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

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

Bowen, J.M., R.J. Gibson, A.G. Cummins, and D.M. Keefe. 2006. Intestinal mucositis: the role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. *Support Care Cancer*: 1-19.

Bowen, J.M., R.J. Gibson, D.M. Keefe, and A.G. Cummins. 2005. Cytotoxic chemotherapy upregulate pro-apoptotic Bax and Bak in the small intestine of rats and humans. *Pathology*. 37:56-62.

Publications submitted for publication or in preparation

Bowen, J.M., R.J. Gibson, A.G. Cummins, A. Tyskin, D.M.K. Keefe. 2006. Irinotecan changes gene expression in the small intestine of the rat with breast cancer. *Cancer Chemo Pharmacol.* (submitted).

Bowen, J.M., R.J. Gibson, A.G. Cummins, D.M.K. Keefe. 2006. The role of p53 in irinotecan-induced intestinal mucositis (in preparation).

Contributions made by Co-authors

Assoc. Prof. Dorothy M.K. Keefe

Bowen, J.M., R.J. Gibson, A.G. Cummins, and **D.M. Keefe**. 2006. Intestinal mucositis: the role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. *Support Care Cancer*: 1-19.

Bowen, J.M., R.J. Gibson, **D.M. Keefe**, and A.G. Cummins. 2005. Cytotoxic chemotherapy upregulate pro-apoptotic Bax and Bak in the small intestine of rats and humans. *Pathology*. 37:56-62.

Bowen, J.M., R.J. Gibson, A.G. Cummins, A. Tyskin, **D.M.K. Keefe.** 2006. Irinotecan changes gene expression in the small intestine of the rat with breast cancer. *Cancer Chemo Pharmacol,* (submitted).

Bowen, J.M., R.J. Gibson, A.G. Cummins, **D.M.K. Keefe.** 2006. The role of p53 in irinotecan-induced intestinal mucositis (in preparation).

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

Bowen, J.M., R.J. Gibson, **A.G. Cummins**, and D.M. Keefe. 2006. Intestinal mucositis: the role of the Bcl-2 family, p53 and caspases in chemotherapy-induced damage. *Support Care Cancer*: 1-19.

Bowen, J.M., R.J. Gibson, D.M. Keefe, and **A.G. Cummins**. 2005. Cytotoxic chemotherapy upregulate pro-apoptotic Bax and Bak in the small intestine of rats and humans. *Pathology*. 37:56-62.

Bowen, J.M., R.J. Gibson, **A.G**. **Cummins,** A. Tyskin, D.M.K. Keefe. 2006. Irinotecan changes gene expression in the small intestine of the rat with breast cancer. *Cancer Chemo Pharmacol,* (submitted).

Bowen, J.M., R.J. Gibson, **A.G**. **Cummins,** D.M.K. Keefe. 2006. The role of p53 in irinotecan-induced intestinal mucositis (in preparation).

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

Bowen, J.M., **R.J. Gibson**, D.M. Keefe, and A.G. Cummins. 2005. Cytotoxic chemotherapy upregulate pro-apoptotic Bax and Bak in the small intestine of rats and humans. *Pathology*. 37:56-62.

Bowen, J.M., **Gibson, R.J.,** Cummins, A.G., Tyskin, A., Keefe, D.M.K. 2006. Irinotecan changes gene expression in the small intestine of the rat with breast cancer. *Cancer Chemo Pharmacol*, (submitted).

Bowen, J.M., **R.J**. **Gibson,** A.G. Cummins, A. Tyskin, D.M.K. Keefe. 2006. The role of p53 in irinotecan-induced intestinal mucositis (in preparation).

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

Bowen, J.M., R.J. Gibson, A.G. Cummins, **A. Tyskin,** D.M.K. Keefe. 2006. The role of p53 in irinotecan-induced intestinal mucositis (in preparation).

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.

Yeoh, A.S., **J.M. Bowen**, R.J. Gibson, and D.M.K. Keefe. 2005. Nuclear factor kappaB (NFkappaB) and cyclooxygenase-2 (Cox-2) expression in the irradiated colorectum is associated with subsequent histopathological changes. *Int J Radiat Oncol Biol Phys*. 63:1295-1303

Gibson, R.J., **J.M. Bowen**, A.G. Cummins, and D.M.K. Keefe. 2005a. Relationship between dose of methotrexate, apoptosis, p53/p21 expression and intestinal crypt proliferation in the rat. *Clin Exp Med*. 4:188-95.

Gibson, R.J., **J.M. Bowen**, and D.M. Keefe. 2005b. Palifermin reduces diarrhea and increases survival following irinotecan treatment in tumor-bearing DA rats. *Int J Cancer*. 116:464-70

Gibson, R.J., **J.M. Bowen**, M.R. Inglis, A.G. Cummins, and D.M. Keefe. 2003. Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. *J Gastroenterol Hepatol*. 18:1095-100.

Sonis, S., Anthony, L., **Bowen, J**., Garden, A., Hewson, I., New Thoughts on the Pathobiology of Regimen-related Mucosal Injury. *Support Care Cancer.* (accepted March 2006).

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.