Liver and muscle protein metabolism in cachexia

The effects of nutritional interventions in preclinical models of cachexia

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Contents

Chapter 1

General Introduction

Up to 50% of cancer patients suffer from a progressive atrophy of adipose tissue and protein (skeletal muscle) reserves, resulting in loss of body weight (1). This process is part of a syndrome which is called cachexia or wasting syndrome. Anorexia often accompanies cachexia, but appears to be only partly responsible for the accelerated loss of, particularly lean, body mass (1). Cachexia is a complicated syndrome, regulated by different, mostly proinflammatory mediators like cytokines, catabolic hormones and prostaglandins (2-4). These are synthesized in the body in response to disease, infection or, in cancer, by tumor presence (2-4).

The most recent definition of cachexia has been described by Evans *et al.* (5) and has been defined at a special consensus conference about cachexia in Washington (DC) in 2008:

> *"Cachexia is a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass. The prominent clinical feature of cachexia is weight loss in adults (corrected for fluid retention) or growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity."*

If missed in treatment, cachexia inevitably leads to death due to catabolism of respiratory and cardiac muscle tissue, resulting in cardiac and respiratory failure. Due to these complications cachexia has been estimated to be responsible for more than 20% of overall deaths in cancer patients (6,7).

In cachexia, the loss of skeletal muscle protein is associated with an increase in the synthesis of acute phase proteins (APP) by the liver (8). The acute phase response is not mentioned in the definition of cachexia by Evans *et al.* (5), probably because the induced synthesis of APP is not directly correlated to increased morbidity and decreased survival in patients. Nevertheless, the acute phase response plays an important role in infection and the process

leading to cachexia. Proinflammatory cytokines play a prominent role in the genesis of cachexia, since they both induce the acute phase protein response in the liver and the induction of skeletal muscle protein breakdown (7). This introduction will give insight in the relationship between and the mechanisms leading to the acute phase protein (APP) response in the liver and the loss of skeletal muscle mass in cachexia. In addition, it will give a short summary of the possibilities of nutritional intervention in cachexia. The introduction will finish with an overview of the experiments that were carried out in this thesis. The influences of proinflammatory cytokines on both muscle and liver protein metabolism were investigated in different liver cell models and a skeletal muscle cell model. Finally, an *in vivo* model was used to investigate whether nutritional supplementation with the essential amino acid leucine can inhibit the cancer cachectic process in a severe cancer cachexia mouse model.

The acute phase response (APR)

In a 'normal inflammatory response', inflammation and trauma lead to the induction of inflammatory stimuli (*e.g.,* proinflammatory cytokines), that leads to a local response, inflammation, and, if severe enough, to a large number of accompanying systemic changes, referred to as the acute phase response (APR) (8,9). The APR aims to prevent ongoing tissue damage, to isolate and destroy the infectious organisms and to activate a repair process to restore the normal functions, *i.e.*, homeostasis, of the organism (10). The APR is characterized by leukocytosis, fever, anorexia, alterations in functions of many organs and changes in the plasma concentrations of various acute phase proteins (APP) (10-12). APP are synthesized almost exclusively by the liver and serve important functions in restoring homeostasis after infection or inflammation (10,13,14). These include haemostatic functions (*e.g.,* fibrinogen), microbiocidal and phagocytic functions (*e.g.,* complement components, C-reactive protein), anti-thrombotic properties (*e.g.*, α1-acid glycoprotein), and anti-proteolytic actions which are important to inhibit protease activity at sites of inflammation (*e.g.,* α2-macroglobulin, α1-antitrypsin, and α1-antichymotrypsin) (9). The acute phase protein response in the liver has been described as a re-prioritization of liver protein metabolism, where the available synthetic capacity and amino acid resources

are shifted from constitutive protein production (*e.g.*, albumin and transferrin) to increased APP production (9,15). The APR may be relatively transient, reverting to recovery. However, in certain circumstances, like chronic disease or cancer, the acute phase response can be rather persistent. In the face of significant tissue injury and infection, defense mechanisms must apparently take priority over the usual homeostatic states (13).

One would expect that the extent and quality of the APP response by the liver is dependent on the host's nutritional state and the severity of the infection. Severe malnutrition may affect the acute phase protein response by reducing the availability of precursors (amino acids) needed for acute phase protein synthesis (16,17). However, in severe malnutrition, patients can still mount an acute phase protein response (18). In malnutrition, however, the prolonged elevated synthesis of APP by the liver is associated with skeletal muscle protein breakdown, in which the protein pool in skeletal muscle serves as a reservoir of amino acids serving as substrates for the synthesis of acute phase proteins by the liver, leading to a negative protein balance in the body. This process is designated as cachexia (1,4,7,8).

Acute phase protein response and amino acid balance

One might explain weight loss associated with cancer cachexia as an imbalance in protein metabolism, in which priority of amino acid substrate delivery to the liver is preferred at the expense of skeletal muscle protein mass. During periods in which dietary supply is insufficient for the demand of APP synthesis by the liver, skeletal muscle protein may be mobilized to meet this demand. The unusual amino acid composition of acute phase proteins, in relation to that of skeletal muscle protein, may be relevant to our understanding of the mechanism of tissue wasting in cachexia. The imbalance in the amino acid composition of APP proteins and skeletal muscle protein may, thus, be detrimental to the body's nitrogen economy. Reeds *et al.* (19) have reviewed the theoretical amino acid exchange between skeletal muscle and the liver in a typical acute phase protein response. They estimated the quantities of amino acids incorporated into acute phase proteins during an acute phase protein response to uncomplicated surgery in adult humans and compared

these with the amino acid composition of skeletal muscle (see Table 1). If assumed that skeletal muscle is the sole source of amino acids for APP synthesis, the excess of amino acid release of the muscle over amino acid incorporation in acute phase proteins could be calculated (see Table 1). From these calculations Reeds *et al*. concluded that a significant proportion of the net loss of body nitrogen could be the result of the excessive demands for aromatic amino acids used in the synthesis of acute phase proteins, with phenylalanine being the first 'limiting' amino acid. From Table 1 the calculation has been made that the phenylalanine content of 850 mg of the mixture of acute phase proteins represents the quantity of 1980 mg of muscle protein. Hence, for each gram of acute phase proteins produced, 2.3 g of muscle protein has to be broken down. The surplus of the other (essential) amino acids, released by the muscle, cannot be incorporated in other APP (since the essential amino acid phenylalanine is limiting), hence, they will probably be catabolized (19). Table 1 does deliberately not correct for eventual sparing of amino acids due to in cancer cachexia observed hypoalbuminemia, since this does not appear to be a result from a decreased hepatic albumin synthesis (20,21). Plasma phenylalanine concentrations, however, do not appear to decrease in fasted cancer patients with an acute phase response. The only decrease of essential amino acids that is observed in these patients is that of tryptophan (18). Tryptophan is the second limiting skeletal muscle amino acid for acute phase protein synthesis according to the article of Reeds *et al.* (19). If the same calculation will be carried out as described above with tryptophan being the 'limiting' amino acid for APP synthesis, then the calculation leads to the conclusion that 2.6 gram of skeletal muscle has to be broken down for 1 gram of fibrinogen synthesis (18).

The theoretical approach of Reeds *et al.* (19) does not seem to be in accordance with the observed plasma amino acid profiles found in patients with an acute phase protein response. This could be due to the fact that the acute phase proteins, in turn, will be degraded to their subsequent amino acids. For example, fibrinogen can be degraded by different protease pathways, resulting in the release of its amino acids in the plasma (22,23). Thus, the levels of the plasma amino acids will not solely depend on the interaction between the amino acid pools of muscle and acute phase proteins, but also depend on the

turnover of the acute phase proteins themselves. Moreover, the substrate need for amino acids by the liver is not only dictated by the acute phase protein synthesis in patients with severe metabolic stress. Also the substrate needs for glutathione synthesis and gluconeogenesis have to be considered.

Table 1. Estimates of the quantities of amino acids incorporated into acute-phase proteins during a "typical" acute response to uncomplicated surgery in adult humans[1]

	Incorporated into acute phase proteins	Muscle protein equivalent To the acute-phase incorporation[2]	Excess of release over incorporation[3]	
	mg amino acid/ kg body wt	g protein/ kg body wt	Amino acids mg/kg	Nitrogen mg/kg
Leu	89	1.09	72	8
Ile	54	1.12	41	4
Val	67	1.24	40	5
Lys	90	0.92	104	20
His	33	0.64	68	11
Phe	79	1.98	0	0
Tyr	55	1.53	16	1
Trp	24	1.85	2	<1
Met	23	0.91	26	2
Cys	14	1.08	12	1
Thr	65	1.38	28	4
Arg	54	0.78	83	28
Pro	50	1.32	25	3
Gly	50	1.11	39	7
Ser	70	1.70	11	1
Ala	51	0.86	66	10
Asx[4]	121	1.32	61	12
Glx[4]	147	1.01	140	15

[1]assuming that the typical acute phase response consists of an increase (mg/kg body wt) of C-reactive protein (250), fibrinogen (200), α1 acid glycoprotein (50), α1-antitrypsin (200), haptoglobin (50), amyloid A (100).

[2]the quantity of muscle protein containing the amount of each amino acid that is incorporated into the mixture acute phase proteins.

[3]the difference between the quantity of each amino acid mobilized in 1.98 g muscle protein (i.e., the amount of muscle protein containing 79 mg Phe) and the quantity incorporated into the acute-phase proteins.

[4]ASX= aspartate + asparagine; GLX= glutamate + glutamine.

Glutathione is the tripeptide γ-glutamyl-cysteinyl-glycine (GSH) and is an endogenous reducing agent and antioxidant (24-26). Glutathione synthesis occurs in virtually all cells, but mainly in the liver (24). The major determinants for glutathione synthesis are the availability of cysteine, the sulfur amino acid precursors, and the activity of the rate limiting enzyme γ-glutamyl-cysteine synthetase (GCS) (26). The availability of cysteine in the liver is regulated by several factors, including diet, membrane transport activities of the three sulfur amino acids cysteine, cystine and methionine, and the conversion of methionine to cysteine via the transsulfuration pathway (27). Of course, also the glutamine levels can be limiting in glutathione synthesis by the liver. The liver appears to be the major organ of glutamine uptake in severe infection; studies in endotoxemic rodents have shown net hepatic glutamine uptake to increase by as much as 8 to 10-fold (28). Experiments in rats under inflammatory conditions have shown that hepatic synthesis of glutathione may account for 60% of hepatic glutamine uptake, which will lead to the loss of amide nitrogen (29). In these rats, the induction of an inflammatory response increases hepatic glutathione production by the equivalent of 60 mg N [per kg body wt per day], a value which, if transferred to humans, would represent about a third of the nitrogen loss following uncomplicated surgery (19). Also in humans decrease in plasma glutamine has been observed in severe illnesses and, therefore, glutamine supplementation has been suggested to reverse catabolic states (30,31).

Skeletal muscle, the major repository of glutamine, exhibits a two-fold increase in glutamine release during infection, which is associated with a significant increase in endogenous glutamine biosynthesis (28). Despite an increase in glutamine synthetase activity in skeletal muscle, the intracellular glutamine pool becomes depleted, indicating that release rates exceed rates of synthesis. Simultaneously, the circulating pool of glutamine does not increase, indicating accelerated uptake by other organs. The liver appears to be the major organ of glutamine uptake in severe infection (28). In addition to serving as a substrate for glutathione synthesis in the liver, glutamine is also a gluconeogenic precursor. Overall, gluconeogenesis from glutamine is responsible for ~5% of systemic glucose and renal production of glucose

from glutamine accounts for approximately 75% of all glucose produced from glutamine (32).

In conclusion, taking the above mentioned amino acid imbalance in cancer cachexia into consideration, it seems logical to intervene in the cachectic process by nutritional intervention focused on restoring protein balance by either supporting acute phase protein synthesis in the liver or by supporting skeletal muscle protein synthesis in order to decrease skeletal muscle protein breakdown. In focusing on the skeletal muscle, the first-pass extraction of amino acids for the increased demand for APP and glutathione synthesis by the liver should be taken into account. This means that sufficient nutritional protein (amino acids) should be supplied to increase the plasma amino acid concentrations which are necessary for skeletal muscle protein synthesis (33).

Nutritional intervention in cachexia

Poor nutritional status, often seen in cachectic patients, worsens the net whole body protein state, resulting in a decline in the quality of life, eventually resulting in death through erosion of skeletal and respiratory muscles (1,7,8). Therefore, the aim of nutritional intervention in cachectic patients should be to prevent weight loss and to improve functional capacity and quality of life (1,7,8). The interventions should be focused, primarily on maintaining protein balance and, more specifically, skeletal muscle protein balance. To compose an optimal nutrition from a substrate point of view, the effects of the substrates on both the liver and skeletal muscle in the cachectic state have to be considered. Firstly, regarding the amino acid balance between liver protein synthesis and skeletal muscle protein breakdown, aromatic amino acids appear to be limiting (18,19). However, since the real complications in cancer cachexia are associated with loss of skeletal muscle protein mass, the muscle should be the primary organ to focus on with the nutrition. Previous research has proven that, at least in healthy individuals, only essential amino acids are primarily responsible in skeletal muscle protein growth (34,35). A study by Paddon-Jones *et al.* (36) shows that essential amino acid ingestion improved muscle protein anabolism during acute hypercortisolemia and may help minimizing muscle loss following debilitating injury, implying that during

stress situations, indeed an essential amino acid product could delay the catabolic response of the muscle. The effects of this composition have not been studied in cachectic patients yet. It has to be kept in mind that cachectic patients will not always respond well to nutrition, since these patients seem to be less sensitive for anabolic signals such as insulin (37-39). Indeed, clinical studies in cachexia patients have continued to demonstrate that it is very hard to obtain an effect on the loss of muscle weight by protein supplementation (40). Therefore, more insight is needed in the role of cytokines and hormones involved in the induction of cachexia, since these seem to play a role in the decreased anabolic response of the muscle to amino acids.

Cytokines and hormones involved in cachexia

In cachectic patients, a complex interrelation of the uncontrolled actions of pro-inflammatory cytokines (IL-6. IL-1β, TNFα, and IFN-γ) (3,41,42), catabolic and anabolic hormones (synthesized in response to *e.g.* stress and meal consumption) (43,44), prostaglandins (41,45), and actions of proteolysis inducing factor (PIF) (44,46), is able to induce a muscle wasting state of the body together with an 'anabolic' effect on the liver by inducing acute phase protein synthesis (4). An overview of the main cachectic mediators is shown in Table 2. Hence, skeletal muscle wasting is not caused simply by a shortage of amino acids needed for acute phase protein synthesis, but the main catabolic signal for the skeletal muscle consists of an orchestra of different catabolic, proinflammatory mediators. These are seemingly the same mediators that induce acute phase protein synthesis in the liver (Figure 1). Meal consumption also functions to induce an up-regulation of hormone secretion and has a profound effect on cytokine production, leading to net protein anabolism under normal circumstances (47-49). The effects of meal consumption with regard to hormone synthesis, cytokine production and other cachectic mediator production will be discussed below, and an extensive overview of their effects is given in Table 2.

Table 2. Roles of hormones and cytokines in cachexia

Mediator	Meal consumption	Overall action	Action on liver	Action on muscle
PGE-2		Up-regulation of adrenalin and corticosterone (43) Promotes: tumor cell survival, tumor cell apoptosis, and tumor cell proliferation (72)	Indirect (via adrenalin and corticosteron) upregulation of IL-6 (43) Indirect induction (via IL-6) of acute phase protein response (43)	Stimulates protein degradation (41, 45)
15-HETE				Stimulates protein degradation (41, 45)
TNFα		Involved in insulin resistance (43, 48) Induces cytokine production (42)	Induction of IL-6 synthesis and inducer of acute phase protein synthesis (43, 48)	Promotes catabolism in mature muscle cells *in vitro* (41) Stimulates protein loss via NF-κB (42) Increases glycogen breakdown in myotubes (43, 48)
IL-6		Activates STAT-3 (46)	Primary cytokine in full acute phase protein response (16,41, 73)	Plays role in muscle proteolysis (41)
IL-1β	There is an inverse association between IL1-β production and dietary intake (49)		Induction of synthesis of only few acute phase proteins (43, 73) Induction of IL-6 synthesis, amplifying the acute phase response (43,73)	Stimulates protein degradation in intact muscle (41)
IFN-γ		Activates macrophages (41,74)		Plays a role in muscle proteolysis (41)

Mediator	Meal consumption	Overall action	Action on liver	Action on muscle
PIF		Activates STAT-3 faster than does IL-6 (46) Induces increased adhesion molecule production (41) Induces release of arachidonic acid (which is metabolised to PGE2 and 15-HETE) (41,45)	Regulates hepatic gene expression via NF-κB and STAT-3 (46) Induces acute phase protein response (41)	Induces muscle atrophy, as co-factor to cytokines (44, 46)
CTNF			Increases acute phase protein response (41)	Induces lean tissue wasting (41)
Gluco-corticoids		Decreases insulin level (44)	Attenuates IL-6, IL-1β, and TNFα action in acute phase protein response (43,44,73-77)	Attenuates skeletal muscle atrophy (19,43,44,73)
Glucagon	Down-regulated by meal consumption (47)		Amplifies acute phase protein response (44,75-77)	Involved in cachectic process (77)
IGF-1	Up-regulated by meal consumption (47)			Increases protein synthesis (47)
Insulin	Up-regulated by meal consumption (47)			Decreases protein breakdown (47)

CTNF ciliary neurotrophic factor; 15-HETE 15-hydroxyeicosatertraenoic acid; IGF-1 insulin-like growth factor; IL-1β Interleukin-1β; IL-6 Interleukin-6; PGE-2 prostaglandin E2; PIF proteolysis inducing growth factor; TNFα tumor necrosis factor-α

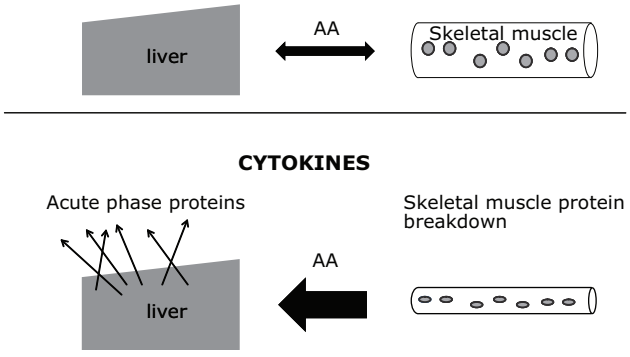

Figure 1. Role of cytokines in protein metabolism in cachexia. In homeostasis there is an equilibrium in protein metabolism in the body between the liver and skeletal muscle. However, during inflammation, among other inflammatory mediators, cytokines induce both acute phase protein synthesis in the liver and skeletal muscle protein breakdown (lower part of the figure). Skeletal muscle protein breakdown seems to serve for providing amino acids (AA) for acute phase protein synthesis in the liver. The net effect of cytokines is a negative protein balance in the skeletal muscle, leading to decreased volume and mass, and ultimately in decreased function of muscles.

Nutritional influence on hormonal balance in cachexia

Hormones are important regulators of protein turnover. In cachexia, the various hormonal concentrations in blood plasma, with their mutual interactions, and interactions with other cachectic mediators, play pivotal roles in the metabolic derangements (47) This results in loss of sensitivity to anabolic signals like insulin and, subsequently, in protein catabolism (1,7,50,51). Nutrition itself also induces an accelerated hormone secretion, resulting in a net anabolic period of time (postprandial phase) which is important in regulating the body's protein balance (47). The metabolic actions of the various individual hormones on protein metabolism, and the hormonal response to meal consumption are summarized in Table 2. Anabolic hormones stimulate human muscle growth mainly by increasing protein synthesis (growth hormone (GH), insulin-like growth factor-1 (IGF-1)) or by decreasing protein breakdown (insulin) (Table 2). A combination of stress hormones (glucagon, glucocorticoids, and

catecholamines) on the other hand, play a role in the induction of muscle wasting (41,52-54). The catabolic stress hormones are elevated in clinically inflammatory conditions and are thought to be involved in the net protein catabolism observed under these conditions. However, catabolic hormones contribute, but are not the sole mediators of the catabolic response during disease (47).

Another aspect of the catabolic response in skeletal muscle is the decreased sensitivity to the anabolic effects on protein metabolism of insulin. Insulin plays dual roles in mounting anabolism in skeletal muscle protein metabolism. If enough amino acids are available, insulin plays an important part of mounting an anabolic response in the skeletal muscle (35,55-60). If no amino acids are available, insulin still has an effect on skeletal muscle protein metabolism, namely inhibiting protein breakdown. Hence, insulin's main anabolic effect on muscle protein is not only by increasing protein synthesis, but also by inhibition of muscle protein breakdown (47,61). The problem in cancer cachexia is that the sensitivity of the muscle to the net anabolic effects of insulin is decreased, leading to catabolism (4,5,62-64).

In conclusion, nutrition *per se* induces an anabolic environment by providing nutrients and indirectly by inducing the production of anabolic hormones which results, under normal circumstances, in protein anabolism and skeletal muscle protein accretion. Catabolic signalling, induced in cancer cachexia, predominates the anabolic effects of nutrition and, in addition, decreases the sensitivity of skeletal muscle to anabolic signals, leading to net skeletal muscle protein breakdown.

Summary

Cachexia is a complicated process, regulated by many inflammatory mediators, synthesized in the body in response to disease. Several endocrine and cytokine mediators interact in the body, to induce a central anabolic (liver APP synthesis) and peripheral catabolic (skeletal muscle protein breakdown) response in cachectic patients. This process can be compared to a circulatory shock when, with respect to blood supply, only the vital organs in the body will

be supplied after severe injury. Actually, exactly the same occurs in protein balance in cachexia. In inflammation and injury recovery from the stress event is essential for survival. This results in the secretion of acute phase proteins by the liver. This APP synthesis by the liver has priority to protein synthesis in the skeletal muscle, resulting in muscle wasting. So, like in circulatory shock, also in cachexia, the priority of protein metabolism shifts to vital organs needed for survival after stress, namely in the case of cachexia the liver.

Because of the wasting of skeletal muscle in cachexia, the quality of life deteriorates for the patient and often results in an early death (1,7,8). The cachectic syndrome involves an increase in liver acute phase protein synthesis, directly associated with a decrease in muscle mass. Therefore, loss in muscle strength, fatigue, will eventually lead to loss in efficiency of respiratory muscle with hyperventilation, respiratory failure, increased risk of pulmonary infections and decrease in heart contractility with decreased ejection fraction and cardiac pump failure (65). Cachexia is encountered in many malignant and nonmalignant chronic, ultimately fatal, illnesses. This is due to the complexity of the cachectic process (66). Cachexia is associated with exceedingly high mortality once the syndrome has fully developed. It is not simply an ancillary event. Limited studies in humans suggest that targeted antiinflammatory interventions may ameliorate the catabolic response of the skeletal muscle (67). The therapeutic and nutritional standard of care for cachexia remains undefined to date. Among the recognized approaches, nutritional interventions appear promising, but much more research is needed to obtain the optimal nutrition for these patients. Therefore, further research efforts are warranted to gain more insight in the mechanisms leading to cancer cachexia (68).

This thesis

In this thesis special emphasis is given to the use and evaluation of different *in vitro* cell culture techniques to study the process leading to cachexia. In the first part of this thesis (chapters 2, 3 and 4) the focus will be on the acute phase protein response of the liver in cancer. The most commonly used *in vitro* model to study acute phase protein metabolism is the human HepG2

liver cell line. Two aspects of the acute phase protein response were studied in this cell line, namely the effects of different cytokines and hormones on the induction of acute phase protein synthesis on the one hand and the effects of substrate delivery (amino acids) on the synthesis of acute phase proteins on the other hand (Chapter 2). We found that secretion of acute phase proteins from this cell line is a result from interactions of combinations of cytokines and hormones. The observations suggest that the acute phase protein response is not as general as it was assumed but that it depends on the nature of the cytokines that are produced at the site of inflammation. In addition, we found that not only essential amino acids were needed to mount an acute phase protein response, but that also three nonessential amino acids, namely cysteine, serine and arginine were essential in the HepG2 model.

In chapter 3 the effects of IL-6 are described on fibrinogen and albumin synthesis in primary rat hepatocytes. This study was carried out to determine if the effects found in the HepG2 cells can be translated to primary rat hepatocytes and *vice versa*. Different primary hepatocyte cell cultures were compared for this purpose, namely a conventional monolayer primary hepatocyte cell culture, a coculture of primary hepatocytes with rat liver epithelial cells and a collagen sandwich cell culture. It was found that upon IL-6 stimulation fibrinogen secretion is increased in all cell cultures and that albumin secretion is decreased in primary cells, in contrast to HepG2 cells. Moreover, both basal and IL-6 induced fibrinogen synthesis were much higher in the co-cultures of hepatocytes with rat liver epithelial cells.

In the primary hepatocyte cell cultures, it was found that arginine was depleted in the cell culture supernatants (Chapter 4). This was due to arginase leakage from, probably broken cells, into the media. This resulted in total depletion of arginine in the media, making the *ex vivo* system not the best model to study the effects of amino acid composition on acute phase protein synthesis. In addition, it was shown that some methods using urea synthesis as viability parameter in primary hepatocyte cultures needs to be re-evaluated (Chapter 4).

Since both the liver and skeletal muscle are involved in the cachectic process, also the effects of cytokines on a skeletal muscle model were investigated (Chapter 5). For this purpose the C2C12 mouse skeletal muscle cell line was used. Muscle atrophy occurs at different levels, starting from repressed gene expression and ending with accelerated protein degradation (4). In the C2C12 model it was found that, like in the liver, the response of the skeletal muscle cells depend on the nature of the cytokines and the concentrations in which they were present in the media. The most important conclusion from that part of the thesis is that, from a concept point of view, cachexia should not solely be interpreted as a negative protein balance *per se*. When combining all results from our model and models used in literature, it was found that muscle catabolism can also be described by a decrease of number of nuclei in skeletal muscle fibers.

Finally, the *in vivo* C26 cancer cachexia mouse model was used to investigate whether it is possible to intervene with the cachectic process with leucine-enriched nutrition (Chapter 6). In this model, the effects of different quantities of leucine on skeletal muscle catabolism were tested. Leucine is known to have the propensity to mount an anabolic effect on skeletal muscle protein synthesis, in addition to the anabolic effect of insulin (69-71). We found a skeletal muscle specific anti-catabolic effect of leucine in cancer cachectic mice. Although anabolic effects were found on the skeletal muscles of the mice, no effects were found on body mass of the mice.

The thesis concludes with a general discussion in which all results are discussed and recommendations are given for future research (Chapter 7). Recommendations are given with respect to the findings in the models used in this thesis. But more importantly, recommendations are given for composing better nutritional products for cancer cachexia patients. These concepts should be further evaluated and repeated in the described cachectic mouse model and, subsequently, tested in a clinical setting in cancer cachectic patients.

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Chapter 2

Availability of essential amino acids, plus serine, cysteine and arginine determines the magnitude of acute phase protein production of HepG2 cells

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Abstract

The acute phase response is a reaction to inflammation and is characterised by an increased secretion of different acute phase proteins (APP) by the liver. APP play a role in restoring homeostasis. Different aspects of APP synthesis have been investigated in the human HepG2 liver cell line. The effects of different cytokines, hormones and amino acids on APP secretions were investigated. It was found that cytokine- and hormone-induced secretion of two positive acute phase proteins, haptoglobin and fibrinogen, is regulated differently. In addition, transferrin acted as a negative APP while no effects were found on albumin secretion. In the second part of the study, effects of amino acid quantity and quality were investigated. Firstly, amino acid consumption from the media was determined. From the essential amino acids, leucine, isoleucine and valine were consumed to a high extend. In addition, serine, arginine and cysteine were found to be essential amino acids for the production of acute phase proteins in HepG2 cells. In conclusion, besides the inflammatory mediators, both the amino acid concentration and the amino acid composition in the cell culture media are independent regulators of acute phase protein synthesis in HepG2 cells. The results from this study in combination with findings that malnutrition can affect the acute phase protein response *in vivo,* suggest that nutrition and the amino acid composition of the protein content, indeed, are determinants of protein synthesis in the liver. Further research is warranted to determine whether these *in vitro* results apply to the *in vivo* situation and may lead to optimal nutrition for patients with a metabolic imbalance in nitrogen distribution.

Introduction

Mammals respond to inflammation and tissue damage by implementing the acute phase response, which comprises a series of specific physiological reactions, including the induction the synthesis of acute phase proteins by the liver (1). The acute phase protein response has been described as a reprioritization of liver protein synthesis, where the available synthetic capacity and amino acid resources are shifted from constitutive protein production (*e.g.,* albumin and transferrin) to increased acute phase protein (APP) production (*e.g,.* fibrinogen, haptoglobin and C-reactive protein (CRP)) (2,3). The latter ones are designated as positive acute phase proteins and play a role in restoring homeostasis (1,2). The synthesis of acute phase proteins is predominantly regulated by interleukin-6 (IL-6) and interleukin-1ß (IL-1ß) (4-6). Consequently, the classifications are made into type I and type II acute phase proteins. Type I acute phase proteins (*e.g.*, CRP, serum amyloid A and α_2 acid glycoprotein), *in vivo*, are maximally induced by the synergistic action of IL-1ß and IL-6, whereas type II proteins (*e.g.,* fibrinogen, haptoglobin and α_2-macroglobulin) are maximally induced by IL-6 alone (1,7,8). In addition, glucocorticoids are able to directly stimulate the synthesis of some acute phase proteins, but, the main action of glucocorticoids is to enhance the effects of IL-1ß and/or IL-6 in a synergistic way (9). Thus, different signalling pathways are involved during an acute phase protein response. This suggests that there is a different response to inflammation, dependent on which cytokines or hormones are secreted during the inflammatory response.

The human HepG2 cell line is capable to produce various APP (10,11). The first part of the study was carried out to test the nature of the APP response in HepG2 cells. Therefore, the effects of different cytokines and hormones on APP secretion of the HepG2 cells were investigated. In the second part of the study, the effects of either the amino acid concentration or composition of the media on the acute phase protein response were studied. For this purpose, the secretion of fibrinogen and albumin were chosen to investigate, since these are, from a quantitative point of view, the most important APP of the HepG2 model. In addition, the relationship between amino acid consumption of the hepatocytes and the effects of amino acid concentration in the media are discussed with respect to APP production of the HepG2 cells.

Materials and Methods

Cells

Human hepatoma HepG2 cells (ATCC HB-8065) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Breda, the Netherlands) with 2 mM L-glutamine and streptomycin/penicillin in Costar (Corning-Costar, Schiphol-Rijk, the Netherlands) T75 flasks at 37°C in a humidified atmosphere of 95% air-5% CO_2. All experiments were carried out in 6-well Costar plates.

Cytokines and reagents

Human IL-6, human tumour necrosis factora (TNFα), and human IL-1β were obtained from Sanguin CLB (Amsterdam, the Netherlands). Human insulin and dexamethasone were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands).

Incubations

HepG2 cells were grown in 6-well plates to confluence, washed with DMEM and subsequently incubated during 24 hours with cytokines and/or hormones in serum-free media. After incubation, the supernatants were collected and stored at –20°C until analysis. The cells were washed with phosphate-buffered saline (PBS) and lysed with 0.1 M NaOH. The protein content was determined using the Bio-Rad Protein assay (Dye Reagent Concentrate, Bio-Rad Laboratories, Inc., Hercules CA, USA) using bovine albumin (Sigma Aldrich) as standard. The data are presented as mg acute phase protein per milligram protein cell content. RPMI-1640 media was used to determine the effects of omission of individual amino acids. An RPMI-1640 Select Amine kit was provided by Life Technologies, Breda, the Netherlands. The kit consisted of all cell culture media components, including all amino acids and, thus, media could be prepared with omissions of individual amino acids. All omissions of amino acids were isonitrogenously compensated for to a total amino acid concentration of 6 mM.

Antibodies

Rabbit anti-human fibrinogen and horse-radish peroxidase (HRP)-conjugated rabbit anti-human fibrinogen antibodies were obtained from DakoCytomation

(Glostrup, Denmark). Monoclonal anti-human haptoglobin antibody (clone HG-36, mouse ascites fluid) was obtained from Sigma Aldrich. Polyclonal sheep anti-human haptoglobin, anti-human albumin, HRP-conjugated anti-human albumin, and rabbit anti sheep antibodies were obtained from Dade Behring (Leusden, the Netherlands). Anti human transferrin, HRP-conjugated anti-human transferrin, sheep anti-human CRP, and HRP-conjugated sheep anti-human CRP antibodies were obtained from Biogenesis (Poole, England).

Antigens

Human fibrinogen, human haptoglobin, human albumin, human transferrin, and human CRP were obtained from Sigma Aldrich.

ELISAs

CRP/albumin/fibrinogen/transferrin: 96-well flat-bottom Costar EIA/RIA plates were coated overnight with primary antibody in PBS at predetermined optimal concentrations. After each incubation step, the plates were washed with 0.1% Tween-20 (Merck Eurolab, Roden, the Netherlands) in PBS. After washing, the plates were blocked with 5 % Protifar (Nutricia, Zoetermeer, the Netherlands) in PBS during 90 min. Subsequently, the samples and antigen were incubated in 0.1% Tween-20 in PBS during 90 min. Next, the plates were incubated with HRP-conjugated antibodies in 0.1% Tween-20 in PBS during 90 min. The colorimetric reaction was carried out with undiluted 1-Step Ultra TMB-ELISA (Pierce, Rockland, IL, USA). The reaction was stopped with 2 M sulphuric acid. The absorbances of the samples were measured at l = 450 nm. For the haptoglobin ELISA, the primary antibody was monoclonal anti-human haptoglobin, the secondary antibody was polyclonal antibody to human haptoglobin and the labelling antibody was HRP-conjugated rabbit anti-sheep antibody.

Amino acid analysis

The amino acid concentrations in the media were determined with HPLC, using *ortho*-phtaldialdehyde as derivatization reagent and L-norvaline as internal standard. The method was adapted from van Eijk *et al*. (12).

Statistics

To determine significant differences between values, multiple pairwise comparisons were conducted with Student's *t*-test. P-values below 0.05 were interpreted as statistically significant.

Results

Figures 1A and 1B show the fibrinogen and haptoglobin secretion in the cell culture supernatants as a function of IL-6 concentration in the media. The secretions of both proteins reached highest levels at IL-6 concentration of 10 ng/ml in the media. It was therefore decided to use IL-6 at a concentration of 10 ng/ml for incubations to study the effects of other cytokines and hormones on IL-6 induced APP secretion of the HepG2 cells.

The effects of the cytokines IL-1β and TNFα, and the hormones insulin and dexamethasone on basal and IL-6 induced fibrinogen secretion are shown in Figure 2A. IL-1β inhibited the inducible effect of IL-6 on fibrinogen secretion by approximately 25% ($p<0.001$), both in the presence or absence of additional supplementation with TNFα. When added in combination with IL-6, insulin had an inhibitory effect (-50%) ($p<0.01$), and dexamethasone enhanced the effect (+20%) of IL-6 induced fibrinogen secretion ($p<0.001$).

TNFα alone had an inhibitory effect (-75%) on basal haptoglobin (Figure 2B) secretion ($P<0.05$). However, no effect of TNFα was observed when added with IL-6. In addition, IL-1β induced (+75%) haptoglobin secretion ($p<0.05$). In contrast, IL-1β inhibited (-20%) the IL-6 induced haptoglobin secretion ($p<0.05$). Insulin had an inhibitory effect (-55%) on IL-6 induced haptoglobin secretion ($p<0.01$).

IL-6 alone had no significant effect on transferrin secretion (Figure 2C). Under all conditions, IL-1β decreased transferrin secretion by approximately 30%. IL-6 together with dexamethasone induced (+40%) the secretion of transferrin ($p<0.05$). Insulin alone induced (+50%) the secretion of transferrin ($p<0.05$). This effect was attenuated by IL-6 ($p<0.05$).

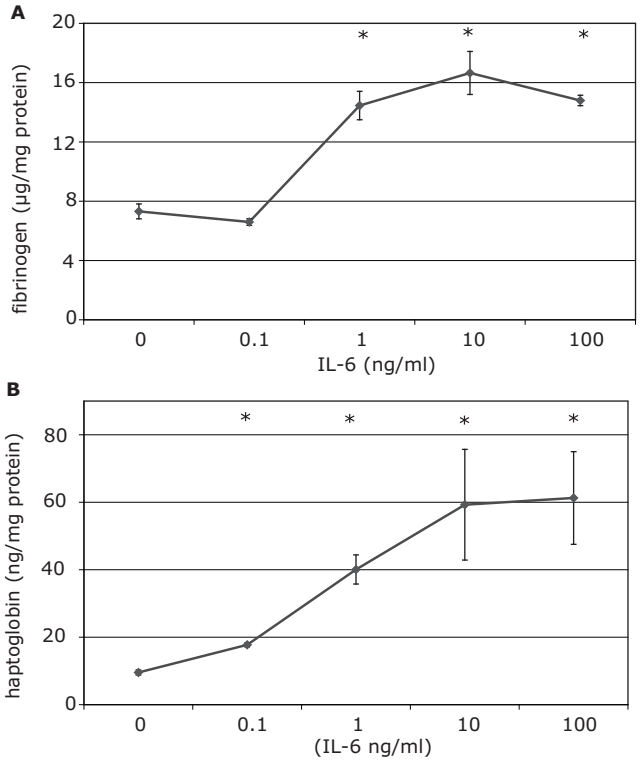

Figure 1. Fibrinogen (µg/mg protein 24-h) (A) and haptoglobin (ng/mg protein 24-h) (B) secretion by HepG2 cells as function of interleukin-6 (IL-6) concentration in the cell culture media. Each data point represents mean ± STD (n=6) (*p<0.01 versus IL-6=0 ng/ml).

The HepG2 cells did not produce detectable amounts of CRP. Albumin was secreted by the HepG2 cells at a rate of approximately 1.5 mg/mg protein per 24-h. There were no significant effects on albumin secretion observed after any of the (mixtures) cytokines or hormones.

The amino acid consumption of the HepG2 cells from the media is shown in Table 1. After a 24-h incubation in FCS free media, a decrease in concentration in the cell culture supernatants was observed of aspartate, glutamate, alanine, valine, leucine and isoleucine and a significant increase was observed of glutamate. No significant differences of other amino acids were observed in the media.

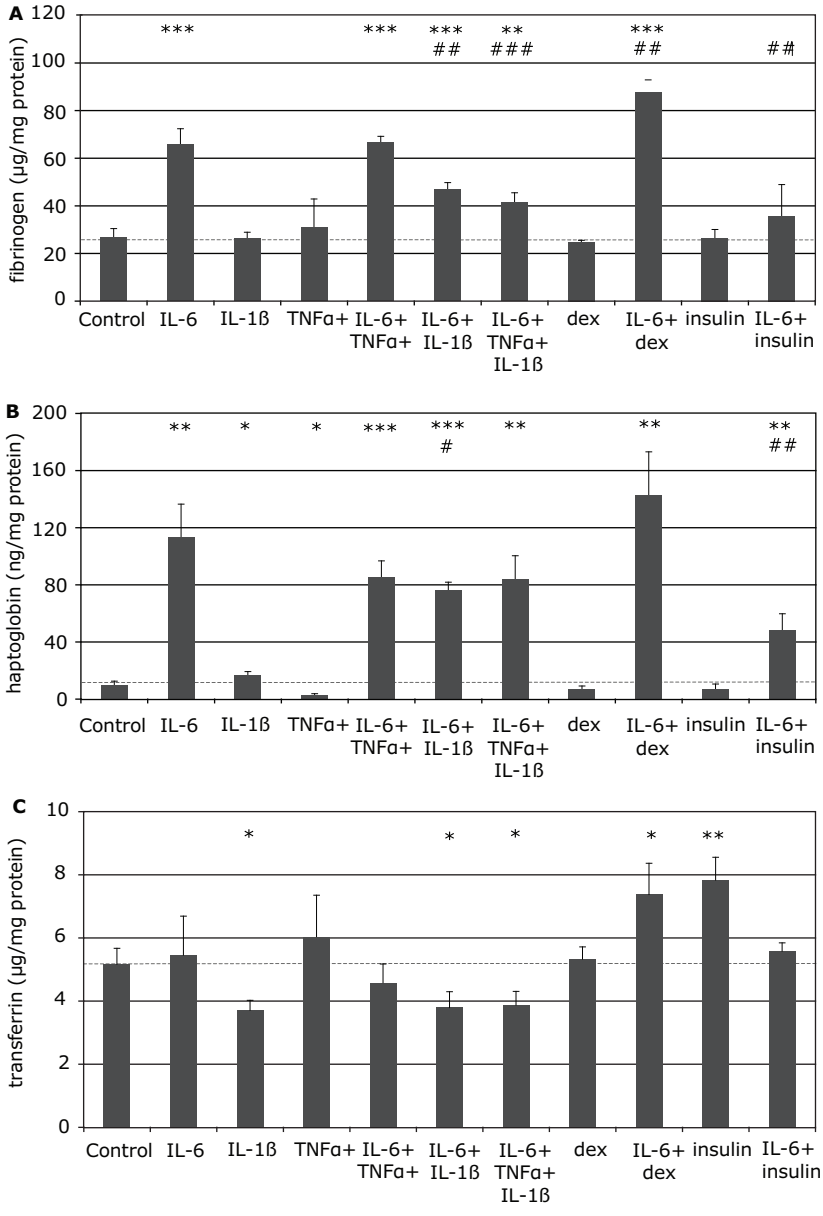

Figure 2. Effects of cytokines, dexamethasone and insulin on fibrinogen (µg/mg protein 24-h) (A), haptoglobin (µg/mg protein 24-h) (B), and transferrin (µg/mg protein 24-h) (C) secretion by HepG2 cells. (10 ng/ml IL-6; 10 ng/ml IL-1β; 10 ng/ml TNFα; 1 mM dexamethasone (dex) and 1 µM insulin. Each data point represents mean ± STD (n=6). (*p<0.05; **p<0.01; ***p<0.001 compared with control; #p<0.05; ##p<0.01; ###p<0.001 compared with IL-6).

Table 1. Concentrations (μM) of amino acids in RPMI-1640 supernatants of HepG2 cells at t=0 and t=24 h. Each data point represents mean ± STD (n=6). Of the essential amino acids, only the BCAA (valine, leucine and isoleucine) concentrations decreased after a 24-h incubation. Of the nonessential amino acids aspartate, glutamine and alanine concentrations decreased and the glutamate concentration increased after a 24-h incubation. Each data point represents mean ± STD (n=6) ($^{*}p<0.05$; $^{**}p<0.001$).

	Concentration of amino acids in the media of HepG2 cells (μM)	
	T = 0	**T = 24**
Asp	377 ± 18	339 ± 15 *
Glu	408 ± 20	465 ± 23 *
Asn	358 ± 17	331 ± 15
Ser	356 ± 14	344 ± 17
His	278 ± 7	260 ± 12
Gln	392 ± 14	212 ± 3 **
Gly	661 ± 40	607 ± 37
Thr	327 ± 17	294 ± 13
Arg	292 ± 11	267 ± 12
Ala	213 ± 5	144 ± 8 *
Tyr	274 ± 12	246 ± 24
Val	383 ± 15	328 ± 13 *
Met	362 ± 11	360 ± 16
Trp	253 ± 8	248 ± 11
Phe	326 ± 16	298 ± 13
Ile	426 ± 12	324 ± 11 *
Leu	368 ± 8	277 ± 10 *
lys	202 ± 13	181 ± 11

An experiment was carried out to determine whether the concentration of total amino acids in the media is an independent regulator of acute phase protein synthesis. Figure 3 shows the effects of increasing plasma amino acid concentrations on fibrinogen and albumin secretion in the HepG2 cells. It is shown that both albumin and fibrinogen secretion increase with increasing total amino acids in the cell culture media, although not to the same extent. When total amino acid concentrations were increased from 3 to 6 mM, fibrinogen

secretion increased approximately by 5-fold, while albumin secretion almost doubled. In media where all amino acids were omitted (0 mM), both albumin and fibrinogen concentrations were still within the calibration curve of the respective ELISAs, *i.e.*, the secretion of fibrinogen and albumin still persisted during the incubation.

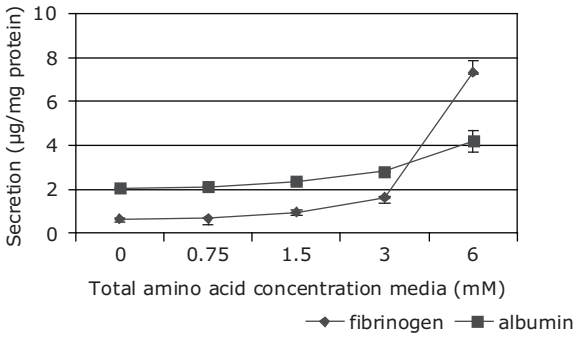

Figure 3. **Fibrinogen (µg/mg protein 24-h)(A) and albumin (µg/mg protein 24-h)(B) secretion of HepG2 cells as function of total amino acid concentration in the media.** Each data point represents mean ± STD (n=6).

Next, a medium with only essential amino acids present (isonitrogenously compensated for until 6 mM total amino acids) was prepared, to investigate whether essential amino acids were sufficient to mount an acute phase protein response in the HepG2 cells. The results are shown in Figure 4. The secretion of both fibrinogen and albumin were significantly decreased in cell culture media with only essential amino acids. However, both proteins were still detectable by ELISA in the cell culture supernatants.

Subsequently, an experiment was carried out to investigate what other non-essential amino acids were essential for acute phase protein synthesis in the HepG2 cell line. Therefore, different media were prepared in which one of the nonessential amino acids was omitted and isonitrogenously compensated for by the other amino acids. If arginine, cysteine or serine were omitted

from the media, the secretion of both fibrinogen and albumin was significantly decreased (to levels not significantly different from media without amino acids) and designated as essential for acute phase protein synthesis in HepG2 cells. In contrast, if any of the other nonessential amino acids was omitted from the media, no effects were observed on acute phase protein secretion of the HepG2 cells. Finally, an experiment was carried out with all essential amino acids plus arginine, cysteine and serine. Unexpectedly, under these conditions a significant reduction was observed for both albumin and fibrinogen secretion; this reduction was comparable to the reduction observed in media only containing EAA (Figure 5).

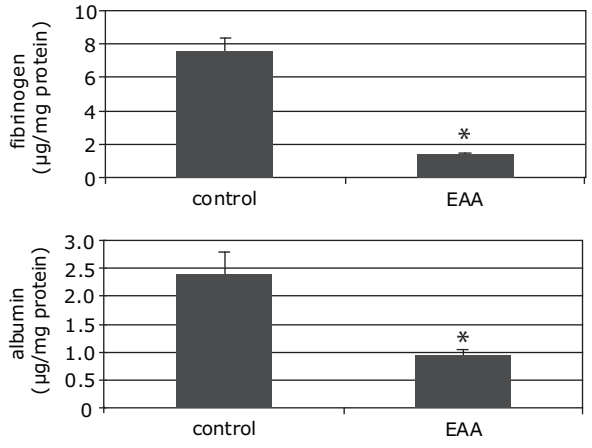

Figure 4. Fibrinogen (µg/mg protein 24-h)(A) and albumin secretion (µg/mg protein 24-h) (B) in complete media and media with only essential amino acids (EAA). The secretion of both proteins in the media decreased significantly when only essential amino acids are present in the media. Each data point represents mean ± STD (n=6)(*fibrinogen $p<0.01$; albumin $p<0.001$).

Discussion

Figures 6 A, B and C depict the effects of the cytokines IL-6, IL-1β and TNFα and the hormones insulin and dexamethasone on the secretion of fibrinogen, haptoglobin and transferrin of the HepG2 cells, respectively. The induction of

fibrinogen and haptoglobin secretion in HepG2 cells by cytokines appeared to be differently regulated, as is illustrated in Figures 6A and 6B. It was shown that IL-1β has an inhibitory effect on the IL-6 induced induction of both haptoglobin and fibrinogen. However, IL-1β alone did not appear to have an effect on fibrinogen excretion, but it did induce haptoglobin secretion. Dexamethasone, on its turn, enhanced the IL-6 inducing effect on fibrinogen secretion, but it did not have any effect on haptoglobin secretion. TNFα alone inhibited haptoglobin secretion of the HepG2 cells, but it did not interfere with any of the cytokines or hormones used in this study.

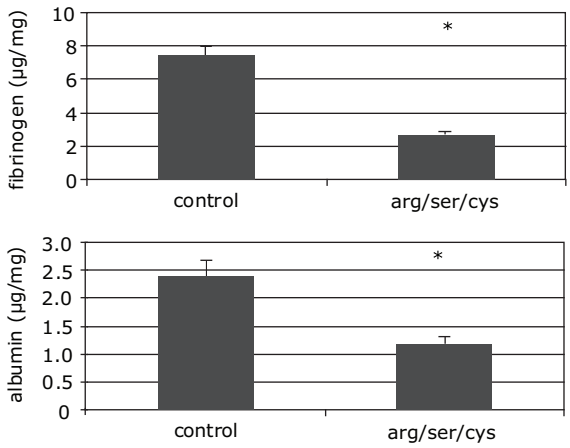

Figure 5. Fibrinogen (µg/mg protein 24-h) (A) and albumin (µg/mg protein 24-h)(B) secretion in complete media and media or EAA with arginine, cysteine and serine. The secretion of both proteins in the media decreases significantly when only essential amino acids plus arginine, cysteine and serine are present in the media. Each data point represents mean ± STD (n=6) (*fibrinogen $p<0.01$; albumin $p<0.001$).

Also the effects of the cytokines and hormones on the constitutively expressed liver proteins albumin and transferrin were determined. No effect at all was observed on albumin secretion. Figure 6C depicts the effects of the cytokines and hormones on transferrin. Insulin had an inducible effect on transferrin

secretion. This effect was inhibited by IL-6, which is an inducer of positive acute phase proteins. This indicates that IL-6 plays a role in the reprioritization of acute phase protein synthesis in the liver: it reduces the synthesis of the negative acute phase protein transferrin on the one hand, and induces the synthesis of positive acute phase proteins on the other hand.

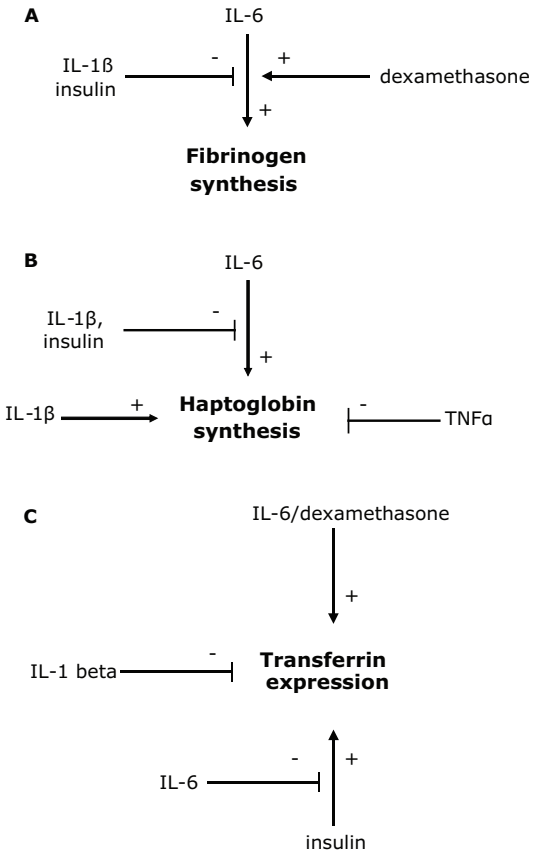

Figure 6. Schemes of the regulation of (A) fibrinogen, (B) haptoglobin, and (C) transferrin synthesis by cytokines and hormones in HepG2 cells. Overview of effects and interactions of cytokines, insulin and dexamethasone on fibrinogen, haptoglobin and transferrin, respectively. The schemes are based on the result in Figure 2A, B, and C.

IL-6 plays a major role in the induction of the APP response, since it has the ability to stimulate a whole spectrum of positive acute phase proteins (13, 14). This study shows different interactions of cytokines, dexamethasone and insulin on the IL-6 induced secretion of APP by HepG2 cells. Type I APP are induced mainly by IL-1β, and type II APP mainly by IL-6 (2,4,15). This study focussed on Type II APP, like fibrinogen and haptoglobin, because quantitatively these are the most important APP in the HepG2 cells. Our observations are in accordance with those by Mackiewicz *et al*. (16), who showed that IL-6 – induced fibrinogen synthesis is mainly inhibited by IL-1β, and that TNFα is responsible for the inhibitory effects on IL-6 induced haptoglobin synthesis in HepG2 cells. Hence, there appears to be a cross-talk between the effects of IL-1β and IL-6 on acute phase protein synthesis. Shen *et al.* (2,7) have presented the first mechanistic evidence for cross-talk between the IL-1β and IL-6 signalling pathways. They showed that IL-1β exerts its inhibitory action on IL-6 dependent expression of type II acute phase proteins by selectively down-regulating the induction of phosphorylation of the factor STAT1 (signal transducer and activator of transcription) by IL-6.

Insulin inhibited both IL-6 induced fibrinogen and haptoglobin secretion. In critically ill patients is has been reported that high-plasma glucose concentrations are a risk factor for infectious complications (17,18). High glucose will result in high insulin and as shown in our results, high insulin will decrease the acute phase protein response in the liver, resulting in suppression of fibrinogen and haptoglobin, both proteins involved in the inflammatory response in infection. This could result in less capacity to cope with infection or tissue injury.

Recent studies have already shown that the cytokine response itself is differentially regulated, dependent on the interactions of different pro- and anti-inflammatory stimuli and/or the cause of inflammation or infection (19-22). The conclusion of the first part of this study is that the acute phase protein response is a differential response, with an outcome dependent on the sum of stimuli from cytokines and hormones produced during a stress response. The effects of the cytokine/hormone combinations on the acute phase proteins studied varied; each protein exhibited a unique and specific pattern of response

to the cytokine and hormone combinations. Further investigation should address the physiological relevance of the acute phase protein response in *in vivo* models in order to establish the specificity of the regulation and signalling of the APP reaction. In addition, the term negative acute phase protein is not as explicit as it seems to be, since some proteins like transferrin can act in a positive or negative way dependent on experimental conditions and albumin secretion did not respond to the cytokine and hormones at all.

In the second part of this study, another aspect of the acute phase protein model in the HepG2 cells was investigated, namely the effects of amino acid composition and quantity in the cell culture media. For this purpose, the HepG2 cells were cultured for 24-h in cell media, supplemented with 10 ng/ml IL-6 in the media to obtain a maximal fibrinogen secretion, since this is, in quantitative amounts, the major inducible acute phase protein which is secreted by HepG2 cells (see results).

During a 24 hour incubation, the consumption of amino acids from the media of HepG2 cells was studied. Of the essential amino acids only the branched-chain amino acids (BCAA) leucine, isoleucine and valine were consumed in significant amounts (Table 1). The consumption of the BCAA, cannot solely be attributed to APP synthesis, because if this would be the case, the other essential amino acids would be expected to disappear too and especially the aromatic amino acids, since these are the main components of APP (23). Although in healthy individuals BCAA normally are catabolised in the skeletal muscle, there are circumstances that the liver can adapt its metabolism and starts to catabolize BCAA (24,25): liver cirrhosis and chronic liver disease are characterized by decreased plasma BCAA concentrations (26). This might indicate that in specific occasions the liver is able to metabolize BCAA. Whether this effect is inflammation-driven remains to be investigated. From the nonessential amino acids, aspartate, alanine and glutamine concentrations in the media decreased, while the glutamate concentration increased in the media. Hence, it seems that aspartate, glutamine and alanine are catabolized by the HepG2 cells. All these amino acids can be catabolized by the liver for, among others, gluconeogenesis (27) or can be used to supply in the energy demand of the HepG2 cells and glutamine is also used for the production

of glutathione (28,29). The increase in glutamate concentrations can be explained by deamination processes of glutamine in the liver (30).

Secondly, the effect of the total amino acid concentration of the cell culture media on acute phase protein synthesis was studied. There exists an almost linear relationship between the total amino acid concentration in the media and the secretion of fibrinogen and albumin of the HepG2 cells until concentration of 3 mM of total amino acids. When the total amino acid concentration then doubled to 6 mM, albumin secretions doubled and fibrinogen secretions increased approximately 5-fold (Figure 3). Hence, the extracellular concentration of amino acids determines the rate of albumin and fibrinogen synthesis, independent of the amino acid consumption from the media. In addition, still significant amounts of acute phase proteins were detectable in the supernatants in the absence of amino acids. This might indicate that the HepG2 cells are able to use intracellular free amino acids to produce acute phase proteins during the 24-h period. These free intracellular amino acids might be the product of autophagocytotic processes within the HepG2 cells (31,32).

In the third part of this study the effects of amino acid depletions of the media were determined on albumin and fibrinogen secretion. This part of the study was carried out to determine which amino acids are essential in the HepG2 model to mount an acute phase protein response. For further investigations on acute phase protein secretion in this model we used the concentration of 6 mM total amino acids in the media. Hence, when amino acids were depleted from the media, they were isonitrogeneously compensated for until 6mM of total amino acids in the media. The results of figure 4 show that when only essential amino acids are supplied in a complete cell culture medium, neither fibrinogen nor albumin secretion show the same values as in complete media. Hence, some non-essential amino acids have to be essential in the HepG2 model to induce an acute phase protein response. From incubations of HepG2 cells in media in which one nonessential amino acid was omitted, respectively, it was concluded that arginine, serine and cysteine are essential, at least for acute phase protein synthesis in the HepG2 model. HepG2 cells have defects in the urea cycle, due to missing the enzymes ornithine transcarbamylase and

arginase I (33). It might be expected that this defect is the explanation of arginine being essential in this model. Liver cells normally have an enormous flux of intracellular arginine thanks to the urea cycle activity (34,35). In a final experiment, where the essential amino acids plus these three nonessential amino acids were included in the media, albumin and fibrinogen secretions still were attenuated. When one nonessential amino acid was omitted from the media, the cells were able to compensate for the amino acids, probably by synthesizing the amino acid. However, when more amino acids were omitted at once, is seemed that the HepG2 cells were not able to produce sufficient amino acids to compensate for the omitted non-essential amino acids. Whether this is due to the metabolic interrelationships between these nonessential amino acids remains unclear.

In conclusion, besides the inflammatory mediators, both the amino acid concentration and the amino acid composition in the cell culture media are independent regulators of acute phase protein synthesis in HepG2 cells. The results found in this study in combination with findings that malnutrition can affect the acute phase protein response *in vivo,* suggest that nutrition and the amino acid composition of the protein content, indeed, may determine protein synthesis in the liver. Further research is warranted to determine whether these *in vitro* results apply to the *in vivo* situation and may lead to optimal nutrition for patients with a metabolic imbalance in nitrogen distribution.

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Chapter 3

Co-culture of primary rat hepatocytes with rat liver epithelial cells enhances the interleukin-6 induced acute phase protein response

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Abstract

Three different primary rat hepatocyte culture methods were compared for their ability to secrete fibrinogen and albumin under basal and IL-6-stimulated conditions. These culture methods comprised a co-culture of hepatocytes with rat liver epithelial cells (CC-RLEC), a collagen type I sandwich culture (SW) and a conventional primary hepatocyte monolayer culture (ML). Basal albumin secretion was most stable in time in the SW. In all cell culture models fibrinogen secretion was induced by IL-6. Compared to ML, CC-RLEC showed an almost threefold higher fibrinogen secretion in both the control and the IL-6 stimulated condition. Induction of fibrinogen release by IL-6 was lowest in the SW cell culture technique. Albumin secretion was decreased after IL-6 stimulation in both ML and CC-RLEC. In conclusion, different primary hepatocyte cell culture techniques react differently to IL-6 stimulation regarding acute phase protein secretion. CC-RLEC is the most preferred method to study cytokine induction of acute phase proteins, because of its pronounced stimulation of fibrinogen secretion upon IL-6 exposure.

Introduction

The acute hepatic response to systemic injury has been described as a re-prioritization of liver protein synthesis: the available synthetic capacity and amino acid resources are shifted from constitutive protein production (like *e.g.* albumin and transferrin) to increased non-constitutive, acute phase protein production (like *e.g.* fibrinogen and haptoglobin). The latter are designated as positive acute phase proteins and play important roles in the restoration of homeostasis after injury and metabolic stress. Acute phase protein synthesis is induced by a multitude of stress factors like cytokines and stress hormones (1-3), with the main inducer being interleukin-6 (IL-6) (4-6).

Most *in vitro* studies on the acute phase protein response are carried out in a human hepatocellular cell-line, HepG2 cells (ATCC number HB-8065) (7-11). However, the HepG2 cell line is a hepatocellular carcinoma cell line and might exhibit different metabolic properties from those observed in primary hepatocytes. Therefore, it is of interest to investigate the effects of IL-6 on acute phase protein synthesis in primary hepatocytes.

Primary hepatocyte cultures have been used successfully to study detoxification processes by cytochrome-P450 activity (12,13) and to a minor extent to study acute phase protein expression (14,15). In addition, monolayer hepatocyte cultures been used to study cytokine-induced acute phase protein expression in rat (16) and human hepatocytes (17,18).

In addition to cytokines, also the extrahepatocellular environment might play a role in the establishment of acute phase response in primary cell cultures. Whether or not the differentiated state of cultured hepatocytes is maintained in time depends on a complex environment in which exogenous factors, cell-matrix and cell-cell interactions play key roles (19,20). These conditions can be mimicked by co-culturing hepatocytes with non-parenchymal rat liver epithelial cells of primitive biliary origin (CC-RLEC), in order to mimic the *in vivo* situation in which hepatocytes also make in contact with liver epithelial cells via the perisinusoidal space of Disse (12,21). Results from Guillouzo et al suggest that this method might improve acute phase protein synthesis (Guillouzo *et al*., 1984). A second approach to provide an extracellular matrix

is the addition of a collagen bilayer. Type I rat tail collagen is generally used as the coating material for hepatocyte cultures, as it is an important component of the hepatocytic basal membrane (22-24).

To investigate the effects of the extracellular environment on acute phase protein synthesis *in vitro*, three different cell culture techniques were used to study the effect of IL-6 on the expression of fibrinogen (a positive acute phase protein) and albumin (a constitutively expressed protein and / or negative acute phase protein). These cell cultures were: a conventional primary monolayer culture of hepatocytes (ML), a co-culture of hepatocytes with rat liver epithelial cells (CC-RLEC) and a collagen type I sandwich culture (SW). This study is the first approach to compare effects of IL-6 stimulation of fibrinogen and albumin secretion in three different cell culture models of hepatocytes of the same batch.

Materials and methods

Chemicals

Crude collagenase type I, bovine serum albumin fraction V, insulin, and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). William's medium E (without L-glutamine), fetal bovine serum (FBS) and trypsin-EDTA solution were from Invitrogen (Brussels, Belgium). Hydrocortisone was from Upjohn S.A. (Puurs, Belgium).

Animals

Adult male outbred Sprague-Dawley rats (± 200 g; food and water *ad lib*) (Iffa Credo Brussels, Belgium) were anaesthetised by *i.p*. injection of sodium pentobarbital solution (0.1 ml/100 g body weight).

Isolation and culture of hepatocytes

Intact rat hepatocytes were isolated by collagenase perfusion according to De Smet *et al.* (25). After testing for cell integrity by trypan blue exclusion, $1.6x10^6$ hepatocytes were cultured in 4 ml DMEM containing 10% FBS (v/v), 2.5 µg/ml bovine insulin, 0.005% kanamycin monosulphate, 0.005% streptomycine sulphate and 0.0045% penicillin on 6 cm Ø culture dishes,

either as a conventional monolayer (ML) (21), as a co-culture with rat liver epithelial cells (CC-RLEC) (21) or as a collagen type I gel sandwich culture (SW) (24). Co-cultures were set up, after 4h, by adding a fourth part of a confluent flask (75 cm^2) of epithelial cells. These were isolated from livers of 8 weeks old Sprague Dawley rats and cultured as described by Henkens *et al.* (26). The renewing media were supplemented with $7x10^{-5}$ M or $7x10^{-6}$ M hydrocortisone hemicuccinate for ML and CC-RLEC, respectively. After 24 h, the cells were incubated with 10 ng/ml recombinant rat IL-6 (Peprotech Inc, Heerhugowaard, the Netherlands). Both IL-6 and the medium were refreshed every 24 h. After 24 and 48 h, media samples were taken and analysed for the amount of albumin and fibrinogen secreted during 24 h.

ELISA for Fibrinogen and Albumin

Sheep anti-rat fibrinogen purified IgG and Sheep anti-rat fibrinogen horse radish peroxidase (HRP) were purchased from Affinity Biologicals (Ancaster, Ontario, Canada). Fibrinogen from rat plasma was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sheep anti-rat albumin, sheep anti-rat albumin HRP labelled and rat albumin antigen were from Biogenesis Ltd. (Pool, England). A 96-well flat bottom EIA/RIA plate (Corning Life Sciences B.V., Schiphol-Rijk, the Netherlands) was coated overnight with primary antibody diluted in phosphate-buffered saline (PBS). Plates were washed after every step with 0.1% (v/v) Tween-20 (Merck Eurolab B.V., Roden, the Netherlands) in 0.1 M PBS pH 7.4. Subsequently, the plates were blocked with 5% (w/v) Protifar (Nutricia, Zoetermeer, the Netherlands) in 0.1 M PBS pH 7.4 during 90 min. Then samples and antigen were incubated in 0.1% (v/v) Tween-20 in 0.1 M PBS pH 7.4 for 90 min. Subsequently, the plates were incubated with HRP antibodies in 0.1% (v/v) Tween-20 in 0.1 M PBS pH 7.4 during 90 min. Finally, a colorimetric reaction was carried out with 100 μl undiluted 1-Step Ultra TMB-ELISA (Pierce, Rockland IL, USA). The reaction was stopped with 50 μl of 2 M sulfuric acid and absorbance was measured at λ = 450 nm.

Protein assay

Protein levels of the cell lysates were measured using a "Bio-Rad protein assay kit" (Bio-Rad, Brussels, Belgium) with bovine serum albumin as a standard. To be able to correct for protein content in RLEC, the percentage

of RLEC was determined as follows. After detaching the hepatocytes from the epithelial cells by use of 4 ml collagenase in PBS buffer (1 mg/ml) at 37°C, the remaining RLEC were scraped off and subjected to the protein assay. By making the ratio of the protein content of the RLEC in co-cultures versus the protein content of the hepatocytes + the RLEC, the percentage of RLEC in coculture was estimated.

Statistics

To determine significant differences between values, multiple pairwise comparisons were conducted with Student's *t*-test. P-values below 0.05 were interpreted as statistically significant.

Results

Hepatocyte morphology in conventional monolayer culture (ML), co-culture (CC-RLEC) and collagen gel sandwich culture (SW)

Hepatocyte morphology in ML, CC-RLEC and SW after 24 h incubation are shown in figures 1A-1C. In the ML hepatocytes cluster on the culture dish (figure 1A); in the CC-RLEC the cells also are clustered but the RLEC fill in the empty spaces on the culture dishes (figure 1B); in the SW the cells are present between a collagen bilayer, with the cells on all sides in contact with collagen as extracellular matrix (figure 1C).

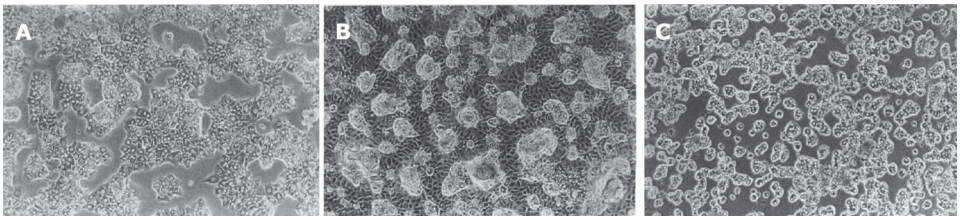

Figure 1. Hepatocyte culture systems. 1A: Hepatocytes in monolayer cell culture (ML). 1B. Co-culture of hepatocytes and rat liver epithelial cells (CC-RLEC). 1C. Collagen sandwich culture (SW) of hepatocytes.

Effects of IL-6 on fibrinogen secretion

Fibrinogen secretion in the media of the different cell cultures after a 24-h incubation with IL-6 is shown in Figure 2. The basal fibrinogen secretion is significant higher in CC-RLEC, when compared to values in the other cell culture models (P<0.0001). The basal secretion of fibrinogen in SW and ML were comparable. When incubated with IL-6, both ML (P<0.001) and CC-RLEC (P<0.001), showed an approximately three-fold increase in fibrinogen secretion compared to basal. In contrast, the effect of IL-6 on fibrinogen secretion in SW was minor but significantly (P<0.01). The IL-6-induced fibrinogen secretion of CC-RLEC was significantly higher (P<0.00001 vs ML); the IL-6 response in SW was significantly lower (P<0.0001) than the value found in ML.

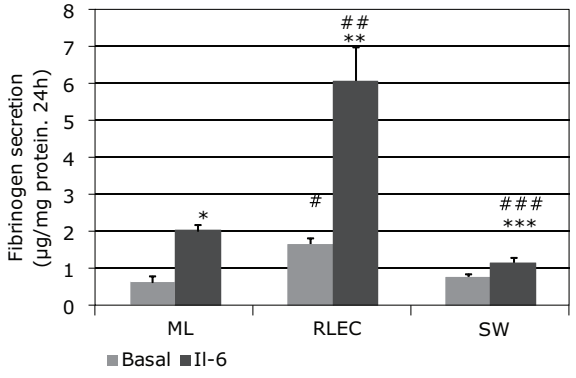

Figure 2. Fibrinogen secretion of primary rat hepatocyte cultures. Control vs. IL-6 (10 ng/ml) stimulated hepatocytes (n = 6). ML: monolayer, CC-RLEC: hepatocytes co-cultures with rat liver epithelial cells, SW: Collagen type I hepatocyte sandwich culture. IL-6 vs. basal : *: *P*<0.001; **: *P*<0.0001; ***: *P*<0.01. #: *P*<0.0001 vs basal ML. ##: *P*<0.00001 vs IL-6 induced ML. ###: *P*<0.0001 vs IL-6 induced ML.

Effects of IL-6 on albumin secretion

In Figure 3 the basal albumin secretion rates of the hepatocyte cultures are shown to be equal for all cell cultures. When incubated with IL-6, albumin secretion in both ML and CC-RLEC decreased significantly ($P<0.01$) and to the same extent. The response to IL-6 was negligible in SW. As control, also albumin secretion was determined in the RLEC (without hepatocytes). No albumin was present in the media of the RLEC. In addition, in earlier experiments we did not find albumin expression in the RLEC as determined by immunostaining (27).

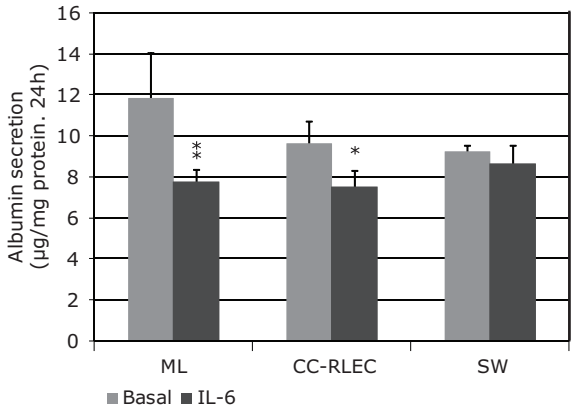

Figure 3. Albumin secretion by primary rat hepatocyte cultures. Control vs. IL-6 (10 ng/ml) stimulated hepatocytes (n=6). ML: monolayer, CC-RLEC: hepatocytes co-cultures with rat liver epithelial cells, SW: Collagen type I hepatocyte sandwich culture (n=6). *: $P<0.01$ *vs* basal.

Stability and reproducibility of the cell cultures

In order to determine the stability and reproducibility of measurements in the cell cultures, all incubations were extended for an additional 24-h period. The albumin secretions after two consecutive days in the different cell cultures are shown in Table 1. Both ML and CC-RLEC showed a significant decrease of albumin secretion in both basal and IL-6-stimulated conditions, whereas in SW secretion levels of albumin did not change. Similar results were observed for fibrinogen secretion. ML and CC-RLEC showed a decrease in basal fibrinogen

secretion rates, whereas basal fibrinogen secretion stayed constant in SW (Table 2). The capacity of IL-6 to induce fibrinogen secretion or to reduce albumin secretion remained in ML and CC-RLEC, also after 48 h incubation.

Table 1. Stability of albumin secretion in three cell cultures after 24 and 48-h incubations. Il-6: incubated with 10 ng/ml Interleukin-6; ML: monolayer, CC-RLEC: hepatocytes co-cultures with rat liver epithelial cells, SW: Collagen type I hepatocyte sandwich culture. (n=6) * $P<0.01$ *vs* 24h basal; ** $P<0.01$ vs 24h IL-6.

	Albumin secretion (µg/mg protein)			
	24-h		48-h	
	Basal	IL-6	Basal	IL-6
ML	11.8 ± 2.2	7.7 ± 0.7	7.4 ± 1.9*	2.5 ± 1.4**
RLEC	9.6 ± 1.1	7.5 ± 0.8	6.2 ± 1.2*	3.6 ± 0.6**
SW	8.2 ± 0.4	8.6 ± 1.2	8.2 ± 1.3	8.1 ± 1.0

Table 2. Stability of fibrinogen secretion in three cell cultures after 24 and 48-h incubations. Il-6: incubated with 10 ng/ml Interleukin-6; ML: monolayer, CC-RLEC: hepatocytes co-cultures with rat liver epithelial cells, SW: Collagen type I hepatocyte sandwich culture (n=6). * Decreased when compared to Control 24 h ($P<0.01$); ** Decreased when compared to IL-6 24 h ($P<0.01$); *** Increased when compared to Control 24-h ($P<0.01$).

	Fibrinogen secretion (µg/mg protein)			
	24-h		48-h	
	Basal	IL-6	Basal	IL-6
ML	0.62 ± 0.16	2.0 ± 0.16	0.26 ± 0.04*	1.29 ± 0.13**
RLEC	1.63 ± 0.18	6.1 ± 0.94	0.85 ± 0.08*	2.63 ± 0.30**
SW	0.74 ± 0.11	1.1 ± 0.16	0.98 ± 0.07***	1.26 ± 0.16

Discussion

In the present study, the effects of different culture conditions on the secretion of albumin and fibrinogen by primary hepatocytes of the same batch have been studied under basal and IL-6 induced conditions. IL-6 is the most important

inducer of the acute phase protein response (2,6). Fibrinogen is an inducible acute phase protein and, quantitatively, one of the most abundant acute phase proteins (28). Fibrinogen secretion is induced by IL-6 (4,8,29,30).

Basal and IL-6-stimulated fibrinogen secretion was higher in CC-RLEC, when compared to ML. This might be due to an increased secretion by the hepatocytes or to an additional secretion by the epithelial cells. A recent study by Lawrence and Simpson-Haidaris showed that extrahepatic epithelial cells are also able to secrete fibrinogen under inflammatory conditions, but not under basal conditions (31). However, no fibrinogen synthesis has been observed from hepatic liver epithelial cells. Our results showed that fibrinogen secretion under both control and inflammatory conditions was increased. α_2-Macroglobulin is another positive acute phase protein. In a co-culture of hepatocytes and endothelial cells Talamini *et al.* found that the interaction between hepatocytes and endothelial cells in co-culture induced the expression of α_2-macroglobulin. They concluded that endothelial cells can modulate hepatocyte acute phase gene expression by cell-cell contact (15). Also on co-culture with epithelial cells there are indications that acute phase protein responses might be more pronounced (Guillouzo *et al*., 1984). Our results confirm these observations that also epithelial cells can modulate both the basal and the acute phase protein response of fibrinogen; since both the basal and the IL-6 induced fibrinogen secretion in CC-RLEC were significantly induced when compared to ML. Whether the increased fibrinogen synthesis is caused by cell-cell interactions of hepatocytes and epithelial cells, by fibrinogen secretion by epithelial cells, or by mutual stimulation via soluble factors remains to be investigated.

The IL-6 induced secretion of fibrinogen in SW is marginal but significant. The induction of fibrinogen secretion by IL-6, however, is significantly less if compared to ML. Earlier experiments have shown that cell-cell interactions between hepatocytes in collagen bilayer cultures are critical regarding albumin synthesis (32). Basal albumin and fibrinogen secretion is not affected by the collagen bilayer in the SW when compared to the monolayer culture. Hence, cell conditions seem to be the same regarding monolayer and SW cultures. Another possible explanation for the minimal effects of IL-6 in SW might be

the physical barrier that is created by the collagen bilayer of the culture, which may cause a decreased interaction of IL-6 with the cells or a decreased secretion of albumin of fibrinogen. The latter does not seem to be the case, since both albumin of fibrinogen secretion is not inhibited by the collagen-bilayer, when compared to ML.

In both ML and CC-RLEC, albumin turned out to be a negative acute phase protein, *i.e.*, its synthesis decreased after IL-6 exposure. These findings are in accordance with the classic literature, referring to albumin as a negative acute phase protein (33,34). From separate studies it was found that the RLEC in monoculture (so without hepatocytes) were not able to secrete albumin (our results and Snykers *et al*. (27). Indeed, the CC-RLEC did not show an increase in albumin secretion. In contrary, a decrease was observed.

In cancer cachexia it was reported that the observed hypo-albuminemia is not linked to a decrease in the hepatic albumin synthetic rate (35,36). This finding changed the idea of albumin being a negative acute phase protein. However, in *in vitro* experiments by Mackiewicz et al. in a human hepatocellular cell line (HepG2 cells) (37) demonstrated a reduced albumin secretion. Also Kasza *et al*., reported a negative effect of IL-6 on albumin synthesis in long term experiments in HepG2 cells (38). Our findings also confirm the hypothesis of albumin being a negative acute phase protein. These contradictory observations in *in vivo* and *in vitro* conditions might be explained by the fact that in the *in vivo* situation a range of factors affect and mediate the hepatic response to stress, such as the many types of cytokines and hormones that are present during a stress response.

Co-culture of hepatocytes with either Kupffer cells (39, 40), Ito cells (41, 42), and rat liver epithelial cells (RLEC) (43,44), demonstrated prolonged maintenance of specific hepatic functions by hepatocytes. Two potential mechanisms have been proposed to explain these findings: (I) intercellular communication mediated by gap junctions (41, 44) and (II) synthesis of extracellular matrices (43,45). Moreover, rat hepatocyte proliferation is inhibited when these cells are co-cultured with epithelial cells (46). This might be due to secreted growth inhibitors by the epithelial cells.

In conclusion, both the conventional monolayer and the co-culture technique of hepatocytes with RLEC are suitable to study acute effects of cytokines on acute phase protein secretion. In contrast, SW is less preferable, because the interaction of IL-6 with the hepatocytes may be impaired due to the collagen barrier. When extracellular rat liver epithelial cells are provided to the hepatocytes, like in CC-RLEC, the basal fibrinogen secretion is significantly higher when compared to a conventional monolayer cell culture technique. An extracellular matrix *per se* does not seem to be the explanation for the increased basal fibrinogen expression, since an increase of fibrinogen synthesis is not observed in SW, where an extracellular matrix is provided by a collagen type I bilayer. In addition, although SW is less sensitive to IL-6 stimulation, the production of albumin in time is more stable. In conclusion, in this study three rat hepatocyte cell culture techniques of one batch of primary hepatocytes have been compared regarding IL-6 induced acute phase protein synthesis. In future studies, the effects of the RLEC on acute phase protein synthesis need to be investigated to determine the role of RLEC on the increased basal fibrinogen secretion. Nevertheless, since the fibrinogen induction is most pronounced in the CC-RLEC, the CC-RLEC is the most preferred method to study cytokine effects on acute phase protein synthesis in primary hepatocytes, when compared to the ML and SW.

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Chapter 4

Arginase release by primary hepatocytes and liver slices results in rapid conversion of arginine to urea in cell culture media

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Abstract

Precision-cut liver slices and primary hepatocytes constitute suitable model systems for studying liver function. Frequently, urea cycle activity is used as a parameter to determine hepatocyte viability. Liver cells contain high levels of the urea cycle enzyme arginase, which converts arginine into urea and ornithine. Arginase can leak from the cells into the supernatants, converting arginine directly to urea and in this way circumventing the urea cycle. In this study, a hepatocellular cell line (HepG2 cells), a primary rat hepatocyte culture, and precision-cut rat liver slices were compared with respect to arginase leakage in the media by determining the arginine conversion into urea. HepG2 cells did not show arginine conversion to urea during 24-h incubations. In contrast, in both precision-cut liver slices and primary rat hepatocytes all arginine was converted to urea. Arginase activity was confirmed by showing that freshly added arginine to the cell-free supernatants again was converted to urea. In conclusion, when choosing urea production of primary hepatocytes cultures as a viability indicator, one has to take into account that arginase can leak from the cells into the supernatant. This can lead to an overestimation of the viability of the cells, since arginase converts arginine into urea without involvement of the urea cycle. We suggest using an extra incubation in an arginine-free buffer supplemented with ornithine and NH_4Cl. In addition, arginase leakage can lead to depletion of the supernatant of arginine in primary hepatocytes cell cultures. This might have implications for studying cellular activities where arginine is involved, like e.g. nitric oxide (NO) production.

Introduction

Isolated and cultured primary hepatocyte cell systems constitute suitable model systems for studying liver functions such as acute phase protein synthesis and phase I and II detoxification reactions. Frequently, urea synthesis is used as a viability indicator for primary hepatocyte cell cultures (1-5) and precision-cut liver slice cultures (6-11). Urea synthesis from primary hepatocytes seems to be a valid viability indicator, since mitochondrial trans-membrane transport of the urea cycle intermediates ornithine and citrulline is involved in the urea cycle (12-14). Accordingly, the rate of appearance of urea is correlated with urea cycle activity and, hence, the viability of the cell system.

So far, two methods have been used to determine urea synthesis as a viability indicator in primary hepatocyte cultures. One involves the production of urea by hepatocytes in an arginine-free ornithine-containing buffer. Urea production is measured and correlated with cell viability (6,9). The other one involves the determination of urea concentrations in the supernatants of arginine-containing media of cell cultures (2,3,7).

One of the enzymes involved in the urea cycle is arginase (L-arginine ureohydrolase EC 3.5.3.1), which hydrolyses arginine to ornithine and urea. Rat liver contains a potent arginase in the cytoplasm of the periportal hepatocytes, which is known as the A1 isoenzyme of arginase, also known as 'liver type' (15). Since hepatocytes contain high levels of arginase, it is possible that arginase is released from the cytoplasm of dying cells. If present in the supernatants of the hepatocytes it might deplete the media of arginine by its hydrolysation into urea. This will result in high levels of urea and an overestimation of urea cycle activity. This study shows the presence of arginase in cell culture media of primary hepatocyte cultures and precision-cut liver slice cultures.

Materials and methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM, containing 0.398 mM arginine), RPMI-1640 (containing 1.15 mM arginine), Fetal Calf Serum (FCS), streptomycin/penicillin and glutamine were purchased from Life Technologies, Breda, the Netherlands.

HepG2 cell culture

Human hepatoma HepG2 cells (ATCC HB-8065) were maintained in DMEM supplemented with 10% heat-inactivated FCS with 2 mM glutamine and streptomycin/penicillin in Costar T75 flasks (Corning-Costar, Schiphol-Rijk, the Netherlands) at 37°C in a humidified atmosphere of 95% air-5% CO_2. Before each experiment, HepG2 cells were incubated during 24-h in RPMI-1640 media with 10% heat-inactivated FCS. All experiments were carried out in 6 wells plates.

Primary hepatocyte culture

Adult male outbreed Sprague-Dawley rats (about 200 g; food and water *ad lib*) (Iffa Credo Brussels, Belgium) were anaesthetised by *i.p*. injection of sodium pentobarbital solution (0.1 ml/100 g body weight). Intact hepatocytes from adult male outbreed Sprague-Dawley rats were isolated by collagenase perfusion according to De Smet *et al*. (16). Hepatocytes ($1.6 \cdot 10^6$ cells; viability checked by the trypan blue exclusion assay) were seeded in 5 ml DMEM containing 10% heat-inactivated FCS (v/v) and penicillin/streptomycin on 6 cm Petri dishes. The cells were allowed to attach to the Petri dishes during 4 h at 37°C in an atmosphere of 95% air and 5% CO_2 at a relative humidity of 100%. Subsequently, the cultures were incubated during 24-h in the same atmosphere and media. Albumin secretion was used as a viability indicator.

Precision-cut liver slice culture

Precision-cut liver slices (thickness 100 mm) were prepared from adult male Wistar rat liver. Rats were anaesthetized by *i.p*. injection of sodium pentobarbital solution (0.1 ml/100 g body weight). Before extraction, the liver was perfused with ice-cold PBS. Subsequently, cores with a diameter of 6

mm were prepared and inserted in the tissue slicer (Brendal Vitron Tissue Slicer) containing RPMI-1640 with penicillin/streptomycin. All media were oxygenated with carbogen. During the incubations the media contained 10% heat-inactivated FCS. After a pre-incubation of 2 h the media (2 ml) were replaced with fresh media and incubated for 24 h. Tissue morphology was checked by light microscopy and cell viability was determined by albumin secretion using ELISA.

Arginase activity assay

Directly after the incubations the supernatants were divided in 2 portions. One portion was immediately frozen and stored at -80°C for urea determination. The second portion was collected in 1.5 ml Eppendorf tubes and centrifuged at 13,000 rpm during 15 minutes. Subsequently, the media were separated from the pellets and frozen at -80°C. For the arginase activity assay, the supernatants were thawed and arginine was added (1.4 mM to the RPMI-1640 supernatants and 0.4 mM to the DMEM supernatants). The media were incubated during 8 hours at 37°C followed by urea and arginine determinations.

Urea assay

The urea concentrations in the media were determined colorimetrically after reaction with diacetyl monoxime, using a kit provided by Sigma Procedure No. 535 (Zwijndrecht, the Netherlands).

Arginine determination by HPLC

The arginine concentrations in the media were determined with HPLC, using *ortho*-phtaldialdehyde as derivatisation reagent and L-norvaline as internal standard (both from Sigma Aldrich). The method was adapted from van Eijk et al. (17). The detection level for arginine was 2 pmol/L

Albumin ELISAs

Albumin levels in cell culture supernatants were determined by enzyme-linked immuno sorbent assay (ELISA). Sheep anti-rat albumin-IgG, HRP-labelled anti-rat albumin-IgG and rat albumin antigen were from Biogenesis Ltd. (Pool England). Rabbit anti-human albumin and HRP-labelled rabbit anti-human albumin were from Dade Behring, Leusden the Netherlands. Human

Albumin was abotained from Sigma Chemical Co., Aalsmeer, the Netherlands. A 96-well flat bottom EIA/RIA plate (Corning Life Sciences B.V., Schiphol-Rijk, the Netherlands) was coated overnight with primary antibody diluted in phosphate-buffered saline (PBS). Plates were washed after every step with 0.1% (v/v) Tween-20 (Merck Eurolab B.V., Roden, the Netherlands) in 0.1 M PBS, pH 7.4. Subsequently, the plates were blocked with 5% (w/v) Protifar (Nutricia, Zoetermeer, the Netherlands) in 0.1 M PBS, pH 7.4 during 90 min. Then samples and antigen were incubated in 0.1% (v/v) Tween-20 in 0.1 M PBS, pH 7.4 during 90 minutes. Subsequently, the plates were incubated with HRP antibodies in 0.1% (v/v) Tween-20 in 0.1 M PBS, pH 7.4 during 90 min. Finally, a colorimetric reaction was carried out by addition of 100 ml undiluted 1-Step Ultra TMB-ELISA (Pierce, Rockland IL, USA). The reaction was stopped with 50 ml of 2 M sulphuric acid and the absorbances were measured at λ=450 nm.

Statistical evaluation

Statistical analysis was performed by using multiple pair-wise comparisons between groups using the Student's *t*-test. P-values below 0.05 were considered statistically significant.

Results

Cell culture supernatants (n=6) were analysed for both arginine and urea concentrations. No significant urea synthesis was observed in the HepG2 cell cultures. Approximately 2.3% of arginine which was present in the media (original concentration 0.398 mM in DMEM), was consumed during the incubation.

In the media of the precision-cut liver slice incubations and incubations of primary hepatocytes, arginine concentrations were below detection limit of the HPLC method (<2 pM). The initial concentration of arginine was 1.15 mM in RPMI-1640 for precision-cut liver slices and 0.40 mM in DMEM for primary hepatocytes. Different media were used for precision-cut liver slice culture, because cell cultures were optimised in different media.

When the reduction of arginine levels in both supernatants of the primary hepatocytes culture and the precision-cut liver slice culture were compared to the urea concentrations of the media it was found that arginine was completely converted into urea (Figure 1 and Table 1). To confirm the presence of arginase activity, arginine was added to the arginine-depleted supernatants to restore the initial concentration of arginine in the media. After 8-h incubations at 37°C, arginine and urea concentrations were determined. In the supernatants of precision-cut liver slices, the arginine concentrations were decreased to below detection limits. All arginine was again converted to urea (Table 2). In addition, 79% of arginine in the primary hepatocyte supernatants was converted to urea (Table 2).

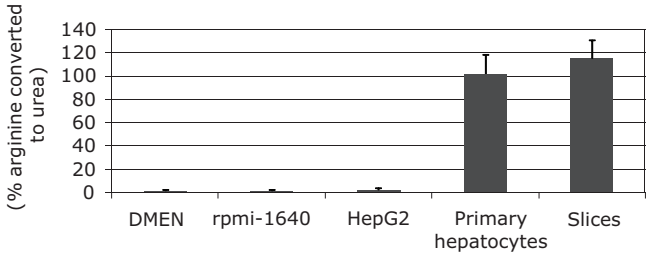

Figure 1. Percentage of arginine conversion to urea in three different hepatocytes cell culture systems after 24-h incubations (n=6). Because different cell culture media were used, the conversion of arginine to urea was expressed as a percentage of the total amount that was present. For concentrations: see Table 1 and 2. In the primary and liver slice cultures the conversion of arginine to urea is not significantly different from 100%. The conversion of HepG2 cells and control media is not significantly different from 0%.

Cell viability of the hepatocyte cultures was determined by liver specific cell morphology by light microscopy for the presicion-cut liver slices and the trypan blue exclusion test, followed by cell counting of viable cells for the primary hepatocytes. More than 99% of the hepatocytes were viable according to the trypan blue test. In addition, all cultures were tested for albumin secretion. In the primary rat hepatocyte culture albumin synthetic rates were 7.4 ± 2.3 µg/mg protein per hour. No albumin synthesis was observed in the precision-cut liver slices. Human HepG2 cells produced 7.5 ± 0.8 µg/mg protein per hour.

Table 1. Concentrations of arginine in the media and urea in the supernatants after 24-h incubation (n=6). HepG2 cells and primary hepatocytes were incubated in DMEM (0.4 mM arginine) and precision-cut liver slices were incubated in RPMI-1640 (1.14 mM arginine). No statistically significant amount of arginine was converted to urea in the HepG2 cell culture. All arginine was converted in both primary hepatocytes and precision-cut liver slices. The percentage of arginine converted to urea is given in the third column, because different media were used.

	Arginine (mM)	Urea (mM)	% conversion of arginine into urea
DMEM	0.389 ± 0.076	0.016 ± 0.022	1.4. ± 0.88
RPMI-1640	1.14 ± 0.268	0.014 ± 0.012	1.2 ± 1.06
HepG2	1.14 ± 0.268	0.026 ± 0.014	2.3 ± 1.26
Primary cells	0.389 ± 0.076	0.393 ± 0.08	98.7 ± 19
Precision-cut liver slices	1.14 ± 0.268	1.31 ± 0.15	115 ± 6.8

Table 2. Concentrations of arginine and urea in the supernatants after 8-h incubation in the media of the first experiment (n = 6). To confirm arginase activity, arginine was added to arginine-depleted media. Again, all arginine was converted to urea in the media of the precision-cut liver slices and 79% of the arginine of the primary hepatocytes. The percentage of arginine converted to urea is given in the third column, because different media were used.

	Arginine (mM)	Urea (mM)	% conversion of arginine into urea
Primary hepatocytes	0.380 ± 0.082	0.301 ± 0.092	79.2 ± 3.0
Precision-cut liver slices	0.983 ± 0.182	1.13 ± 0.24	115 ± 6.4

Discussion

Arginase (L-arginine ureohydrolase EC 3.5.3.1), which is highly prevalent in hepatocytes (18), hydrolyses arginine into urea and ornithine (19). It has been suggested as a parameter for liver damage, since arginase can escape from damaged cells into the plasma and its activity is relatively stable (20, 21). Hence, when carrying out *in vitro* studies with hepatocytes, cell damage might result in arginase leakage in cell culture media (18,19).

In this study it was shown that arginase leakage from primary hepatocytes and precision-cut liver slices into the supernatants of the cell cultures has a dramatic effect on the arginine concentration in the media. Arginase depleted the supernatants of arginine, which was completely converted to urea.

The presence of arginase in the supernatants was confirmed by showing its activity in an additional incubation in the arginine-depleted supernatants of the cell cultures. All arginine was converted into urea in the supernatants of the precision-cut liver slices and 79 % of arginine was converted to urea in the supernatants of the primary hepatocytes (Table 1).

HepG2 cells are known to be deficient in two essential urea cycle enzymes, namely transcarbamylase and arginase I (22). In the incubations in HepG2 cells no arginine was converted to urea. The incubations in HepG2 cells were carried out to show that (i) arginine is not broken down during normal incubations and (ii) arginine is stable during sample preparation for HPLC.

To check hepatocyte viability, albumin was determined in the supernatants of the cell cultures. It was shown that HepG2 cells as well as primary hepatocytes showed significant secretion of albumin, although the supernatant of the latter was depleted of arginine. This may suggest that arginine is not essential for acute phase protein synthesis. In addition, these findings showed that both cell cultures were apparently in good condition. However, the precision-cut liver slice cultures show no significant albumin secretion. So we concluded that, despite the good morphology, the liver slices might have been less viable. Nevertheless, the urea concentrations in the supernatants were very high. In conclusion, in the presented experimental setting viable and less viable hepatocyte cell cultures showed high urea concentrations in the supernatants. Therefore, a high urea concentration in the media is not necessarily a valid marker of cell viability.

Two different techniques have been employed so far that use urea as viability indicator. The most straightforward method is to determine the urea concentration in the supernatants of hepatocyte cultures and, subsequently, to correlate urea concentration in the media to urea cycle activity (2,3,7).

However, we discovered that, when using this method, results can be biased by the formation of urea by arginase, which has leaked from the cells into the supernatants.

In our opinion, the best method to use urea synthesis as a viability indicator is to incubate the hepatocytes in arginine-free Krebs-Henseleit-Hepes buffer supplemented with ornithine and NH_4Cl. These are the substrates forcing the urea cycle to go through a complete cycle to produce urea. This method has been used successfully by several groups (6,23). By using this method, leakage of arginase into the buffer will not bias urea production, since transmembrane transport of the intermediates ornithine and citrulline is essential in the urea cycle and the hydrolysation of arginine by arginase is the final and not the rate-limiting step in the cycle.

One study by Maas *et al*. (8) used the combination of the two methods by adding ornithine and NH_4Cl to the arginine containing cell culture media. They interpreted their results as significant, however, not being able to discriminate between urea formed from the urea cycle or from arginine cleavage in the media.

In conclusion, when choosing urea production of primary hepatocytes cultures as a viability indicator, one has to take into account that arginase can leak from the cells into the supernatant. This can lead to an overestimation of the viability of the cells, since arginase converts arginine into urea without involvement of the urea cycle. We suggest using an extra incubation in an arginine-free buffer supplemented with ornithine and NH_4Cl. In addition, arginase leakage can lead to depletion of the supernatant of arginine in primary hepatocytes cell cultures, which might have consequences for experiments studying cellular activities, like e.g. nitric oxide (NO) production (24). Further research is needed to clarify the exact rate of the reaction in order to estimate the magnitude of the effect in relation to cell culture characteristics like acute phase protein synthesis and NO synthesis.

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Chapter 5

TNFα and IFNγ disrupt C2C12 myotube integrity and inhibit fusion of myoblasts

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Abstract

During cachexia or wasting syndrome there is a negative balance between skeletal muscle protein synthesis and breakdown, resulting in net skeletal muscle wasting. The C2C12 cell line has been frequently used to study muscle metabolism. No unambiguous results of proinflammatory cytokines on protein metabolism in this cell line have been found so far. Myoblast fusion resulting in addition of nuclei to the myotubes has been reported to play an essential role in myotube formation, just like fusion of progenitor cells is essential for skeletal muscle maintenance. The C2C12 cell line was used to study the effects of the cytokines TNFα and IFNγ (interferon-γ) on cell morphology and protein metabolism. These cytokines inhibited the differentiation of C2C12 myoblasts to myotubes and inhibited myoblast fusion into the myotubes. After 48-h TNFα and IFNγ did affect neither protein content nor protein degradation in C2C12 myotubes. However, within this 48-h time span the cytokine mixture caused nuclear clustering and derangements of structural proteins (α-actinin, myosin and tubulin) in the differentiated myotubes. It is concluded that a combination of TNFα and IFNγ reduces the number of cell nuclei in C2C12 myotubes by (1) a direct effect on myotube integrity and (2) inhibition of the fusion of myoblasts with myotubes. The results from these *in vitro* experiments suggest that the catabolic process of cachexia should not only be seen as a negative protein balance, but also as a negative nuclei balance in skeletal muscle myofibers.

Introduction

Cancer cachexia is life-threatening and accounts for approximately 20% of deaths in neoplastic patients (1,2). One of the main characteristics of cachexia is the loss of skeletal muscle mass, which may result in functional impairment, fatigue and respiratory complications, leading to increased morbidity and mortality (3-6). Hence, inhibition of the cachectic process by decreasing skeletal muscle catabolism is an important feature in maintaining quality of life and increasing longevity in cancer patients (3,7-10).

Skeletal muscle is a unique tissue which consists of multinucleated fibers with adjacent dormant satellite cells. These satellite cells can be activated to proliferate and fuse with the fibers during a hypertrophic response to exercise or during regeneration after catabolic periods (11-13). The daughter cells of the activated satellite cells, called myogenic precursor cells (mpcs), undergo multiple rounds of division prior to fusion with the existing or new myofibers (14). Hence, satellite cells are essential in maintaining and, if needed, restoring muscle volume and mass (12,13).

A frequently used model to study skeletal muscle metabolism is the murine skeletal muscle cell line C2C12. These cells are able to undergo differentiation by fusing into myotubes when cultured upon growth factor withdrawal (15, 16). This cell line has been used to study the effects of cachexia-related inflammatory cytokines. However, no unambiguous results have been reported so far. For example, Alvarez *et al.* found divergent effects of TNFα on skeletal muscle protein synthesis measured by incorporation or release of radioactively labeled phenylalanine in C2C12 cells. Depending on the concentration of TNFα, the effects were catabolic (low concentrations) or anabolic (higher concentrations) (17). In addition, Los and Haagsman found that TNFα induced proliferation of myoblasts in fully differentiated cultures in low serum media and inhibited adult fast myosin accumulation. Moreover, TNFα caused a proliferation dependent increase in total cell protein (18). Finally, TNFα and IFNγ can specifically inhibit adult fast myosin synthesis in myotubes *in vitro* (9), suggesting that proinflammatory cytokines not only induce protein breakdown, but also inhibit protein synthesis.

Taken together, the aforementioned studies led to the hypothesis that in both atrophy and hypertrophy satellite cells play an essential role: without fusion into muscle fibers, no growth is to be expected. The confusing effects of TNFα on protein metabolism in C2C12 cells are likely to be, at least partly, explained by effects on the contribution of myoblasts to the total protein content. Therefore, more insight is needed in the effects of proinflammatory mediators on both protein metabolism and on the role of myoblasts, *i.e.,* the fusion process of new nuclei into the myotubes. In this study, we investigated the effects of the cytokines TNFα and IFNγ on protein metabolism, myofibrillar and nuclear configuration (by confocal microscopy) in the myotubes on the one hand and on the differentiation of C2C12 myoblasts to myotubes on the other hand. Moreover, a comparison was made between the effects of the cytokines on protein content and nuclei content of the cells.

Material and Methods

Cell culture and differentiation

The murine skeletal muscle cell line C2C12 (ATCC# CRL 1772) is able to undergo differentiation into myotubes upon growth factor withdrawal (15, 16). The C2C12 myoblasts were grown in growth medium: low glucose (2 g/L) Dulbecco's Modified Eagle's Medium (DMEM) with 50 U/ml penicillin and 50 µg/ml streptomycin and 10% heat inactivated FBS (all Life Technologies). Myogenic differentiation was induced by plating cells in 24 wells-plates, coated with Matrigel (Becton Dickinson Labware, Bradford MA; 1:50 diluted in PBS) at 80% confluence. After 2 h incubation at 37ºC in growth medium, the plates were washed with PBS and, subsequently, cultured in differentiation media, which was the same as growth media, but with 0.4% Ultroser G (UG) instead of FBS. After differentiation cells were treated with 10 ng/ml IFNγ and/or 10 ng/ml TNFα. All media and cytokines were refreshed daily.

Determination protein breakdown rate with ^{14}C-phenylalanine labeling

For the measurement of protein degradation, the cells were grown in 6-well plates, and proteins were radiolabeled by incubating 4 day-old myotubes with 1 µCi/ml L-^{14}C-phenylalanine (^{14}C-Phe; 143 Ci/mmol, Amersham) for 24

hours in DMEM supplemented with 10% FCS. The cells were rinsed twice in 2 ml PBS (Life Technologies) containing 2 mM non-radioactive phenylalanine (PBS-Phe) and then incubated for 2 hours in a chase medium, composed of DMEM containing 10% FBS and 2 mM non-radioactive phenylalanine, to allow the degradation of short-lived proteins. The cells were rinsed twice in PBS containing 2 mM Phe and then transferred to the degradation medium (3 ml), consisting of DMEM + 2 mM Phe, supplemented with or without cytokines. After 24 and 48 hours, 0.5 ml aliquots were sampled to measure the radioactivity of [^{14}C] in the non-TCA-precipitable fraction in the medium released from the cells using a liquid scintillation counter (LSC A2200/01 Packard).

Determination of total protein content of cell cultures

After the incubations the cells were washed with PBS and dissolved in 0.1 M NaOH. Subsequently, the total protein content of the cells was determined using the Bio-Rad protein assay (Dye Reagent Concentrate, Bio-Rad Laboratories, Inc., Hercules CA, USA).

Confocal microscopy

C2C12 cells for confocal microscopy were grown on acid treated cover glasses. Before fixation cells were washed twice with PBS (Invitrogen). For microtubule staining (antibody B-5-1-2, Sigma) cells were fixed with methanol (-20°C). For staining with phalloidin, sarcomeric α-actinin (antibody EA53, Sigma), or adult fast myosin (antibody MY32, Sigma) cells were fixed with 4% paraformaldehyde (Merck) in PBS for 10 min, rinsed in Tris-buffered saline pH 7.6 (TBS), permeabilized in 0.1% Triton X-100 (Sigma) in TBS for 5 min, rinsed in TBS, and blocked with 10% goat serum in TBS for 30 min at room temperature. Cells were incubated for 1 hour with primary antibody diluted 1:100 in TBS with 0.05% (w/v) BSA (Roth). Samples were rinsed in TBS and incubated with a 1:300 dilution of secondary antibody coupled to Alexa Fluor 488 or 568 (Molecular Probes) in TBS-BSA for 1 h. Phalloidin and DAPI (4'-6-diamidino-2-phenylindole) staining were performed directly after the antibody incubations. Phalloidin was diluted 1:25 in PBS/Triton X-100, DAPI was diluted to 300 nM with PBS. After 30 minutes incubation, cells were washed 3 times with TBS and mounted with Fluorosave (Calbiochem). Fluorescent signals were visualized using a Bio-Rad Radiance 2100MP confocal and multi-

photon system equipped with a Nikon TE300 inverted microscope (Uvikon, Bunnik, Netherlands). Excitation of the Alexa 350 and DAPI probes was achieved by multiphoton excitation at 750 nm and 770 nm respectively using a mode locked Titanium: Sapphire laser (Tsunami; Spectraphysics, Mountain View, CA) pumped by a 10W solid state laser (Millennia XS; Spectraphysics). Alexa 488 and 568 probes were excited by confocal lasers.

Bromodeoxyuridine (BrdU) labelling and staining

C2C12 cells were incubated with 10 μM BrdU (Sigma) during treatment with TNFα and IFNγ. After 48 hrs incubation cells were fixed with 3.7 % formaldehyde (Sigma). Cells were subsequently permeabilized with PBS (Invitrogen) with 1% Triton X-100 (Sigma). Permeabilised cells were incubated with 1M HCl for 10 min at 4°C. This was followed by incubation in 2M HCl for 10 min at room temperature. Immediately after the acid washes borate buffer (0.1M, pH 8.5) was added to buffer the cells for 12 min at room temperature. Samples were then washed 3 times 5 min with PBS/1% Triton X-100. Samples were blocked with PBS/1% Triton X-100, 1M glycine and 2% BSA prior to overnight incubation with anti-BrdU antibody (Invitrogen) diluted 1:50 in blocking solution. Samples were washed 3 times 5 min with PBS/1% Triton X-100. This was followed by a 1-hr incubation with goat anti mouse antibody (Dako cytomation) diluted 1:100 in blocking solution. Finally cells were washed 6 times 5 min with PBS, stained with AEC (3-amino-9-ethylcarbazole), counterstained with hematoxilin and mounted with Fluorsave (Calbiochem).

Caspase-3 activity

Caspase-3 activity was assessed with the colorimetric assay kit (Sigma Aldrich). This assay is based on the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase-3, resulting in the release of p-nitroaniline which is detected at λ=405 nm.

Statistical evaluations

To determine significant differences between values, multiple pair-wise comparisons were conducted with Student's *t*-test. P-values below 0.05 were interpreted as statistically significant.

Results

Effects cytokines on differentiation

When C2C12 cells were incubated in differentiation media in the presence of TNFα (10 ng/ml) and/or IFNγ (10 ng/ml), the cells did not differentiate to myotubes in the differentiation protocol while in the absence of cytokines they did differentiate.

Protein breakdown C2C12-myotubes

Net protein breakdown was determined by the release in the cell culture supernatants of ^{14}C-phenylalanine from the protein content of C2C12 myotubes. No significant effects were found between control myotubes and myotubes that were incubated with TNFα (10 ng/ml) or TNFα/IFNγ (10 ng/ml) after 48-h of incubation with the cytokines (figure 1). In addition, no effects of TNFα and/or IFNγ were observed on total protein content of the C2C12 myotubes as determined by the protein Bradford assay (Control, TNFα, IFNγ or TNFα/IFNγ; 25.2 ± 0.6; 25.6 ± 0.5; 26.8 ± 1.4; 27.3 ± 2.0 mg/well respectively).

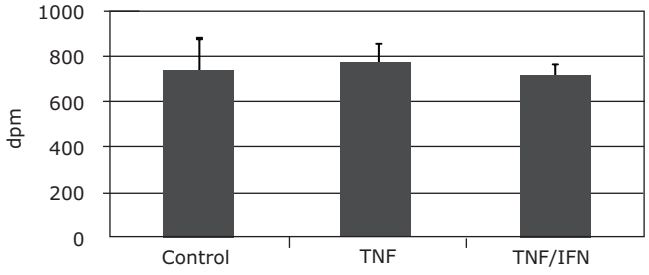

Figure 1. Protein breakdown in C2C12 myotubes. ^{14}C-phenylalanine leakage from the protein content of pre-labeled C2C12 myotubes in the cell culture supernatants. Each data point represents mean ± STD (n=6).

Morphological structural studies of C2C12 myotubes

Fully differentiated C2C12 myotubes showed aligned nuclei throughout the differentiated myotubes. When the myotubes were treated with TNFα and IFNγ, the alignment of the nuclei throughout the myotubes disappeared and the nuclei clustered (figures 2-4). Structural proteins in the myotubes were

stained with specific antibodies against tubulin, α-actinin and adult myosin. Control myotubes showed a cross-striated pattern with all stainings (figures 3-4). After 48h homogeneous α-actinin was observed in the control myotubes, while the myotubes that were treated with the combination of TNFα/IFNα showed aggregates of α-actinin (figure 3B). The cross-striated pattern that was found after myosin labeling could not be found after 48h treatment with the combination of TNFα/IFNγ (figure 4B). BrdU was added to the media to determine if the cytokines had an effect on proliferation and whether this would play a role in the nuclear clustering. After 48-h BrdU incorporation was evident in control myotube cultures (figure 5A). In myotube cultures treated with TNFα the number of labeled nuclei was higher then in the controls (figure 5B). In myotube cultures treated with IFNγ the number of labeled nuclei was lower to that in controls (figure 5C). In myotube cultures treated with the combination of TNFα and IFNγ no BrdU incorporation into the myotubes was visible, only the nuclei of the myoblasts were stained (figure 5D).

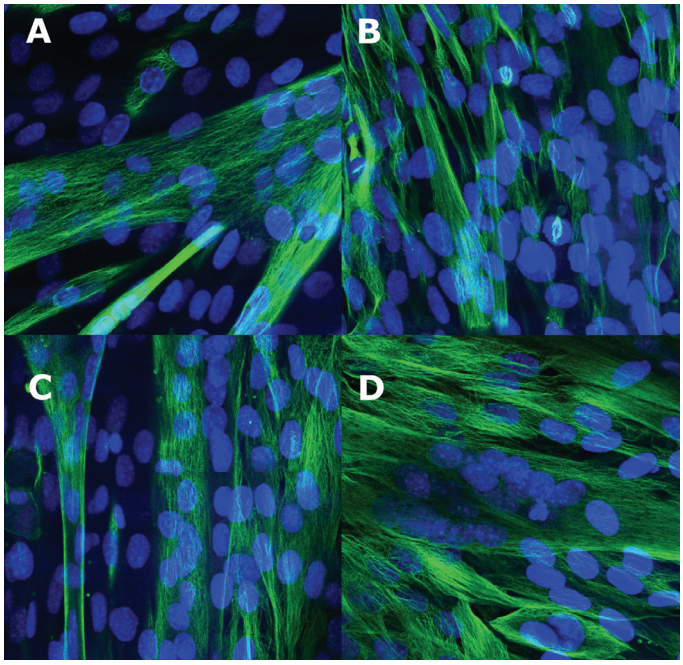

Figure 2. Confocal images of myotube cultures stained for DNA (blue) and beta tubulin (green). Myotubes were treated for 48h with no cytokines (A), TNFα (B), IFNγ (C), or TNFα and IFNγ (D). In myotubes treated with both TNFα and IFNγ a large cluster of nuclei is present in the myotube and tubulin staining is absent in that area.

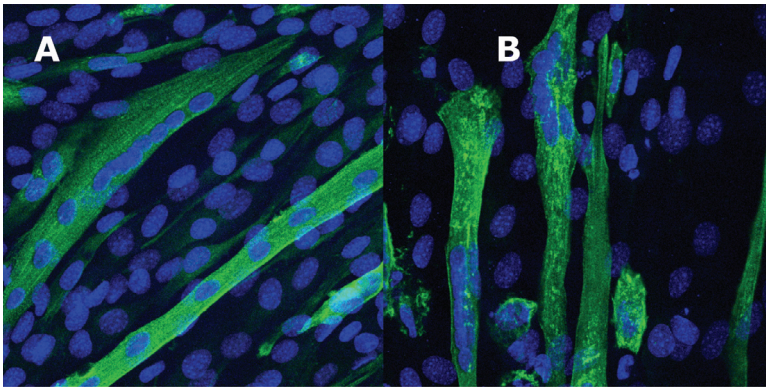

Figure 3. Confocal images of myotubes stained for DNA (blue) and sarcomeric α-actinin (green). Control (A) and myotubes treated for 48-h with both TNFα and IFNγ (B). The treated myotubes show clustered nuclei and the striated pattern in the control cells (A) has disappeared in the treated myotubes (B).

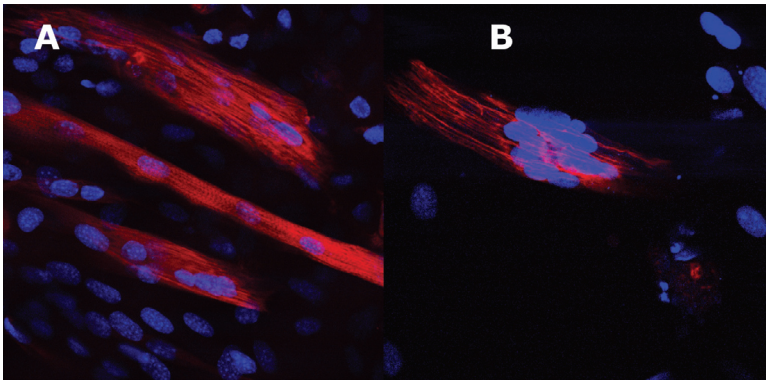

Figure 4. Confocal images of myotubes stained for DNA (blue) and myosin (red). Control (A) and myotubes treated for 48-h with both TNFα and IFNγ (B). The treated myotubes show clustered nuclei and the striated pattern in the control cells (A) has disappeared in the treated myotubes (B).

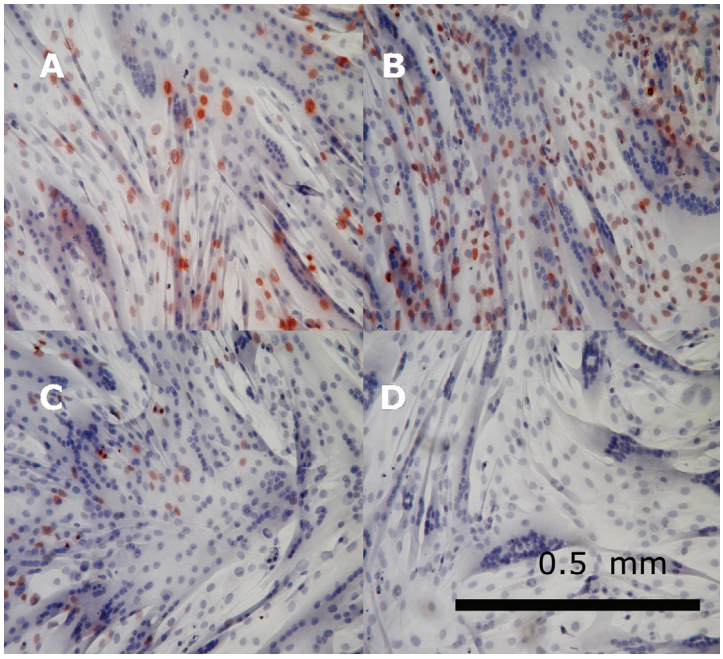

Figure 5. BrdU incorporation in myotubes. Nuclei stained red-brown have incorporated BrdU. Remaining nuclei are stained blue. Control (A); TNFα (B); IFNγ (C); TNFα/IFNγ (D). IFNγ treatment resulted in less BrdU incorporation (C). Cells that were treated with the combination of TNFα/IFNγ showed no uptake of BrdU labeled nuclei in the myotubes (D).

Caspase-3 activity C2C12 myotubes

Caspase-3 activity is increased in 48-h myotubes when incubated with the combination of TNFα/IFNγ. This increase in activity was not observed when the myotubes were incubated with only TNFα (Figure 6).

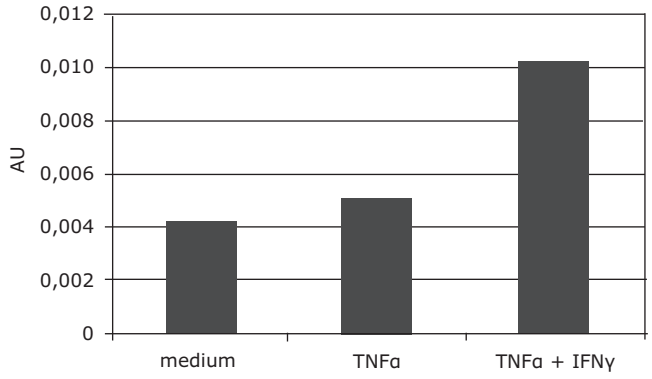

Figure 6. **Caspase-3 activity in cell cultures of C2C12 myotubes.** Activity given in Arbitrary Units (AU).

Discussion

Cancer cachexia can be defined as a wasting syndrome with the loss of skeletal muscle mass as one of the main features (19). The induction of pro-inflammatory cytokines like TNFα, IFNγ, IL-1β and IL-6 by tumor presence is associated with net skeletal muscle mass loss (20-23). The murine skeletal muscle cell line C2C12 is a frequently used model for studying skeletal muscle metabolism: when cultured upon growth factor withdrawal C2C12 cells are able to undergo differentiation by fusing into myotubes, resembling multinucleated skeletal muscle fibers (15,16). The model has frequently been used as a model for cachexia to study the effects of cytokines on protein metabolism in myotubes (24-27). However, no unambiguous results have been reported so far. For example, Alvarez *et al.* found divergent effects of TNFα on skeletal muscle protein synthesis measured by incorporation or release of radioactively labeled phenylalanine in C2C12 cells. Depending on the concentration of TNFα, the effects were catabolic (low concentrations) or anabolic (higher concentrations) (17). In addition, Los and Haagsman found that TNFα induced proliferation of myoblasts in fully differentiated cultures in low serum media and inhibited adult fast myosin accumulation. Moreover, TNFα caused a proliferation dependent increase in total cell protein (18). In this study we investigated the effects of the cytokines TNFα and IFNγ on protein metabolism, proliferation and fusion of nuclei into the myotubes. If these

processes are taken into account, some confusing results regarding protein metabolism in the C2C12 model can be explained. Moreover, the results of this study fit in the hypothesis that atrophy cannot solely be explained by protein loss alone, but the loss of nuclei from skeletal muscle fibers should be considered also.

When C2C12 myotubes were incubated with TNFα and/or IFNγ no net effects were found on total protein content of the cell cultures and, in addition, no effects were found on ^{14}C-phenylalanine leakage in the cell culture supernatants, serving as a more sensitive protein breakdown parameter. Thus, no effects were found on net protein breakdown in the C2C12 myotube cultures. However, some effects on the protein content in the myotubes were readily observed, namely that the cross-striated pattern of the structural proteins myosin, α-actinin and tubulin disappeared after treatment with the cytokines, next to a clustering of the myotube nuclei. Together, these findings fit in the hypothesis of Pajak *et al*. (28) who propose the next model of atrophy regarding protein catabolism: 'Muscle atrophy occurs at different levels, starting from repressed gene expression and ending with accelerated protein degradation. Muscle growth (myogenesis) is severely compromised and disruption of sarcomere architecture heralds the proteolysis of the contractile apparatus.' This is exactly what is observed in the C2C12 myotube model. Although cytokines induced disruption of the cross-striated patterns of the structural proteins, the 48-h protocol was probably too short to detect significant effects on net protein breakdown, because no effects of the cytokines on net total protein mass of the cell cultures were found.

Previous work of our group showed that TNFα has a dual role on protein content in C2C12 myotubes, since it induces proliferation of myoblasts that are still present in fully differentiated C2C12 myotube cultures and, in addition, inhibits adult fast myosin accumulation in the myotubes. Moreover, TNFα caused a proliferation dependent increase in total cell protein (18). Alvarez *et al.* (17) reported that TNFα could either decrease or increase protein content in muscle cell cultures, depending on its concentration. At concentrations below 1 U/ml it stimulated protein breakdown, whereas at higher concentrations (100 U/ml) it favored myofibrillar protein accumulation. In addition, when

TNFα was added together with aphidicolin, a proliferation inhibitor, they found that TNFα was catabolic at all concentrations used (24). These findings suggest that the increase in protein content induced by high TNFα concentrations were related to myoblast proliferation and not to increase of structural protein from the myotubes. Therefore, we also focused on the role of the C2C12 cells as satellite cell model in atrophy.

When added at the beginning of the differentiation protocol all cytokines inhibited terminal differentiation of the C2C12 cells into myotubes. In addition, when added to a myotube culture TNFα and IFNγ induced proliferation of the present myoblasts, however, these did not fuse into the existing myotubes (figure 5D). In addition, effects of the cytokines were found on nuclear organisation in the myotubes: TNFα and IFNγ induced a process of nuclear clustering, *i.e.,* the nuclei that are normally aligned though the myotubes, clustered (figure 2-4). From these results a simple model regarding nuclei balance can be constructed. Proinflammatory cytokines like TNFα and IFNγ inhibit myoblast differentiation, which results in a negative nuclear balance in the myotubes, since no new nuclei are incorporated into the myotubes. Hence, using the C2C12 myotube model does not appear to be the most ideal one for studying protein metabolism in cachexia, because negative protein balance is hard to observe in the myotubes. During the first 48-h of incubations, rearrangements of nuclei and proteins were observed. However, this time period seems to be too short to observe net protein breakdown measured by total protein mass or labeled amino acid leakage from the C2C12 myotubes. Unfortunately, longer incubations that 48-h were not feasible with the C2C12 myotubes. After longer incubations than 48-h the myotubes started to detach from the wells.

Adult skeletal muscle consists of fully differentiated multinucleated myofibers (29, 30). Satellite cells, stem cells of adult skeletal muscles, reside beneath the basal lamina closely juxtaposed against the muscle fibers. Satellite cells make up about 2-7% of the nuclei associated with a particular myofiber. Satellite cells are normally mitotically quiescent but are activated and re-enter the cell cycle in response to stress induced by weight-bearing exercise or trauma, including injury (29-31). The daughter cells of the activated

satellite cells, called myogenic precursor cells (mpcs), undergo multiple rounds of division prior to fusion with existing or new myofibers (29,32,33). These multinucleated cells can be compared to the myotubes, as used in the C2C12 model. Satellite cells appear to form a population of cells that are biologically and biochemically distinct from their descendant mpcs (29,32,34). The total amount of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, suggesting that a self-renewal of the satellite cell compartment maintains a population of quiescent cells (14). Myofibers are generated in the embryonic state of the organism and seem to exist during the whole life of the organism. Hence, in homeostasis there exists a continuous exchange of nuclei in the myofibers. Existing nuclei are removed through local apoptotic processes and replaced by fusion of satellite cell nuclei (35,36). However, a negative nuclei balance exists in atrophy (35,36). This has been observed in the C2C12 model. On the one hand, after the incubations with the cytokines, the cell nuclei, which are normally aligned through the myofibers, clustered. On the other hand, caspase-3 activity is increased in 48-h myotubes when incubated with the combination of TNFα/IFNγ. This increase in activity was not observed when the myotubes were incubated with only TNFα (figure 6). Caspases play essential roles in the initiation of apoptosis and caspase-3 activity is closely related to myofibrillar protein degradation in muscle in cachexia (37,38). This confirms the suggestion that the apoptotic machinery is initiated in the myotubes or residing myoblasts in reaction to the combination of cytokines.

The control of cell size and fiber size is poorly understood. Most cells have a precisely controlled cell size achieved by balancing synthesis and degradation of cellular constituents. Skeletal muscle fibers, however, are able to alter cell size during life, with, *e.g.*, increased cell size after exercise and decreased cell size after inactivity. Since muscle fibers are multinucleated syncitia, the concept of nuclear domain size (cytoplasmatic volume per nucleus) is useful in the interpretation of muscle growth and atrophy. A change in fiber size could be due to alterations in nuclear numbers, nuclear domain size or both (39, 40). In homeostasis new nuclei have to be incorporated in the myofibers to maintain protein content of the skeletal muscle. However, when satellite cell differentiation is inhibited by inflammatory cytokines, homeostasis cannot be

maintained. The intracellular apoptotic machineries are still running, resulting in loss of nuclei and, hence, of muscle mass. This loss in muscle mass *in vivo* will be interpreted as a negative protein balance. The current point of view regarding atrophy is that catabolic stimuli trigger net protein breakdown in skeletal muscle (41). The hypothesis, that is used so far, is that proteolysis is the main cause of atrophy by decreasing the domain size, resulting significant changes in nuclear number in the fibers (33,42-44). In addition, after denervation-induced atrophy, satellite cells are more susceptible to apoptosis (35). Here, we state that the reverse hypothesis is also a valid one: a decrease in the number of cell nuclei in the fiber, due to cytokine action, could result in an increase in nuclear size domain, which is corrected for by proteolysis (Figure 7). For the treatment of cachexia, it might be of less importance which one of the two hypotheses is the most relevant. However, the hypotheses offer a new perspective and new targets for interventions, since proteolysis and loss of cell nuclei occur at the same time in the fibers.

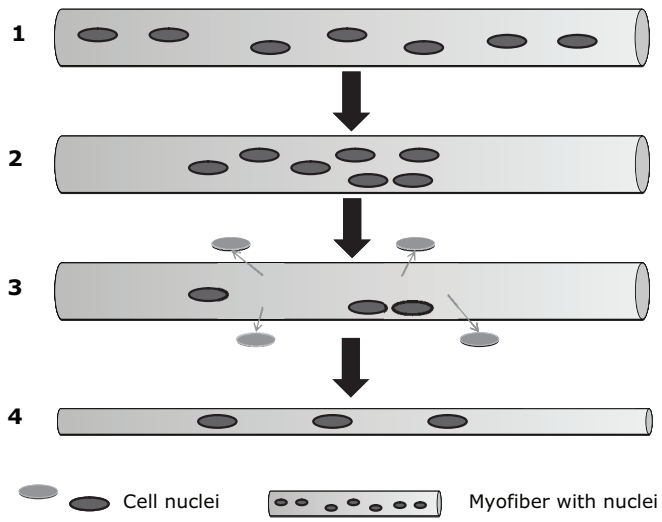

Figure 7. Schematic representation of negative nuclei balance in myofibers as based on the C2C12 model. 1. Normal myotube with aligned nuclei 2. Cell nuclei cluster after cytokine stimulation. 3. Cell nuclei are 'expelled' by apoptotic processes within the myotubes (represented by light cell nuclei). 4. After nuclei have been cleared away, the myotubes will decrease in volume by protein catabolism to correct for nuclear domain size (see text).

In conclusion, to reverse the catabolic process called cachexia, the focus should not be solely on restoring the protein pool of the atrophic skeletal muscle, but also on intervening with the signaling that induces the net loss of nuclei from the muscle fibers. This signaling prevents fusion of satellite cells into myofibers. This fusion is the prerequisite of skeletal muscle maintenance and growth and when it is inhibited it will lead to atrophy. Our observations are in accordance with those of Brack *et al*. (42). They propose a model in which a decline in satellite cell function and/or number during aging leads to a loss of nuclei from fibers and an associated domain size increase that triggers cytoplasmatic atrophy through the normal cell-size-regulating machinery. The effects in cancer cachexia can be compared to age-related atrophy, but as a more severe one in which additional catabolic signaling exists through proinflammatory mediators. These cytokines enhance the decrease in cell nuclei, not only by increasing the loss of cell nuclei from the fiber, but, in addition, by preventing the fusion of progenitor cells with the fibers as is shown in this study. Hence, the catabolic process designated as cachexia should not only be seen as a negative protein balance, but also as a negative nuclei balance in the skeletal muscle myofibers.

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Chapter 6

Dose-dependent effects of leucine supplementation on preservation of muscle mass in cancer cachectic mice

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Abstract

Rationale Cancer cachexia, characterized by muscle wasting, is associated with increased morbidity and mortality. Leucine supplementation has been reported to increase muscle protein synthesis and decrease protein breakdown. The present study aims at assessing the effects of dietary supplementation with leucine on muscle weight and markers of muscle protein breakdown (mRNA of Atrogin and Murf) in the C26 tumor-bearing cachectic mouse model.

Methods Male CD2F1 mice were subcutaneously inoculated with tumor cells (1×10^6 cells) or sham injected (Control C). The mice received standard or leucine (1 or 8 gram supplemented leucine per 100 g feed) supplemented diets : 8.7% (TB), 9.6% (TB1Leu) and 14.6% (TB8Leu) Leu/g protein. After 21 days body weights, plasma amino acid concentrations, tumor size and muscle mass of Gastrocnemius (mG), Tibialis Anterior (mTA), Extensor Digitorum Longus (mEDL) and Soleus (mS) muscles were determined.

Results Tumor bearing (TB) mice showed reduced carcass and skeletal muscle masses and increased levels of Atrogin and Murf mRNA in the mEDL. Muscle mass loss was counteracted by leucine supplementation, in a dose-dependent way; for TB8Leu: mG +23% and , mTA +22% ($p<0.05$) versus TB. However, Atrogin and Murf mRNA levels were unchanged by leucine supplementation. Total plasma amino acid concentrations were increased in TB, especially for taurine, lysine, arginine and alanine. Leucine supplementation attenuated the increase of total plasma amino acid concentrations.

Conclusion Leucine supplementation reduced muscle wasting in tumor-bearing cachectic mice and attenuated changes in plasma amino acids, unrelated to changes in protein breakdown markers.

Introduction

Cancer cachexia is characterized by a progressive loss of both fat mass and skeletal muscle mass, related to the presence of a systemic inflammation caused by the tumor presence (1-3). Therefore, the metabolic changes that occur in cancer cachexia differ from starvation (4). The observed skeletal muscle wasting in cancer cachexia is associated with increased morbidity and mortality (4,5); its severity is inversely related to the survival time of the patient (6). The essential amino acid leucine controls skeletal muscle protein balance by stimulating protein synthesis and inhibiting protein breakdown (7-10). In diabetic and somatostatin-treated fasted rats oral leucine at 1.35 g/kg BW enhanced skeletal muscle protein synthesis via both insulin-dependent and -independent mechanisms (11,12).

In weight losing cachectic cancer patients, the decrease of skeletal muscle protein synthesis is the immediate cause of muscle wasting (13). A potential approach to stimulate net muscle protein synthesis in cancer patients, therefore, is nutritional intervention with leucine. Namely, in tumor-bearing cachectic rats a diet containing 17% vs. 9% leucine altered proteasome activity (14) and was able to reduce loss of lean body mass, gastrocnemius mass and myosin content of skeletal muscle (10).

The C26 cancer cachectic model is a validated cachexia model in which CD2F1 mice are inoculated subcutaneously with murine colon adenocarcinoma (C26) cells (15-18) and subsequently loose body weight, starting after about 14 days. Recently, we observed a synergistic effect of a dietary intervention combining fish oil, high protein and leucine on maintaining body composition in the cancer cachectic model (19,20). Both fat and muscle mass were preserved when the combination was used, while the individual components did not preserve either fat or muscle mass. Moreover, combining specific oligosaccharides, fish oil, high protein and leucine improved muscle function, daily activity and improved immune function (19,20).

In this study, the effects of leucine in the C26 cancer cachectic mouse model are presented. This study aims to unravel the mechanisms behind the effect

of leucine supplementation on cancer cachexia related muscle mass loss and to define the parameters of muscle breakdown involved.

Materials and methods

Animals

Male CD2F1 mice at 6-7 weeks of age, (BALB/c x DBA/2, Harlan / Charles River, Horst, the Netherlands) were individually housed in a climate-controlled room (12:12 dark-light cycle with a constant room temperature of 21 ± 1ºC). After acclimatization for one week, mice were divided into weight-matched groups (n=10): (i) a control group (C) receiving control chow (AIN93M (21), containing 8.7% Leu per g of protein), (ii) a tumor-bearing group (TB) receiving control chow, (iii) a tumor-bearing group (TB1Leu) receiving low leucine (AIN93M + 1 g leucine per kg feed, containing 9.6% Leu per g protein) and (iv) a tumor-bearing group (TB8Leu) receiving high leucine (AIN93M + 8 g leucine per kg feed, containing 14.8% leucine per g protein). The AIN93M control diet contained per kg feed: 126 g protein (100% casein), 727 g carbohydrates and 40 g fat (100% soy oil) (Research Diet Services, Wijk bij Duurstede, the Netherlands). All experimental procedures were approved by the Animal Ethics Committee (DEC consult) and complied with the principles of laboratory animal care.

Tumor model

Murine C-26 adenocarcinoma cells were cultured *in vitro* with RPMI 1640 (Life Technologies, Merelbeke, Belgium) supplemented with 5% fetal calf serum and 1% penicillin-streptomycin (15). Tumor cells were trypsinized in a sub-confluent state and, after washing, suspended in Hanks' balanced salt solution (HBSS) (Life Technologies, Merelbeke, Belgium) at a concentration of 2.5×10^6 cells/ml. Under general anesthesia (isoflurane/N_2O/O_2), tumor cells (1×10^6 cells in 0.2 ml) were inoculated subcutaneously into the right inguinal flank of the mice. Control (C) animals received a sham injection with 0.2 ml HBSS. Following inoculation of tumor cells or sham treatment body mass, food intake and tumor size (length and width) were assessed three times per week. Animals were anaesthetized at day 21 after tumor inoculation, blood was sampled by heart puncture and tumor and skeletal muscles mEDL (Extensor

Digitorum longus), mG (Gastrocnemius), mS (Soleus) and mTA (Tibialis) were dissected and weighed.

HPLC analysis plasma amino acids

Plasma and feed amino acid concentrations and plasma 3-methylhistidine were determined with HPLC, using *ortho*-phtaldialdehyde as derivatization reagent and L-norvaline as internal standard (both from Sigma Aldrich). The method was adapted from van Eijk *et al*. (22).

RNA isolation

Total RNA from EDL skeletal muscle tissue was isolated using the RNeasy kit (Qiagen Benelux B.V., Venlo, The Netherlands) according to the manufacturer's instructions. Briefly, a minimum of 300 µl of lysis buffer was used to homogenize the muscle using a low-volume glass potter. After complete lysis, the lysate was diluted and subjected to a proteinase K (20 mg/ml) treatment. Debris was pelleted by centrifugation. Ethanol was added to the cleared lysate. RNA was bound to the silicagel membrane, and traces of genomic DNA were removed by DNAse I treatment of the RNeasy column. After washing the column the RNA was eluted in 30 µl of RNAse-free water.

Total RNA quantification and Real time PCR

The total RNA content was measured using the Ribogreen RNA quantitation kit (Invitrogen, Leiden, The Netherlands) procedure as outlined in the manufacturer's instructions with the supplied RNA as a standard. Approximately 500 ng of total RNA was transcribed into cDNA using 100 ng/µl of random hexamers (Roche Diagnostics, Almere, The Netherlands) and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Invitrogen, Leiden, The Netherlands). Real-time PCR experiments were carried out in a 25 µl reaction comprised of TaqMan Universal Mastermix (TUM, Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), 5 µl of cDNA, 900 nm of each forward and reverse primer and 200 nm Probe. A comparative Ct method was used to obtain a relative quantification of gene expression. GAPDH was used as a reference gene.

Statistics

All data are expressed as means ± SEM. Statistical analyses were performed using SPSS 15.0 (SPSS Benelux, Gorinchem, the Netherlands). Data were compared between groups with analysis of variance (ANOVA) and post-hoc LSD.

Results

Body mass, tumor weight and food intake

Leucine concentrations in the feeds were determined by HPLC and found to be 8.7% w/w protein for C and TB, 9.6% w/w in the low leucine group (TB1Leu) and 14.8% w/w in the high leucine group (TB8Leu). The average weight of the mice on day 0 was 23 g and on average they consumed 4 g chow per day, resulting in total daily leucine intakes of 348, 384 and 592 mg, respectively. Daily leucine intake, therefore, was 1.85, 2.06 and 3.17 g/kg BW, respectively, resulting in a leucine supplementation on top of the normal diet of 0.20 and 1.31 g/kg BW in the TB1Leu and TB8Leu groups, respectively. On day 21 after tumor inoculation, tumor-bearing mice (TB) had a lower body and carcass weight (carcass weight = body weight – tumor weight) when compared to control mice (Table 1A). Leucine addition to the diet did not have an effect on body weight 21 days after tumor inoculation. Furthermore, leucine supplementation did not have any effect on tumor weight. On day 20 the food intake in the TB group was significantly lower than in the C group. The food intake between tumor-bearing groups did not differ (Table 1B).

Muscle weights

At autopsy the tumor-bearing group (TB) had reduced mG, mTA, mEDL and mS weight (-28%; -28%; -32%; -22% vs C $p<0.05$, respectively). When compared to TB, muscle mass was higher for TB8Leu: mG +23% $p<0.05$, mTA +22% $p<0.05$ (Figure 1).

Plasma amino acid concentrations

At day 21 (Table 2) plasma concentrations of total amino acids (AA), total essential amino acids (EAA) and total non-essential amino acids (NEAA) in TB were higher compared to control mice. All amino acids were increased

in tumor-bearing mice, except for methionine. Taurine, lysine, arginine and alanine showed the highest increase (360%, 204%, 200% and 195%, respectively). Leucine supplementation in the high-leucine group (TB8Leu) resulted in a significant increase in plasma leucine. In contrast, in TB8Leu, total amino acids, total essential amino acids minus leucine and total non-essential amino acids were lower compared to TB. Of the individual amino acids, isoleucine, valine, lysine, alanine, asparagine, serine and glycine were significantly lower in TB8Leu compared to TB. Plasma AA levels in the TB1Leu group were also lower compared to TB animals, but for serine and glycine significant lower levels were observed when compared to TB. No effect on the ratio tryptophan/large neutral amino acids (Table 2) was observed in the tumor-bearing and leucine groups.

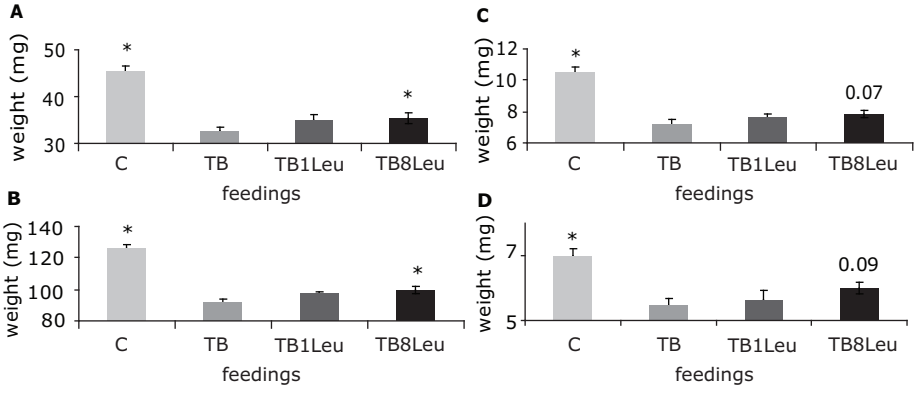

Figure 1. Muscle mass (mg) at day 21 after different interventions

A) muscle Tibialis Anterior (mTA), B) muscle Gastrocnemius (mG), C) muscle Extensor Digitorum Longus (mEDL), D) muscle Soleus (mS). C = mice receiving control diet (AIN93M), TB = tumor-bearing mice receiving control diet, TB1Leu = tumor-bearing mice receiving control diet supplemented with 1 g leucine /kg, TB8Leu = tumor-bearing mice receiving control diet supplemented with 8 g leucine /kg. Data as means ± SEM (n=10): * significantly different from TB ($p<0.05$) .

Skeletal muscle breakdown parameters and MyoD

Muscle (mEDL) mRNA levels of Murf and Atrogin, two skeletal muscle-specific ubiquitin ligases essential for protein breakdown (23) were increased in the TB group compared to the C group. No effects of the tumors were found on myoD mRNA expression, a skeletal muscle differentiation marker (24). Compared to the C group, plasma 3-methylhistidine concentrations were increased in the TB group (Table 3). Leucine supplementation had no effect on any markers of muscle catabolism in mEDL.

Table 1 Parameters in control and tumor-bearing mice with or without leucine supplementation. C = mice receiving control diet (AIN93M), TB = tumor-bearing mice receiving control diet, TB1Leu = tumor-bearing mice receiving control diet supplemented with 1 g leucine /kg, TB8Leu =tumor-bearing mice receiving control diet supplemented with 8 g leucine /kg. BW = body weight; CW = carcass weight, TW = tumor weight. Data are presented as means ± SEM (n=10): * significantly different from TB ($p<0.05$).

A	**Carcass, Body and Tumor Weights**						
Treatment	**N**	**CW**	**p**	**BW**	**p**	**TW**	**p**
C	10	25.6 ± 0.3	0.000*	25.6 ± 0.3	0.000*	±	
TB	9	19.4 ± 0.4	-	22.2 ± 0.3	-	2.8 ± 0.2	-
TB+1gLeu	9	20.2 ± 0.4	0.156	23.3 ± 0.4	0.046	3.0 ± 0.1	0.095
TB+8gLeu	10	19.2 ± 0.4	0.709	21.9 ± 0.4	0.522	2.6 ± 0.1	0.373

B	**Food Intake (per day)**					
Treatment	**N**	**1**	**6**	**10**	**13**	**17**	**20**
C	10	3.9 ± 0.1	3.4 ± 0.1	3.2 ± 0.1	3.5 ± 0.1	3.7 ± 0.2	3.3 ± 0.1*
TB	9	3.9 ± 0.2	3.5 ± 0.1	3.2 ± 0.1	3.6 ± 0.1	3.5 ± 0.2	2.1 ± 0.3
TB+1gLeu	9	3.7 ± 0.1	3.4 ± 0.1	3.0 ± 0.1	3.3 ± 0.1	2.6 ± 0.2	2.5 ± 0.4
TB+8gLeu	10	4.2 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.7 ± 0.2	3.4 ± 0.3	2.0 ± 0.3

Discussion

The C26 cancer cachectic model is a validated cachexia model in which CD2F1 mice are inoculated subcutaneously with murine colon adenocarcinoma (C26) cells (15-18) and, subsequently, lose body weight, starting after about 14

days. In the model the cachexia was followed by anorexia after approximately 19-20 days: the food intake in the tumor-bearing mice (TB) was significantly decreased at the end of the study (day 21). Leucine did not have an effect on food intake. On day 21, TB mice showed a decreased total carcass weight and the masses of mG, mTA, mEDL and mS were significantly decreased in TB mice. Leucine supplementation did not have an effect on the carcass weights of the mice. However, high-leucine (TB8Leu) supplementation decreased the loss of weight of mG and mTA. In the mEDL of the TB mice both Atrogin-1 and Murf RNA expression were up-regulated. Atrogin-1 and Murf are muscle-specific ubiquitin ligases, which play an essential role in skeletal muscle protein breakdown (23). Leucine supplementation did not have an effect on the expression of both ligases. High leucine supplementation appeared to decrease the cachexia-associated hyperaminoacidemia (Table 2). In short, although no effects were observed of leucine supplementation on whole body and carcass weight of the TB mice, high leucine supplementation attenuated the weight loss of the mTA and mG, suggesting a skeletal muscle-specific effect of leucine. Hence, leucine supplementation was shown to dose-dependently reduce the loss of skeletal muscle mass in the TB mice.

The data of leucine on skeletal muscle mass support the findings from healthy human volunteers studies, that show that leucine can have an anabolic effect on skeletal muscle protein synthesis both in young (25) and elderly men (26-28). In addition, in animal models it has been shown that leucine stimulates signaling pathways leading to protein translation through insulin-dependent and insulin-independent pathways in rats (11,12). These experiments were carried out in diabetic and somatostatin-treated fasted rats receiving 1.35 g leucine per kg BW by oral gavage, which was slightly higher than the highest concentration used in the present experiment (1.31 g/kg BW supplemented on top of the normal diet). Hence, in accordance with Anthony *et al*. (11,12) and other studies reviewed in (29-31), our study confirms that leucine can, at least partly, compensate for the loss of anabolic effects of insulin on skeletal muscle during cancer cachexia.

Table 2 Day 21 levels of plasma amino acids in control (C) and tumor-bearing (TB) mice with or without leucine supplementation. C = mice receiving control diet (ain93M), TB = tumor-bearing mice receiving control diet, TB1Leu = tumor-bearing mice receiving control diet supplemented with 1 g leucine per kg protein, TB8Leu = tumor-bearing mice receiving control diet supplemented with 8 g/kg leucine. AA = amino acid. EAA = essential amino acid, NEAA = non-essential amino acid. Data are means ± SEM (n=10): * significantly different from TB ($p<0.05$). # Plasma amino acids that increase to a higher extent in TB animals relative to the total amino acid increase of protein-based amino acids ($p<0.05$).

	Plasma amino acid concentrations of protein based AA (µM)			
EAA	**C**	**TB**	**TB1Leu**	**TB8Leu**
Histidine	93 ± 3 *	152 ± 6	144 ± 8	140 ± 5
Isoleucine	114 ± 9 *	184 ± 8	165 ± 11	152 ± 8 *
Leucine	162 ± 11 *	280 ± 13	298 ± 21	367 ± 24 *
Valine	327 ± 18 *	483 ± 19	446 ± 29	392 ± 20 *
Lysine	274 ± 15 *	561 ± 23	549 ± 20	488 ± 20 *
Methionine	81 ± 5	88 ± 4	83 ± 6	82 ± 4
Phenylalanine	56 ± 3 *	104 ± 5	94 ± 8	98 ± 6
Threonine	310 ± 22 *	472 ± 47	422 ± 38	415 ± 34
Tryptophan	46 ± 2 *	65 ± 4	62 ± 4	56 ± 3
NEAA	**C**	**TB**	**TB1Leu**	**TB8Leu**
Alanine	1105 ± 77 *	2156 ± 136	1967 ± 185	1711 ± 125 *
Glutamate	43 ± 4 *	78 ± 4	70 ± 9	66 ± 5
Glutamine	551 ± 17 *	752 ± 34	712 ± 48	646 ± 34
Aspartate	9 ± 1 *	13 ± 1	13 ± 1	12 ± 1
Aspargine	74 ± 6 *	93 ± 5	78 ± 8	74 ± 6*
Serine	214 ± 13 *	322 ± 37	253 ± 24 *	246 ± 16*
Glycine	235 ± 11 *	375 ± 22	326 ± 22 *	295 ± 16*
Arginine	80 ± 6 *	160 ± 4	149 ± 4	147 ± 8
Tyrosine	68 ± 5 *	101 ± 8	87 ± 10	84 ± 5
Totals protein based AA	**C**	**TB**	**TB1Leu**	**TB8Leu**
total EAA	1462 ± 79 *	2389 ± 92	2263 ± 125	2190 ± 94
total EAA w/o Leu	1301 ± 69 *	2109 ± 86	1965 ± 106	1823 ± 72*
total NEAA	2379 ± 116 *	4048 ± 193	3639 ± 296	3152 ± 189*
total BCAA	603 ± 37	947 ± 35	909 ± 57	911 ± 49

Totals protein based AA	**C**	**TB**	**TB1Leu**	**TB8Leu**
Total LNAA	683 ± 42	1035 ± 32	992 ± 59	995 ± 53
tryptophan/total LNAA	0,070 ± 0,006	0,062 ± 0,003	0,063 ± 0,002	0,057 ± 0,003
total AA	3841 ± 191	6437 ± 252	5902 ± 412	5342 ± 272
Non-protein based amino acids (µM)	**C**	**TB**	**TB1Leu**	**TB8Leu**
Taurine	516 ± 47 *	1858 ± 90	1662 ± 195	1797 ± 141
Citruline	71 ± 2 *	125 ± 7	122 ± 8	107 ± 5
total AA	3841 ± 191	6437 ± 252	5902 ± 412	5342 ± 272

Although food intake was reduced in the TB mice, leucine supplementation did not change food intake. This is in contradiction to clinical data indicating that BCAA supplementation might increase food intake in cancer patients, malnourished patients and patients with liver cirrhosis ((32,33)(34-36) reviewed by (37)). One of the working mechanisms suggested for this orexigenic effect of leucine is a decrease in the tryptophan to large neutral amino acid ratio (33). In the C26 mouse model the tryptophan to large amino acid ratio did not change after supplementation with leucine. So, if the hypothesis of the orexigenic effects of leucine is correct, the leucine supplementation in this study would the be too low to induce a leucine (or BCAA) induced increase in food intake.

MyoD is one of the factors that controls cell cycle propagation and induction of differentiation of skeletal muscle cells (24,38) and, hence, plays a role in skeletal muscle growth and recovery. Neither the presence of a tumor nor supplementation with leucine had an effect on MyoD mRNA expression in mEDL, suggesting that mRNA levels of the skeletal muscle differentiation marker (of anabolism) MyoD did not play a significant role in the cachectic process (Table 3). In contrast, mRNA levels of Atrogin-1 and Murf, both muscle specific ubiquitin protein ligases involved in proteolysis (23), were increased in the mEDL muscles of TB compared to C mice. These data indicate that, at least in the mEDL, markers of protein breakdown are up-regulated in tumor bearing mice. No effects of leucine supplementation were observed on mRNA levels of both ubiquitin ligases (Table 3).

Table 3 Markers for proteolysis from plasma or mEDL

C = mice receiving control diet (AIN93M), TB = tumor-bearing mice receiving control diet, TB1Leu = tumor-bearing mice receiving control diet supplemented with 1 g/kg leucine, TB8Leu = tumor-bearing mice receiving control diet supplemented with 8 g/kg leucine. Messenger RNA levels represent the 'fold induction', compared to C. For total RNA quantification, mRNA of GAPDH was used. Data are means ± SEM (n=10): * significantly different from TB ($p<0.05$).

Marker for whole body catabolism				
plasma concentration (μM)	**C**	**TB**	**TB1Leu**	**TB8Leu**
3 methyl histidine (μM)	5.7 ± 0.2*	9.7 ± 0.8	8.6 ± 0.9	9.9 ± 1.0
Marker for muscle catabolism				
Fold induction (units)	**C**	**TB**	**TB1Leu**	**TB8Leu**
mRNA Murf (units)	1.0 ± 0.1*	14.9 ± 5.5	7.1 ± 2.9	16.2 ± 3.9
mRNA Atrogin (units)	1.1 ± 0.2*	15.1 ± 2.6	8.7 ± 2.4	18.2 ± 4.8
Muscle differentiation marker				
Fold induction (units)	**C**	**TB**	**TB1Leu**	**TB8Leu**
mRNA MyoD (units)	1.0 ± 0.1	0.9 ± 0.2	1.3 ± 0.2	1.0 ± 0.2

Another protein breakdown marker is 3-methylhistidine. 3-Methylhistidine in urine is usually used as a marker for skeletal muscle protein breakdown (39,40). The concentration of plasma 3-methylhistidine has also been used as skeletal muscle protein breakdown marker in mice (41). Although the specificity of 3-methylhistidine concentrations for skeletal muscle breakdown is challenged, because also other organs might contribute to its plasma levels (42, 43), it is still considered a valid indicator when used together with other muscle-specific catabolism markers. Plasma 3-methylhistidine concentrations were increased in the cachectic mice when compared to C and correlated to muscle mass of all four muscles measured (R=-0.509 (mEDL), -0.583 (mS); -0.584 (mG); -0.541 (mTA); all $p<-0.001$), suggesting increased protein breakdown. In addition, skeletal muscle contains high amounts of taurine. When skeletal muscle decreases in volume, taurine will be expelled from the intracellular and

interstitial spaces (44), leading to increased plasma taurine levels. In the C26 model plasma the taurine level indeed increased dramatically in the cachectic mice (Table 2), and was strongly (negatively) correlated with muscle mass (R=-0.780 (mEDL), -0.607 (mS); -0.757 (mG); -0.729 (mTA); all p<-0.001).

The lack of effects of leucine supplementation on plasma taurine and 3-methylhistidine concentrations and the ubiquitin ligases Murf and Atrogin-1, in combination with the finding that leucine supplementation can increase skeletal muscle protein mass in cancer cachectic mice, suggests that the main effect of leucine on skeletal muscle protein balance is on the protein synthesis side and not on protein breakdown side.

In the TB cachectic mice, an increase in plasma total amino acids was observed when compared to control mice, this was shown for all individual amino acids, except for methionine (Table 2). This increase in amino acids in the TB group could be due to either differences in food intake, enhanced protein breakdown increased transamination and conversion or to diminished plasma clearance (by reduced protein synthesis or organ dysfunction) (45). An increase in plasma amino acids due to food intake is not likely, because food intake was reduced in TB mice and food intake between all TB mice was similar, while leucine supplementation even reduced the amino acid levels. Of both leucine groups, only TB8Leu showed a significant increase in plasma leucine concentration. In addition, in TB8Leu, total plasma amino acids decreased significantly when compared to TB. Hence, when provided in sufficient amounts, there seems to be a relationship between leucine supplementation and reduction of the measured hyperaminoacidemia. This decrease after leucine supplementation was predominant for the total non-essential amino acids, of which alanine decreased most. Alanine is a product of intracellular transamination of free amino acids, which occurs when there is an increase of intracellular free amino acids, *e.g.* during proteolysis (46). Therefore, the increase in plasma amino acids, with alanine in particular, might reflect an increase in transamination of amino acids, released from the muscle during protein breakdown. In summary, high leucine supplementation resulted in a reverse or inhibition of cancer cachexia-associated skeletal muscle mass loss

and, in addition, a reduction in the increase of total plasma amino acid levels, suggesting a return to homeostasis.

The only plasma amino acid concentration that did not change was methionine. Methionine might be rate-limiting for whole body protein synthesis on the one hand, which previously has been suggested, based on a study in AIDS patients (47) or, on the other hand, that plasma methionine levels are kept in very narrow ranges in the body. This latter hypothesis is supported by the finding that methionine concentrations do not correlate with muscle mass. The exact explanation why methionine did not change in this model needs further investigation.

The net effect of leucine on the muscle masses was small, but statistically significant. However, the effects found in this study do not appear to be clinically significant, since no effects were found on total body weight in the tumor bearing mice. Since leucine alone has not enough effects, it would be wise to combine different nutritional components with suggested efficacy in cachexia. Therefore, to further inhibit the cachectic process, we recently combined supplementation of leucine with other nutritional components aiming at restoration of the negative protein balance in skeletal muscle and body mass. Eicosapentaenoic acid (EPA) has been reported to decrease the protein catabolic signal in cachexia (48-52) and a high supply of essential amino acids has been described to be essential for increasing protein synthesis (53). Therefore, the effect of the combination of leucine, high protein and fish oil was tested on both body composition parameters as well as functional parameters. In addition, for stimulation of immune function a specific oligo-saccharide mixture (SOM) was added. This combination was tested in a milder C26 model for cachexia: one that did not result in anorexia at the end of the study period. This was obtained by injecting half amount of tumor cells and by decreasing the study period to 20 days instead of 21 days (19,20). This model still resulted in reduction of fat, muscle and body mass. In addition, muscle performance and total daily activity were impaired in tumor-bearing mice. Addition of single nutrients resulted in no or modest effects on muscle wasting (19,20). However, supplementation of the diet with the combination of high protein, leucine and fish oil significantly reduced loss of carcass,

muscle and fat mass. The specific combination of high protein, leucine, fish oil and SOM improved muscle performance, daily activity and immune function (Th1 response). These results support the need for a balanced combination of ingredients to achieve multiple effects on the complex conditions of cachexia.

In summary, cachectic and anorectic mice showed an increase in skeletal muscle protein breakdown in the C26 model involving Murf and Atrogin-1, and a decrease in carcass weight. Leucine supplementation inhibited muscle wasting dose-dependently. The positive effect of leucine on muscle metabolism was reflected in its effects on the reduction of increased plasma amino acid levels. However, the net effects of leucine on muscle mass were not substantial enough, to obtain a clinically relevant effect on whole body mass. Therefore, a multi-target nutritional approach, targeting both anabolism and catabolism is preferred.

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Chapter 7

General discussion

Introduction

Up to 50% of cancer patients suffer from cachexia, a progressive atrophy of adipose tissue and skeletal muscle, which results in weight loss, a reduced quality of life, and a shortened survival time (1). Cachexia has been estimated to be responsible for approximately 20% of deaths in cancer (2,3) and it may impair responses to antineoplastic drug treatments (4). Anorexia often accompanies cachexia, but appears only partly to be responsible for the loss of body mass (1,5). Another important feature in cancer cachexia is the presence of the acute phase response, with the secretion of acute phase proteins by the liver as one of the most prominent features (6-8). Hence, cachexia is a complex process and it is regulated by a multitude of mediators like cytokines, catabolic hormones and prostaglandins (6,8,9). In the cancer cachectic process, cytokines seem to play a major role, with the proinflammatory cytokines TNFα, IL-1β and IL-6 as the most important ones (6,10). These cytokines play dual roles in cachexia. On the one hand, they play a role in the induction of skeletal muscle protein breakdown (11-14). On the other hand, proinflammatory cytokines are important in the initiation of the acute phase protein response in the liver (6-8). Under normal circumstances, the acute phase response (APR) acts as an aspecific defense mechanism against, *e.g.,* inflammation or tissue injury, and is a rather transient reaction (15, 16). In cancer patients, however, the presence of the acute phase protein response (APR) or high circulating levels of pro-inflammatory cytokines is rather persistent and, like skeletal muscle protein breakdown, related to adverse outcome (7,17).

In the introduction of this thesis the interdependence of protein metabolism between both the muscle and the liver has been explained from a physiological perspective. This thesis focuses on gaining more insight in the mechanisms behind cancer cachexia by studying the effects of cytokines on protein metabolism in either the liver or skeletal muscle. This thesis consists of three parts. The first part (Chapters 2 and 3) focuses on the effects of cytokines on the acute phase protein response of the liver in different *in vitro* hepatocyte models, namely the human hepatocellular HepG2 cell line (Chapter 2) and three different primary rat hepatocytes cell culture techniques (Chapter 3). Two aspects of the acute phase protein response were studied in the

HepG2 model, namely the effects of different cytokines and hormones on the synthesis of different acute phase proteins. Secondly, the effects and role of substrate (amino acids) consumption and delivery on the synthesis of acute phase proteins were studied. Finally, different primary hepatocyte cell cultures were compared and validated to investigate whether these are suitable to study the effects of cytokines on acute phase protein (APP) synthesis.

The second part of the thesis focuses on the role of skeletal muscle in cancer cachexia (Chapter 5). The mouse skeletal muscle cell line C2C12 was used for this purpose. These cells are able to undergo differentiation by fusing into multinucleated myotubes. These myotubes resemble skeletal muscle, since skeletal muscle consists of multinucleated fibers (18,19). This makes C2C12 cells suitable for studying effects of cytokines on the catabolic processes in skeletal muscle. However, no unambiguous results have been reported, so far, on the effects of cytokines on skeletal muscle protein metabolism in this cell line. In this thesis the results of earlier studies in this cell line were evaluated. Based on literature and on own results, a model could be constructed, which not only can explain the confusing results which are obtained in the C2C12 model, but also gives more insight into the effects of stress mediators on skeletal muscle tissue. This means that one should not only consider protein metabolism as parameter for skeletal muscle protein breakdown, but one should take into account the complex cellular structure of skeletal muscle: skeletal muscle protein content seems to be interdependent on structural protein content, the nuclear cellular domain ratio and the contribution of satellite cells to the maintenance of net protein mass (Chapter 5).

The third and final part of the thesis (Chapter 6) describes an animal study (the C26 mouse cachexia model) in which a nutritional intervention was carried out in a severe cachectic mouse model. In this study the effect of leucine on different parameters for cachexia was investigated. This study was used to see if an effect can be observed of nutritional intervention with leucine in cancer cachexia. Leucine has been described as an amino acid that has anabolic effects on skeletal muscle protein synthesis (20-22). If this nutritional intervention would be successful, the model could also be used to study more complex nutritional interventions.

Results from this thesis

Liver models

The most commonly used *in vitro* model to study acute phase protein metabolism is the human HepG2 liver cell line (23-25). Two aspects of the acute phase protein response were studied in this model (Chapter 2), namely the effects of different cytokines and hormones on the induction of APP synthesis and the effects of amino acids on the secretion (interpreted as synthesis) of acute phase proteins. An overview of the results is given in Figures 6 A, B and C of Chapter 2. The results indicate that the acute phase protein response is dependent on the nature and sum of cytokines produced at the site of inflammation and the related hormonal response.

The first part of the studies in HepG2 cells served as a validation of the HepG2 model to study the effects of amino acid supplementation and depletion on APP synthesis. To determine the optimal cell culture conditions for studying effects of amino acids on the APP response, it was decided to focus on albumin and on fibrinogen. From the results in the first part of the HepG2 study it is concluded that these APP, from a quantitative point of view, are the most relevant in the HepG2 model. During the incubations, also IL-6 (10 ng/ml) was added to the supernatants, because IL-6 approximately doubles the fibrinogen secretion, making the APP response in the HepG2 model more suitable to study eventual effects of amino acids on the APP response. It was found that not only essential amino acids were needed to mount an acute phase protein response, but that also three nonessential amino acids, namely cysteine, serine and arginine acted as essential amino acids in the HepG2 model. However, when all essential amino acids were added, together with the three non-essential amino acids arginine, serine and cysteine, the decreased APP secretion was not corrected for to a normal response. The dependence of this model on arginine is likely to be model related. HepG2 cells are known to have defects in the urea cycle, due to missing the enzymes ornithine transcarbamylase and arginase I (26). Liver cells normally have an enormous flux of arginine thanks to the urea cycle activity, generating enough intracellular arginine to mount normal protein responses.

The results found in this study in combination with findings that malnutrition can affect the acute phase protein response *in vivo* (27), suggest that the protein content and amino acid composition of nutrition are determinants of protein synthesis in the liver. Further research is warranted to determine whether these *in vitro* results apply to the *in vivo* situation and may lead to optimal nutrition for patients with a metabolic imbalance in nitrogen distribution.

The effects of IL-6 on fibrinogen and albumin synthesis in primary rat hepatocytes were investigated to see if the effects found in the HepG2 cells were the same as in primary rat hepatocytes (Chapter 3). Different primary hepatocyte cell cultures were compared for this purpose, namely a conventional primary hepatocyte cell culture, a co-culture of primary hepatocytes with rat liver epithelial cells and a collagen sandwich cell culture. It was found that upon IL-6 stimulation fibrinogen synthesis is increased in all three cell cultures and that albumin synthesis (in contrast to HepG2 cells) was decreased. Moreover, both basal and IL-6 induced fibrinogen synthesis were much higher in the co-cultures of hepatocytes with rat liver epithelial cells.

The main difference between the HepG2 model and the primary hepatocyte cultures was that albumin acted as a negative acute phase protein in the primary hepatocytes, regarding its decreased secretion in the supernatants. These findings are in accordance with literature, referring to albumin as a negative acute phase protein (28,29). In cancer cachexia, however, it was reported that the observed hypoalbuminemia was not linked to a decrease in the hepatic albumin synthetic rate (30,31). This finding changed the idea of albumin being a negative acute phase protein. In our studies, however, albumin secretion decreased after IL-6 stimulation in primary hepatocytes. This observation seems difficult to reconcile with the studies in patients. However, it cannot be excluded that also albumin acts differently under different circumstances. As is shown in the HepG2 cells line, different cytokines and hormones can act differently on APP expression in the liver. The same might be the case *in vivo* and for albumin synthesis in the liver. It is possible that IL-6 alone can lead to a decrease in albumin synthesis, while other cytokines can inhibit this effect.

In the same model of primary hepatocyte cultures, it was found that arginine, in contrast to all other amino acids in the cell culture supernatants, was depleted in the cell culture supernatants of the primary hepatocyte cultures, but also from the supernatants of precision-cut liver slices (Chapter 4). This was due to arginase leakage from (probably broken) cells into the cell culture supernatants. This suggests that the *ex vivo* system is not the best model to study acute phase protein synthesis. However, although the cell culture media in primary hepatocytes was depleted in arginine, the cells were still able to mount an APP response. In contrary, HepG2 cells were not able to mount a complete APP response, since the secretion of both albumin and fibrinogen was dramatically decreased upon arginine depletion of the cell culture media. The APP production of the primary hepatocytes in arginine-depleted media, confirms the hypothesis that the urea cycle produces enough intracellular arginine to be supplied as substrate for APP synthesis. HepG2 cells were not able to compensate for arginine depletion, since these are seemingly not able to mount a complete urea cycle (26).

In addition, it was shown that when using urea synthesis as viability parameter in primary hepatocyte cultures, one should realize that arginase can escape from the cells and leak into cell culture media. The arginase will split arginine into ornithine and urea, resulting in false-positive results which can be interpreted as positive cell viability (32), since in the urea cycle mitochondrial transmembrane transport of urea cycle intermediates (ornithine and citrulline) is involved (33-35). So, based on the results in this thesis, one cannot simply conclude that urea appearance in cell culture supernatants is a sign of viability. The only right way to use urea cycle activity as a viability indicator, is to culture the primary hepatocytes or precision-cut liver slices in a separate cell culture in arginine free media, supplemented with ornithine and NH_4Cl (33-35). These are the substrates forcing a complete urea cycle, including mitochondrial transmembrane transport, which is the basis of the viability parameter. Hence, the cells first have to produce endogenous arginine, before it can be converted to urea (and ornithine) in the final step of the urea cycle (Chapter 4) (32).

Nutrition in cancer cachexia: from liver towards skeletal muscle

The introduction of this thesis gave a short historical account from the beginning of the 1980s until now of the changing ideas of nutritionists about the intervention of the cachectic process. Before the 1980s cancer cachexia was mainly interpreted as malnutrition. And if patients lost weight, this was interpreted as a negative energy balance. When studying the literature, special emphasis towards protein-energy malnutrition arises from the end of the 1970s and beginning of the 1980s (36-38). The intervention parameters in these studies were either body weight (39) or biochemical parameters like plasma albumin concentrations (40,41).

In the recent two decades, focus of nutritional intervention in cancer cachexia shifted from supporting APP synthesis (42-46) towards trying to maintain skeletal muscle protein mass (47-50). Regarding nutritional support for liver APP synthesis, two aspects are relevant in the scope of this thesis. The first one is that during an acute phase response (APR) the body lays priority on liver APP synthesis, resulting in net loss of skeletal muscle protein mass. Even in malnourished circumstances, the body is still able to mount an APP response, with only small differences between well-nourished and malnourished patients (42). A second aspect is that the acute phase response fulfils a useful role in inflammatory conditions like wound healing or infection (51). Also, in cancer cachexia, the inflammatory mediators that are initiated by presence of the tumor induce an APP response. However, APP do not seem to play a role in reversing the underlying processes of cancer cachexia, namely tumor presence. Evidently, supporting the APP response in cancer cachexia seems to be quite contradictionary since APP do not seem to play a major role in reversing cancer or cachexia. This is in contrast to *e.g.* post-operative nutrition, where the APP play an important role in recovery (42,52). However, if one wants to support skeletal muscle protein synthesis in cancer cachexia, enough amino acids should escape the increased first-pass extraction of amino acids by the liver to be available for the skeletal muscle. This means that sufficient nutritional amino acids should be provided and this implies that automatically APP synthesis will be supported, which is unnecessary in cancer patients. In summary, when composing a nutrition for cancer patients, the APR response

should not be the priority. The focus should be on retaining skeletal muscle protein mass.

Role skeletal muscle in cancer cachexia

Inflammatory cytokines such as IL-6, IFNγ and TNFα are likely to, together with other catabolic hormones and inflammatory mediators, contribute to the effects of inflammation on muscle protein metabolism through several pathways (10,53,54). In this thesis the effects of cytokines on skeletal muscle protein were chosen to investigate because they can act as catabolic mediators on skeletal muscle on the one hand and on the other hand, they are the same mediators that can induce APP synthesis in the liver. In the introduction of this discussion, it was already mentioned that cachexia can be seen as a kind of 'biochemical shock' in which the priority of protein metabolism lays more in the splanchnic area, with the liver being the most important anabolic organ regarding protein synthesis. The advantage of this mechanism, from a survival point of view, is that the body gives priority to the synthesis on proteins that play a role in wound healing and restoring homeostasis. After the organism has returned to homeostasis, the skeletal muscle protein pool will be replenished (55). Nevertheless, during the APR skeletal muscle seems to function as a reservoir for amino acid supply for APP synthesis (7,56). If the duration of the APR is so long that skeletal muscle protein will exhaust, this will lead to complications, because, *e.g.,* respiratory muscles will be depleted, resulting in respiratory failure: cachexia, is responsible for more than 20% of the overall deaths in cancer patients (2,3). Hence, one of the main targets in the treatment of cancer, besides antineoplastic therapy, is retainment of the skeletal muscle protein pool, either by drug treatment or nutritional intervention (3,47,57).

When taking into consideration whole body protein metabolism in cachexia, it is obvious that the basis of a clinical nutrition for cancer cachectic patients should be a high-protein content. First, because amino acids are required to restore protein content in the skeletal muscle. Second, but as important, a high protein content is essential, because the first-pass extraction of amino acids in the liver is increased significantly, due to increased amino acid demands for *e.g.* gluconeogenesis (58), acute phase protein synthesis (59)

and glutathione synthesis (60). Therefore, enough protein should be supplied to the patient to overcome first-pass extraction of amino acids by the liver, resulting in a significant increase in the plasma concentrations of amino acids. Namely, muscle protein synthesis is modulated by extracellular amino acid availability in a dose-dependent matter (61,62).

The C2C12 skeletal muscle model

Since the skeletal muscle protein breakdown should be avoided in the cachectic process, also the effects of cytokines on a skeletal muscle model were investigated (Chapter 5). For this purpose the C2C12 mouse skeletal muscle cell line was used. In the C2C12 model it was found that, like in the liver, the response of the skeletal muscle cells depends on the nature of the cytokines and the concentration in which they were present in the media. The most important conclusion from this part of the thesis is that, from a concept point of view, cachexia should not solely be interpreted as a negative protein balance *per se*, but also as a 'negative nuclei balance' in the skeletal muscle myofibers (for explanations see figures 1 and 2). To reverse the catabolic process one should not only focus on restoring protein mass of the atrophic skeletal muscle, but also on intervening with the catabolic mediators that interfere with cell fusion, which is required for muscle maintenance and growth.

These observations are in accordance with the model proposed by Brack *et al.* (63). They propose a model in which a decline in satellite cell function and/or number during aging leads to a loss of nuclei from fibers and an associated domain size increase that triggers cytoplasmic atrophy through the normal cell-size-regulating machinery. The effects in cancer cachexia can be compared to age-related atrophy (sarcopenia) (64-66), but as more severe one, in which extra catabolic signaling exists through proinflammatory mediators. These cytokines enhance the decrease in cell nuclei per muscle fiber, not only by inducing the loss of cell nuclei from the fiber, but in addition by preventing fusion of satellite cells with existing fibers as is shown in Chapter 5. The use of the C2C12 myotube model for studying protein metabolism in cachexia appears not to be the most ideal one, since net protein breakdown is hard to observe in the myotubes. During the first 48-h, rearrangements of nuclei

and proteins are observed after incubations with TNFα and IFNγ. However, this time period seems to be too short to observe net protein breakdown measured by total protein mass or labeled ^{14}C-phenylalanine release from the C2C12 myotubes.

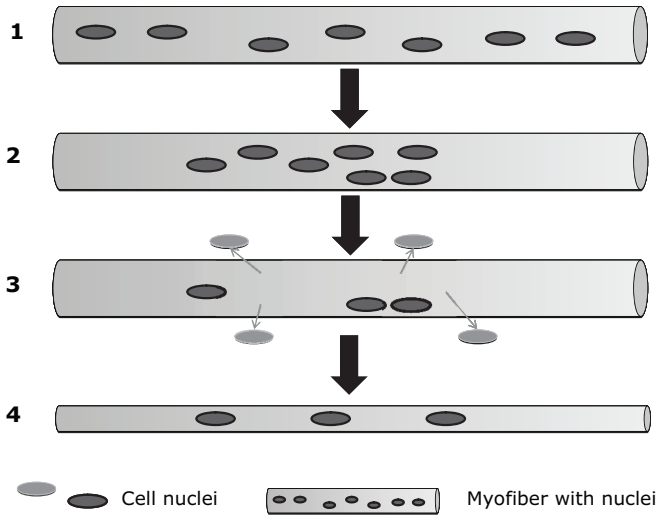

Figure 1 Model: effect of cytokines on negative nuclei balance, leading to atrophy (based on observations in C2C12 myotubes. 1. A normal myotube has aligned nuclei 2. Cell nuclei cluster after cytokine stimulation. 3. Subsequently, cell nuclei are 'expelled' by apoptotic processes within the myotubes (represented by light cell nuclei). 4. After nuclei have been cleared away, the myotubes will decrease in volume by protein catabolism to correct for nuclear domain size, resulting in atrophy.

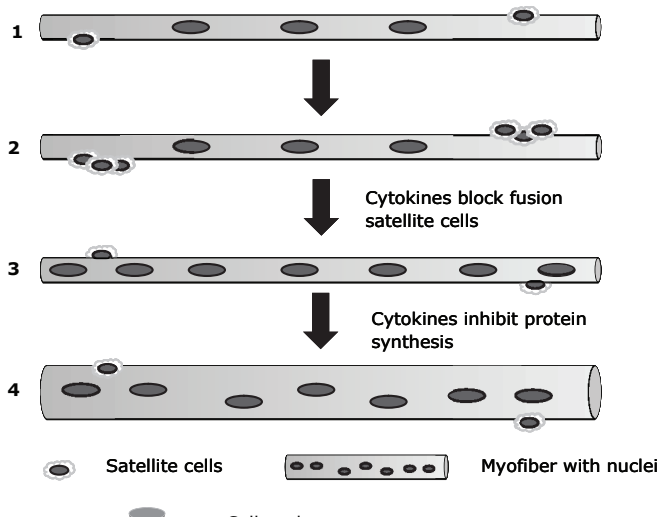

Figure 2. Model: satellite cells in skeletal muscle growth, maintenance and recovery. Muscle fibers are unique tissues, because they are syncitia that are formed by fusion of myogenic precursor cells. Adult skeletal muscle fibers have aligned nuclei throughout the fiber, with adjacent satellite cells (1). These are dormant cells that can be activated by myotrauma, exercise or recovery to proliferate (2) and subsequently fuse into the fibers (3). When fused into the fiber, protein synthesis will be induced for skeletal muscle growth (4). From experiments in the C2C12 myotube model it is concluded that cytokines have the ability to inhibit fusion of satellite cells into myotubes and, therefore, inhibit net protein synthesis in the skeletal muscle and, hence, muscle growth.

Muscle atrophy occurs at different levels, starting from repressed gene expression and ending with accelerated protein degradation. Muscle growth (myogenesis) is severely compromised and disruption of sarcomere architecture heralds the proteolysis of contractile apparatus (8). The disruption of the sarcomere architecture was observed in the C2C12 myotubes: tubulin and actin filament staining showed clear rearrangements of the contractile (cross-striated) proteins in the myotubes.

In vivo modeling: the mouse C26 cachexia model

In chapter 6 of the thesis, an *in vivo* C26 mouse cachectic model was used to investigate whether it was possible to intervene with cancer cachexia with the essential amino acid leucine. Leucine is known to have the propensity to mount an anabolic effect on skeletal muscle protein synthesis, additional to the anabolic effect of insulin (67-69). In the aforementioned studies, high concentrations of leucine were given in the animals. The study in this thesis is used to investigate whether it is possible to intervene with the cachectic process by feasible amounts of leucine in nutrition. We found a skeletal muscle specific anti-catabolic effect of leucine in the cancer cachectic mice. Although skeletal muscle-specific anabolic effects were found in the cachectic mice, no effects were found on whole body mass of the mice.

In conclusion, the nutritional intervention of cancer cachexia by leucine in the C26 model is not sufficient enough to obtain effects on whole body weight of the animals. Hence, a multi-target approach is preferred to obtain a clinically relevant effect on whole body mass. Therefore, the effects of a combination of leucine, high protein and fish oil was tested on both body composition parameters as well as functional parameters (70). In addition, for immune stimulation a specific oligosaccharide mixture was added. Muscle performance and total daily activity were impaired in tumor-bearing control animals. Addition of the single nutrients resulted in no or modest effects on muscle wasting. However, supplementation of the diet with the combination of the nutritional components resulted in a significant reduction of loss of carcass weight, muscle and fat mass. In addition, also improvements on muscle performance and daily activity) were observed (70). So the results of this thesis in combination with those of van Norren et al. (70) support the hypothesis for the need of a well-balanced combination of nutritional components to achieve multiple effects to counteract the cachectic process.

Conclusions of this thesis

The acute phase protein response by the liver is a complex result of interactions of cytokines and hormones and might not be a general reaction, but one that

is dependent on the amount and nature of cytokines and hormones that are secreted during inflammatory reactions (Chapter 2).

Essential amino acids are not sufficient to mount an acute phase protein response in the human liver HepG2 cell line; at least arginine, cysteine and serine act as essential amino acids in the HepG2 model (Chapter 2).

Co-culture of primary rat hepatocyte with rat liver epithelial cells was found to be the best to study fibrinogen synthesis by primary hepatocytes (Chapter 3). In primary hepatocyte cell cultures albumin acts as a negative acute phase protein, *i.e.*, its secretion significantly decreases after IL-6 stimulation (Chapter 3).

Leakage of the urea cycle enzyme arginase from primary hepatocytes may deplete the cell culture supernatant of arginine (Chapter 4).

Arginine does not act as an essential amino acid in primary hepatocyte cell cultures, since in arginine depleted media in primary hepatocyte cultures, the cells are still able to mount an acute phase protein response (Chapter 4 and Chapter 7).

C2C12 cell differentiation to myotubes is dependent on cell fusion. Cell fusion and thus differentiation is inhibited by the proinflammatory cytokines TNFα and IFNγ (Chapter 5).

Skeletal muscle mass is dependent on fusion of 'new nuclei' from satellite cells into existing muscle fibers. When inclusion of new nuclei into the fiber is blocked, this will result in atrophy of the fiber (Figures 1 and 2)(Chapter 5). Leucine is able to, dose-dependently, reduce skeletal muscle mass loss in the C26 cancer cachectic mouse model (Chapter 6).

Supplementation of nutrition with 14.3% leucine did not result in a net effect on body weight in C26 cancer cachectic mice (Chapter 6).

Both skeletal muscle protein metabolism and liver acute phase protein synthesis are to be taken into account for composing an optimal nutrition for cancer cachectic patients. Although skeletal muscle should be the organ to focus on, the increased first pass extraction of amino acids by the liver should be overcome to provide sufficient amino acids to promote skeletal muscle growth.

In order to reverse cancer cachexia with nutrition it is advised to apply a multi-factorial approach in which, at least, sufficient amino acids are supplied to overcome first-pass extraction by the liver in order amino acids to be available for skeletal muscle protein synthesis. In addition, the nutritional design should encompass components that stimulate protein synthesis (*e.g.* leucine), components that inhibit inflammation or inflammatory mediators, and components that decrease acute phase protein synthesis by the liver (*e.g.* n3-fatty acids) (Chapter 7).

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Chapter 8

Nederlandstalige samenvatting

Deze samenvatting bestaat uit twee delen. In het eerste deel zal ik proberen uit te leggen wat cachexie precies is om in het tweede deel de uitkomsten van mijn onderzoek samen te vatten.

Cachexie, wat is dat nu eigenlijk?

Cachexie. Het is een woord dat verschrikkelijk klinkt en als je deze samenvatting hebt gelezen, zal je begrijpen dat het ook over een verschrikkelijk proces gaat. Het komt bij een aanzienlijk deel van de kankerpatiënten voor en dan voornamelijk bij patiënten in de terminale fase van de ziekte. Ik heb het over het proces dat ten grondslag ligt aan het typische verschijnsel dat kankerpatiënten ineens veel in gewicht afvallen. Zo veel zelfs, dat ze er door verzwakt raken, bedlegerig worden of nog erger. Dit proces, van onvrijwillig gewichtsverlies en verzwakking noemen we cachexie. Het woord cachexie is van het Grieks afgeleid. Als je het zou vertalen betekent het iet van ´slechte conditie´. Maar dan is dat wel zwak uitgedrukt. De mate van het gewichtsverlies in cachexie heeft zelfs een voorspellende waarde voor overlevingskansen en kansen op complicaties tijdens de ziekteduur. Hiermee bedoel ik: hoe meer gewichtsverlies, hoe kleiner de kans op overleven. Dus het is belangrijk om dit gewichtsverlies tegen te gaan.

Als mensen gewicht verliezen zou je dat tegen kunnen gaan door ze veel energierijke voeding te geven. Gezonde mensen komen daar namelijk van aan, zoals je weet. Lekkere vette hap dus. Heel vroeger werd dit ook wel gedaan. Het probleem is echter dat het bijvoeden met energierijke voeding het proces van gewichtsafname en verzwakking in cachexie helemaal niet stillegt. De patiënten blijven gewoon afvallen. Hoeveel vette hap ze ook eten. Dus dat helpt niet. Laat staan dat de patiënt er vitaler van wordt of er zich beter van gaat voelen. Er is dus meer aan de hand dan dat de patiënt alleen maar te weinig energie binnen krijgt. Nee, het is niet het vetverlies dat voor de complicaties zorgt, maar het is het verlies van ander weefsel dat voor de problemen zorgt bij cachexie (en dus bij veel kankerpatiënten), namelijk verlies aan spierweefsel. Dit geeft ook meteen het verschil aan tussen afvallen door cachexie en met afvallen met diëten. Bij diëten (eigenlijk vasten) verlies je voornamelijk vetmassa. Bij cachexie verlies je (naast

vetmassa) aanzienlijk veel van je spiermassa en omdat de spier voornamelijk uit eiwit bestaat, eiwitmassa dus. Juist dat verlies aan spiermassa geeft de problemen bij de patiënt. Patiënten die erg 'cachectisch' zijn (dus erg veel gewicht – en dus spiermassa – verliezen) raken soms zo verzwakt dat ze zelfs ademhalingsproblemen kunnen krijgen. Eén groep van spieren die als laatste wordt afgebroken, zijn namelijk de ademhalingsspieren. De patiënt kan zo verzwakt raken dat er soms zelfs ondersteuning bij de ademhaling moet worden gegeven. Het is dus erg belangrijk dit proces van cachexie tegen te houden, want in cachexie verbruikt de patiënt zo'n beetje alle reserves van zijn lichaam.

Kortom, de truc in de behandeling van cachexie is om de spierafbraak tegen te gaan. Dat is niet erg makkelijk. De onderliggende processen achter cachexie zijn namelijk erg complex en niet zo maar tegen te houden. Wat er eigenlijk tijdens cachexie gebeurt, is dat het lichaam in een status van een op hol geslagen ontstekingsreactie is geschoten. Hierbij zijn verschillende organen en cellen betrokken. Om nu uit te kunnen leggen wat nu precies cachexie is, leg ik je eerst iets anders uit. Ik ga je uitleggen hoe het lichaam reageert op een simpele operatie. Na een operatie komt er een ontstekingreactie op gang, die lijkt op die bij cachexie. Deze reactie noemen we de acute fase reactie. Er is echter één verschil tussen de acute fase reactie na een operatie en die bij kanker: die na een operatie optreedt, zorgt voor genezing. Die bij kanker optreedt, zorgt niet voor genezing. Als je de volgende alinea's hebt doorgelezen en begrijpt wat de acute fase reactie is, ben je al aardig op weg om te begrijpen wat cachexie is.

De acute fase reactie

Tijdens een operatie wordt je lichaam door middel van een incisie opengemaakt door een chirurg. Eigenlijk maakt hij een wond in je lichaam. Je weet dat na het hechten van de wond, deze uiteindelijk geneest. Hoe komt dit nu precies? Op het moment dat er in het lichaam iets aan de hand is, zoals in dit geval een wond door een operatie, komen er cellen uit het bloed bij de wond terecht. Die cellen (afweercellen) merken dat er iets aan de hand is en nemen de regie in handen om de schade die is ontstaan te herstellen. Dit doen ze op twee manieren. De cellen gaan helpen bij het opruimen van de ontstane schade.

Ze 'eten' de troep op en voeren het af. Daarnaast doen ze nog iets anders. Ze gaan een heleboel verschillende stoffen uitscheiden, signaalstoffen. Die stoffen gaan de bloedbaan in en worden in het lichaam verspreid. De belangrijkste stoffen die ze uitscheiden zijn de zogenaamde cytokines. Deze cytokines zijn eigenlijk boodschappers die op verschillende plaatsen in het lichaam gaan vertellen dat er ergens anders 'iets aan de hand is'. Ze vertellen niet wat er aan de hand is, maar gewoon: 'er is iets niet goed en er is hulp nodig'. Op het moment dat deze stoffen verschijnen, gaat de rest van het lichaam over op het inzetten van hulptroepen. Met andere woorden, het lichaam gaat door deze cytokines over van het 'stationair draaien' op de 'ontstekingsmode'. Alles wordt nu in het werk gesteld om problemen elders in het lichaam op te lossen. Dit proces noemen de we *acute fase reactie*. Bekende tekenen hiervan zijn onder andere koorts, pijn en minder honger (anorexie). Dit heeft allemaal te maken met het herstel van de, in het geval van de operatie, ontstane wond.

Een ander orgaan dat de signalen van de cytokines opvangt, is de lever. Zodra de lever de signalen binnen krijgt, weet hij dat er ergens in het lichaam iets aan de hand is. De lever start meteen met het maken van allerlei soorten eiwitten en scheidt die uit in het bloed. De eiwitten die de lever uitscheidt, spelen zowat allemaal een rol in het herstellen van de wond. Zo maakt de lever fibrinogeen, dat belangrijk is voor bloedstolling. Hij maakt het zogenaamde C-reactive protein (CRP), wat helpt bij het opruimen van bacteriën en ontstekingen te voorkomen. Hij maakt haptoglobine dat wordt gebruikt bij het opruimen van (rode) bloedcellen. En zo maakt de lever tientallen verschillende soorten eiwitten die allemaal een bepaalde rol spelen bij het herstel van de wond, maar ook ter bestrijding van de infectie, het opruimen van troep enz. enz. De verzamelnaam van deze eiwitten is: Acute fase eiwitten. Deze acute fase eiwitten worden dus eigenlijk door de lever het lichaam ingegooid nadat het signalen heeft gekregen dat er iets aan de hand is in het lichaam, zonder dat hij precies weet wat er aan de hand is. Het gaat hier om een zogenaamde 'aspecifieke' reactie.

In het geval van de wond die voor de operatie is gemaakt, zorgen de acute fase eiwitten ervoor dat de wond geneest. Het bloed wordt gestold, bacteriën worden opgeruimd en de ontstane troep wordt netjes afgevoerd. Op het

moment dat dit is gebeurd (na genezing), gaan de afweercellen weg en gaat het lichaam weer terug op 'stationair draaien'. Die 'aspecifieke afweerreactie' wordt dus de acute fase respons genoemd. De reden dat ik heb uitgelegd wat de acute fase respons precies is, komt omdat deze respons ook een rol speelt bij kanker cachexie. Hierover gaan de volgende alinea's.

De acute fase respons en cachexie

Wat gebeurt er nu precies bij kanker cachexie? Eigenlijk start het ongeveer het zelfde als bij de acute fase reactie na een operatie. Bij het groeien van een tumor, hebben de afweercellen in de gaten dat er iets aan de hand is. Ze gaan vervolgens cytokines uitscheiden. De lever vangt het signaal op en gaat de acute fase eiwitten in het lichaam uitscheiden. Er is nu echter een probleempje. Als je naar de functies van die eiwitten kijkt, dan zie je dat die acute fase eiwitten die de lever uitscheidt, niets kunnen doen aan de tumor. Er worden geen eiwitten uitgescheiden die de tumor op kunnen ruimen. Het gevolg is dat de afweercellen cytokines blijven uitscheiden en de lever alsmaar die acute fase eiwitten blijft maken. De tumor blijft groeien en het de reactie wordt steeds heviger. Eigenlijk is er een soort van kortsluiting in het eiwitmetabolisme.

Zoals hierboven beschreven, is de acute fase reactie een proces dat is gemaakt om problemen op te lossen en het blijft doorgaan totdat het probleem is opgelost. In het geval van kanker wordt het probleem echter niet opgelost. De tumor blijft gewoon groeien. De acute fase respons blijft aanstaan. Dat het proces blijft aanstaan hoeft op zich geen probleem te zijn. Echter, één van de verschijnselen van acute fase respons is onder andere ook verminderde eetlust. De vraag is nu of je met die verminderde eetlust via de voeding wel voldoende eiwit binnen krijgt om te voorzien in bouwstoffen (aminozuren) voor de aanmaak van acute fase eiwitten in de lever. Het antwoord hierop is nee. Toch vindt het lichaam dit proces zo belangrijk, dat het alles in het werk stelt om de acute fase eiwit productie door te laten gaan. Het lichaam wil het probleem koste wat het kost oplossen. Hoe krijgt het lichaam dit dan voor elkaar?

Voor de acute fase eiwit synthese zijn bouwstoffen nodig. De bouwstoffen van eiwitten zijn aminozuren. Het lichaam heeft een enorme voorraad aan aminozuren. Die zitten namelijk in de eiwitten in spieren. Spieren bestaan voornamelijk uit eiwit. Het lichaam gaat nu spiereiwitten afbreken. Daar komen de aminozuren bij vrij. Die worden door de spier uitgescheiden in het bloed en komen zo in de lever terecht. De lever neemt ze op en kan zo weer acute fase eiwitten maken. Dit proces loopt niet 100% efficiënt. Niet alle aminozuren die door de spier worden uitgescheiden komen in de acute fase eiwitten terecht. De aminozuren die over zijn, worden door de lever opgeruimd. Dit heeft tot netto effect dat het lichaam eiwit aan het afbreken is. Dit proces ligt ten grondslag aan cachexie. De spieren blijven eiwit afbreken tot een moment dat alle eiwitten, en dus de spieren afgebroken zijn. De patiënt wordt dan erg mager en raakt dan verzwakt. Bij terminale patiënten waar dit proces al een tijd op gang is, kan het zelf zo erg zijn dat zelfs de ademhalingsspieren worden afgebroken, met alle gevolgen van dien. Dit is dus cachexie.

Samenvattend: Cachexie kan je definiëren als de afbraak van spieren, die aminozuren leveren voor acute fase eiwitten die door de lever worden gemaakt. De patiënt valt hierdoor enorm in gewicht af en raakt door al zijn reserves heen. In extreme gevallen kan dat zelf leiden tot de dood. Dit proces wil je dus te allen tijde tegenhouden. Om het proces tegen te houden, moet je wel weten wat er precies gebeurt in het lichaam tijdens cachexie. Dat is de reden waarom het onderzoek uit dit proefschrift is gedaan. Door beter te weten wat er aan de hand is in de lever en de spier tijdens cachexie, kan je misschien wel een bepaalde voeding ontwikkelen die het proces van cachexie kan tegen houden.

Goed, na deze lange inleiding, kan ik nu beter uitleggen wat ik heb gedaan in mijn promotieonderzoek en wat ik heb ontdekt. In het tweede deel van deze samenvatting, zal ik kort de belangrijkste bevindingen van mijn onderzoek proberen uit te leggen.

De belangrijkste bevindingen uit dit proefschrift

Hierboven heb ik beschreven wat kanker cachexie ongeveer is. Hieronder ga ik puntsgewijs weergeven wat ik heb gevonden in het onderzoek dat staat beschreven in dit proefschrift. Hoofdstuk 2 tot en met 4 van dit proefschrift gaan over onderzoeken die ik met levercellen heb gedaan. Die experimenten zijn uitgevoerd om te kijken hoe je de acute fase eiwit synthese (productie) kan beïnvloeden en of modellen met verschillende soorten levercellen wel representatief zijn voor wat er in de lever in het lichaam gebeurt.

In hoofdstuk 2 zijn experimenten beschreven waarin gebruik gemaakt werd van levercellen van menselijke oorsprong, zogenaamde HepG2 cellen. Dit zijn cellen die ooit eens uit een levertumor zijn gehaald bij een mens en vervolgens zijn doorgekweekt om in het laboratorium te kunnen gebruiken. Tumorcellen blijven altijd delen en daarom kan je deze cellen heel lang in leven houden om ze voor experimenten te gebruiken. Hoewel ze niet precies hetzelfde reageren als echte menselijk levercellen, kunnen ze wel worden gebruikt om de acute fase eiwit synthese te bestuderen, omdat ze veel van deze eiwitten produceren. Wat ik met de HepG2 experimenten heb gevonden, is dat het mogelijk lijkt om met een bepaalde samenstelling van de voeding (eiwitten) de acute fase eiwit synthese te beïnvloeden. Althans, in deze levercellen (HepG2 cellen). Of dit ook geldt voor de patiënt, moet nog uitgezocht worden. Eerder heb ik omschreven dat de acute fase eiwit respons een aspecifieke respons was. Maar als je verschillende cytokines aan de HepG2 cellen toevoegt, dan zie je dat al die combinaties aan cytokines andere effecten hebben op de acute fase eiwit synthese. Dat kan betekenen dat de respons specifieker wordt aangestuurd dan eerder was aangenomen. Met andere woorden: verschillende cytokines worden afgescheiden na bijvoorbeeld een operatie of na een infectie met bacteriën. Die verschillende cytokines zorgen er dan voor dat er andere acute fase eiwitten worden uitgescheiden door de lever, misschien meer gericht op de oorzaak van het probleem. Dus bijvoorbeeld dat er meer fibrinogeen wordt uitgescheiden bij een verwonding om het bloed te stollen dan bij een infectie met bacteriën. Om deze conclusie te rechtvaardigen is nog wel meer onderzoek nodig.

In het derde hoofdstuk is een studie beschreven naar welke methode het beste is om acute fase eiwit synthese te bestuderen in zogenaamde primaire levercellen. Dit zijn levercellen die direct uit de lever van een rat zijn gehaald. Dit is een ander soort cellen dan de HepG2 cellen. Deze cellen zijn namelijk gezond en geen tumorcellen, zoals HepG2 cellen. Er zijn verschillende soorten van celkweek geprobeerd. Wat bleek, is dat cellen veel meer acute fase eiwitten produceren op het moment dat ze met een andere celsoort worden gekweekt, zogenaamde epitheelcellen, die ook in de lever voorkomen. In de echte lever, liggen de levercellen tussen deze epitheelcellen. Dus het kweken van levercellen met epitheelcellen lijkt veel meer op de situatie in de echte lever, dan het kweken van levercellen alleen. De resultaren in dit hoofdstuk laten zien dat deze manier van celkweken het beste is om acute fase eiwit synthese te bestuderen.

Als je cellen kweekt, dan neem je altijd bepaalde controles mee om te kijken of de kweken goed verlopen. Een van die controles is de vorming van ureum. Dit is een stof die zowat alleen in de lever wordt gevormd. Om ureum te maken, moet er in de lever een heel proces van verschillende stappen worden doorlopen. Dus als er ureum wordt gevormd, betekent dit dat het hele proces is doorlopen en dat de cellen in goede conditie zijn. Wat we in het vierde hoofdstuk laten zien, is dat deze controle in sommige studies verkeerd wordt toegepast. In verschillende studies wordt ureum direct in het medium na de incubaties (celkweek) gemeten: aan het einde van de kweek wordt er een monster van het medium genomen en hierin wordt ureum bepaald. In hoofdstuk 4 laat ik zien dat je niet zomaar een monster uit kweekmedium kan halen om ureum te bepalen. Het probleem is namelijk dat ureum niet alleen in de levercellen wordt gevormd (via dat complexe proces), maar dat er uit de levercellen een enzym kan lekken dat één van de aminozuren (arginine) in het celkweekmedium in tweeën knipt, waarbij ook ureum ontstaat. Dit enzym heet arginase. Als arginase uit de cellen vrij komt, betekent dit juist dat de cellen kapot zijn en niet meer levend zijn. Dus je meet dan ureum dat op de goede wijze in de levercellen wordt gemaakt, plus het door arginase gevormde ureum uit het medium, wat je juist niet wilt meten omdat dat juist een maat is voor kapotte, dode cellen. Kortom, je krijgt een grote overschatting van de ureum vorming en dus van de controle voor levensvatbaarheid van de cellen..

In het hoofdstuk wordt uitgelegd hoe je ureumproductie op een correcte manier kan gebruiken als teken van leven van de levercellen. Dit moet je dan doen door een aparte kweek van de cellen te doen, in een medium zonder arginine. Dan kan het uit de kapotte cellen vrijgekomen arginase niet het arginine in tweeën knippen en krijg je geen 'verkeerd ureum'. Nu kan je alle ureum dat wordt gevormd, gebruiken als controle.

Het onderwerp van het tweede deel van het proefschrift is de spier, omdat je bij cachexie spierafbraak wilt remmen en wilt weten wat er nu precies gebeurt in de spier bij cachexie.

Hoofdstuk 5 gaat over de spier, het andere weefsel dat een belangrijke rol speelt in cachexie. Voor dit deel zijn spiercellen gebruikt, zogenaamde C2C12 cellen. Het leuke aan die cellen is dat ze erg op de spier lijken. Het lichaam bestaat uit cellen, zoals je weet. Allemaal losse cellen. Op de spier na. De spier bestaat namelijk uit bijzonder weefsel, namelijk zogenaamde gefuseerde cellen. Dit zijn cellen die zijn samengesmolten en zo hele lange vezels vormen. Soms wel een bijna een halve meter lang, zoals de spiervezels die de gehele lengte van je dijbeen kunnen beslaan. De C2C12 cellen zijn eigenlijk voorlopercellen van de spier. Tijdens het kweken kan je ze laten samengaan of fuseren zodat ze een soort van spiervezels vormen. Het zijn dan langwerpige cellen. Het feit dat ze zijn gefuseerd, kan je zien aan alle kernen die in de vezels op een rijtje liggen (normaal heeft elke cel maar 1 kern). Op die lange vezels liggen zogenaamde satellietcellen. Dit zijn eigenlijk 'slapende cellen', die wakker worden op het moment dat de spier weer gaat groeien. Ook zorgen deze cellen er voor dat als er een stukje spiervezel worden afgebroken (dat zie je doordat er kernen uit de spier verdwijnen) er nieuwe celkernen naar binnen komen. Wat is nu het leuke aan dit hoofdstuk? Zoals ik in de inleiding heb uitgelegd, is cachexie een proces van eiwitafbraak in de spier. Toch is het zo dat als je er voor zorgt dat je veel eiwit aanbiedt aan de spier via de voeding, je de afbraak niet zo maar kan remmen. Uit dit hoofdstuk blijkt dat je de spier ook niet alleen maar moet bekijken als een 'vat' met eiwit, maar als een samenspel van de verschillende cellen die in de spier zitten. Dus niet alleen het eiwitgehalte en eiwitaanbod via de voeding zijn bepalend voor de spiergroei, maar ook of de satellietcellen wakker kunnen worden en kunnen bijdragen aan de spiergroei.

Wat blijkt nu uit dit hoofdstuk? De cytokines die tijdens kanker vrijkomen, zorgen er niet alleen voor dat er eiwit wordt afgebroken in de spier. Ze zorgen er ook voor dat de satellietcellen niet 'wakker' worden als ze nodig zijn. Ze fuseren dan niet met de spiervezels en de spier kan dan de verhoogde afbraak tijdens cachexie niet compenseren met groei. Dus eigenlijk moet je cachexie niet alleen zien als een negatieve eiwitbalans (eiwitafbraak) maar ook als een negatieve 'kernenbalans' of negatieve 'cellenbalans'. Dit is belangrijk om je te realiseren, want als je de spier wilt laten groeien tijdens cachexie, moet je er niet alleen voor zorgen dat je veel eiwit geeft aan de patiënt, maar moet je er ook voor zorgen dat je de cytokines en andere signaalstoffen die de satellietcellen laten slapen, moet proberen te remmen. Bij het remmen van die signaalstoffen kunnen de satellietcellen weer wakker worden en hun werk doen om de spier te laten groeien

Als allerlaatste is er ook nog een dierstudie gedaan. Hierin is gekeken of het mogelijk is om alleen met voeding cachexie te kunnen remmen. We hebben muizen die kankercachexie hadden een specifieke voeding gegeven. In die voeding zat relatief veel eiwit, plus het aminozuur leucine. Dat leucine fungeert niet alleen als bouwstof van eiwitten, maar het kan ook nog een zogenaamd 'anabool' signaal geven. Dat betekent dat het eiwitproductie in de spier kan aanzetten. Dat is nodig in cachexie, want in cachexie is de spier veel minder gevoelig voor een andere stof die normaliter de eiwitproductie in de spier aanzet: insuline. We hebben in de studie laten zien dat leucine inderdaad een anabool signaal kan geven, door de spierafbraak een beetje te remmen tijdens cachexie. Echter het proces van spierafbraak wordt niet helemaal geremd. Daartoe zijn inmiddels, volgend op deze studie, al weer andere experimenten gedaan, die nog succesvoller waren dan mijn studie, omdat de onderzoekers bovenop leucine nog andere voedingsstoffen hebben gegeven aan de zieke muizen. Zo zie je maar, dat een losse studie weer nieuwe ideeën geeft. En dit kan weer leiden tot goede en werkende producten voor kankerpatiënten met cachexie.

Dat was in een overzicht wat ik heb gevonden. Het doel van dit project was om een bijdrage te leveren aan de onderbouwing van voedingsproducten voor kankerpatiënten. Die bijdrage is denk ik goed geleverd. Bovendien heb ik

nog aanvullende suggesties gedaan voor zogenaamde 'volgende generatie producten' voor kankerpatiënten. Die suggesties staan in de Engelstalige discussie, het laatste hoofdstuk. Samenvattend komt het er op neer dat als je voor cachectische mensen een specifieke voeding wilt maken je er moet rekening mee moet houden (1) dat je voldoende eiwit moet geven in de voeding, en wel zoveel dat de lever er niet alle aminozuren uithaalt voor de acute fase eiwit synthese. Anders blijft er voor de spier niets over. (2) Bovendien moet je er voor zorgen dat naast het stimuleren van de eiwitproductie in de spier ook de cytokines en andere signaalstoffen die cachexie veroorzaken, uitgedoofd worden. Als je dat doet, dan blijven de satellietcellen actief en kunnen zij hun bijdrage leveren aan de spiergroei of spierbehoud. Dit laatste is essentieel om problemen en complicaties ten gevolge van cachexie voor de patiënt te voorkomen.

Curriculum Vitae

Stephan Peters werd geboren op 8 januari 1971 te Oud Gastel. In 1994 ontving hij zijn Getuigschrift Hoger Beroepsonderwijs voor de Hogere Laboratoriumopleiding, afstudeerrichting Medische Biotechnologie aan de Hogeschool Brabant te Etten-Leur. Na zijn diensttijd is hij gaan werken bij TNO Voeding in Zeist (1995-2000) op de afdeling bestrijdingsmiddelenanalyse en later bij TNO Farma. In de tussentijd heeft hij een half jaar in het Wilhelmina Kinderziekenhuis te Utrecht, laboratorium voor erfelijke metabole stoornissen gewerkt. Van 2000-2006 heeft hij gewerkt bij het toenmalige Numico Research (nu Danone Research), waar hij het onderzoek voor dit proefschrift heeft gedaan. In diezelfde periode heeft hij zijn WO-opleiding Voeding en Toxicologie aan de Open Universiteit te Heerlen afgerond (2003). Na een jaar gewerkt te hebben als productontwikkelaar van voedingssupplementen bij MCO Health in Almere, werkt hij sinds 2007 bij het Voedingscentrum. Daar is hij nu werkzaam als Kennisspecialist Voedselveiligheid. Bij Numico Research is onder leiding van Dr. Klaske van Norren het onderzoek voor dit proefschrift verricht. Prof. Dr. Henk P. Haagsman is de promotor namens de Universiteit Utrecht.

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Na een kort uitstapje van een jaar bij MCO Health, waarin het werk voor dit proefschrift een beetje heeft stilgelegen, ben ik nu al weer bijna 3 jaar werkzaam bij het Voedingscentrum. Ik wil al mijn collega's bij het VC bedanken voor jullie interesse in het verloop van dit 'project'.

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Paranimfen. Tja, wie moet je dan uitzoeken? In mijn ogen mensen die altijd onvoorwaardelijk achter je staan. En wie kunnen dat anders zijn dan mijn ouders? Bergen en dalen hebben we met elkaar meegemaakt. Gelukkig op 17 december weer een hoogtepunt. Ik ben er trots op dat jullie mijn paranimfen willen zijn.

Als laatste, hoe kan het ook anders. Mimoun. Hier geen woorden, maar gewoon een kus.

Stephan

Abbreviations

AA	Amino acids
APP	Acute phase proteins
APR	Acute phase response
BCAA	Branched-chain amino acids
BW	Body weight
CHS	Contact hypersensitivity test
CC-RLEC	Co-culture of primary hepatocytes with rat liver epithelial cells
CRP	C-Reactive protein
CTNF	Ciliary neuroptrophic factor
CW	Carcass weight
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagles Medium
EAA	Essential amino acids
FCS	Fetal calf's serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCS	γ-Glutamyl-cysteine synthetase
HBSS	Hank's balanced salt solution
HRP	Horse radish peroxidase
IFNγ	Interferon-γ
IGF-1	Insulin-like growth factor-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
LSC	Liquid scintillation counter
mEDL	Extensor Digitorum Longus muscle
mG	Gastrocnemius muscle
ML	Monolayer hepatocyte culture
mS	Soleus muscle
mTA	Tibialis muscle
MyoD	Myogenic differentiation factor-1
Murf	Muscle ring finger protein
NEAA	Non-essential amino acids
NO	Nitric oxide

PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PIF	Proteolysis-inducing factor
RLEC	Rat liver epithelial cells
RPMI	Roswell Park Memorial Institute medium
S	Sham injected (control) mice
SOM	Specific oligo-saccharides
SW	Sandwich culture of primary hepatocytes in a collagen bilayer
TB	Tumor-bearing mice
TB1Leu	Tumor-bearing mice receiving 1g/kg leucine-enriched feed
TB8Leu	Tumor-bearing mice receiving 8g/kg leucine-enriched feed
TNFα	Tumor necrosis factor-α
TW	Tumor weight
15-HETE	15-hydroxyeicosatetraenoic acid

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