oral Presentation, August 2010.

Abbreviations

ArgI	Arginase I
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCL2	Chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CCR7	C-C chemokine receptor 7
CDs	Cluster of differentiation
Col 1	Collagen 1
COX2	Cyclooxygenase 2
CRP	C-reactive protein
CSF1	Clony stimulating factor 1
CSF1R	Clony stimulating factor 1 receptor
DAB	3,3 diaminobenzadine
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DMBA	7,12-Dimethylbenz (a) anthracene
DNA	Deoxyribonucleic acid
Dox	Doxycylcine
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
FBXW7	F-box/WD repeat-containing protein 7
HRP	Horseradish peroxidase
IFNG	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kb	Kilo base
LAP	Latency-associated peptide
LOX	Lysyl oxidase
LPS	Lipopolysaccharide
LTBP	Latent TGFB binding protein
LTGFB1	Latent transforming growth factor 1
MD	Mammographic density

MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MMTV	Mouse mammary tumour virus
MMTV-LTR	Mouse mammary tumour virus long terminal repeat
NO	Nitric oxide
PBS	Phosphate buffered saline
PCNA	Proliferating cellular nuclear antigen
PCR	Polymerase chain reaction
PyMT	Polyoma middle T antigen
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
SEM	Standard error of the mean
SOCSI	Suppressor of cytokine signalling 1
TAM	Tumour-associated macrophages
TGFB1	Transforming growth factor beta 1
TGFBRI	Transforming growth factor beta type I receptor
TGFBRII	Transforming growth factor beta type II receptor
TIMPs	Tissue inhibitors of matrix metalloproteinases
TLR	Toll-like receptor
TNFA	Tumour necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WAP	Whey acid protein