SMALL INTESTINAL PROTEIN METABOLISM DURING CANCER CACHEXIA AND CHEMOTHERAPY IN MICE

by

ANDREW LLEWELLYN KNOWLES

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ABSTRACT

Cachexia is the leading cause of morbidity and mortality in patients with cancer. Mechanisms The effects of cancer cachexia and chemotherapy on small intestinal protein metabolism and the mechanisms regulating recovery are currently not known. Compromises to small intestinal protein metabolism may impair normal digestive and immune functions within the intestine and may negatively impact the whole body. Colon 26 adenocarcinoma induces cancer cachexia in mice, characteristic to the human condition, and this tumour can be cured with 100% efficacy using the experimental alkylating agent, cystemustine. In the present study, both healthy mice and colon 26 adenocarcinoma bearing mice were given either a single i.p. injection of N-(2-Chloroethyl)-N-[2-(methylsulfonyl)ethyl]-N-nitrosourea (cystemustine)(20 mg/kg) or saline 3 d following the onset of cachexia in colon 26 adenocarcinoma bearing mice. The rate of protein synthesis was determined in vivo using the flooding dose method. The possible involvement of proteolysis was assessed through northern blot hybridization of mRNA encoding components of the major proteolytic systems (lysosomal, calcium dependent, ATP-ubiquitin dependent). Villus and crypt morphology was studied through histological analysis.

In tumour bearing mice not treated with chemotherapy, cancer cachexia reduced the rate of protein synthesis in the small intestine relative to healthy mice on a fractional (-15 to -20%, $P<0.05$) and an absolute basis (-20 to -35%, $P<0.05$) resulting in a 25% loss of protein mass ($P<0.05$), and minor alterations to villus and crypt morphology. Northern

blot hybridization suggested that protein degradation may not have contributed to wasting as an up-regulation in mRNA levels were not detected ($P>0.05$).

In treated mice, acute cytotoxicity of chemotherapy did not promote further wasting of small intestinal protein mass, nor did it result in damage to intestinal morphology. In contrast, mucosal damage and a 17% reduction in small intestinal protein mass ($P<0.05$) was evident in healthy mice treated with cystemustine, suggesting that the small intestine in cachectic mice responds differently to chemotherapy than in normal healthy mice. Complete and rapid recovery of small intestinal protein mass in cured mice resulted from an increase in the rate of protein synthesis compared to healthy mice on a fractional (+25%, $P<0.05$) and absolute basis (+35%, $P<0.05$); northern blot hybridization suggested no involvement of proteolysis during recovery.

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PREFACE

A human being is part of the whole, called by us the universe. A part limited in time and space. He experiences himself, his thoughts and feelings, as something separate from the rest, a kind of optical delusion of his consciousness. This delusion is a kind of prison for us, restricting us to our personal desires and to affection for a few persons nearest to us. Our task must be to free ourselves from this prison by widening our circle of compassion to embrace all living creatures.

—Albert Einstein

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INTRODUCTION

Cancer is one of the most common diseases in developed countries world wide. One in five people living in North America will die from this disease. Morbidity and mortality in cancer patients is often the direct result of cancer cachexia (DeWys et al. 1980, Inagaki et al. 1974, Kern & Norton 1988, Warren 1932). Cancer cachexia is a complex metabolic syndrome characterized by progressive wasting of host tissue (Tisdale 1997a, 1997b). This deterioration of body mass results in weakness, lowered responsiveness to chemotherapeutic treatment and an overall lowered survival time compared to cancer patients without weight loss (Pisters & Pearlstone 1993). The interactions between cancer cachexia and host tissue are not fully understood.

Chemotherapy is used widely as an anti-cancer treatment that attacks proliferating tumour cells. Distinguishing between tumour cells and host cells is difficult, making any proliferating cells vulnerable to the cytotoxic effects of therapy. Non-specific cytotoxic damage to host tissue remains to be a serious complication in the treatment of cancer (Schwartsmann et al. 1995). Currently, there is no treatment available that destroys cancer cells without affecting host cells to a certain degree. The treatment of patients suffering from cancer cachexia becomes even more problematic. Body wasting prior to chemotherapeutic treatment reduces expected patient survival (DeWys et al. 1980) and chemotherapy may even worsen the cachexia.

Central to the process of cancer cachexia is the gradual loss of host protein mass. Reduction of skeletal muscle mass can be as high as 75% (Preston et al. 1987). Almost nothing is known about the effect of cancer cachexia on protein metabolism in other

tissues such as the small intestine. The small intestine is responsible for the digestion and absorption of nutrients, serves as a physical barrier to endotoxins, bacteria, viruses as well as being involved in apolipoprotein metabolism, mucous secretion and immune function (McBurney 1994). Any condition that compromises the integrity and function of the small intestine may interfere with these processes, and therefore, may impact the rest of the body. Numerous studies have addressed the clinical effects of chemotherapy including nausea, vomiting, diarrhoea and in more extreme circumstances, septicaemia (Berg 1983, Mercadante 1995, Sandovsky-Losica et al. 1990, 1992, Wadler et al. 1998). However, the effects of cancer cachexia and chemotherapy on small intestinal protein metabolism and the mechanisms regulating recovery remain unknown.

An understanding of how cancer cachexia and chemotherapy affect small intestinal protein metabolism may lead to more effective therapies and the design of effective treatment strategies. Promoting the recovery of the small intestine following treatment of cancer could be vital for recovery of the whole body. Therefore, the purpose of this thesis was to study the effect of cancer cachexia and chemotherapy on small intestinal protein metabolism. Specifically, the acute and chronic effects of cancer cachexia on small intestinal protein metabolism were assessed. Toxic effects of chemotherapy were determined, and some of the mechanisms regulating recovery of small intestinal protein metabolism were elucidated.

LITERATURE REVIEW

A. Cancer cachexia

Cancer cachexia is one of the leading causes of morbidity and mortality in patients with advanced cancer (DeWys et al. 1980, Inagaki et al. 1974, Kern & Norton 1988, Warren 1932). As many as two thirds of all patients with advanced cancer show symptoms of this complex metabolic syndrome (Pisters & Pearlstone 1993). Some of the associated characteristics of cachexia can be present even before other symptoms of cancer become evident. This syndrome is associated with lower responsiveness to chemotherapeutic treatment and an overall lowered survival time compared with non-cachetic cancer patients. Weakness, anorexia, hyperlipidaemia, depleted lipid stores, abnormal carbohydrate metabolism, skeletal muscle atrophy and negative energy balance are associated with cancer cachexia (DeWys et al. 1980, Kern & Norton 1988, Nelson et al. 1994, Pisters & Pearlstone 1993, Tisdale 1993, 1997a, 1997b, Toomey et al. 1995).

Factors responsible for causing the cachexia associated with cancer are starting to be elucidated. A variety of cytokines including tumor necrosis factor-alpha, interleukin-6, interferon-gamma and leukemia inhibitory factor as well as tumor-derived factors such as the lipid and protein mobilizing factors have been linked to the development of cancer cachexia (Tisdale 1999). Significant advances have also been made in the treatment of the cachexia. For example, eicosapentaenoic acid has been shown to inhibit the progress of cachexia in patients with pancreatic cancer (Wigmore et al. 1996) and the combination of megestrol acetate/ibuprofen appeared to improve quality of life and to reverse weight

loss in patients with advanced gastrointestinal cancer (McMillan et al. 1999).

B. Chemotherapy

Chemotherapy is used widely as an antineoplastic agent representing therapies involving single agents or combinations of drugs and is often used in conjunction with radiation. The basic principle of chemotherapy involves the inhibition of cellular proliferation. Cytostatic drugs are classified according to their mechanism of action. The four general groups of drugs include alkylating agents, antimetabolites, plant alkaloids and antitumour antibiotics. These groups are then be divided into subclasses with each subclass consisting of several variations of drugs with similar modes of action (Cleton 1995).

There are several limitations to chemotherapy. There is a lack of specific identifiable markers distinguishing tumour cells from host cells. Cytotoxic effects of chemotherapy will therefore affect host cells, as well as tumour cells, introducing serious complications in the treatment of cancer (Schwartsmann et al. 1995). As the dose of cytostatic drugs increase, cell survival decreases exponentially. For a drug to be used during chemotherapy, the tolerance of the host cells to the drug must exceed that of the tumour cells (Cleton 1995). Related to cell sensitivity is tumour cell resistance. Intrinsic or acquired resistance to cytostatic drugs can often impede treatment. Research, therefore, is partly dedicated to inhibiting this resistance before chemotherapy as a way to increase the effectiveness of the treatment (Buchdahl et al. 1998, Cussac et al. 1994, Debiton et al. 1997, Ludlum 1997, Waxman 1990). Another major limitation to the use

of chemotherapy is the associated toxic effects experienced as a result of the drugs themselves. Symptoms include nausea, vomiting, diarrhoea, myelosuppression, mucositis, thrombocytopenia and in more extreme cases, septicaemia (Berg 1983, Mercadante 1995, Sandovsky-Losica et al. 1990, 1992, Schwartsmann et al. 1995, Sonis 1993, Wadler et al. 1998). There is also evidence to suggest that chemotherapy may directly contribute to the cachexia observed during cancer, further complicating the treatment of this disease and subsequent recovery by the patient (Le Bricon et al. 1995a). The precise effect of chemotherapy on host and tumoural tissue is currently not well understood. A better understanding of the effects of chemotherapy may offer new insights towards treatment strategies.

C. Protein metabolism

Protein turnover

To understand how cancer cachexia and chemotherapy affect tissue protein mass, it is necessary to have an understanding of the processes contributing to protein mass. Protein in the body is dynamic and is continually being broken down and rebuilt. This process is referred to as protein turnover. Constant turnover of body proteins is important and necessary for survival. Protein turnover is mediated through the processes of protein synthesis and degradation. Together, these two processes determine the protein mass of the body, replace damaged or abnormal proteins, contribute to heat production and maintain circulating amino acid pools (Sugden & Fuller 1991).

To maintain protein at a constant level in tissues, the dynamic nature of this system requires that the rates of protein synthesis and degradation must be in balance with each other. If the rate of synthesis is greater than breakdown, growth will occur. The opposite is also true. If the rate of protein degradation is greater than synthesis, atrophy or wasting of body protein will occur. Detecting changes in protein mass within a given tissue will indicate an altered balance between the processes regulating protein mass, but measuring the rates of synthesis and degradation will elucidate the processes responsible for the change (Sugden & Fuller 1991).

Protein synthesis

One of the processes involved in protein turnover is protein synthesis. Protein synthesis is the process by which amino acids are incorporated into proteins. Initiation of this process requires the information encoded within DNA to be transferred to RNA. This process of transcription produces ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA), each representing about 85%, 10% and 3% of the total RNA produced respectively (Griffiths et al. 1993, Sugden & Fuller 1991). The mRNA serves as a template from which cytosolic ribosomes (comprised of rRNA and protein) can use aminoacyl tRNAs as building blocks to synthesize long polypeptide chains. These polypeptides then fold into three dimensional structures forming protein (Griffiths et al. 1993, Lehninger et al. 1993).

There are different methods available to measure the rate of protein synthesis, and all are based on the same general principle (Sugden & Fuller 1991). The rate of protein synthesis can be determined by monitoring the incorporation of an isotopically labelled

amino acid into protein, over a specific period of time. The flooding dose method is commonly used to determine the rate of protein synthesis in vivo (Garlick et al. 1980). This method involves the injection of a flooding dose of a radiolabelled amino acid of known specific radioactivity, followed by measuring the amount of radiolabelled amino acid incorporated into the tissue of interest over a short period of time.

Certain criteria must be met and assumptions made when using this method in order to determine the rate of protein synthesis. Firstly, rapid equilibration of specific radioactivity of all amino acid pools must occur. Rapid equilibration of specific activity within plasma, intracellular and injected amino acid pools has been previously shown to occur (Attaix et al. 1986, Garlick et al. 1980, Obled 1989, Samuels et al. 1996) and presumably occurs in the aminoacyl-tRNA pool (Sugden et Fuller 1991). The direct precursor pool for protein synthesis is the aminoacyl-tRNA pool, but specific radioactivity in the aminoacyl-tRNA pool is difficult to measure (Sugden & Fuller 1991). The specific radioactivity of the free amino acid pool, however, is technically easier to measure, and is used to represent that of the aminoacyl-tRNA pool (Garlick et al. 1980). Secondly, the specific activity of the free amino acid pool must be relatively constant over the short time of the experiment and the rate of incorporation into protein must be linear. Specific radioactivity in the free amino acid pool has been shown to reach a plateau shortly following injection of a flooding dose of various radiolabelled amino acids (Garlick et al. 1980, Obled et al. 1989). Linear incorporation of label has also been shown in a variety of studies (Attaix et al. 1986, Obled et al. 1989, Samuels et al. 1996). Therefore, the flooding dose method meets these criteria and has become the method of choice to measure the in vivo rate of protein synthesis in tissues from small animals. The

major limitation to this method is that the time given for incorporation of radioactivity into protein is usually about 15 min and is assumed to be representative of the rate of protein synthesis throughout that day.

Protein degradation

Another process that is fundamental to protein turnover is protein degradation. The four major proteolytic pathways include the lysosomal, calcium dependent, ATP-ubiquitin dependent and ATP-ubiquitin independent pathways (Attaix et al. 1994). Lysosomal proteases, such as cathepsins B, D, H and L degrade extracellular, intracellular, membrane and secretory proteins (Attaix et al. 1994, Lee & Marzella 1994) and generate peptides for presentation on major histocompatibility complex (MHC) class II molecules (Mitch & Goldberg 1996). Lysosomes are particularly abundant in the liver and kidney (Lee & Marzella 1994). The calcium dependent proteases, known as calpains, are regulated by levels of cytoplasmic calcium (Melloni & Pontremoli 1991). The specific functions of calpains are still unclear. They do not seem to play a major role in skeletal muscle protein breakdown but may be involved in mediating the degradation of short lived proteins (Attaix et al. 1994, Pontremoli & Melloni 1986).

The ATP-ubiquitin dependent proteolytic pathway involves a 26S proteasome consisting of a 20S proteolytic core and two 19S flanking regions containing an ATPase (Attaix et al. 1997, Dubiel et al. 1995, Koster et al. 1995). Proteins that are degraded by this pathway are first marked with ubiquitin (Hilt & Wolf 1995). Ubiquitinylation of proteins requires at least two enzymes. The ubiquitin activating enzyme (E_1) and the

ubiquitin carrier (E_2) work together to attach a ubiquitin molecule onto the protein. Some proteins are multiubiquitinylated by ubiquitin protein ligase (E_3)(Attaix et al. 1994). Ubiquitinylated proteins are preferentially degraded by the 26S proteasome. This pathway is responsible for the degradation of short lived proteins, abnormal proteins and myofibrillar proteins (Attaix et al. 1994, 1998, Dubiel et al. 1995, Koster et al. 1995) and has a role in MHC class I antigen presentation (Coux et al. 1996, Goldberg et al. 1997). The ATP-ubiquitin dependent proteolytic system is particularly important in skeletal muscle and is believed to be the key pathway regulating muscle protein degradation during fasting, disuse atrophy and pathological conditions such as sepsis and cancer (Attaix et al. 1994, Baracos et al. 1995). Much less is known about ATP-ubiquitin independent proteolysis, but the 20S proteolytic core of the proteasome has been shown to function independently from the 26S proteasome (Coux et al. 1996).

There are a few different methods available to determine the rate of protein degradation (Attaix et al. 1994, Sugden & Fuller 1991). The rate of protein degradation in vivo can be measured as the difference between protein synthesis and growth (protein accretion) or by using prelabelling techniques. Protein degradation can be measured directly in vitro by measuring the release of an amino acid such as tyrosine (Sugden & Fuller 1991). However, this method will only allow degradation to be measured in very small or thin tissues (example: extensor digitorum longus from mice), as it is necessary for the incubation media to reach all cells (Fulks et al. 1975). These methods will measure total degradation but offer little information as to the involvement and importance of the various proteolytic pathways. To obtain this information in vitro, incubation of tissues with inhibitors specific for the major proteolytic systems, will

enable the relative contribution of each system to be estimated (Attaix et al. 1994), but is again limited to thin tissues only.

The identification and possible involvement of each of these pathways can also be determined through northern blot hybridization of mRNAs encoding proteases and/or subunits of the major proteolytic systems. Analysis of these mRNAs may provide information as to the relative importance of each proteolytic pathway. Levels of these mRNAs in skeletal muscle correlate well with measured proteolytic activity (Attaix et al. 1994). For example, increased mRNA levels for the ATP-ubiquitin dependent proteasome in skeletal muscle occur with an increase in proteasome content and an increase in the rate of proteolysis in cachectic rats (Baracos et al. 1995). Although there is a correlation between mRNA levels and proteolysis, increased mRNA levels do not necessarily indicate increased protein levels and/or increased proteolysis. The major advantage to this approach, however, is that it can indicate transcriptional regulation of the different proteolytic pathways and may suggest the qualitative involvement of proteolysis during different conditions, such as starvation and cancer cachexia.

D. The small intestine and protein turnover

There is almost no information regarding the effects of cancer cachexia and chemotherapy on small intestinal protein metabolism. This is surprising as the small intestine is the organ responsible for digestion and absorption of nutrients, serves as a physical barrier to endotoxins, bacteria, viruses and is involved in apolipoprotein metabolism, mucous secretion and immune function (McBurney 1994). Any condition

that compromises the integrity and function of the small intestine may interfere with these processes, and therefore, may impact the rest of the body. It has been shown that small intestinal protein mass, in comparison to other tissues, is very sensitive to stressors such as starvation. For example, small intestinal protein mass in the rat was reduced by about 25% after 1 d of starvation and close to 50% after 5 d (Samuels et al. 1996). This is in contrast to the rest of the body where no reductions in protein mass were observed after 1 d of starvation and only an 8% reduction in whole body protein mass after 5 d.

The small intestine is one of the most metabolically active organs in the body and has one of the highest rates of protein turnover (McBurney 1994, Reeds et al. 1993). One of the factors contributing to protein turnover is protein synthesis. The rate of protein synthesis can be measured in vivo, using the flooding dose method (Garlick et al. 1980), and provides valuable information as to the importance of protein synthesis in the small intestine. The small intestine accounts for approximately 19% of whole body protein synthesis in growing rats while only representing 7% of total body protein mass (McNurlan & Garlick 1980). The fractional rate of protein synthesis in the small intestine in rats has been estimated to be as high as 100%/d, demonstrating that over the course of one day, protein mass equivalent to the entire protein mass in the small intestine can be synthesized (Samuels et al. 1996).

Small intestinal protein mass is also influenced by protein degradation. Unfortunately, there is very little known concerning the rate of protein degradation and the relative importance of the different major proteolytic pathways in the small intestine. Measuring the rate of protein degradation in the small intestine is difficult. Indirect, in vivo measurements cannot be made by subtracting the growth rate from the rate of

protein synthesis. This method assumes that the difference between the growth rate and the rate of protein synthesis is due to protein degradation, an assumption that does not apply to the small intestine. In the small intestine, cells are continually sloughing off into the lumen (McBurney 1994), reducing the protein content. Secretory proteins account for a significant proportion (30-40%) of total small intestinal protein synthesis (Reeds et al. 1993). These proteins are secreted both lumenally and systemically thereby reducing the protein apparent content of the small intestine (Reeds et al. 1993). Therefore, determining the rate of protein degradation using an indirect method is problematic because protein accretion in the small intestine is dependent upon several factors. There are no in vitro techniques available for determining the rate of protein degradation in the small intestine.

Gene expression of the proteolytic pathways can be determined through northern blot hybridization. Levels of mRNA encoding proteases and subunits of the major proteolytic pathways (lysosomal, calcium dependent and ATP-ubiquitin dependent), in the small intestine, have been shown to increase during starvation (Samuels et al. 1996). Using northern blot hybridization, the relative importance of each proteolytic pathway in the small intestine during cancer cachexia and chemotherapy can be estimated.

E. Cancer cachexia, chemotherapy and protein turnover

Cancer cachexia and protein turnover

Body wasting associated with cancer cachexia is the result of depletion of fat

stores and lean tissue atrophy. Characteristic to this wasting is an increased rate of whole body protein turnover (Kern & Norton 1988). Studies in patients with advanced cancer show that this increased whole body protein turnover results from an increase in both the rates of synthesis and degradation, with a greater increase in degradation resulting in net protein catabolism (Borzotta et al. 1987, Jeevanandam et al. 1984, Pisters & Pearlstone 1993). Lean tissue atrophy includes wasting in skeletal muscle and visceral organs. Wasting of skeletal muscle has been shown in animal models to be the result of an increased rate of protein degradation (Temparis et al. 1994), a reduced rate of protein synthesis (Emery et al. 1984) or the result of both an increased rate of protein degradation and a reduced rate of protein synthesis (Baracos et al. 1995, Lopes et al. 1989, Smith & Tisdale 1993, Strelkov et al. 1989). Skeletal muscular atrophy resulting from cancer cachexia can be independent of reduced food intake as the rates of protein synthesis and degradation in pair-fed rats was not significantly different from healthy controls (Baracos et al. 1995, Strelkov et al. 1989).

Wasting of visceral organs such as the liver and small intestine is poorly documented, but has been shown to occur in tumour bearing rats. Le Bricon et al. (1995b) showed a reduction in small intestinal wet weight from Morris Hepatoma 7777 bearing rats relative to pair-fed rats in the early stages of cachexia (11% tumour burden). In a subsequent study, wasting of small intestinal protein mass was shown in rats bearing Morris Hepatoma 7777 but the study was complicated by a severe reduction in food intake (50%) and a large tumour burden (25%) (Le Bricon et al. 1996). It is difficult in the latter study to determine the extent to which wasting was the result of cachexia and not reduced food intake. Wasting has not been demonstrated in other tumour models.

More importantly, there is nothing known concerning the mechanisms responsible for possible intestinal atrophy. The rate of small intestinal protein synthesis and the importance of proteolysis have not been determined during cancer cachexia.

Treatment of cancer

To treat cancer, the tumour must be removed from the host. One way this can be achieved is through surgical excision of the tumour. Direct excision of tumours in rats has been shown to reverse some of the symptoms of cachexia (Le Bricon et al. 1996). Positive nitrogen balance and an increase in body weight were observed in rats after tumour excision. Ten days following tumour excision, small intestinal protein mass had returned to healthy levels (as small intestinal protein mass prior to tumour excision was lower in tumour bearing rats compared to healthy). The other common treatment for cancer is the use of antineoplastic treatments including chemotherapy and radiation.

Chemotherapy is used widely in the treatment of cancer. The ultimate goal of chemotherapy is to cure cancer, and allow for recovery of host protein that was lost as a result of cancer cachexia. There is, however, growing evidence indicating that chemotherapy itself has a negative impact on protein metabolism. In cancer patients, it is difficult to assess the effects of chemotherapy independently from cancer cachexia and it is therefore difficult to determine the interactions between chemotherapy and host tissue. It has been suggested that chemotherapy may be involved in the actual development of cachexia (Le Bricon et al. 1995a). Two days following the injection of cisplatinum, an alkylating agent, body weights of tumour bearing rats were significantly reduced

compared to tumour bearing rats that received no treatment (Le Bricon et al. 1995a, Le Bricon 1997). Body weights continued to decrease up to a week post-treatment when muscle mass was significantly lower in tumour bearing rats treated with chemotherapy compared to untreated tumour bearing rats. Similar results were found in the same study using healthy rats given an injection of cisplatinum (Le Bricon et al. 1995a). In a study by Temparis et al. (personal communication), treatment with cystemustine, an alkylating agent, on tumour bearing mice resulted acutely in a decrease in the rate of protein synthesis in skeletal muscle with no affect on protein degradation as compared to untreated tumour bearing mice. This resulted in further skeletal muscle atrophy in the tumour bearing mice in addition to the atrophy already present as a result of the cancer cachexia. Chemotherapy, as seen in these studies, contributes to and enhances cachexia. The possibility that chemotherapy may contribute to cachexia needs to be explored further, especially in tissues like the small intestine where there is almost nothing known concerning the effects of chemotherapy on protein metabolism during cancer cachexia.

Chemotherapy and small intestine

Evidence of cytotoxicity in the small intestine resulting from chemotherapy is well established (Potten 1995). Clinicians have long been aware of the several toxic side effects including nausea, vomiting, diarrhoea and in more extreme circumstances, septicaemia (Mercadante 1995, Sandovsky-Losica et al. 1990, 1992, Wadler et al. 1998). These side effects impair the treatment of cancer and if sufficiently severe, are fatal (Sandovsky-Losica et al. 1992). Cytotoxic damage to the small intestine has been linked

to each one of these side effects. A complete understanding of how chemotherapeutic agents damage the small intestine is imperative to improving the treatment of cancer.

Nausea and vomiting are regulated primarily through hormonal and neuronal signals and have less to do with localized intestinal cytotoxic damage (Veyrat-Follet et al. 1997). There is stronger evidence for a direct link between cytotoxic damage and diarrhoea. Acutely following therapy, there is a shift in balance between secretory and absorptive cells leading to altered secretions of fluids and electrolytes. Damage to brush-border enzymes resulting in incomplete digestion of carbohydrates and proteins increase the osmotic flow of fluids into the lumen. These extra nutrients may also promote colonic microbial proliferation (Mercadante 1995, Wadler et al. 1998). A more extreme case could occur when cytotoxic agents induce holes in the epithelial lining exposing regions of the lamina propria to the lumen of the intestine. With no epithelial lining, water and electrolyte balance would be impaired leading to loss of fluid and ultimately diarrhoea (Potten 1995). Furthermore, such damage could also facilitate more serious complications, with impaired digestive functions and bacterial translocation being of primary concern.

The small intestine is the portal of entry for several major nutrients into the body. Impaired nutrient intake could directly influence the nutritional status of all tissues in the body. Absorptive and digestive capacity could be reduced with a decreased in villus surface area. Other abnormalities include increased passive permeability of the intestine to disaccharides shown in human breast cancer patients 2 d following anthracycline therapy. Restoration of normal permeability occurred by day 8 (Parrilli et al. 1989). A different study in humans showed that passive permeability remained high for a week

following chemotherapy but was normal 3 wk later (Keefe et al. 1997). Although no systemic infections were reported in these studies, the potential for cytotoxic therapy to affect nutrient digestion and absorption in the small intestine is illustrated. More research is needed to determine the extent to which nutrient absorption is altered during antineoplastic therapy.

Opportunistic infections are a major problem associated with antineoplastic therapy (Sandovsky-Losica et al. 1990, 1992). These infections are the result of (1) compromised integrity of the intestinal barrier and (2) compromised immune system. Compromised barrier function is thought to arise from induced lesions that facilitate the translocation of indigenous microflora from the lumen of the gastrointestinal tract to lymph nodes and visceral organs where they replicate and can cause sepsis or even death. Systemic candidiasis results from the translocation of *Candida sp.* and occurs in human patients as well as rodent models (Berg 1983, Sandovsky-Losica et al. 1990, 1992). Methotrexate, an antimetabolite, caused increased intestinal permeability and bacterial translocation resulting in death of rats (Shou et al. 1991). Compromises to the immune system are also well documented. Methotrexate and 5-fluorouracil, both antimetabolites, reduced white blood cell counts by 30% and spleen weights by 50% in rats (Sandovsky-Losica et al. 1990). Cyclophosphamide, an alkylating agent, caused a 30% reduction in gut-associated lymphoid tissue in the lamina propria with a concomitant 65% reduction in lumenal IgA secretions. This impairment of IgA secretion may alter normal digestive microflora and facilitate the proliferation and colonization by pathogenic microbes (Cozon et al. 1991). This is thought to contribute to diarrhoea, but more importantly, to

bacterial translocation leading to septicaemia. Systemic infections of this nature still remain problematic and complicate the treatment of cancer.

Morphological alterations

Some of the most metabolically active cells in the body reside in the mucosal layer of the small intestine. Of the 250 cells housed within a murine crypt, 150 can be designated as proliferating or actively progressing through the cell cycle. Cellular migration at the average rate of 1.5 cell positions per hour replaces 1500 of the 3500 cells covering a murine villus every day (Potten 1995). Since chemotherapy is effective against rapidly proliferating tissue, crypt cells are clear targets to the non-specific cytotoxicity of chemotherapy. Reductions in crypt cell number or cell proliferation rate will have profound effects on mucosal homeostasis and could result in reduced crypt depth, decreased villus height and impaired barrier function (Potten 1995). Assessment of small intestinal morphology indicates if small intestinal surface area has been reduced and if the epithelial lining has been severely damaged.

There is very little current information in human cancer patients regarding morphological alterations resulting from chemotherapy. Earlier studies showed that chemotherapy reduced crypt proliferation. Methotrexate treatment in human cancer patients caused severe reductions of mitotic frequency in crypt cells (Trier 1962). Mitoses in crypts from intestinal biopsies were virtually absent just 12 hr after treatment and remained low for 2 d. Mitotic counts in crypts were 80% lower in human patients

after 2-5 d following various chemotherapeutic regimes (Smith et al. 1979). These rates returned to normal after 2 wk.

The vast majority of research pertaining to cytotoxic effects of chemotherapy on small intestinal morphology has been completed in healthy animals and has mainly involved two general groups of drugs: alkylating agents and antimetabolites. Alkylating agents form covalent cross-links between DNA strands. Once formed, only a few cross-links within the entire genome are required to inhibit cell proliferation (Brent et al. 1987, Konopa 1988). Antimetabolites inhibit DNA synthesis. This is achieved by direct inhibition through incorporation into DNA or indirectly by inhibition of purine and pyrimidine synthesis (Cozon et al. 1991). Early studies in laboratory animals looking at the effects of these chemotherapies on the small intestinal morphology involved excessive lethal doses (Altmann 1974). Although these studies are of little clinical relevance, they do outline the extreme condition. Morphological analysis of intestines from these animals revealed that the intestine is extremely sensitive to cytotoxic therapies. In one experiment, rats were given a lethal dose of chemotherapy and intestinal atrophy was measured (Altmann 1974). After 3 d, villi were reduced to 85% and crypts were reduced to a staggering 5% of healthy crypt area, eliminating any possibility of regeneration. Two conclusions can be made from this and from other early experiments: (1) sterilization of crypts is possible after a single large dose of therapy and (2) the gastrointestinal tract is one of the most sensitive tissues to cytotoxic therapy. It is surprising that any animal can even survive to a point were such extreme intestinal atrophy develops.

The next phase of research was dedicated to defining the cytotoxic effects of more realistic therapeutic regimes. A variety of chemotherapies have been tested in healthy animals at varying doses. Moore (1984) used 1,3-bis(2-chloroethyl)-1-nitrosourea, mechlorethamine hydrochloride, cyclophosphamide, adriamycin, 5-fluorouracil and actinomycin at different doses. All drugs tested in this study caused crypt sterilization at higher doses. At low doses, peak cell death in the crypts occurred after 1 d and was followed by recovery of crypt depth on the fourth day. Cisplatinum has also been shown to cause crypt sterilization at high doses. At lower doses, cell proliferation within the epithelial lining was reduced 2 d following treatment with subsequent compensatory hyperplasia by day four. There was no permanent effect of cisplatinum as cellular proliferation returned to normal by day seven and remained normal for at least a month (Kovacs et al. 1982). These studies indicated that crypt sterilization was dose dependent, and that recovery of healthy small intestinal crypt homeostasis occurred following low doses of chemotherapy.

Other researchers began to look in more detail at the effects of individual cytotoxic agents. Methotrexate was administered to rats as a single injection and comparisons were made to pair-fed healthy controls. One day following treatment, crypt depth was reduced by 25% and crypt cells had enlarged irregular nuclei; the mitotic index was drastically reduced. On day two, villus height was 40% lower than in controls, with 35% fewer crypt cells that were now cuboidal in shape (not columnar). Mitosis was still reduced (50% lower). On day four, evidence of recovery was noticeable. Villus height was only 20% lower and crypts were now significantly deeper (25% more cells). Mitotic counts were 30% higher and migration rate doubled. On day eight, recovery of villus

height was complete (Taminiau et al. 1980). These studies further illustrate that while the healthy small intestine is acutely sensitive to cytotoxic chemotherapies, complete recovery of morphological parameters can occur rapidly, and is initiated through crypt hyperplasia.

While the effects of chemotherapy on healthy small intestinal morphology from experimental animals have been extensively documented, studies involving tumour bearing animals are surprisingly scarce. The metabolic and hormonal milieu is very different during cancer and may affect homeostasis in the small intestine. The effect of antineoplastic therapy on small intestinal morphology needs to be addressed in the context of cancer cachexia.

Recovery from cancer cachexia following chemotherapy

Recovery from cancer following treatment with chemotherapy requires repletion of whole body and tissue protein mass. There is very little known concerning the processes involved during the recovery from cancer cachexia following chemotherapy. Recently, an experimental murine model has been developed in which a solid tumour, colon 26 adenocarcinoma, causes cachexia (Corbett et al. 1975, Tanaka et al. 1990) and can be cured with 100% efficacy using the newly developed alkylating agent, cystemustine (Bourut et al. 1986, Madelmont 1994). The development of this model allows for recovery from cancer cachexia to be studied in detail. The only study reported that has used this model for assessing protein metabolism in skeletal muscle during the recovery from cancer cachexia was done by Temparis et al. (personal communication).

Recovery from cachexia, in terms of increases in body weight, began within 1 wk of treatment. Skeletal muscle mass, although initially lower in the tumour bearing mice following treatment, was significantly greater within 1 mo following cystemustine treatment compared to untreated tumour bearing mice. Reversal of muscle wasting, in tumour bearing mice treated with cystemustine, resulted from an increase in the rate of protein synthesis and a reduction in the rate of protein degradation as compared to untreated tumour bearing mice. Further analysis to identify which proteolytic systems were involved in this reduction, indicated that the reduced proteolysis was a result of an inhibition of the ATP-ubiquitin dependent pathway. Body weights of tumour bearing mice treated with cystemustine, however, was still lower than body weights of healthy mice 1 mo post treatment (even though the tumour regressed within the first 2 wk). This suggests that cystemustine may be affecting the long term recovery of body weight and skeletal muscle protein mass in mice.

The precise effects of cancer cachexia on small intestinal protein metabolism has not been determined. However, if protein metabolism is compromised, it is not known if recovery in the small intestine occurs following chemotherapeutic treatment. Recovery of small intestinal protein mass and function may be of vital importance for recovery of tissues throughout the body. Currently, no study has defined the mechanisms mediating recovery of the small intestine from cancer cachexia following chemotherapy. The rate of small intestinal protein synthesis and the role of degradation during recovery are not known. Further studies will help to define the interaction of chemotherapy with cancer cachexia on the small intestine. This information may be of value in designing therapies which promote or enhance recovery from cancer cachexia following chemotherapy.

F. Summary

Cancer cachexia refers to the progressive wasting of host tissue observed in the majority of patients with advanced cancer and complicates existing treatment strategies. The effect of cancer cachexia on host tissue protein metabolism is only partially understood. There is also very little known concerning the interactions between cancer cachexia and chemotherapy on protein metabolism. In particular, there is almost nothing known concerning the effects of cancer cachexia and chemotherapy on the small intestine. The small intestine is responsible for the digestion and absorption of nutrients, serves as a physical barrier to bacteria and other pathogens and has a role in immune function. Compromises to the integrity and function of the small intestine may interfere with these processes. Clearly, studies determining the effects of cancer cachexia and chemotherapy on small intestinal protein metabolism are required. Furthermore, an understanding of the mechanisms regulating recovery of small intestinal protein metabolism may lead to more effective therapies and the design of effective treatment strategies.

HYPOTHESIS

The governing hypothesis central to this thesis was that cancer cachexia and chemotherapy affect small intestinal protein metabolism through modifications of the rates of protein synthesis and degradation. The specific hypotheses were as follows:

1) cancer cachexia, independent of food intake, causes protein wasting in the small intestine by reducing the rate of protein synthesis and increasing proteolysis,

2) partial or full recovery of small intestinal protein mass from cancer cachexia following chemotherapy occurs, and is mediated through restoration of the rates of protein synthesis and degradation, and

3) chemotherapy itself will promote small intestinal protein wasting and will cause morphological damage to crypts and villi.

OBJECTIVES

The overall objective of this thesis was to study the effect of cancer cachexia and chemotherapy on small intestinal protein metabolism.

1) The primary objective of this thesis was to study how treatment of cancer, through chemotherapy, affects protein metabolism in the small intestine during cancer cachexia. Specifically, the following parameters were determined following chemotherapy:

a) intestinal protein mass,

b) the rate of intestinal protein synthesis,

c) mRNA levels of proteins participating in specific proteolytic pathways, and

d) histology of crypts and villi.

2) A secondary objective was to study the acute and chronic effects of cancer cachexia on small intestinal protein metabolism, without chemotherapeutic treatment. The same parameters outlined in the primary objective were determined.

3) A third objective of this thesis was to characterize the effects that chemotherapy itself may have on small intestinal protein metabolism. The same parameters outlined in the primary objective were determined.

MATERIALS AND METHODS

A. Animals, housing and diet

All animal studies were completed in accordance with the guidelines of the Canadian Council of Animal Care. Male balb/c ByJ mice (20 g, 4-5 wk old, Jackson Laboratories, ME, USA) were housed in individual cages and maintained at 22-23 °C on a 12-h light-dark cycle commencing at 0800h. Mice were given at least 4 d to adjust to their new environment and diet before experimental treatments were imposed. Mice were given free access to water and food (standard rodent chow, UBC Animal Care Centre) with the exception of pair-fed mice. Pair-fed mice received one third of their allotted food between 1000 - 1100h. The remaining two thirds was given between 1700 - 1800h.

B. Tumour model

In order to study the effect of cancer cachexia and chemotherapy on the intestine, an appropriate model must be used to represent the condition observed in human cancer patients. Therefore, the tumour must be able to induce cachexia and the associated physiological abnormalities while maintaining a reasonably small tumour burden, as human tumour mass does not generally exceed 1% of the total body weight (Bibby et al. 1987). Colon 26 adenocarcinoma meets these requirements. It is an undifferentiated transplantable murine carcinoma that was first isolated in 1975 from one of 82 colon

tumours (Corbett et al. 1975). Weakness, abnormal carbohydrate metabolism, hypercorticism, involved in initiating protein catabolism and growth inhibition, impaired hepatic function, leukocytosis and elevated interleukin 6 levels are observed (Fujiki et al. 1997, Tanaka et al. 1990). All are characteristic of human cancer cachexia and were observed in these mice with a tumour burden of 3-6% of total body weight (Kern & Norton 1988, Nelson et al. 1994, Tanaka et al. 1990, Toomey et al. 1995). Earlier work using colon 26 adenocarcinoma showed that 2 wk after inoculation, extensive losses in body weight (about 20 % of the initial body weight) occurred as a result of significant reductions in adipose and skeletal muscle tissue weight (Fujita et al. 1996, Tanaka et al. 1989, 1990, Temparis et al., personal communication). Of particular interest, colon 26 adenocarcinoma was reported to have no affect on food intake by mice while extensive weight loss occurs (Tanaka et al. 1990). This is of experimental importance as it allows cachexia to be studied without the confounding factor of decreased food intake observed in other cachexia models.

C. Tumour inoculation and transplantation

Stock cells of colon 26 adenocarcinoma were obtained from Dr. Didier Attaix (INRA de Theix, Ceyrat, France) and used to generate the first tumour passage. These cells were shipped on dry ice and stored in liquid nitrogen. Cells were thawed in a water bath at 37°C, centrifuged for 5 min at 1000 g and washed twice with 2 ml sterile, non-pyrogenic saline (centrifuged between washes for 5 min, 1000 g). Cells were resuspended in 1 ml sterile, non-pyrogenic physiological saline and injected

subcutaneously into the upper dorsal region of the mouse, developing a solid tumour at the location of injection.

Subsequent tumour passages were generated by serial transplantation from solid tumours. Tumours were grown in mice for 1 mo prior to transplantation. Tumour bearing mice were anaesthetised with halothane and killed by cervical dislocation. Tumours were excised and weighed. The hard, whitish core of the tumour was weighed, sliced into small pieces and homogenized in sterile, non-pyrogenic saline (1 ml saline per 0.5 g core) using a 5 ml glass-to-glass tissue homogenizer. The homogenate was filtered through a sterile 70 μm nylon filter (Falcon 2350, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Immediately, 0.1 ml of filtrate was injected subcutaneously into recipient mice using a 21G needle. Each tumour-bearing mouse provided enough tumoural tissue to inoculate 15-20 recipient mice. Tumour bearing mice were housed separately to avoid cannibalization.

Mice from passages three and four were used for the experiments described below. All tumour bearing mice used experimentally displayed evidence of cancer cachexia characteristic of this model. A detailed description of these characteristics is provided in the results section.

D. Chemotherapeutic agent

Specific criteria must be met for selection of an appropriate chemotherapeutic drug. Firstly, the drug has to cure the cancer thereby allowing recovery from cancer and the associated cachexia. Secondly, the drug should have a certain degree of

pharmacological importance and relevance to human cancer patients. N-(2-Chloroethyl)-N-[2-(methylsulfonyl)ethyl]-N-nitrosourea ($C_6H_{12}ClN_3O_4S$), referred to as cystemustine, a member of the nitrosourea family of alkylating antineoplastic agents, has been recently identified and meets both of these criteria.

Cystemustine has been shown to cure a number of murine tumours including colon 26 adenocarcinoma with a 100% efficacy (Bourut et al. 1986, Madelmont 1994). The cytotoxic mechanism of cystemustine is common to other nitrosoureas used clinically and is mechanistically similar to many commonly used alkylating agents (Godeneche et al. 1990, 1993). Therefore, recovery from cancer cachexia was studied using a chemotherapeutic agent representative of commonly used therapies.

Phase II clinical trials have already been completed using cystemustine in advanced head and neck, colorectal and melanoma cancer patients (Cappelaere et al. 1995, Chauvergne et al. 1995, Cure et al. 1998, Kerbrat et al. 1993, Urosevic et al. 1996). Results from these studies indicate that cystemustine is an ineffective treatment for head and neck and colorectal patients but may have anti-cancer activity in patients with advanced melanoma. Current research is focused on reducing tumour resistance to cystemustine, as well as other nitrosoureas, thereby enhancing the antineoplastic activity of these drugs (Buchdahl et al. 1998, Debiton et al. 1997, Ludlum 1997).

E. Chemotherapeutic regime

Cystemustine was obtained from Dr. Didier Attaix (INRA de Theix, Ceyrat, France) and was maintained desiccated in the dark. A stock solution of cystemustine was

made 30 min prior to use at a concentration of 1 mg per ml sterile, non-pyrogenic saline. A single intraperitoneal (i.p.) injection of cystemustine was given at a dose of 20 mg per kg body weight between 1015h and 1045h. The timing of injection was important, as cystemustine is highly chronotoxic (Martineau-Pivoteau et al. 1996). This dose has previously been shown to cure colon 26 adenocarcinoma in mice (Temparis et al., personal communication). Details of tumour regression are given in the results section.

F. Experimental design

Treatment groups

There were four principle treatment groups used in this experiment: (1) healthy mice, (2) healthy mice treated with cystemustine, (3) tumour bearing mice and (4) tumour bearing mice treated with cystemustine. Mice were randomly designated to one of the four treatment groups. Tumours were transplanted into mice in the tumour-bearing groups, while mice in the healthy groups received a 0.1 ml subcutaneous injection of sterile, non-pyrogenic saline to their upper dorsal region. Shortly after the onset of cachexia, chemotherapy was administered to one of the tumour bearing groups of mice and one healthy group. All other mice received a 0.5 ml i.p. injection of sterile, non-pyrogenic saline. Halothane was used to anaesthetize mice in each experimental group before any injections were given.

An additional group of mice (healthy pair-fed mice) was used to control for any possible effects of reduced food intake by tumour bearing mice resulting from chemotherapy. Healthy mice were initially fed at the rate of food intake of tumour

bearing mice. Once the chemotherapeutic agent was administered, healthy pair-fed mice received the food intake of tumour bearing mice treated with cystemustine. Pair-fed mice received a 0.5 ml i.p. injection of sterile, non-pyrogenic saline on the day of tumour transplantation and on the day of chemotherapy.

Parameters

The parameters measured in these experimental groups included: body weight, food intake, small intestinal protein mass, rate of protein synthesis, mRNA encoding components of proteolytic systems and small intestinal histology. Body weights and food intake of individual mice were measured between 0800h and 1000h on a daily basis starting 8 d before chemotherapy was administered until the end of the experiment. Food intake was determined as the difference between food given and food remaining 24 h later. The other parameters were determined on the experimental days described below.

Experimental time points

Chemotherapy was administered on day 0 (about 3 d after the onset of cachexia and 18 d following tumour transplantation). Animals were killed on days 2, 4 and 11 following chemotherapy. Days 2 and 4 were used to determine the acute effects of chemotherapy and the mechanisms initiating recovery. By day 11, the tumour had fully regressed and therefore served as a good indicator of long term recovery. Healthy mice,

tumour bearing mice and tumour bearing mice treated with cystemustine were killed on days 2, 4 and 11.

Healthy mice treated with cystemustine and healthy pair fed mice were killed on days 2 and 4 only. Preliminary experiments showed that chemotherapy caused acute damage to the small intestine of healthy mice, but recovery of small intestinal protein mass was complete by day 4. Preliminary experiments also showed that food intake in tumour bearing mice had returned to normal by day 4. Therefore, mice from these two groups were not killed on day 11.

Two supplemental time points were chosen to meet specific objectives. Tumour bearing mice were killed 2 d prior to chemotherapy (day –2) to determine the acute mechanisms responsible for initiating wasting in the small intestine. A second group of tumour bearing mice was killed 1 d following chemotherapy (day 1) to further define the acute effects of the chemotherapeutic treatment. Both of these groups of mice were compared to healthy controls killed on the same days.

The experiment was completed in two replicates with all treatments and time points represented in both replicates. All measurements were combined. The total sample size of all treatment groups on each experimental day is given in Table 1.

Table 1.

Sample size, treatment groups and experimental time points

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.	Pair-fed
-2	5	6			
1	5		7		
2	10	7	7	8	12
4	10	6	6	8	6
11	10	9	6		

Values are number of mice killed on each experimental day. Tumour was colon 26 adenocarcinoma. Chemotherapy was cystemustine (cyst) administered on day 0. Pair-fed mice were fed the food intake of tumour bearing mice treated with cystemustine.

G. Protocol used on experimental days

Protein synthesis was measured in vivo using a flooding dose injection as described previously (Garlick et al. 1980, McAllister et al. 1995). Between 1100h and 1400h, mice were injected i.p. with 150 μmol per 100 g body weight of phenylalanine with a specific radioactivity of 0.6 μCi L-[2,6-^{3}H]phenylalanine per μmol (Amersham Canada Limited, Oakville, Canada). After precisely 15 min from time of injection, each mouse was killed by cervical dislocation, immediately submerged in ice water and the visceral cavity was opened. The carcass was kept on ice for at least 1 min to inhibit protein turnover. During this time, the small intestine was removed and flushed with ice cold saline. Four consecutive samples weighing between 100 and 150 mg were taken starting 10 cm distal to the pyloric sphincter, blotted, weighed and immediately placed in liquid nitrogen. These samples were used to determine the rate of protein synthesis and

for RNA isolation. A fifth sample was removed immediately distal to the four previous samples, weighed and placed in buffered formalin. This sample was used to measure histological parameters. The remaining small intestine, representing on average 65.1 ± 0.4% of small intestinal wet weight, was weighed, blotted and placed in liquid nitrogen and used later to determine the protein content of the small intestine. Intestinal samples were stored at –70°C until analyzed except for the histology samples which were stored at room temperature. At the same time, tumours were excised, blotted and weighed.

H. Analyses

Protein mass

Small intestinal samples were solubilized in 2 ml 1 M NaOH. Samples were placed in a water bath at 40°C for 48 h and periodically vortexed. A sub-sample was taken and percent nitrogen was determined using a LECO analyzer (model FP428; Leco Instruments Ltd. Mississauga, Ontario, Canada)(Sweeney 1988). Protein content in the samples was calculated by multiplying the weight of nitrogen in the sample by 6.25. Protein mass in samples of small intestine used to measure the rate of protein synthesis, for northern blot hybridization and to assess histology was estimated using the ratio of wet weight to protein mass. This estimate assumes the ratio of wet weight to protein mass is constant throughout the intestine.

In vivo rate of protein synthesis

Small intestinal samples were analysed in a manner similar to that reported by McAllister et al. (1995) and Garlick et al. (1980). Small intestinal samples (150 mg) were homogenized in 1.5 ml 2% $HClO_4$ using a 5 ml glass-to-glass tissue homogenizer. The homogenizer was rinsed with 0.5 ml 2% $HClO_4$. Homogenate and rinse were combined and placed on ice for at least 30 min to facilitate protein precipitation. They were then centrifuged at 3000 g for 15 min at 4°C. The supernatant was decanted and the protein precipitate was placed on ice. Half a volume (1 ml) of saturated potassium citrate was added to the supernatant, which was then centrifuged at 3000 g for 15 min. Supernatants were used for the estimation of free phenylalanine specific radioactivity. The protein precipitate was washed four times using 8 ml ice cold 2% $HClO_4$ (centrifuged between washes at 3000 g for 15 min at 4°C). Protein precipitates were then hydrolyzed in 3 ml 6 M HCl for 24 h at 110°C. HCl was removed by vacuum centrifugation (Dugan et al. 1992) and the amino acids were resuspended in 1 ml 0.5 M sodium citrate at pH 6.3. Hydrolysates were used to determine the specific radioactivity of protein-bound phenylalanine. Phenylalanine was converted to β-phenylethylamine by adding 0.25 ml L-tyrosine decarboxylase (0.1 units/ml) suspended in 0.5 M sodium citrate (pH 6.3), containing pyridoxal phosphate (1 mg/ml) (Sigma Chemical Company, St. Louis, MO, USA). The enzyme suspension was added to 1 ml of supernatant or 0.5 ml of hydrolysate and incubated in a waterbath for 20 h at 50°C.

β-Phenylethylamine was isolated from the incubated samples by adding 0.5 ml 3 M NaOH and 5 ml chloroform:n-heptane (1:3, v/v). Samples were shaken for 2-3 min

and centrifuged at 200 g for 5 min. The upper organic phase was removed and 2.5 ml chloroform and 2 ml 0.05 M H_2SO_4 were added. Samples were shaken for 2-3 min and centrifuged at 200 g for 5 min. The top aqueous phase was removed and placed in a waterbath at 65°C for 30 min to remove any residual chloroform. The concentration of β-phenylethylamine in the aqueous layer was determined by reverse phase HPLC analysis (Waters 717plus autosampler, Waters Limited, Mississauga, Ontario, Canada) using ethanolamine as the internal standard (McAllister et al. 1995). Radioactivity in the aqueous layer was determined using liquid scintillation counting (Beckman LS5600, Fullerton, CA, USA). These values were necessary to determine the specific radioactivity within each sample.

The fractional rate of protein synthesis (k_s)(%/day) was calculated using the following equation: $k_s = (S_B \times 100) / (S_A \times t)$ (Garlick et al. 1980), where S_B is the specific radioactivity of protein-bound phenylalanine, S_A is the specific radioactivity of free phenylalanine and t is the time between injection and slaughter (in days). The absolute rate of protein synthesis (mg/day) was calculated by multiplying the fractional rate of protein synthesis by the total protein mass of the small intestine.

Northern blot hybridization

Total RNA was isolated from small intestinal samples by phenol-chloroform extraction (Chomczynski & Sacchi 1987). Northern blot hybridizations were done in a manner similar to Temparis et al. (1994). Fifteen micrograms of RNA was run on a 1% agarose gel containing formaldehyde and was electrophoretically transferred to a nylon

membrane (GeneScreen, NEN Research Products, Boston, MA, USA). RNA was covalently bound to the membrane using an ultraviolet crosslinker. The RNA on the membrane was then hybridized overnight at 65°C with [^{32}P]cDNA probes labelled by random priming (Pharmacia Biotech, Oligolabelling Kit, Baie d'Urfe, Quebec, Canada) (Temparis et al. 1994). The probes used encoded rat cathepsin B (Sengudo et al. 1985), human m-calpain (Imajoh et al. 1988), chicken polyubiquitin (Agell et al. 1988), rat 14 kDa ubiquitin conjugating enzyme E2 (Wing & Bandville 1994), and C8 (Tanaka et al. 1990) and C9 (Kumatori et al. 1990) rat proteosome subunits. These probes were selected as they encode proteases and/or subunits of the major proteolytic systems (lysosomal, calcium dependent and ATP-ubiquitin dependent). Probed membranes were washed four times in 0.1% SDS at 65°C for 15 min and the stringency of washes was varied between 2x SSC to 0.2x SSC, depending on the probe. Washed membranes were placed in a phosphorimaging cassette and analysed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). After stripping the different probes, membranes were reprobed with a ribosomal RNA probe. The 18S ribosomal band was quantified using the phosphorimager to test for loading differences among wells.

Histology

Intestinal samples were stored in buffered formalin before being stained with hemotoxylin and eosin. Briefly, samples were dehydrated with ethanol and saturated with xylene. Samples were embedded in paraffin wax. Two transverse and one longitudinal sections (5 μm thick) were cut from the paraffin blocks and fixed on glass

slides. Hemotoxylin and eosin staining were used to differentially stain nuclear and cytoplasmic membranes.

Villus height, villus width, crypt depth and muscularis width were measured in micrometers at 200 fold magnification, using a light microscope. A minimum of ten consecutive villi and crypts were measured per animal. Treatment designation of slides were blinded at time of measurement. Villus contour length was calculated using the following equation: villus contour length = (2 x villus height) + (0.3 x villus width) (de Roy van Zuidewijn et al. 1992).

Statistics

The effect of treatment was tested by analysis of variance (SAS 1996). Differences among means were assessed by Student's *t*-tests. Variability was expressed as SEM. Differences were considered significant at $P < 0.05$.

RESULTS

A. Characterization of the experimental model

Cancer cachexia

Tumours were first palpable in mice 1 wk following tumour transplantation (day –18). Symptoms of cachexia began about 4 or 5 d after tumours were detected and developed in a predictable manner. Cancer cachexia in this study was characterized by three phases: (1) pre-cachexia from days –7 to –3, (2) early cachexia from days –3 to 2 and (3) late cachexia from day 2 onward.

A plateau in body weight of tumour bearing mice defined the start of the pre-cachectic period (Fig. 1). Onset of the early cachexia phase began once tumours grew to a critical mass of approximately 0.5 g (tumour weight measured on day –2, Fig. 2). Early cachexia was characterized by a rapid loss of body weight. This phase of cachexia lasted for 4-5 d with an average loss in body weight of 0.6 g per day (2-3% loss per day). By day 2, body weights of tumour bearing mice were nearly 20% lower than healthy controls ($P<0.05$). The late phase of cachexia was characterized by a more gradual loss of body weight. By the end of the experiment on day 11, tumour-bearing mice had lost almost 20% of their pre-cachectic weight and weighed 30% less than healthy controls ($P<0.05$).

Figure 1. Effect of cancer cachexia and chemotherapy on body weight of mice

Values for body weight are means ± SEM (g) with $n \geq 6$ mice per mean. *Values are significantly different from healthy controls on the same experimental day ($P<0.05$). ‡ Values are significantly different from both healthy controls and tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. H = healthy mice; T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine; HC = healthy mice treated with cystemustine.

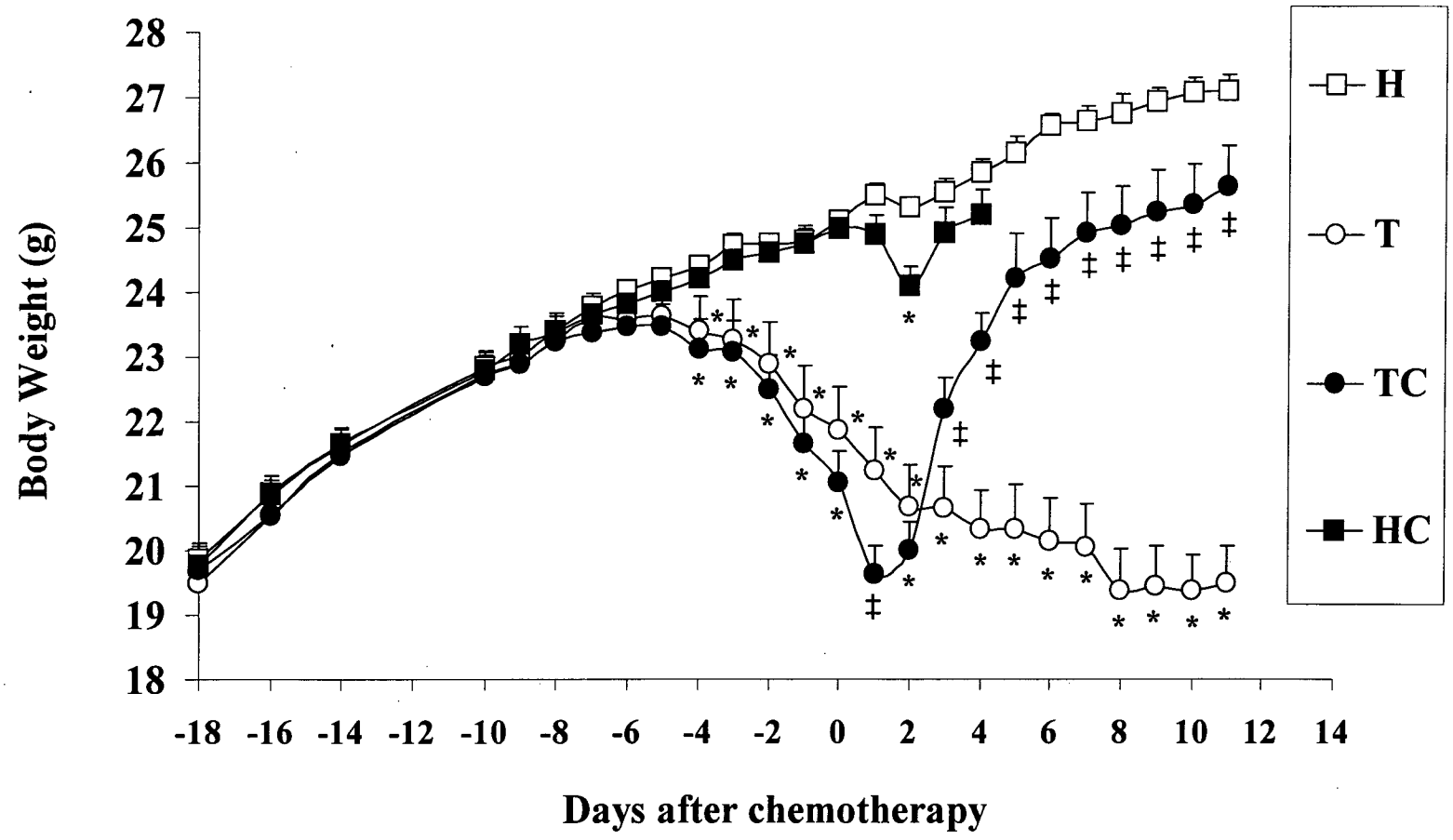
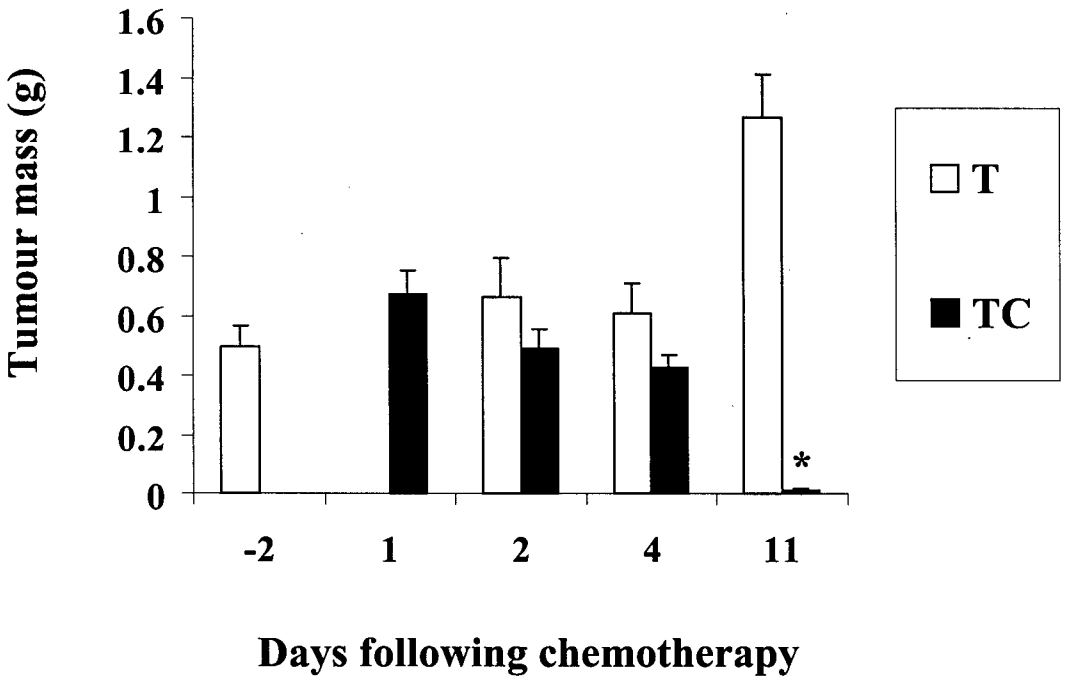

Body Weight (g)
28
27
26
25
24
23
22
21
20
19
18
-18 -16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14
Days after chemotherapy
H
T
TC
HC

Figure 2. Tumour mass on experimental days

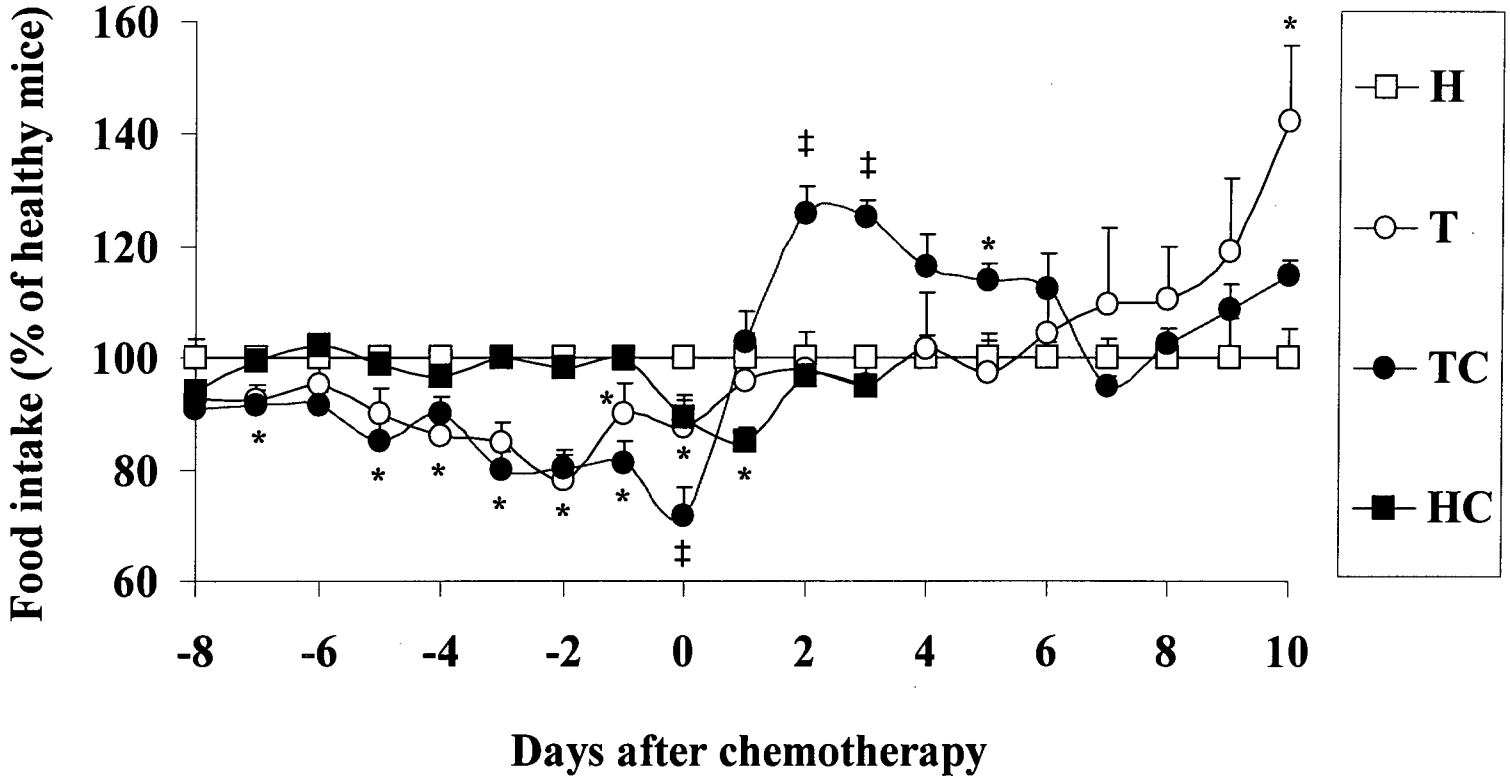
Values for tumour mass are means ± SEM (g) with n = 6 to 9 mice per mean (see Table 1). *Tumour mass values are significantly different from untreated tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine.


Tumour mass (g)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-2
1
2
4
11
*
T
TC
Days following chemotherapy

During the pre-cachexia phase, food intake of tumour bearing mice was ~10% lower than in healthy mice (Fig. 3). As mice became cachectic, food intake was reduced by ~20% but returned to healthy levels near the end of the early cachexia phase (day 1). Food intake continued to rise in tumour bearing mice and was higher than healthy animals by the end of the study ($P<0.05$). Food intake was not different between tumour bearing mice and healthy mice on a cumulative basis between days –8 and 10 (111.7 ± 6.3 g versus 111.1 ± 2.5 g respectively, $P<0.05$).

Figure 3. Effect of cancer cachexia and chemotherapy on food intake in mice

Values for food intake are means ± SEM (% of healthy control food intake) with n ≥ 6 mice per mean. Average food intake of healthy mice between days –8 and 10 was 5.85 ± 0.11 g. *Values are significantly different from healthy controls on the same experimental day ($P<0.05$). ‡ Values are significantly different from both healthy controls and tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. H = healthy mice; T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine; HC = healthy mice treated with cystemustine.


Food intake (% of healthy mice)
160
140
120
100
80
60
-8
-6
-4
-2
0
2
4
6
8
10
Days after chemotherapy
H
T
TC
HC

Chemotherapy

Cystemustine was administered to tumour bearing mice 3 d after the onset of cancer cachexia. Acute toxicity was evident in treated tumour bearing mice 1 d following chemotherapy. Body weight of treated tumour bearing mice was 7% lower than that of untreated tumour bearing mice ($P<0.05$) at this time. Food intake was also reduced compared to untreated tumour bearing mice (-20%; $P<0.05$). There was no evidence of tumour regression.

Recovery from cancer cachexia began by day 2. Body weight of treated mice started to increase and food intake returned to healthy levels. Tumour mass in treated mice was 25% smaller compared to untreated tumour bearing mice ($P>0.05$) (Fig. 2).

Mice were well into a state of recovery by day 4. Treated tumour-bearing mice were 15% heavier than untreated tumour bearing mice ($P<0.05$) but were still 10% lighter than healthy mice ($P<0.05$). Food intake of treated mice was ~25% higher than that of both healthy mice and untreated tumour bearing mice ($P<0.05$). By day 11, tumour regression was complete with only trace amounts of tumoural tissue visually present in some of the mice. Body weights of cured mice were 5% lower than those of healthy mice ($P<0.05$) and food intake had returned to normal.

Healthy mice treated with cystemustine showed similarities to tumour bearing mice treated with cystemustine. Body weight of healthy mice given cystemustine was lower compared to healthy controls on day 2 ($P<0.05$), but was not different on days 3 and 4. Food intake of healthy mice treated with cystemustine was lower than food intake of healthy mice 2 d following treatment ($P<0.05$) and returned to normal by the third and fourth day following chemotherapy.

B. Small intestinal protein mass

Small intestinal protein mass of tumour bearing mice at the onset of cachexia (day –2) was not different from healthy controls (Table 2; $P>0.05$). However, evidence of wasting was present by day 2 as small intestinal protein mass of tumour bearing mice was reduced by 25% compared to healthy controls ($P<0.05$). This wasting was not the result of decreased food intake, as pair-fed controls showed no evidence of wasting compared to healthy controls (178.1 ± 8.9 mg protein vs. 171.7 ± 6.1 mg protein respectively, on day 2, $P>0.05$). Small intestinal protein mass remained chronically lower in tumour bearing mice compared to healthy controls but the difference diminished by the end of the experiment on day 11, where this difference was 13% ($P<0.05$).

Small intestinal protein mass of tumour bearing mice treated with cystemustine on days 1 and 2 was almost 20% lower than that of healthy controls ($P<0.05$). However, there was no evidence indicating that chemotherapy had any negative effect on protein mass because small intestinal protein mass of treated tumour-bearing mice on days 1 and 2 was not different from untreated tumour bearing mice on day 2 ($P>0.05$). In contrast, evidence of acute toxicity from chemotherapy was detected on day 2 in healthy mice treated with cystemustine; small intestinal protein mass was 17% lower compared to healthy controls ($P<0.05$).

Protein mass in the small intestine from treated tumour bearing mice on day 4 was significantly higher (10%) than that in healthy controls ($P<0.05$). Small intestinal protein mass was still higher in cured mice at the end of the study on day 11 ($P<0.05$).

Table 2.

Effect of cancer cachexia and chemotherapy on small intestinal protein mass

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
-2	156.4 ± 5.8 *	149.8 ± 3.9 *		
1	165.8 ± 6.0 *		133.6 ± 2.8 ‡	
2	171.7 ± 6.1 *	128.7 ± 9.5 ‡	140.0 ± 7.1 ‡	142.5 ± 6.1 ‡
4	175.1 ± 4.2 *	137.3 ± 9.6 ‡	193.9 ± 10.1 §	174.7 ± 2.3 *
11	167.2 ± 4.8 *	145.3 ± 5.3 ‡	183.9 ± 8.2 §	

Values for small intestinal protein mass are means ± SEM (mg) with n = 5 to 10 mice per mean (see Table 1). *‡§ Values on the same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine (cyst) administered on day 0.

Small intestinal protein mass was also expressed relative to body weight to assess the degree of wasting and recovery in the small intestine relative to the whole body (Table 3). Protein mass expressed in this manner was never lower in tumour bearing groups of mice compared to healthy mice and was actually 20% higher on day 11 in untreated tumour bearing mice ($P<0.05$) compared to healthy controls. In tumour bearing mice treated with cystemustine, this ratio was higher than in healthy mice on days 1, 4 and 11 ($P<0.05$).

In contrast, small intestinal protein mass relative to body weight in healthy mice treated with cystemustine was significantly lower (-13%) on day 2 compared to all other groups ($P<0.05$). By day 4, this ratio was not different from healthy controls.

Table 3.

Small intestinal protein mass expressed relative to body weight

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
-2	6.19 ± 0.23 *	6.12 ± 0.21 *		
1	6.15 ± 0.19 *		6.79 ± 0.17 ‡	
2	6.82 ± 0.25 *	6.81 ± 0.24 *	7.07 ± 0.31 *	5.95 ± 0.18 ‡
4	6.78 ± 0.10 *	7.08 ± 0.27 *	8.34 ± 0.28 ‡	6.95 ± 0.11 *
11	6.16 ± 0.14 *	7.46 ± 0.20 ‡	7.17 ± 0.17 ‡	

Values for small intestinal protein mass (mg) expressed relative to body weight (g) are means ± SEM with n = 5 to 10 mice per mean (see Table 1). *‡ Values on same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine (cyst) administered on day 0.

C. In vivo rate of protein synthesis

In tumour bearing mice, the fractional rate of protein synthesis was lower (-18%) compared to healthy controls at the onset of cachexia (day –2, Table 4; $P<0.05$), before losses in protein mass were detected (Table 2). The fractional rate of protein synthesis was still lower on day 2 ($P<0.05$). This decrease in the fractional rate of protein synthesis was not due to the small reduction in food intake because the fractional rate of protein synthesis in pair-fed mice was not different from that of healthy control mice (95.2 ± 5.6 %/d vs. 104.4 ± 3.5 %/d respectively, on day 2, $P>0.05$). On days 4 and 11, the fractional rate of protein synthesis in tumour bearing mice was lower than in healthy control mice but the difference was not statistically significant ($P>0.05$).

One day following chemotherapy, the fractional rate of protein synthesis in treated tumour bearing mice was 25% lower than in healthy mice ($P<0.05$). To determine if there were any acute effects of cystemustine on the fractional rate of protein synthesis in treated tumour bearing mice, a comparison was made to untreated tumour bearing mice. The fractional rate of protein synthesis in treated tumour bearing mice on day 1 was 15% lower than in untreated tumour bearing mice on day 2 ($P<0.1$; Students *t*-test).

The fractional rate of protein synthesis was the same on day 2 and 18% higher on day 4 in treated tumour bearing mice compared to healthy controls ($P<0.05$). This increase in the fractional rate of protein synthesis corresponds to the day hypertrophy of small intestinal protein mass was first detected. The rate returned to normal by day 11. No changes in the fractional rate of protein synthesis were detected in healthy mice treated with cystemustine on days 2 or 4 ($P>0.05$).

Table 4.

The fractional rate of protein synthesis in the small intestine during cancer cachexia and chemotherapy

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
-2	103.6 ± 4.6 *	84.9 ± 6.0 ‡		
1	100.4 ± 13.9 *		75.2 ± 5.7 ‡	
2	104.4 ± 3.5 *	90.6 ± 5.5 ‡	108.3 ± 6.2 *	105.9 ± 3.5 *
4	103.7 ± 3.9 *	99.4 ± 7.5 *	127.8 ± 10.5 ‡	104.8 ± 8.3 *
11	101.4 ± 7.7 *	92.3 ± 5.3 *	106.2 ± 9.2 *	

Values for the fractional rate of protein synthesis in the small intestine are means ± SEM (%/day) with n = 5 to 10 mice per mean (see Table 1). *‡ Values on same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine (cyst) administered on day 0.

The absolute rate of protein synthesis (Table 5) showed similar trends as the fractional rate of protein synthesis. The absolute rate of protein synthesis in tumour bearing mice was significantly lower compared to healthy controls on all experimental days ($P<0.05$). The greatest difference was on day 2 (-34%).

In tumour bearing mice treated with cystemustine, the absolute rate of protein synthesis was 40% lower than in healthy controls on day 1 ($P<0.05$). On day 2, the absolute rate of protein synthesis in treated tumour bearing mice was higher (~ 25%) than in untreated tumour bearing mice but was still lower than in healthy controls ($P<0.05$); by day 4, the rate was 35% higher compared to healthy mice and was almost normal by day 11 (+15%, $P<0.05$).

The absolute rate of protein synthesis in healthy mice treated with cystemustine was 15% lower than healthy controls on day 2 and returned to normal on day 4 ($P>0.05$).

Table 5.

The absolute rate of protein synthesis in the small intestine during cancer cachexia and chemotherapy

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
-2	162.4 ± 11.4 *	128.1 ± 11.9 *		
1	167.6 ± 24.7 *		100.4 ± 7.4 ‡	
2	179.6 ± 9.5 *	118.5 ± 14.0 ‡	151.5 ± 11.7 §	151.4 ± 9.5 §
4	182.7 ± 11.4 *	136.1 ± 12.2 ‡	245.6 ± 20.4 §	182.7 ± 13.9 *
11	170.4 ± 14.8 *	134.3 ± 9.1 ‡	194.1 ± 16.2 §	

Values for the absolute rate of protein synthesis in the small intestine are means ± SEM (mg/day) with n = 5 to 10 mice per mean (see Table 1). *‡§ Values on same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine (cyst) administered on day 0.

D. Northern blot hybridization

Northern blot hybridization of cathepsin B (lysosomal) and m-calpain (calcium dependent) is shown in Figure 4. Cathepsin B mRNA in the small intestine from tumour bearing mice was ~25% lower than in healthy mice on day –2 and day 4 ($P<0.05$) and ~15% lower on day 2 ($P>0.05$). Tumour bearing mice treated with cystemustine had similar levels of cathepsin B mRNA as healthy mice on all experimental days. There

were no differences in levels of m-calpain mRNA in the small intestine from any treatment groups on the days analyzed ($P<0.05$).

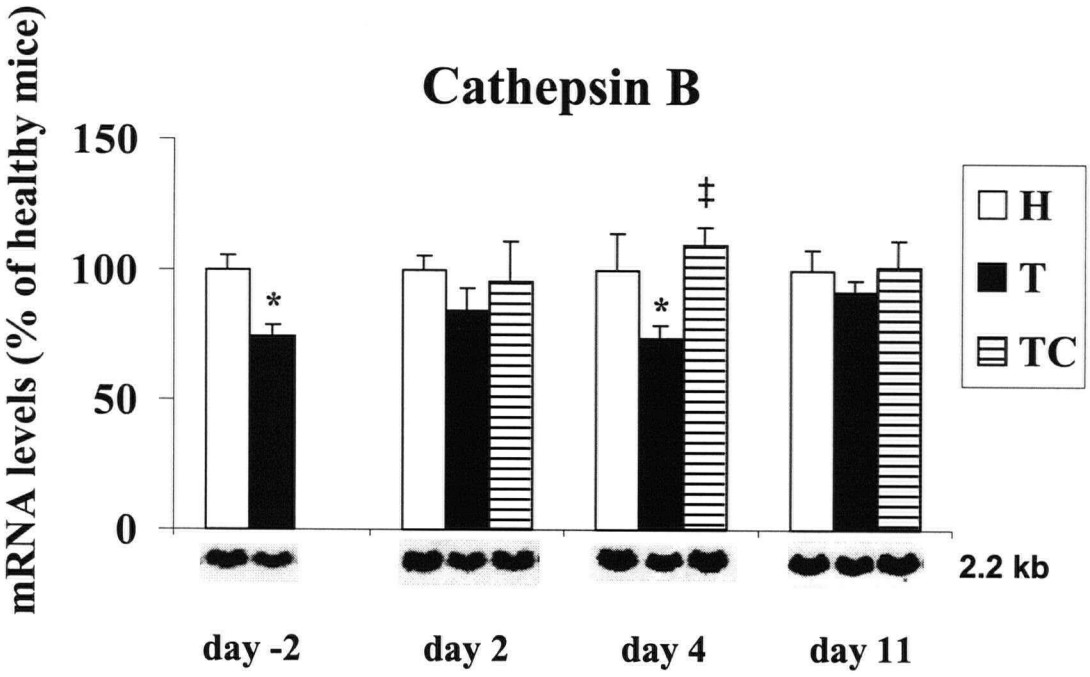
Several proteins are involved in the ATP-ubiquitin dependent proteolytic pathway. Of these, mRNA encoding the 14-kDa E2 ubiquitin conjugating enzyme, ubiquitin, and C8 and C9 proteasome subunits where probed. mRNA encoding ubiquitin was ~30% lower in tumour bearing mice compared to healthy mice on days –2, 2 and 4 ($P<0.05$) (Fig. 5). mRNA encoding the 14 kDa E2 ubiquitin conjugating enzyme appeared to follow a similar trend as ubiquitin mRNA but there were no significant differences (Fig. 5). There were no differences in mRNA levels encoding the 14-kDa E2 ubiquitin conjugating enzyme or ubiquitin between treated tumour bearing mice and healthy mice on any of the experimental days analyzed ($P>0.05$).

Northern blot hybridization of mRNAs encoding C8 and C9 subunits of the 26S proteasome is shown in Figure 6. Levels of mRNAs encoding C8 and C9 in tumour bearing mice were lower (25-50%)($P<0.05$) than in healthy control mice on days –2, 2 and 4 but were not different from healthy mice on day 11 ($P>0.05$). Restoration of mRNA levels for C8 and C9 in treated tumour bearing mice occurred on day 2 ($P<0.05$). Levels of mRNA encoding C8 in treated tumour bearing mice was higher than in healthy mice on day 4, with a similar upregulation of mRNA levels encoding C9 on day 11 ($P<0.05$).

No differences were detected among levels of mRNAs from healthy mice treated with cystemustine, healthy pair-fed mice and healthy controls for all mRNA species probed on all experimental days analysed ($P>0.05$)(data not shown).

Figure 4. mRNA levels for cathepsin B and m-calpain during cancer cachexia and chemotherapy

Values are means ± SEM of phosphorimager signals expressed as a percentage of healthy controls with n = 5 to 6 mice per mean. No differences in loading were detected, determined by 18S rRNA signal analysis ($P>0.05$). Representative northern blots are shown below the corresponding bar. *Values are significantly different from healthy controls on the same experimental day ($P<0.05$). ‡ Values are significantly different from tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. H = healthy mice; T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine.


Cathepsin B
mRNA levels (% of healthy mice)
150
100
50
0
*
*
‡
H
T
TC
2.2 kb
day -2
day 2
day 4
day 11

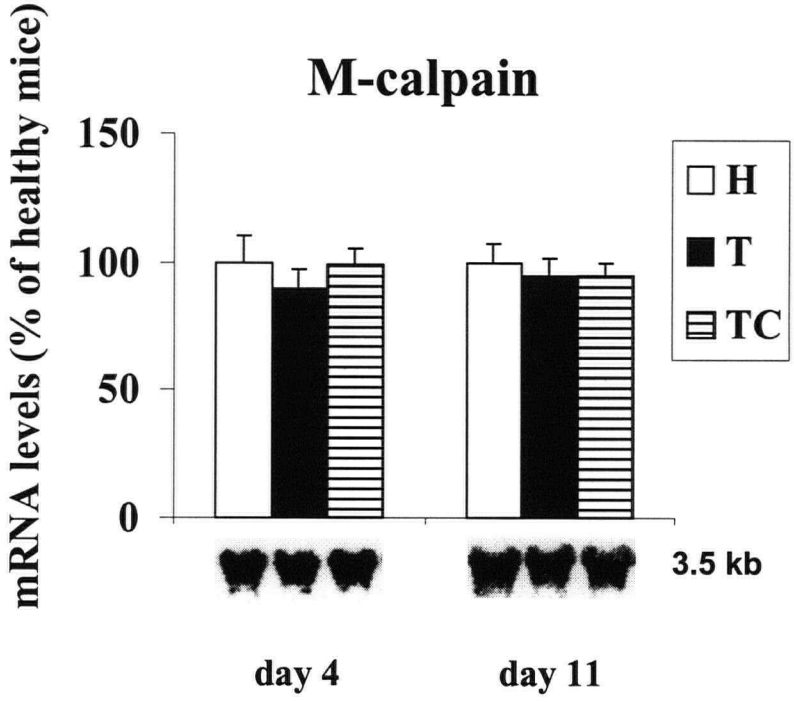
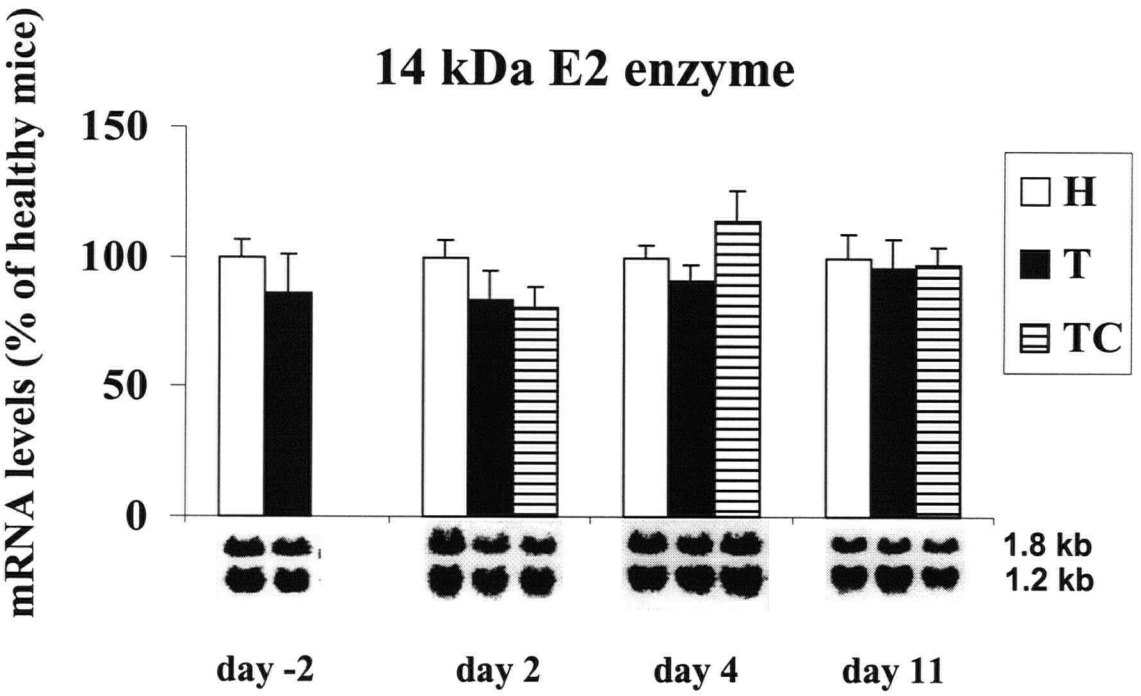

M-calpain
mRNA levels (% of healthy mice)
150
100
50
0
H
T
TC
3.5 kb
day 4
day 11

Figure 5. mRNA levels for 14-kDa E2 ubiquitin conjugating enzyme and ubiquitin during cancer cachexia and chemotherapy

Values are means ± SEM of phosphorimager signals expressed as a percentage of healthy controls with n = 5 to 6 mice per mean. No differences in loading were detected, determined by 18S rRNA signal analysis ($P>0.05$). Representative northern blots are shown below the corresponding bar. *Values are significantly different from healthy controls on the same experimental day ($P<0.05$). ‡ Values are significantly different from tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. H = healthy mice; T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine.


14 kDa E2 enzyme
mRNA levels (% of healthy mice)
150
100
50
0
H
T
TC
1.8 kb
1.2 kb
day -2
day 2
day 4
day 11

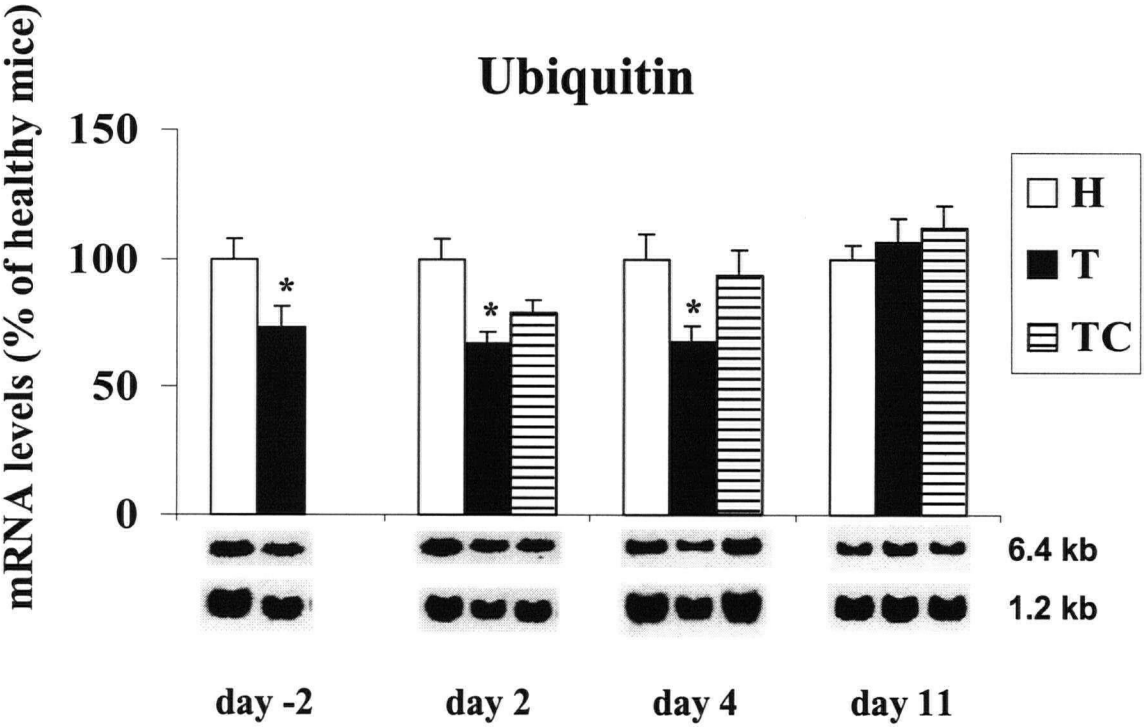
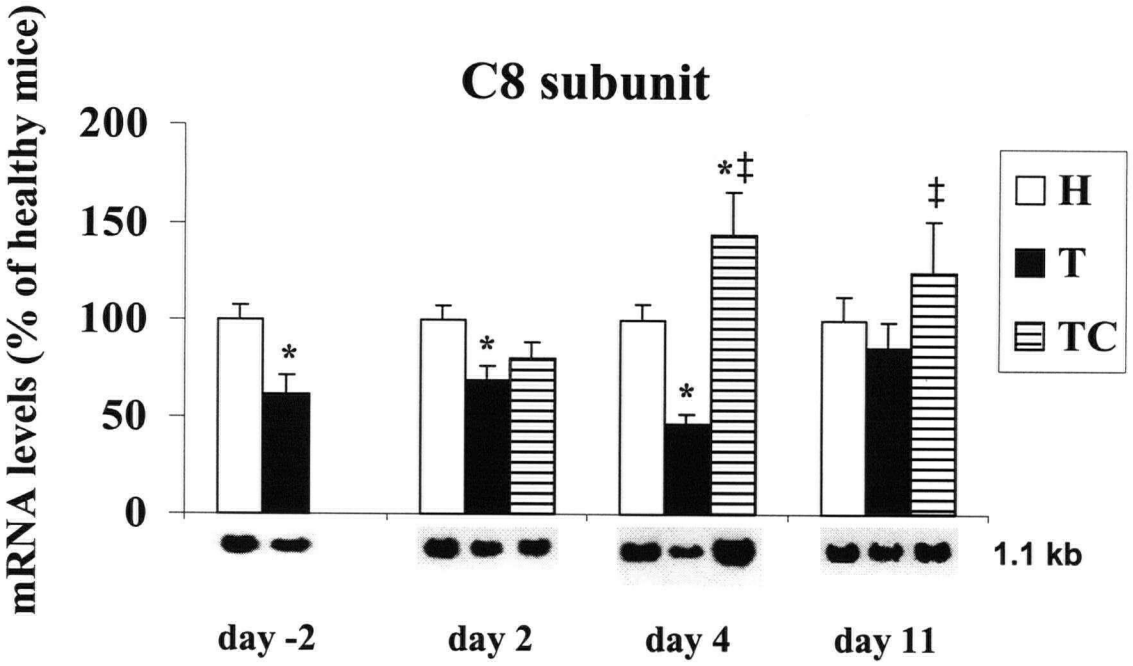
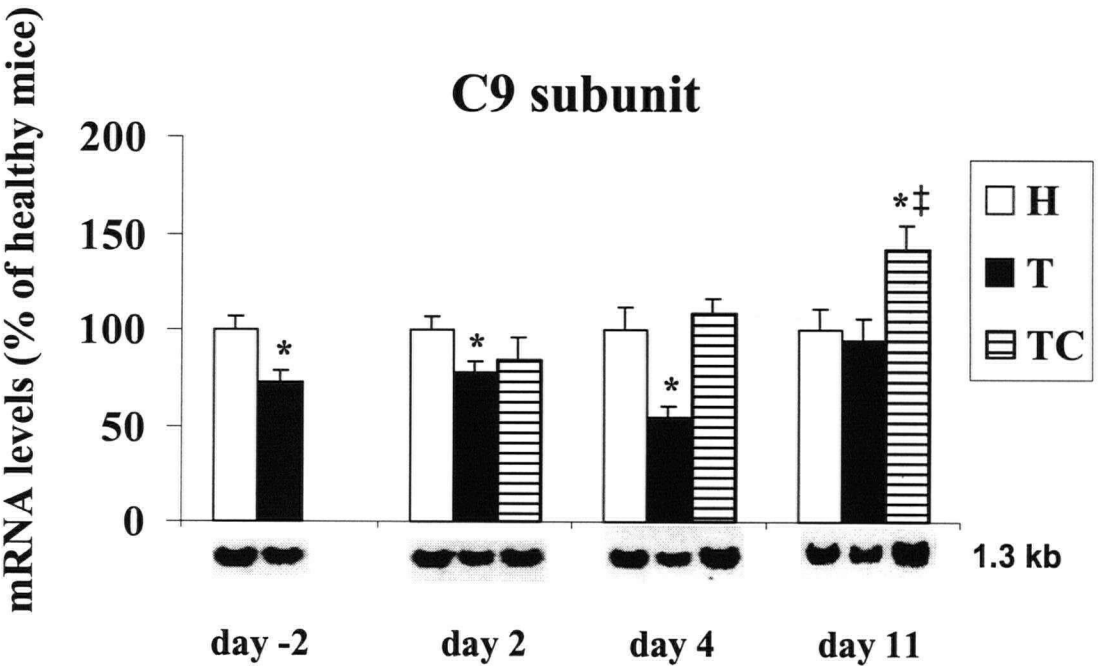

Ubiquitin
mRNA levels (% of healthy mice)
150
100
50
0
*
*
*
H
T
TC
6.4 kb
1.2 kb
day -2
day 2
day 4
day 11

Figure 6. mRNA levels for C8 and C9 proteasome subunits during cancer cachexia and chemotherapy

Values are means ± SEM of phosphorimager signals expressed as a percentage of healthy controls with n = 5 to 6 mice per mean. No differences in loading were detected, determined by 18S rRNA signal analysis ($P>0.05$). Representative northern blots are shown below the corresponding bar. *Values are significantly different from healthy controls on the same experimental day ($P<0.05$). ‡ Values are significantly different from tumour bearing mice on the same experimental day ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine administered on day 0. H = healthy mice; T = tumour bearing mice; TC = tumour bearing mice treated with cystemustine.


C8 subunit
mRNA levels (% of healthy mice)
200
150
100
50
0
*
*
*
*‡
‡
H
T
TC
1.1 kb
day -2
day 2
day 4
day 11


C9 subunit
mRNA levels (% of healthy mice)
200
150
100
50
0
*
*
*
*‡
H
T
TC
1.3 kb
day -2
day 2
day 4
day 11

E. Histology

Cancer cachexia induced fairly consistent alterations to morphological parameters of the small intestine. Villus height and contour length were only slightly lower in tumour bearing mice than in healthy mice ($P>0.05$), whereas villus width and crypt depth showed substantial evidence of atrophy ($P<0.05$) (Table 6, Fig. 7). Wasting of smooth muscle in the muscularis layer was indicated by a reduction in muscularis width on days 2 and 11 ($P<0.05$), but not on day 4 ($P>0.05$).

No evidence of acute morphological damage was detected in tumour bearing mice treated with cystemustine on day 2; villus height, contour length and width were not different from untreated tumour bearing mice ($P>0.05$) (Table 6, Fig. 7). Crypt depth in treated tumour bearing mice was higher ($P<0.05$) than in untreated tumour bearing mice and not different from healthy mice ($P>0.05$). This is in contrast to healthy mice treated with cystemustine; villus height and contour length were drastically reduced (30%; $P<0.05$)) compared to healthy control mice (Table 6, Fig. 7). Villus width was also reduced (~10%) in treated healthy mice compared to controls ($P<0.05$). Evidence of crypt hypertrophy in healthy mice treated with cystemustine was indicated by a 30% increase in crypt depth ($P<0.05$) compared to healthy control mice. Muscularis width in healthy mice treated with cystemustine was not different from healthy controls ($P>0.05$).

Recovery of the mucosa was complete by day 4; villus height, contour length, width and crypt depth in treated tumour bearing mice were not different from healthy mice ($P>0.05$). Muscularis width was not different from healthy mice on day 2, but was ~20% lower on day 4 ($P<0.05$) in treated tumour bearing mice.

Table 6.

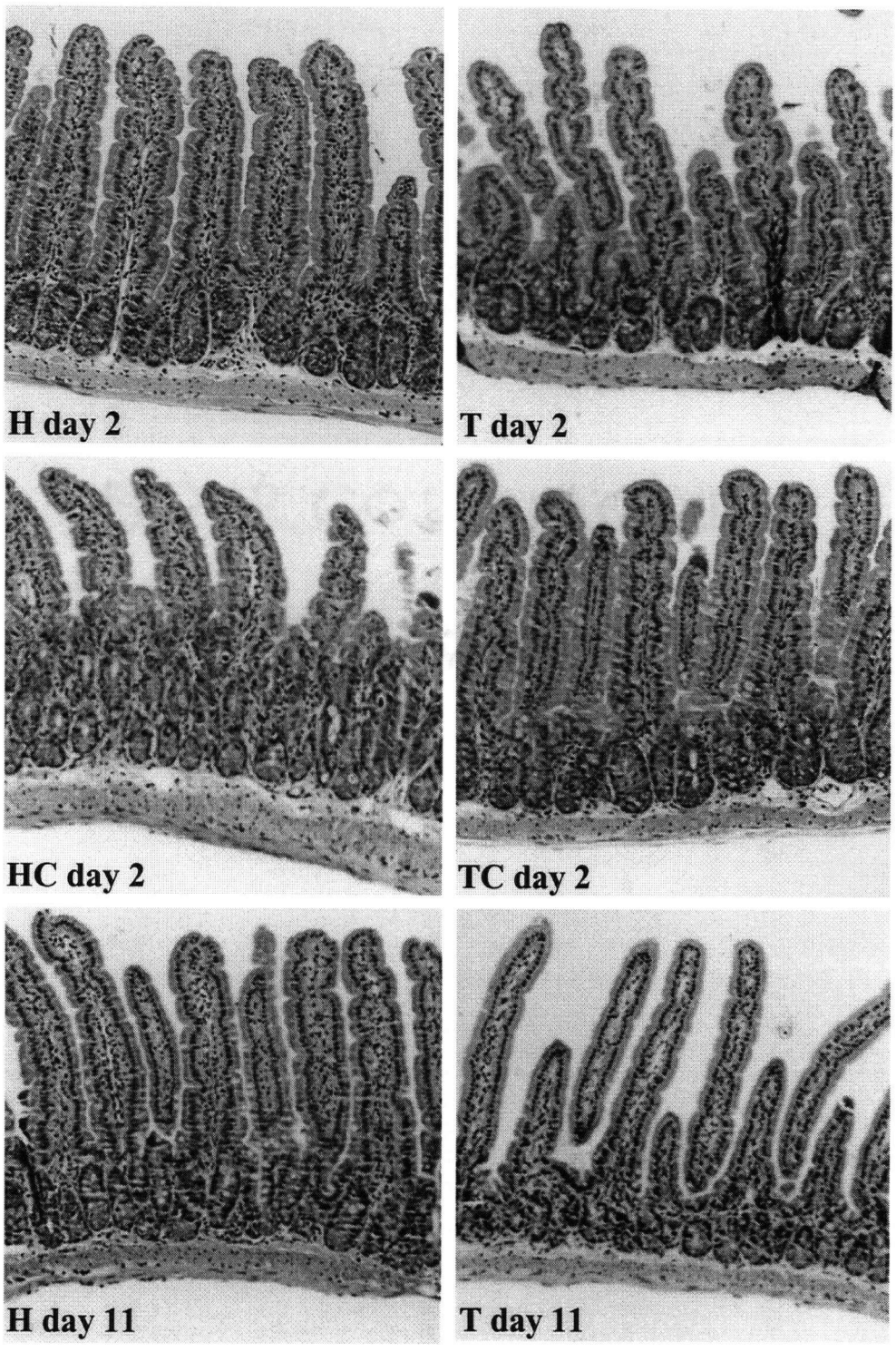
Histological parameters of the small intestine during cancer cachexia and chemotherapy

Day	Parameter	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
2	Villus height	342 ± 19 *	312 ± 19 *	321 ± 21 *	241 ± 20 ‡
	Villus width	81 ± 3 *	72 ± 1 ‡	72 ± 4 ‡	71 ± 3 ‡
	Crypt depth	121 ± 7 *	99 ± 4 ‡	111 ± 6 *	156 ± 9 §
	Villus contour Length	708 ± 39 *	646 ± 39 *	663 ± 42 *	503 ± 40 ‡
	Muscularis width	46 ± 3 *	36 ± 2 ‡	40 ± 5 *‡	47 ± 4 *
4	Villus height	367 ± 16 *	336 ± 18 *	382 ± 24 *	
	Villus width	80 ± 2 *	68 ± 1 ‡	77 ± 3 *	
	Crypt depth	124 ± 2 *	98 ± 3 ‡	120 ± 4 *	
	Villus contour Length	757 ± 33 *	692 ± 36 *	786 ± 47 *	
	Muscularis width	43 ± 2 *	42 ± 2 *	34 ± 2 ‡	
11	Villus height	354 ± 18 *	345 ± 34 *		
	Villus width	80 ± 2 *	63 ± 3 ‡		
	Crypt depth	117 ± 3 *	88 ± 5 ‡		
	Villus contour Length	732 ± 36 *	708 ± 69 *		
	Muscularis width	47 ± 4 *	34 ± 3 ‡		

Values of histological parameters are means ± SEM (micrometers). A minimum of ten consecutive villi and crypts were measured per animal and the mean used as the animal's value. Sample size ranged from 5-6 mice per treatment group per experimental day. *‡§ Values within a given parameter on the same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day –18. Chemotherapy was cystemustine (cyst) administered on day 0.

Figure 7. Morphological features of the small intestine during cancer cachexia and chemotherapy

Representative histological slides of the small intestine from healthy mice (H), healthy mice treated with cystemustine (HC), tumour bearing mice (T) and tumour bearing mice treated with cystemustine (TC) are shown. Slides at 200 fold magnification from day 2 illustrate the acute effects of chemotherapy; day 11 represents the chronic effect of cancer cachexia (2 wk after onset of cachexia). Jejunal segments were embedded in paraffin wax. Longitudinal sections were cut, fixed, and stained with hemotoxylin and eosin. Table 6 summarizes means values for morphological parameters.


H day 2
T day 2
HC day 2
TC day 2
H day 11
T day 11

DISCUSSION

This thesis showed for the first time that wasting of small intestinal protein mass occurs in tumour bearing mice as a result of a decrease in the rate of protein synthesis, independent of reductions in food intake. Acute toxicity of chemotherapy did not induce further wasting of small intestinal protein mass or alter histological parameters in tumour bearing mice. In contrast, chemotherapy did promote wasting of small intestinal protein mass in healthy mice and caused characteristic morphological damage to small intestinal crypts and villi. The rapid recovery of small intestinal protein mass, in mice cured of cancer, was the result of an increase in the rate of protein synthesis.

A. Cancer cachexia

Cancer cachexia is an abnormal metabolic syndrome resulting in wasting of host tissue. While numerous studies have shown wasting of skeletal muscle in both humans and animals (Baracos et al. 1995, Pisters & Pearlstone 1993, Temparis et al. 1994), the effect of cancer cachexia on the small intestine remains almost unknown. The small intestine serves one primary function: to provide a selectively permeable barrier between the exterior of the body and the internal tissues. Effectively, the intestine is responsible for allowing nutrients to enter the body while preventing entry of pathogens. Compromises to the integrity of the small intestine may impair these functions, thus limiting nutrient availability and rendering the body more susceptible to disease (Berg

1983, Potten 1995, Sandovsky-Losica et al. 1990, 1992). Very little is known concerning the extent to which wasting occurs in the small intestine during cancer cachexia.

Cancer cachexia caused wasting of small intestinal protein mass in colon 26 adenocarcinoma bearing mice. Atrophy of small intestinal protein mass occurred shortly after the onset of cachexia. Colon 26 adenocarcinoma induced a state of cachexia causing wasting of small intestinal protein mass, with a relatively small tumour burden (2-3% of body weight), and there was no atrophy detected in the small intestine from healthy pair-fed controls. In a study by Le Bricon et al. (1995b), small intestinal wet weight of Morris Hepatoma bearing rats, shortly after the onset of cachexia (11% tumour burden), was lower than in pair-fed rats. In a subsequent study, wasting of small intestinal protein mass was shown in rats bearing Morris Hepatoma 7777 but this time was complicated by a severe reduction in food intake (50%) and a large tumour burden (25%) (Le Bricon et al. 1996). It is difficult in the latter study to determine the extent to which wasting was the result of cachexia or reduced food intake.

Central to maintaining a functional small intestine is the protection of the mucosal layer. Compromises to the mucosal layer could impair digestive and immunological functions. Histological analysis of the small intestine indicated that cancer cachexia induced morphological damage. Most notably, villus width and crypt depth in tumour-bearing mice were lower than in healthy control mice. Villus and crypt atrophy was present both acutely and 2 wk following the onset of cachexia. To my knowledge, no other study has assessed the morphology of the small intestine during cancer cachexia. These results suggest that while protein mass is wasted, the intestinal barrier may not be seriously compromised during cancer cachexia. The difference in protein mass detected

between cachectic and healthy mice, therefore, cannot be fully explained by mucosal damage. Wasting of the muscularis in cachectic mice detected on days 2 and 11 would have contributed to a reduction in protein mass. The difference could also be explained in part by a reduction in small intestinal length, although length was not measured in the present study.

Chronic wasting of small intestinal protein mass did not occur. This is in contrast to skeletal muscle mass. Acute wasting of skeletal muscle mass in tumour bearing mice occurred (Appendix I) to the same extent as small intestinal protein mass. However, skeletal muscle mass continued to show evidence of wasting by the end of the experiment and was reduced by 40% 2 wk after the onset of cachexia (Appendix I). Chronic wasting of skeletal muscle mass is well established. Several clinical studies (Borzotta et al. 1987, Jeevanandam et al. 1984, Pisters & Pearlstone 1993) and other animal models of cancer cachexia (Baracos et al. 1995, Emery et al. 1984, Lopes et al. 1989, Smith & Tisdale 1993, Strelkov et al. 1989, Temparis et al. 1994) have indicated varying degrees of chronic wasting of skeletal muscle mass. The lack of chronic wasting of small intestinal protein mass suggests an adaptive conservation by the small intestine during cancer cachexia. Conservation of the small intestine relative to skeletal muscle has also been demonstrated in protein malnourished neonatal pigs (Ebner et al. 1994). Conservation of the small intestine during cancer cachexia is further suggested by the ratio of small intestinal protein mass to body weight. In tumour bearing mice, the ratio was similar to healthy mice acutely into the cachectic phase and higher at the end of the experiment. This indicates that other body tissues wasted to a greater extent than small intestine. Conservation could be very important to the host, but also to the tumour, since the small

intestine is a portal of entry for nutrients needed to meet the elevated metabolic demands for both the host and tumour.

B. Mechanisms of wasting

Protein mass in the small intestine is dependent on several factors. Protein accretion is the result of protein synthesis. Protein loss is dependent on cell sloughing, export protein secretion (systemic and luminal), and protein degradation (McBurney 1994). Wasting will occur when the rate of protein synthesis is lower than the combined rates of cell sloughing, export protein secretion, and proteolysis. While procedures for measuring the rate of protein synthesis in the small intestine are well established (Garlick et al. 1980), there are currently no methods available for measuring the rates of all the components contributing to protein loss.

This study was the first to measure the rate of protein synthesis in the small intestine during cancer cachexia. Reductions in the rate of protein synthesis were detected in the small intestine from tumour bearing mice at the very onset of cachexia. A decrease in the fractional rate of protein synthesis was present before any atrophy of small intestinal protein mass was detected. Therefore, acute wasting of small intestinal protein mass was likely the result of a decrease in the rate of protein synthesis. However, the rate of protein synthesis in this thesis was measured in whole small intestine, and it is not possible to define which cells in the small intestine are being most affected.

Protein degradation may also be involved in wasting of small intestinal protein mass. There are no methods available to measure the rate of protein degradation in the

small intestine. It is possible, however, to assess changes in the expression of mRNA encoding components of the major proteolytic systems in the small intestine through northern blot hybridization (Samuels et al. 1996). Treatment differences among relative levels of mRNA may suggest directional changes in protein degradation as well as indicate transcriptional regulation or modulation of mRNA catabolism. Changes in levels of mRNA have been shown to be associated with similar changes in the rate of proteolysis of skeletal muscle under a variety of conditions (Attaix et al. 1994). Since the rate of protein degradation in the small intestine remains unknown, it is difficult to determine the true value of relative changes in mRNA encoding components of the major proteolytic systems.

Overall, northern blot hybridization indicated that there was a down regulation of mRNA encoding proteases and/or subunits of the lysosomal and ATP-ubiquitin dependent proteolytic pathways in the small intestine from cachectic mice. If protein degradation had contributed to wasting of small intestinal protein mass in tumour bearing mice, mRNA levels would likely have been up-regulated. Therefore, northern blot hybridization suggests that proteolysis was unlikely to have contributed to wasting. Down regulation of mRNA may suggest a reduction in the rate of protein degradation consistent with protein conservation and a new steady state in homeostatic balance with the reduced rate of protein synthesis. Interestingly, during starvation, mRNA levels suggested an up-regulation of proteolysis (Samuels et al. 1996). This could indicate that while proteolysis may not have contributed to wasting during colon 26 adenocarcinoma induced cachexia, proteolysis could be up-regulated in other catabolic conditions.

The mechanism of wasting small intestinal protein mass during cancer cachexia through reduced protein synthesis may not occur in all types of cancer. Wasting of skeletal muscle during cancer cachexia has been shown to result from reduced rates of protein synthesis (Emery et al. 1984), increased rates of protein degradation (Temparis et al. 1994), or the combination of both (Baracos et al. 1995). Variability in the mechanism of wasting of skeletal muscle illustrates the necessity for measuring the rate of small intestinal protein synthesis in other tumour models to determine if the reduction in the rate of protein synthesis is unique to this model, or a mechanism common to cancer cachexia in general. In addition, new techniques need to be developed to accurately assess the importance of proteolysis within the small intestine.

C. Recovery from cancer cachexia following chemotherapy

The basic premise of all antineoplastic therapy is inhibition of cellular proliferation. Alkylating agents constitute one of the most common families of chemotherapeutic drugs and inhibit DNA synthesis directly, by forming covalent cross-links between complementary strands of DNA (Brent et al. 1987, Konopa 1988). Other commonly used drugs inhibit metabolic pathways involved in nucleotide synthesis (Cleton 1995). These drugs all have one element in common, the inhibition of DNA synthesis. Unfortunately, this mechanism is fairly non-specific causing chemotherapy to be cytotoxic to any rapidly dividing cells, including host tissue. Cytotoxicity resulting from chemotherapy seriously complicates treatment strategies and may impede recovery (Potten 1995, Wadler et al. 1998).

The small intestine is of particular interest when studying recovery from cancer cachexia following chemotherapy. This tissue is one of the most metabolically active tissues in the body. As a result, the small intestine may be particularly sensitive to non-specific cytotoxicity of chemotherapy. Clinical studies have reported several toxic side-effects related to the small intestine. Diarrhoea, impaired nutrient intake and in more extreme cases, septicaemia, are all symptoms associated with chemotherapy (Mercadante 1995, Sandovsky-Losica et al. 1990, 1992, Wadler et al. 1998). Several studies using healthy animals have shown morphological damage to crypts and villi as well as impaired immune function within the intestine following chemotherapy (Altmann 1974, Cozon et al. 1991, Kovacs et al. 1982, Moore 1984, Taminiau et al. 1980). Surprisingly, there have been almost no studies determining the effects of chemotherapy on the small intestine from tumour-bearing animals. The metabolic and hormonal milieu is very different during cancer (Tisdale 1997a, 1997b, 1993) and may affect homeostasis in the small intestine. Thus, the effect of chemotherapy on small intestinal metabolism and function needs to be addressed in the context of cancer cachexia.

Cystemustine is an experimental alkylating agent that cures murine colon 26 adenocarcinoma with 100% efficacy (Bourut et al. 1986, Madelmont 1994) and is currently being optimized for use in humans (Buchdahl et al. 1998, Debiton et al. 1997). Signs of acute cytotoxicity were present following treatment in both healthy and tumour bearing mice; body weights and food intakes were reduced. Surprisingly, chemotherapy did not induce further wasting of small intestinal protein mass in tumour bearing mice. This is in contrast to numerous studies reporting intestinal atrophy in healthy mice following chemotherapy (Altmann 1974, Cozon et al. 1991, Kovacs et al. 1982, Moore

1984, Taminiau et al. 1980). Indeed, healthy mice injected with cystemustine in my study showed significant reductions in small intestinal protein mass. It is possible that the reduced rate of protein synthesis in cachectic mice rendered the small intestine less susceptible to the cytotoxic effects of chemotherapy, which normally targets the rapidly proliferating intestinal tissue. This study has shown for the first time that the small intestine in a state of atrophy induced by cancer cachexia responds differently to chemotherapy than the normal, healthy small intestine. Since the overwhelming majority of research on the small intestine has been done in healthy animals, there is clearly a need to further define the effects of chemotherapy on the small intestine in cachectic animals.

It is well established that antineoplastic agents inadvertently target the epithelial lining of the small intestine in healthy animals. Atrophy is initiated by a reduction in crypt size and followed by a reduction in villus height resulting from the decreased proliferation in the crypts. Recovery from this state of atrophy is driven by crypt hyperplasia (Taminiau et al. 1980). Remarkably, in this study, chemotherapy did not alter morphological features of the small intestine in treated tumour bearing mice. Again, this is in direct contrast to the effects of chemotherapy on the small intestine from healthy mice. Severe compromises to villus height were detected in healthy mice treated with chemotherapy and crypt hyperplasia indicated previous cytotoxic damage to crypt depth. The lack of cytotoxicity in treated tumour bearing mice further indicates that the small intestine from cancer cachectic mice may in some way, be more resistant to chemotherapy than the healthy, normal small intestine.

Complete recovery of the small intestine requires repletion of protein mass. Rapid recovery of small intestinal protein mass did occur in colon 26 adenocarcinoma

bearing mice cured with cystemustine. In fact, protein mass was higher relative to healthy controls just 4 d following chemotherapy. Rapid recovery of small intestinal protein mass also occurred in healthy mice treated with cystemustine. In a study by Le Bricon et al. (1995a), three commonly used chemotherapeutic drugs induced hypertrophy in small intestinal weight 6 d following treatment in healthy rats indicating that the chemotherapy did not permanently impair small intestinal protein mass. Protein mass has also been shown to recover in rats bearing Morris Hepatoma 7777 following tumour excision (Le Bricon et al. 1994, 1995b). These studies illustrate the resilient nature of the small intestine and indicate that complete recovery occurs quickly. Furthermore, cytotoxicity due to chemotherapy did not impede full recovery of small intestine in cured mice.

Small intestinal protein mass recovered well before body mass and skeletal muscle mass. In fact, skeletal muscle mass had still not fully recovered by the end of the experiment (data not shown). Slower recovery of skeletal muscle mass compared with small intestine has also been reported during recovery from infection (Samuels & Baracos 1995). This may suggest that priority was given to recovery of the small intestine in order to facilitate the recovery of other tissues. Alternatively, recovery of body mass and skeletal muscle may have been limited by factors other than recovery of the small intestine.

D. Mechanisms of recovery

Chemotherapy, in tumour bearing mice, acutely reduced the rate of protein synthesis in the small intestine for just 1 d, but did not promote further wasting of small intestinal protein mass. Kaibara et al. (1994) also measured the rate of protein synthesis in the small intestine from tumour bearing rats following chemotherapy. High doses of mitomycin C were administered to rats bearing AH109A ascites hepatoma. The rate of protein synthesis was reduced compared to the rate in untreated tumour bearing rats.

The present study was the first study to investigate the mechanisms mediating recovery of the small intestine following chemotherapy during cancer cachexia. Small intestinal recovery was evident by the second day following chemotherapy; the rate of protein synthesis had risen to healthy levels and subsequently surpassed healthy values. This augmentation in the rate of protein synthesis indicated that recovery of small intestinal protein mass was driven primarily by an increase in the rate of protein synthesis, resulting in hypertrophy. Le Bricon (1997) measured the rate of protein synthesis in the small intestine 1 wk after cisplatinum treatment and no differences between healthy and tumour bearing rats were detected. Chemotherapy, however, in the study by Le Bricon (1997) was administered before onset of cancer cachexia and the rate of protein synthesis was not measured at any other time point. In another study by Le Bricon et al. (1995b), recovery of rat small intestinal protein mass following excision of Morris Hepatoma 7777 occurred through an increase in the rate of protein synthesis. More studies are required using different tumours and antineoplastic therapies to determine if there are common mechanisms regulating intestinal recovery.

The possible involvement of proteolysis in mediating recovery was assessed using northern blot hybridization. Levels of mRNA encoding proteases and/or subunits of the major proteolytic pathways in the small intestine of treated cachectic mice were essentially restored to normal. If protein degradation had enabled recovery of small intestinal protein mass, mRNA levels would likely have been down-regulated, suggesting that proteolysis was not involved during recovery. By the end of the experiment, normalization of the rate of protein synthesis and mRNA levels suggested that homeostasis of protein metabolism in small intestine of cured mice was not permanently affected by chemotherapy. It also illustrates that chemotherapy was capable of reversing the deleterious effects of cancer cachexia on small intestinal protein metabolism.

Recovery of skeletal muscle has been studied in colon 26 adenocarcinoma bearing mice cured with cystemustine (Temparis et al., personal communication). Partial recovery of skeletal muscle protein mass in cured mice involved restoration of the rate of protein synthesis to healthy levels, which was previously depressed during cancer cachexia, with a subsequent reduction in proteolysis. This indicates that mechanisms promoting recovery in the small intestine are distinct from those in skeletal muscle under the same conditions. Therefore, mechanisms of recovery must be established on a tissue basis, as different tissues may utilize different strategies to promote recovery.

FUTURE DIRECTIONS

Cancer remains one of the most common diseases in developed countries. Research must be continued in all directions of science to widen our understanding of this disease in an attempt to cure cancer. The present study has further defined the interactions among cancer cachexia, chemotherapy and small intestinal protein metabolism, but has also revealed many more areas that need to be explored.

Wasting of small intestinal protein mass was mediated through a reduced rate of protein synthesis in this tumour model. Other tumours have different characteristics in regard to the severity of induced anorexia and cachexia, the associated cytokine environment, and the release of cachexia inducing factors from the tumour itself (Tisdale 1997a, 1997c, 1998). These variations will affect host tissue differently and often in an unpredictable manner. The rate of protein synthesis needs to be measured in other tumour models to ascertain if altered intestinal protein synthesis is common during cancer cachexia. In addition, further research is necessary to identify the factors, secreted by host and tumour, responsible for initiating changes in small intestinal protein synthesis. There may exist, however, common elements among different types of cancer, which could be exploited and used to conserve host tissue (Baracos 1998, Fujiki et al. 1997, Tisdale 1997a). Elucidating the factors and mechanisms responsible for wasting in small intestine during cancer cachexia may be useful for designing strategies aimed at minimizing wasting. Conservation of host tissue during cancer greatly increases expected survival time and tolerance to antineoplastic therapy (DeWys et al. 1980, Kern & Norton 1988, Tisdale 1993).

A totally unexpected finding was that the small intestine in a state of atrophy was less susceptible to cytotoxic effects of cystemustine than the normal, healthy small intestine. The next phase of research will need to determine if these results are corroborated using different antineoplastic therapies. There is tremendous variability among cytotoxic responses from the various antineoplastic agents due to different mechanisms of action and effective doses. In addition, antineoplastic agents selectively target specific cells in the lower crypts that is somewhat distinct from one agent to the next (Ijiri & Potten 1983, 1987, Potten et al. 1992). It would be of extreme interest to establish if the small intestine is less susceptible to other antineoplastic therapies, not only during colon 26 adenocarcinoma induced cachexia, but also in other cancer cachexia models. Common mechanisms enabling this possible enhanced resistance in small intestine during cachexia may exist among different cachectic conditions. The reduced rate of protein synthesis in cachectic mice could be one factor, however, cell proliferation and other humoral and tumoural factors may also be involved. Studying the effects of different antineoplastic therapies on the small intestine in the context of cancer cachexia may provide novel insights into strategies for reducing the cytotoxic impact of antineoplastic therapy, thus promoting tissue conservation.

Rapid recovery of small intestinal protein mass resulted from an increase in the rate of protein synthesis. No other study to my knowledge has assessed the mechanisms regulating recovery of small intestine during cancer cachexia, following chemotherapy. Recovery needs to be addressed in other models with different tumours and antineoplastic therapies. Again, an understanding of the mechanisms and factors, which promote recovery of the small intestine, could offer direction for new treatment strategies.

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APPENDIX I

Effect of cancer cachexia and chemotherapy on gastrocnemius muscle mass

Day	Healthy	Tumour	Tumour + Cyst.	Healthy + Cyst.
-2	261.6 ± 7.8 *	255.0 ± 13.1 *		
1	261.0 ± 8.8 *		179.4 ± 7.1 ‡	
2	249.6 ± 8.4 *	174.7 ± 14.6 ‡	179.9 ± 11.3 ‡	258.1 ± 6.5 *
4	256.2 ± 7.7 *	178.7 ± 12.9 ‡	193.3 ± 9.3 ‡	250.3 ± 7.9 *
11	260.8 ± 5.9 *	152.9 ± 9.4 ‡	231.2 ± 12.4 §	

Values for gastrocnemius muscle mass are means ± SEM (mg) with n = 5 to 10 mice per mean (see Table 1). *‡§ Values on same experimental day with different superscripts are significantly different ($P<0.05$). Tumour was colon 26 adenocarcinoma transplanted on day -18. Chemotherapy was cystemustine (cyst) administered on day 0.