Chemotherapy-induced intestinal mucositis:
The role of apoptosis regulators.

Joanne M. Bowen BHSc(Hons)

Thesis submitted for degree of
Doctor of Philosophy

Department of Medicine
The University of Adelaide
Australia

March 2006
Declaration

“This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to 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.”

I give consent for this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying.

Joanne Marie Bowen

March 2006
# Chapter 1: Literature Review

1.0 Introduction  
1.1 The intestinal epithelium  
1.1.1 Structure and function  
1.1.2 Cell proliferation and loss  
1.1.3 Stem cells  
1.2 Apoptosis  
1.2.1 The role of apoptosis  
1.3 Chemotherapy, apoptosis and mucositis  
1.3.1 The treatment of cancer  
1.3.2 Chemotherapy treatment and mucositis  
1.3.3 Methotrexate and the intestine  
1.3.4 Irinotecan and the intestine  
1.4 Molecular control of cell death  
1.5 The Bcl-2 family  
1.6 Caspases  
1.7 p53  
1.8 Bcl-2 family and the apoptotic pathway  
1.8.1 Mitochondria and the Bcl-2 family in apoptosis  
1.8.2 ER and the Bcl-2 family in apoptosis  
1.9 Bcl-2 and apoptosis in the intestine  
1.9.1 Protein expression of Bcl-2 members in the intestine
1.9.2 The role of Bcl-2 in apoptosis in the intestine 26
1.9.3 Differential sensitivity to apoptosis in intestine 26
1.10 Effect of chemotherapy agents on p53 and Bcl-2 expression 27
  1.10.1 Chemotherapy and Bcl-2 27
  1.10.2 Chemotherapy and p53 27
1.11 Prevention of mucositis, p53 and pifithrin-α 29
  1.11.1 Anti-mucositis agents 29
  1.11.2 p53 and pifithrin-α 30
1.12 Summary 33
1.13 Specific aims and hypothesis 33

Chapter 2: Investigation of Bcl-2 family protein expression in the crypts of the small and large intestine of the DA rat with breast cancer

2.0 Introduction 34
2.1 Methods and Materials 36
  2.1.1 Laboratory animals 36
  2.1.2 Preparation of tumour inoculum 36
  2.1.3 Experimental design 36
  2.1.4 Detection of Bcl-2 family proteins 37
  2.1.5 Quantitative immunohistochemistry 37
  2.1.6 Statistical analysis 38
2.2 Results 41
  2.2.1 Histology 41
  2.2.2 Bcl-2 41
  2.2.3 Bcl-xL 41
  2.2.4 Bcl-w 41
  2.2.5 Mcl-1 41
  2.2.6 Bax 42
  2.2.7 Bak 42
  2.2.8 Bim 42
  2.2.9 Bid 42
  2.2.10 Small intestinal crypts v large intestinal crypts 43
2.3 Discussion 48
Chapter 3: The effect of cancer chemotherapy treatment on expression of Bcl-2 family genes in the small intestine

3.0 Introduction 51
3.1 Methods and Materials 53
   3.1.1 Laboratory animals 53
   3.1.2 Preparation of tumour inoculum 53
   3.1.3 Experimental design 53
   3.1.4 RNA extraction 54
   3.1.5 Nucleic acid quantification 54
   3.1.6 cDNA synthesis 55
   3.1.7 Primer design 55
   3.1.8 Polymerase chain reaction 57
   3.1.9 Investigation of protein expression 57
   3.1.10 Detection of Bcl-2 family proteins 58
   3.1.11 Quantitative immunohistochemistry 58
   3.1.12 Qualitative assessment of staining 59
   3.1.13 Statistical analysis 59
3.2 Results 63
   3.2.1 RNA expression 63
   3.2.2 Protein expression in rat 66
   3.2.3 Protein expression in patients 73
3.3 Discussion 77

Chapter 4: Effect of chemotherapy treatment on apoptosis, proliferation and protein expression in intestinal cells

4.0 Introduction 80
4.1 Methods and Materials 82
   4.1.1 Cell lines 82
   4.1.2 Cell culture 82
   4.1.3 Drug treatment 83
   4.1.4 Cell viability assessment 83
   4.1.5 Cell number assessment 83
   4.1.6 Cell proliferation assessment 84
   4.1.7 Immunohistochemistry on intestinal cells 84
   4.1.8 Protein extraction 85
4.1.9 Determination of protein concentration 85
4.1.10 Western Blotting 86
4.1.11 Statistical analysis 86

4.2 Results 87
4.2.1 Initial dose-finding experiments 87
4.2.2 Effect of chemotherapy on cell adherence 91
4.2.3 Effect of pifithrin-α on cell survival 95
4.2.4 Effect of chemotherapy on p53 and p21 expression 100
4.2.5 Effect of chemotherapy on Bcl-2 family expression 100

4.3 Discussion 104

Chapter 5: The effect of irinotecan on the intestine in the rat with breast cancer
5.0 Introduction 107
5.1 Methods and Materials 110
5.1.1 Laboratory animals 110
5.1.2 Preparation of tumour inoculum 110
5.1.3 Experimental design 110
5.1.4 Histological examination 111
5.1.5 Intestinal morphometry 111
5.1.6 Apoptosis measurement 112
5.1.7 Immunohistochemical detection of proteins 112
5.1.8 Statistical analysis 113
5.2 Results 115
5.2.1 Effect of irinotecan on tumour-bearing rats 115
5.2.2 Histopathological changes induced by irinotecan 115
5.2.3 Morphological changes induced by irinotecan 119
5.2.4 Apoptosis in intestinal crypts 119
5.2.5 Crypt protein expression following irinotecan 123
5.3 Discussion 130

Chapter 6: Effect of irinotecan treatment on gene expression profiles in the small intestine of the rat with breast cancer
6.0 Introduction 133
6.1 Methods and Materials 135
6.1.1 Laboratory animals 135
Abstract

Mucositis is the damage that occurs to the alimentary canal from anti-cancer therapies. It is caused by chemotherapy, radiotherapy and combination therapy and affects a large proportion of patients. Despite its prevalence, an effective anti-mucositis agent has yet to be developed that protects the whole tube, although the use of keratinocyte growth factor (Amgen’s Palifermin) has recently been approved for the prevention of oral mucositis. It is important to understand mechanisms controlling mucositis so that treatment can be targeted appropriately. This thesis has investigated some of the key components identified as being involved in mucositis as well as identifying new genes which contribute to chemotherapy-induced intestinal injury. The research chapters investigated:

1) Gene expression of the apoptosis-regulating Bcl-2 family, p53 and caspase-3, and the changes which occur in the intestine following chemotherapy treatment for cancer.

2) The effect of different chemotherapeutic agents on intestinal cells in vitro and the role p53 plays.

3) The mucositis caused by single dose irinotecan in the rat with breast cancer and the role of p53 in induction of intestinal damage.

4) The early gene changes that occur in the small intestine of the rat with breast cancer following irinotecan treatment.

Firstly, to investigate the difference in susceptibility to damage between the small and large intestine, the protein expression of 8 members of the Bcl-2 family (4 pro-apoptotic; Bax, Bak, Bid, Bim and 4 anti-apoptotic; Bcl-2, Bcl-xL, Bcl-w, Mcl-1) was quantified in jejunal and colonic sections taken from rats inoculated with breast cancer. It was found that there was significantly higher expression of the pro-apoptotic proteins, Bax, Bak, Bim and Bid, in the crypts of the jejunum compared to the colon. Furthermore, expression of the anti-apoptotic proteins, Bcl-2, Bcl-xL and Bcl-w, was significantly lower in jejunal crypts compared to colonic crypts. Mcl-1 expression was similar in both regions. Thus, the small intestine is an environment balanced to favour apoptosis through specific Bcl-2 family protein expression profiles.

The Bcl-2 family regulates apoptosis in response to a variety of chemotherapy agents. However, it is unknown how Bcl-2 family gene expression changes along with other
apoptogenic factors following cytotoxic therapy in the normal intestine. To investigate
this, sections of rat jejunum treated with methotrexate and duodenal biopsies from
chemotherapy patients treated with various regimens for cancer were subjected to
quantitative immunohistochemistry to detect Bcl-2 family proteins, p53 and caspase-3.
Treatment caused expression of p53 and caspase-3 to increase within the crypts and follow
a similar pattern to apoptosis levels. Pro-apoptotic Bcl-2 family members, Bax and Bak,
were increased, while the anti-apoptotic protein, Mcl-1, was significantly reduced. A
significant increase in mRNA expression for Bax and Bak was noticed at 6 h, without a
concurrent decrease in Mcl-1. Thus, Bcl-2 family genes were altered in the small intestine
in both humans and rats, and this was irrespective of chemotherapy agent or regimen used.

The best characterised changes which occur during chemotherapy-induced damage in the
intestine are in the epithelial layer, although it is thought that pan-mucosal alterations are
involved. Two intestinal cell lines were chosen to investigate changes in apoptosis,
proliferation and protein expression following cytotoxic treatment with various
chemotherapeutic agents. These were the rat IEC-6 and human FHs 74 cell lines, which
represent untransformed epithelial cells. The human breast carcinoma cell line, MCF-7,
was also used as a positive control. Intestinal cells were resistant to the occurrence of
methotrexate toxicities within 24 h of treatment, modestly affected by irinotecan and
extremely sensitive to doxorubicin. Doxorubicin caused a marked increase in p53 and p21
expression, which for irinotecan was less pronounced. The effect of cytotoxic treatment on
Bcl-2 family expression in intestinal cells varied, however the pro-apoptotic proteins, Bax
and Bak, were generally upregulated following doxorubicin. Temporary inhibition of p53
using pifithrin alpha resulted in a significant improvement in cell survival in cancerous cell
only and did not alter Bcl-2 family expression. It was concluded that cultured epithelial
cells exhibit varying sensitivities to different chemotherapeutic agents which is dependent
on induction of p53 gene expression.

The topoisomerase I inhibitor, irinotecan, is a chemotherapeutic agent commonly used in
the treatment of colorectal cancer. It often induces severe mucositis with the most
common symptom being diarrhoea. Previous research has shown that irinotecan damages
the small and large bowel equally, which is unusual. This is characterised by an increase
in apoptosis and a reduction in proliferation within epithelial crypts, an increase in
inflammatory cell infiltrate in the lamina propria and excess mucin production. These
investigations used two sequential doses of irinotecan. The early effect of a single dose of
irinotecan on the intestine have yet to be studied. Thus the primary aim of this experiment was to examine in detail the changes caused by irinotecan at 6 and 48 h in the rat. A secondary aim was to investigate the role of p53 on induction of apoptosis and cell cycle arrest within intestinal crypts and the effect of temporary inhibition of the protein. Single dose irinotecan caused a decrease in body and small intestinal weight by 48 h after treatment. This was accompanied by crypt and villous degeneration, increased apoptosis and reduced proliferation within crypt epithelium as well as inflammatory infiltrate throughout lamina propria. An increase in Bax expression was seen at 6 h, however p53 protein levels remained relatively low until 48 h. Rats also treated with pifithrin alpha to inhibit p53 and had a significantly lower peak in apoptosis in the colon at 6 h, however did not show improvements in any other parameters tested. It was concluded that irinotecan-induced damage in the rat intestine is primarily p53-independent, and that pifithrin alpha acts to inhibit apoptosis in the large intestine via a p53-independent pathway.

A study was designed to investigate the early genome-wide changes which occur following irinotecan treatment in the rat small intestine. Microarray analysis found that regulation of many genes was altered at 6 h following dual dose irinotecan. These genes were involved in apoptosis, cell cycle regulation, immune function, calcium homeostasis and protein turnover. Multiple genes from the MAP kinase pathway were also activated by irinotecan. The cystine protease, caspase-1 was upregulated and was chosen for further investigations due to its role in apoptosis and inflammation. Real time PCR analysis confirmed the increase in gene expression at 6 h and also showed a return to baseline levels by 24 h which was followed by another modest increase at 48 h. It was concluded that irinotecan induces a wide range of gene changes within the intestine and that apoptosis and inflammatory damage pathways are activated during treatment.

This thesis described key molecules in apoptosis and their role in induction of chemotherapy-induced intestinal mucositis. It has provided evidence of the importance of apoptosis in mucosal injury and also highlighted areas requiring further research. Results presented herein show that the Bcl-2 family is involved in intestinal damage following many chemotherapy agents, whereas p53 is agent-specific. It has also shown that irinotecan causes intestinal damage via a mainly p53-independent manner in the rat. It can be concluded that gastrointestinal mucositis is complex and activates multiple pathways to induce damage. Findings from this thesis will aid targeting of new anti-mucotoxic agents.
Acknowledgments

I extend my thanks and gratitude to my two supervisors, Assoc. Prof. Dorothy Keefe and Dr. Adrian Cummins for giving me the opportunity to undertake this PhD, reading the many drafts of this thesis and always providing encouragement while completing the studies.

In addition I must thank Dr. Rachel Gibson for her continuous help throughout my candidature.

Thanks also to members of the Mucositis Research Group, Dr. Richard Logan, Andrea Stringer and Ann Yeoh for their input over the years.

Thank you, to Dr. Randall Grose and Dr. Fiona Thompson from the Department of Gastroenterology and Hepatology at the The Queen Elizabeth Hospital for your friendship and advice.

To Rupal Pradham and Jim Manavis, thank you for your assistance with the histology.

Thanks also to my entire family, for the support, encouragement and belief in me over the years.
Publications arising from this thesis


Publications submitted for publication or in preparation


Contributions made by Co-authors

Assoc. Prof. Dorothy M.K. Keefe


Assoc. Prof. Keefe was my principle supervisor and therefore was listed as a Co-author on all publications arising from this thesis. Dorothy helped to design and interpret results from the series of experiments, as well as gain funding for the project. In addition she read multiple drafts of the papers.

Dr. Adrian Cummins


Dr. Cummins was my Co-supervisor. Together with Dorothy, helped to design and interpret results from the series of experiments, as well as gain funding for the project. In addition he read multiple drafts of the papers.

**Dr. Rachel J. Gibson**


Dr. Gibson was a member of the Mucositis Research Group during my candidature. Rachel contributed to each of the studies through helping with animal work and reading multiple drafts of the papers.

**Dr. Anna Tyskin**


Dr. Tyskin carried out statistical analysis of microarray data detailed in this thesis. Anna also contributed by reading multiple drafts of the paper.
Additional studies and publications

During my candidature, I was involved in several other studies investigating intestinal mucositis, not presented in this thesis. These have resulted in Co-authorship of several other manuscripts as shown below.


Thesis explanation

The format of this thesis is as follows: literature review, five distinct research chapters, general discussion, and then references. During my candidature two manuscripts were prepared from chapters in this thesis and are also included as appendices.