Selective induction of apoptosis via the TNF system in hepatocellular carcinoma

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Tobias Speicher

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1. Referent: Prof. Dr. Albrecht Wendel
2. Referent: Privatdozentin Dr. Elisa Ferrando-May

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Abbreviations

ActD	Actinomycin D
Afc	7-amino-4-trifluoromethylcoumarin
ANOVA	analysis of variance
ATP	adenosine triphosphate
Br-PA	3-bromopyruvate
cAMP	cyclic adenosine monophosphate
CD95	Fas, Apo-1
CD95L	CD95 ligand
cDNA	complementary DNA
Cyt c	cytochrome C
db-cAMP	dibutyryl cyclic adenosine 3′,5′-monophosphate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAXX	death domain associated protein
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynukleosid-triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	etylendiamintetraacetat
EGTA	ethylene-bis(oxyethylenenitrilo)tetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FADD	fas-associated protein with death domain
FCS	fetal calf serum
FLICE	FADD-like IL-1beta-converting enzyme
FLIP	FADD-like IL-1beta-converting enzyme interacting protein
HCC	hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF1	hypoxia inducible factor1

HKII	hexokinase II
HRP	horseradish peroxidase
HSA	human serum albumin
IAP	inhibitor of apoptosis protein
IHP	isolated hepatic perfusion
ILP	isolated limb perfusion
i.p.	intraperitoneal
i.v.	intravenously
JNK	c-Jun N-terminal kinase
kDA	kilodalton
K_m	Michaelis-Menten constant
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
Min	minute
mRNA	messenger RNA
NF-κB	nuclear factor-κB
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	positron emission tomatography
PKA	cAMP-dependent protein kinase
Prim	primary
RAIDD	RIP-associated ICH-1 homologous protein with a death domain
RIP	receptor interacting protein
RT	room temperature
RT-PCR	reverse transcription PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
tBid	truncated form of Bid
TGF-β	transforming growth factor-β

TNF	tumor necrosis factor-alpha
TNFR	TNF receptor
TRADD	TNF receptor-associated protein with death domain
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
Tris	tris-(hydroxymethyl)-aminomethan
XIAP	X-linked inhibitor of apoptosis protein
αCD95	agonistic CD95 antibody

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1 Introduction

1.1 Cancer

Cancer is a group of more than 200 different diseases, characterised by uncontrolled proliferation, invasion and in some cases metastasis. These malignant characteristics differentiate cancers from benign tumors. First descriptions of cancer and medical treatment by surgery or pharmacology were written around 1500 B.C. in Egypt. Today cancer is the second leading cause of death worldwide according to the WHO, and the American Cancer Society expects cancer to become the leading cause of death in the United States if the currents trends continue. In response, cancer research was continuously intensified and became a wide research field. Due to a more extended knowledge, the understanding of cancer development and progression changed from regarding cancer progression solely as an atrocious attitude of malignant cells rather than conceiving this processes as a consequence of abnormalities in the genetic material, which finally leads to disturbance of the balance between cell division and controlled cell death. According to such a view, almost every tissue or organ can develop cancer, because the affected cells loose important control mechanisms.

Surgery as a treatment option plays an important role, but is only applicable for certain forms of mostly solid cancer. Eradication of the malignant tissue by induction of cell death is the main alternative, placing the search for anticancer drugs in a central position of cancer research. Unfortunately, most substances used in the clinics or in experimental settings are relatively unspecific. For example, the widely used chemotherapeutic drug adriamycin (doxorubicin) is associated with cardiomyopathy, probably due to toxic effects on cardiomyocytes [1]. Cytokines as inducers of apoptosis such as TNF or TRAIL are also toxic towards normal cells such as hepatocytes [2]. These examples highlight that the main drawbacks of most anticancer agents are toxic effects on healthy cells. While for some forms of cancer curative treatment options were developed, for others such as cancer confined to the liver, there is no such option at hand. This organ, which may be affected by primary or metastatic cancer, is notably critical in terms of available curative therapeutic approaches and survival rates.

1.2 Hepatocellular carcinoma

The liver can be affected by cancers originating from liver (primary liver cancer) or by metastasis spreading from tumors in other parts of the body, such as colon, lung or breast (secondary liver cancer). Most cases of HCC are secondary to fibrosis and cirrhosis, mainly induced by hepatitis infection, alcoholism or non-alcoholic steatohepatitis or their combinations [3]. In the Unites States HCC accounts for about 90% of primary hepatic cancers while the remaining 10% are intrahepatic cholangiocarcinomas [4]. Hepatocellular carcinoma is the fifth most common cancer worldwide and the third most common tumor-associated cause of death [5]. The current status of advanced HCC therapy is alarming, since on one hand survival rates of surgically incurable HCC patients have not improved significantly over the past 30 years [6], while on the other hand a massive increase in age-adjusted incidence rates for certain parts of the population in the US, particularly among middle-aged men was reported [7]. A main problem in the context of HCC, being associated with the poor survival rates, is the relatively late detection of the disease. Only in a minority of patients HCC is diagnosed in a localized stage, where tumors occur as a clearly defined entity and resection or transplantation are options for treatment. Most patients (>80%) are diagnosed with advanced HCC [8], occuring as poorly defined tumors with multiple spots and infiltrative growth patterns, which are often located in close contact to major blood vessels. These are regarded as non-resectable and are candidates for palliative care, leading to a 5-year survival rate of about 10% [4].

A first flicker of hope is coming from the latest therapeutic approach for treatment of advanced HCC targeting oncogenic kinase signalling involved in tumor cell proliferation and tumor angiogenesis. The orally active multikinase inhibitor sorafenib was reported to provide survival benefit for patients with advanced HCC [9]. For further alternative treatment strategies it is noteworthy that the anatomy of the liver allows regional drug delivery in hepatic cancer treatment by isolated hepatic perfusion (IHP), which opens up the possibility to overcome problems due to systemic toxicity [10]. In this procedure a circulation system *via* catheters allows the perfusion of the liver temporarily separated from the blood circulation system, thereby enabling high dosage of anticancer agents. It was first reported in 1960 [11] and due to refinements as well as improved experience the mortality rates due to the complex procedure reached an acceptable level of below 6 % [12-17]. The method was designed for unresectable, multifocal tumors confined to the liver. This setting allows organ selectivity, but the selective targeting of tumor cells within the organ still remains a largely unsolved problem in liver cancer.

Since the discovery of apoptosis as cellular suicide, the intention to employ it for therapy of various diseases was born. For treatment of hepatic carcinomas selective induction of apoptosis is also considered to be a desirable approach, but has not been realised yet.

1.3 Apoptosis

A delicate balance between cell division and cell death is essential for development and maintenance of multicellular organisms. Malfunctions in either of the two processes leads to severe diseases such as neurodegenerative disorders, autoimmune diseases, and cancer. Apoptosis is a physiological form of programmed cell death characterised by specific events leading to defined self-destruction. The term apoptosis is of Greek origin, describing the falling of leaves from a tree and was introduced by Kerr and co-workers for the morphological changes leading to programmed cell death [18]. Cell death studies in the nematode *Caenorhabditis elegans* led to the discovery of a family of cysteinyl-aspartate-cleaving proteases (caspases), which are major initiators and executors of most forms of apoptotic cell death [19-21]. The hallmarks of apoptosis are nuclear condensation of chromatin, condensation of cytoplasm, rounding up, blebbing of plasma membrane, intranucleosomal cleavage of the DNA, phosphatidylserine exposure and finally the fragmentation of the cell into compact membrane enclosed structures, called apoptotic bodies, which contain cytosol, condensed chromatin and organelles [22]. The apoptotic bodies are taken up by phagocytes or neighbouring cells, thereby preventing inflammation [23].

1.4 Necrosis

Necrosis is in contrast to apoptosis an accidental, pathophysiological form of cell death caused by external factors. This uncontrolled and passive process is characterised by lysis of the nucleus, swelling of the organelles and the cell [24], finally leading to damage of the plasma membrane. As a consequence, intracellular components are released, causing leukocyte infiltration and inflammation [25].

Apoptosis and necrosis have to be considered as two extremes of cell death. Intermediate forms of cell death may appear depending on several factors such as cell type, inducer of apoptosis, external and internal factors.

1.5 Initiation of apoptosis - extrinsic and intrinsic pathway

Induction of apoptosis on the level of cellular interaction occurs in two converse mechanisms:

- Most cells require certain survival signals (trophic factors) to stay alive. In their absence cells will undergo apoptotic cell death.
- On the contrary, presence of specific signals induces apoptosis.

A third possibility for apoptosis induction, but independent of cellular interaction, is *via* internal signals due to cellular stress or injury by environmental stresses such as ionising radiation, heat shock, osmotic stress, oncogenes or drugs.

The molecular mechanism of apoptosis induction is separated in extrinsic and intrinsic pathway. The extrinsic pathway is initiated by trimerisation of death receptors on the cell surface, such as CD95, TNF receptor (TNFR), or TRAIL receptor (TRAILR) by their respective ligands. Interaction of the cytoplasmatic receptor domains with adapter molecules leads to cleavage and activation of initiator caspases-8 and -10, which in turn cleave and activate effector caspases-3 and –7 (Ashkenazi 1998). The extrinsic pathway can be regulated at certain steps e.g. by inhibition of effector caspase activation by Flice-inhibitory protein (cFLIP) or *via* inhibition of initiator and effector caspases by inhibitors of apoptosis protein (IAPs) [26, 27].

In contrast, the intrinsic or mitochondrial pathway is activated in response to intracellular signals. Inducers are DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress, finally leading to permeabilisation of the outer mitochondrial membrane and subsequent release of proapoptotic proteins from mitochondria that activate caspases, which ultimately trigger apoptosis [19, 28-30]. Mitochondrial release of cytochrome C is a main contributor to caspase activation by inducing the assembly of the apoptosome, a complex that consists of cytochrome C, dATP, Apaf-1 and caspase-9 and leads to activation of effector caspases [31, 32]. The intrinsic pathway is mainly regulated by the balance of pro- and antiapoptotic factors of the Bcl-2 superfamily modulating the permeability of the mitochondrial membrane [33]. Between the extrinsic and the intrinsic pathways cross-talk exists. Death receptor-mediated activation of caspase-8 can induce proteolytic activation of cytosolic Bid. The truncated form tBid translocates to mitochondria, facilitating cytochrome release by mitochondrial pore formation leading to amplification of the caspase cascade [34-36].

Depending on the necessity of the described mitochondrial amplification loop for induction of apoptosis *via* death receptors, cells and apoptosis stimuli are classified. In type I cells induction of apoptosis does not rely on the participation of the intrinsic amplification loop. In type II cells, receptor-mediated activation of caspase-8 is insufficient and requires enhancement *via* the intrinsic pathway. Hepatocytes were shown to be an example of type II cells, since inhibition of the intrinsic pathway in receptor-mediated apoptosis was cytoprotective [37]. The involvement of mitochondria in the intrinsic and extrinsic pathway emphasises the central role of this organelle in regulation and execution of apoptosis.

1.6 Death receptors and their ligands

Death receptors belong to the Tumor Necrosis Factor Receptor gene superfamily of which 29 members are currently known. They regulate a variety of biological processes such as proliferation, cell death induction and inflammatory responses [38]. Their ligands are active as trimeric, mostly membrane bound proteins. The receptors are classified in three groups depending on their cytoplasmatic sequences. Well studied members characterised by a cytoplasmatic death domain (DD) inducing a closely related scheme of signalling events are TNFRI, CD95/Fas and TRAILRI/II [39]. Binding of their ligands leads to receptor trimerisation, resulting in activation of the intracellular DD, which functions as a platform for the assembly of the death-inducing signalling complex (DISC), activating the apoptotic signalling cascade [22].

1.6.1 <u>TNF/TNFR</u>

Based on the antineoplastic activity TNF was isolated in 1975 [40]. TNF is produced mainly by macrophages (e.g. Kupffer cells in the liver) [41] as a membrane-bound form, which can be cleaved by the metalloproteinase TNF converting enzyme (TACE)

giving rise to the 17 kDa soluble form [42, 43]. Long before TNF was discovered by Carlswell et al. in 1975, the American surgeon Dr. Coley described an endogenously induced tumor-killing in humans. He developed a “vaccine” of killed bacteria called “Coley´s toxin”, and described strong antitumor efficacy [44]. Today we know that injection of endotoxins leads to endogenous release of TNF, being one central mediator of the described antitumor properties. TNF exerts pleiotropic effects on many cell types and functions as a key mediator in various physiological and pathophysiological situations such as inflammation, sepsis, immunological reactions, apoptosis, rheumatiod arthritis, and liver regeneration [19, 45-50].
The currently accepted concept of TNFRI-mediated signal transduction includes the sequential formation of two signalling complexes activating the three main components of TNFRI signalling: JNK and NF-κB signalling as well as the caspase cascade. Complex one emerges at the plasma membrane and consists of TNFR1, the adaptor TNF-R associated death domain (TRADD), the kinase RIP1 and TNF-R associated factor 2 (TRAF-2) [51]. It leads to activation of the c-Jun N-terminal kinase (JNK) pathway and to activation of the antiapoptotic transcription factor NF-κB functioning as a checkpoint. NF-κB signalling regulates the duration of JNK signalling thereby controlling its function during TNF-induced apoptosis. Prolonged JNK activity is essential for TNF-induced cell death [52-54]. Therefore, inhibition of NF-κB signalling is required for the induction of apoptosis. In a second step complex two is formed by TRADD and RIP1, which associates with Fas-associated protein with death domain (FADD) and caspase-8, responsible for induction of the caspase cascade [51, 55].

1.6.2 CD95L/CD95

The CD95 ligand (CD95L) is expressed mainly on the surface of T-lymphocytes or natural killer cells, and is also constitutively expressed in immune-privileged sites. The membrane bound form can be cleaved, giving rise to the soluble form. Main function of the CD95 system is control of homeostasis in many tissues like kidney, heart, and liver as well as mediation of T cell selection and immunity [56]. Overexpression of CD95L on cancer cells, known as counter attack, was described to delete antitumor-specific lymphocytes, thereby enabling the cancer cells to escape the immune response [57]. On target cells CD95L induces receptor trimerisation and formation of the DISC, which consists mainly of the receptor, FADD and Caspase-8 resulting in activation of the caspase cascade. Interaction of the cytoplasmatic DED with additional proteins such as death domain associated protein (DAXX), receptor interacting protein (RIP) kinase and RIP-associated ICH-1 homologous protein with a death domain (RAIDD) has been shown, but the exact role remains unclear [58].

1.7 TNF in cancer therapy

Cytokine-mediated, tumor selective induction of apoptosis is a main goal of cancer research. TNF was the first cytokine employed for cancer therapy and when recombinant TNF became available to medical oncology the hope for curing cancer rose and clinical investigations started [59-61]. While several studies demonstrated remarkable anti-tumor effects of TNF in murine models [62], subsequent phase I and II studies of systemic TNF demonstrated only a transient partial response [63]. The maximum tolerated doses of TNF undercut those to induce tumor cell death in the mouse models by multiples due to the pronounced toxic effects in humans [64-67]. Since the effective TNF dose needed to exceed the maximum tolerated dose by a factor of 5-25 [68], TNF as such cannot be used for the systemic treatment of cancer patients. Regional drug delivery systems such as isolated hepatic perfusion (IHP) or isolated limb perfusion (ILP) open up the possibility to overcome the limitations in clinical use, which are related to systemic toxicity [67, 69-71]. While combinations of TNF with antineoplastic agents such as melphalan allowed impressive clinical success in the remission of advanced non-resectable soft tissue sarcomas with very poor prognosis in isolated limb perfusion, selectivity for tumor cells remains a largely unsolved problem in liver cancer [72]. A combinational therapy with a compound that specifically reduces cytotoxicity in healthy cells (hepatocytes) seems worth to be developed. Fructose-mediated hepatic ATP depletion prevents TNF-induced toxicity *in vitro* and *in vivo* [73], and is discussed as a potential tool to protect healthy hepatocytes against toxic conditions mediated by TNF [74].

1.8 Fructose metabolism and its influence on cell death

1.8.1 <u>Liver-specific fructose metabolism</u>

D-fructose was used as a source of energy supply during parenteral nutrition for many years, because it was claimed to have advantages over glucose due to its faster and insulin-independent metabolism [75-77]. After the discovery of fructose-induced hepatic ATP depletion, which is due to the liver-specific metabolism after intravenous administration, its use in parenteral nutrition was stopped. The depletion of ATP by ketohexoses was shown to be transient and non-toxic, since a residual, sufficient level of ATP is ensured *via* oxidative phosphorylation and glycolysis, thereby preventing necrosis [78, 79].

In most mammalian cells e.g. muscle, fructose is metabolised the same way as glucose *via* low-K_m-hexokinases to fructose-6P and subsequent cleavage by aldolase A. Due to the specific function of the liver in regulation of energy homeostasis, these low-K_m-hexokinases are nearly absent. The liver-specific fructokinase takes over the phosphorylation of ketohexoses, but in contrast to muscle cells at the position C1. The resulting fructose-1P is then cleaved by liver-specific aldolase B to glyceraldehyde-3P and dihydroxyacetonephosphate. In contrast to liver-specific glucose metabolism, where the activity of glucokinase is tightly regulated by feedback inhibition and the following step catalysed by phosphofructokinase (the key regulatory enzyme in glycolysis) is controlled *via* substrate inhibition, allosteric regulation by energy metabolites or hormone signalling, these regulatory mechanisms are absent in hepatic fructose metabolism. Due to the fact that fructokinase shows a much higher activity than aldolase B, a load of fructose leads to accumulation of fructose-1P in the mammalian liver. The increase in fructose-1P simultaneously initiates a severe reduction of ATP, inorganic phosphate and subsequent degradation of nucleotides to uric acid [80]. Although the total amount of adenosine nucleotides is reduced [81] the energy charge is maintained, since ATP, ADP, and AMP are equally affected [82]. The decrease of adenosine nucleotides leads to degradation of AMP to adenosine or inosinemonophosphate *via* adenosine deaminase or nucleotidase, respectively, leading to a slow increase of inosine, uric acid, and allantoin in the plasma [83]. Formation and release of adenosine during fructose-induced hepatic ATP depletion has so far not been shown.

1.8.2 Fructose-mediated ATP depletion modulates cytokine-induced cell death

ATP depletion by various phosphate-trapping ketohexoses such as fructose was shown to completely block TNF-induced hepatic cell death *in vitro* and *in vivo*, while apoptosis by CD95 was enhanced [73]. Characteristic hallmarks of TNF-induced apoptosis such as mitochondrial cytochrome C release, loss of mitochondrial membrane potential, activation of type II caspases, DNA fragmentation and cell lysis

were prevented by fructose-induced ATP depletion [73]. The protective mechanism could be extended to models of TNFRI-dependent apoptotic and necrotic liver injury in mice [84]. The inverse modulation of fructose on cell death induced by the two related cytokines CD95L and TNF, which was also observed in animal experiments, emphasis that ATP depletion does not prevent cytokine-induced cell death in general. Interference with a cellular master switch of TNF-induced hepatocyte apoptosis seems to be a reasonable explanation for the cytoprotective effects. Early TNFRI-mediated signalling like activation of the NF-κB pathway as well as receptor internalisation was not affected by the presence of fructose [73, 84]. This implies general receptor aggregation and recruitment of adapter molecules to be functional in general in the presence of fructose, which is further supported by the fact that CD95-induced apoptosis was not blocked. In contrast, the mitochondrial amplification loop, which is required for full activation of effector caspases leading to apoptosis was prevented by fructose [73]. Interference of ATP depletion with apoptotic signalling upstream of mitochondrial cytochrome C release, preventing lethal insults was also shown in other models [85, 86], but the point of interaction between ketohexose-induced ATP depletion and cytokine-induced cell death has not been elucidated.
However, for many therapeutic applications such as intervention, or control of liver failure, as well as improved chemotherapy, the selective modulation of cytokine-induced cell death is desirable. Inhibition of TNF-induced hepatotoxic side effects in cancer therapy would be a conceivable application. In this context, the question arises whether tumor-specific alterations in energy metabolism, which have been described as typical features of most fast growing tumors, have an impact on the described liver-specific fructose metabolism and the associated effects on apoptosis induction.

1.9 Energy metabolism of malignant cells

ATP as the primary form of energy is required for most cellular processes like biosynthesis, transport and signalling mechanisms, turning ATP into a limiting factor under certain growth conditions. Glycolytic degradation of monosaccharides as a source of ATP is providing only a relatively small amount due to its low energy efficiency but is oxygen independent. The rate-limiting reaction is catalysed by hexokinases. Under physiological conditions the major part of ATP is generated by oxygen-dependent mitochondrial oxidative phosphorylation. This highlights the importance of providing sufficient availability of oxygen as well as nutrients to serve the energy demand. This also applies for tumors, since angiogenesis and increased rate of glycolysis facilitate development and growths of malignant tissue, turning these tumor-specific alterations into targets for cancer therapy.

1.9.1 Warburg effect

In the 1920s the German biochemist Otto Warburg described that one of the most characteristic phenotypes of highly malignant cancers is their increased rate of glycolysis, thereby satisfying high amounts of their energy demands by glycolysis even under normoxic conditions [87-93], which is accepted today as a key metabolic hallmark of cancers [94]. These alterations, known as the Warburg effect, are worth being reconsidered also in modern cancer research. By now we know that hexokinase II plays a central role in the Warburg effect [94, 95]. In many highly malignant rapidly growing tumors increased expression of hexokinases was detected. Since, as previously mentioned, ketohexoses such as fructose are metabolised the same way as glucose by hexokinases in most cells of the mammalian body, a potential influence of increased expression of hexokinases on fructose metabolism has to be considered. But almost none of the studies addressing Warburg's observations discriminated between the metabolisms of these sugars.

1.9.2 Hexokinases and their role in cancer

Hexokinases catalyse the phosphorylation of certain sugars e.g. glucose or fructose at position C6, being the initial and rate-limiting step of glycolysis. Four isoforms with tissue-specific expression pattern have been described in mammalian cells [96-98]. Isoforms I-III are also called low-K_m hexokinases due to their high affinity for glucose (K_m ~ 0.2 mM). While these isoforms are nearly silent in the liver, isoform IV (glucokinase), characterised by a high K_m (K_m ~ 5 mM), is expressed and enables the energy storage function of the liver. The organ regulates blood glucose levels, by storing glucose as glycogen in phases of excessive sugar availability (resorption phase) [96-98], whereas glucose is released during post-resorption phase to ensure sufficient glucose plasma concentrations.

Enhanced glycolytic activity during cancerogenesis was shown to be due to increased expression of hexokinases [91]. Further studies of hepatomas, and malignant cells of other origin revealed that hexokinase isoform II is the main contributor to the cancer phenotype of increased glycolytic activity [99-101]. Enhanced expression of HKII by promoter activation, gene amplification, epigenetic regulation, as well as elevated binding to mitochondria *via* a hydrophobic amino acid sequence in the N-terminal region of HKII are the initiators of increased glycolytic activity [102-107]. Binding of HKII to mitochondria leads to improved access of mitochondrial ATP as substrate [102], reduced product inhibition by glycose-6P [99, 108, 109], and reduced proteasomal degradation, resulting in reinforced activity of HKII. The increased rate of glycolysis ensures to cover the high energetic demand of cancer cells for cell division and growth, and warrants an advantage for survival and proliferation in tumor microenvironment. Warburg´s pioneering studies found their way into clinical applications, since upregulation of HK resulting in increased rate of glycolysis is exploited daily in the clinics for detection of tumors by positron emission tomography (PET), visualising uptake of 18F-2-deoxyglucose [110]. In this context, upregulation of HKII as adaptive mechanism to hostile hypoxic tumor environment has to be mentioned. Hypoxic microenvironment is only one inducer besides others such as certain oncogenes, like the signalling molecule Ras, Akt kinase as well as the transcription factor Myc driving changes in energy metabolism [94].

1.9.3 Hypoxia as tumor environment

The moment tumors outgrow their blood supply, regions of hypoxia develop. This leads to an oxygen gradient within malignant tissues, diminishing towards the less vascularised centre of the tumor [111, 112]. Hypoxia as a tumor environment was shown to induce an aggressive phenotype, increasing metastatic potential, promoting tumor progression, and limiting the effectiveness of radiation therapy and some chemotherapeutic agents [113, 114]. Even in case of hepatic carcinogenesis hypoxia plays an important role, since it was reported to regulate proliferation, angiogenesis, metastasis, chemo- and radioresistance, apoptosis and differentiation of HCC [115]. Additionally, hypoxia was described to stimulate growth of HCC by increased expression of HKII [116]. The pleiotropic effects of reduced oxygen availability are mainly orchestrated by the master transcription factor hypoxia-inducible factor1 (HIF1). The main targets of HIF1 signalling are induction of angiogenesis e.g. *via* vascular endothelial growth factor (VEGF) expression and adaption to hypoxia by increased anaerobic glycolysis e.g. *via* HKII upregulation. Advanced energy supply of cancerous tissue is the effect of most HIF1 targets. It is noteworthy that cytokines, growth factors and oncogenes are also potential activators of HIF1 [117]. Influence of reduced oxygen availability itself or of its regulators, also in presence of oxygen, has been postulated to induce changes in the energy metabolism of cancers of various origins [118, 119]. However, stabilisation of HIF1 during hepatic carcinogenesis was shown in animal experiments, and its accumulation was reported in more than 50% of human primary cancers and metastasis. Increased levels of HIF1, of hexokinase II and glycolytic activity were related to aggressiveness of HCC and patients survival time [118, 120, 121].

In context of therapeutic cancer targets, the changes in the energy metabolism are enforced, attracting attention leading to a resurrection of Warburg´s theory. Discussions whether a shift in the energy metabolism is a requirement of transformation or a byproduct of the cell´s transformation are still going on. Whether increased rate of glycolysis according to Warburg is a cause or a consequence of cancer is a minor concern in our context, if it opens up new therapeutic approaches for cancer therapy.

2 Aims of the study

HCC represents a prime cancer worldwide, and most cases are diagnosed at an advanced stage where no curative treatment options are available. TNF was discovered by its antitumor properties and it was the first cytokine employed for cancer treatment. Due to its severe toxic effects TNF cannot be used for systemic treatment. Regional drug delivery systems prevented systemic toxicity and thereby enabled its application. In isolated limb perfusion TNF in combination with alkylating agents allowed impressive clinical success in the remission of advanced non-resectable soft tissue sarcomas with very poor prognosis, and is since then in clinical use for treating tumors located on the legs. However, in liver cancer, such a local treatment is elusive until now, because toxicity towards healthy tissue represents the dose-limiting factor for TNF in this setting. The obvious question arises, how to make the killer molecule TNF tumor-specific in the liver.

The goal of this thesis was to explore whether and how the metabolism of the naturally occuring sugar fructose could introduce selectivity into TNF-mediated cancer cell killing by selective protection of healthy hepatocytes. Fructose-mediated ATP depletion prevents TNF-induced hepatic apoptosis *in vitro* and *in vivo*, thereby highlighting fructose as a potential candidate to introduce selectivity into TNF-mediated cancer cell killing, provided that malignant cells of hepatic origin are lacking this protective mechanism. The transient, non-toxic ATP depletion that leads to the cytoprotection is due to the liver-specific fructose metabolism by fructokinase and aldolase B.

- The aim of the first project was to analyse if changes in the energy metabolism of transformed cells of hepatic origin prevent liver-specific fructose metabolism, thereby inhibiting fructose-induced ATP depletion and protection against TNF-induced apoptosis. In case that hepatoma cells show this phenotype, identification of the alterations preventing liver-specific fructose metabolism was a further goal. Therefore, biochemical and functional differences of fructose metabolism were compared between primary liver cells and hepatic tumor cell lines by biochemical, pharmacological and molecular biological approaches.

Fructose-induced ATP depletion does not inhibit cytokine-induced apoptosis in general, since CD95-induced cell death is enhanced, while TNF-induced cell death is prevented. The link between fructose-induced ATP depletion and cytokine-induced apoptosis has not been elucidated.

- The second part of this thesis aims to elucidate the molecular mechanism of protection against TNF-induced apoptosis in healthy hepatocytes by fructose-mediated ATP depletion. Therefore, downstream events following fructose-induced ATP depletion and their effects on MAP Kinase signalling, relevant in cytokine-induced apoptosis, were investigated.

3 Malignant but not naïve liver cells are killed by TNF after metabolic depletion of ATP by fructose

Tobias Speicher, Annika Foehrenbacher, Isabelle Pochic, Timo Weiland,

and Albrecht Wendel*

Chair of Biochemical Pharmacology, Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany
* To whom correspondence should be addressed
Albrecht Wendel, Faculty of Biology, University of Konstanz, M667, D-78457, Konstanz, Germany.
Phone: +49-7071-45802; FAX +49-7071-44464; E-mail: albrecht.wendel@uni-konstanz.de

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The Abbreviations used are: Br-PA, 3-bromopyruvate; HCC, hepatocellular carcinoma; HIF1, hypoxia inducible factor1; HKII, hexokinase II;
IHP, isolated hepatic perfusion; TNF, tumor necrosis factor-alpha

3.1 Summary

Our aim was to identify metabolic differences between normal and malignant liver cells to exploit for cancer therapy. Primary mouse, rat and human hepatocytes depleted of ATP in the presence of fructose, were fully protected against TNF-induced cytotoxicity. However, rodent or human hepatic tumor cell lines lack a fructose-mediated ATP depletion mechanism and remain susceptible to TNF. We demonstrate that increased expression of hexokinase II (HKII) in transformed cells of hepatic origin shifts liver-type to muscle-type fructose metabolism, in which fructose-induced ATP depletion does not occur and as a consequence sensitivity to TNF/ActD-induced cell death is maintained. Inhibition of hexokinases by 3-bromopyruvate restored fructose-induced ATP depletion in HepG2 cells, and stabilisation of hypoxia-inducible factor1 (HIF1) by Desferal increased the expression of HKII in primary murine hepatocytes, which in turn prevented fructose-induced ATP depletion. Furthermore, inhibition of TNF-induced apoptosis by fructose was prevented by overexpression of HKII in primary murine hepatocytes. We propose to take advantage of the increased expression of HKII in liver tumors to transiently protect healthy liver cells against TNF-mediated cell death by depleting their ATP stores with fructose while simultaneously targeting malignant cells for receptor-mediated apoptotic cell death.

3.2 Introduction

Recombinant Tumor necrosis factor-alpha (TNF) became available to medical oncology in 1985, however, its clinical usefulness was very limited due to severe adverse reactions [68], which disqualified it for the systemic treatment of cancer patients [122]. As an alternative, its local delivery e.g. *via* isolated limb perfusion of combinations of TNF with antineoplastic agents such as melphalan, allowed an impressive clinical success in the remission of soft tissue tumors with very poor prognosis [123].

Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide and, unfortunately, its incidence has doubled within the past twenty years [124, 125]. In spite of some progress to improve survival by surgical, immunological and radio / X-ray therapies, the multiple wide-spread tumors in the liver characteristic for advanced stages of the disease cannot be eradicated effectively by these means. Since the anatomy of the liver allows a selective local entry, regional drug delivery systems, such as isolated hepatic perfusion (IHP), enable the organ-targeted administration of TNF and other antineoplastic agents without the problems related to systemic toxicity [10]. However, on top of the organ selectivity, the target cell selectivity for tumor cells remains a largely unsolved problem in liver cancer. Many attempts to solve it started from the rationale to hit tumor cells lethally and healthy cells sub-lethally by more or less toxic compounds. Here, we followed the strategy of identifying and exploiting biochemical differences between healthy liver cells and liver carcinoma cells to selectively protect healthy cells from a death receptor ligand-induced killing.

We previously showed that in the healthy murine liver, ATP depletion by phosphate-trapping ketohexoses such as fructose completely blocks TNF-induced hepatotoxicity *in vitro* and *in vivo*. This protection against TNF-induced hepatotoxicity by non-toxic doses of fructose was transient and reverted within a time frame of some hours [73]. Decreased total liver ATP following dosage of fructose has also been reported in humans [77, 81, 126, 127]. The biochemical rationale for fructose-induced ATP depletion is the free permeability of hepatocytes for fructose and the instant usage of ATP by the very rapid conversion of the hexose *via* fructokinase to fructose-1-phosphate. In organs other than the liver, fructose is converted primarily *via* hexokinases to fructose-6-phosphate, which then enters glycolysis.

We noticed that malignantly transformed hepatic cell lines were not susceptible to fructose-mediated ATP depletion and remained sensitive to TNF-induced cell death in the presence of fructose. Consequently we investigated here the hypothesis whether dedifferentiated hepatoma cells are lacking this genuine liver-typical fructose metabolising capability, which in turn means that upon fructose exposure, the intracellular ATP depletion and protection is impaired, while healthy hepatocytes are protected due to their ATP depletion.

Already Warburg described that one of the most characteristic features of highly malignant cancers is their increased glycolytic activity, which we know today is mainly caused by the increased expression of type II hexokinase (HKII), the rate-limiting glycolytic enzyme [92, 128]. Gene amplification [129] and promoter activation (e.g. *via* hypoxia inducible factor1, HIF1) [130, 131] during carcinogenesis are considered the main inducers of HKII overexpression. Hardly any of the studies addressing Warburg's observations discriminated between fructose and glucose metabolism, because most mammalian cells, except the liver, metabolise ketohexoses using the same enzymes as for glucose (Fig. 1). In muscle cells, fructose is initially phosphorylated to fructose-6-phosphate by low-K_m hexokinases, followed by aldolase A-mediated cleavage, while in the liver these hexokinases are weakly expressed and the fructokinase pathway is preferred.

In this study, we elucidate the mechanism underlying a tumor-specific switch in the hepatic fructose metabolism explaining the loss of fructose-induced ATP depletion capacity in hepatoma cell lines compared to normal primary hepatocytes.

3.3 Materials and Methods

Reagents

Cell culture plates were from Greiner (Nürtingen; Germany), Dulbecco's modified Eagle's medium/Ham's F12 medium, and ITS insulin/transferrin/selenium mixture was from Gibco (Invitrogen, Carlsbad, CA), other culture media, FCS, penicillin, streptomycin, Accutase and Hepes were purchased from PAA (Pasching, Germany), collagen was obtained from Serva (Heidelberg, Germany), Percoll was purchased from Pharmacia Biotech (Uppsala, Sweden). Unless further specified, all other reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell cultures

Primary human hepatocytes were isolated from pathologically inconspicuous specimens obtained from patients undergoing surgery of hepatic tumors. The cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% Pen/Strep and maintained in a humidified incubator at 5% CO_2 and 95% air at 37°C in 24 well plates. Primary murine hepatocytes were isolated from 8-wk-old C57Bl6 wild type mice by the two step collagenase perfusion method and cultured as described [132]. Hepatocytes were plated in 500 µl RPMI 1640 medium containing 10% FCS in collagen-coated 24-well plates at a density of 9 x 10^5 hepatocytes per well, or in collagen-coated 6-well plates at a density of 3 x 10^6 hepatocytes per well. Cells were allowed to adhere to culture dishes for 4 h before the medium was changed to RPMI 1640 without FCS. HepG2 cells were maintained in RPMI 1640 containing 10% FCS, Huh7 and Hepa 1-6 in DMEM high glucose containing 10% FCS, and AML-12 in Dulbecco's modified Eagle's medium/Ham's F12 medium supplemented with 10%

FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone as previously described [133, 134]. Cell lines were maintained in 70 mm^2 flasks, the day before experiments were carried out cells were harvested with Accutase and plated in 200 µl medium containing 10% FCS in 24-well plates (1.2 x 10^6 cells/well). Incubation of murine hepatocytes or cell lines was started directly after medium exchange for RPMI 1640 without FCS. Cell lines were sensitised with 1 µg/ml, primary hepatocytes with 400 ng/ml ActD 15 minutes before treatment with 100 ng/ml recombinant murine TNF (Innogenetics, Ghent, Berlgium). All substances were diluted in saline supplemented with 0.1% HSA. Incubations of primary hepatocytes were carried out at 37°C in a humidified atmosphere of 5% CO_2, 40% O_2, and 55% N_2. Incubation of cell lines was carried out in a humidified atmosphere of 5% CO_2 and 95% air for the times indicated.
Cytotoxicity was quantified by measurement of lactate dehydrogenase (LDH) [135] in culture supernatants (S) and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100, and calculation of the percentage of LDH release from the ratio of S/(S + C).
ATP content of cultured cells was determined luminometrically (ATP assay kit, Roche, Mannheim, Germany) according to the manufacturer´s instructions. Luminescence was measured in a $VICTOR_2$ multilabel counter (Wallac Instruments). ATP data are expressed as the percentage of untreated control cells.

Immunoblotting

Cultured cells were lysed with lysis buffer (250 mM sucrose, 50 mM Tris/HCl, 5 mM imidazole, 2.5 mM EDTA, 2.5 mM DTT, 0.1% Triton X-100, pH 7.4). Cellular debris was removed by centrifugation (12,000 g, 20 min, 4°C) and an aliquot of each sample equivalent to 25 µg protein was boiled after addition of the appropriate amount of 5x sample buffer (5 mM EDTA, 162 mM DTT, 5% SDS, 50% glycerol, 0.5% bromophenol blue, 188 mM Tris, pH 8.8). Samples for detection of HIF1α were separated on 12%, for detection of HKII on 8% SDS-polyacrylamide gels (PAGE) by electrophoresis and blotted to nitrocellulose filters. Equal loading and transfer of proteins was verified by Ponceau staining of the membranes.
HKII was detected with a goat polyclonal antibody 1:200 (Santa Cruz Biotechnology, Santa Cruz, USA), HIF1α with a rabbit polyclonal antibody 1:500 (Cayman Chemicals, Ann Arbor, USA) and β-actin with a rabbit polyclonal antibody 1:1000

(Cell Signalling, Boston, USA). Antigen-antibody complexes were visualized with HRP-coupled secondary antibodies (goat anti-rabbit, Dianova, Hamburg, Germany), (rabbit anti-goat, Dako, Glostrup, Denmark) and an ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.015% H_2O_2, pH 8.5).

Preparation of Nuclei

For the preparation of nuclei according to Lazebnik [136], 5 x 10^6 hepatocytes were plated in 10 ml RPMI, 10% FCS and 1% Pen/Strep on 10 cm dishes. After exchange of the media cells were treated as depicted. After 20 h cells were washed once in PBS, once in Buffer A (10 mM Pipes pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitor cocktail) and scraped off in 5 ml Buffer A. After a swelling period of 20 min, cells were lysed by dounce homogeniser and layered on 10 ml Buffer A containing 30% sucrose. Nuclei were pelleted by centrifugation (800 g, 10 min, 21°C), washed in 1 ml Buffer A, centrifuged (400 g, 10 min, 21°C), resuspended and stored in 500 µl Buffer B (10 mM Pipes pH 7.4, 80 mM KCl, 20 mM NaCl, 1mM DTT, 5 mM EGTA, 250 mM sucrose, 50 % glycerol, protease inhibitor cocktail). Preparation of nuclei was confirmed by Hoechst staining. The suspension of nuclei was diluted in 1500 µl Buffer A, centrifuged (1500 g, 10 min, 21°C) and the pellet lysed in 100 µl Western blot lysis buffer.

Enzyme Activity Assays

Fructokinase

Activity of fructokinase was determined by coupled enzymatic assay based on the principle described by Adelman and Heinz [137-140]. Lysates were prepared in assay buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 20 mM KCl) containing 0.1% Triton X-100 and protease inhibitor cocktail by vigorous pipetting after detachment of cells by a cell scraper. Cellular debris was removed by centrifugation (12,000 g, 10 min, 4°C). 50 µg of supernatant were used for enzymatic assay following centrifugation (20,000 g, 4°C, 20 min). The assay was performed in a total volume of 1 ml containing 0.2 mM NADH, 2 mM fructose, 1 mM phosphoenolpyruvate, 1 U pyruvate kinase and 1 U lactate dehydrogenase. The reaction was started by addition of 2 mM ATP. Extinction was determined for 20 min at 340 nm and values were corrected by substracting background control without substrate.

Aldolase B

The cells were lysed by 4 cycles of freezing and thawing in assay buffer. Cellular debris was removed by centrifugation (12,000 g, 10 min, 4°C). The activity of aldolase B was determined in assay buffer (50 mM Tris/Acetate, 10 mM EDTA, 100 µg/ml BSA, pH 7.6) containing 50 µg of supernatant, 0,15 mM NADH and 2 U glycerophosphate dehydrogenase according to Rellos [141, 142]. The reaction was started by addition of 5 mM fructose-1P. The extinction was determined for 20 min at 340 nm and corrected by substracting background control.

Low-K_m hexokinases

Cells were lysed in assay buffer (0.1 M Tris/HCL, 6.6 mM $MgCl_2$, pH 7.6) containing 0.1% Triton X-100 and protease inhibitor cocktail by vigorous pipetting after detachment of cells by a cell scraper. Cellular debris was removed by centrifugation (12,000 g, 10 min, 4°C). The assay was performed in assay buffer containing 50 µg of lysates, 0.3 mM $NADP^+$, 50 mM fructose and 0.5 U hexose-phosphate isomerase. To avoid the influence of glycogen, phosphorylase and phosphoglucomutase, which are present in the lysates, the reaction was not started by addition of 4.5 mM ATP until all the glycogen was converted. The extinction was determined for 20 min at 340 nm.

Preparation of RNA

RNA preparation and digestion of DNA was performed with RNeasy Mini Kit and RNase-Free DNase Set according to the manufacturer´s instructions (Roche, Hilden, Germany). 9 x 10^4 cells were lysed by vigorous pipetting with a 20-Gauge syringe for the preparation.

Reverse transcription and real-time-PCR

Primers were designed for the liver-specific enzymes fructokinase, aldolase B, hexokinase IV and for the low-K_m hexokinases I-III, as well as for the control gene β-actin (table1). Reverse transcription was carried out with 1 µg RNA using 50 U Superscript III Reverse Transcriptase (Invitrogen, Carsbad, USA) in a final volume of 20 µl containing 1x First-Strand buffer, 0.5 mM dNTPs, 2.5 µM oligo-d(t), 5 mM DTT, 30 U Prime RNase Inhibitor. The RT reaction was performed according to the manufacturer´s instructions at 25°C for 5 min, 55°C for 45 min, and was stopped by heating to 70°C for 15 min.

Amplifications were done using a SYBR Green I system (Roche Diagnostics) combined with a thermocycler (LightCycler; Roche Diagnostics). The real-time PCR reaction was carried out with 1 µl of cDNA in a final volume of 20 µl containing 3 mM $MgCl_2$, 0.5 µM of each respective primer and 2 µl LightCycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The cycling parameters were 15 min initial denaturation, followed by 50 cycles of 15 sec of denaturing at 95°C, 5 sec of annealing at 60°C and 10 sec of elongation at 72°C. Fluorescence was measured at the end of each annealing phase. The relative copy number was determined on the basis of standard curves. For all PCR products a melting point analysis was performed to determine the specificity of the amplified sequences.

Gene	Primer (5´→ 3´)	Product size [bp]
fructokinase	cctgccagatgtgtctgcta aagtgcttggccacatcttt	249
aldolase B	tggcatctgctttttgtctg cgcttcataaaagcctcctg	195
hexokinase I	cctgggagatttcatggaga gtgcccactgtgtcattcac	240
hexokinase II	tctatgccatccctgaggac tctctgccttccactccact	220
hexokinase III	tgatggtgctagaggtgtgc tctgactgcaggaacgtgac	232
hexokinase IV	cactgctgagatgctcttcg ccacgacattgttcccttct	197
β-actin	ggacttcgagcaagagatgg agcactgtgttggcgtacag	234

Table 1 Genes and primers used for real-time-PCR

Cotransfection of EGFP and HKII

Primary murine hepatocytes were cultured in RPMI 1640 with 25 mM Hepes in 6-well dishes for transfection. Cotransfections of EGFP and HKII were performed using the HKII construct (OriGene Technologies, Rockville, USA) and the vector pEGFP-C1

(Clontech, Saint-Germain-en-Laye, France) in a ratio of 3:1. *Lipofectamine2000* (Invitrogen, Carlsbad, CA) was used as transfection reagent. Expression of EGFP was confirmed by fluorescence microscopy (Axiovert 25, Zeiss, Oberkochen, Germany). Expression of HKII was confirmed by Western blot analysis.

Fluorescence Microscopy

Cellular morphology and nuclear condensation were visualized by fluorescence microscopy (excitation 365; emission 450) following staining for 5 min at 37°C with 2 µg/ml Hoechst (Molecular Probes, Leiden Netherlands).

Statistical Analysis

Statistical differences were determined by unpaired t test if applicable, or data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

3.4 Results

Fructose induces ATP depletion in primary hepatocytes but not in hepatoma cell lines and protects only primary cells against TNF-induced apoptosis

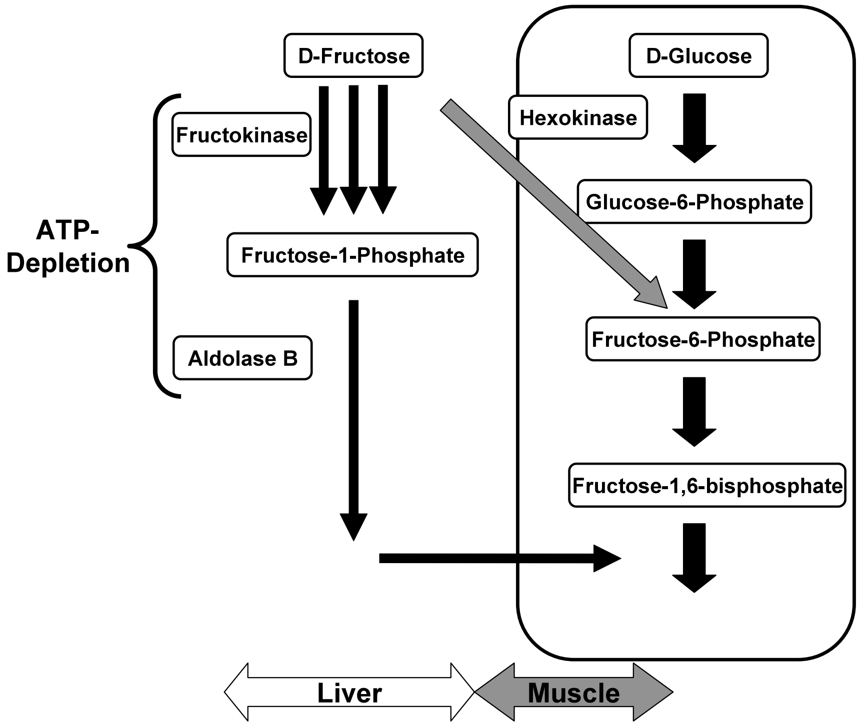

Figure 1. Schematic representation of liver-specific and muscle-specific fructose metabolism. In muscle cells fructose is phosphorylated to fructose-6P by low-K_m hexokinases. Within the liver these hexokinases are weakly expressed. Here fructose is phosphorylated by the liver-specific fructokinase to fructose-1P, followed by aldolase B-mediated cleavage. The high activity of fructokinase leads to an accumulation of fructose-1P, thus trapping phosphate from ATP, with a transient decrease of ATP, without toxic effects, in rat liver (*in vivo)*, in the perfused rat liver (*in vitro)* and in man.

First, we compared the effect of fructose on the ATP level of primary rat, murine and human hepatocytes, the human hepatoma cell lines Huh7 and HepG2, the murine hepatoma cell line Hepa 1-6 , and the TGF-β overexpressing murine cell line AML-12. In the presence of 50 mM fructose, ATP was depleted by 80-90% compared to controls in primary murine, rat and human hepatocytes (Fig. 2A). The mean effective concentration $(EC)_{50}$ of fructose for ATP depletion was 4.1/ 5.7/ 8.5 mM for primary murine, rat and human hepatocytes, respectively. The presence of fructose had no significant effect on basal hepatocyte viability at any concentration tested. In contrast to all primary cells, none of the tested tumor cell lines showed a fructose-mediated decrease in ATP. On the contrary, rather an increased ATP level was observed after fructose treatment, which indicates profound alterations in the anaerobic energy metabolism of these cells compared to primary cells.

Next, we addressed the functional consequences of fructose exposure on TNF-induced apoptosis in ActD-sensitised primary cells compared to malignantly transformed cell lines. Toxicity was assessed by LDH release from dying cells after 18 h. The data in Fig 2B demonstrate that while primary hepatocytes were protected against TNF-induced cell death in the presence of fructose, the transformed cells were prone to TNF-induced apoptosis under these conditions. The data suggest that biochemical changes in the sugar metabolism of hepatoma cell lines are responsible for this phenomenon.

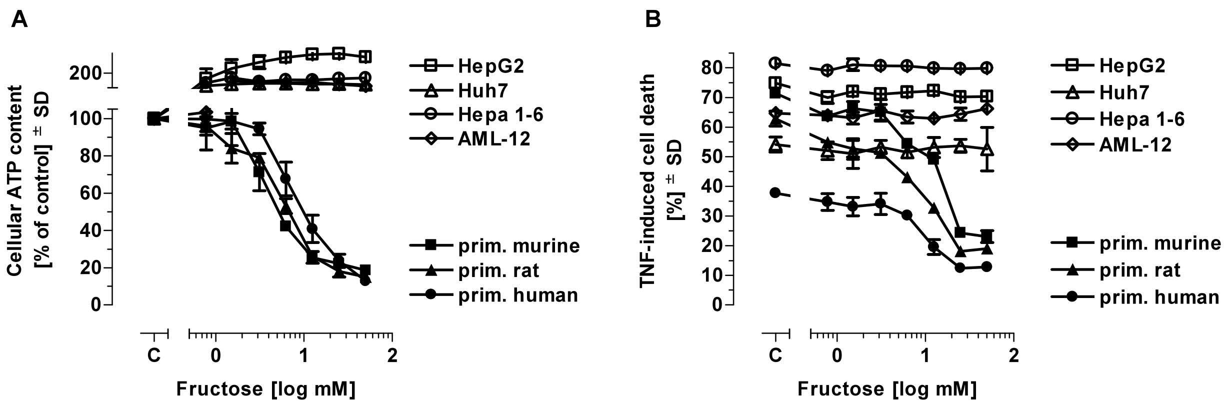

Figure 2. Selective induction of apoptosis by TNF in hepatic tumor cell lines but not in primary hepatocytes under ATP depleting conditions. A) Isolated primary (prim.) hepatocytes derived from mice, rats or humans, or different hepatic tumor cell lines (HepG2, Huh7, Hepa1-6, AML-12) were treated with increasing concentrations of fructose for 30 min, and ATP content was determined. **B)** TNF-induced cell death in ActD-sensitised cells following incubation with increasing concentrations of fructose was assessed by LDH assay after 18 h. Data are means ± SD of triplicate cultures.

The role of fructokinase and aldolase B in fructose-induced ATP depletion

Since the metabolism of fructose *via* fructokinase and the subsequent cleavage of fructose-1-phosphate *via* aldolase B is required to cause fructose-induced ATP depletion, the question arose whether either a reduction of fructokinase or an increase in aldolase B activity might prevent the accumulation of fructose-1-phosphate and hence ATP depletion in the cell lines. To test this possibility we determined the specific activities and the transcription pattern of fructokinase and aldolase B in lysates of primary human hepatocytes in comparison to the hepatoma cell lines HepG2 and Huh7. The results in Fig. 3A show that the specific activity of fructokinase was unchanged compared to primary human hepatocytes in the two human cell lines investigated, while the specific activity of aldolase B was significantly reduced. The transcription analysis of fructokinase did not correlate with the results of the activity determination, which is not surprising, because mRNA level does not necessarily reflect enzyme activity. In case of aldolase B, a reduction of mRNA expression did match the activity data (Fig. 3B). These results suggest that the liver-specific enzymes are unlikely to be responsible for the lack of ATP depletion, because a reduction of aldolase B activity should increase the accumulation of fructose-1P and ATP depletion rather than decrease it.

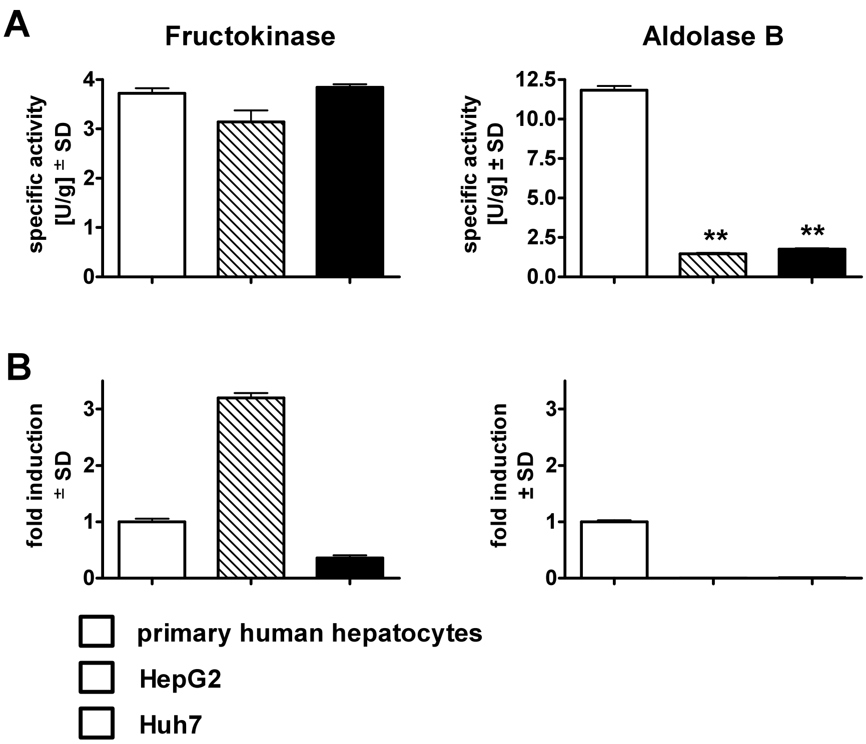

Figure 3. Modulations in the liver-specific enzymes fructokinase and aldolase B are not responsible for the lack of fructose-mediated ATP depletion in the cell lines. A) Specific activities of fructokinase and aldolase B were determined in lysates of primary human hepatocytes originating from 3 different donors, HepG2 and Huh7 cells, as described. Data represent means ± SD of triplicates. *, $p<0.05$ and **, $p<0.01$ compared with primary hepatocytes based on ANOVA followed by the Dunnett's multiple comparison test. **B)** Quantitative real-time-PCR was used to determine the expression levels of fructokinase and aldolase B in primary human hepatocytes and the human hepatoma cell lines HepG2 and Huh7. Data were normalised to β-actin and the ratios compared to primary human hepatocytes, which were set to 1, are illustrated as fold induction in means ± SD of triplicates.

Upregulation of muscle-type hexokinase II in hepatoma cell lines

Reflecting the organ's central function in energy homeostasis, a specific high-K_m HKIV is expressed in liver, while low-K_m hexokinases are only expressed weakly. Because increased expression of these low-K_m hexokinases was described as a typical phenotype of highly malignant cancer cells e.g. hepatocellular carcinoma [143-145], we hypothesised a causal involvement of hexokinase upregulation in the altered fructose metabolism of tumor cells. To test this possibility most directly, we compared the specific activities of hexokinases in primary hepatocytes and cell lines. Figure 4A demonstrates a significant increase in specific low-K_m hexokinase activity in all tested tumor cell lines. HepG2 cells showed an increase in activity by a factor of about 3, and Huh7 by about 4 compared to human hepatocytes, while the murine cell line Hepa 1-6 showed an increase by a factor of 24 and AML-12 by 13 in comparison to primary murine hepatocytes. To clarify which hexokinase isoform is responsible for this increased activity, we examined the transcription pattern of all 4 hexokinase isoforms in the human cell lines compared to the primary hepatocytes. The transcription of HKII was strongly increased in HepG2 and Huh7 cells, while isoforms I and III were not upregulated in these cells (Fig. 4B). The liver-specific high-K_m HKIV showed a weaker expression in the cell lines. We then confirmed that the increased mRNA level of HKII resulted in a stronger protein expression: Western blotting showed a distinct upregulation of HKII expression in all cell lines tested compared to the primary hepatocytes originating from two different individuals. These findings provide uniform evidence, from the transcriptional level to the translational level to the enzyme activity, that the upregulation of HKII is a biochemical hallmark of transformed liver cells.

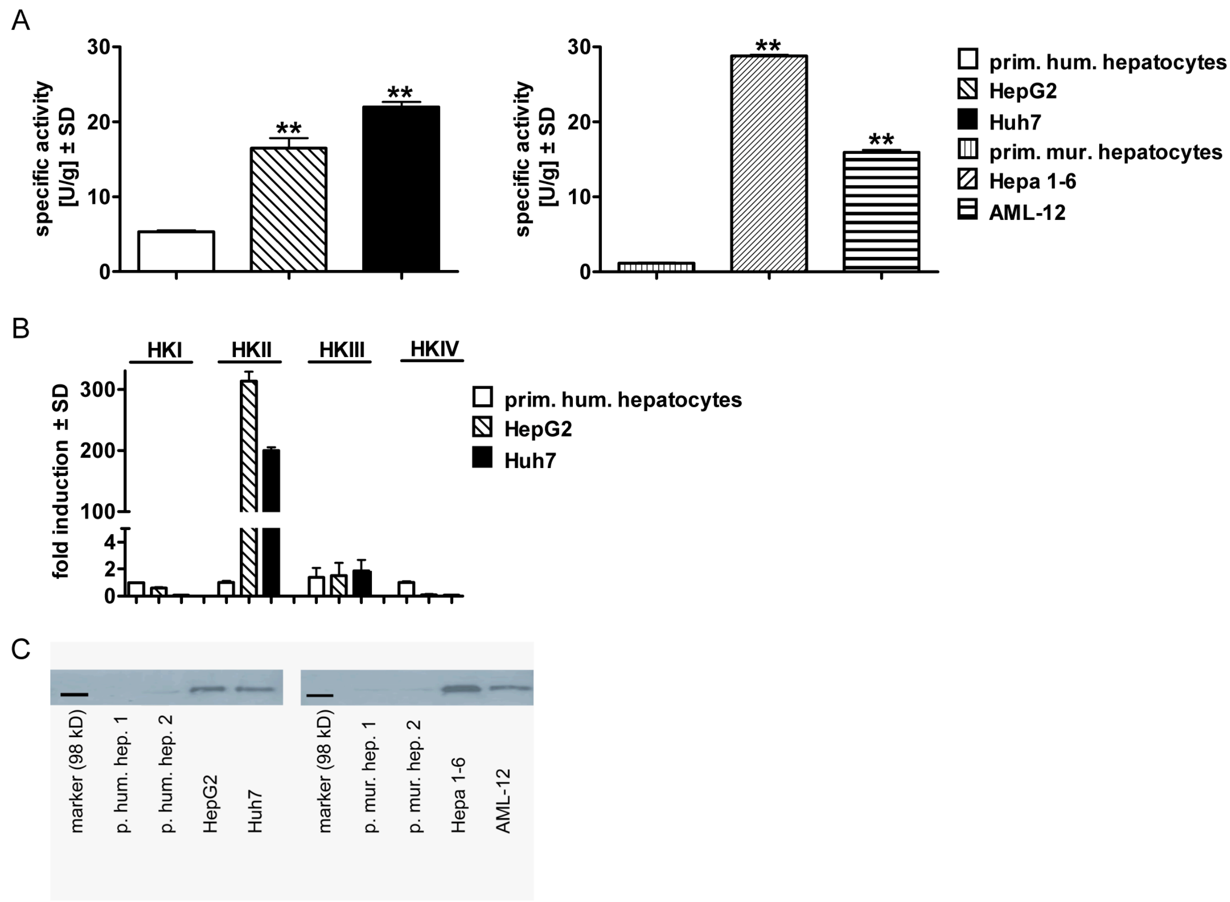

Figure 4. Hexokinase II is upregulated in cell lines that lack ATP depletion. A) Specific activities of low-K_m hexokinases were determined in lysates of primary human hepatocytes of three different donors, the human cell lines HepG2, Huh7, primary murine hepatocytes of three animals, and the murine cell lines Hepa 1-6 and AML-12. Data represent means ± SD of triplicates. *, $p<0.05$ and **, $p<0.01$ compared to primary hepatocytes based on ANOVA followed by the Dunnett's multiple comparison test. **B)** Quantitative real-time-PCR was used to determine the expression profile of the 3 low-K_m hexokinase isoforms and the liver-specific high-K_m isoform IV. Data were normalised to β-actin and the ratios compared to primary human hepatocytes, which were set to 1, are illustrated as fold induction in means ± SD of triplicates. **C)** Expression of HKII was determined by Western blot in primary human hepatocytes of two donors, primary murine hepatocytes of two mice, and the cell lines HepG2, Huh7, Hepa 1-6, AML-12. 25 µg of protein was loaded and equal loading was checked by Coomassie staining.

Inhibition of low-K_m hexokinase allows ATP depletion in HepG2 cells

In order to functionally determine whether or not upregulation of hexokinase leads to muscle-type fructose metabolism and thereby prevents ATP depletion in the cell lines, we used a hexokinase inhibitor. 3-bromopyruvate (Br-PA) is considered a specific inhibitor of HKII and is used in preclinical experiments to target HKII, which has been reported also by others to be a major player in the increased glycolytic activity of highly malignant tumors [146-148]. In lysates of HepG2 cells, we determined and confirmed the inhibition of low-K_m hexokinase activity (IC_{50} = 11 µM) by Br-PA. In the presence of 80 µM, i.e. at nearly complete inhibition, metabolic ATP depletion by fructose was resumed by these cells in a concentration-dependent manner (Fig. 5A). Inhibition of low-K_m hexokinase by Br-PA alone reduced ATP by 13 %, while the presence of 50 mM fructose further reduced ATP to about 50 % of control cells. When we provided pyruvate as an alternative energy source, bypassing glycolysis, ATP depletion (Fig. 5B) and basal toxicity were completely blunted (data not shown) in HepG2 cells in the presence of Br-PA. This shows that pyruvate antagonises the ATP depletion and also prevents the cytotoxicity of Br-PA. Under such conditions, inhibition of low-K_m hexokinase reconstituted the fructose-induced ATP depletion in HepG2 cells (Fig. 5C). These experiments strongly corroborate the causal role of increased HKII expression in preventing fructose-induced ATP depletion.

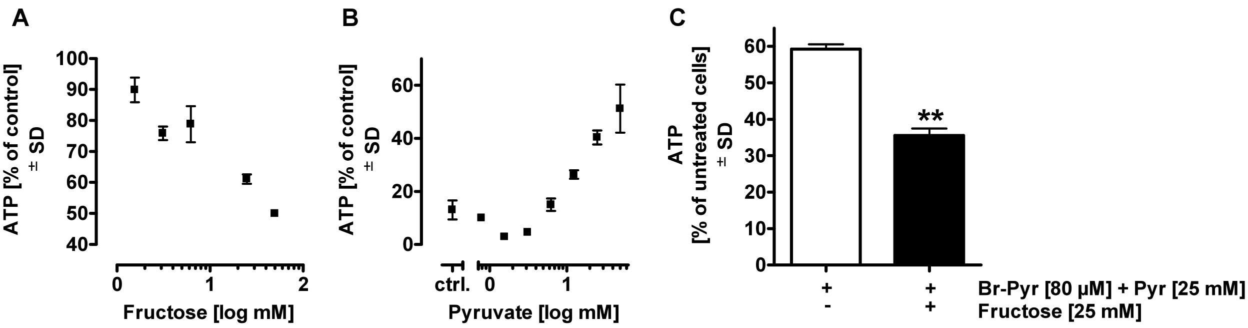

Figure 5. Inhibition of low-K_m hexokinases reconstitutes hepato-specific fructose-induced ATP depletion in HepG2 cells. A) Fructose-induced ATP depletion in the presence of Br-PA. HepG2 cells were incubated with Br-PA for 1 h. Increasing concentrations of fructose were added, and the effect on the ATP level was assessed. Data represent means ± SD of triplicates. **B)** Restoration of ATP levels by increasing concentrations of pyruvate in the presence of Br-PA. Pyruvate was added 1 h before treatment of HepG2 cells with 80 µM Br-PA. After another 90 min, the ATP level was assessed. Data represent means ± SD of triplicates. **C)** Further depletion of the ATP level by fructose in the presence of Br-PA and pyruvate. HepG2 cells were cultured in the presence of pyruvate for 1 h, 80 µM Br-PA was added for another 90 min before 25 mM fructose was added. The effect on the ATP level was determined 30 min after fructose administration. Data are means ± SD of triplicates. **, $p<0.01$ based on t test.

Modulation of fructose metabolism in primary murine hepatocytes by induction of HKII *via* HIF1α prevents ATP depletion

Stabilisation of HIF1 and its influence on the HKII promoter *via* its HIF-response elements is characteristic for malignant cells or tissues and leads to upregulation of HKII [116, 149]. The actual activity of HIF1, composed of an α- and β-subunit, is determined by the degradation of HIF1α, which is induced by proline hydroxylation followed by ubiquitination and proteasomal degradation [150-152]. The relevant prolyl hydroxylase is inhibited by hypoxia and iron chelators (e.g. Desferal), leading to stabilisation of HIF1α [153, 154]. Our experiments, shown in Fig. 6A demonstrate that in the presence of Desferal, primary murine hepatocytes accumulate HIF1α in the nucleus after 20 h and increase expression of HKII (Fig. 6B). To further characterise the role of HKII in fructose metabolism, we tested whether upregulation of HKII prevents the liver-specific ATP depletion by fructose in primary hepatocytes. ATP depletion in hepatocytes by 50 mM fructose was completely blocked by Desferal pretreatment (Fig. 6C). To confirm that the effect of Desferal is due to HKII upregulation, we then inhibited this enzyme by Br-PA. Br-PA completely reversed the effect of Desferal and restored fructose-induced ATP depletion in Desferal pretreated cells (data not shown).

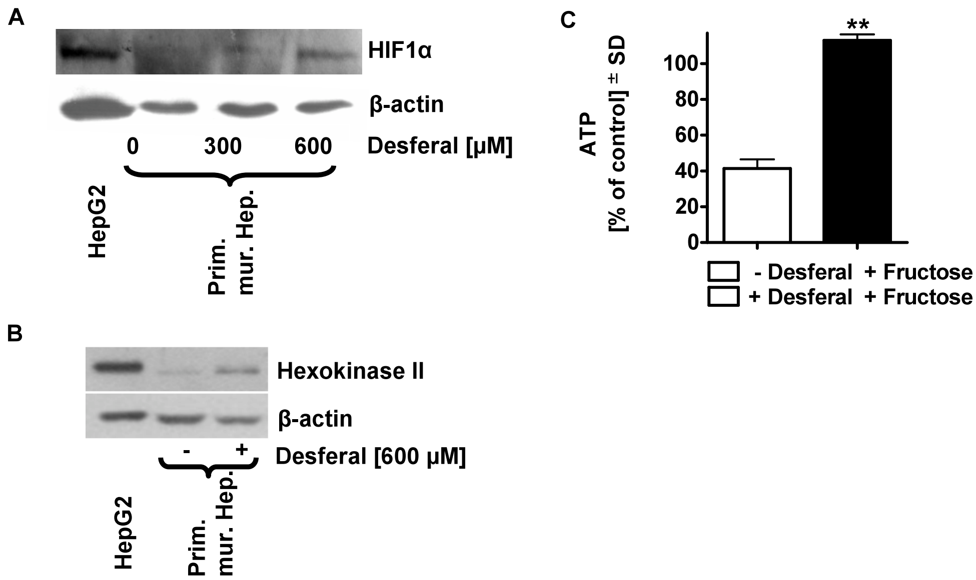

Figure 6. Upregulation of hexokinase II by HIF1α prevents fructose-induced ATP depletion in primary murine hepatocytes. A) Primary murine hepatocytes were cultured in the presence of Desferal. After 20 h nuclei were prepared and the presence of HIF1α was analysed by Western blot. **B)** The effect of Desferal-mediated HIF1α stabilisation on the expression of HKII was analysed by

Western blot. Primary murine hepatocytes were cultured in the presence of 600 µM Desferal for 20 h before cell lysates were prepared. **C)** The influence of Desferal-induced expression of HKII on fructose-mediated ATP depletion was studied. Primary murine hepatocytes were cultured in the presence of 600 µM Desferal for 20 h. 50 mM fructose was added and the ATP level was determined after 30 min. Data represent means ± SD of triplicates. **, $p<0.01$ based on t test.

Overexpression of HKII prevents the cytoprotective effect of fructose against TNF-induced apoptosis

Since our data showed that increased activity of HKII prevents ATP depletion in primary hepatocytes and *vice versa* in the hepatoma cell line HepG2, we asked whether increased HKII activity also prevents fructose-mediated protection against TNF-induced apoptosis. The modulation of HKII expression with Desferal was experimentally not feasible in this context, because Desferal treatment reduced susceptibility for TNF-induced cell death (data not shown). To answer this question we instead transfected primary murine hepatocytes with a HKII expression-vector. We performed a co-transfection of EGFP- and HKII-vector in ratio of 1:3. Transfection efficiency, as determined by fluorescence microscopy of EGFP transfected primary murine hepatocytes was 5-10%. Increased expression of HKII after transfection was verified by Western blot (Fig. 7A). 15 h after transfection, cells were treated with fructose, apoptosis was induced with a combination of ActD and TNF, and chromatin condensation, as a marker of apoptosis, was determined microscopically in EGFP-positive cells by Hoechst staining after 7h. Figure 7B illustrates the reduction by more than 50% of TNF-induced chromatin condensation in the presence of 50 mM fructose in EGFP/mock transfected cells. The transfection with EGFP/HKII reduced the protective effect of fructose in this setting from 10% to 25%. Figure 7C shows the corresponding pictures of the EGFP/mock and EGFP/HKII transfected cells and their chromatin.

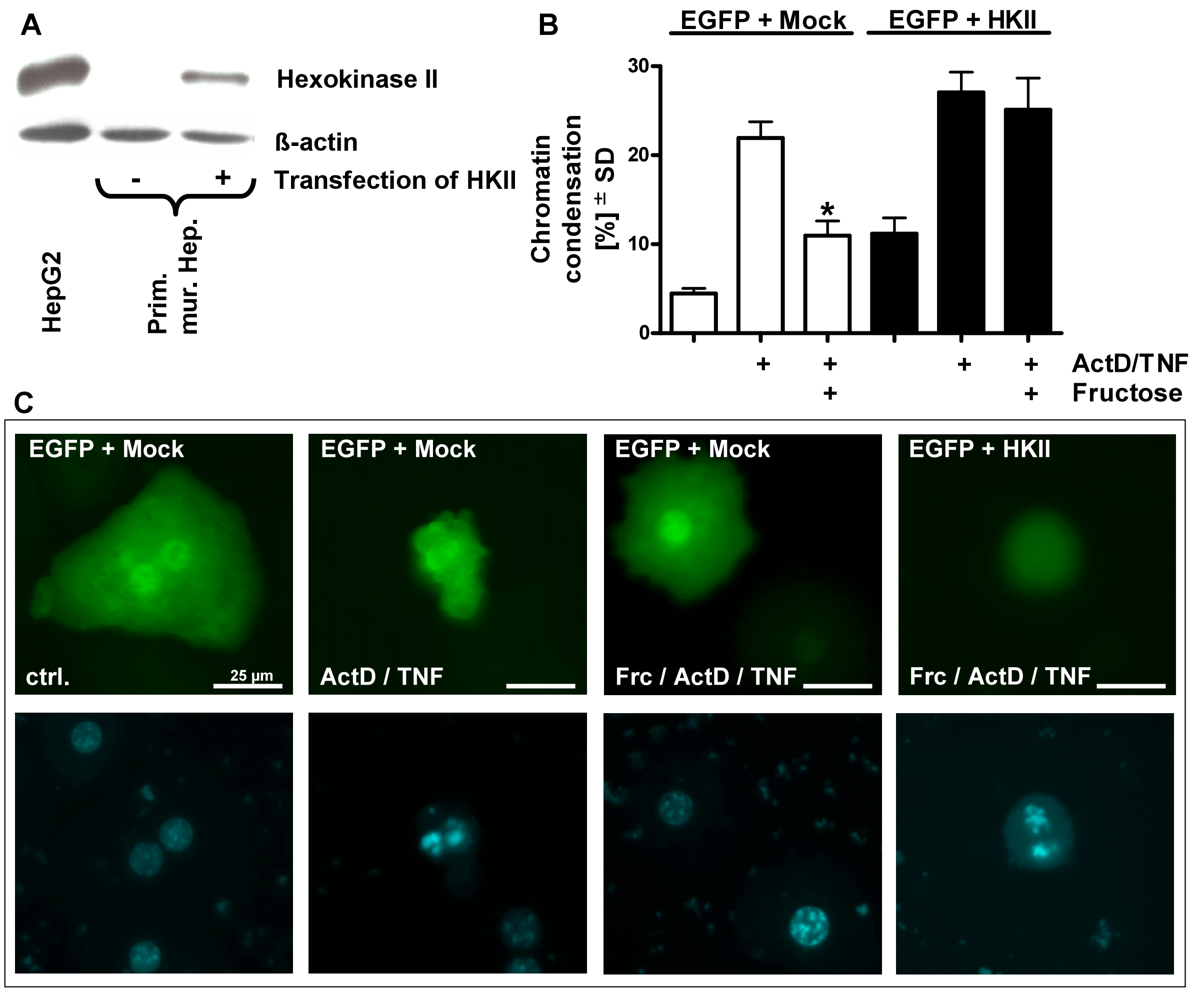

Figure 7. Overexpression of HKII prevents the cytoprotective effect of fructose against TNF-induced apoptosis. A) Primary murine hepatocytes were transfected with HKII vector for 15 h. HKII expression was detected by Western blot. HepG2 cells were used as positive control. **B)** Primary hepatocytes were transfected with EGPF and HKII and incubated for 15 h before treatment with ActD/TNF. The effect of HKII expression on the protective effect of fructose (50 mM) against ActD/TNF-induced apoptosis was determined by analysing chromatin condensation in EGFP-positive cells by Hoechst staining 7 h after TNF was added. Data represent means of three independent experiments ± SD. *, $p<0.05$ compared with primary hepatocytes treated with ActD/TNF based on ANOVA followed by the Dunnett's multiple comparison test. **C)** Exemplary pictures were taken of cells treated according to Figure 7B.

3.5 Discussion

Our initial finding that fructose-induced hepatic ATP depletion protects hepatocytes against TNF-induced cell death *in vitro* and *in vivo* [73] was unexpected and warrants a molecular explanation. The incidental finding that this does not apply *in vitro* to the commonly used transformed liver cell lines HepG2, Huh7, AML-12 and Hepa 1-6, and the fact that alterations in the energy metabolism are characteristic of highly malignant cells [155], prompted us to carry out a detailed study on whether transformed cells lack liver-specific fructose metabolism.

Our results show that all tested hepatoma cell lines lack fructose-induced ATP depletion and protection against TNF-induced cell death, while primary mouse, rat and human hepatocytes demonstrate ATP depletion and protection. This emphasises the potential of the non-toxic and naturally occurring sugar fructose and the liver-specific metabolism to implement selectivity into receptor-mediated tumor cell killing.

Our findings falsified the most obvious explanation for the lack of ATP depletion in tumor cells, i.e. that the activity of aldolase B is upregulated. Warburg's theory, published more than 80 years ago, demonstrated that highly malignant cancer cells had increased glycolytic activity [87], later shown to be associated with increased expression of type II hexokinase [92, 128]. In line with these findings, we demonstrated here that increased expression of HKII in transformed cells of hepatic origin leads to a shift from liver-type to a muscle-type fructose metabolism, thereby preventing fructose-induced ATP depletion under our conditions. These findings are corroborated by showing that inhibition of hexokinases by 3-bromopyruvate restores fructose-induced ATP depletion in HepG2 cells and that stabilisation of HIF1 by Desferal leads to increased expression of HKII, which in turn prevents fructose-induced ATP depletion. Further, we could show that inhibition of TNF-induced apoptosis by fructose is prevented by overexpression of HKII.

It is noteworthy that the increased expression of HKII, as a typical hallmark of rapidly growing tumors, is already exploited in clinical practice for the detection of malignant cells in PET scans owing to the fact that this rate-limiting enzyme causes the accumulation of labelled hexoses in malignant tissue [156]. Evidence accumulates that increased expression of HKII is associated with advanced tumor stage, turning this parameter into a good prognostic marker [145, 157]. These clinical data lend further support to our proposal to take advantage of the increased expression of HKII in liver tumors to transiently protect healthy liver cells against TNF by application of fructose and to simultaneously target malignant cells for receptor-mediated apoptotic cell death. As to the clinical feasibility of such a nowadays only little spectacular proposal, it is noteworthy that liver tumors take their supply of nutrients and oxygen almost exclusively from the liver artery [158, 159], while the surrounding healthy cells are nourished by the portal vein. Thus, this anatomic situation is indeed favourable for introducing fructose *via* its natural entry point and TNF by arterial perfusion.

4 Fructose-induced hepatocyte ATP depletion prevents TNF-induced apoptosis by inhibition of JNK signalling

Tobias Speicher[1], Ulrike Köhler[1], Alexander Chouker[2], Timo Weiland[1] and Albrecht Wendel[1,*]

[1] Chair of Biochemical Pharmacology, Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany
[2] Department of Anesthesiology, Klinikum Grosshadern, Munich, Germany
* To whom correspondence should be addressed
Albrecht Wendel, Faculty of Biology, University of Konstanz, M667, D-78457, Konstanz, Germany.
Phone: +49-7071 45802; FAX +49-7071 44464; E-mail: albrecht.wendel@uni-konstanz.de

Keywords: fructose metabolism, TNF, CD95, adenosine, cAMP, PKA, JNK

FOOTNOTES

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The Abbreviations used are: ActD, Actinomycin D; cAMP, cyclic adenosine monophosphate; HCC, hepatocellular carcinoma; IHP, isolated hepatic perfusion; JNK, c-Jun N-terminal kinase; PKA, cAMP-dependent protein kinase; tBid, truncated form of Bid; TNF, tumor necrosis factor-alpha.

4.1 Summary

We previously reported that fructose-induced hepatic ATP depletion prevents TNF-induced while in enhances CD95-induced apoptosis *in vitro* and *in vivo*. Moreover, transformed liver cells were not protected against TNF due to metabolic alterations. These findings open up the possibility to make the killer molecule TNF specific for liver tumor cells.

Here, we address the molecular mechanism of fructose-mediated protection against ActD/TNF-induced hepatocyte apoptosis. Fructose-induced ATP depletion resulted in transient extracellular accumulation of the degradation product adenosine *in vitro* and *in vivo*. Adenosine induced a cAMP response in an autocrine manner. cAMP as well as adenosine mimicked the modulation by fructose on CD95- and TNF-induced apoptosis, and both effects were sensitive to inhibition of Protein kinase A. Fructose-mediated ATP depletion as well as cAMP signalling prevented the proapoptotic, sustained phase of TNF-induced JNK activation. We explain the dichotomous effects of fructose on CD95- and TNF-induced cell death by the observation that JNK signalling is not required for CD95-, but for TNF-induced apoptosis. Furthermore, fructose-induced inhibition of JNK prevented the activation of the intrinsic apoptotic pathway *via* Bid in response to TNF receptor activation. Cleavage of Bid and tBid translocation to mitochondria which is essential for TNF-induced hepatocyte apoptosis was inhibited by fructose-induced ATP depletion in a PKA-dependent manner. These findings provide a mechanistic rationale for the cytoprotection of hepatocytes from death receptor-induced cell death by non-toxic doses of fructose.

4.2 Introduction

Selective and organ-specific tumor therapy is an equally noble as well as ambitious aim of cancer research, which has been partially reached in a variety of malignant diseases such as leukemias, e.g. by specifically attacking neoplastic growth by immunological means. However, in solid tissue cancer of the liver –in particular in HCC- such a therapeutic concept is currently not at hand. TNF was the first cytokine to be used for cancer therapy with biologicals, and such an approach raised the hope for death receptor ligand-induced killing of hepatomas. In contrast to CD95-induced programmed cell death, apoptosis induced by TNF requires inhibition of NF-κB signalling (basically in order to prevent formation of antiapoptotic factors), which can be experimentally achieved by transcriptional inhibition in the presence of actinomycin D (ActD). Inhibition of NF-κB signalling was shown to sustain TNF-induced JNK activation [160, 161], which *via* cleavage of Bid in turn activates the intrinsic apoptosis pathway [52, 53]. Subsequent activation of the caspase cascade finally leads to execution of ActD/TNF-induced apoptosis. To avoid severe dose-limiting toxicity of TNF, which prevents its systemic use, locoregional drug delivery systems such as isolated hepatic or isolated limb perfusion were experimentally studied or proposed [74]. While combination of TNF with antineoplastic agents such as melphalan in isolated limb perfusion allowed impressive clinical success in the remission of advanced non-resectable soft tissue sarcomas with very poor prognosis, selectivity for tumor cells remains a largely unsolved problem in liver cancer [72]. As an alternative approach to a selective tumor cell hit, we propose a selective protection of healthy hepatocytes against death receptor ligand-induced killing.

We previously showed that hepatic ATP depletion by various phosphate-trapping ketohexoses such as fructose inversely controls CD95- and ActD/TNF-induced apoptosis. While ATP depleted hepatocytes are fully protected against TNF-induced apoptosis *in vitro* and *in vivo*, sensitivity for CD95-induced cell death is increased [73]. Interestingly, malignantly transformed hepatoma cell lines are not depleted of ATP by fructose and hence fail to be protected against ActD/TNF-induced toxicity upon fructose treatment. As the biochemical rationale, we found type II hexokinase expression to be upregulated in malignant hepatocytes, which in turn induces a switch of the hepatocyte-specific fructose metabolism *via* fructose-1-phosphate to the ubiquitous metabolism *via* fructose-6-phosphate (first part of the thesis). Increased expression of type II hexokinase was shown to be characteristic for advanced HCC tumor stage in humans [145]. From our findings and those of others we proposed a metabolic approach for a hepatic tumor therapy based on the selective protection of healthy hepatocytes from death receptor ligand-induced toxicity (first part of the thesis) However, the factor(s), which ultimately and causally protected healthy hepatocytes after fructose-induced ATP depletion against TNF-induced cell death, remained still elusive.

In this study, we investigated the downstream events following fructose-induced ATP depletion in order to elucidate the molecular mechanism of protection against ActD/TNF-induced toxicity in healthy hepatocytes. We show here, that massive ATP depletion results in a transient accumulation and release of the degradation product adenosine from hepatocytes *in vitro* and *in vivo*, which in turn leads to increased levels of intracellular cAMP and hence protection. We provide pharmacological supporting evidence that adenosine and cAMP-analogues mimic the effects of fructose-induced ATP depletion on ActD/TNF- and CD95-induced apoptosis, and that inhibition of cAMP-dependent protein kinase A abolishes them. In order to explain the dichotomy between enhanced CD95-induced toxicity and suppressed ActD/TNF-induced toxicity under these conditions, we studied the role of JNK activation in signal mediation in our system. We show here, that fructose as well as cAMP-analogues prevents the late and sustained part of JNK activation, that is required for TNF-induced cell death. Finally, we show that fructose-induced JNK-inhibition prevents the activation of the intrinsic apoptotic pathway in response to TNF receptor activation. These results provide a plausible mechanistic rationale for the strategy to selectively protect healthy hepatocytes from death receptor-induced cell death by non-toxic doses of fructose.

4.3 Materials and Methods

Reagents

Agonistic CD95 antibody (αCD95, clone Jo-2) was purchased from BD PharMingen. (San Diego, CA, USA), recombinant murine TNF from Innogenetics (Ghent, Belgium). KT 5720 was obtained from Alexis (Lausen, CH), db-cAMP from Tocris Cookson (Bristol, UK) and 8-Br-cAMP-AM from Biolog Life Science (Bremen, Germany). Cell culture plates and co-culture inserts (ThinCert) were purchased from Greiner (Nürtingen; Germany), culture medium, FCS, penicillin, streptomycin from PAA (Pasching, Germany), collagen was obtained from Serva (Heidelberg, Germany), Percoll from Pharmacia Biotech (Uppsala, Sweden). Unless further specified, all other reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Cell cultures

Hepatocytes were isolated from 8-wk-old C57Bl6 wild type mice by the two step collagenase perfusion method and cultured as described [132]. In brief, hepatocytes were plated in 500 µl RPMI 1640 medium containing 10% FCS in collagen-coated

24-well plates at a density of 9 x 10^5 hepatocytes per well, or in collagen-coated 6-well plates at a density of 3 x 10^6 hepatocytes per well. Cells were allowed to adhere to culture dishes for 4 h before the medium was exchanged to RPMI 1640 without FCS. The cells were cultured in RPMI1640 supplemented with 10% FCS, 1% Pen/Strep and maintained in a humidified incubator at 5% CO_2 / 95% air at 37°C in 24 well plates. HepG2 cells were maintained in RPMI 1640 medium containing 10% FCS. HepG2 were maintained in 70-mm^2 flasks, the day before experiments were carried out they were harvested with Accutase and plated in 200 µl media containing 10% FCS in 24-well plates (1.2 x 10^6 cells/well) or in 100 µl in inserts for co-culture (0.3 x 10^6 cells/insert). Incubation of murine hepatocytes or cell lines was started directly after medium exchange for RPMI 1640 without FCS. HepG2 were sensitised with 1 µg/ml, primary hepatocytes with 400 ng/ml ActD 15 minutes before treatment with recombinant murine TNF (100 ng/ml). All substances were diluted in saline supplemented with 0.1% HSA. Incubations of primary hepatocytes were carried out at 37°C in a humidified atmosphere composed of 5% CO_2, 40% O_2, and 55% N_2. Incubation of cell lines were carried out in a humidified atmosphere of 5% CO_2 and 95% air for the times indicated.

Cytotoxicity was quantified by measurement of lactate dehydrogenase (LDH) [135] in culture supernatants (S) and in the remaining cell monolayer (C) after lysis with 0.1% Triton X-100, and calculation of the percentage of LDH release from the ratio of S/(S + C). In co-culture experiments cytotoxicity was measured by the reduction of the tetrazolium dye Alamar Blue (BioSource, Solingen, Germany) by viable cells. Untreated cells were used to set the basal level of cytotoxicity (i.e. 0% cytotoxicity), cells lysed with 0.1% Triton X-100 were used to set its maximum level (i.e. 100% cytotoxicity).

ATP content of cultured cells was determined luminometrically (ATP assay kit, Roche, Mannheim, Germany). In brief, cells were incubated in 24-well plates with different stimuli. At the indicated time points, cells were lysed in 150 µl ATP lysis buffer, immediately frozen at -80°C and stored until measurement. Before the assay, frozen samples were thawed on ice. Luminescence was measured in 96-well plates using an automated procedure (VICTOR$_2$ multilabel counter; Wallac Instruments). Data were compared with calibration solutions, and ATP data are expressed as the percentage of untreated control cells.

Intracellular cAMP was determined by Direct cAMP Enzyme Immunoassay Kit according to the manufacturer's instructions (Assay Designs, Ann Arbor, Michigan, USA).

MAPK Assay

To test the effects of treatments on JNK activity 5 x 10^6 primary hepatocytes were washed twice with ice-cold 2 mM Na-vanadate in PBS followed by lysis in buffer containing 10 mM Tris, pH 7.6, 1% Triton X-100, 0.05 M NaCl, 5 mM EDTA, 2 mM Na-vanadate, and 20 mg/ml aprotinin. After centrifugation for 15 min at 4°C at 14,000 g, protein concentration was determined and 100 µg of protein was incubated for immunoprecipitation overnight with agarose conjugated JNK1, 2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 2 washing steps in lysis buffer, followed by 2 washing steps in assay buffer immune-complex kinase assays were carried out as described [162, 163]. In brief, reactions were performed in a final volume of 30 µl of kinase buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM DTT, 20 mM ATP). Reactions were initiated by addition of 2 µg c-Jun 1-79 (Calbiochem, Schwalbach, Germany) and 6 µM [γ-32P]ATP (5.000 Ci/mmol). After 30 min at 25°C, the reactions were terminated by the addition of Laemmli sample buffer, and the phosphorylation level of c-Jun was evaluated by SDS-PAGE followed by autoradiography and phosphorimage analyses.

Caspase-3/-7 activity assay

Cells were washed three times with phosphate-buffered saline (PBS) and lysed with hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PEFA-block plus 0.1% Triton X-100). After centrifugation (15 min, 13,000 g, 4°C) supernatants were frozen at -80°C. 7-amino-4-trifluoromethylcoumarin (afc) formation was followed in 50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT, 50 µM fluorogenic substrate DEVD-afc (N-acetyl-asp-glu-val-asp-afc, Biomol, Hamburg, Germany) for 30 min at 37°C. The fluorometer plate Victor² (Wallac Instruments, Turku, Finland) was set at 385 nm excitation and 505 nm emission wavelength. Protein was determined with the Pierce-Assay (Pierce, Rockford Illinois, USA). Specific caspase-3/-7-like protease activity was calculated in pmol free afc per min (µU) and mg protein using serially diluted standards (0-5 µM afc).

Animals

Specific pathogen-free male C57Bl6 mice (25 g) were from the in house Animal Breeding Facility of the University of Konstanz. Animals were held at 22°C and 55% humidity, and given a constant day–night cycle of 12 h. Mice were starved overnight before the *in vivo* experiments started. Experiments generally commenced at 8 AM. All steps of animal handling were performed according to the Guidelines of the European Council (directive 86/609/EEC) and the national German authorities and followed the directives of the University of Konstanz Ethical Committee.

In Vivo Experiments

Fructose (10 g/kg) was injected intraperitoneal in a volume of 300 µl pyrogen-free water. Blood samples were obtained after lethal intravenous anaesthesia of mice with 150 mg/kg pentobarbital plus 0.8 mg/kg heparin. After midline laparotomy and opening of the chest, blood samples were drawn. Due to a short half life of adenosine, the syringes were pre-filled with ice cold physiological saline containing dipyridamole 2 x 10^{-4} M, EHNA (erythro-9-(2-hydroxy-3-nonyl)adenine) 2 x 10^{-5} M, EDTA-Na 2 x 10^{-2} M, EGTA 2 x 10^{-2} M, and DL-α-glycerophosphate 2 x 10^{-2} M to prevent degradation or additional formation of plasma adenosine by inhibition of ecto-nucleotidases and non-specific phosphatases. Samples were immediately centrifuged for 2 min at 4°C at 5,000 g to separate the plasma from the cellular fraction. The samples were processed and plasma concentrations of adenosine determined by high-performance liquid chromatography (HPLC) as described previously [164, 165].

Immunoblotting

Translocation of Bid from cytosol to mitochondria was analysed using a selective digitonin permeabilisation method. To separate cytosol- and mitochondria-containing fraction for western blotting 5 x 10^6 cells per 6 well were used. After washing the cells with PBS, the cell membrane was permeabilised in buffer A (10 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA pH 7.4) containing 0.05% digitonin by gentle shaking for 8 min. Cells were scraped of and centrifuged for 10 min, 10,000 g, 23°C. Protein from the supernatants was separated on 15% polyacrylamide gels for cytosolic detection of Bid. Integrity of the mitochondria membrane was confirmed by cytochrome C Western blot. The pellet was resuspended in buffer A containing 2 %

CHAPS, incubated for 30 min on ice and centrifuged for 15 min, 10,000 g, 23°C. Supernatant was used for detection of tBid translocated to mitochondria. Protein concentration was determined and samples were boiled with laemmli buffer for SDS-PAGE. Bid and tBid were detected with anti-Bid antibody from New England Biolabs (Frankfurt am Main, Germany). Loading control was performed with rabbit polyclonal anti-β-actin antibody 1:1000 (Cell Signalling, Boston, USA). Antigen-antibody complexes were visualized with HRP-coupled secondary antibodies (goat anti-rabbit, Dianova, Hamburg, Germany), (rabbit anti-goat, Dako, Glostrup, Denmark) and ECL detection system (2.5 mM luminol, 0.4 mM para-coumaric acid, 10 mM Tris base, 0.015% H_2O_2, pH 8.5).

Statistical Analysis

Statistical differences were determined with an unpaired t test if applicable, or data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

4.4 Results

Fructose-induced hepatic ATP depletion leads to release of adenosine *in vitro* and *in vivo*

First, our previous findings on the concentration-dependent effect of fructose on cell death induced by ActD/TNF or by agonistic CD95 antibody were confirmed. Primary murine hepatocytes were treated with various concentrations of fructose for 30 min before addition of TNF or αCD95 antibody. For TNF-induced apoptosis cells were sensitised with ActD 15 min before TNF application. Toxicity was assessed by LDH release from dying cells after 18 h. Fructose prevented ActD/TNF-induced but enhanced CD95-induced cell death in primary hepatocytes in a concentration-dependent manner, while fructose alone did not affect basal hepatocyte viability at any concentration tested (Fig. 1A). Next, we focused on secondary effects of fructose-induced hepatic ATP depletion, which is due to the preferential metabolism of ketohexoses by the liver-specific fructokinase, aldolase B pathway [81, 166]. Since a decline of the total amount of adenosine nucleotides has been described during fructose-induced ATP depletion [81], we addressed the metabolic consequences of fructose-induced ATP depletion on production and release of adenosine from primary hepatocytes *in vitro*. In the presence of 50 mM fructose, hepatic ATP was depleted by more than 70% within 20 min. Concomitantly, adenosine was released from hepatocytes into the supernatant, where within 20 minutes the concentration doubled from 24 to 49 nM (Fig. 1B). In order to extend these *in vitro* findings to the *in vivo* situation, we examined whether injection of fructose into mice leads to the accumulation of plasma adenosine. Intraperitoneal administration of 10 g/kg fructose, which we previously showed to induce hepatic ATP depletion and protection against TNF-induced hepatotoxicity [73], induced a 9-fold increase in plasma adenosine with a maximum adenosine concentration of 900 nM (Fig. 1C). 30 min after fructose administration, this increase in adenosine concentration returned to basal level.

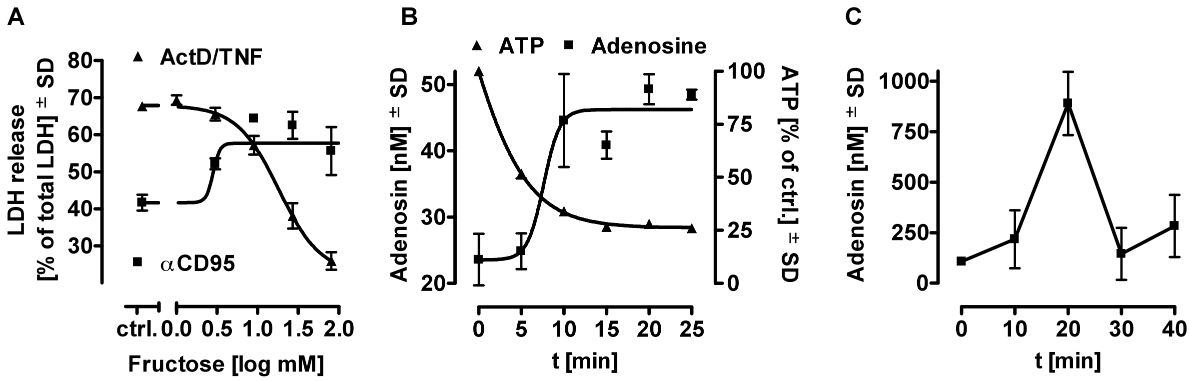

Figure 1. Fructose-induced ATP depletion inversely controls CD95- and TNF-induced cell death and induces hepatocyte adenosine release *in vitro* and *in vivo*. A) Cell death induced by ActD/TNF or by agonistic CD95 antibody in isolated primary murine hepatocytes following incubation with increasing concentrations of fructose for 30 min was assessed by LDH assay after 18 h. Data are means ± SD of triplicate cultures. B) Primary murine hepatocytes were treated with 50 mM of fructose. Intracellular ATP-level and concentration of adenosine in the supernatant was determined every 5 minutes. Data represent means ± SD of triplicate cultures. C) Fructose (10 g/kg) was injected intraperitoneally into mice. Concentration of adenosine in plasma was quantified. Values are means ± SD from 2 animals per time point.

ATP depleting carbohydrates as well as adenosine induce a cAMP response in primary murine hepatocytes

For further mechanistic details, we used the *in vitro* system of isolated primary hepatocytes. Previous studies by others had shown that adenosine induces cytoprotection during cell stress or injury mainly *via* adenylate cyclase-activating receptors in an autocrine manner [167]. Therefore, we monitored the levels of intracellular cAMP in response to ATP depleting ketohexose and adenosine, respectively. While 50 mM of fructose induced a cAMP response to 180% of control cells within 40 min, the non-ATP depleting sugar glucose did not increase the cAMP level (Fig. 2B). Incubation of primary hepatocytes with adenosine (100 µM) also induced a time-dependent cAMP response. Within 10 min after adenosine administration the level of cAMP reached a maximum of 170 % compared to control cells (Fig. 2B).

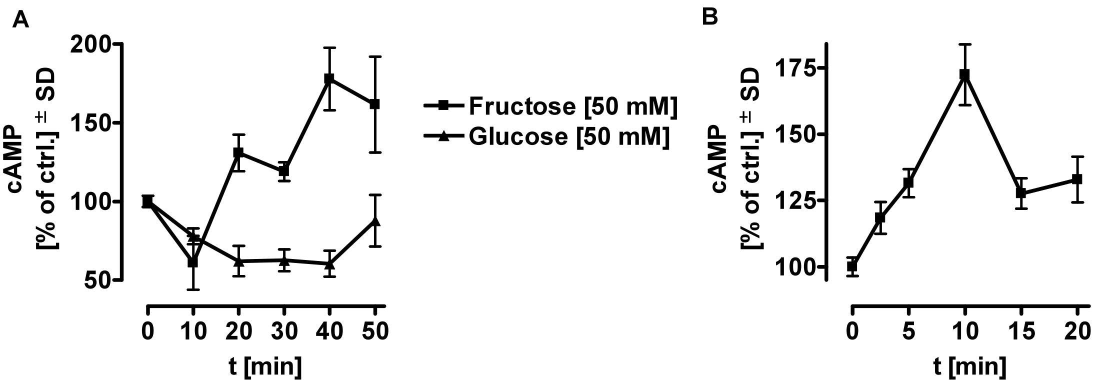

Figure 2. ATP depleting carbohydrate as well as adenosine induce a cAMP response in primary murine hepatocytes. A) Primary murine hepatocytes were incubated with 50 mM of ATP depleting carbohydrate fructose or the non-depleting sugar glucose. Cellular cAMP content was quantified at the indicated time points. B) Primary murine hepatocytes were incubated with adenosine (100 µM) and the influence on intracellular cAMP level was assessed. Date represent means ± SD of triplicate cultures.

Adenosine and cAMP mimic the effects of fructose on cytokine-induced apoptosis

Since fructose-induced ATP depletion leads to formation of adenosine, and fructose as well as adenosine induced a cAMP response in hepatocytes, the question arises whether these downstream metabolites show the same modulating effects as fructose on cytokine-induced apoptosis. To test this possibility we pretreated hepatocytes with fructose, adenosine or a cAMP analogue for 15, 5, 45 min, respectively, before cells were sensitised with ActD. After additional 15 min cell death was induced with TNF. After 18 h, toxicity was quantified by LDH release. Similar to fructose, treatment with adenosine or one of the two stable cAMP analogues db-cAMP and Br-cAMP protected primary hepatocytes from ActD/TNF-induced cell death in a concentration-dependent manner (Fig. 3A). In analogy, treatment of primary hepatocytes with adenosine or cAMP analogues, respectively, enhanced CD95-induced cell death in a concentration-dependent manner (Fig. 3B) which was associated with increased activation of the effector caspases 3 and 7 (data not shown).

Since our ultimate clinical aim is to selectively protect primordial hepatocytes in order to introduce selectivity into TNF-induced cancer therapy, we determined whether or not this also holds true in co-culture with malignantly transformed hepatocytes. Primary murine hepatocytes were seeded in 24-well dishes, while HepG2 cells were seeded in permeable co-culture inserts within the 24-wells. Cytotoxicity in the presence of ActD/TNF was analysed by the MTT assay, and as an apoptotic parameter, the activation of effector caspases was analysed. To test a potential influence of one cell type on the other, we monitored the effect of fructose on ActD/TNF-induced toxicity in this setting under co-culture conditions as well as under control conditions with one of the cell types at a time. We found that fructose-mediated inhibition of Act/TNF-induced caspase activation and cell death in healthy hepatocytes was independent of the presence of HepG2 cells. In contrast, HepG2 cells alone were sensitive to ActD/TNF-induced apoptosis even in the presence of fructose. Under co-culture conditions the susceptibility of HepG2 cells for TNF-induced apoptosis was unaffected. Thus, fructose-mediated ATP depletion and protection restricted to healthy hepatocytes allowed the selective killing of HepG2 cells by ActD/TNF in our setting (Fig. 3C, D).

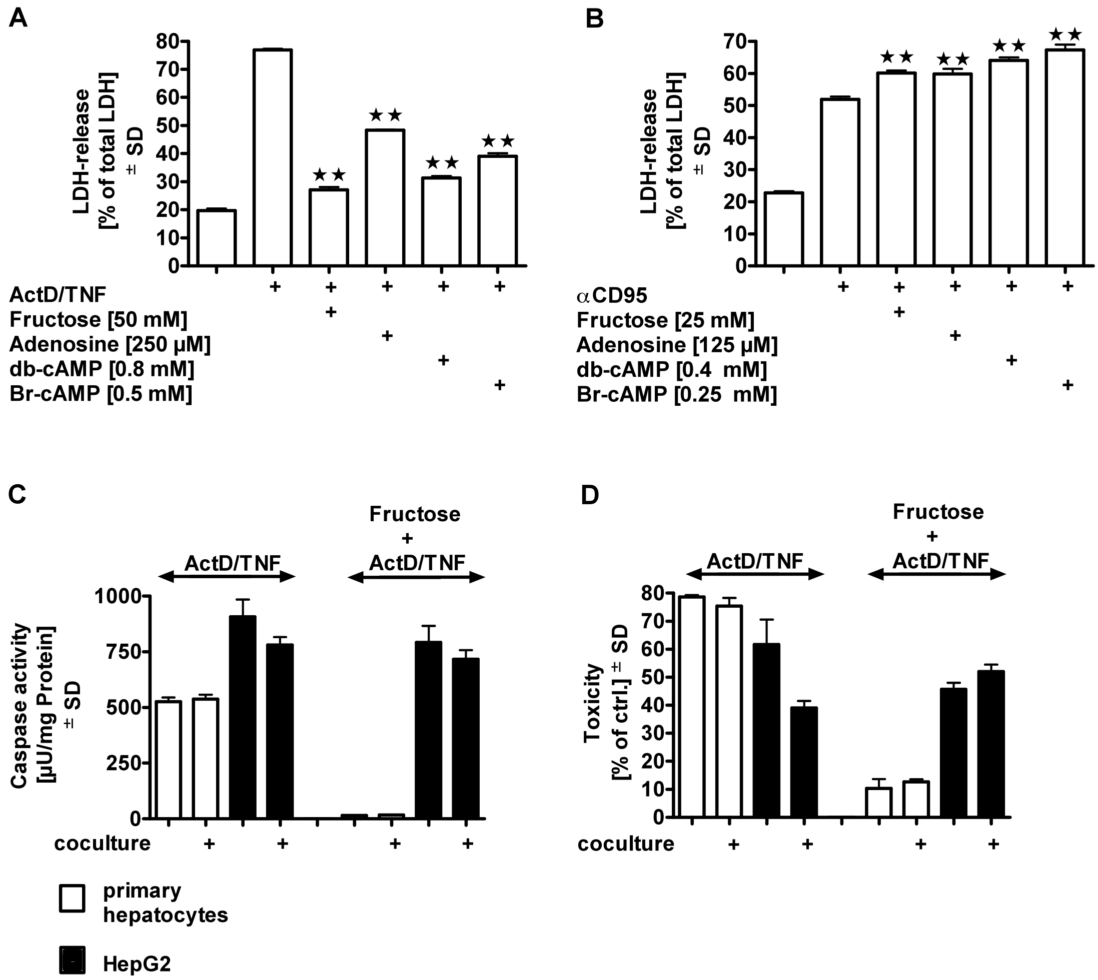

Figure 3. Adenosine and cAMP show the same effects as fructose on TNF- and CD95-induced cell death A) Primary hepatocytes were pretreated with fructose, adenosine or one of the cAMP analogues for 15, 5, 45 min before cells were sensitised with ActD. After additional 15 min TNF was added and after 18 h toxicity was analysed by LDH release. B) Primary cells were incubated according to setting 3 a). Cell death was induced with αCD95 antibody without ActD. Data represent means ± SD of triplicate cultures. *, $p<0.05$ and **, $p<0.01$ compared to ActD/TNF-/ or CD95-induced cell death based on ANOVA, followed by Bonferronis´s multiple comparison test. C-D) Primary murine hepatocytes were cultured in 24 well dishes while HepG2 cells were cultured in co-culture inserts. The effect of fructose on ActD/TNF-induced apoptosis on each cell population and under co-culture conditions was analysed. Toxicity was examined after 18 h by MTT assay and as apoptotic parameter caspase 3, 7 activity was determined. Data represent means ± SD of triplicate cultures.

Fructose- and cAMP-mediated modulation of CD95- and TNF-induced apoptosis is sensitive to PKA inhibitors

Protective effects of cAMP in hepatic pathophysiological situations, such as cold preservation or ischemia-reperfusion injury during organ transplantation, have been described to be mediated in a PKA-dependent manner [167]. We therefore investigated the possibility, whether PKA is the downstream target of fructose-induced cAMP signalling, which finally leads to modulation of receptor-mediated apoptosis. To address this issue, we analysed the effects of the specific PKA inhibitor KT5720 on ActD/TNF-induced cytotoxicity modulated by fructose or db-cAMP, respectively. Primary hepatocytes were treated with KT5720 30 min prior to addition of fructose or db-cAMP, respectively. After another 15 min, alternatively 45 min in case of db-cAMP ActD was added, and again 15 min later death receptor activation was induced by TNF. After 18 h, toxicity was analysed by measurement of LDH release, and as an apoptotic parameter, the activity of effector caspases was assessed. Fructose- and cAMP-mediated inhibition of ActD/TNF-induced caspase activity and LDH-release were completely reversed in the presence of the PKA inhibitor KT5720 (Fig. 4A-D). This reverting effect of KT5720 was concentration-dependent and was also observed using the structurally unrelated PKA inhibitor H-89 (data not shown).

In the following experiment we analysed whether PKA inhibition also modulates the sensitising effects of fructose and cAMP on CD95-induced cell death. Therefore, primary hepatocytes were pretreated either with KT5720 for 30 min before fructose or alternatively 60 min before db-cAMP, and after additional 30 min, agonistic CD95 antibody was added. Toxicity was analysed after 18 h by detection of LDH release and as apoptotic parameter activity of effector caspases was assessed. An analogous experimental design was used for the experiment with CD95-induced apoptosis instead of TNF. Pretreatment with KT5720 reversed fructose and db-cAMP-mediated sensitisation of CD95-induced caspase activity and LDH-release (Fig. 4E-F).

To summarise these results, we showed that fructose leads to a cAMP response in hepatocytes, and that fructose metabolism (upstream) or db-cAMP (downstream) inversely control CD95- and TNF-induced apoptosis. This dichotomous modulation of cytokine-induced cell death was sensitive to inhibitors of PKA. The fact that the inverse effects of fructose and cAMP on CD95- and TNF-induced apoptosis were revertible by two structurally different inhibitors of PKA makes it unlikely to be due to unspecific effects of the inhibitors. Therefore, we propose to consider PKA as the modulator of the fructose-mediated modulation of CD95- and TNF-induced apoptosis.

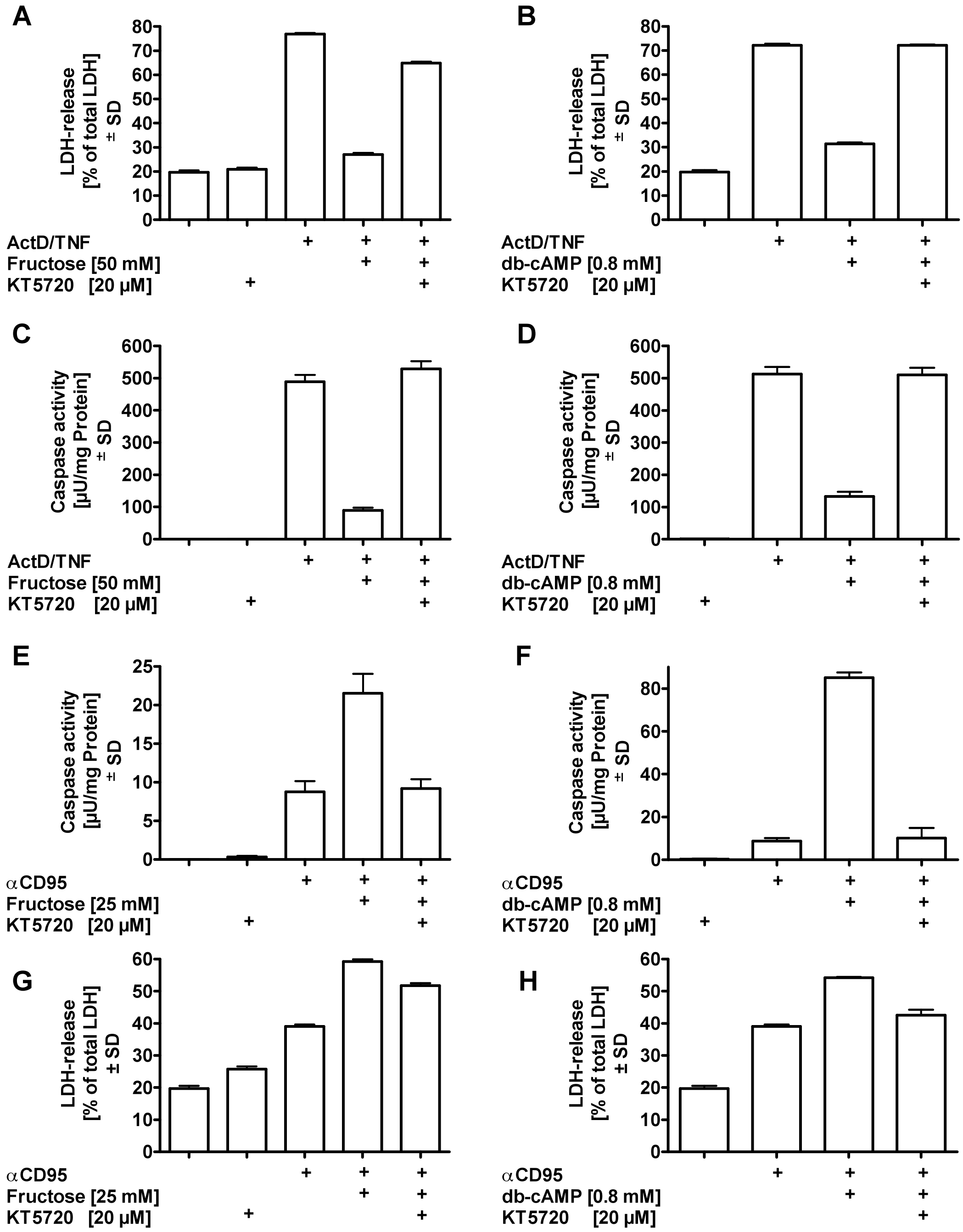

Figure 4. PKA inhibition reverses the inverse effects of fructose or cAMP on CD95- and TNF-induced apoptosis A-D) Effect of pretreatment with the PKA inhibitor KT5720 on fructose- or db-cAMP-mediated protection against ActD/TNF-induced apoptosis was analysed in primary murine hepatocytes. Activity of effector caspases and LDH-release was determined. E-H) Effect of pretreatment with the PKA inhibitor KT5720 on fructose- or db-cAMP-mediated sensitisation against CD95-induced apoptosis was analysed in primary murine hepatocytes. Activity of effector caspases and LDH-release was determined. Data represent means ± SD of triplicate cultures.

Inhibition of JNK attenuates ActD/TNF- but not CD95-induced hepatocyte apoptosis

Since fructose, adenosine as well as cAMP inversely control CD95- and TNF-induced apoptosis, we checked the possibility whether or not JNK activation represents a major difference in the apoptotic machinery of the two death receptor-ligands with respect to the influence of fructose and its downstream events. Schwabe *et al.* recently showed in hepatocytes that in contrast to CD95-induced apoptosis TNF-induced cell death requires activation of JNK [52, 53]. We therefore analysed the effect of JNK inhibition in our system. Pretreatment of primary hepatocytes with the JNK inhibitor SP600125 attenuated ActD/TNF-induced cell death in a concentration-dependent manner, while the inhibitor did not significantly reduce CD95-induced cell death compared to control cells (Fig. 5A).

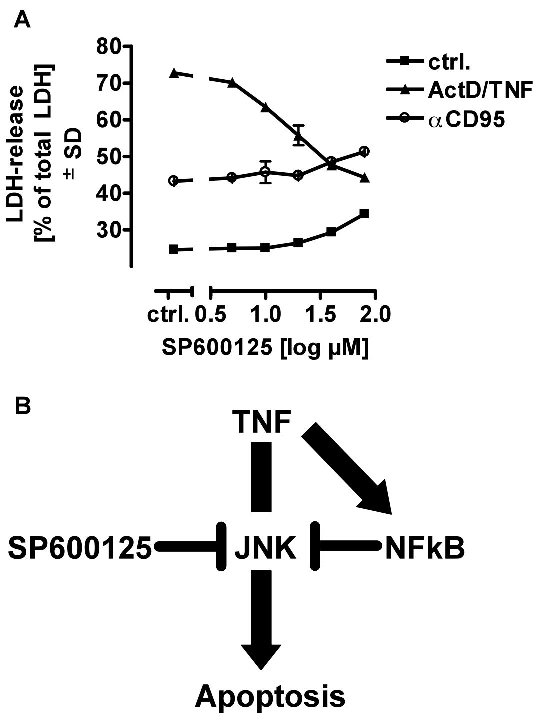

Figure 5. ActD/TNF- but not CD95-induced cell death is sensitive to the JNK inhibitor SP600125 A) Primary hepatocytes were pretreated with SP600125 for 90 min before αCD95 antibody or TNF was added. For TNF-induced cell death cells were sensitised by ActD 15 min before TNF stimulus. After 18 h LDH release was determined. Data represent means ± SD of triplicate cultures. B) Schematic presentation of the role of JNK in TNF-induced cell death and its interplay with NF-κB and SP600125.

Fructose and cAMP-analogues prevent ActD/TNF-induced prolonged JNK activity and toxicity

TNF-induced JNK activity occurs in two phases. The early transient phase (< 1h) was described to mediate cell survival while the sustained phase (>1h) is thought to mediate proapoptotic signalling [160, 168]. TNF-induced NF-κB activation is described to prevent apoptosis by inhibiting prolonged JNK activity [160, 161], unless expression of NF-κB genes is inhibited e.g. by transcriptional arrest.

We therefore analysed the JNK activation pattern in ActD/TNF-induced signalling modulated by fructose or SP600125 by *in vitro* kinase assay. TNF alone induced a maximum JNK activity after 30 min, followed by a constant reduction reaching the basal level after 120 min (Fig. 6A) while the corresponding analysis of LDH release showed that TNF alone did not induce toxicity (Fig. 6B). Sensitisation of hepatocytes by ActD lead to sustained TNF-induced JNK activity and to cell death in over 80% of primary hepatocytes (Fig. 6A, B). Pretreatment with SP600125 attenuated early, and completely blocked late phase of ActD/TNF-induced JNK activity and reduced cell death to 44%. Fructose pretreatment strongly suppressed ActD/TNF-induced prolonged JNK activity, and subsequent toxicity, in a concentration- and time-dependent manner (Fig. 6A-D). Thereby, fructose restricted the course of JNK activation to the early, transient phase, which like in case of TNF alone does not induce cell death. The concentration- and time-dependent course of fructose-mediated inhibition of ActD/TNF-induced sustained JNK activation in hepatocytes strongly correlates with the cytoprotective effects (Fig. 6C, D). According to our hypothesis that cAMP might mediate the protective effects of fructose in ActD/TNF-treated hepatocytes, we also examined the effect of Br-cAMP on JNK activity. In analogy to fructose, Br-cAMP reduced ActD/TNF-induced prolonged JNK activation along with its protective effects in a concentration and time-dependent manner (Fig. 6E). The extent of inhibition of JNK activity by Br-cAMP was comparable to the one of fructose.

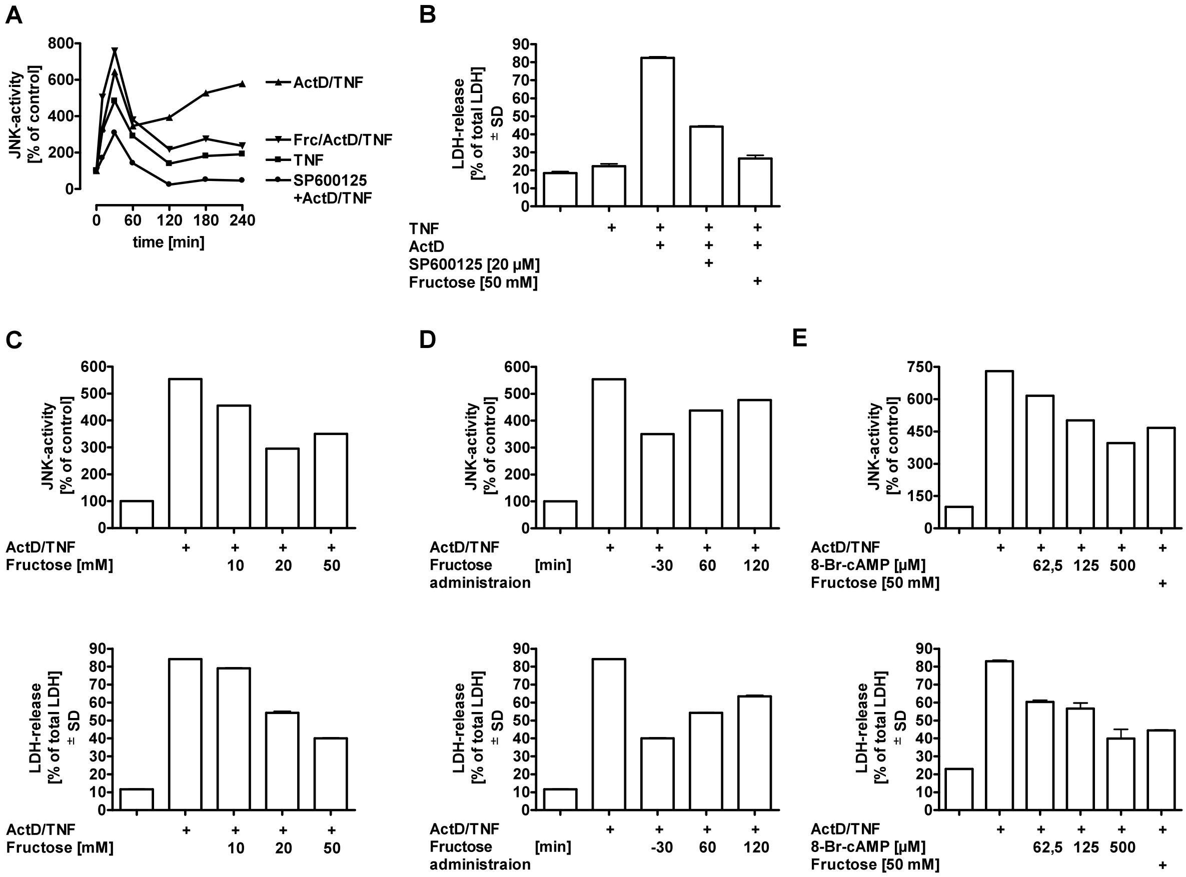

Figure 6. Fructose and Br-cAMP prevent ActD/TNF-induced prolonged JNK activation and toxicity A) Primary hepatocytes were treated as indicated. At various time points JNK activity was determined and B) after 18 h toxicity was detected by LDH release. C) Effect of 0, 10, 20 or 50 mM fructose 30 min before TNF and B) of 50 mM fructose 30 min before, 60 or 120 min after TNF application on JNK activity detected 3 h after TNF stimulus and on toxicity after 18 h was analysed. E) Effect of various concentrations of Br-cAMP was analysed according to C-D. Toxicity data represent means ± SD of triplicate cultures.

Fructose prevents ActD/TNF-induced cleavage of Bid and translocation of tBid to mitochondria in a PKA-dependent manner

JNK was shown to be essential for TNF-induced apoptosis in hepatocytes and to induce cleavage of the “BH3-domain only protein” Bid to truncated Bid (tBid), which leads to release of mitochondrial cytochrome C and thereby mediates amplification of the caspase cascade [169, 170]. Since Bid-deficient primary hepatocytes have been shown to be resistant against ActD/TNF-induced apoptosis and our previous data showed that fructose-induced ATP depletion prevents TNF-induced cytochrome C release [73], we analysed the influence of fructose on cleavage of Bid and translocation of tBid to mitochondria. In the presence of fructose or SP600125, ActD/TNF-induced reduction of full length Bid in the cytosol, which is probably due to cleavage of Bid to active tBid, was inhibited (Fig. 7A). Fructose pretreatment completely prevented ActD/TNF-induced tBid translocation to mitochondria between 3 to 6 h after TNF-stimulation, as shown by Western blot analysis (Fig. 7B). Complete fructose-induced inhibition of tBid translocation to mitochondria by fructose after 6 h after TNF-stimulation was reversed by the PKA inhibitor KT5720. Additionally, inhibition of JNK activity by SP600125 strongly reduced ActD/TNF-induced tBid translocation to mitochondria.

In total, our finding provide strong evidence for the conclusion that fructose-mediated protection of hepatocytes against TNF-induced apoptosis is due to ATP depletion-induced adenosine release, which then evokes a cAMP response that blocks sustained JNK activation and subsequent mitochondrial activation *via* PKA (Fig. 7C).

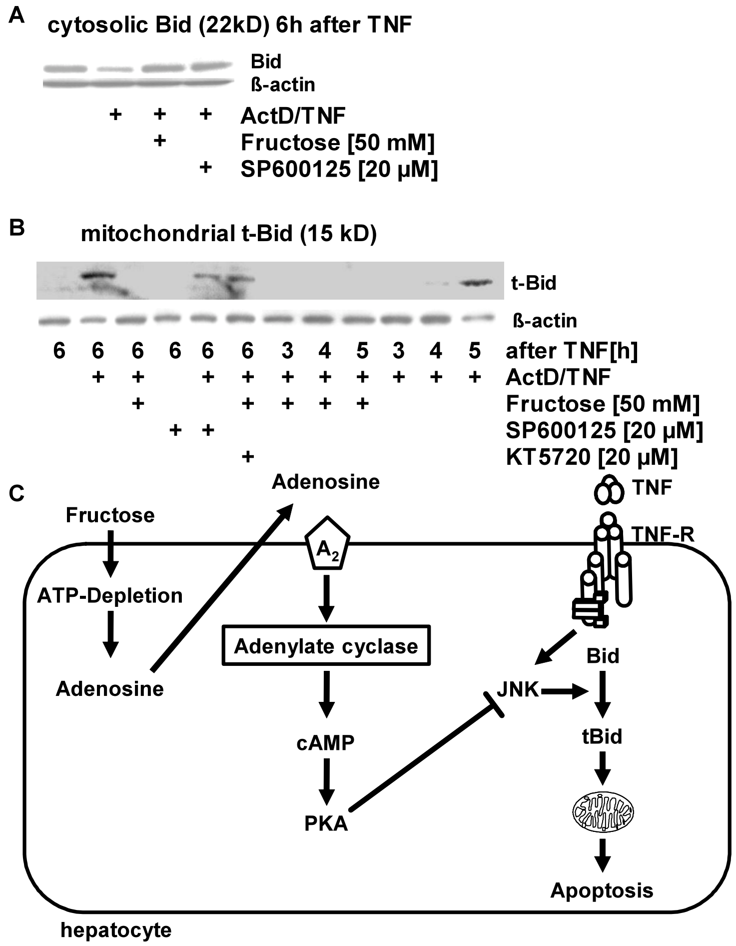

Figure 7. ActD/TNF-induced cleavage of Bid as well as tBid translocation to mitochondria is inhibited by fructose in a PKA-dependent manner A) Apoptosis was induced in primary murine hepatocytes by ActD/TNF. Fructose (50 mM) was added 30 min, SP600125 90 min before TNF

stimulus. After 6 h full length Bid was detected in the cytosolic fraction. B) Apoptosis was induced in primary murine hepatocytes by ActD/TNF. 30 min before TNF stimulus fructose or 90 min before TNF SP600125 was added. KT5720 was added 30 min before fructose. tBid was detected in mitochondrial fraction at the indicated time points. C) Schematic representation of the proposed molecular mechanism of apoptosis prevention by fructose.

4.5 Discussion

Selective cell death-inducing targeting of transformed cells by immunological approaches represents a desirable main aim in cancer therapy, which has not been verified yet for solid hepatic cancer such as hepatocellular carcinoma. TNF as the first cytokine to be used for cancer therapy is still an interesting killer molecule, whose use, however, is restricted by severe side effects on healthy tissue and thus is not given systemically. This problem of limited applicability was tackled by different approaches originating from multiple disciplines. Molecular biologists investigated certain possibilities, such as cell based therapy with tumor-infiltrating TNF expressing lymphocytes or TNF antibody complexes [171]. The objective was to increase local concentrations to make TNF tumor-specific, but clinical applicability remains to be awaited. On the other hand, surgical approaches allowed to circumvent the potentially harmful action of TNF by locoregional drug delivery systems, such as isolated limb perfusion, enabling its application in practice. For treatment of solid limb tumors, a combination of TNF and melphalan turned out to be a very potent treatment regimen with outstanding response rates [172-175]. However, in liver cancer, selectivity for tumor cells remains a largely unsolved problem

We have previously shown that fructose-induced hepatic ATP depletion prevents TNF-induced hepatocyte apoptosis *in vitro* and *in vivo* [73]. Interestingly, fructose did not induce ATP depletion in malignantly transformed hepatoma cell lines, which was due to tumor-specific alterations in the energy metabolism. These alterations were described by others as characteristics of highly malignant cells such as advanced stage HCC [145]. As a consequence hepatoma cells in contrast to healthy hepatocytes remained susceptible to TNF-induced cell death in the presence of fructose (first part of the thesis). We therefore proposed to take advantage of the selective protection by the naturally occurring sugar fructose, as a potential tool to selectively target malignant cells for receptor-mediated apoptotic cell death.

Since we reported TNF-induced hepatotoxicity to be prevented by fructose-induced ATP depletion, while CD95-induced hepatotoxicity was enhanced [73] we investigated the downstream events following fructose-induced ATP depletion on these two cell death machineries in order to elucidate the molecular mechanism of protection against ActD/TNF-induced apoptosis in healthy hepatocytes. Here we show that fructose-induced ATP depletion leads to transient accumulation and release of the degradation product adenosine from hepatocytes *in vitro* and *in vivo*, temporally corresponding to the decline of hepatocyte ATP.

The anti-inflammatory potential of adenosine in certain hepatic pathophysiological settings, such as ischemia reperfusion injury, is attributed to adenosine receptor 2 activation that leads to adenylate cyclase-induced cAMP signalling [176]. Based on this and in line with these reports we demonstrate that fructose as wells as adenosine lead to increased levels of intracellular cAMP in primary murine hepatocytes. Furthermore, we provide evidence that both, adenosine and cAMP, as putative downstream mediators of fructose-induced ATP depletion, mimic the dichotomous effects of fructose on CD95- and TNF-induced apoptosis. This view is corroborated by the observation that two structurally unrelated PKA inhibitors reversed fructose- and cAMP-mediated effects on cytokine-induced cell death.

Taken together, our data strongly suggest the involvement of an adenosine/cAMP/PKA signalling in the apoptosis modulating property of fructose metabolism. Remarkably, down to the level of PKA we found the fructose-mediated signalling mechanism to be identical for modulation of TNF- and CD95-induced apoptosis, although fructose metabolism affects CD95- and TNF-induced apoptosis in an opposite way. In search of a candidate downstream of PKA, we examined the involvement of stress-activated protein kinase (JNK) which is only required for TNF-induced, however not for CD 95 induced hepatocyte apoptosis. TNF induced a non-toxic, transient activation of JNK, which was sustained by sensitisation *via* ActD-mediated transcriptional arrest i.e. when the apoptotic power of TNF was facilitated. Fructose or cAMP prevented the late and sustained part of ActD/TNF-induced JNK activation as well as toxicity in a concentration- and time-dependent manner.

Recently, the JNK pathway was shown to be a key regulator of TNF-induced mitochondria-mediated hepatocyte cell death [52, 53]. JNK was shown to activate the proapoptotic protein Bid, which leads to amplification of the caspase cascade *via* the intrinsic pathway of TNF-induced hepatocyte apoptosis [37, 169, 170]. In our experiments, the presence of fructose completely prevented ActD/TNF-induced Bid-cleavage and tBid translocation to mitochondria in a PKA-dependent manner, i.e. activation of the intrinsic apoptotic pathway was blocked. This is in line with our previous report of fructose-mediated inhibition of TNF-induced cytochrome C release from mitochondria in primary hepatocytes [73], which is the main result of mitochondria activation leading to activation of caspases.

In conclusion, our findings are consistent with the uniform conception that fructose-induced ATP depletion leads to adenosine release, which in turn induces cAMP signalling in an autocrine manner. Subsequent PKA activation inversely modulates CD95- and TNF-induced apoptosis. Downstream from the adenosine/cAMP/PKA sequence, we propose the antiapoptotic mechanism of fructose on TNF-induced apoptosis to be due to the inhibition of the sustained phase of ActD/TNF-induced JNK signalling, finally preventing formation of tBid and mitochondrial amplification of the caspase cascade. These results provide a plausible mechanistic rationale for the strategy to selectively protect healthy hepatocytes from death receptor-induced cell death by non-toxic doses of fructose and also demonstrate the potential of fructose to induce a liver-specific release of endogenous adenosine.

5 Summarising Discussion

Cancer is a group of more than 200 different diseases sharing the characteristics of uncontrolled proliferation, invasion and in some cases metastasis. Apart from these typical properties that occur in most diseases of this group, each of them is confined by specific attributes, which determine the scope of action for individual therapeutic intervention. As a consequence, development of a universal treatment for the many different forms and stages of cancer is indeed very unlikely. Eradication of malignant tissue by induction of cell death represents the main alternative to surgery. Therefore, search for anticancer drugs is a main focus of cancer research, but most substances used in the clinics or in experimental settings are relatively unspecific. Since toxicity on healthy tissue is the main drawback of most antineoplastic agents, increased specificity leading to selective induction of preferentially apoptotic cell death in malignant cells is the ultimate goal.

The requirements for selective induction of cell death are:

- Cell death targeted to malignant cells
- Low / no toxicity on healthy tissue

Compounds combining both properties of killing and selectivity represent the holy grail of cancer research but are pretty rare. Uncoupling of the two requirements by task sharing is an alternative and may enhance chances. In this case a compound that induces apoptosis in combination with a second one that either selectively increases susceptibility for the toxic compound in malignant cells or selectively protects healthy cells would lead to the desired goal.

Although TNF was the first cytokine employed for cancer therapy, the molecule unfortunately turned out not to be the holy grail of cancer research due to severe systemic toxicity [171]. Regional drug delivery systems such as isolated limb or isolated hepatic perfusion (IHP) opened up the possibility to overcome limitations of systemic treatment, thereby enabling the application of TNF [67, 69-71]. IHP was designed for unresectable, multifocal tumors confined to the liver, which is characteristic of most hepatocellular carcinomas [12, 172]. The liver is notably critical due to the fact that primary cancers and metastasis are often the life-limiting component in human cancer affecting this organ [15]. Combination of TNF with chemotherapeutics such as melphalan exhibits high tumoricidal properties and response rates in this setting [15, 172], but tolerance of the perfused healthy tissue represents the dose-limiting factor in this setting [172].

In conclusion, TNF turned out to be a potent killer molecule and thereby fulfils the first demand of an anticancer agent but is often lacking the second one of selectivity towards malignant tissue [171, 172]. Therefore, since decades the biomedical community is searching for an option to make the killer molecule tumor-specific [171]. Selectivity is always based on differences between cells leading to converse reactions to the same stimulus. In case of introducing selectivity into TNF-mediated cancer killing the difference between healthy and malignant cells should determine susceptibility towards TNF-induced cell death. Since fructose-mediated hepatic ATP depletion was shown to prevent TNF-induced apoptosis [73], we evaluated whether fructose could introduce selectivity into TNF-mediated cancer cell killing. The protective effect of fructose is due to the liver-specific fructose metabolism leading to ATP depletion [73]. Since certain alterations in the energy metabolism, known as the Warburg effect, are accepted today as a key metabolic hallmark of cancer [94, 95], we evaluated whether tumor-specific alterations in the energy metabolism affect hepatoma fructose metabolism and thereby prevent ATP depletion and protection against TNF-induced apoptosis in these cells.

5.1 Malignant but not naïve liver cells are killed by TNF after metabolic depletion of ATP by fructose

In this study we addressed the question whether fructose has the potential to introduce selectivity into TNF-induced killing of transformed cells of hepatic origin by selective protection of healthy hepatocytes. Therefore, we compared the biochemical and functional differences in fructose metabolism between rodent or human primary liver cells and rodent or human hepatic tumor cell lines.

In agreement with our previous results that fructose-induced ATP depletion protects hepatocytes against TNF-induced cell death *in vitro* and *in vivo* [73], we extended the functionality of ATP depletion and the protective effect of fructose to hepatocytes of human and rat origin. In contrast, all tested hepatoma cell lines were lacking fructose-induced ATP depletion and protection. Our findings demonstrate the potential of fructose to selectively protect healthy hepatocytes against TNF-induced apoptosis in this setting. We assumed alterations in the energy metabolism of the transformed cell lines to be responsible for the lack of ATP depletion. Since the metabolic rationale for fructose-induced ATP depletion is directly linked to fructokinase catalysed rapid conversion of fructose to fructose-1-phosphate, which accumulates and functions as a phosphate trap [77, 81, 83, 126], we analysed conceivable transformations that could prevent this process. Neither a reduced activity of fructokinase, nor an increased activity of aldolase B was detected in the transformed cells compared to healthy cells. Therefore, we concluded that alterations of these enzymes are not responsible for the lack of ATP depletion. Instead we identified an increased activity of low-K_m hexokinases in all tested cell lines. In agreement with the present understanding of Warburg´s theory we identified upregulation of hexokinase isoform II on transcriptional and protein level. Taking these findings and the knowledge that low-K_m hexokinases catalyse the conversion of fructose to fructose-6-phosphate in most cells of the mammalian body into account, we hypothesised that HKII induces a bypass of the liver-specific enzymes, thereby preventing ATP depletion in the transformed cells.

To verify this hypothesis we tackled the question from either side:

- Inhibition of hexokinase in transformed cells should restore fructose-induced ATP depletion by fructose.
- Introduction of the cancer phenotype of upregulated HKII in primary hepatocytes should prevent fructose-mediated ATP depletion and protection against TNF-induced apoptosis.

We showed that the pharmacological inhibitor of HKII, 3-bromopyruvate, restored fructose-induced ATP depletion in HepG2 cells. On the other hand we induced expression of HKII in primary murine hepatocytes *via* stabilisation of HIF1 with Desferal, which in turn prevented fructose induced ATP depletion. Desferal-mediated targeting of HIF1 hydroxylases regulating HIF1 stability is due to chelating the cofactor Fe(II). To exclude unspecific effects of Desferal we used 3-bromopyruvate in this setting. The HKII inhibitor reversed the effects of Desferal on fructose-induced ATP depletion, suggesting the effects to be due to HKII upregulation.

Increased expression of HKII *via* transfection of hepatocytes verifying the potential of HKII to prevent fructose-induced ATP depletion is not feasible due to the low transfection efficiency of this cell type and the fact that there is no accepted method for single cell ATP quantification. In contrast, such methods are available for the detection of apoptotic parameters in single cells. Therefore, we analysed the effect of HKII transfection on fructose-mediated protection in primary hepatocytes and showed that overexpression of HKII prevented inhibition of TNF-induced apoptosis by fructose in primary murine hepatocytes.

In summary, we elucidated a tumor-specific switch from liver-specific to muscle-specific fructose metabolism by upregulation of HKII. As a consequence fructose-induced ATP depletion and protection is prevented in transformed cells, allowing selective protection of healthy hepatocytes. In this context upregulation of HKII in transformed cells is a requirement for introduction of selectivity into TNF-mediated cancer cell targeting by fructose. It is noteworthy that increased expression of HKII is a key player in the Warburg effect, which is accepted today as a key metabolic hallmark of rapidly growing cancers [94, 95, 100, 143] and is in daily use in clinical practice for the detection of malignant cells by PET scan. In context of our results the transformation of the energy metabolism, preventing liver-specific fructose metabolism and protection, is a prerequisite for selective targeting of malignantly transformed hepatocytes residing in the liver. Therefore, the alterations in the energy metabolism of malignantly transformed hepatocytes might be used as a prognostic marker to evaluate the applicability of the approach in specific cases.

In contrast to tumor cells of hepatic origin, metastases originating from other tissues are unlikely to exhibit liver-specific fructose metabolism and the associated protective mechanism of fructose. Therefore, selective protection of healthy hepatocytes should be independent of the energetic phenotype of these malignant cells. Since unresectable metastases confined to the liver are often the life-limiting component of the disease further research is required.

5.2 Fructose-induced hepatocyte ATP depletion prevents TNF-induced apoptosis by inhibition of JNK signalling

After we proposed to take advantage of the increased expression of HKII in liver tumors to selectively target malignant cells, the question about the molecular mechanism of fructose-mediated protection against TNF-induced apoptosis arose. We previously showed that fructose-induced ATP depletion does not inhibit cytokine-induced apoptosis in general, since fructose-induced ATP depletion in hepatocytes increases sensitivity for CD95-induced cell death, while the cells are fully protected against TNF-induced apoptosis *in vitro* and *in vivo* [73]. To elucidate the molecular mechanism of fructose-mediated protection against ActD/TNF-induced cell death we investigated downstream events of fructose-induced ATP depletion and their effect on cytokine-induced cell death.

High loads of fructose lead to a strong, transient, and untoxic reduction of hepatic ATP in humans and rodents, resulting in a decline of the total amount of adenosine nucleotides [81]. We identified transient accumulation of the purine nucleoside adenosine as a consequence of fructose-induced hepatic ATP depletion *in vitro* and *in vivo*, temporally corresponding to the decline of hepatocyte ATP. Adenosine has been described as a metabolite with retaliatory action in inflammation due to its autocrine signalling following extracellular accumulation during cell stress or injury [167]. The anti-inflammatory potential in certain hepatic pathophysiological settings is mainly assigned to G-Protein-coupled adenosine receptors inducing cAMP-signalling [176]. In line with these reports we could show that fructose as well as adenosine induced a cAMP response in hepatocytes. Both downstream events of fructose-induced ATP depletion (adenosine and cAMP) mimicked the inverse effects of fructose on TNF- and CD95-induced apoptosis in a PKA-dependent manner. Whether this line of events, induced by fructose-induced ATP depletion, leads to cytoprotection or in contrary to sensitisation depends on the cytokine used for induction of apoptosis. In case of fructose-mediated sensitisation towards CD95-induced hepatocyte cell death there is an enormous number of potential factors being responsible for this effect. On the other hand we expected that fructose downstream effects interfere with a cellular master switch of TNF-induced hepatocyte apoptosis because of the powerful protective effect. Previous studies showed that early TNFR-mediated signalling like activation of the NF-κB pathway as well as receptor internalisation are not affected by the presence of fructose, while activation of mitochondria leading to cytochrome C release and subsequent activation of caspases was prevented [73, 84]. Therefore we hypothesised the mechanistic point of action linking fructose-induced ATP depletion and TNF-induced cell death to exist downstream of early TNFR signalling but upstream of mitochondria activation. Additionally, due to the opposite effects of fructose on apoptosis induced by CD95 or TNF we expected major differences in their apoptotic machinery as the underlying mechanism. TNF exerts its biological functions through activating of distinct signalling pathways such as NF-κB, JNK and caspases [177, 178]. These signalling pathways are connected and may influence each other by cross talk so that the cell fate is determined by the interaction. Hepatocytes, for example, show resistance to TNF-induced apoptosis, which is dependent on activation of the transcription factor NF-κB [161]. This was underlined by the finding that mice deficient in activation of NF-κB were not viable due to TNF-induced excessive hepatocyte apoptosis. For a long time the antiapoptotic effects were solely assigned to NF-κB-induced expression of antiapoptotic proteins. In the meantime inhibition of TNF-induced JNK signalling by NF-κB was shown to be involved in the antiapoptotic mechanism [161, 179]. While transient activation of JNK by TNF is associated with cellular survival, prolonged JNK signalling is proapoptotic and required for TNF-induced hepatocyte apoptosis [168]. Several studies showed activation of NF-κB to inhibit the sustained phase of TNF-induced JNK activation and thereby prevent TNF-induced cell death [161, 180, 181]. Therefore, the crosstalk between NF-κB and JNK plays a major role in determining the cell fate during TNF-induced signalling [181]. Recently, pharmacological inhibition of JNK was shown to prevent TNF-induced cell death in hepatocytes sensitised towards TNF either *via* transcriptional arrest by actinomycinD or by expression of dominant negative c-Jun [53]. This suggested the apoptotic function of JNK to be independent of transcription and c-Jun [52, 53]. Furthermore inhibition of JNK was shown to block tBid formation and cytochrome C release by TNF, demonstrating its function upstream of the intrinsic mitochondrial amplification loop, which was shown to be required for TNF-induced hepatocyte apoptosis [37]. In contrast, JNK signalling as well as inhibition of NF-κB signalling was described not to be required for CD95-induced apoptosis. This demonstrates that JNK activation is a major difference in the apoptotic machinery of TNF and CD95 [53] and therefore attracted our attention.

We could show fructose-induced ATP depletion to prevent ActD/TNF-induced sustained activity of JNK in a concentration- and time-dependent manner corresponding to the cytoprotective effects. cAMP-analogues showed the same effects on ActD/TNF-induced prolonged JNK signalling and cytoprotection. Since we previously reported fructose-induced ATP depletion to prevent the activation of the mitochondrial pathway, thereby inhibiting cytochrome C release, we investigated Bid as an essential inducer of the mitochondrial amplification loop in TNF-induced hepatocyte apoptosis. ActD/TNF-induced cleavage of Bid as well as tBid translocation to mitochondria was prevented by fructose in a PKA-dependent manner. PKA was described as a negative regulator of JNK signalling in different cell types but if PKA influences JNK itself or upstream kinases is not described [182-185].

In summary, our data strongly suggest the involvement of adenosine/cAMP/PKA signalling in the apoptosis modulating property of fructose. Surprisingly, we found the opposing effects of fructose on CD95- and TNF-induced apoptosis to be identical down to the level of PKA. In case of TNF, PKA prevented the sustained, proapoptotic phase of JNK signalling and subsequent activation of the intrinsic mitochondrial pathway by tBid formation, leading to cytoprotection against TNF-induced hepatocyte cell death.

6 Summary

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common tumor-associated cause of death. Most patients are diagnosed with advanced stage HCC, regarded in the majority of cases as non-resectable due to the infiltrative growth pattern, classifying these patients as candidates for palliative care. The fact that current treatment standards for primary liver cancer have limited clinical benefit and the lack of curative therapy for HCC highlights the need for more effective liver-directed regional cancer therapies.

TNF was the first cytokine used for cancer treatment due to its tumoricidal properties, but first clinical trials showed that the effective TNF dose needed to exceed the maximum tolerated dose by a factor of 5-25, thereby preventing the systemic use. Isolated hepatic perfusion, which was designed for the treatment of unresectable liver cancer, enabled the application by preventing systemic toxicity, but even in this regional use selectivity for malignant cells is an unsolved problem. Tolerance of the healthy tissue to anticancer agents such as TNF represents the dose-limiting factor in isolated hepatic perfusion. Introduction of selectivity into TNF-based cancer cell killing by selective protection of healthy tissue would make the killer molecule TNF tumor-specific. Fructose-induced hepatic ATP depletion, which is due to the liver-specific fructose metabolism, was shown to prevent TNF-induced cell death *in vitro* and *in vivo*. In the first part of this thesis, the potential of fructose to selectively protect healthy hepatocytes against TNF-induced apoptosis was analysed and the alterations in the energy metabolism of the transformed cells preventing the protection in these cells were characterised.

- All tested hepatoma cell lines lacked fructose-induced ATP depletion and protection against TNF-induced cell death, while primary mouse, rat and human hepatocytes showed ATP depletion and protection. Alterations in the specific activity of fructokinase and aldolase B catalysing the liver-specific metabolism were not responsible for the lack of ATP depletion in the cell lines. Activity of low-K_m hexokinases was increased in all cell lines compared to primary hepatocytes and an upregulation of hexokinase isoform II was detected on the level of mRNA and protein. Pharmacological inhibition of hexokinases by 3-bromopyruvate restored fructose-induced ATP depletion in HepG2 cells. In contrast, increased expression of HKII *via* stabilisation of the transcription factor HIF1 prevented fructose-induced ATP depletion in primary

murine hepatocytes. Fructose-mediated cytoprotection of primary murine hepatocytes against TNF-induced apoptosis was prevented by overexpression of HKII. These findings show that increased expression of HKII in transformed cells of hepatic origin leads to a shift from liver-type to muscle-type fructose metabolism, thereby preventing fructose-induced ATP depletion and protection against TNF-induced apoptosis.

Increased expression of HKII is a typical hallmark of rapidly growing tumors such as HCC and plays a central role in the Warburg effect. In context of this study, upregulation of HKII in malignant cells is a requirement for selective targeting of malignantly transformed hepatocytes by TNF in the presence of fructose. Based on the obtained data we propose to take advantage of the increased expression of HKII in liver tumors for transiently protecting healthy liver cells. Since cytoprotection of fructose-induced ATP depletion against TNF-induced hepatocyte apoptosis is the crucial point, the second part of the thesis addressed the molecular mechanism(s) mediating this protection downstream of ATP depletion.

- Fructose-induced ATP depletion resulted in transient extracellular accumulation of the ATP degradation product adenosine *in vitro* and *in vivo.* Adenosine induced a cAMP response in an autocrine manner. cAMP and adenosine mimicked the inverse effects of fructose on CD95- and TNF-induced apoptosis. Both effects were sensitive to inhibition of PKA. Analysis of TNF-induced MAP kinase signalling revealed that fructose-mediated ATP depletion as well as cAMP signalling prevented the proapoptotic, sustained phase of TNF-induced JNK activation. This may explain the dichotomous effects of fructose on apoptosis induced by TNF or CD95, since JNK is only essential for TNF-induced apoptosis. Fructose-mediated inhibition of prolonged JNK signalling prevented activation of the intrinsic mitochondrial amplification loop by Bid, since Bid cleavage as well as tBid translocation to mitochondria was prevented in a PKA-dependent manner. Based on these data we propose fructose-induced ATP depletion to modulate TNF- and CD95-induced apoptosis in an adenosine/cAMP/PKA-mediated process. PKA inhibits TNF-induced prolonged JNK activation, thereby preventing Bid cleavage, which is essential for TNF-induced apoptosis. These findings provide a mechanistic rationale for the modulation of cytokine-induced hepatocyte apoptosis by fructose.

In summary, this thesis contributes to the understanding how tumor-specific alterations in the energy metabolism of malignant cells of hepatic origin circumvent liver-specific fructose metabolism. As a consequence, these cells loose the mechanism of fructose-mediated protection from TNF-induced apoptosis, since it is based on the liver-specific fructose metabolism. Additionally, the presented data elucidate the molecular mechanism of TNF-induced apoptosis prevention by fructose. As a pragmatic corollary from these findings, a new concept of selective tumor therapy in the liver is proposed, which exploits selective induction of apoptosis in malignant hepatic cells combined with specific transient protection of healthy liver cells.

7 Zusammenfassung

Das Hepatozelluläre Karzinom (HCC) ist der fünfthäufigste Tumor weltweit und die dritthäufigste tumorassoziierte Todesursache. Das HCC ist charakterisiert durch infiltrierendes und multifokales Wachstum, es wird i.a. erst in fortgeschrittenen Stadien diagnostiziert, ist meist nicht resektabel, und wird palliativ behandelt. Daraus folgt die Notwendigkeit einer effektiveren zielgerichteten regionalen Krebsbehandlung innerhalb der Leber.

TNF war das erste Zytokin, das basierend auf seinen antitumoralen Eigenschaften für Krebstherapie angewendet wurde. Es stellte sich heraus, dass eine systemische Anwendung von TNF nicht möglich ist, da die benötigte antitumorale Dosis die maximal tolerierte Dosis um ein Vielfaches übersteigt. Die *in situ* isoliert perfundierte Leber ermöglicht zwar die Applikation von TNF ohne systemische Toxizität, jedoch trotzdem mit Schädigung gesunden Lebergewebes.

Im Gegensatz zum klassischen Ansatz des selektiven Tumor-Treffers verfolgt diese Arbeit die Alternative des selektiven Schutzes der gesunden Zellen vor TNF-induzierter Apoptose. Fruktose-induzierte ATP Depletion, die auf den leberspezifischen Fruktosestoffwechsel zurückzuführen ist, verhindert TNF-induzierten Zelltod *in vitro* und *in vivo.* Es wurde zunächst untersucht, inwieweit dieser zytoprotektive Mechanismus selektiv gesunde Hepatozyten vor TNF-induziertem Zelltod schützt, und ob Veränderungen im Energiemetabolismus transformierter Zellen diesen Schutz verhindern.

- Die verwendeten Hepatomzelllinien wiesen keine durch Fruktose hervorgerufene ATP Depletion und den damit verbundenen Schutz gegenüber TNF auf, während primäre Hepatozyten der Maus, Ratte und des Menschen durch Fruktose-induzierte ATP Depletion geschützt waren. Veränderungen der spezifischen Aktivität der am leberspezifischen Fruktosestoffwechsel beteiligten Enzyme Fruktokinase und Aldolase B konnten das Ausbleiben der ATP Depletion in den Zelllinien nicht erklären. Die Aktivität der low-K_m Hexokinasen war in allen Zelllinien im Gegensatz zu den entsprechenden Primärzellen erhöht, und eine verstärkte Expression der Hexokinase Isoform II (HKII) auf mRNA- und Proteinebene wurde nachgewiesen. Pharmakologische Inhibition der Hexokinasen durch 3-Brompyruvat ermöglichte fruktoseinduzierte ATP Depletion in HepG2-Zellen. Auf der anderen Seite wurde Fruktose-vermittelte ATP Depletion durch HIF1-induzierte

Hochregulation der HKII in primären murinen Hepatozyten verhindert. Entsprechend konnte die Protektion der primären murinen Hepatozyten mittels Fruktose gegenüber TNF-induzierter Apoptose durch Überexpression der HKII aufgehoben werden. Diese Ergebnisse zeigen, dass die erhöhte Expression der HKII in transformierten Zellen hepatischen Ursprungs zu einem Übergang vom leberspezifischen zu einem muskelspezifischen Fruktosemetabolismus führt, wodurch Fruktose-vermittelte ATP Depletion und der daraus resultierende Zellschutz vor TNF-induzierter Apoptose verhindert wird.

Die erhöhte Expression der HKII ist ein typisches Kennzeichen aggressiver Tumore, wie des HCC, und übernimmt eine Schlüsselrolle im sog. Warburg Effekt. Daraus folgt, dass die Hochregulation der HKII in malignen Zellen hepatozellulären Ursprunges eine Voraussetzung darstellt, um selektiv diese Zellen durch TNF in Anwesenheit von Fruktose zu attackieren und gleichzeitig selektiv gesunde Hepatozyten zu schützen. Im zweiten Teil dieser Arbeit wurde der molekulare Mechanismus untersucht, welcher durch ATP Depletion diesen Schutz verursacht.

- Fruktoseinduzierte ATP Depletion führte zu transienter extrazellulärer Akkumulierung des ATP Abbauproduktes Adenosin *in vitro* und *in vivo.* Adenosin induzierte in einem autokrinen Mechanismus die Produktion des second Messenger cAMP. Adenosin und cAMP führten in Analogie zu Fruktose zu inversen Effekten auf CD95- und TNF-induzierte Apoptose. Beide Effekte waren sensitiv gegenüber Inhibitoren der PKA. Die Analyse des TNF-induzierten MAP-Kinase Signalweges zeigte auf, dass ATP Depletion mittels Fruktose, sowie cAMP die proapoptotische, verlängerte Phase der TNF-induzierten JNK Aktivierung inhibieren. Dies könnte die konträren Effekte von Fruktose auf CD95- und TNF-induzierte Apoptose erklären, da JNK nur im TNF-vermittelten Zelltod essentiell ist. Inhibition des proapoptotischen JNK Signalweges durch Fruktose verhinderte die Aktivierung des mitochondrialen Verstärkungsmechanismus durch Bid, da sowohl die Spaltung von Bid, als auch die Translokation von tBid zum Mitochondrium unter Mitwirkung der PKA gehemmt wurde. Basierend auf diesen Daten postulieren wir, dass ATP Depletion durch Fruktose die TNF- und CD95-induzierte Apoptose über einen Adenosin-/cAMP-/PKA-vermittelten Prozess moduliert. Des Weiteren hemmt PKA die TNF-induzierte verlängerte und proapoptotische JNK Aktivierung, die als essentiell für TNF-vermittelte Apoptose beschrieben wurde. Diese Daten erklären das mechanistische Prinzip der Modulation der zytokininduzierten Apoptose in Hepatozyten durch Fruktose.

Zusammenfassend zeigt diese Arbeit auf, dass tumorspezifische Veränderungen im Energiemetabolismus maligner Zellen hepatozellulären Ursprunges ihren leberspezifischen Fruktosestoffwechsel verlieren. Dies bedingt, dass diese Zellen nicht durch Fruktose vor TNF-induzierter Apoptose geschützt werden, da der zytoprotektive Effekt direkt auf dem Fruktosestoffwechsel basiert. Der molekulare Mechanismus, der zum Schutz vor TNF-induziertem Zelltod durch Fruktose führt, wurde aufgeklärt. Die Befunde führen zu einem neuen Konzept der Machbarkeit selektiver Tumortherapie innerhalb der Leber, indem in malignen Hepatozyten Apoptose induziert wird, während die gesunden Leberzellen spezifisch und transient geschützt werden.

8 Declarations of authors' contributions

- **T. Speicher**, A. Foehrenbacher, Isabelle Pochic, T. Weiland, and A. Wendel: Malignant but not naïve liver cells are killed by TNF after metabolic depletion of ATP by fructose

The experiments were mainly performed by T. Speicher and A. Foehrenbacher under the supervision of A. Wendel. The study was designed by A. Wendel and T. Speicher. Further contributions were made of T. Weiland. The manuskript was written by T. Speicher.

- **T. Speicher**, U. Köhler, A. Chouker, T. Weiland and A. Wendel: Fructose-induced hepatocyte ATP depletion prevents TNF-induced apoptosis by inhibition of JNK signalling

The experiments were mainly performed by T. Speicher and U. Köhler, with minor contributions by A. Chouker under the supervision of A. Wendel. The study was designed by A. Wendel and T. Speicher. The manuskript was written by T. Speicher.

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