

# **Regulation of Bovine Mammary Epithelial Cell Response by Autocrine IGF-I and by Collagen I Gel**

Rose Marie Robinson

## **ABSTRACT**

Understanding how insulin-like growth factor-I (IGF-I) signaling in mammary epithelial cells may be modified or interrupted by modifications in the cellular environment may lead to 1) methods to increase the growth and proliferation of normal mammary epithelial cells for an increase in the amount of milk produced on a per animal basis or to 2) the development of medical interventions to disrupt the growth and proliferation of cancerous mammary epithelial cells. IGF-I, a signaling protein provided by stromal cells and through the bloodstream, stimulates the proliferation of mammary epithelial cells and is crucial for mammary development. Collagen I is an extracellular matrix protein (ECM) found in skin and in other connective tissues throughout the body. The guiding question in this dissertation was how IGF-I signaling and how binding protein profile were influenced by autocrine IGF-I and by collagen I. The MAC-T cell line was chosen as the cell model utilized in these investigations because it is an immortalized bovine mammary epithelial cell line known to retain hormonal responsiveness to IGF-I.

It was hypothesized that the production of IGF-I by mammary epithelial cells (autocrine secretion) would alter the response of these cells to additional IGF-I by de-sensitizing the IGF-I receptor on the cell surface. The normal mammary epithelial cell does not produce IGF-I and responds to IGF-I supplied either by stromal cells (paracrine pathway) or through the bloodstream (endocrine pathway). The IGF-I secreting bovine mammary epithelial cell line was investigated for the response of the cells to autocrine IGF-I, and the response was compared to the normal, parental cell line. To examine the effect of autocrine IGF-I on the cells, IGF-I was added both to MAC-T cells and to cells transfected to secrete IGF-I (SV40-IGF-I). The cell response of the two cell lines was compared using microphysiometry, a tool that measures IGF-IR stimulation by detecting resultant extracellular acidification. It was found that the SV40-IGF-I cell line retains IGF-I receptor sensitivity, yet, unlike the parental cell line, does not proliferate in response to IGF-I. Both cell lines exhibited increased protein synthesis in response to IGF-I as measured by amino acid uptake (AIB incorporation), but the lack of a proliferation response to additional IGF-I in the SV40-IGF-I cell line suggested that the autocrine cell line exhibited an un-coupling of IGF-IR stimulation with downstream cell proliferation. Both autocrine IGF-I and added IGF-I increased the amount of IGFBP-3 secreted by the cells into growth media.

Additionally, it was hypothesized that the presence of collagen I, an important ECM protein, would alter the cell production of insulin-like binding protein-3 (IGFBP-3), a protein that modulates IGF-I interaction with the IGF-I receptor (IGF-IR). The literature reports that surface substrate can affect the phenotypic expression of cells, presumably

via interaction with integrins, the cell surface receptors that connect cells to ECM proteins and that are responsible for cell adhesion and for cell migration. It was hypothesized that the MAC-T cells would interact with a collagen I surface (possibly via the  $\alpha 2\beta 1$  integrin) and that the stimulation of this transmembrane signaling molecule would in turn impact the IGF-I signaling pathway. Comparison studies on tissue culture plastic, collagen I BIOCOAT, and collagen I gel were performed. It was found that collagen I gel increased IGFBP-3 secretion and decreased insulin-like binding protein-2 (IGFBP-2) secretion in MAC-T cells. The collagen I BIOCOAT did not induce this response.

Additional studies were performed to determine if there were differences in IGF-IR phosphorylation, exogenous IGF-I utilization, and IGFBP mRNA production by cells cultured on the three different substrates. IGF-IR phosphorylation was only evident following the addition of IGF-I to MAC-T cells on all three substrates. Measurement of residual IGF-I present in the cultured media of cells on all three substrates by radioimmunoassay did not reveal any differences in the amount of IGF-I present. Northern blot analysis revealed that the addition of IGF-I caused an increase in detected IGFBP-3 mRNA and a decrease in detected IGFBP-2 mRNA across all three surfaces. As measured by ligand blot analysis, cells cultured on all three surfaces showed an increase in IGFBP-3 protein in the media with IGF-I addition, and the collagen I gel showed more IGFBP-3 protein than the other two surfaces. However, cells cultured on collagen I gel showed a decrease in IGFBP-2 protein expression compared to cells cultured on tissue culture both with and without the addition of IGF-I. Cells cultured on

tissue culture plastic and on collagen I BIOCOAT did not show a decrease in IGFBP-2 to correspond with the decreased IGFBP-2 mRNA detected in the presence of IGF-I on all three substrates. DNA assays to detect cell proliferation revealed no differences in cell DNA content in the absence of exogenous IGF-I and revealed similar increases in response to IGF-I addition on all three substrates.

In conclusion, it was found that autocrine IGF-I un-couples increased IGF-IR stimulation by exogenous IGF-I from a downstream cell proliferation response. IGFBP-3 inhibits the ability of IGF-I to interact with the IGF-IR in MAC-T cells and inhibits subsequent cell proliferation. Collagen I gel increases IGFBP-3 secretion and decreases IGFBP-2 secretion by MAC-T cells.

The relevance of this work is that it adds to the body of knowledge in understanding cellular function in mammary epithelial cells. It is known that the growth and the maintenance of living tissue are dependent on an intricate system of intercellular and intracellular responses which are orchestrated by the movement and secretion of proteins and other molecules. Goals of understanding mammary epithelial cell function include having the means to find ways to increase cell functionality via bioengineering and having the means to find ways to restore cells to normal function in disease processes such as cancer.

## ACKNOWLEDGMENTS

The completion of this dissertation would not have been possible without the help and support of a number of people during the years of graduate research. Thanks to all of the members of the graduate committee: Kimberly Forsten Williams (Chair), R. Michael Akers, Richey M. Davis, William R. Huckle, and Kevin Van Cott.

Thanks are due to Dr. R. Michael Akers and his students for access to his lab and the sharing of materials and equipment. Patricia Boyle provided her expert technical support in learning cell culture techniques as well as in learning numerous assays in Dr. Akers' lab in Dairy Science. Dr. William R. Huckle of the school of veterinary science shared his expertise in developing a protocol for detecting IGF-IR phosphorylation using Western blots. Thanks to Delbert Jones at the school of veterinary sciences for access to the labs and equipment. Lucy Gray provided technical support on the Cytosensor® Microphysiometer System. Laura Delo completed the last Western blots for IGF-IR phosphorylation. Dr. Joyce Wong of Boston University and her graduate student Christy Gaudet prepared the polyacrylamide gels for cell seeding experiments using these gels.

Chapters 2 and 3 are reprinted with permission. This work was supported by the Whitaker Foundation Biomedical Engineering Research Grant.

Thanks are due my family and friends for their support and friendship. Thank you Gary for your support during these years.

# TABLE OF CONTENTS

LIST OF FIGURES	x
LIST OF TABLES	xiii
CHAPTER 1	
1.1 Introduction	1
1.1.1 Objective	5
1.1.2 Hypotheses	5
1.1.3 Specific Aims	5
1.1.3.1 Aim 1	6
1.1.3.2 Aim 2	7
1.1.3.3 Aim 3	8
1.1.4 Summary	8
1.2 Literature Review	10
1.2.1 Mammary physiology	10
1.2.2 MAC-T cell line	12
1.2.3 Insulin-like growth factor-I (IGF-I)	14
1.2.4 Insulin-like growth factor-I receptor (IGF-IR)	15
1.2.5 Insulin-like growth factor binding protein –3 (IGFBP-3) and –2 (IGFBP-2)	15
1.2.6 Autocrine IGF signaling	18
1.2.7 Signaling via the insulin-like growth factor-I (IGF-I) axis	20
1.2.8 Extracellular matrix (ECM)	24
1.2.9 Collagen I	28
1.2.10 Other important ECM proteins	28
1.2.11 Integrins	29
1.3 References	32
CHAPTER 2	
Real-time detection of insulin-like growth factor–1 stimulation of the MAC-T bovine mammary epithelial cell line. (2000) <i>Endocrine</i> <b>13</b> , 345-352	47
2.1 Abstract	48
2.2 Introduction	49
2.3 Results	52
2.4 Discussion	62
2.5 Materials and Methods	66
2.6 Acknowledgments	69
2.7 References	70

CHAPTER 3	
IGF-I stimulation of extracellular acidification is not linked to cell proliferation for autocrine cells. (2001). <i>Endocrine</i> <b>15</b> , 205-211	77
3.1 Abstract	78
3.2 Introduction	79
3.3 Results	80
3.4 Discussion	91
3.5 Materials and Methods	95
3.6 Acknowledgments	99
3.7 References	100
CHAPTER 4	
Collagen I gel substrate increases the IGFBP-3/IGFBP-2 ratio of secretion for a bovine mammary epithelial cell line	106
4.1 Abstract	107
4.2 Introduction	108
4.3 Results	111
4.4 Discussion	127
4.5 Materials and Methods	134
4.6 Acknowledgments	139
4.7 References	140
CHAPTER 5	
Topics related to detection of IGFBP-2 and IGFBP-3 from MAC-T cells cultured on collagen I	146
5.1 Part 1: Ligand blot detection of IGFBP-2 and IGFBP-3 from MAC-T cells on tissue culture plastic plates, collagen I BIOCOAT plates, and collagen I gels	146
5.1.1 Introduction/Methods	146
5.1.2 Results	151
5.1.3 Discussion	175
5.2 Part 2: Effect of 10% fetal bovine serum (FBS) on MAC-T cell attachment and growth	177
5.2.1 Introduction/Methods	177
5.2.2 Results	178
5.2.3 Discussion	181
5.3 Part 3: Detection of IGFBP-2 and IGFBP-3 from conditioned media of MAC-T cells seeded without FBS for 24 h, without FBS for the first 8 h of 24 h, or with FBS the full first 24 h on tissue culture plastic	182
5.3.1 Introduction/Methods	182
5.3.2 Results	182

5.3.3 Discussion	183
5.4 Part 4: Polyacrylamide gels (PAGE) as an alternate collagen I presentation	184
5.4.1 Introduction/Methods	184
5.4.2 Results	185
5.4.3 Discussion	189
5.5 Part 5: IGFBP-2 and IGFBP-3 secretion by cells on a porous polycarbonate membrane	189
5.5.1 Introduction/Methods	189
5.5.2 Results	191
5.5.3 Discussion	194
5.6 Part 6: Other extracellular matrix proteins: laminin and fibronectin BIOCOAT plates	196
5.6.1 Introduction/Methods	196
5.6.2 Results	196
5.6.3 Discussion	201
5.7 References	203
CHAPTER 6	
Summary of results and recommendations for further research	204
6.1 Effect of autocrine IGF-I on MAC-T cell response	206
6.1.1 Summary of results in this dissertation for autocrine IGF-I	206
6.1.2 Recommendations for future work with IGF-I signaling	207
6.2 Effect of collagen I gel substrate on MAC-T cell response	208
6.2.1 Summary of results in this dissertation for collagen I gel	208
6.2.2 Recommendations for future work with ECM proteins	210
6.3 Reference	215
APPENDIX A: Calculation of parameters from binding studies	216
APPENDIX B: Procedure for detection of IGFBP proteins by ligand blot	220
APPENDIX C: Procedure for mRNA detection by Northern blot	231
APPENDIX D: Procedure for detection of IGF-IR by Western blot	240
APPENDIX E: Radioimmunoassay (RIA) procedure for IGF-I detection	247
APPENDIX F: Statistical analyses of results	251
VITA	252

## LIST OF FIGURES

CHAPTER 1	
Figure 1.1: Detail of a single milk-producing alveolus	10
Figure 1.2: Detail of a mammary epithelial cell	11
Figure 1.3: Intracellular signaling pathways of the IGF-IR	21
CHAPTER 2	
Figure 2.1: Representative plot of microphysiometer measured response by MAC-T cells to 5 ng/mL of IGF-I	53
Figure 2.2: Effect of IGF-I on microphysiometer measured MAC-T response	56
Figure 2.3: Effect of insulin and IGFBP-3 on microphysiometer measured MAC-T response	58
Figure 2.4: Mean [ <sup>3</sup> H]thymidine incorporation by MAC-T cells in response to IGF-I	60
Figure 2.5: Comparison of response measurements for the microphysiometer and [ <sup>3</sup> H]thymidine incorporation by MAC-T cells	61
CHAPTER 3	
Figure 3.1: Representative plot of ECAR response by SV40-IGF-I cells to IGF-I (5ng/mL)	81
Figure 3.2: Concentration-dependent effect of IGF-I on ECAR in SV40-IGF-I cells	82
Figure 3.3: Amino acid uptake determined by [ <sup>14</sup> C]AIB incorporation	85
Figure 3.4: [ <sup>3</sup> H]thymidine incorporation in response to IGF-I	86
Figure 3.5: Cell seeding density effect on [ <sup>3</sup> H]thymidine incorporation	88
Figure 3.6: [ <sup>3</sup> H]thymidine incorporation by SV40-IGF-I cells ( $2.6 \times 10^4$ cells/cm <sup>2</sup> ) grown on transwell polycarbonate membranes	89
Figure 3.7: BrdU incorporation by SV40-IGF-I cells exposed to IGF-I (50 ng/mL) under either stagnant or flow conditions	90
CHAPTER 4	
Figure 4.1: DNA content of cells seeded at 50,000 cells/well and 200,000 cells/well	112
Figure 4.2: Representative ligand blots showing IGFBP-3 and IGFBP-2 bands	113
Figure 4.3: Representative Western blot of IGF-IR stimulation is shown	122
Figure 4.4: Representative Northern blot for four experiments to detect IGFBP-2 mRNA and IGFBP-3 mRNA	125
Figure 4.5: Analysis of IGFBP-2 mRNA and IGFBP-3 mRNA from Northern blots as measured by densitometry	126

## CHAPTER 5

Figure 5.1.1: Ligand blot with lanes corresponding to the configuration shown in Table 5.1.1 for conditioned media from 200,000 MAC-T cells per well on tissue culture plastic	151
Figure 5.1.2: Ligand blot with lanes corresponding to the densitometry measurements shown in Table 5.1.2 for conditioned media from 50,000 MAC-T cells per well on collagen I BIOCOAT	152
Figure 5.1.3: Ligand blot with lanes corresponding to the densitometry measurements shown in Table 5.1.2 for conditioned media from 50,000 MAC-T cells per well on collagen I BIOCOAT	153
Figure 5.1.4: Average intensities of IGFBP-3 and IGFBP-2 bands on ligand blot as measured by densitometry from two experiments is summarized	172
Figure 5.2.1: DNA content of plastic tissue culture wells seeded with 200,000 MAC-T cells/well	179
Figure 5.2.2: DNA content after 48 h from MAC-T cells seeded at 100,000 cells/well in the absence of FBS (open columns) and in the presence of 10% FBS (solid columns) on three different surfaces: tissue culture plastic, collagen I BIOCOAT, and collagen I gel	180
Figure 5.3.1: Ligand blot for conditioned media from MAC-T cells seeded without FBS for 24 h (lanes 1-3), from MAC-T cells seeded without FBS for the first 8 h of 24 h (lanes 4-5), and from MAC-T cells seeded with 10% FBS for the full first 24 h period (lanes 7-9)	183
Figure 5.4.1: Light microscope photos of MAC-T cells on PAGE preparations 24 h after initial cell seeding (1,000,000 cells/well, 6-well plate)	187
Figure 5.4.2: Light microscope photos of MAC-T cells adhered on PAGE preparations following media replacement 24 h after initial cell seeding (1,000,000 cells/well, 6-well plate)	188
Figure 5.5.1: Transwell placed in the well of a 12-well tissue culture plate	191
Figure 5.5.2: Ligand blot to detect IGFBP-3 and IGFBP-2 from Transwell system for MAC-T and SV40-IGF-I cells	193
Figure 5.5.3: Ligand blot to detect IGFBP-3 and IGFBP-2 from Transwell system for co-cultures of MAC-T and SV40-IGF-I cells	194
Figure 5.6.1: Ligand blot for conditioned media from MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, fibronectin BIOCOAT, and laminin BIOCOAT	198
Figure 5.6.2: IGFBP-3 band intensities from ligand blot as measured by densitometry for tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT	199
Figure 5.6.3: IGFBP-2 band intensities from ligand blot as measured by densitometry for tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT	200

Figure 5.6.4: DNA content per well for MAC-T cells cultured on tissue culture plastic, collagen I BIOCOAT, laminin BIOCOAT, and fibronectin BIOCOAT

201

## LIST OF TABLES

CHAPTER 1	
Table 1.1: Selected components of the IGF-I signaling pathway	23
CHAPTER 2	
Table 2.1: Decreased response by MAC-T cells with repeated IGF-I stimulation	54
Table 2.2: Effect of short term exposure to IGF-I on IGF-IR	55
CHAPTER 3	
Table 3.1: Response by SV40-IGF-I cells with repeated IGF-I stimulation	83
CHAPTER 4	
Table 4.1: Relative intensity of IGFBP-3 bands on three separate blots from three independent experiments	116
Table 4.2: Relative intensity of IGFBP-2 bands on three separate blots from three independent experiments	117
Table 4.3: Relative intensity of IGFBP-3 bands on separate blots from two independent experiments	118
Table 4.4: Relative intensity of IGFBP-2 bands on separate blots from two independent experiments	119
Table 4.5: IGF-I concentration measured by radioimmunoassay (RIA) technique	121
CHAPTER 5	
Table 5.1.1: Example configuration of lanes for a single ligand blot	150
Table 5.1.2: IGFBP-2 and IGFBP-3 band intensities as measured by densitometer from two separate ligand blots for MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well	153
Table 5.1.3: Ligand blot 1: IGFBP-2 and IGFBP-3 band intensities on a per ng/mL cellular DNA basis from MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well	154
Table 5.1.4: Ligand blot 1: Cell-free collagen I gel control normalized IGFBP-2 and IGFBP-3 band intensities on a per ng/mL cellular DNA basis from MAC-T cells cultured on collagen I BIOCOAT at 50,000 cells/well	155
Table 5.1.5: IGFBP-3/IGFBP-2 ratio in MAC-T conditioned media from cells cultured on tissue culture plastic or on collagen I gel	157
Table 5.1.6: Set A: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on tissue culture plastic	159
Table 5.1.7: Set A: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I BIOCOAT	160
Table 5.1.8: Set A: IGFBP-2 band intensities on ligand blots from	

conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I gel	161
Table 5.1.9: Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on tissue culture plastic	162
Table 5.1.10: Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I BIOCOAT	163
Table 5.1.11: Set B: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 50,000 cells/well on collagen I gel	164
Table 5.1.12: Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on tissue culture plastic	165
Table 5.1.13: Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I BIOCOAT	166
Table 5.1.14: Set C: IGFBP-2 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I gel	167
Table 5.1.15: Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on tissue culture plastic	168
Table 5.1.16: Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I BIOCOAT	169
Table 5.1.17: Set D: IGFBP-3 band intensities on ligand blots from conditioned media of MAC-T cells initially seeded at density of 200,000 cells/well on collagen I gel	170