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Cystine-Import and Regulation of Apoptosis in B-Lymphocytes

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LIST OF ABBREVIATIONS

α-TG	α-thioglycerol
β-ME	β-mercaptoethanol
µl	microliter
µM	micromolar
A	Adenine
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BAP	Bacterial alkaline phosphatase
BL	Burkitt´s Lymphoma
bp	base pair
BSO	Buthionine sulfoximine
C	Cytosine
cDNA	complementary DNA
Cys	Cysteine
Cyss	Cystine
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxid
DNA	2'-Deoxyribonucleic acid
dNTP	3'-Deoxyribonucleoside-5'-triphosphate
DTNB	5,5'-DITHIO-bis(2-NITROBENZOIC ACID)
DTT	Dithiothreitol
E.coli	*Escherichia Coli*
EBV	Epstein Barr Virus
EDTA	Ethylenediamine –N,N,N',N'-tetra-acetic acid
EtOH	Ethanol
FCS	Foetal Calf Serum
G	Guanosine
GFP	Green Fluorescent Protein
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)

h	human
HPLC	High Pressure Liqid Chromatography
Hyg	Hygromycine
IRES	Internal Ribosomal Entry Site
LMP agarose	Low Melting Point agarose
mM	millimolar
MOPS	3-(N-morpholino) propansulfonic acid
SDS	Sodium dodecylsulfate
NADH	Nicotinadeninedinucleotide
NADPH	Nicotinadeninedinucleotide phosphate
nM	nanomolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
Pur	Puromycine
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SDS-PAGE	Sodiumdodecylsulfate- polyacrylamide gel electrophoresis
-SH	sulfydryl group
SSC	Sodium chloride-sodium citrate buffer
TAE	Tris-Acetate-EDTA
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE	Tris-chloride/EDTA (10 :1)
TEMED	N,N,N',N''-tetramethylethylendiamine
TRIS	Tris-(hydroxymethyl)-ammoniummethan
V	Volt
UV	Ultraviolet
RT	Room Temperature
w/v	Percentage weight to volume

INTRODUCTION

"The aerobic life style offers great advantages, but is fraught with danger" *(51)*

1.1 Oxygen Radicals and Antioxidative Defense System

Utilizing oxygen enables living cells to use much more energy than anaerobic organisms, but this is a two-edged sword. In one way it offers great advantages regarding energy metabolism and host defense, but products of its metabolism (*reactive oxygen species, ROS*) may also lead to damage of cells and tissues. ROS are mainly generated as a side product of the respiratory chain and can cause modifications of proteins, lipids, and DNA especially when present in high concentrations. ROS are not only detrimentary to cells, they are also involved in cell signaling and host defense. To maintain the balance of cellular ROS, a complex defense system (*Antioxidative defense system or AOS*) against high levels of ROS has been developed allowing aerobic organisms to live in such an environment.

Owing to its own electronic configuration, oxygen is prone to gain electrons and is thus a potent oxidant. During the respiratory process, O_2 is progressively reduced by a controlled supply of four electrons to yield water:

$$O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O$$

Incomplete reduction of O_2, with a gain of one, two or three electrons is, however, possible and leads to the formation of chemical entities (ROS) that are still potent oxidants. The usually well controlled enzymatic systems that use electron transfer reactions may exhibit some leakage, and in the presence of oxygen any such electron leakage may result in ROS production. ROS are generated either exogenously or produced intracellularly from several different sources. Exogenous sources generating ROS are ultraviolet light, ionizing radiation, chemotherapeutic agents, inflammatory stimuli and environmental toxins (50). The majority of intracellular ROS are generated during respiration in mitochondria. Electron transport through the mitochondrial respiratory chain is extraordinarily efficient, only 1-2% of electrons leak out and are partly reduced to superoxide anion. The production of mitochondrial superoxide

radicals occurs primarly at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at complex III (ubiquinon-cytochrom c reductase). Under normal metabolic conditions, complex III is the main site of ROS production (135). The leaking point of this system is the formation of free radical semiquinone anion species ($^{\cdot}$Q-) that occurs as an intermediate in the regeneration of coenzyme Q. Once formed, $^{\cdot}$Q- can readily and non-enzymatically transfer electrons to molecular oxygen with the subsequent generation of superoxide radicals. This activated oxygen molecule can react with organic substances by noncatalytic means. ROS are also formed in the endoplasmatic reticulum, liposomes, cell membranes, macrophages, peroxisomes and in the cytosol (33). Enzyme systems contributing to oxidative stress include NADPH cytochrome P450 reductase in the endoplasmatic reticulum, hypoxanthine/xanthine oxidase, glucose oxidase, monoamine oxidase, lipoxygenases, cyclooxygenase and NADPH oxidase (50, 80, 96).

The most common forms of ROS include superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), the highly reactive hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), peroxyl radical ($RO_2^{\cdot}$), alkoxyl radical ($RO^{\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$) (96). The production of the various radicals is linked via chemical or enzymatic reactions and ROS can give rise to secondary reactive products such as lipid peroxides. The most common intracellular forms of ROS are listed in Table 1, together with their main cellular sources of production and the relevant enzymatic antioxidant systems scavenging these ROS molecules.

Table 1: The major ROS molecules and their metabolism

ROS molecule	Main source	Enzymatic defense systems	Product(s)
Superoxide ($O_2^{\cdot -}$)	Leakage of electrons	superoxide dismutase (SOD)	$H_2O_2 + O_2$
	from the electron transport chain	superoxide reductase	H_2O_2
	Activated phagocytes,		
	Xanthin oxidase, flavoenzymes		
Hydrogen peroxide (H_2O_2)	From $O_2^{\cdot -}$ via SOD	Glutathione peroxidase	H_2O_2 + GSSG
	NADPH-oxidase	Catalase	$H_2O + O_2$
	Xanthine oxidase	Peroxiredoxin (Prx)	H_2O
	Glucose oxidase		
Hydroxyl radical ($^{\cdot}OH$)	From $O_2^{\cdot -}$ and H_2O_2 via		
	transitions of metals (Fe and Cu)		
Nitric oxide (NO)	Nitric oxide synthase	Glutathione/TrxR	GSNO

Adopted from Nordberg and Arner, Free Radical Biology & Medicine, 2001 (100)

Free radicals and nonradical reactive molecules derived from radicals exist in cells and tissues at low but measurable concentrations. Their concentration is determined by the balance between their rates of production and their rates of clearance by various antioxidant compounds and enzymes (41). When they are present in low (physiological) concentration, ROS have been implicated in the regulation of diverse cellular functions including defense against pathogens, intracellular signaling, transcriptional activation, proliferation, and apoptosis (34). Upon bacterial activation, phagocytes produce ROS (HOCl and $^{\cdot}OH$) in amounts sufficient to kill invading bacteria (129) and this is an essential host defense mechanism necessary to combat infection.

ROS stimulate cell proliferation in smooth muscle cells, fibroblasts, amnion cells, prostate cancer cells and aortic endothelial cells (119), and are thought to act as subcellular messengers for certain growth factors (IL-1β, IL-6, IL-3, TNF-α). ROS also function as physiological mediators of transcriptional control. Redox-sensitive transcription factors are Nuclear Factor-κB (NF-κB) and Activation Protein-1 (AP-1) (100), Sp-1, c-Myb, p-53, egr-1 and hypoxia-inducible factor (Hif-1) (119). Several proto-oncogenes are known to be transcriptionally activated by increased cellular oxidation such as *jun-B*, *jun-D*, *c-fos*, and *fos-B* (2). In the regulation of apoptosis ROS have a dual function. They induce apoptotic pathways through the production of ceramide (137), the activation of JNK (73, 137), p53 (133, 153), ASK-1 (21), p38 (21) and the induction of the regulatory domain of PI 3-kinase, p85 (153), or the combined activation of several pathways, but may also prevent apoptosis through the activation of NF-κB including NF-κB target genes (103), MAP kinase (55), Akt (81) and Hsp 27 (62), or by NO-mediated supression of caspases through a cGMP-dependent mechanism and direct inhibition of caspases by protein S-nitrosylation (NO activity) by NO (80). Thus, ROS perform their dual role in cell survival and cell death through a cross talk between the apoptotic machinery and cellular signaling mechanisms.

All these observations suggest that free radicals have an influence on molecular and biochemical processes and may directly affect cellular processes (2).

Imbalance of cytoplasmic ROS may result in potentially cytotoxic “oxidative stress”. Under these pro-oxidant conditions, highly reactive radicals are formed prone to cause damage, and thereby to be potentially toxic, mutagenic, or carcinogenic. The targets for ROS damage include all major groups of biomolecules: DNA, RNA, proteins, and lipid components. ROS such as $^{\cdot}OH$ and peroxynitrite ($ONOO^{-}$) cause alterations of DNA, e.g. cleavage of DNA, oxidation of and nitration of DNA bases, and generation of DNA-protein cross links. Chemical modification of bases may result in mutations due to erroneous base paring during replication.

ROS may also damage lipids through the reaction with polyunsaturated fatty acids, a process known as lipid peroxidation. The radical chain reaction starts with segregation of a hydrogen atom from polyunsaturated fatty acids, is followed by formation of highly toxic lipid ($L^{\cdot}$) and peroxyl radicals ($LOO^{\cdot}$), and ends with damage of biological membranes.

To protect themselves against increased levels of ROS, referred to as oxidative radical stress, cells possess several antioxidant systems that tightly regulate and determine a balance between the rate of ROS production and clearance. Antioxidants are defined (58) as substances that are able to compete at relatively low concentration with other oxidisable substrates. They are thus able to significantly delay or inhibit the oxidation of these substrates.

Antioxidant defense systems (AOS) can be divided in two major groups, enzymatic and nonenzymatic systems. Antioxidant enzyme systems include the enzymes superoxide dismutase (SOD), superoxide reductase (SOR), catalase (Cat), thioredoxins (Trx) and thioredoxin reductases (TrxRs), peroxiredoxines (Prx), glutathione peroxidase (GPx), glutathione reductase (GR) and other GSH-related enzyme systems. Nonenzymatic compounds active in AOS are α-tocopherol (vitamine E), retinol (vitamine A), β- carotene, coenzyme Q, lipoic acid, ascorbate (vitamine C) and thiol reducing systems consisting of small molecules with redox active sulfhydryl moieties such as glutathione (GSH). In addition there are compounds that have a relatively low specific antioxidative activity on a molar basis, but can contribute significantly to the overall antioxidant defense system when present at high concentration. The most prominent examples of such high-level, low-efficiency antioxidants are free amino acids, metabolic intermediates and small molecules (e.g. cysteine, histidine, pyruvate, bilirubin, α-thioglycerol, β-mercaptoethanol), peptides and proteins (e.g. albumin, transferrin, metallothioneins, ceruplasmine etc.) (41).

This work is going to deal with an antioxidant defense system that regulates the uptake and intra- and extracellular concentration of cysteine.

1.2 Burkitt Lymphoma Cells and Oxidative Stress

Burkitt lymhoma (BL) is a malignant human B-cell tumor occurring at high incidence in children in tropical areas of Central Africa and New Guinea (29) and with a lower incidence all over the world (86). Virtually all the cases arising in high incidence areas are associated with Epstein-Barr virus (i.e. they carry the genetic viral information), whereas only 10 to 20 %

of the cases in low incidence areas are associated with EBV. EBV positive BL cells *in vivo* only show expression of EBNA1, a viral antigen required for maintaining multiple episomal copies of the viral genome in the tumor cells (107). Induction of EBV latent genes causes a shift in the phenotype of the cells from that of BL cells (group I- phenotype) in vivo to a phenotype similar to that of EBV-immortalized lymhoblastoid cell lines (LCL) (group-III phenotype). A hallmark of Burkitt lymphomas are translocations of the proto-oncogene *c-myc* into one of the immunoglobulin loci resulting in constitutive activity of this transcription factor. Moreover, mutations of the gene encoding the tumor suppressor p53 are found in one third of the cases of Burkitt lymphoma (24, 52), and there is also evidence for genetic alterations of the putative tumor suppressor gene retinoblastoma-like 2 (RB2) in most cases of endemic Burkitt lymphoma and some sporadic cases (31).

During the attempt to establish cell lines from BL cases from low-incidence areas, Lenoir et al. (1985) noted that the use of feeder layer of irradiated human fibroblasts greatly improved the frequence of outgrowth of cell lines (87). Most of these cell lines became feeder-independent upon prolonged *in vitro* cultivation. However, some cell lines (EBV-positive group I and EBV-negative BL cells) retained a significant feeder dependence when cultured at suboptimal conditions (low cell density or low FCS concentration) (44). Modulating the susceptibility of BL cells to apoptosis by changing the culture conditions has become an important model system to study the role of EBV and viral gene products in the regulation of apoptosis (45).

Burkitt Lymphoma (BL) cells are highly sensitive to suboptimal growth conditions in vitro and undergo apoptosis when seeded at reduced serum concentration or low cell density. However they are protected from apoptosis and proliferate on a feeder layer of irradiated fibroblasts (45). Irradiated fibroblasts can protect BL cells from apoptosis through secretion of a survival- and proliferation-promoting activity that is soluble and labile.

At the beginning of the seventies it had been shown that a mouse lymphoma cell line was dependent for its growth on the presence of "an activated serum component". This growth factor was found to be inactive in foetal calf serum (FCS) but could be activated by β-mercaptoethanol (2-ME) and by macrophages (61). A similar observation was made by Broome and Jeng (1973) (27) who found that 13 out of 22 leukemic and neoplastic lymphoid cell lines were dependent on the presence of a thiol-containing compound for their growth in vitro.

Detailed biochemical studies by Bannai and his coworkers have elucidated the molecular mechanism how 2-ME acts as a growth factor for murine B cell lymphoma lines

(11-13, 65, 67-69, 71). Murine lymphoma cells have a limited uptake capacity for cystine. As cystine is the only avalaible source of cystine/cysteine in the medium, the limited uptake capacity of cystine thus limits their growth. 2-ME forms a mixed disulfide with cystine. This mixed disulfide is taken up into the cell via an amino acid transport system for bulky amino acids that is shared with methionine. Intracellularly, the mixed disulfide is reduced releasing cysteine and 2-ME. As the supply of cysteine is the rate limiting step for the synthesis of glutathione, cysteine is rapidly incorporated into glutathione. 2-ME is secreted and can react extracellularly with another cystine molecule to initiate another round of cysteine import. Even if the growth medium is completely oxidized and lacks any free sulfhydryl groups, 2-ME is able to sustain a highly efficient amino acid uptake shuttle system for cysteine in murine B cells.

For a long time the effect of 2-ME has been believed to be restricted to mouse lymphoma cells and not to operate in human cells. Paradoxically, addition of 2-ME to human BL cells induced cell killing rather than survival and cell proliferation (45). It could be shown in our laboratory, however, that α-thioglycerol (α-TG) as well as 2-ME and dithiothreithol (DTT) can indeed efficiently promote the uptake of cysteine into BL cells provided that formation of ROS through reduction of molecular oxygen by the thiol-containing compound was prevented by the addition oxygen scavengers, like sodium pyruvate, catalase or bathocuproine disulfonate (BCS) (26). Sulfhydryl containing compounds can efficiently reduce molecular oxygen by the Fenton reaction if trace amounts of transition metals are present in the medium or serum. Addition of oxygen radical scavengers and/or chelators of transition metals was thus necessary to dissect the cell death inducing effect of thiol compounds from their survival and growth promoting effect. Similarly to mouse B lymphoma cells, also in human BL cell the availability of cysteine turned out to be the rate limiting factor for the synthesis of glutathione and thus for the ability of the cells to cope with oxidative stress (72). Similarly to mouse B cells, BL cells have a low capacity for uptake of cystine which is the only available source of cysteine in the medium and is taken up through the cystine-glutamate exchange transporter also called system x_c^-. On the other hand irradiated fibroblasts take up cystine readily from the medium through system x_c^-, reduce it intracellulary to cysteine and secrete the surplus of cysteine into the medium thus providing to cocultured BL cells sufficient amounts of cysteine that is taken up by the ASC system, a shared amino acid transporter for alanine, serine and cysteine (Fig. 1).

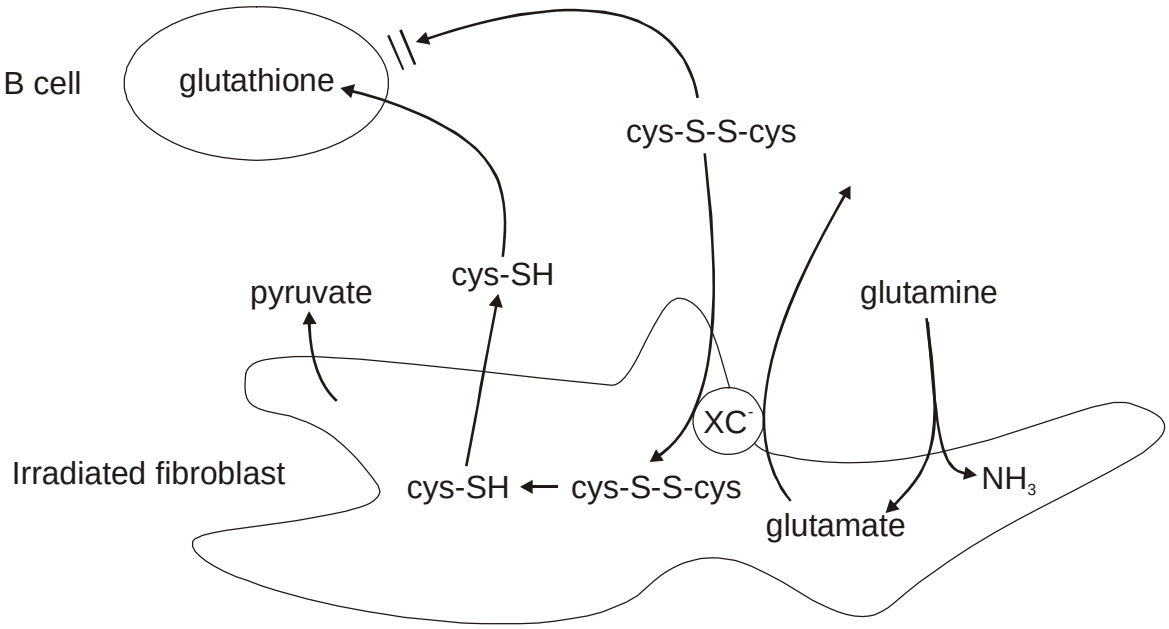

Figure 1. Metabolic interaction between fibroblasts and B Cells

1.3 The Heterodimeric Amino Acid Transporters

The heterodimeric amino acid transporters belong to a group of cell surface antigens of leukocytes that are involved in diverse processes such cell adhesion and activation through integrins, migration, differentiation, regulation of cell fusion by viral proteins as well as regulation of proliferation and apoptosis (38).

The cystine glutamate exchange transporter belongs to the glycoprotein- associated amino acid transporter family (gpaAT). Generally, amino acid transporters together with peptide transporters accomplish the uptake of amino acids and are involved in basic cellular functions like regulation of cell volume, synthesis of glutathione (GSH), provision of amino acids for protein synthesis, and energy metabolism (144). According to their specialized functions amino acid transporters are divided into different families that are distinguished by the functional properties (specificity of amino acids transported, transport mechanism, coupling to ions) and their molecular similarity or dissimilarity. Recently, a new large family of heterodimeric amino acid transporters has been discovered that is unique in its functional and molecular properties.

These transporters are heterodimers of aproximately 120 kDa composed of two subunits linked by a disulfide bond (Fig. 2).

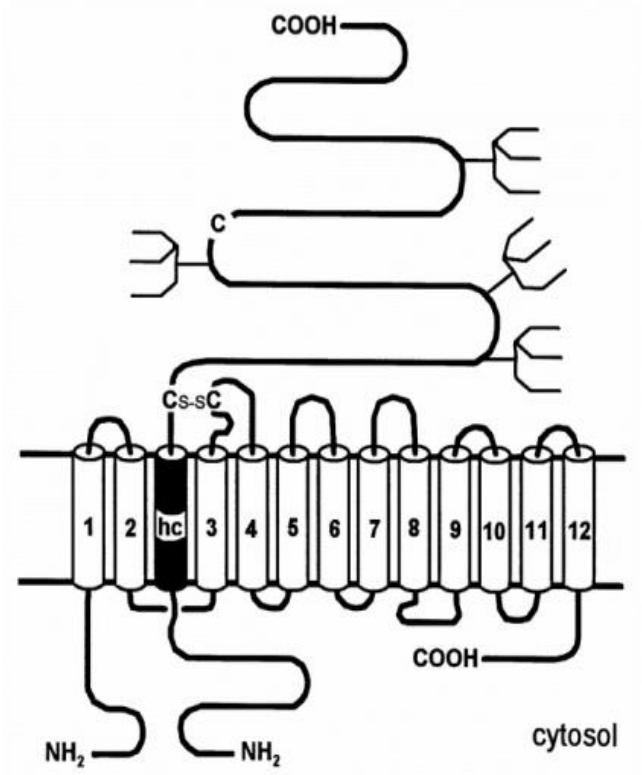

Figure 2. Structure of glycoprotein-associated amino acid transporters

One subunit is referred to as heavy chain. The heavy chain is a highly glycosylated polypeptide of 80 kDa (529 aa). The other subunit is referred to as the light chain, a protein of 40 kDa with 12 putative transmembrane domains. The substrate specificity depends on the nature of the light chain. The name "glycoprotein-associated" amino acid transporters is based on a common feature of the members of this family that their functional cell-surface expression depends on the association of the light chain with a heavy chain. The heavy chain is a type II surface glycoprotein, either 4F2hc (CD98) or rbAT (D2) (139).

1.3.1 The Heavy Chains rbAT and 4F2hc

Two different types of heavy chains were described. The first identifed heavy chain of the heterodimeric amino acid transporter family was rbAT, independently isolated by three groups by expression cloning (22, 127, 147). This heavy chain is a component of $b^{o,+}$ transporters of neutral and dibasic amino acids. The second identified heavy chain subunit is the 4F2 heavy chain (4F2hc) (22, 148), previously identified and cloned as a surface antigen

expressed on activated lymphocytes and later renamed as CD98 (59, 90, 105, 128). The two heavy chains rbAT and 4F2hc exhibit about 30% identity and 50% similarity and are associated with different light chains thus forming different amino acid transporters (22). Analysis of the amino acid sequences suggests that both heavy chains contain a single transmembrane domain (helix) (83-106 amino acids), with the NH_2 terminus intracellulary localized (22, 23). The COOH terminus is presented as a globular glycosylated extracellular domain. Both proteins have therefore been classified as type II membrane proteins. In both heavy chains, cysteine 109 forms the disulfide bond with the light chain.

1.3.2 The Light Chains

The light chains of the heterodimeric amino acid transporters belong to a family of homologous proteins with similar molecular weight and membrane topology. The light chains are markedly hydrophobic proteins of 502-535 anmino acids and contain 12 putative transmembrane domains (see figure 2) with NH_2 and COOH termini located intracellularly (38). So far, seven cDNAs encoding distinct light chains have been identified. These are LAT1, LAT2, y^+LAT1, y^+LAT2, ascAT1, b^{0+}AT1 and xCT. All light chains identified to date function as amino acid transporters when associated with a heavy chain and the substrate specifity of the heterodimer depends on the nature of the light chain. A heavy chain is needed for trafficking a light chain to the plasma membrane.

Interaction between heavy and light chains occurs via a disulfide bridge between Cys 109 of the heavy chain and a conserved cysteine residue between transmembrane helixes TM3 and TM4 in the different light subunits (91, 97, 104, 146). The functional role of the disulfide bridge has, however, remained elusive. It is neither required for trafficking of the transporter to the membrane nor for the amino acid transport function (37, 97, 104, 143). The conservation of the cysteine residues involved in the formation of the disulfide bond in all heavy and light chains, suggests, nevertheless a functional role. Given the functional heterogeneity of proteins sharing 4F2hc, disulfide bond formation may be important to ensure that less abundant heterodimers with distinct function can be formed at sufficient rate and that these heterodimers exhibit sufficient stability to fulfill their function (37, 48). There are still several open questions regarding the functional role of glycoprotein associated amino acid transporters in cell proliferation, integrin function and cell adhesion, cell fusion and possibly other important processes (139, 142).

1.4 The Cystine Glutamate Antiporter

The Cystine Glutamate Antiporter (x_c^- system) is one of the seven members of the glycoprotein associated amino acid transporter family. It consists of two polypeptide chains, xCT light chain and 4F2heavy chain (4F2hc). The light chain (xCT) determines the substrate specificity. The heavy subunit 4F2hc is responsible for the trafficking of the heterodimer to the plasma membrane. The membrane topology of the cystine glutamate antiporter is probably very similar to that of other amino acid transporters that share the 4F2 heavy chain subunit (Figure 3), although the substrate specificity is markedly different.

The system has been first described as system x_c^- by Bannai and Kitamura in 1980 (16) and has finally been molecularly identified by expression cloning from mouse activated macrophages by Sato and Bannai in 1999 (116). The cystine glutamate antiporter is a plasma membrane-localised, Na^+ independent, anionic amino acid exchange transport system highly specific for glutamate and cystine. Under physiological conditions the antiporter mediates the influx of cystine and the efflux of glutamate, because the cystine concentration is very low in the cytosol due to its rapid reduction to cysteine, and conversely, the concentration of glutamate is much higher inside the cell than in the extracellular fluid due to the activity of different isoforms of the amino acid transport system X_{AG} and the uptake of glutamine which is converted intracellularly to glutamate by deamination (14, 20). The gradient of substrate concentration across the plasma membrane determines the direction of the exchange reaction through system x_c^-. Cystine is transported only in its mono-anionic form in a molar ratio of 1:1 with glutamate. The cystine/glutamate exchange by system x_c^- is thus electroneutral (114).

As mentioned, the xCT light chain determines the substrate specificity of system x_c^-. The cDNA for mouse xCT encodes a putative protein of 502 amino acids and a relative molecular mass of 55.5 kDa. Three different xCT transcripts (12, 3.5 and 2.5 kb) have been identifed after treatment of macrophages for 8 hours with LPS and/or diethylmaleate (116). The different transcripts are formed by alternative splicing, use of alternative polyadenylation sites or a combination of both.

The conserved cysteine residue at position 158 of xCT is the putative site of disulfide bond formation with Cys^{109} of 4F2hc (116). Cysteine at position 327 (Cys327) in the middle of the eighth transmembrane domain of the xCT light subunit has recently been shown to be a functionally important residue accessible to the aqueous extracellular environment that is presumably structurally linked to the permeation pathway and/or the substrate binding site (75).

Two clones for human xCT were isolated from a cDNA library derived from human fibroblasts. The first clone consists of 1,861 bp and contains a single open reading frame that encodes a putative protein of 501 amino acids. Another clone is composed of 5,626 bp and contains the same single open reading frame. This second clone has a long 3'-untranslated region. The human and murine xCT mRNAs of different size have 3'-untranslated regions of different lengths suggesting that the untranslated region might be involved in regulating the expression of xCT at a post-transcriptional level (117). The human xCT gene is localized at chromosome 4q28-31. The xCT gene is composed of twelve exons. The activity of the cystine glutamate exchange transporter contributes to the maintenance of intracellular glutathione levels (19). The activity of system x_c^- is significantly induced by various stimuli, including diethylmaleate (DEM), arsenite, cadmium chloride, hydrogen peroxide, bacterial lypopolisacharide, and TNF-α in mouse peritoneal macrophages (18, 113). A consequence of induction of x_c^- activity is an increase in the level of intracellular glutathione suggesting that system x_c^- belongs to the category of stress proteins that are required for the metabolic response to oxidative stress. It has been shown recently that EpRE-like sequences (electrophile response elements like sequences) in the 5' flanking region of xCT gene are responsible for DEM induction of xCT (112). Sequence analysis identified four (EpRE)-like sequences (designated EpRE-1 to EpRE4), between –230 and –1 in the 5' flanking region in front of the first exon.

1.4.1 Physiological Role of the Cystine Glutamate Antiporter in the Cellular Antioxidative Defense System

The availability of cysteine is the rate-limiting step for the synthesis of glutathione (GSH), the major cellular antioxidant (19). Transport of cystine across the cell membrane is essential for the synthesis of glutathione. Outside of cells, cysteine is unstable and readily oxidized to cystine with a half life of 0.5-1 hour in the culture medium (66). Therefore cystine is predominant in plasma as well as in cell culture medium, and is the only available source of cysteine for the cells. The cystine glutamate antiporter imports cystine into the cells in exchange with glutamate in a molar ratio of 1:1. Intracellularly, cystine is immediately reduced to cysteine, which in turn is used for the synthesis of glutathione and protein synthesis (Fig. 3).

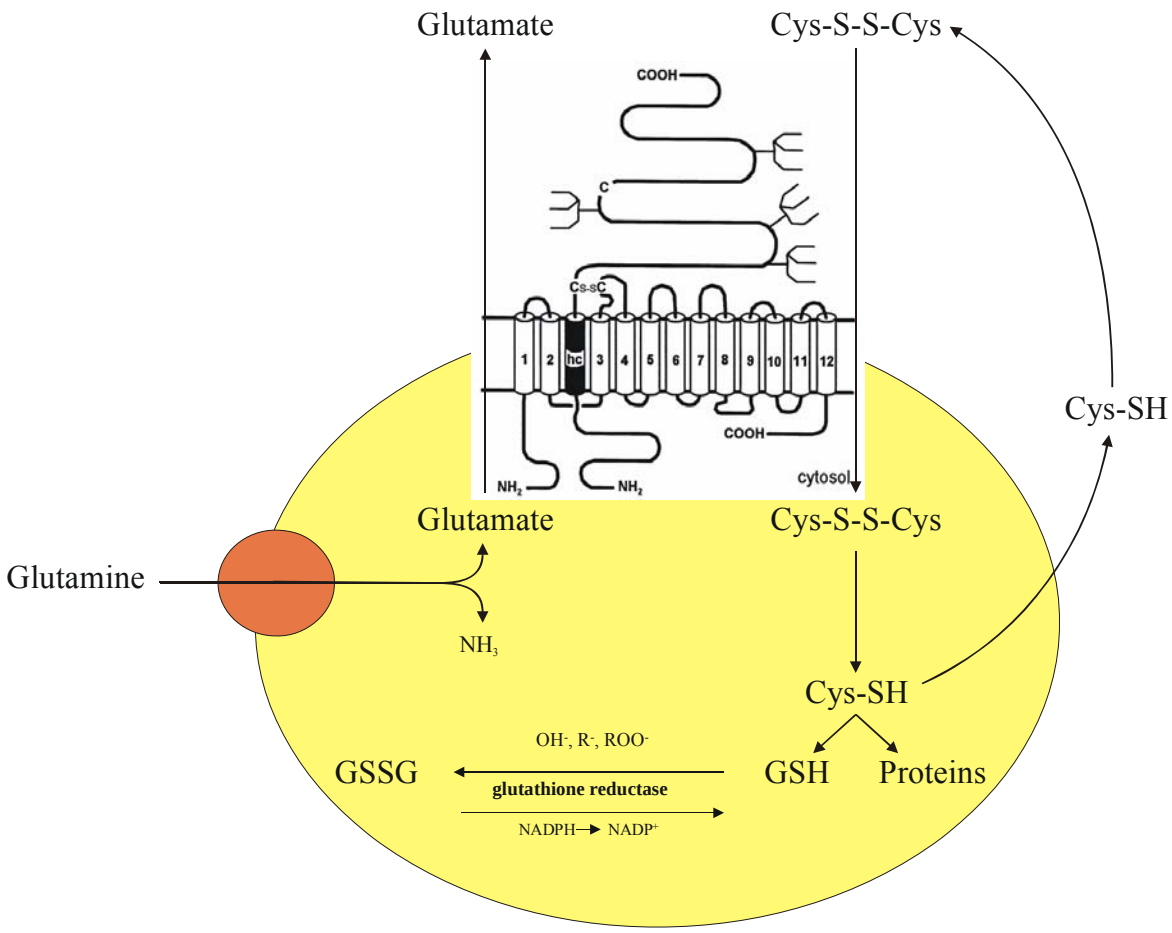

Figure 3. Physiological role of cystine glutamate antiporter

Part of cysteine is released back into medium via the neutral ASC amino acid transport system and reoxidized to cystine by oxygen (17). Sato et al. have thus proposed that system x_c^- drives a cycle, called the cystine/cysteine cycle, that maintains the balance between cystine and cysteine in the culture medium (114). Thereby system x_c^- contributes to the maintaince of the redox state in the cerebral spinal fluid (CSF). Cysteine and cystine, and glutathione and glutathione disulfide, are the critical components that are acting as cellular redox buffers in metabolism and homeostasis (36). Electrophilic agents as well as chemical and physical stress induce the activity of the cystine glutamate antiporter in a variety of cells such as macrophages, neuronal or glial cell, fibroblasts, kidney cells, pancreas cells, and hepatocytes (9, 10, 17, 70, 94, 115). The activity of system x_c^- directly regulates the intracellular GSH concentration (114) and thus can be categorized as part of the cellular antioxidant defense system.

1.5 Glutathione (GSH)

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) and the predominant non-protein sulfhydryl compound in the cell. Inside the cell, glutathione is present in millimolar concentration (1-10 mM) predominantly in reduced form (GSH) and to a lesser extent (about 1% of GSH) in disulfide form (GSSG) ((3, 82). GSSG is formed by disulfide bond formation of two GSH molecules (Fig. 4).

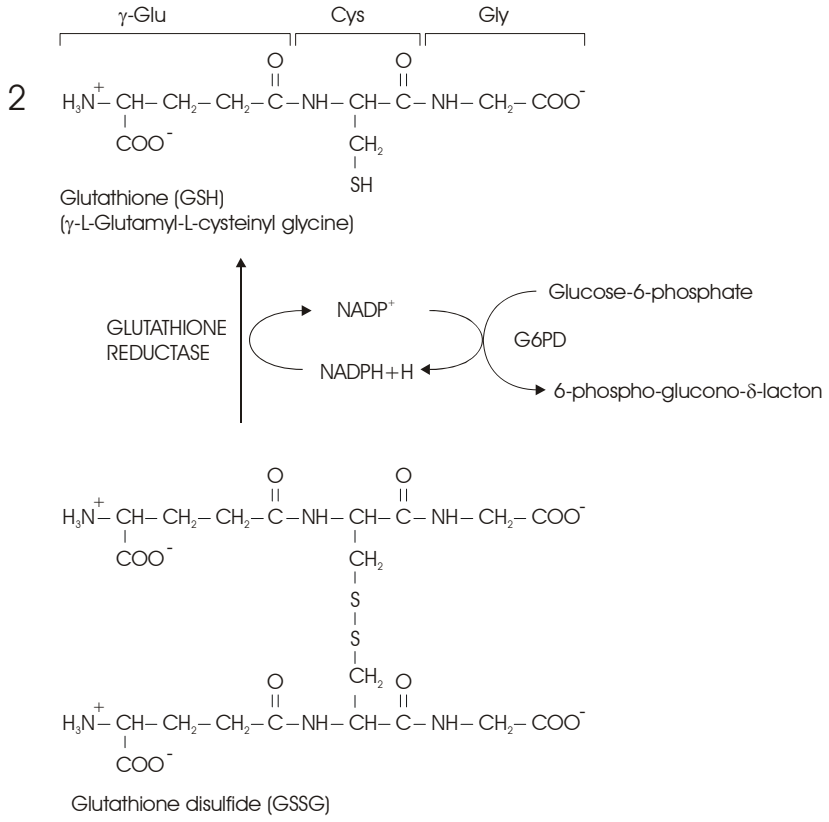

Figure 4. Structure of the reduced (GSH) and dissulfide (GSSG) form of glutathione

1.5.1 Role of Intracellular Glutathione

Glutathione has several functions intracellulary. One of the most important functions of glutathione is the protection against oxidative damage caused by reactive oxygen species (ROS). As shown in figure 5, glutathione is the major scavenger of hydroxyl radicals, peroxides and lipid peroxides inside the cell. Glutathione reacts with ROS and during this

process the reduced form of glutathione (GSH) is converted to the disulfide form. Glutathione disulfide (GSSG) is again reduced to GSH by NADPH through a reaction catalyzed by glutathione reductase (GR). NADPH is formed through the activity of glucose 6-phosphate and 6-phosphogluconate dehydrogenase in the pentose phosphate cycle.

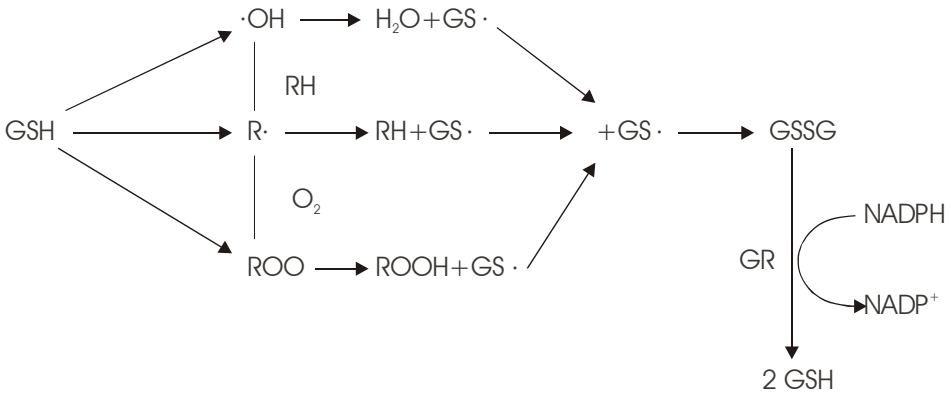

Figure 5. Reactions of glutathione with ROS

Glutathione does not react directly with hydroperoxides, but is used as a substrate for glutathione peroxidases (GSHPx) which catalyze the destruction of H_2O_2 and lipid peroxides at the expense of reduced glutathione (3, 40):

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

$$ROOH + 2GSH \rightarrow 2ROH + GSSG$$

The disulfide form of glutathione (GSSG) is normally present in the cell at a level of about 1% of total glutathione. During oxidative stress the concentration of GSSG increases, but this is transient as reduction of GSSG by glutathione reductase is a rapid process. GSSG can also exchange sulfhydryl groups with proteins leading to protein-glutathione mixed disulfides (40):

$$GSSG + \text{protein-SH} \Leftrightarrow \text{protein-SSG} + GSH$$

Mixed disulfides of proteins with glutathione (protein-SSG) have a longer half-life than GSSG, and a significant basal level of glutathionylated proteins is found in cells (88). By keeping proteins in different states, this exchange reaction may provide a mechanism for the action of GSH in cell signaling.

Glutathione has several other functions in cells (3, 35, 93, 121): in amino acid transport through the action of the γ-glutamyl cycle, in synthesis of nucleotides as a donor of hydrogen (125), in the biosynthesis of leukotrienes, in the detoxification of xenobiotics, and as a reservoir for cysteine. Glutathione´s role in detoxification processes is linked to the activity of the glutathione-S-transferases. This group of enzymes catalyzes the conjugation of the reduced form of glutathione (GSH) to a variety of electrophilic compounds thus rendering them water-soluble. Conjugation with GSH is an essential step in the detoxification of hepatic toxins including xenobiotics, epoxides and carcinogens. The reaction is performed as follows:

$$R\text{-}X + GSH \rightarrow R\text{-}SG + HX$$
$$R\text{-}SG + GSH \rightarrow RH + GSSG$$

X is an electrophilic compound of exogenous origin.

1.5.2 Compartmentation of Glutathione

The majority of glutathione in cells is synthesized and present in the cytoplasm in two forms, as a free glutathione and bound glutathione. Glutathione is also utilized for a variety of physiological functions in other cellular compartments, including the nucleus, mitochondrial matrix, endoplasmic reticulum, microsomes and extracellular space (124).

The intracellular compartmentation of glutathione is important for cells that are exposed to toxic compounds or to oxidative stress, because the various cellular organelles appear to have their own GSH pool independent from the cytosol.

The nuclear glutathione pool represents 5 to 10% of the total cellular glutathione. In the nucleus, glutathione maintains the redox state of critical protein sulfhydryl groups, which are necessary for DNA repair and gene expression (5). Glutathione in the nucleus is critical to the cell's well-being and is therefore maintained as a distinctly regulated pool that is protected from depletion when levels of GSH in the cytosol fluctuate. Although, the majority of the glutathione concentration in the nucleus is equivalent to that in the cytosol and most of the GSH present in the nucleus is likely to be in free equilibrium between the cytosolic and nuclear compartments, probably exchanged via nuclear pores (124), some studies (42, 74) have suggested that at least a portion of the nuclear glutathione pool is "protected" from depletion and can maintain its levels. This subnuclear pool of GSH is not in equilibirum with the cytosolic GSH pool and presents about 1-2% of the total cellular GSH pool and is believed to

play an important role in protecting biochemical processes when the cell is faced with fluctuations in cytosolic GSH concentration.

The mitochondrial pool of glutathione is approximately 30% of total cellular glutathione (~ 5mM) (124) and it plays the major role in the maintenance of the mitochondrial thiol-disulfide status. Different mitochondrial proteins like dehydrogenases, ATPases, and transport proteins, contain essential sulfhydryl groups that must be reduced to be active. Glutathione may be transported into the mitochondria from the cytosol, or may be *de novo* synthesized in mitochondria.

In contrast to mitochondria and the nucleus, the endoplasmatic reticulum is a compartment with a more oxidizing environment than the cytosol. The ratio of GSH/GSSG appears to range from 1:1 to 3:1 (64) which is very low compared to the overall ratio in the cell of 30:1 to 100:1. The more oxidizing environment of the endoplasmatic reticulum ensures proper protein folding and disulfide bond formation in proteins.

Glutathione is also present in extracellular pools (78) in the concentration range of 1-10 μM. This suggests that the extracellular surface of most cells is potentially more vulnerable to oxidants from extracellular exposure. There are several proposed physiological roles for extracellular glutathione. Extracellular glutathione functions in detoxication and protection of cells from chemical- and oxidant-induced injury, and it may control the extracellular oxidation-reduction state and may thus contribute to the protection of cell membranes (92). Hwang and Sinskey (1991) (63) showed that cultured cells maintain their extracellular redox states and that there is a linear correlation between extracellular thiols and viable cell density. They found that thiol-disulfide ratios were an important component of the redox potential and that thiol release was a universal characteristic feature of cells in culture. But, very little information is available on the relative efflux rates of glutathione and cysteine and it is unclear whether the GSH/GSSG redox couple is equilibrated with other redox active components in extracellular fluids.

An important function of extracellular glutathione is to serve as a source of cysteine in the overall sulfur amino acid balance. GSH cannot be broken down intracellulary and it must be released to be degraded by ecto-enzyme γ-glutamyltranspeptidase to yield cysteine. Cysteine is a critical amino acid for synthesis and function of many cellular proteins, and it is highly reactive so that only low concentrations of cysteine can be tolerated inside the cell. Thus, cysteine is transiently stored in cells as GSH in a less reactive form and one of the functions of extracellular glutathione is therefore to supplement the endogenous synthesis (7, 28, 56, 57, 84). It is also used as a substrate for extracellular GSH-dependent enzymes (124), for protection of critical thiol groups of transporters and receptors present on the extracellular

surface of cells (126), for reduction of iron for iron absorption, for the reduction of disulfides to control mucus fluidity, and for the maintenance of other antioxidant systems (e. g. ascorbate).

1.6 The Multiple Roles of Cysteine in Cells

Thiols are organic compounds that contain the –SH functional group (called the thiol-, mercapto- or sulfhydryl group). Thiols, because of their ability to be reversibly oxidised, are recognised as key components involved in the maintenance of the redox balance. Thiol groups located in various molecules act as a redox sensitive switch and thereby provide a common target for a variety of reactive oxygen and reactive nitrogen species (95). The most abundant biologically occurring thiol is the amino acid cysteine together with its disulfide cystine. Extracellular cysteine is oxidized within minutes to cystine, the disulfide form of cysteine (Fig. 6).

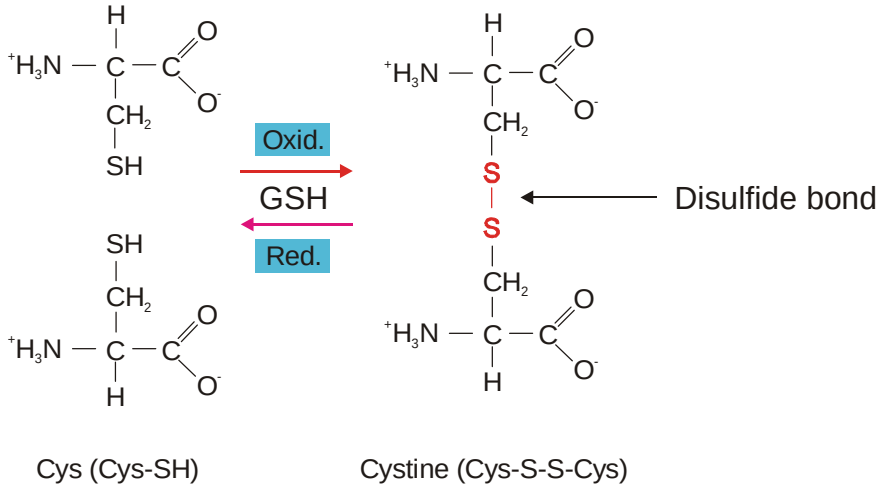

Figure 6. Structure of cysteine (Cys) and its disulfide cystine (Cys-S-S-Cys)

Cysteine´s ability to occur in up to 10 different sulfur oxidation states in vivo allows the formation of a range of cysteine modifications in peptides and proteins including thiols, thiolates, thiyl radicals, disulfides, sulfenic (RSOH), sulfinic (RSO_2H) and sulfonic acids (RSO_3H), disulfide-S-oxides and selenodisulfides. Each of these cysteine modifications has its own chemical and biochemical properties such as stability, redox behaviour, metal binding, acidity, nucleophilicity and catalytic activity. The chemical properties of the neighbouring amino acids have furthermore a strong impact on the behaviour and pH-dependence of these

cysteine modifications. This versatility enables cysteine to fulfill a wide range of different functions in proteins including disulfide formation, metal-binding, electron donation, hydrolysis, and redox-catalysis.

Cysteine-based redox systems are exceptional since sulfur can participate in several mechanistically distinct redox-reactions. These reactions (Fig. 7) can be classified as thiol/disulfide exchange reactions (thioredoxin, Trx); electron transfer reactions (glutathione reductase, GR); thiol/thiyl hydrogen radical transfer reactions (in ribonucleotide reductase, RNRase); oxygen atom transfer reactions (in peroxiredoxins, NADH oxidase (Nox) and NADH peroxidase (Npx)); and different hydride transfer reactions (in Npx and glyceraldehyde 3-phosphate dehydrogenase, GAPDH).

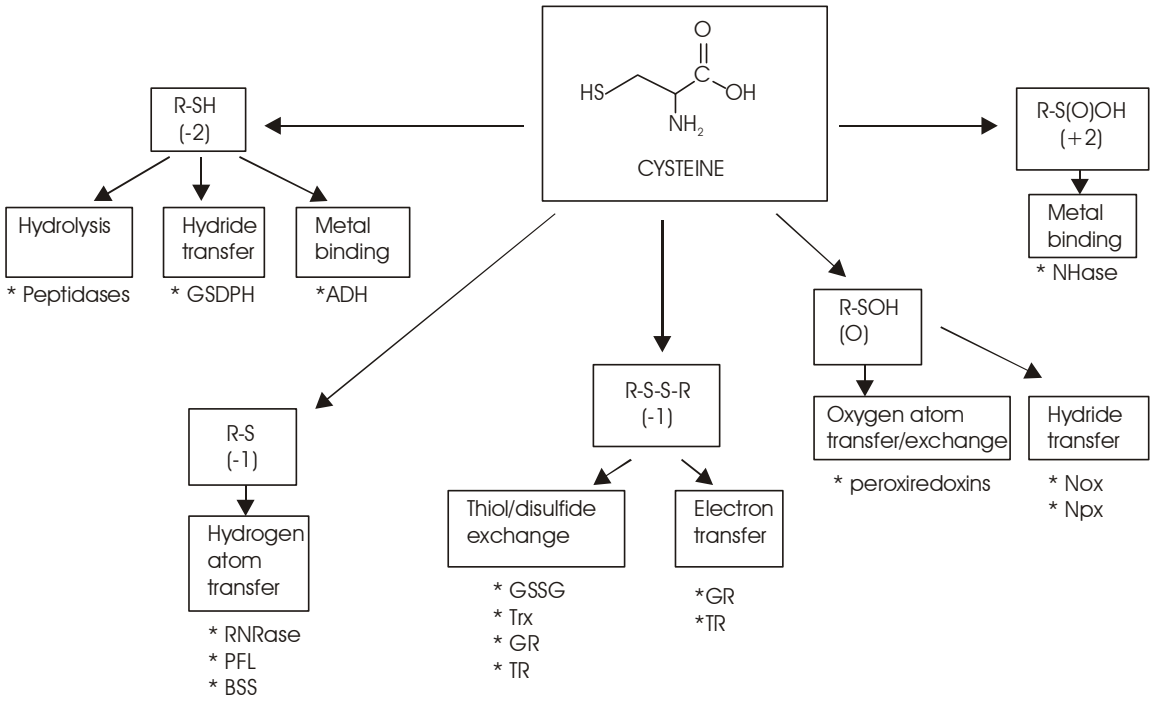

Figure 7. Oxidation states, properties, reactivity, and occurrences of different cysteine modifications in vivo. Oxidation states of sulfur are given for R in oxidation state +1 (Giles et al., 2003)

Besides the role of cysteine in catalytic reactions, disulfide bonds play also an important role in determining the tertiary structure and the metabolism of proteins. Molecules containing cysteine are among the most easily metabolized compounds. They are easily oxidized by transition metals and participate in thiol-disulfide exchange reactions. The concentration of protein-sulfhydryl groups in cells and tissues is much higher than that of glutathione (134). These groups are present in proteins as thiols (-SH), disulfides (PS-SP) and mixed disulfides (PS-SG) when conjugated with GSH. Protein S-thiolation or dethiolation are

dynamic processes that are reversible and occur at different rates depending on the protein and nature of the thiol groups in the protein (39). The oxidation of protein sulfydryl groups to mixed disulfides is an early cellular response to oxidative stress (130).

Cysteine and its disulfide form cystine also constitute an important extracellular thiol-disulfide system (99), that have important role in the regulation of different cellular processes such as cell proliferation, differentiation and apoptosis (98). Despite the oxidizing nature of the extracellular environment, the presence of reduced thiol (-SH) groups may be supported and maintained on the cell surface by the specific cell surface microenvironment. Thiol containing substances like GSH, thioredoxin and glutaredoxin participate in the regulation of the redox state on the cell surface (110).

2 THE AIM OF THE WORK

From the previous work of the group it was known that Burkitt lymphoma cells are highly sensitive to suboptimal grow conditions *in vitro* and grow very poorly when seeded in tissue culture at low cell density or at reduced serum concentration in the absence of irradiated fibroblasts (25, 45). In the absence of feeder cells the cells undergo apoptosis at low cell density. The high sensitivity towards induction of apoptosis is caused by increased oxidative stress at low as compared to high cell density. One limiting factor appeared to be the uptake capacity of B cells for cystine. Cystine uptake is the limiting step in the synthesis of glutathione, a central player in the antioxidant defense system of the cell. Feeder cells take up cystine from the medium, reduce it intracellularly and secrete the surplus as cysteine that can be efficiently taken up by cocultivated Burkitt lymphoma cells by the amino acid transport system with specificity for alanine, serine and cysteine (ASC-system). As similar observations have been made for murine B cells and mouse and human T lymphocytes, these findings suggested that one important factor in the network of regulation of apoptosis in the lymphoid system might be the uptake capacity for cystine.

The murine and human amino acid uptake system for cystine has been cloned by Sato and Bannai in 1999 and 2000 (116, 117). It is an antiporter importing cystine in exchange for glutamate. It is a heterodimeric membrane protein consisting of two chains, xCT, specific for the cystine-glutamate antiporter, and 4F2heavy chain (4F2hc), a multifunctional protein present as the common chain in many different amino acid transport systems.

The aim of this work was to express the cystine-glutamate antiporter ectopically in Burkitt lymphoma cells and to study (i) the susceptibility versus resistance of transfected cells to apoptosis induced by growth at low cell density and by depletion of glutathione; and (ii) to elucidate the nature of the pathway induced by overexpression of the xCT light chain that controls cell survival under non-permissive conditions.

3 MATERIALS

3.1 Chemical reagents

α-Thioglycerol	Sigma-Aldrich Chemie GmbH, Deisenhofen
Acrylamide	Carl Roth & Co., Karlsruhe
Agarose	Invitrogen Life Technologies, Karlsruhe
Ampicillin	Merck KgaA, Darmstadt
Bacto-agar	Difco Laboratories, Michigan, USA
Bacto-yeast extract	Difco Laboratories, Michigan, USA
Bacto-tryptone	Difco Laboratories, Michigan, USA
DL-Buthionine -[S, R]-sulfoximine	Sigma-Aldrich Chemie GmbH, Deisenhofen
DNA 1Kb Plus Ladder	Invitrogen Life Technologies, Karlsruhe
RNA ladder 0.24-9.5 kb	Invitrogen Life Technologies, Karlsruhe
dNTPs	GibcoBRL Life Technologies GmbH, Karlsruhe
DNA linkers	New England Biolabs GmbH, Schwalbach
Ethanol p.a.	Merck KgaA, Darmstadt
Ethidiumbromide	Merck KgaA, Darmstadt
Fetal bovine serum	Biochrom KG, Berlin
	PAN, Biotech GmbH
	Sigma-Aldrich Chemie GmbH, Deisenhofen
	GibcoBRL Life Technologies GmbH, Karlsruhe
LMP-agarose	MBI FERMENTAS, St. Leon-Rot
L-glutamine	Invitrogen LifeTechnologies, Karlsruhe
L-Pyruvate	Invitrogen LifeTechnologies, Karlsruhe
Sulfosalicylic acid dihydrate	Sigma-Aldrich Chemie GmbH, Deisenhofen
Mineral Oil	Sigma-Aldrich Chemie GmbH, Deisenhofen
5, 5'-Dithio-bis (2-nitrobenzoic acid)	Sigma-Aldrich Chemie GmbH, Deisenhofen
β-NADPH tetrasodium salt	Sigma-Aldrich Chemie GmbH, Deisenhofen
L-Cystine	Sigma-Aldrich Chemie GmbH, Deisenhofen
Di-n-buthyl Phthalate	Sigma-Aldrich Chemie GmbH, Deisenhofen
Phosphate Buffered Saline (tablets)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Phosphate Buffer Saline	Merck KgaA, Darmstadt

Glutathione (reduced form)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Diethyl Ether p.a.	Merck KgaA, Darmstadt
Penicillin-streptomycin	Invitrogen LifeTechnologies, Karlsruhe
RPMI-1640 cell culture medium	GibcoBRL Life Technologies GmbH, Karlsruhe
Puromycin	Sigma-Aldrich Chemie GmbH, Deisenhofen
Hygromycin B	Invitrogen LifeTechnologies, Karlsruhe
Propidium iodid	Sigma-Aldrich Chemie GmbH, Deisenhofen
Annexin-V-FITC	BD PharMingen, Heidelberg, Germany

3.2 Enzymes

Glutathione reductase from Baker's Yeast (500 U)	Sigma-Aldrich Chemie GmbH, Deisenhofen
Bacterial Alkaline Phosphatase	GibcoBRL Life Technologies GmbH, Karlsruhe
Klenow fragment	New England Biolabs GmbH, Schwalbach
T4 DNA Ligase	New England Biolabs GmbH, Schwalbach
Restriction endonucleases	New England Biolabs GmbH, Schwalbach MBI FERMENTAS, St, Leon-Rot
Pfu DNA polymerase	Promega Corp., Wisconsin, USA
RED-Taq DNA polymerase	Sigma-Aldrich Chemie GmbH, Deisenhofen

3.3 Antibodies

The mouse monoclonal antibodies:

anti-Bax YTH-2D2 (1:10000)	Trevigen Inc. Gaithersburg, MD
anti-Bcl-2 100/D5 (1:100)	Novocastra, Dossenheim, Germany

The rat monoclonal antibody:

anti Bcl-w 16H12 (1:1000)	ALEXIS GmbH, Gruenberg, Germany

The plyclonal goat antibodies: Anti-PUMA N-19 (1:100) , anti-Bid C-20 (1:1000) and anti-Nbk/Bik N-19 (1:1000) Santa Cruz Biotechnology, Santa cruz, California

The following antibodies were polyclonal rabbit antibodies used ina dilution 1:1000:

Anti-Bak	Dako, Hamburg, Germany
Anti-Bcl-x and anti-Bad	BD Transduction Laboratories, Heidelberg, Germany

Anti-Bim and anti-p53	BD PharMingen, Heidelberg, Germany
Anti-β-actin	Sigma-Aldrich Chemie GmbH, Deisenhofen

3.4 Radioactive Isotopes

[α-^{32}P] dCTP (3000 Ci/ mmol, 10m Ci/ ml)	Amersham Pharmacia Biotech, Freiburg, Germany
L- [^{14}C (U)] Cystine (250 mCi/ mmol/ 0.02 mCi/ ml)	Perkin Elmer Life Sciences, Inc., Boston, USA

3.5 Disposables and Kits

3MM-paper	Whatman Ltd., Kentucky, USA
Cell culture plasticware	Greiner GmbH, Frickenhausen
General laboratoryware	GLW GmbH, Würzburg
DNA Midi/ Maxi kits	Qiagen GmbH, Hilden
DNA gel extraction kit	Qiagen GmbH, Hilden
PCR purification kit	Qiagen GmbH, Hilden
Rneasy Mini/ Midi kit	Qiagen GmbH, Hilden
Jet Star 2.0 Plasmid Purification kit	Genomed, Bad Oeynhausen
Carboxylfluoroscein FLICA Apoptosis Detection Kit Caspase Assay	ALEXIS GmbH, Gruenberg, Germany
Electroporation cuvettes, 4 mm	Bio-Rad Laboratories, München
Oligonucleotides	Metabion, München
Parafilm	Dynatech, Denkendorf
Polypropilene tubes 15 ml, 50 ml	Becton-Dickinson GmbH, Heidelberg
Centrifugation sampling tube (0.4 ml, 46.8 mm x 5.7 mm with lid)	Bender & Hobein GmbH, Bruchsaal
Reactione vials 1.5 ml, 2 ml	Eppendorff-Nertheler-Hinz, Hamburg
Readyprime ^{TM} II DNA labelling kit	Amercham Pharmacia Biotech, Freiburg
Sephadex G-50 (NICK^{TM} Column)	Amersham Pharmacia Biotech, Freiburg
X-ray film (XAR5, BIOMAX.MS)	Eastman Kodak Co, New York, USA

3.6 Bacteria

DH10B: *E. coli* strain obtained from Invitrogen GmbH, Karlsruhe. Used for the routine cloning steps of plasmid DNA.

E.coli K12, GM 2163 (dam- and dcm – strain) used for maintaining unmethylated plasmid DNA, obtained from New England Biolabs GmbH, Schwalbach, Germany.

3.7 Cell line

HH514 is a human, EBV positive Burkitt's Lymphoma cell line, a subclone of the P3HR-1 cell line.

3.8 Stably transfected cell lines

All transfections were performed using the BL cell line HH514 as a parental cell line.

3.9 Parental cell line

Name		*Plasmid*	*Resistance*
HH514	AB1	m4F2hc-pREP7	Hyg
HH514	AB2	IRES-m4F2hc-pREP7	Hyg
HH514	AB3	IRES-EGFP-pINCO (control)	Pur
HH514	AB4	mxCT-IRES-EGFP-pINCO	Pur
HH514	AB5	mxCT-IRES-m4F2hc-pREP7	Hyg
HH514	AB6	pREP7 + IRES-EGFP-pINCO (control)	Hyg + Pur
HH514	AB7	m4F2hc + IRES-EGFP-pINCO (control)	Hyg + Pur
HH514	AB8	mxCT-IRES-EGFP + pREP7 (control)	Hyg + Pur
HH514	H (1 and 2)	hxCT-p141	Pur
HH514	M (1 and 2)	mxCT-p141 (control)	Pur
HH514	141 (1 and 2)	p141 (control)	Pur

3.10 Materials for cloning

3.10.1 Oligonucleotides

All oligonocletides were obtained from Metabion GmbH, Martinsried, Germany.

MC 1: 5’ ATT TCA TTC GAA CGG AGA TCT TG 3’ (MC linker for cloning)
MC 2: 5’ CTA GCA TGA TCT CCG TTC GAA TG 3’ (MC linker for cloning)

hxCT 3.1: 5’GAG GGA ACG AGG AGG TGG AG 3’ (for the generation of a human xCT specific probe used in Northern Blots)
hxCT 5.1: 5’GTG ATG ACG AAG CCA ATC CC 3’ (northern blot probe as above)

m4F2 3.1: 5’ AGG CTG TCC AGG AAG GGC ACC 3’ (used for sequencing)
h4F2 3.2: 5’ CCG TAC AGG CCT AGA AGT AA 3’ (used for sequencing)
h4F2 3.3: 5’ GGT CCA GAA TGA CAC GGA TGC 3’ (used for sequencing)
h4F2 3.4: 5’ ACC TTG ATC TTC ACC AGA CC 3’ (used for sequencing)
h4F2 5.1: 5’ GAA GAT TTT GAC AGT CTC TTG C 3’ (used for sequencing)
h4F2 5.2: 5’ CTG CAT GGT GAA GTC TTC C 3’ (used for sequencing)
h4F2 5.3: 5’ AGT CAT GCT GTG GGA TGA GTC C 3’ (used for sequencing)
mxct 5.3: 5’ CAT TCT GGA GGT CTT TGG TCC 3’ (used for sequencing)
hxct 5.5: 5’ GTG GAA CTC CTC ATA ATA CGC CC 3’ (used for sequencing)

3.10.2 Linkers for cloning

All linkers were ordered from New England Biolabs GmbH, Schwalbach, Germany.

BclI linker 5’ d(pCTGATCAG) 3’
EcoRI linker 5’ d(pGGAATTCC) 3’
HindIII linker 5’ d(pCAAGCTTG) 3’

3.10.3 Vectors used for cloning

All basic vectors used for the cloning steps of both genes (human and mouse xCT) are listed:

1) 141-pCAG-3SIP: eucaryotic expression vector carrying an IRES–puromycin phosphotransferase gene (gift from Timm Schroeder) (Fig. 8)
2) pINCO: eucaryotic expression plasmid harbouring a retroviral packinging system, and conferring puromycin resistance (gift from P. G. Pelicci) (Fig. 9)
3) pREP7: eucaryotic expression plasmid conferring hygromycine resistance (Invitrogen LifeTechnologies, Karlsruhe, Germany) (Fig. 10)
4) pMC30: IRES containing vector (gift from Marcus Conrad)
5) pIRES2-EGFP: IRES-EGFP containing vector (Clontech, Heidelberg, Germany)
6) pSPORT-mxCT: vector containing mxCT sequence (gift from S. Bannai)
7) pSPORT-m4F2hc: vector carrying mouse 4F2hc gene (gift from S. Bannai)
8) pBlueScript-hxCT: vector harbouring human xCT gene (gift from S. Bannai)

Figures 8 ,9 and 10 are shown at the end of this chapter.

List of intermediate and final clones generated in this work

Intermediate clones

1) pINCO-IRES-EGFP (introduction of IRES-EGFP in pINCO vector in order to generate the expression plasmid mxCT-IRES-EGFP-pINCO)
2) IRES-m4F2hc-pREP7 (insertion of IRES 5´from m4F2hc for the generation of mxCT-IRES-m4F2hc-pREP7 expression vector)
3) pSPORT-mxCT-*EcoRI* linker (introduction of an *EcoRI* site 5´ from the mCXT ORF allowing the transfer of mxCT as an *EcoRI* fragment in p141-pCAG-3SIP and in pINCO-IRES-EGFP)
4) pSPORT-mxCT-*HindIII* linker (insertion of a *HindIII* site for the transfer of mxCT in pREP7-IRES-m4F2hc to generate the expression plasmid mxCT-IRES-m4F2hc-pREP7)
5) p141-pCAG-3SIP-MC linker

Final expression plasmids used for transfection of HH514 cells

1) m4F2hc-pREP7
2) mxCT-pINCO
3) mxCT-IRES-EGFP-pINCO
4) mxCT-IRES-4F2hc-pREP7
5) mxCT-pREP7
6) mxCT-p141-pCAG-3SIP
7) hxCT-p141-pCAG-3SIP

All cloning steps are described in detail in chapter 5.4.

3.10.4 Maps of vectors used for the expression of mouse and human xCT and mouse 4F2 hc

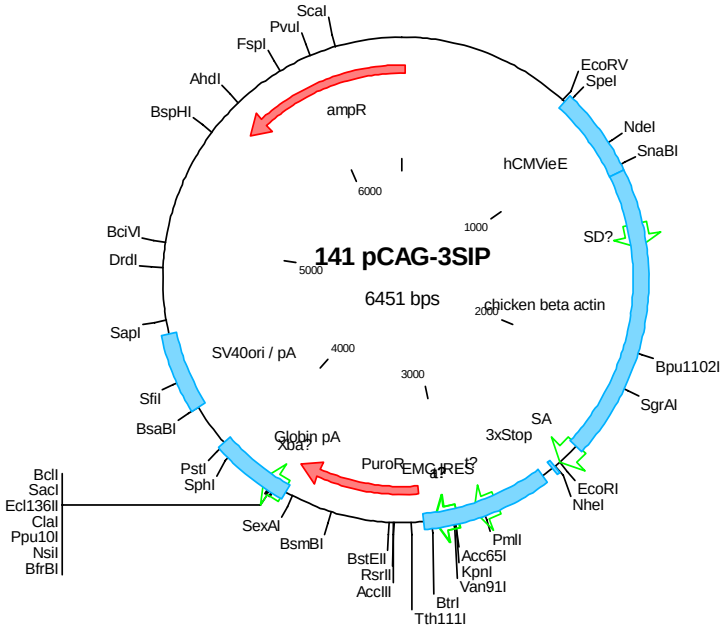

Figure 8. Restriction map of 141 pCAG- 3 SIP (CAG = CMV, chicken beta actin, globin; 3 SIP = translational stop element in every possible ORF followed by IRES and puromycine phosphotransferase gene)

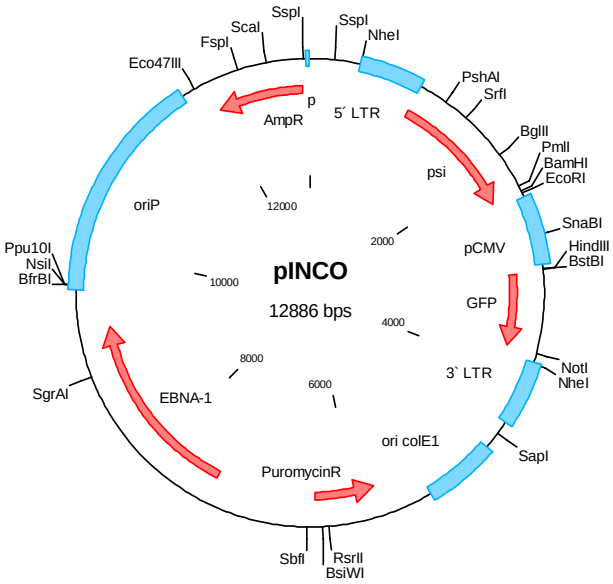

Figure 9. Restriction map of the pINCO vector (This plasmide carries retroviral packinging system: 5'LTR and 3'LTR sequences (long terminal repeats) and conferring puromycin and ampicilin resistances)

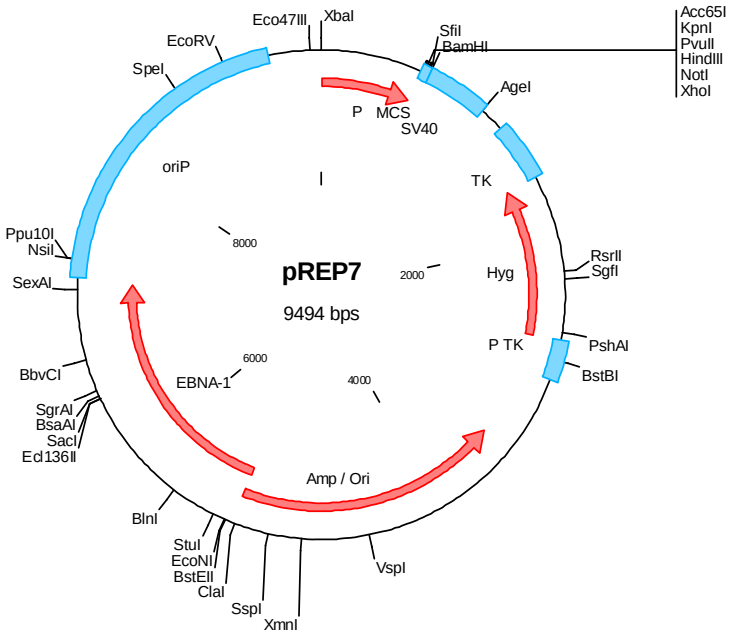

Figure 10. Restriction map of the pREP 7 vector (High-level of constituve expression is driven by CMV or RSV promoters. The Epstein-Barr Virus replication origin (oriP) and nuclear antigen (encoded by the EBNA-1 gene) is carried by this plasmid to permit extrachromosomal replication in human, primate and canine cells. This plasmid also carries the hygromycine B resistance gene.)

4 METHODS

During this work the standard protein and molecular biology techniques were used (6, 111), and described in following text.

4.1 Culture of Bacteria

Bacteria were cultured either on LB agar plates or in liquid LB medium in an incubator at 37°C overnight. Selection of transformed bacteria was achieved by adding the corresponding antibiotics.

LB medium	LB agar
20 mM $MgSO_4$	20 mM $MgSO_4$
10 mM KCl	10 mM KCl
1% (w/v) Bacto-Tryptone	1% (w/v) Bacto-Tryptone
0.5% (w/v) Bacto yeast extracts	0.5% (w/v) Bacto yeast extracts
0.5% (w/v) NaCl	0.5% (w/v) NaCl
	1.2% (w/v) Bacto-agar

4.1.1 Preparation of competent bacteria

To increase the efficiency of plasmid DNA uptake (transformation), bacteria were treated with solution of di-valent cations. A LB plate was first inoculated with a probe from bacterial stock (XL-1 blue *E. coli* strain) and grown overnight at 37°C. A single colony was used to inoculate 2 ml of LB medium, which was then incubated overnight at 37°C in a loose-capped vessel, with shaking. The following day, the entire overnight culture was used to inoculate 100 ml of LB medium containing 100mM KCl and 200mM $MgCl_2$. Bacteria were grown at 37°C in a 1l flask, with shaking, until absorbance at 600 nm (A_{600}) reached 0.4-0.55 (approximately 2-4 hours). The bacterial pellets were collected by centrifugation on 4000 rpm, for 10 min., at 4 °C. The pellets were resuspended in 15 ml TFB1 buffer and incubated for 5 min. on ice. After another centrifugation (4000 rpm/10 min./4°C) the pellet was resuspended in 2ml of ice-cold TFB2 buffer. Aliquots of 200-300 µl bacteria were snap-frozen in dry ice and removed to storage at –80°C.

TFB1

30 mM Potassium acetate

50 mM $MnCl_2$

100 mM RbCl

10 mM $CaCl_2$

15 % (w/v) glycerin

Adjust to pH 5.8 with 0.2N acetic acid; sterile filter (0.2 μm)

TFB2

10 mM MOPS pH 7.0

75 mM $CaCl_2$

10 mM RbCl

15% (w/v) glycerin

Adjust to pH 6.5 with 1M KOH; sterile filter (0.2 μm)

4.1.2 Transformation of bacteria

For sub-cloning and production of large amounts of cloned DNA, the *Escherichia coli* strain, DH5-α 10B was used. For transformation 1 ng to 0.1 μg of plasmid DNA in a 10 μl volume of ligation mixture was added to 50-100 μl of competent bacteria suspension and incubated on ice for 30 min. The bacterial cells were subjected to heat schock at 42°C for 90 sec., before returning them on ice for another 2 min. 900μl of SOC medium (LB recovery medium) was added to the cells and incubated for 1 hour at 37°C to allow the cells to express resistance genes conferred by the plasmid. For routine transformations, 100 μl of suspension was plated onto LB-agar plates containing ampicillin and grown at 37°C overnight.

4.1.3 Miniprep of plasmid DNA

Plasmide DNA was purified using JETstar kit (GENOMED GmbH, Bad Oeynhausen Germany), according to the manufacturer's protocol. Procedure is based on a modified alkaline/SDS method. High molecular weight chromosomal DNA is denaturated when cells are lysed at pH 12.0-12.6, whereas low molecular weight supercoiled plasmid DNA remains unaffected. Neutralisation of the pH in the presence of high salt concentrations subsequently precipitates chromosomal DNA, which then can be separated from the mixture. A single colony obtained from transformation bacteria was used to inoculate 2 ml of LB medium

containing the appropriate antibiotics (ampicillin), and incubated overnight in 20 ml loose-capped tube with vigorous shaking at 37°C. The following day the bacterial pellet was collected by centrifugation at 14000 rpm for 1 min, and resuspended in 200 µl of solution E1 supplemented with RNAse by vigorous vortexing. Bacterial cells were lysed in alkaline lysis solution E2 (200µl) and cell debrises were precipitated with E3 solution (200 µl) and removed by centrifugation at 14000 rpm for 10 min. at room temperature. Plasmide DNA from the resulting supernatant was precipitated with 500 µl of isopropanol (centrifugation at 15000 rpm for 15 min at 4°C) and resuspended in 50 µl of TE buffer.

Solution E1 (Cell Resuspending)
50 mM Tris-HCl pH 8.0
10 mM EDTA
RNase 20 µg/ml

Solution E2 (Cell Lysis)
200 mM NaOH
1% SDS (w/v)

Solution E3 (Neutralization)
3.1 M potassium acetate pH 5.5

4.1.4 Maxi-prep of plasmid DNA

Large quantities of plasmids were purified using Qiagen Maxi prep protocols based on modified alkaline lysis procedure. Plasmid DNA is recovered by running the bacterial lysate through an anion exchange column under appropriate low-salt and pH conditions. Following washing, the DNA can be eluted by a high-salt buffer. All steps have been done according to the manufacturer's protocol.

Briefly, a single colony was used to inoculate a starter culture of 2-8 ml of selective LB medium and incubated overnight at 37°C. The following day the starter culture was then diluted 1:500 into 100-300 of selective LB medium and grown overnight at 37°C with shaking. Bacterial cells were harvested by centrifugation, and were resuspended in 10 ml of chilled alkaline lysis solution I containing 100 µg/ml Rnase A. 10 ml of alkaline lysis solution II was added to the suspension and mixed gently by inverting. Following the incubation on ice and

centrifugation at 20000 rpm for 30 min. at 4°C, the supernatant was applied to a Qiagen column equilibrated with 10 ml of equilibration buffer and washed twice with 30 ml of wash buffer. The bound plasmid was eluated in 15 ml elution buffer and precipitated by adding 10.5 ml (0.7 volumes) of isopropanol and resuspended in 300 µl of TE buffer.

Equilibration buffer

50mM MOPS pH 7
750 mM NaCl
15% ethanol

Wash buffer

50mM MOPS pH 7
1M NaCl
15% Ethanol

Elution buffer

50 mM Tris-HCl pH 8.5
1.25 M NaCl
15% Ethanol

4.2 Molecular biology techniques

4.2.1 Cloning of plasmid DNA

Restriction digests were carried out according to the manufacturer's instructions. In brief, for the cloning steps 10 µg of plasmid DNA were digested with 20 u of enzyme for 2 h at the given temperature in a final volume of 50 µl. In case of a double digest, the primary digest was precipitated with 2.5 volumes of 100% EtOH and 0.3 M NaCl. The DNA was recovered by centrifugation at 13000 rpm, 10 min, and 4°C. The pellet was washed with 70% EtOH and the DNA was briefly air-dried and resuspended in 1 x TE. After the second restriction digest, the DNA was either subjected to further modification, e.g. dephosphorylation, and then loaded on a LMP agarose gel. Electrophoresis on a LMP gel was performed on 70V (higher voltage

induces melting of a gel). Elution of DNA from LMP gel are described in the chapter "Isolation of DNA for cloning and analysis".

4.2.2 Dephosporylation of 5' ends

Deposphorylation of 5´ ends was performed to prevent religation of linearized vector with compatible cohesive ends. Following digestion, DNA fragments were incubated with 150 u of bacterial alkaline phosphatase (BAP) for 1 hour at 65°C in final volume of 50 µl.

4.2.3 Filling in DNA overhangs

To enable the ligation of non-compatible ends, overhanging (sticky) ends were filled in to generate universally-compatible (blunt) ends. Linearized DNA was treated with Klenow DNA polymerase for 25 min at room temperature, as recommended by the manufacturer's protocol. The reaction was stopped either by phenol treatment or by loading on a LMP agarose gel.

4.2.4 Ligation of DNA ends /or with DNA linkers

DNA fragments were ligated by using T4 DNA ligase (400000 u/ml) and 10 x ligase buffer (supplemented with 10 mM ATP). In all experiments phosphorylated linkers were purchased from Biolabs New England, Schwalbach, Germany. The concentration of the linkers was 0.4 µg/µl, and 1 µg for the vector. Ligations were performed overnight at 16°C.

4.2.5 Hybridisation of complementary oligonucleotides

For the production of double-stranded linker DNA, equimolar amounts of 5' phosphorylated complementary oligonucleotides were mixed, heated to 95°C in a thermo-mixer for 5 min, and then allowed to slowly cool to RT.

4.2.6 Polymerase chain reaction (PCR)

The standard polymerase chain reaction (PCR) was performed essentially as recommended by the manufacturer. For standard (analytical) applications, *Taq* polymerase was used, whereas the proof-reading DNA polymerase, *Pfu*, was used for cloning purposes. The DNA template (20 ng) to be amplified was mixed with 20 µM of forward and reverse primers, 0.5 mM dNTPs (dATP, dGTP, dCTP, dTTP), and 2.5 U *Taq* DNA polymerase in a volume of 50 µl and 1 x polymerase buffer. PCR was performed in a thermocycler. The PCR cycles consisted of an initial denaturation step for 4 min at 95°C, followed by the amplification cycles (20 sec 95°C; 20 sec annealing temperature; 30 sec.-1min. elongation time at 72-73°C; 30-35 cycles) and a final incubation time for 7 min at 72°C. The elongation temperature, and elongation time used was dependent on the DNA polymerase used (*Taq* polymerase 500 bp/min at 72°C; *Pfu* polymerase has an optimal elongation rate of 500 bp/min at 73°C).

4.2.7 Isolation of DNA for cloning and analysis

The DNA PCR products and DNA fragments were isolated (purifed) by using a Qiagen quick protocol. The protocol combines convenience spin-column with selective binding properties of a uniquely-designed silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt concentration, while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluated with Tris buffer or water. The procedure has been done according to the manufacturer's protocol. For the PCR purification buffer PB (guanidine chloride and isopropanol) was used. Salts were washed away by the ethanol containing PE buffer. Elution of DNA provided by buffer EB or water with low concentration of salt and under basic conditions.

Purification of DNA fragments also has been done by phenolisation, followed by ethanol precipitation. For purification of DNA, low melting type of agarose was used. A band of interest was removed form the gel and melted at 68°C in a 1.5 ml reagent tube. An equal volume of phenol (pH 8.0) was then mixed with the melted gel, before centrifugation at 15000 rpm to remove protein contaminations. The resulting supernatant was repeatedly phenolised (2-3 times) before precipitation and resupended in a suitable volume of TE buffer pH 8.0.

TE buffer
10 mM Tris-HCl pH 8.0
1 mM EDTA

4.2.8 Generation of eucaryotic expression vectors for human and murine xCT and murine 4F2hc

1) Eucaryotic expression vector for m4F2 heavy chain (m4F2hc-pREP7)

Vector pREP7 was digested with *KpnI* and *NotI,* dephosporylated with BAP and ligated with the m4F2 fragment isolated from pSPORT-m4F2 by *KpnI* and *NotI.*

2) Eucaryotic expression vector for mxCT (mxCT-pINCO)

Vector pINCO was digested with *EcoRI* and *NotI,* dephosporylated with BAP, and ligated with the mxCT ORF that was isolated from pSPORT-mxCT with the same enzymes.

3) Eucaryotic pINCO-IRES-EGFP expression vector

pIRES2-EGFP was digested with *EcoRI* and *NotI* in order to generate the IRES-EGFP insert that was subsequently inserted in pINCO linearized with the same enzymes (*EcoRI*, *NotI*).

4) Eucaryotic mxCT-IRES-EGFP-pINCO expression vector

pSPORT-mxCT was digested with *HindIII*, blunt-ended with Klenow, thereby generating a *NheI* site. This plasmid was ligated with an *EcoRI* linker in order to generate pSPORT-mxCT-*EcoRI* linker. The coding region of mxCT, obtained by *EcoRI* digestion of pSPORT-mxCT-*EcoRI* linker, was finally cloned in the *EcoRI* site of pINCO-IRES-EGFP.

5) Eucaryotic mxCT-IRES-m4F2hc-pREP7 expression vector

pREP7-m4F2hc was linearized with *NheI*, blunt-ended, and dephosporylated. The IRES sequence was excised from pMC30 by *EcoRI* digestion, blunt-ended, and inserted in the *NheI* digested, blunt-ended site of pREP7-m4F2hc. In parallel, pSPORT-mxCT was digested with

EcoRI, blunt-ended with Klenow, that will lead to an *AseI* site and ligated with a *HindIII* linker in order to generate the plasmid pSPORT-mxCT-*HindIII* linker. The mxCT open reading frame was isolated from pSPORT-mxCT-*HindIII* linker by *HindIII* digestion that was inserted in the *HindIII* site of pREP7-IRES-m4F2hc. The final vector was mxCT-IRES-m4F2hc-pREP7.

6) Eucaryotic expression vector for mxCT (mxCT-pREP7 and mxCT-pINCO)

6.1 mxCT-pREP7

mxCT-pSPORT was grown in dam- bacteria (*E. coli* K12, GM2163, BioLabs, New England, Schwalbach, Germany) digested with *BclI*, filled-in with Klenow, digested with *HindIII*, and finally ligated in pREP7 previously digested with *PvuII* and *HindIII*. Resulting plasmid was mxCT-pREP7. *BclI* digestion was necessary to remove a first non-sense open reading frame in the 5'UTR.

6.2 mxCT-pINCO

A *BclI* linker was inserted in mxCT-pINCO (see also cloning step No. 2) after linearization with *EcoRI*, Klenow-treatment and dephosphorylation. After passaging in a dam- bacterial strain, the plasmid was digested with *BclI*, filled-in with Klenow polymerase, and religated. This step was necessary to remove a putative first non-sense open reading frame in the 5´UTR.

7) Insertion of mouse xCT in the expression vector141pCAG-3SIP

Firstly, mouse xCT-pSPORT clone was digested with *BclI*, blunt-ended, dephosphorylated and then ligated with an *EcoRI* linker. Subsequently, the *HindIII* site was converted to a *NheI* site (digestion with *HindIII*, Klenow-treatment and religation). Finally, murine xCT cDNA was transferred as a *HindIII*/*NheI* fragment in 141 pCAG- 3 SIP. The final plasmid was named mxCT-IRES-Pur-141 pCAG- 3 SIP.

8) Cloning of human xCT into 141pCAG-3SIP (IRES-Puromycine) vector

The synthetic linker MCa/b(MCa: 5' ATT TCA TTC GAA CGG AGA TCT TG 3' and MCb: 5' CTA GCA TGA TCT CCG TTC GAA TG 3'), harbouring *EcoRI*, *BstBI*, *BglII* sites, was cloned in the expression vector 141 pCAG- 3 SIP linearized with *EcoRI* and *NheI*. Human xCT

cDNA was isolated from pBlueScriptSK+ hxCT as a *ClaI*/*BglII* fragment and inserted in 141 pCAG- 3 SIP + MC between the *BstBI* and *BglII* sites. The expression plasmid was, thus, referred as hxCT-IRES-Pur-141 pCAG- 3 SIP.

4.3 Eucaryotic cell culture

4.3.1 Cultivation of Burkitt's lymphoma cells

Cells were cultivated in suspension culture at 37°C and 6% CO_2 under water saturated atmosphere. Optimal growth of HH514 was achieved at a density of 3 x 10^5. Cells were cultured in standard medium (see below) and routineously splitted at a ratio of 1:3 every third day.

Standard cell culture medium
RPMI 1640-medium
10% FCS
2 mM L-glutamine
100 U/ml penicillin
100 mg/ml streptomycin
In some experiments medium was supplemented with 100µM α-thioglycerol and 1 mM sodium pyruvate.

4.3.2 Assessments of cell number

The number of living and dead cells in a given volume was calculated using Fuchs-Rosenthal or Neubauer haemocytometer. 30 µl of cell suspension was mixed with an equal volume of 0.5% (w/v in PBS) trypan blue solution. By this method, dead cells can easily be discriminated from living cells by the blue staining of the cells due to leakage of the plasma membrane. Living cells are unstained.

4.3.3 Cryo-conservation and thawing of eucaryotic cell(s) lines

Stocks of cell lines can be stored for several years in liquid nitrogen. Cells were split 1:1 with fresh medium one day before storage. Cells were collected by centrifugation (1500

rpm, 5 min.), the supernatant removed the cell pellet resuspended in storage medium (approximately $1x10^7$ cells/ml), and transferred as 1 ml aliquots in 1.5 ml cryotubes. Cells were firstly frozen overnight at –80°C before and then transferred in liquid nitrogen.

Storage medium was: 10% RPMI 1640; 10% DMSO and 80% FCS.

Cells were thawed in a waterbath at 37°C, immediately transferred to 10 ml RPMI 1640 medium. Cells were collected by centrifugation at 1500 rpm for 5 min and resuspended in 3 ml of 10% RPMI 1640 medium supplemented with 100 μM α-thioglycerol and 1 mM sodium pyruvate.

4.3.4 Stable transfection of BL cells

For the production of stably expressing cell lines, expression plasmids were introduced into cell lines selected with the appropriate antibiotic for at least two weeks after transfection. To achieve optimal transfection efficiencies, cells were split 1:1 one day prior to transfection. Around $2x10^7$ cells were used per transfection.

Cells were collected by centrifugation (1500 rpm for 5 min) and resuspended at a density of 2 x 10^7 cells in 300 μl of RPMI 1640 medium. Cells were transferred to a 0.4 cm electroporation cuvette on ice, mixed with 20 μg of plasmide DNA, and electroporation was carried out at a voltage of 240 V and capacitance charge of 950 μF at room temperature. In case of integrating plasmids such as 141-pCAG-3SIPmxCT and 141-pCAG-3SIPhxCT, plasmid DNA was linearized prior to transfection. Immediately after electroporation, cells were resuspended in 3 ml of 10% FCS RPMI 1640 containing 100 μM α-thioglycerol and 3 mM pyruvate and cultivated in 6 well plates. Cells were recovered for 24 h. After one day, cells were splitted 1:3 and selection was started by adding the appropriate antibiotic (e.g. puromycine at final concentration 2 μg/ml). Proliferation of the cells was monitored microscopically and stably transfected cell lines were obtained usually within two weeks.

4.3.5 Dilution experiments

To test for the capability of proliferation of untransfected or transfected HH514 cell lines dilution experiments were performed. Cells were seeded in 96 well plates at different cell densities and at a final volume of 200 μl per well. The initial cell concentration was $1x10^5$ cells/ml, and by serial dilution cells were diluted to a density of 97 cells/ml. In all experiments cells were cultured in RPMI 1640 medium, 2 mM glutamine and 5% FCS (PAN, Biotech,

GmbH, Germany). In some experiments medium was additionally supplemented with 100 μM α-thioglycerol and 1 mM sodium pyruvate. Outgrowth of the colonies was monitored by microscopic inspection up to 40 days post-plating. The colour change of the medium from red to yellow was used as an initial indication to monitor cell proliferation.

4.3.6 BSO treatment

In order to investigate the influence of glutathione depletion on the viability of untransfected and transfected HH514 cells, cells were treated with different concentrations of BSO ranging from 0, 5, 10, 20, 30, 50 to 100 μM. Cells were seeded at a concentration of $1x10^5$ cell/ml in final volume of 2-3 ml per well in 6 well plates in triplicates. The number of viable and dead cells was determined by the trypan blue exclusion method over period of 6-8 days. Cells were cultured in RPMI 1640 medium, 2 mM glutamine and 10% FCS (Biochrom KG, Germany).

4.3.7 Co-culture assay

To investigate whether xCT-overexpressing cells could promote the outgrowth of vector-transfected cells under conditions non-permissive for the control cells, co-culture experiments were performed. xCT-overexpressing cells and control cells were seeded at a density $5x10^4$ cells/ml each in six well plates in 2 ml of standard medium supplemented with 10 μM BSO. As a control, cells from either cell line were seeded at densities of $5x10^4$ cells/ml or 10^5 cells/ml. The number of viable cells and total cells was monitored over a period of 8 days.

4.4 Methods for the analysis of DNA, RNA and proteins

4.4.1 Radioactive labelling of DNA fragments

The multi prime method (46, 47) was used for labelling of DNA fragments. Usually, 30-50 ng DNA were labelled using the Redprime ™ II randome prime labeling system (Amersham Biosciences UK, Buckinghamshire, England) and 50 μCi [α-^{32}P]-dCTP (3000 Ci/mmol, 10 mCi/ml), according to the manufacturer's protocol. Non-incorporated nucleotides were separated from the labelled probe by using a Sephadex G-50 column (NICK™ Column,

Amersham Biosciences). The labelling products were measured using a bench-top radioisotope counter (Bioscan, Washington D. C., USA). Before hybridisation in church buffer, the probe was heat-denatured at 95°C, for 5 min.

Church buffer

200 mM NaH_2PO_4

400 mM Na_2HPO_4

7% SDS (w/v)

1 mM EDTA

pH 7.1-7.2

4.4.2 Isolation of total RNA

Total cellular RNA (of approximately 0.5-1 x 10^7 cells) was extracted by using RNeasy Mini and Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's specification. The cells were splitted one day before extraction. Total RNA was dissolved in 50-300 µl DEPC treated water (DEPC was added into water the day before in order to inhibit RNAse activity) and the samples were stored at –80°C.

4.4.3 Northern blot analysis

10 µg of total RNA was lyophilised in a speed vac (Speed Vac Concentrator SVC100H, Savant Instruments NC, Farmingdale; N. Y., USA), dissolved in 15 µl RNA loading buffer and incubated at 70°C for 10 min to minimize secondary structures. RNA was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde in MOPS buffer overnight with a voltage of 40 V. Upon separation, the amount of loading of the RNA samples was estimated by visualisation of the gels using a UV (254 nm) trans-illuminator. RNA was transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech) using 10 x SSC solution. The gel was placed on a glass plate covered with two Whatman filter papers (dipped in reservoirs with 10 x SSC solution) and overlaid with a Hybond-N^+ membrane. The membrane was covered with several layers of blotting paper ("Zellstoff") and RNA was transfered to the membrane by capillary forces. The transfer of the RNA was controlled by UV light. RNA was covalently linked to the membrane by baking the membrane at 80°C for 1 hour. Before hybridisation of the probe, the membrane was pre-hybridised at 65°C for 3 hours in church buffer.

Hybridisation was performed at 68°C overnight in 15-20 ml of fresh church buffer containing around $1x10^7$ dpm of radioactively labelled probe. Membranes were washed twice for 15 minutes at 68°C with 2 x SSC, 1% sodium dodecyl sulfate (SDS) and then twice for 10 minutes at 68°C with 0.1 x SSC, 0.5% SDS. Membranes were then dried, and exposed to an X-ray film (Kodak, BioMAx, U. S. A) usually over night at - 80°C.

RNA loading buffer	1% Formaldehyde-agarose gel
50% (v/v) formamide	2.2 M formaldehyde
2.2 M formaldehyde	1 x MOPS buffer
1 x MOPS buffer	1% (w/v) agarose
50 µg/ml ethidium bromide	
0.2% (w/v) bromphenol blue	

20 x SSC	10 x MOPS
3 M NaCl	0.4 M MOPS (free acid)
300 mM Tris-sodium citrate/NaOH pH 7.0	100 mM Na-acetate
	10 mM EDTA/NaOH pH 8.0
	pH 7.0 (adjusted with 10 N NaOH)

4.4.4 SDS-PAGE Electrophoresis

Denaturing (SDS) polyacrylamide gel electrophoresis (PAGE) was performed essentialy as described previously (109). 10 µl of cell suspension (approximatelly10-30 µg of protein solubilised in SDS-PAGE loading buffer) were denaturated by boiling at 95°C before loading on a SDS-PAGE gel. Pre-stained markers (Bio-Rad Laboratories, Hercules, CA) were used to estimate the molecular weight. The amount of acrylamide in the separating gel depended on the size of the proteins to be analysed usually ranging between 10- 15%. (106). Electrophoresis was performed at constant current of 30 mA/gel in 1 x SDS running buffer. After SDS-PAGE separation, gels were subjected for Western blotting.

Separating gel 15% (60 ml)	12.5 % (60 ml)	Separating gel 12.5 % (60 ml)
30 % Acrylamide 25 ml	30 ml	30 % Acrylamide 25 ml
4 x Tris/SDS pH 8.8 15 ml	15 ml	4 x Tris/SDS pH 8.8 15 ml
Aqua Dest. 20 ml	20 ml	Aqua dest. 20 ml
10% APS 200 µl	200 µl	10% APS 200 µl
TEMED 40 µl	40 µl	TEMED 40 µl

4 x Tris/SDS pH 8.8 (500 ml)	4 x Tris/SDS pH 6.8 (100 ml)
Tris 91 g	Tris 6.05 g
SDS 2 g	SDS 0.4 g
adjust to pH 8.8 with HCl	adjust to pH 6.8 with HCl

10 x SDS-PAGE Running buffer

0.25 M Tris-base

2.5 M glycine

0.5% (v/v) SDS

4.4.5 Protein extraction and immunobloting

After treatment under the respective conditions, cells were washed twice with PBS and lysed in buffer containing 10 mM Tris/HCl pH 7.5, 300 mM NaCl, 1% Triton X-100, 2 mM $MgCl_2$, 5µM EDTA, 1µM pepstatin, 1µM leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined using the bicinchonic acid assay (Pierce, Rockford, IL), and equal amounts of protein (20 µg per lane), were separated by SDS-PAGE. Immunoblotting was performed as described (149). Membranes (Schleicher & Schull, Dassel, Germany) were swollen in CAPS-buffer, and blotting was performed at 1 mA/cm^2 for 1 hour in a transblot SD cell (Bio-Rad, Muenchen, Germany). The membrane was blocked for 1 hour in PBST (PBS, 0.05 % Tween-20) containing 3% non-fat dry milk and incubated with the primary antibody for 1 hour. After washing in PBST, the respective secondary antibodies conjugated to horse radish peroxidase (Promega, Madison, WI) were applied for 1 hour. Finally, the membrane was washed in PBST, and protein bands were detected using the enhanced chemiluminescence system (Amersham Buchler, Braunschweig, Germany).

CAPS buffer

10 mM 3-[cyclohexylamino]propane-1-sulfonic acid, pH 11,10

10% Methanol

4.5 Biochemical methods

4.5.1 Measurement of L-cystine uptake activity

Uptake of cystine was measured by techniques essentially as described by Sagara et al., 1993 (108, 109) and Novogrodsky et al., 1979 (101). About 2 x 10^6 cells were washed in PBS and resuspended in 0.125 ml of pre-warmed PBS containing 0.1% glucose and 0.01% Ca^{2+}, Mg^2. 125 µl of isotope labeled cystine (0.1 µCi/sample) (L- [^{14}C (U)] Cystine (250 mCi/ mmol/ 0.02 mCi/ ml) (Perkin Elmer Life Sciences, Inc., Boston, USA) uptake solution was added to the cell suspension, and suspension was incubated at 37°C. 100 µl aliquots were taken after the given time intervals and layered on a mixture of mineral oil and di-n-butyl phthalate 15:85 (vol/vol; total 200 µl) previously put in a small plastic tube. After centrifugation at 13000 rpm for 10 sec in a microfuge, the tip of the tube containing the cell pellet was cut off, and the cells were solubilized in 200 µl of 0.5 M NaOH in a scintillation vial overnight at 37°C. Afterwards 3 ml of scintillator liquid and 100 µl of Tris-HCL were added to the vials and the radioactivity was determined using a scintillation counter (Beckman Instruments, LIQUID SCINTILATION SYSTEM, LS 5000 TA, Fullerton, CA). To compare basic activity of uptake solution with uptake activity of the cells, 5 µl of isotope labeled uptake solution was taken and mixed with 200 µl of 0.5 M NaOH for determination of specific activity.

Uptake solution (for 10 samples)	1% (w/v) Ca^{2+}, Mg^{2+}
50 µl [^{14}C] Cyss (0.1 µCi/sample)	$MgCl_2$ x 6 H_2O 1 g
5 mM Cyss 15 µl *	$CaCl_2$ x 2 H_2O 1.324 g
5 x PBS 50 µl	dissolved in 100 ml of H_2O
10% (w/v) glucose 2.5 µl	
1% (w/v) Ca^{2+}, Mg^{2+} 2.5 µl	
H_2O 130 µl	

*5 mM Cyss dissolved in 0.05 M HCl and stirring for few hours at room temperature

4.5.2 Determination of intracellular glutathione

Total cellular glutathione (GSH + GSSG) was measured as described by Bannai and Ishii, 1982 (15). This method is based on the glutathione reductase mediated catalytic reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (Fig. 11) by glutathione (132).

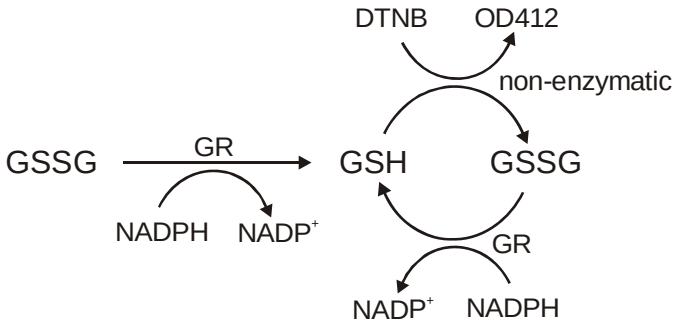

Figure 11. Glutathione reduction of DTNB by the glutathione reductase system

Around 1 x 10^6 cells were collected by centrifugation (1500 rpm/ 10 min), washed with PBS and on ice for 30 min in 1 ml of 5% TCA (trichloracetic acid). This incubation step leads to extraction of total cellular glutathione. Subsequently, proteins and cellular debris was removed by centrifugation (1500 rpm, 5 min) and the supernatant including the glutathione was transferred to a fresh glass tubes. To remove TCA from the samples, ether extraction was performed. Ether saturated with 0.01 M HCl in volume four times bigger than TCA ressolved sample (1.5-2 ml), was added to a sample, vortexing and upper layer was removed by aspiration. The final step was incubation at 37°C for 10 min, to be sure that all ether were removed.

For the glutathione determinations, a mixture of 1.2 ml of 0.2 M K-phosphate buffer, 100 µl DTNB, 700 µl H_2O, and 50 µl of glutathione reductase (from Baker's Yeast (500 U), Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was added to 500 µl of a sample. The reaction was started by adding of 50 µl NADPH (β-NADPH tetrasodium salt, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) and the increase of DTNB reduction was measured at λ_{412} and two different time points (30 sec and 5 min). For the protein measurements, the protein pellet, obtained from the centrifugation step as described above, was dissolved in 500 µl of 0.5 mM NaOH and the proteins extracted overnight. 100 µl of the extracts were used to determine the protein concentration according to the Lowry (89).

Reagents for glutathione determination:

1) 0.2 M K-phosphate buffer (KH_2PO_4 + K_2HPO_4 = 2:5); 10 mM EDTA pH 7.2
2) DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)) 10 mM (dissolved 4 mg in 1 ml of 0.1 M K-phosphate buffer; 5 mM EDTA)
3) Glutathione reductase (GR) dilute 1 U/ 500 µl water final concentration 20 U/ml and for each sample 50 µl should be used
4) NADPH 4 mM in water (0.2 µmol/50 µl), 50 µl per sample

Solutions 2, 3, and 4 have to be freshly prepared.

4.5.3 Determination of total thiol containing compounds

4.5.3.1 Determination of total extracellular thiols

For the determination of total thiols secreted into medium, a method previously described by Bannai and Ishii 1980 (13) was applied/used. The method is based on the chemical reduction of DTNB by thiol containing compounds that can be quantified by the increase of absorbance at 412 nm. About 2 x 10^6 cells were washed in serum free medium, resuspended in 2 ml of serum free medium and incubated for 2 hours at 37°C. Afterwards, cells were harvested by centrifugation (1500 rpm/5 min) and 1.2 ml of the supernatant was mixed with 1.2 ml 0.2 M K-phosphate buffer and 100 µl DTNB (0.4 mg/ml). The increase in absorbance was measured at 412 nm during 2 min and GSH was used as a standard. All solutions were prepared as described in chapter 5.6.2.

4.5.3.2 Determination of acid soluble thiols

The procedure is based on the same protocol (13) used for the determination of secreted thiols (5.6.3.(b)). About 2 x 10^6 cells were cultivated for 24 hours in 20 ml (final concentration of the cells was 1x10^5cellc/ml) standard medium in the presence or absence of 50 µM BSO. 600 µl of the cell-free supernatant were mixed with 200 µl 15% sulfo-salicylic acid and incubated on ice for 30 min in order to precipitate the proteins. Precipitated proteins were removed by centrifugation (10000rpm/ 10 min) and 700 µl of the supernatant were mixed with 400 µl 0.6 M NaOH and 1.2 ml of 0.2 M K-phosphate saline. Finally, 100 µl DTNB (0.4 mg/ml) were added to the mixture and the increase in absorbance was measured at 412 nm during a period of 2 min., GSH was again used as a standard.

4.5.4 HPLC determination of extra- and intracellular cysteine

Determination of cysteine in cell culture supernatants was performed by the HPLC method described by Feussner et al. (49) with slight modifications. In brief, about 2 x 10^6 cells were washed, resuspended in 2 ml of serum free medium, and incubated for 2 hours at 37°C. Cells were collected by centrifugation and 100 µl of the supernatant were mixed with 100 µl 2M borate buffer (containing 5 mM Na_2EDTA, pH 10.05) and 100 µl SDBF (7-fluoro-benzo-2-oxa-1,3-diazole-4-sulfonate) (1mg/ml in borate buffer) and incubated at 60°C for 60 min. The reaction was performed without any reducing agent to measure the free reduced cysteine only. The reaction was stopped by incubation on ice for 5 min. 200 µl of $HClO_4$ containing 0.5M EDTA were added to precipitate proteins. The sample was centrifuged and 50 µl of the supernatant were applied on a HPLC column (Separation column - LiChroCART 250-4 and precolumn -LiChrospher 60 RP-select B [5 µM], MERCK KGgA (Darmstadt, Germany); Beckman detector System Gold, Pump Model 126 and Beckman counter Autosampler System Gold 508). The mobile phase consisted of a 30 mmol/l ammonium-formiate – 40 mmol/ ammonium-nitrate buffer (pH 3.65) and acetonitrile (95 + 5, by vol) at a flow rate of 1 ml/min. The column effluent was monitored by fluorescence detection (Fluorescence Detector RF 10Axl (Shimadzu, Japan)) with an excitation wavelength of 385 nm and an emission wavelength of 515 nm.

For the determination of intracellular cysteine levels, cell pellets were resuspended and sonified by adding 20 µl of tri-n-butylphosphine for 30 min at 4°C, deproteinized using $HCLO_4$ and centrifuged again. 100 µl of the supernatant was mixed with 250 µl of 2M borate buffer and thiol groups were derivatized using SBDF. HPLC was performed as described above.

4.6 FACS™-Analysis

FACS analysis was used to measure the percentage of dead cells in some of experiments. Cells were stained with PI (propidiumiodide) in PBS. About 3-5x10^5 were collected by centrifugation at 1500 rpm for 5 min and resuspended in 200-500 µl of FACS buffer (PBS with 2% FCS) including PI at a final concentration of 2 µg/ml. Data were evaluated in a FACS-Scan, Cell Quest Software (Becton-Dickinson, Heidelberg, Germany).

4.7 Apoptotic assays

4.7.1 Measurement of cell death by Annexin-V-FITC and propidium iodide staining

Cell death was determined by staining cells with annexin-V-FITC and counterstaining with PI. Annexin-V-FITC binds to phosphatydilserine (PS) on the outer side of the plasma membrane. PI is excluded by cells with intact membranes. PI positivity is therefore a sign of necrosis, whereas positive for annexin-V, but negative for PI are generally defined as apoptotic (138). Cells were washed twice with cold PBS and resuspended in buffer (10 mM N-(2-hydroxyethyl)piperazin-N'-3(propansulfonicacid)/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Next, 5 µl of annexin-V-FITC (BD PharMingen, Heidelberg, Germany) and 10 µl PI (20 µg/ml, Sigma-Aldrich) were added. Analyses were performed using a FACScan and CellQuest analysis software (Becton Dickinson, Heidelberg, Germany).

4.7.2 Assessment of genomic DNA fragmentation by flow cytometric measurement of nuclear DNA content

To measure genomic DNA fragmentation, nuclear DNA content was determined by flow cytometry. $2x10^5$ cells were pelleted in a 96 well U-bottom plate and fixed in 200 µl of 2 % (vol/vol) formaldehyde in PBS on ice for 30 min. After fixation, DNA was precipitated with 100% Et-OH for 15 min., pelleted and resuspended in PBS containing RNAseA (40 µg/ml). Following incubation at 37°C for 30 min, the pellet was resuspended in 200 µl of propidium iodide (PI) (50 µg/ml) and incubated overnight in the dark at 4°C. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypoploid DNA. Data were collected and analysed using FACScan flow cytometer and CellQuest analysis software (Becton Dickinson, Heidelberg, Germany). Nuclei displaying a hypodiploid, sub-G1 DNA content were identified as apoptotic. Cell debris, characterized by low forward and side scatter values, was excluded from the analysis.

4.7.3 Detection of active caspases

Active caspases were determined by the FLICA Apoptosis Detection Kits (Alexis GmbH, Gruenberg, Germany) according to the manufacturer's protocol. The methodology is

based on the covalent binding of a fluorochrome-labelled inhibitor of caspases (FLICA) to the active site (43). These inhibitors (VDVAD-AFC for caspase 2; DEVD-AFC for caspase 3; IETD-AFC for caspase 8 and LEHD-AFC for caspase 9) are cell permeable and non-cytotoxic. The number of cells with active caspase was determined by FACScan flow cytometer using CellQuest analysis software (Becton Dickinson, Heidelberg, Germany). FITC-labelled FLICAs were used for staining and positive cells were measured in the FL-1 channel.

4.7.4 Determination of the mitochondrial membrane potential

Cells were collected by centrifugation at 1500 rpm for 5 min, and the mitochondrial membrane potential was determined by staining the cells with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolycarbocyanin iodide (JC-1; Biotium, Inc. Hayward California) as described by von Haefen et al., 2004 (141). $2x10^5$ cells were resuspended in 500 µl PBS containing JC-1 at a final concentration of 2.5 µg/ml, incubated for 30 min at 37°C and moderately shaken. Cells were washed and resuspended in 200 µl PBS. The mitochondrial membrane potential was measured by flow cytometry using a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with CellQuest software. Data are presented as percentage of cells with lowered membrane potential ($\Delta\Psi m$).

4.7.5 Determination of intracellular reactive oxygen species (ROS) levels

Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes) was used to detect the generation of reactive oxygen intermediates. H_2DCFDA is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterase after incorporation into cells. H_2DCF is membrane impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS . $4x10^5$ cells were incubated with 5 µM H_2DCFDA for 30 min at 37°C. Following the incubation time cells were washed and resuspended in 200 µl PBS. The number of cells with the higher levels of intracellular ROS was determined by FACScan flow cytometer using CellQuest analysis software (Becton Dickinson, Heidelberg, Germany) and were measured in the FL-1 channel.

5 RESULTS

5.1 Strategies for the stable overexpression of human and murine xCT chains in the BL cell line HH514

Several cloning strategies, including different eucaryotic expression systems, were established in order to achieve stable overexpression of the human cystine-glutamate-antiporter (x_c^- system) in the BL cell line HH514.

5.1.1 Initial strategy for cloning of murine xCT and 4F2hc in the expression plasmids pINCO and pREP7

Initially, the idea was pursued to express both polypeptide chains, xCT and 4F2 heavy chain (4F2hc), either independently in two different episomal vectors encoding different antibiotic genes, or both chains as a bicistronic message consisting of murine xCT and 4F2hc (or GFP) separated by an internal ribosomal entry site (IRES) in one vector.

a) For independent expression of xCT and 4F2hc, two different episomal vectors, pINCO and pREP7 encoding puromycine and hygromycine resistance genes, respectively, were used, as described in MATERIALS (Chapter 3, figures 9 and 10). Murine xCT was cloned into pINCO expression vector conferring puromycine resistance (pINCO-mxCT) and murine 4F2 heavy chain was cloned into pREP7 expression vector conferring hygromycine resistance (pREP7-4F2hc).

b) An IRES was used for several approaches: (i) expression of both chains in one vector (pREP7-mxCT-IRES-m4F2hc), (ii) to monitor xCT expression by a reporter gene such as EGFP (pINCO-mxCT-IRES-GFP), or (iii) to produce stably xCT expressing cell lines by using the selectable marker gene puromycine phosphotransferase. A schematic diagram of the translation of the bicistronic RNA is presented in figure 12.

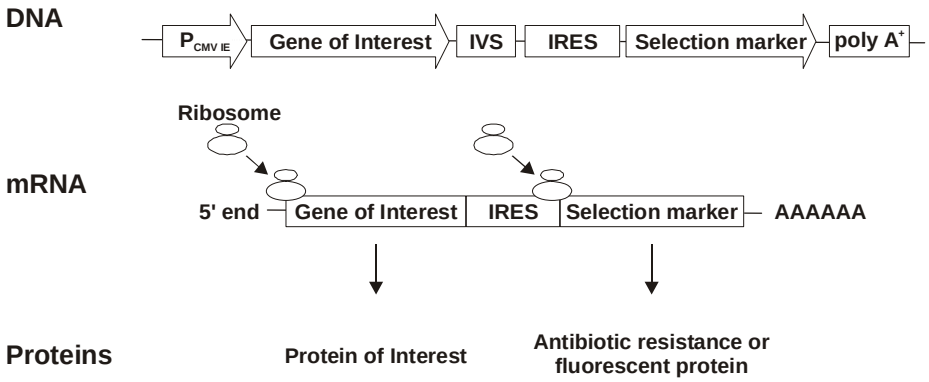

Figure 12. Schematic diagram of the translation of a bicistronic mRNA. The internal ribosome entry site (IRES) permits a protein of interest and an antibiotic resistance gene or fluorescent protein used as selection marker to be translated from the same mRNA. IVS = synthetic intron.

The HH514 BL cell line was transfected with the following plasmids: pREP7-m4F2hc, pINCO-mxCT, pINCO-mxCT-IRES-GFP, pREP7-mxCT-IRES-m4F2hc. Transfected clones were selected by puromycine and/or hygromycine and maintained under selection pressure. However, very soon it became obvious that it was not possible to establish stably expressing cell lines by using the pINCO expression plasmids and its derivatives. Cells transfected with pINCO-mxCT-IRES-EGFP could be selected with puromycine, however, EGFP expression was transient and only high during the first two weeks after transfection. EGFP expression finally disappeared, presumably as a result of silencing of the promoter.

Stably transfected cell lines could be obtained with clone pREP7-mxCT. Four independent clones were selected and maintained under hygromycine selection, but Northern blot analyses showed that murine xCT was only weakly expressed in HH514 cells (data not shown). These experiments prompted us (i) to use a vector with a hybrid CMV-chicken β-actin promoter that is known to show no or little silencing (150), and (ii) to use a bicistronic expression vector that allows selection for expression of xCT.

5.1.2 Final strategy for cloning of murine and human xCT light chain in 141pCAG-3SIP and stable expression of xCT in HH514

Since its discovery as a surface antigen in lymphocytes (59), 4F2 heavy chain was found to be present in all established human tissue culture cell line tested and in most malignant human cell lines (20, 38, 60, 105, 140). The expression of 4F2hc was tested in several different cell lines by northern blot experiments revealing that 4F2hc is ubiquitously expressed in all tested cell lines (Fig. 13). This indicates that the critical determinant of

expressing system x_c^- is the expression of xCT light chain, and that 4F2 expression does not limit cystine-glutamate-antiporter activity.

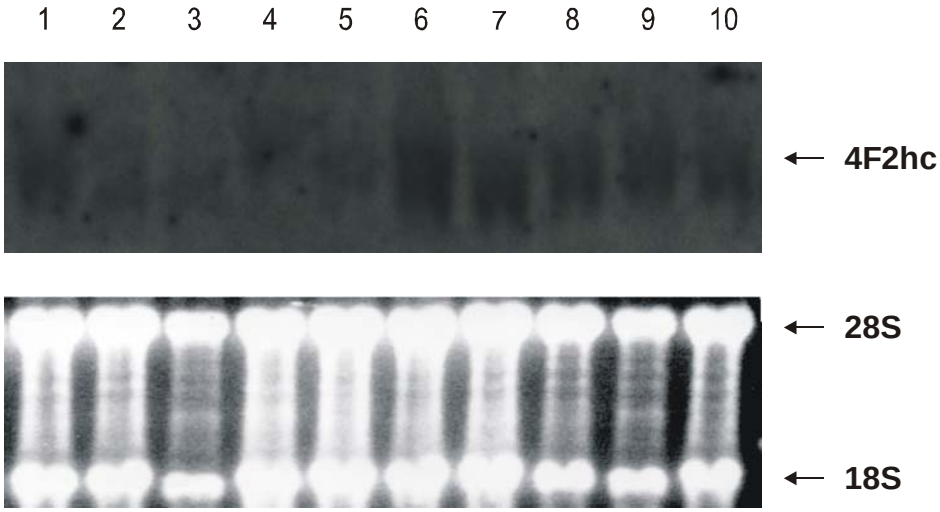

Figure 13. Expression of human 4F2 heavy chain in different B cell lines. Northern blot revealing ubiquitous expression of 4F2hc (marked with an arrow) in different cell lines: 1-A1; 2-Raji; 3-BL-29; 4-LCL 1.24; 5-LCL1.26; 6-HH514; 7-Akata; 8-BL41; 9-BL41-P3HR1; 10-BL41-95.8. Lower panel is the Et-Br control of the agarose gel.

Therefore, further work was focused on expression vectors expressing xCT only. An alternative strategy was designed to establish cell lines stably expressing xCT light chain using the non-episomal eucaryotic expression vector 141pCAG-3SIP. This vector was chosen for two reasons. Firstly, the hybrid CMV-chicken β-actin promoter driving expression of the gene of interest is known to be a strong promoter that is not prone to silencing in mouse ES cells and transgenic mice, and secondly, the puromycine phosphotransferase gene is expressed from an IRES allowing selection of xCT expression by puromycine. As no antibody is available to monitor expression of xCT, this indirect selection strategy turned out to be mandatory.

Human and murine xCT light chain cDNAs were cloned in 141 pCAG-3SIP and transfected in HH514. The following clones were used:

1) hxCT-IRES-Pur-141 pCAG-3SIP
2) mxCT-IRES-Pur-141 pCAG-3SIP
3) 141 pCAG-3SIP was used as an empty vector control

Transfected clones were selected by puromycine at a final concentration of 2 μg/ml and maintained under selection pressure. At least three independent clones per transfection were

selected. Stably transfected cell lines were obtained after two weeks. Northern blot analyses of total RNA isolated from control cells (empty vector transfected cells) and xCT-overexpressing cells were performed to study expression levels of xCT. To study expression of human xCT, a specific probe (1.4 kb) was generated by PCR using the primers hxCT3.1 and hxCT5.1 (see Materials). To detect murine xCT by northern blotting, a probe was isolated from pSPORT-mxCT by *XhoI* and *BamHI* digestion. Strong hybridisation signals were obtained corresponding to bicistronic human xCT-IRES-puromycine (Fig. 14a and 14b) and bicistronic murine xCT-IRES-puromycine (Fig. 14c) mRNAs of 2.7 kb each. As expected, no signals were observed in control cells. After long exposure, a weak signal corresponding to the endogenous xCT mRNA of 12 kb became visible (Fig. 14b).

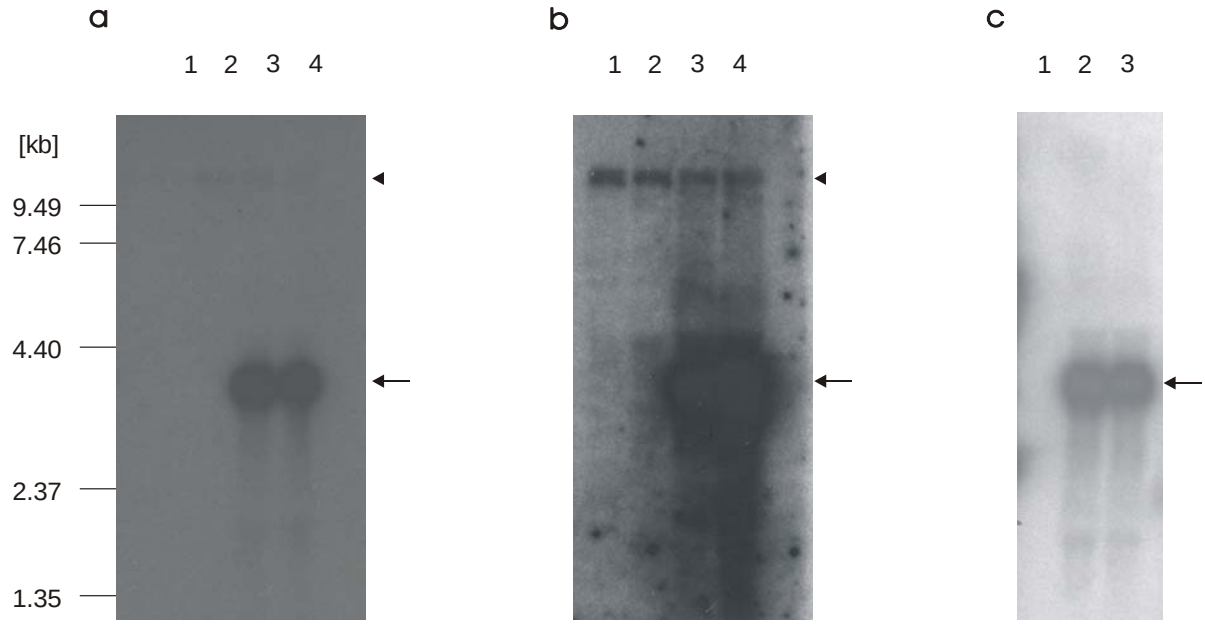

Figure 14. Overexpression of human and murine xCT in HH514. Northern blot revealing hxCT (a and b) and mxCT expression (c) in BL cells. Two representative cell clones transfected with human (lanes 3 and 4 in a and b) and murine xCT (lanes 2 and 3 in c) show high overexpression of xCT (marked with an arrow) in contrast to cells transfected with the empty vector (lanes 1 and 2 in a and b, lane 1 in c). Panel (b) is a long exposure of (a) to visualize the low level of endogenous xCT expression (arrowhead).

5.2 Phenotype of xCT overexpression in HH514

5.2.1 Cystine uptake activity in cells overexpressing xCT

L-cystine uptake activity was measured in control and xCT transfected cells to confirm that overexpression of xCT leads to enhanced expression of a functional protein and thus, to

increased uptake activity. Figure 15 shows a time course of L-cystine uptake (0.1 μCi/sample) in non-transfected cells, empty vector transfected cells, and hxCT-overexpressing cells for the indicated time intervals (1, 2, and 3 minutes). L-cystine uptake was linearly increasing in overexpressing cells during the period of 3 minutes and remained unchanged in control cells (Fig. 15).

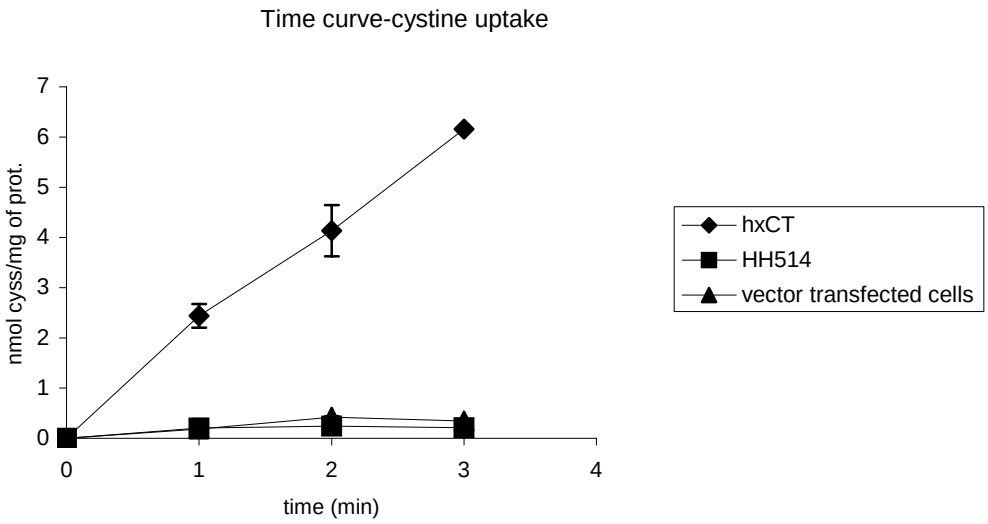

Figure 15. Overexpression of xCT promotes specific L-cystine uptake. Uptake of radioactively labelled L-cystine was measured in non-transfected cells (■), empty vector transfected cells (▲), and hxCT-overexpressing cells (◆) for the indicated time intervals. The data represent mean values of two independent experiments with duplicate measurements +/- standard deviation and are presented as nmol L-cystine/mg of protein.

L-cystine uptake in xCT-overexpressing cells was dramatically increased as compared to control cells (Fig. 15 and Table 2).

Table 2 **Time course of L-cystine uptake in xCT-overexpressing and control cells**

Sample	1 minute	2 minutes	3 minutes
Non-transfected cells	0.203 ± 0.025	0.203 ± 0.025	0.213 ± 0.057
Empty vector transfected cells	0.183 ± 0.047	0.423 ± 0.075	0.347 ± 0.015
xCT transfected cells	2.44 ± 0.08	4.14 ± 0.23	6.16 ± 0.509

Values are given as nmol L-cystine/mg of protein ± S.D.

To verify that L-cystine has in fact been taken up by the cystine-glutamate antiporter system, uptake of L-cystine was measured in the presence of the substrate L-glutamate that efficiently blocks L-cystine uptake when present in high concentrations. As shown in figure 16, transport of L-cystine in control and xCT-overexpressing cells was significantly inhibited by 2.5 mM L-glutamate.

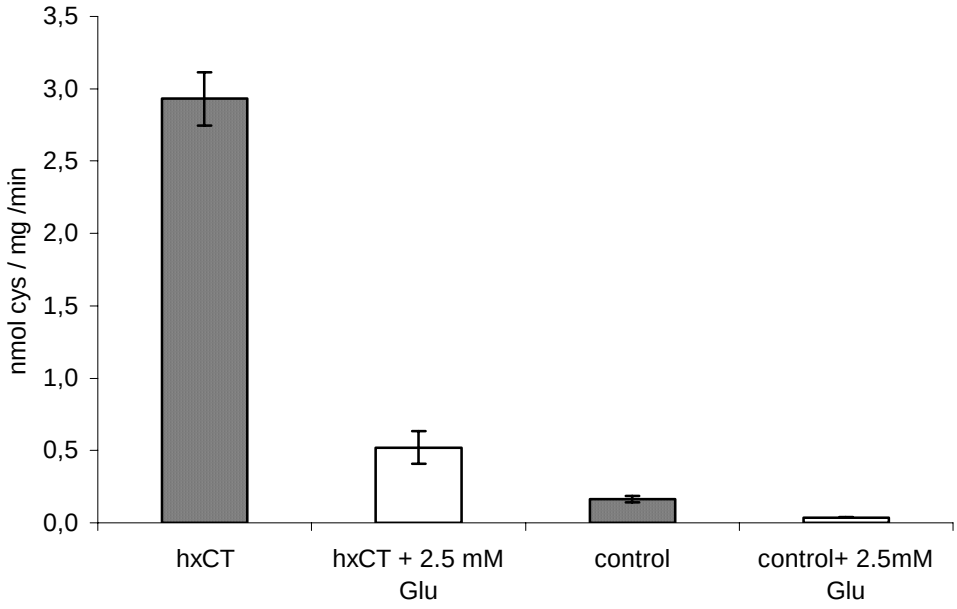

Figure 16. Overexpression of xCT promotes specific cystine uptake. To evaluate the specificity of cystine uptake through the cystine-glutamate antiporter, L-cystine uptake was inhibited by glutamate, a specific inhibitor of cystine uptake by xCT. Measurements were performed in the absence (filled bars) or presence (empty bars) of 2.5 mM glutamate (Glu) within 1 minute of incubation. A fivefold reduction of specific L-cystine transporter activity could be observed in xCT-overexpressing cells in the presence of 2.5 mM glutamate. A clear decrease of L-cystine uptake was also noticed in empty vector-transfected cells. Data are presented as mean value of two independent experiments with duplicate measurements +/- standard deviation, presented as nmol L-cystine/ mg of protein/ min.

In the absence of excess L-glutamate, the activities of L-cystine uptake in xCT-overexpressing and vector-transfected cells was 2.93 ± 0.18 and 0.163 ± 0.021 nmol L-cystine/min/mg protein, respectively. In the presence of 2.5 mM L-glutamate, uptake of L-cystine was decreased to 0.52 ± 0.11 in xCT-overexpressing cells and to 0.0373 ± 0.002 nmol L-cystine/min/mg protein in vector-transfected cells corresponding to an inhibition of 83% and 77%, respectively.

5.2.2 Determination and evaluation of the proliferation behaviour of HH514 cells

From previous work in our laboratory (45) it has been known that BL cells are highly sensitive to suboptimal growth conditions. BL cells readily undergo apoptosis when seeded e.g. at reduced serum concentration or low cell density (non-permissive conditions). Supplementation of the growth medium with thiol containing compounds such as α-thioglycerol or dithiothreithol (DTT) in the presence of oxygen scavengers like sodium pyruvate (26) and the metal chelator bathocuproine sulfonate (BCS), efficiently prevented apoptosis under non-permissive conditions. In addition to the cell density, the batch of serum plays also an important role in defining the growth conditions of the cells. Antioxidants, free thiol groups, and/or trace elements (selenium, copper) in the serum are deemed to be the most critical factors affecting the survival and proliferation of the cells. The experiments in which the permissive and nonpermissive conditions for proliferation and survival of BL cells had been initially defined, had been performed in our lab about 10 years ago. Unfortunately, the batches of serum that had been used to define nonpermissive and permissive conditions in the papers of Falk et al. (1993, 1998) (44, 45) and Brielmeier et al. (1998, 2001) (25, 26) were no longer available. It was thus necessary to test a number of sera and to identify a serum which recapitulates the features that had been described by Falk et al. and Brielmeier et al. before. Nine sera from different suppliers were tested at different concentrations of serum (1, 2, 4, 6, 8 and 10%) for their supportive effect on survival and proliferation of HH514 cells. Additionally, all experiments were performed in the presence or absence of 100 µM α-TG and pyruvate. Cells were seeded in a 96 well plates at serial dilutions ranging from 100,000 cells per ml to 100 cells per ml in total volume of 200 µl per well. Cell survival was monitored by the medium colour change (acidification due to production of lactate) from red to yellow, three weeks after plating. All sera tested supported survival and proliferation more efficiently than the previously used batch. For the experiments, serum “PAN 7” was chosen as it did not maintain cell growth of HH514 cells at a cell density of less than 6000-3000 cells/ml at a concentration of 10%. As shown in figure 17, cells could easily survive and proliferate at low cell density when cultured in medium containing 100 µM α- thioglycerol plus pyruvate and BCS (Fig. 17a; e.g. 100 cells/ml), whereas they were not capable of proliferating and died at a cell concentration of about 6000 cells/ml in the absence of supplements (Fig. 17b).

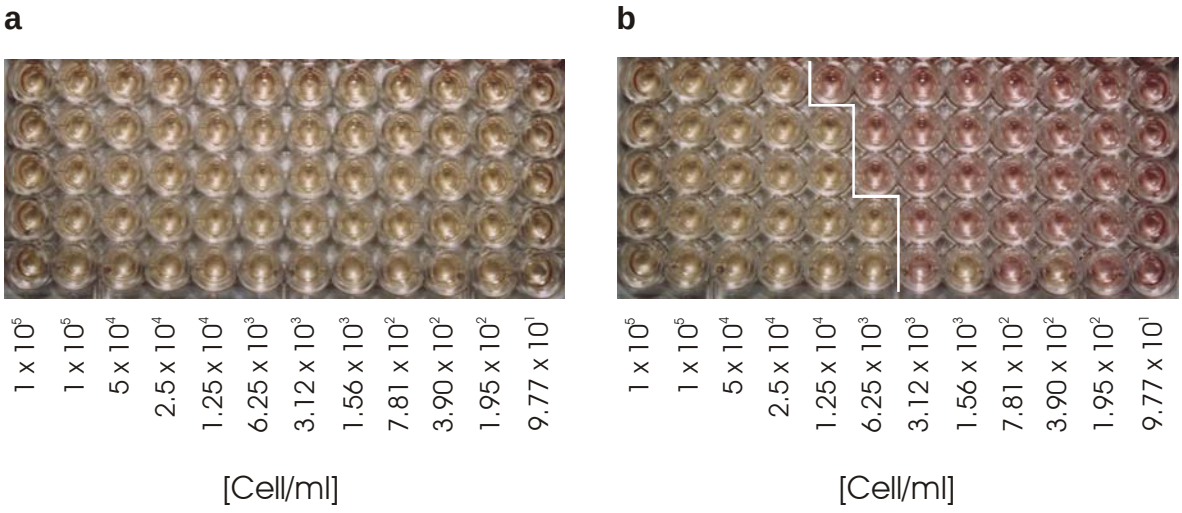

Figure 17. Supplementation of growth medium with α-thioglycerol allows BL cells to grow at low cell density. Three weeks after seeding, cell proliferation and survival were monitored by the medium colour change from red to yellow. Red medium colour indicates no proliferation (no production of lactate and pyruvate) whereas yellow colour indicates proliferation of HH514 cells during the given time period. In growth medium supplemented with α-thioglycerol, pyruvate, and BCS, cells were able to proliferate even at low cell density (100 cells/ml), (a) whereas medium without supplements was unable to support cell proliferation at low cell density. In this experiment the critical cell concentration was 6000 cells/ml (b).

5.2.3 Growth conditions of xCT-transfected HH514 cell clones

In order to examine whether overexpression of human and murine xCT allows BL cells to grow at low cell density, cells were plated in standard RPMI medium containing 10 % FCS (PAN 7) without supplements in 96 well plates at serial dilutions ranging from 100,000 cells per ml to 100 cells per ml.. Three weeks after plating, survival could easily be monitored by the medium colour change from red to yellow, as depicted in figure 18. The critical cell density for survival and proliferation of empty vector-transfected cells and non-transfected cells (the parental HH514 cell line) was between 100,000 and 50,000 cells per ml (Fig. 18a and 18b). Overexpression of human and murine xCT in BL cells supported cell growth at densities of about 6000 cells per ml (Fig. 18c and 18d).

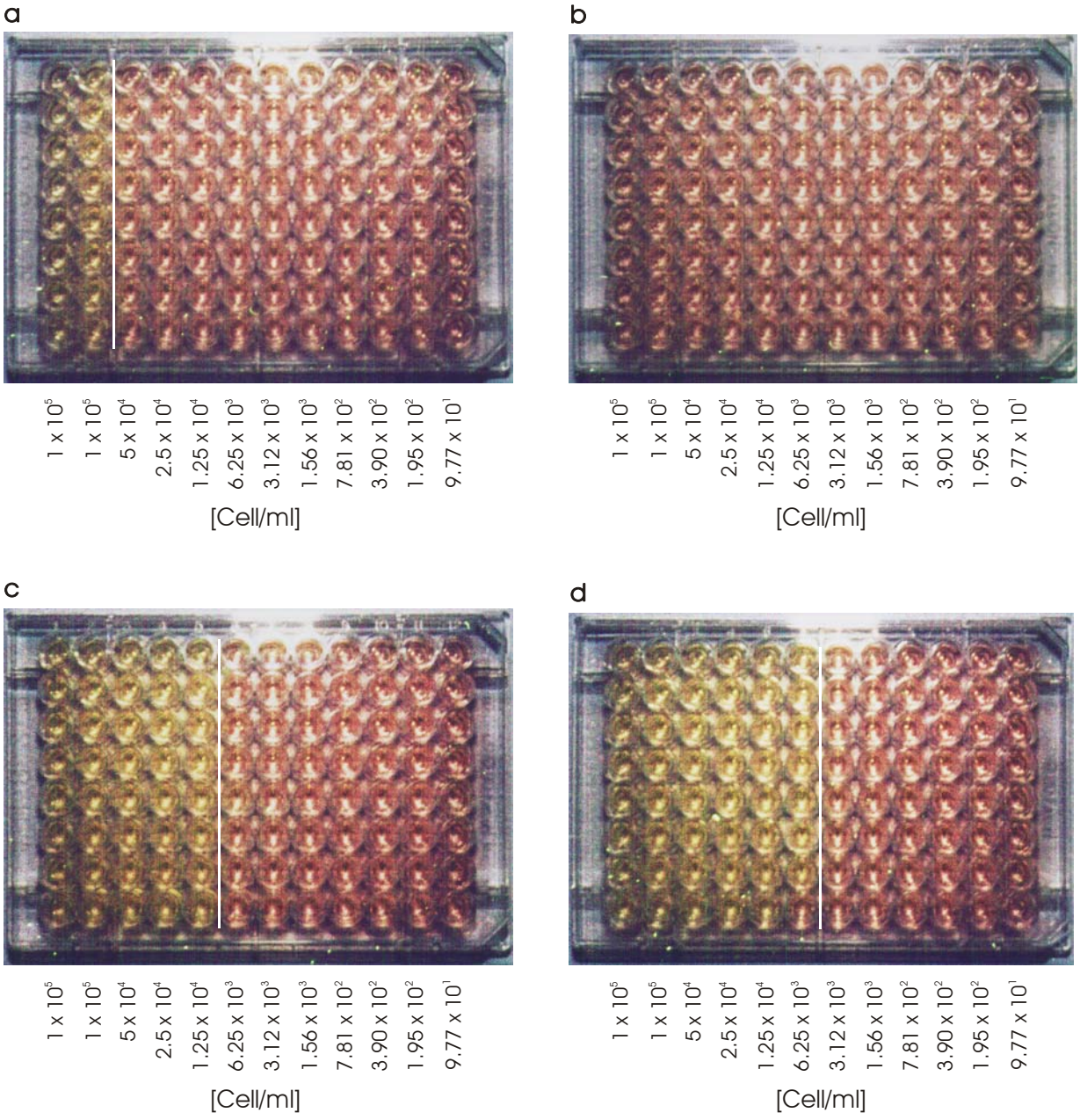

Figure 18. Overexpression of human and murine xCT allows BL cells to grow at low cell densities. Cell proliferation and survival could easily be monitored by the medium colour change from red to yellow three weeks after seeding. Empty vector-transfected cells (a) and non-transfected cells (b) were not able to survive and proliferate at a high cell density (100,000 cells/ml), whereas cells overexpressing human xCT (c) and murine xCT (d) were able to proliferate at a density of 6000 cells/ml.

5.3 Oxidative stress-mediated cell death induced by glutathione depletion

To investigate whether xCT overexpression and, as the consequence, higher uptake of the cystine allows BL cells to survive and proliferate under limiting GSH conditions, xCT-overexpressing cells and control cells were treated with buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamyl-cysteinyl-synthetase, the enzyme that catalyses the first and rate-limiting step in GSH synthesis (54). BSO thus causes rapid depletion of intracellular GSH.

5.3.1 BSO is not a substrate for the cystine-glutamate antiporter

To investigate the possibility that BSO, due to its structural similarity with glutamate, might be a potential substrate for the cystine-glutamate antiporter, we had to rule out the possibility that the growth promoting effect of xCT overexpression is due to the export of BSO rather than due to the increased L-cystine uptake. L-cystine uptake activity was measured in xCT-overexpressing cells and control cells in the absence and presence of 5 mM BSO. No influence of BSO on the L-cystine uptake activity was observed in both cell lines (Fig. 19).

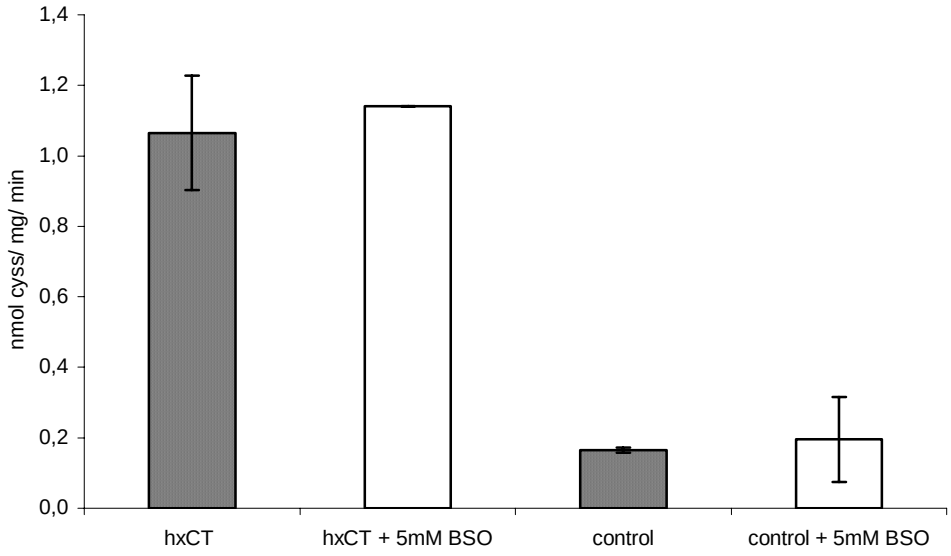

Figure 19. BSO is not a substrate for xCT. L-cystine uptake activity was measured in the absence (filled bars) and presence (empty bars) of BSO. No difference in xCT activity was observed in xCT-overexpressing and vector-transfected cells at a BSO concentration of 5 mM.

5.3.2 Survival and proliferation of glutathione-depleted cells

5.3.2.1 BSO inhibits survival and proliferation of non-transfected cells, but not of xCT-overexpressing cells

To investigate whether xCT overexpression allows BL cells to proliferate under limiting GSH conditions, xCT-overexpressing cells and vector-transfected control cells were treated with different concentrations of BSO (0, 5, 10, 20, 30, 50 and 100 µM) over a period of 8 days. Cell viability was monitored by Trypan Blue exclusion. Overexpression of xCT-light chain strongly supported cell growth even in the presence of 100 µM BSO (Fig. 20a), whereas control cells died at 5 µM BSO (Fig. 20b) during the first 48 hours of treatment. These data show that increased uptake of L-cystine renders cells more resistant to GSH depletion.

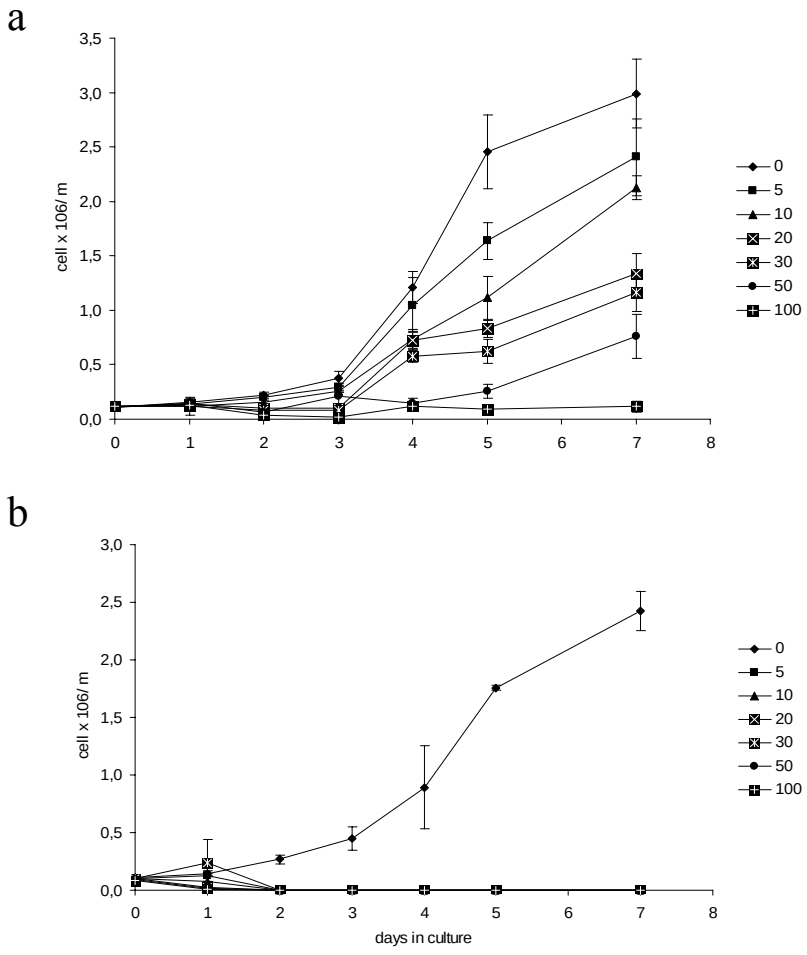

Figure 20. Impact of different concentrations of BSO on viability and proliferation of xCT-overexpressing BL cells and vector-transfected control cells. In xCT-overexpressing and vector-transfected control cells, cell numbers were determined by trypan blue exclusion over a period of 8 days in the presence of different concentrations of buthionine sulfoximine (BSO), a specific inhibitor of glutathione synthesis. Dose dependent impairment of proliferation of xCT-overexpressing BL cells in the presence of 5 to 100 µM BSO (a). Survival of xCT-overexpressing cells was supported up to a concentration of 100 µM BSO, whereas vector-transfected cells died at concentrations of 5 µM and above (b).

5.3.2.2 The glutathione level is not critical for cell survival and proliferation of xCT-overexpressing cells

The availability of cysteine is the rate-limiting step for the synthesis of glutathione (GSH), the major cellular antioxidant and scavenger of hydroxyl radicals, peroxides and lipid peroxides inside the cell (19). Having shown that xCT overexpression supports cell growth of HH514 cells at 100 μM BSO, it was important to answer the question whether xCT-overexpressing cells will show higher levels of intracellular glutathione than control cells under BSO treatment.

To this end, total GSH levels were determined in xCT-overexpressing and control cells after 24 hours in the absence or presence of different BSO concentrations (0, 5, 10, 20, 30 50, and 100 μM) (Fig. 21). As presented in figure 21, no marked difference in GSH levels between xCT-overexpressing cells and control cells could be detected despite the fact that 5 μM BSO induced apoptosis in vector-transfected control cells, but not in xCT-overexpressing cells. The absolute values are presented in Table 3.

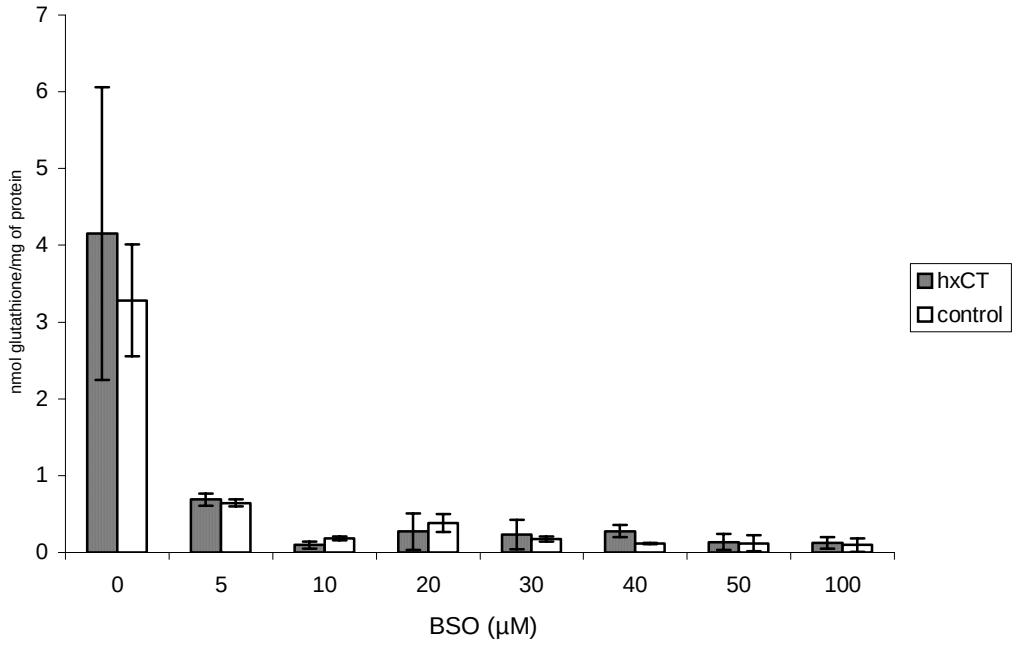

Figure 21. Glutathione levels are strongly decreased in hxCT-overexpressing cells and vector-transfected control cells in the presence of BSO. Total cellular glutathione levels were measured in hxCT-overexpressing (filled bars) and vector-transfected cells (empty bars) after cultivation of cells for 24 hours in the absence of BSO and in the presence of different BSO concentrations. Glutathione levels were strongly decreased in both cell lines treated with different BSO concentrations. These data rule out the possibility that a treshold level of GSH is decisive for cell survival. The data represent mean values of three independent experiments with duplicate measurements ±SD and are shown as nmol of total glutathione/ mg of protein.

Table 3 Glutathione levels in xCT-overexpressing and control cells in the absence or presence of different concentrations of BSO

Sample	xCT-overexpressing cells	control cells
BSO (μM)		
0	4.15 ± 1.90	3.28 ± 0.72
5	0.69 ± 0.07	0.643 ± 0.046
10	0.0975 ± 0.0451	0.183 ± 0.028
20	0.273 ± 0.238	0.383 ± 0.118
30	0.234 ± 0.188	0.178 ± 0.033
40	0.277 ± 0.081	0.17 ± 0.011
50	0.136 ± 0.103	0.120 ± 0.104
100	0.123 ± 0.074	0.0967 ± 0.085

Values are given as nmol of total glutathione/mg of protein ± S.D.

This finding was confirmed by HPLC analysis revealing that the GSH peak has disappeared in control as well as xCT-transfected cells after BSO treatment (Fig. 22).

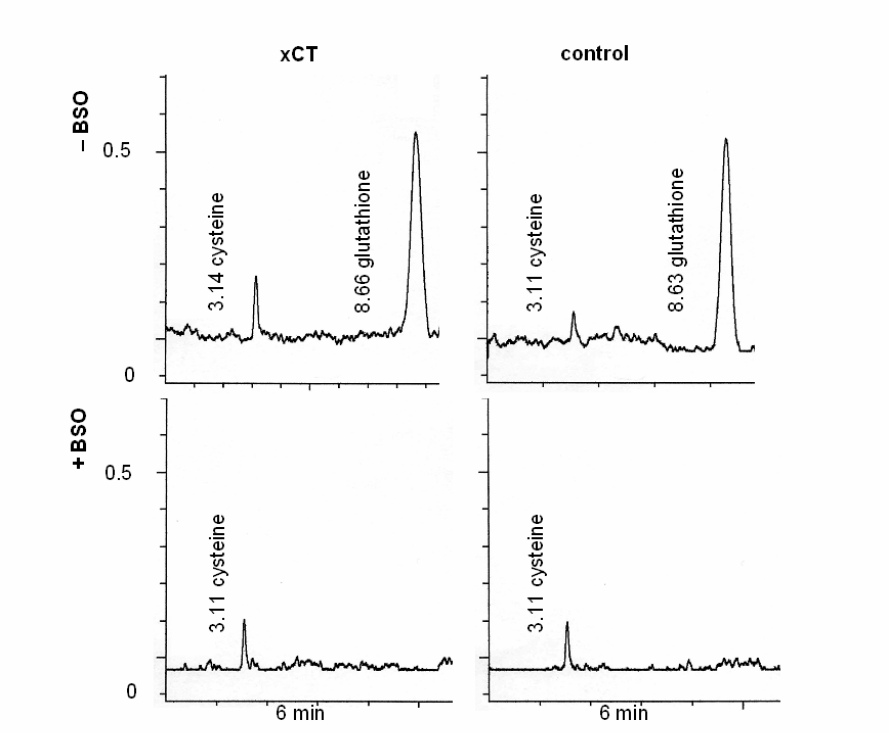

Figure 22. HPLC chromatogram for intracellular cysteine and glutathione. HPLC chromatogram of xCT-overexpressing cells **(**xCT) and vector-transfected cells (control) in the absence (- BSO) and presence of 10 μM BSO (+ BSO). In xCT-overexpressing **(**xCT) and vector-transfected cells (control), the intracellular glutathione level is similarly high in untreated (upper part) and similarly low in BSO-treated cells (lower part).

These data strongly suggested that, to sustain cell survival, xCT overexpression does not increase the GSH levels above a critical threshold. Therefore, another mechanism than an increase in GSH level must be responsible for the supportive effect on cell survival and proliferation at low GSH concentration.

5.3.3 xCT overexpression induces secretion of cysteine

Since intracellular GSH levels in xCT-overexpressing and vector-transfected control cells were unchanged after BSO treatment, intra- and extracellular total mercaptan levels were determined in untreated cells and cells treated with BSO.

5.3.3.1 xCT overexpression increases the intracellular cysteine levels

Intracellular cysteine levels were determined by HPLC in untreated cells and cells treated with 30 µM BSO for 24 and 48 hours. As shown in figure 23a, in the absence of BSO xCT-overexpressing cells exhibited significantly higher levels of intracellular cysteine (4.96 ± 0.65 nmol/mg) than control cells (0.69 ± 0.97nmol/mg). In the presence of 30 µM BSO, intracellular cysteine levels in xCT-overexpressing cells were not changed after 24 hours and were even higher (7.92 ± 0.42) after 48 hours. Intracellular cysteine levels in the control cells remained unchanged.

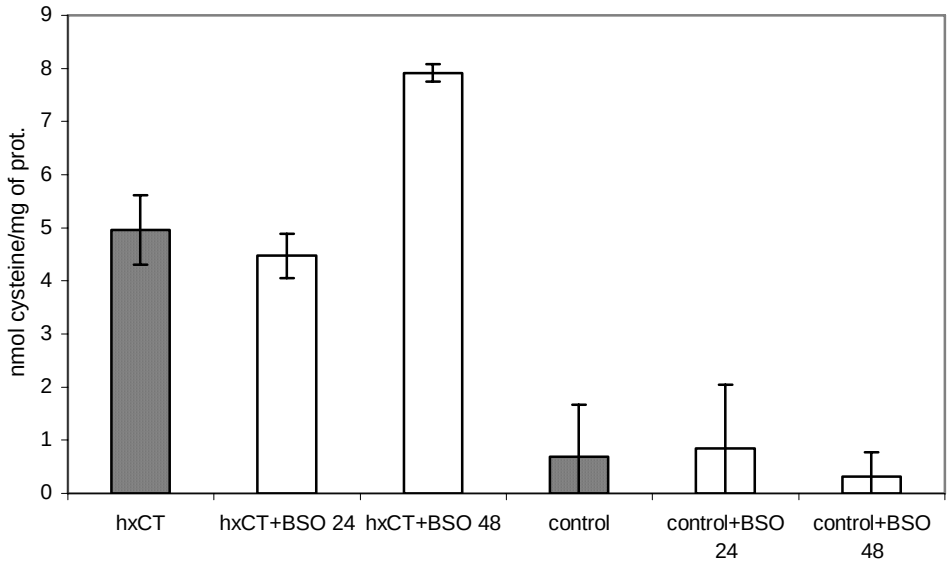

Figure 23a. Increased L-cystine uptake results in higher intracellular cysteien levels. Intracellular cysteine levels were determined in xCT-overexpressing cells and vector-transfected control cells that were either treated for 24 and 48 hours hours with 30 µM BSO (empty bars) or were left untreated (filled bars). xCT-overexpressing

cells have significantly higher levels of total mercaptans than vector-transfected control cells. The data represent mean values of two independent experiments with duplicate measurements ± SD.

5.3.3.2 Extracellular levels of secreted mercaptans are strongly increased by xCT overexpression

A dramatic difference between xCT-overexpressing and vector-transfected controls cells was found when extracellular total mercaptan levels were compared (Fig. 23b).

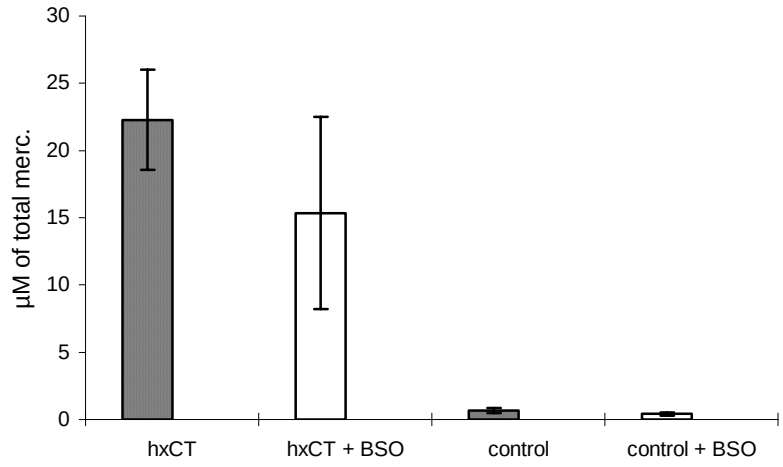

Figure 23b. High levels of total extracellular mercaptans are secreted from xCT-overexpressing cells. hxCT-overexpressing and vector-transfected control cells were treated with 50 µM BSO for 24 hours (empty bars) or left untreated (filled bars) and were plated in serum-free medium at a density of 2 x 10^6/ml. Even after BSO treatment high amounts of extracellular mercaptans are secreted from xCT-overexpressing cells but not from vector-transfected control cells. The data represent mean values of three independent experiments with duplicate measurements ± SD.

xCT-overexpressing cells secreted high amounts of total mercaptans (22.3 ± 3.73 µM), whereas the levels secreted by vector-transfected control cells were by more than an order of magnitude lower (0.661 ± 0.187 µM). In xCT-overexpressing cells, the extracellular total mercaptan levels were slightly reduced after BSO treatment, but the huge difference between xCT-overexpressing cells (15.4 ± 7.15 µM) and vector-transfected control cells (0.407 ± 0.135 µM) was still apparent.

To rule out the possibility that the strong increase in total mercaptans secreted into the medium is the consequence of increased cell death, the percentage of dead cells was determined in xCT-overexpressing and vector-transfected control cells under the same experimental conditions that had been used for the determination of extracellular mercaptans. The percentage of dead cells was determined by FACS analysis (PI staining) or by trypan blue exclusion. No difference in the number of dead cells could be observed in hxCT-overexpressing untreated and BSO treated as well as in non-treated vector-transfected control

cells (data not shown). As expected, the number of dead cells was high in control cells treated with BSO. The increase in the percentage of dead cells did not lead to an increase in the levels of total secreted mercaptans.

5.3.3.3 The majority of small thiol containing compounds in the medium is bound to proteins

Small thiol compounds such as glutathione, cysteine and homocysteine are present in plasma in two different forms: (i) to a major extent as small molecules in form of free thiols or mixed disulfides present in the acid-soluble fraction, and (ii) to a lesser extent as protein-bound fraction. We next asked whether the majority of the thiols is present in the acid-soluble or protein-bound fraction.

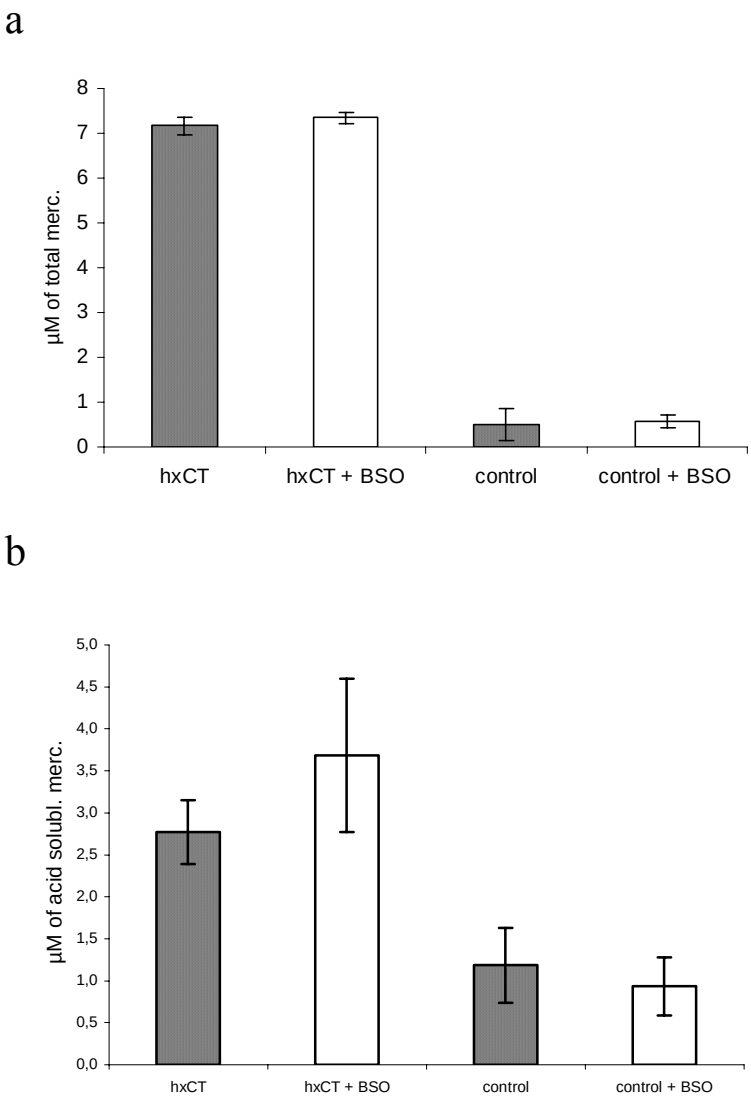

Figure 24. Extracellular thiols are mainly bound to serum proteins. Determination of thiol levels from the supernatant of xCT-overexpressing cells and control cells in medium containing 10% FCS without (a) and with

acid precipitation (b) revealed that elevated levels of mercaptans are secreted by xCT-overexpressing cells (a), and that the majority of these mercaptans is bound to serum proteins (b).

To address this question, the level of total mercaptans determined without and with acidic precipitation of proteins were compared. This experiment showed that small mercaptans are mainly bound to serum proteins or proteins secreted into the medium by the cells, mostly to albumin (95). Without acidic precipitation, high levels of secreted mercaptans were observed in the culture medium of xCT-overexpressing cells (Fig. 23b and 23a). With acid precipitation (Fig. 23b) the difference in total secreted mercaptans was much lower but still significant. This indicates that the majority of small thiols is bound to free SH-groups of proteins present in the serum.

5.3.3.4 The secreted extracellular mercaptan is predominantly cysteine

HPLC analysis was performed to determine which type of mercaptan(s) is responsible for the dramatic difference in total secreted mercaptans of xCT-overexpressing cells versus empty vector-transfected control cells. As shown in figure 25, large part of the mercaptans secreted by xCT-overexpressing cells was cysteine regardless whether the cells had been treated with 50 µM BSO for 24 hours or had been left untreated. In contrast, in the supernatant of vector-transfected control cells L-cysteine was hardly detectable by HPLC analysis, regardless whether the cells had been treated with BSO or had been left untreated.

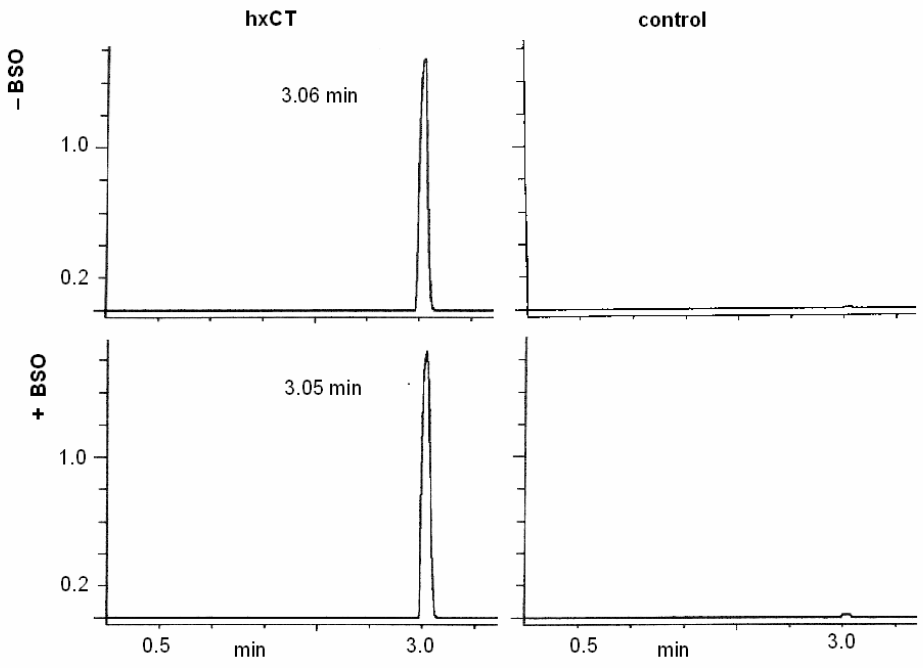

Figure 25 xCT-overexpressing cells secrete cysteine. HPLC chromatogram of the medium of xCT-overexpressing cells (xCT) and vector-transfected cells (control) in the absence (- BSO) and presence of 50 µM

BSO (+BSO). hxCT-overexpressing cells, treated with BSO or left untreated, secrete cysteine. No extracellular mercaptan was found in the supernatant of vector-transfected control cells.

Under the HPLC conditions used, cysteine was the only secreted mercaptan. The concentration of L-cysteine was about 30 µM in the medium of xCT-overexpressing cells with or without BSO treatment as determined by HPLC analysis. This value corresponds very well to the concentration of total extracellular mercaptans determined by the DTNB method in serum-free medium (see Fig. 23b).

5.3.3.5 In a coculture, xCT-overexpressing cells support the growth of control cells at conditions non-permissive for control cells

To test whether xCT-overexpressing cells may provide a "feeder-like" effect on control cells by supplying them with cysteine, coculture experiments were performed (Fig. 26).

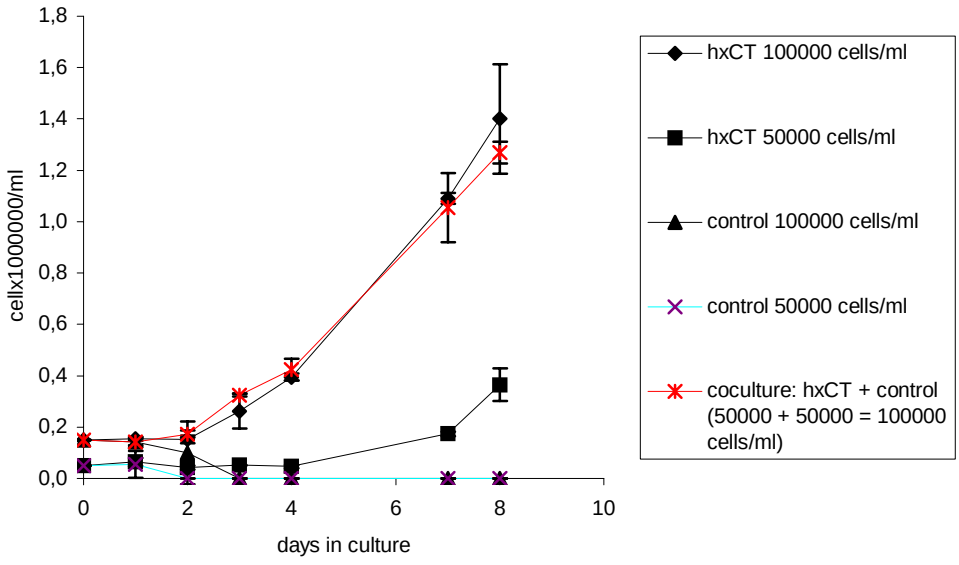

Figure 27. Coculture experiment: xCT-overexpressing cells support the growth of vector-transfected control cells at conditions non-permissive for control cells. xCT-overexpressing cells and vector-transfected control cells were seeded in a coculture, at a density of $5x10^4$ cells/ml each in medium containing 10 µM BSO (✳). As controls, xCT-overexpressing cells and vector-transfected control cells were seeded separately at $5x10^4$ (♦ hxCT; ▲ control) and 1x 10^5 cells/ml (■ hxCT; × control).

xCT-overexpressing cells and vector-transfected cells (control cells) were seeded, together in medium containing 10 µM BSO at a density of $5x10^4$ cells/ml each. This cell density and BSO concentration are non-permissive for vector-transfected control cells. xCT-overexpressing and control cells, seeded, distinctly, at densities of $5x10^4$ cells/ml or 10^5 cells/ml served as controls. The number of viable and total cells was monitored over a period

of 8 days. xCT-overexpressing cells were capable of proliferating at either cell concentration, whereas control cells alone died under these conditions. When both cell types were cocultivated, however, no cell death was observed and the cell number increased with the same kinetics as in cultures containing xCT-overexpressing cells only. This strongly argues that the x_c^- system-mediated supply of cysteine from xCT-overexpressing cells can rescue control cells from cell death induced by 10 µM BSO.

5.3.4 Mode of the cell death induced by BSO

The next question was whether treatment of cells with BSO provokes apoptosis or necrosis. xCT-overexpressing and vector-transfected control cells were treated with 10 µM BSO over a period of 2 days and analysed for intracellular ROS levels, annexin-V and propidium iodide (PI) staining, genomic DNA fragmentation, caspase-2, 3, 8 and 9 activation, and for the change of the mitochondrial membrane potential. hxCT-overexpressing and vector-transfected cells that had been treated with 10 µM BSO in medium supplemented with 100 µM α-TG and 3 mM sodium pyruvate served as additional controls.

5.3.4.1 BSO-induced formation of ROS is decreased in xCT-overexpressing cells as compared to control cells

To address the question whether increased levels of intracellular ROS are responsible for cell death induced by BSO, cells were stained with the fluorescent dye H_2DCFDA.

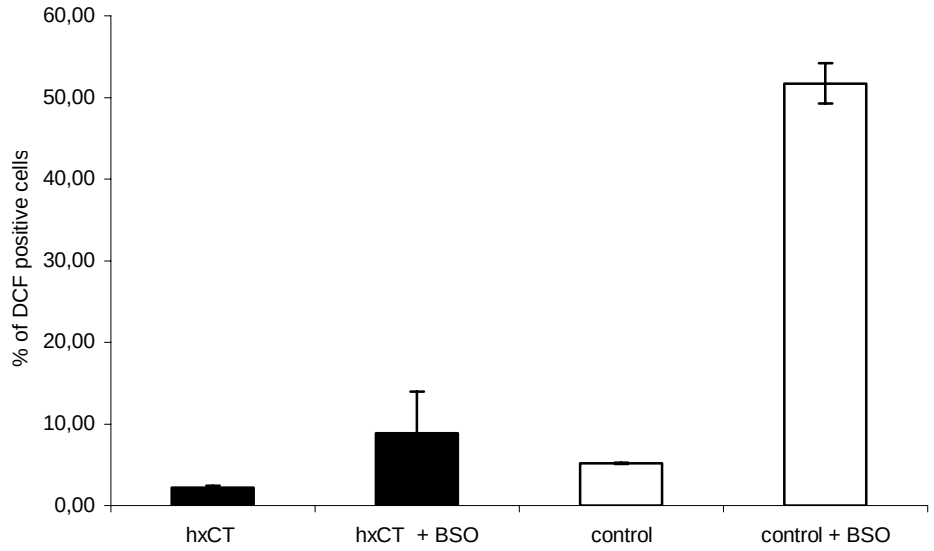

Figure 27. Reduced formation of intracellular ROS in xCT-overexpressing cells upon BSO treatment. BSO (10 μM) treatment for 48 hours induces higher production of intracellular ROS in vector-transfected (empty bars) compared to xCT-overexpressing cells (black bars).

H_2DCFDA is intracellulary converted into the nonfluorescent polar derivate H_2DCF that is rapidly oxidized to the highly fluorescent dye DCF in the presence of the intracellular ROS. xCT overexpresing cells and vector-transfected cells were treated with 10 μM BSO for 48 hours or left untreated. As shown in figure 27, under BSO treatment the level of ROS generated in the vector-transfected cells increased markedly compared with xCT-overexpressing cells.

5.3.4.2 BSO-treated cells die by rapid apoptosis or necrosis

To study the mode of cell death after BSO treatment, Annexin-V-FITC and PI staining were quantified. Generally two distinct ways of cell death are discriminated: necrosis and apoptosis. Necrosis is a passive process, occurring upon tissue injury or after accumulation of toxic reagents within a cell. The damaged cell is enlarged, plasma membrane disrupts, and cytosolic components are released into the extracellular space. In contrast, apoptosis or programmed cell death, is an active process characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry and blebbing associated with membrane inversion and exposure of phosphatidylserine, cell shrinking, chromatin condensation, and chromosomal DNA fragmentation. Changes in the plasma membrane are among the earliest features of cells undergoing apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, while in normal viable cells, PS is located on the cytoplasmic surface. Annexin V is a Ca^{2+}-dependent phospholipid–binding protein that has high affinity for PS, and binds to apoptotic cells with exposed PS. Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. Propidium iodide (PI) is used to distinguish viable from nonviable cells. Viable cells with intact membrane exclude PI, whereas membranes of dead and damaged cells are permeable for PI.

Cells which stained neither positive for Annexin-V nor PI represent viable cells. As already shown in figures 20a and 20b and also documented in figure 28, viability of hxCT-overexpressing cells decreased to a much lesser extent after 24 or 48 hours of BSO treatment (Fig. 28a), whereas the vast majority of empty vector-transfected cells started to die within 48 hours of BSO treatment (Fig. 28b). As shown in figure 28b, control cells could be rescued from BSO-induced cell death also by α- thioglycerol (100μM) and pyruvate treatment.

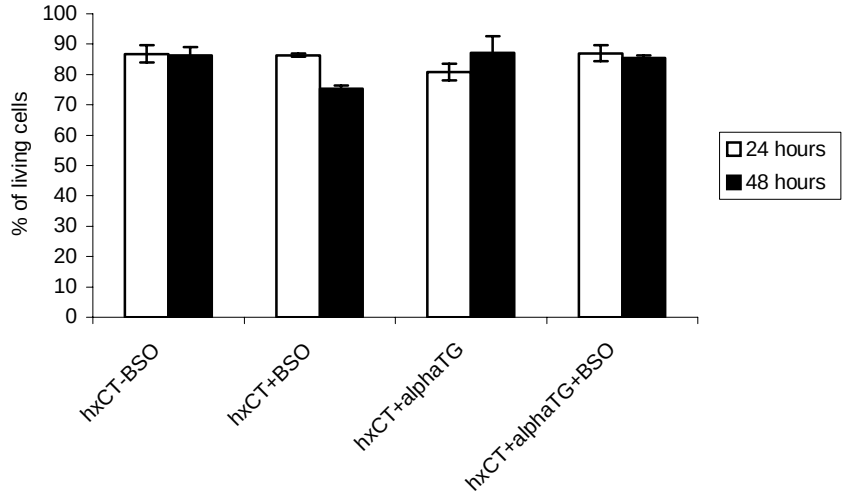

Figure 28a. Percentage of viable hxCT-overexpressing cells after 24 and 48 hours of 10 µM BSO treatment Cells which stained neither positive for Annexin-V nor PI were used to quantitatively determine the percentage of viable cells. hxCT overexpressing cells were treated with 10 µM BSO, 100 µM α-TG, BSO and α-TG together, or left untreated during 24 hours (empty bars) and 48 hours (black bars). Viability of hxCT-overexpressing cells was not affected by BSO treatment.

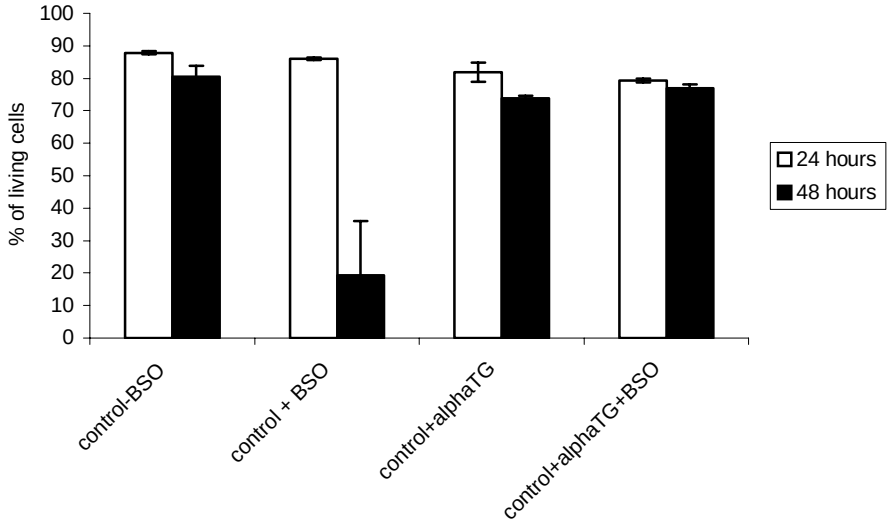

Figure 28b. Percentage of viable empty vector-transfected cells after 24 and 48 hours of 10 µM BSO treatment Cells which stained neither positive for Annexin-V nor PI were used to quantitatively determine the percentage of viable cells. Viability was determined after 24 hours (empty bars) and 48 hours (black bars) culturing with 10 µM BSO, 100 µM α-TG, BSO and α-TG, or without any treatment. Viability of the control cells decreased after 48 hours of BSO treatment, whereas medium supplementation with α-TG, protect cells from BSO induced cell death.

Positivity for Annexin-V-FITC is a characteristic feature of apoptosis (low right quadrant, Fig. 29 and 30), while positivity for PI (upper left quadrant, Fig. 29 and 30) indicates membrane damage and a necrotic type of cell death. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, undergoing necrosis, or are already dead. Only moderate amounts of dead cells undergoing apoptosis-like and necrosis-like death are visible in hxCT-overexpressing cells after 48 hours without (Fig. 29 upper panel) and with BSO treatment (Fig. 29 lower panel). xCT-overexpressing cells are virtually resistant to cell killing induced by 10 µM BSO (Fig. 28a and 29). In contrast, BSO treatment of empty vector-transfected cells for 48 hours resulted in a substantial increase in necrosis-like cell death (Fig. 30 lower panel), while apoptotic cell death, as determined by Annexin-V-FITC positivity and PI negativity, did not increase. BSO-induced necrosis-like cell death was associated with genomic DNA fragmentation (see figures 31a and 31b).

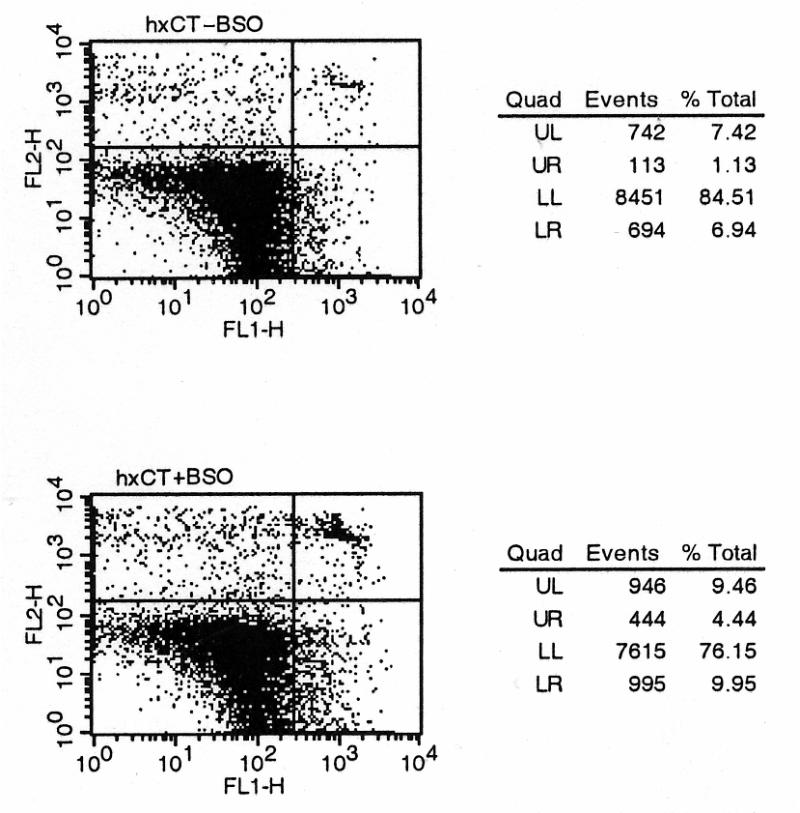

Figure 29. Annexin-V/PI staining of hxCT-overexpressing cells either left untreated (upper panel) or treated with 10 µM BSO for 48 hours (lower panel).

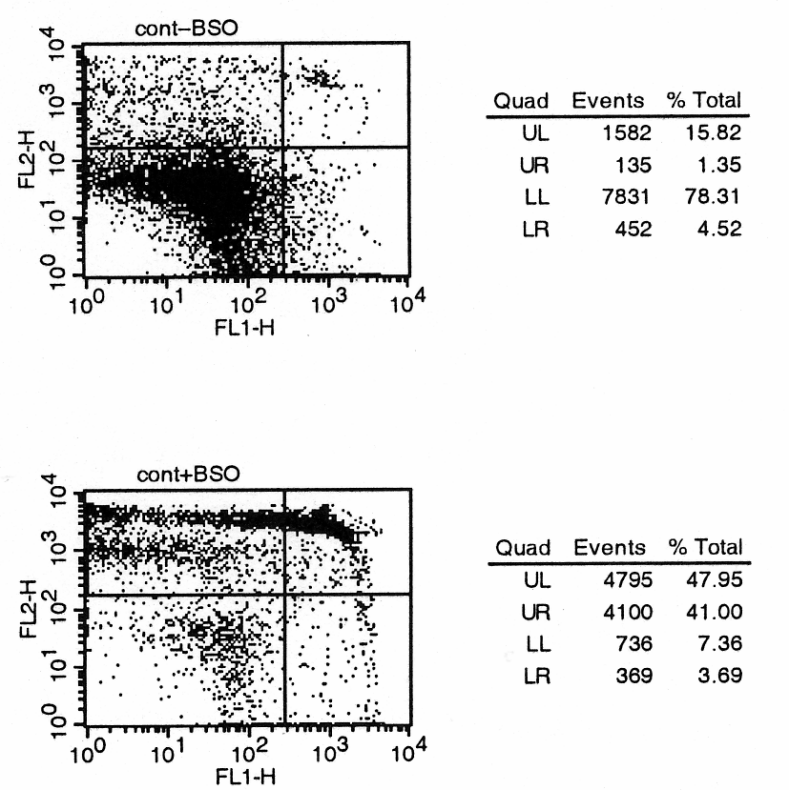

Figure 30. Annexin-V/PI staining of empty vector-transfected cells either left untreated (upper panel) or treated with 10 μM BSO for 48 hours (lower panel).

5.3.4.3 xCT-overexpression or α-TG treatment protects cells from BSO-induced genomic DNA fragmentation

Measurement of DNA fragmentation showed no significant difference in DNA fragmentation of xCT-overexpressing cells, within 24 and 48 hours of 10 μM BSO treatment (Fig.31a).On the other hand, empty vector-transfected cells (control cells) (Fig. 31b) after 48 hours of BSO treatment showed increased DNA fragmentation to 28% of the total cell population. In the conditions where medium was supplemented with α- thioglycerol (100μM) and pyruvate, the empty vector transfected cells were resistant to BSO induced cell death, and the percentage of the apoptotic cells were the same as in the control.

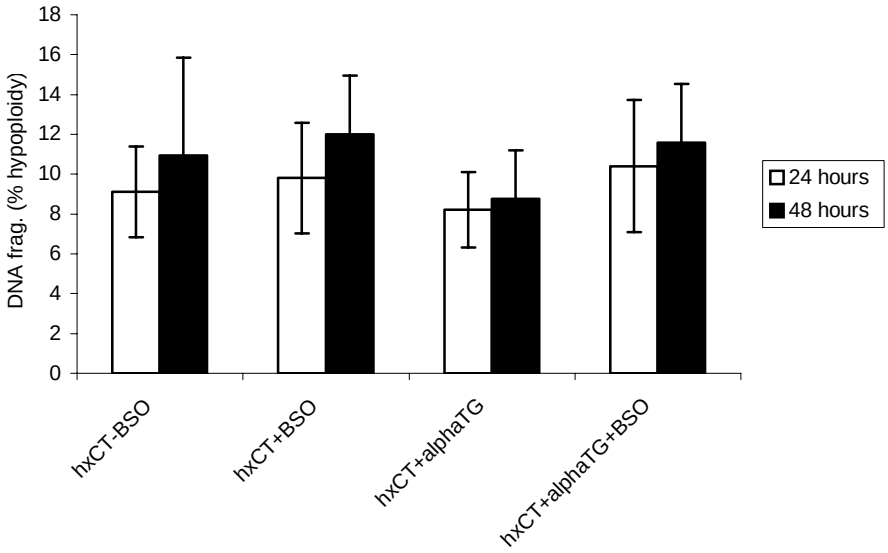

Figure 31a. Percentage of hxCT-overexpressing cells showing hypoploid DNA under BSO treatment. xCT-overexpressing cells were treated with 10 μM BSO, 100 μM α-TG, BSO and α-TG, or left untreated for 24 hours (empty bars) and 48 hours (black bars). No significant DNA fragmentation was obtained under BSO treatment neither after 24 nor after 48 hours.

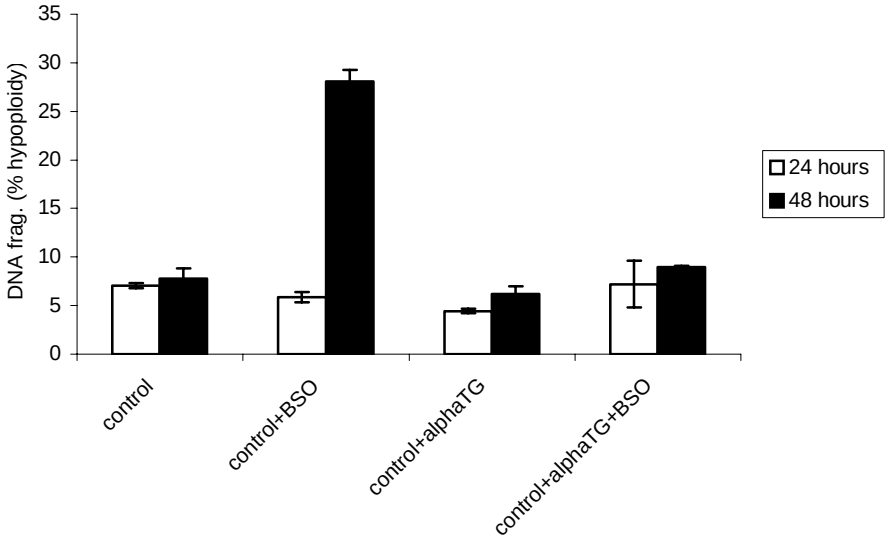

Figure 31b. Percentage of empty vector-transfected cells showing hypoploid DNA under BSO treatment. Empty- vector transfected (control) cells were cultured during 24 hours (empty bars) and 48 hours (black bars) with 10 μM BSO, 100 μM α-TG, both together BSO and α-TG or left untreated. DNA fragmentation increased after 48 hours of BSO treatment, whereas α-TG medium supplementation protected cells from BSO induced apoptosis and no DNA fragmentation was obtained.

5.3.4.4 xCT overexpression protects cells from BSO-induced caspase activation

The molecular hallmark of apoptosis, is the activation of caspases (131), a cascade of proteolytic enzymes. These enzymes participate in a series of reactions that are triggered in

response to proapoptotic signals and result in the cleavage of protein substrates involved in the transmission of signals causing cell death and the disassembly of the cell. Caspases are synthesized as inactive zymogens and must undergo a process of activation during apoptosis. To examine whether caspases are activated under BSO treatment, the cells were stained with fluorochrome-labelled peptides (FLICAs) specifically binding the activated forms of caspase 2 (VDVAD-AFC), caspase 3 (DEVD-AFC), caspase 8 (IETD-AFC) and caspase 9 (LEHD-AFC). Binding was evaluated by flow-cytometry. Analysis of caspases 2, 3, 8 and 9 (Fig. 32) activation showed that all four caspases were activated in empty vector-transfected cells (empty bars) after 48 hours of 10 µM BSO treatment, while hxCT-overexpressing cells (black bars) did not show any caspase activity after 48 hours BSO treatment.

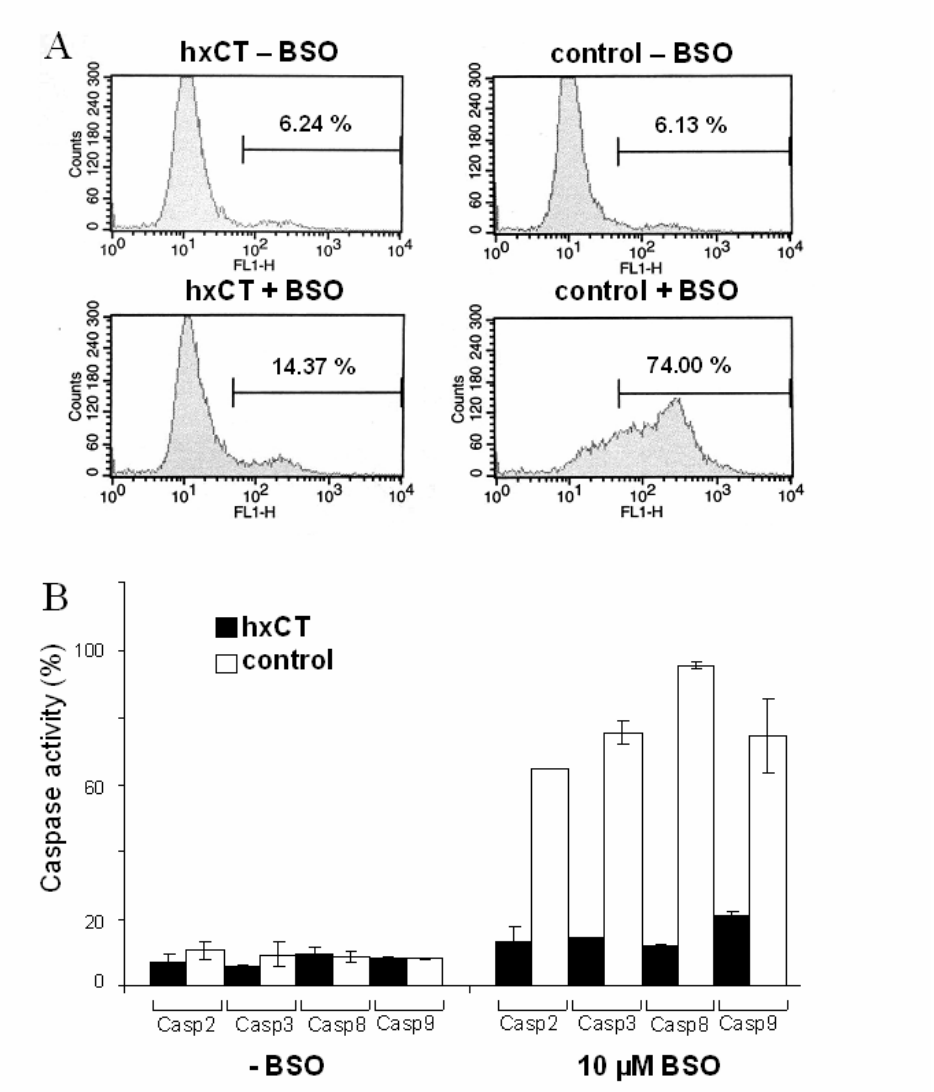

Figure 32. xCT overexpression protects HH514 cells from BSO-induced caspase activation. xCT-overexpressing cells (black bars) and vector-transfected control cells (empty bars) were treated with 10 µM BSO (+BSO) for 48 hours or left untreated. Caspases 3 staining is exemplarily presented in (A). The quantitative evaluation of the FACS-staining for caspases 2, 3, 8 and 9 is presented in (B).

5.3.4.5 xCT-overexpression protects cells from BSO-induced mitochondrial cell death

One of the early features of BSO-induced cell death is the disruption of the mitochondrial membrane potential. To ask whether xCT-overexpression affects the mitochondrial membrane potential of BSO-treated cells, the cells were stained with the cationic mitochondrial dye JC-1. JC-1 looses its red fluorescence in mitochondria with low membrane potential. The number of cells with lowered mitochondrial membrane potential $\Delta\Psi_m$ was determined by flow cytometry. As shown in figure 33, xCT overexpression protected HH514 cells efficiently from breakdown of the mitochondrial membrane potential induced by treatment of the cells with 10 or 50 µM BSO for 48 hours.

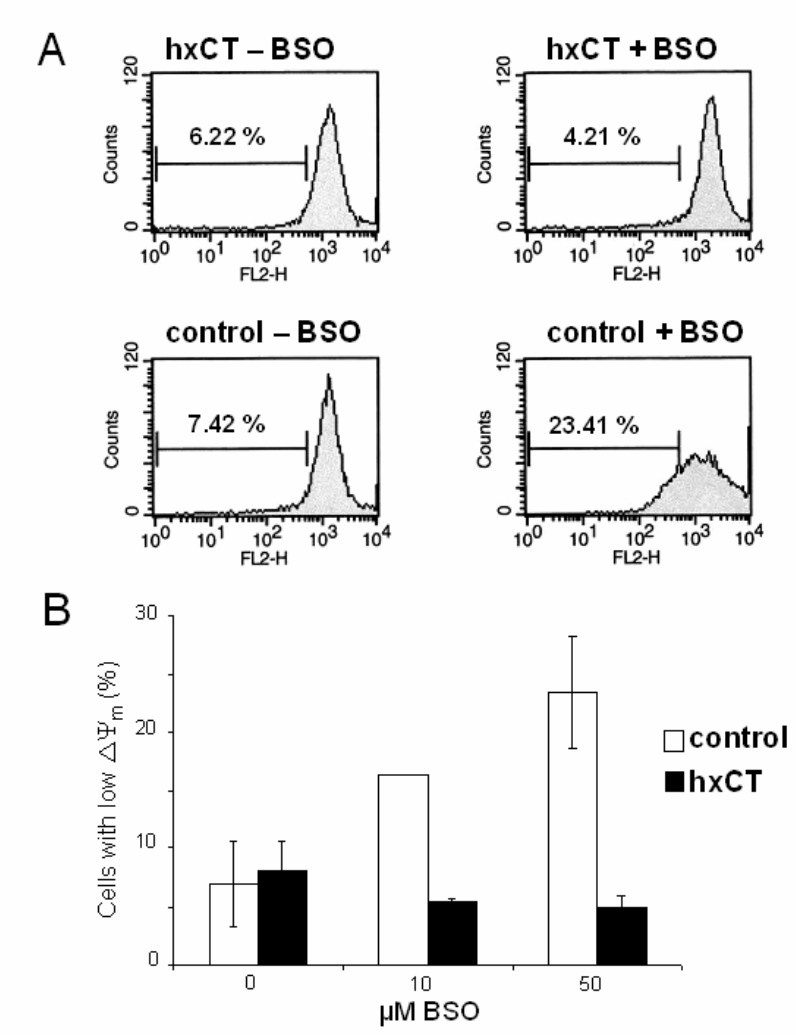

Figure 33. xCT overexpression protects HH514 cells from BSO-induced breakdown of the mitochondrial membrane potential. The number of cells with low $\Delta\Psi_m$ was determined by FACS analysis (A). A quantitative evaluation of the data is presented in (B). BSO treatment abrogates the mitochondrial membrane potential of control cells (empty bars) but not of xCT-overexpressing cells (black bars).

5.3.4.6 Overexpression of xCT does not alter the expression of Bcl-2 and Bcl-2 family members

Bcl-2 and its family members act primarly at the site of mitochondria controlling mitochondrial membrane potential and release of pro-apoptotic factors into the cytosol. Susceptibility to apoptosis is determined to a large extent by the balanced interplay of pro- and anti-apoptotic members of Bcl-2 family. To study a putative impact of xCT-overexpression on the expression of p53, the antiapoptotic proteins Bcl-2 and Bcl-$x_{L,}$ and Bcl-w and the proapoptotic family members Bax, Bak, Bid, Bim and Nbk/Bik, western blot analysis was performed. As shown in figure 34, no difference could be detected in xCT- versus vector-transfected cells.

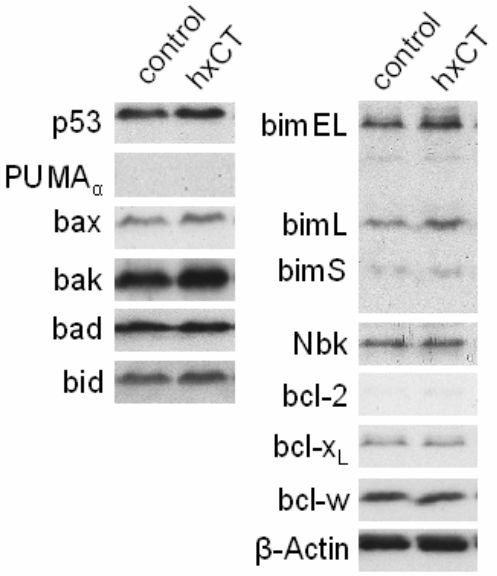

Figure 34. Overexpression of xCT does not affect the expression of proteins that regulate cell death in mitochondria. Western blot analysis of the expression of p53, and pro- as well as antiapoptotic members of the Bcl-2 family that regulate cell death at the mitochondria.

6 DISCUSSION

6.1 Small thiol-containing compounds promote survival and proliferation of normal and malignant cells

The identification of compounds that promote cell survival may provide useful tools for deciphering fundamental mechanisms by which cells live or die and also may give rise to compounds of potential use in clinical application. It has long been known that proliferation of murine normal B cells and leukemic cell lines is dependent on thiol-containing compounds (61). The reason for the requirement of thiol-containing compounds is the limited uptake capacity of the cells for cystine (68). It has been shown in our laboratory that human BL cells are equally dependent for their survival and proliferation on thiol-containing compounds, such as α-thioglycerol, 2-ME and dithiothreithol (DTT) that are added to the medium in conjunction with scavengers of oxygen radicals (44). BL cells have a high propensity to undergo apoptosis when seeded at low cell density in normal medium, but can be single-cell cloned either when thiol-containing compounds are added or when they are cocultured with irradiated fibroblasts as feeder cells (44, 45). Irradiated fibroblasts protect BL cells from apoptosis through secretion of a survival and proliferation-promoting activity which was soluble and labile and has been identified as cysteine (45). They are capable to take up cystine from the medium, reduce it intracellularly and secrete cysteine. Different groups have shown that proliferation of T- cells also requires a "small thiol compound" provided by activated macrophages or dendritic cells (123) that has been identified as cysteine and/or thioredoxin (4). Moreover, thioredoxin has been identified as a growth factor of Adult-Leukemia virus (ATLV-1)- transformed human T cells as well as of EBV immortalized human B cells (145).

The requirement for thiol-containing compounds is not restricted to B and T lymphocytes. Neuronal cells also depend on cysteine that is secreted by glial cells (109) for their proliferation and survival.

Generally, cells that are deficient in the capacity of cystine uptake, such as B and T lymphocytes and neurons, are dependent on thiol-containing compounds that are added to the medium or provided by cocultured fibroblasts, astrocytes, glia cells, macrophages or dendritic cells that are able to take up cystine efficiently and may provide it to their neighbouring cells as secreted cysteine.

One mechanism of the growth-promoting effect of 2-ME on mouse lymphoma cells, that is operating even if the medium has been completely oxidized, was proposed by Ishii et al. (67). The chemical reaction of 2-mercaptoethanol with cystine produces a mixed disulfide of 2-ME and cysteine. The mixed disulfide is taken up by the cells via a transport system for bulky amino acids shared with methionine. Within the cell, the mixed disulfide is rapidly reduced and 2-ME exported into the medium where it initiates another round of reaction with cystine. As the supply of cysteine is the rate limiting step for the synthesis of glutathione, cysteine is rapidly incorporated into glutathione. 2-ME thus maintains a shuttle that guarantees a steady supply of cysteine for the cells (67). It is still unresolved as to whether the proliferative effect of cyst(e)ine is entirely mediated by a change in intracellular glutathione or is dependent on the reaction of thiols with extracellular receptors and the maintenance of an intra- and extracellular redox balance.

To examine whether the restricted capacity for cystine uptake is the cause for the high sensitivity of BL cells to apoptosis, and to study the role of cystine uptake for the regulation of proliferation and apoptosis, we cloned the human and murine xCT-light chain, the component of the dimeric cystine glutamate antiporter conferring substrate specificity, and established stably xCT-overexpressing cell lines. We hypothesized that xCT overexpression would enable the cells to cover their intracellular cystine/cysteine supply by themselves, to use the cysteine for glutathione synthesis and thus to rescue cell survival and proliferation under non-permissive conditions.

6.1.1 Overexpression of xCT increases cystine uptake and compensates for the addition of exogenous thiol-containing compounds

The aim of this project was to investigate the impact of increased availability of L-cysteine on cell survival and proliferation. Due to their growth and proliferation behaviour, EBV-positive BL group I cell lines (i.e. BL lines that have retained the in vivo phenotype of BL cells *in vitro*) were considered most suitable to address this issue (44, 45). One of the reasons for the sensitivity of BL cells to apoptosis seems to be their limited ability to take up cystine from the medium (45). The BL cell line HH514 was selected because of its ability to be efficiently transfected by electroporation (26). The human and murine xCT-light chain was cloned onto an eucaryotic expression vector and transfected into the BL cell line HH514. Thus, a cell line was established that stably expresses the light chain xCT of the heterodimeric cystine glutamate antiporter (x_c^- system). Since the second component of the x_c^- system, 4F2

heavy chain, is constitutively expressed, it was sufficient to transfect the light chain xCT in order to achieve increased L-cystine uptake. xCT was expressed from a bicistronic mRNA that includes an IRES and the selectable marker gene puromycine phosphotransferase to make sure that xCT is indeed expressed when the cells are under puromycine selection. This strategy of xCT expression has been used throughout the studies presented in this work. Overexpression of xCT in the BL cell line HH514 allowed us to address the question whether overexpression of the transporter is able to rescue BL cells from apoptosis under non-permissive growth conditions.

The data presented provide evidence that overexpression of xCT light chain is sufficient to strongly increase the uptake capacity for L-cystine in HH514 cells. xCT-overexpressing cells had dramatically higher uptake capacity for L-cystine than control cells. Increased availability of L-cystine rendered BL cells more resistant to cell death induced either by seeding the cells at low cell density or by depleting glutathione by BSO. xCT-overexpressing cells were protected from cell death induced by seeding the cells at critical cell density. The xCT-overexpressing cells were able to survive and proliferate at low cell density that was non-permissive for control cells. By xCT overexpression we achieved a similar “rescue” effect as described previously for supplementation of the medium with thiol compounds (45) and were able to protect BL cells from oxidative stress-induced cell death. These data provide conclusive evidence for the notion that availability of cystine and cysteine itself is the limiting factor for survival and proliferation of BL cells under our culture conditions *in vitro*.

6.1.2 Overexpression of xCT renders cells resistant to induction of cell death by BSO

The original hypothesis was that under nonpermissive growth conditions uptake of L-cystine or L-cysteine becomes limiting for the survival and proliferation of HH514 cells by limiting the synthesis of glutathione. The question whether glutathione plays indeed such a decisive role in protection of cells from cell death was addressed by experiments in which the synthesis of glutathione was inhibited in xCT-overexpressing cells and control cells. The cells were treated with increasing concentrations of buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl-cysteinyl-synthetase (γ-GCS), the rate limiting enzyme in glutathione synthesis, to establish a putative correlation between cell survival/proliferation and intracellular GSH levels. These experiments provided compelling evidence that xCT-overexpressing cells can survive

and proliferate in the presence of 5 μM to 100 μM BSO, whereas control cells died at a concentration of 5 μM BSO in the first 48 hours of BSO treatment.

6.2 Glutathione is not the key element of the antioxidant defense system that is activated by xCT overexpression in BL cells

It was our first expectation that there is a threshold of glutathione in xCT-overexpressing versus control cells that would discriminate between cell survival and death. If a threshold indeed exists, a prediction would be that xCT-overexpressing cells will have invariably higher glutathione levels than control cells. Determination of glutathione levels in BSO treated cells, however, clearly revealed that the glutathione levels were equally decreased in both, xCT-overexpressing and control cells. Most importantly, the glutathione level of control cells in the presence of 5 μM BSO (non-permissive for cell survival) was higher than that in xCT-overexpressing cells at higher BSO concentrations under which xCT-overexpressing cells still could proliferate. These results rule out a putative threshold for the glutathione concentration that is compatible with cell survival and proliferation, and excludes a critical role for glutathione in the rescue from oxidative stress-induced cell death. Even though glutathione has been considered to be the most important redox and antioxidant defense system, recent data from several laboratories including our own strengthen the point that this long-standing paradigm has to be re-evaluated. Independent evidence that glutathione is indeed dispensable for cell proliferation has been provided by genetic means (127). Deletion of the gene for γ-glutamyl-cysteinyl-synthase (γ-GCS) in mice was found to be associated with early embryonic death around gestational day 8.5. Unexpectedly, the block of proliferation of γ-GCS deficient blastocyst-derived cell lines in culture could be overcome by N-acetylcysteine (NAC) even if glutathione was absent from the medium (122). These data clearly demonstrate that GSH itself is dispensable for proliferation when antioxidants are added to the growth medium. Apparently, beside glutathione several other redox couples participate in intracellular redox regulation. At least four related systems function in redox control: thioredoxine-1/thioredoxine reductase-1 (Trx1/TR1), thioredoxine-2/thioredoxine reductase-2 (Trx2/TR2), glutaredoxin-1/GSH/glutathione reductase (Grx1/GSH/GR), and oxidase-linked protein disulfide isomerases (PDI) (79). In vitro experiments with CaCo2 cells have furthermore revealed that the extracellular redox potential defined by the ratio of extracellular cysteine to cystine is critical for cell proliferation independently from intracellular glutathione (77, 99).

6.3 Role of extracellular cysteine

Determination of the level of secreted mercaptans revealed a dramatic difference between xCT-overexpressing cells and control cells and clearly showed that proliferation of xCT-overexpressing cells under conditions, that are non-permissive for control cells, is associated with a dramatic increase in the level of extracellular small mercaptans. HPLC analysis revealed that the small mercaptan in the exracellular space is virtually exclusively cysteine. The difference in the extracellular cysteine level of xCT-overexpressing and vector-transfected control cells became even more evident when cells were depleted of glutathione. Additionally, co-culture experiments revealed a feeder effect of xCT-overexpressing cells for vector-transfected control cells indicating that cysteine that is secreted by xCT-overexpressing cells is sufficient to maintain survival and proliferation of vector-transfected control cells.

Thus, the question arises as to the protective mechanism of increased extracellular cysteine levels. Cysteine fulfills a wide range of different functions including disulfide formation, metal-binding, hydrolysis, electron donation and redox-catalysis (53). Thiol/disulfide reactions are *in vivo* substitution reactions with the protonated thiol group of cysteine acting as a nucleophile. Intracellularly, these reactions play a crucial role in the maintenance of the cellular redox balance in different redox couples such as GSSG/GSH, thioredoxin (Trx), protein disulfide isomerase (PDI) and glutaredoxin (Grx) (53). Given the pivotally important role of cysteine in redox active peptides and proteins, surprisingly little is known about the contribution of extracellular cysteine levels to the maintenance of the cellular redox balance. The low molecular weight thiol/disulfide pool consists predominantly of cystine and cysteine, and a generalized impact of the extracellular thiol/disulfide redox state on cell proliferation and/or apoptosis has been proposed. For instance, the redox state of the extracellular cysteine/cystine pool may affect cell proliferation by increasing the susceptibility of growth factor receptors to their ligands (77).

Our data indicate the existence of an independent redox cycle, the cystine/cysteine cycle, that by itself protects the cells from cell death under conditions of GSH depletion. These findings support the notion that maintenance of the intra- and extracellular redox balance rather than the glutathione level itself is critical to protect HH514 cells from cell death.

6.4 The cystine/cysteine cycle

The activity of x_c^- system contributes to driving the cystine/cysteine cycle and to maintaining the balance between cystine and cysteine in the cell culture medium. Cystine taken up by cells via x_c^- system, is reduced intracellulary to cysteine and part of cysteine is released back into the medium via the neutral amino acid transport system. Extracellular cysteine is rapidly oxidized to cystine by oxygen in the medium. Cystine uptake, reduction to cysteine, secretion of cysteine, and reoxidization to cystine thus constitute a redox cycle that is driven by the cystine glutamate exchange transporter (118, 152). The data presented provide compelling evidence that the cystine/cysteine cycle is able to protect cells from oxidative stress-induced cell death and to maintain survival and proliferation of xCT-overexpressing cells. The proposed model is presented in figure 35.

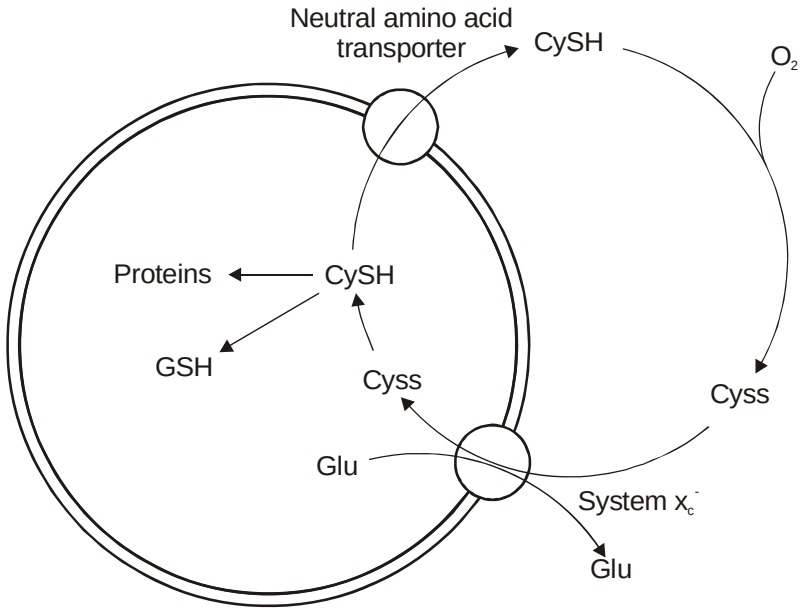

Figure 35. Schematic model for the cystine/cysteine cycle in the xCT-overexpressing HH514 cells.

The question arises which molecules are providing the reducing equivalents for the reduction of cystine to cysteine inside the cells in untreated as compared to BSO-treated cells. In untreated cells, the glutathione system consisting of glutathione (GSH), glutaredoxin, glutathione reductase and NADPH, is the prime candidate. But what is reducing cysteine if glutathione synthesis is blocked by BSO? The cytosolic and mitochondrial thioredoxin-thioredoxin reductase systems are the most likely candidates for taking over this function in BSO treated cells, either completely or in part. Our group has recently demonstrated that the

apoptosis-inducing function of BSO can be partly compensated by the thioredoxin system in fibroblasts derived from thioredoxin reductase 2 (Txnrd2) wild-type mice but not from Txnrd2 knockout mice (32). But a role for glutathione can not be definitively ruled out. Since inhibition of glutathione synthesis by BSO is incomplete and only leads to reduced levels of cellular GSH of around 10-20% as compared to non-treated cells, the data from this work do not rule out the possibility that small amounts of residual glutathione found in BSO treated cells do play a role for the inititation or maintenance of the cystine/cysteine cycle. The fact that BSO interferes with proliferation in a dose-dependent manner might suggest that residual glutathione may indeed play a role in initiating and/or sustaining the cystine/cysteine cycle. Whether glutathione is completely dispensable for reduction of cystine can only be answered by genetic means. If so, cells deficient in γ-GCS that are overexpressing xCT should become independent of NAC. If they remain dependent on NAC, this would indicate that residual glutathione is indeed required to sustain the cystine/cysteine cycle.

The cystine/cysteine cycle also seems to be important in maintaining the redox status in the nervous system and has also been proposed to play a role in the resistance of cancer cells to chemotherapy (118). In the brain, reactive oxygen species are produced at a higher rate because of the high consumption of oxygen, and ROS are involved in the pathogenesis of various neurodegenerative disorders (Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (8). Sato and colleagues showed (118) that x_c^- system is expressed predominantly in regions facing the cerebrospinal fluid (CSF) and contributes to the clearance of cystine from CSF. Clearance of cystine via system x_c^- in the cells facing CSF and release of cysteine from the cells via neutral amino acid transporters may contribute to maintaining the reduced state in the CSF. The cysteine/cystine cycle could thus serve as a major redox buffer in the extracellular medium (118) thus playing an important role in the protection against oxidative stress.

Elevated levels of extracellular glutamate can induce seizure and cause excitotoxic neuronal cell death. This is normaly prevented by astrocyte Na^+-dependent glutamate uptake. However, human malignant glioma cells display strong Na+-independent cystine-glutamate exchange activity (152). In malignant glioma cells, unlimited activity of the cystine/cysteine cycle cycle may thus lead to release of glutamate at concentrations that can induce widespread neurotoxicity.

The most difficult question that arises from this work addresses the mechanism how extracellular cysteine may protect cells from cell death. The cysteine/cystine redox couple participates in the redox balance and in the equilibrium with other redox pairs like GSSG/GSH,

thioredoxin (Trx), glutaredoxin (Grx) and protein thiols/protein disulfides through disulfide isomerase (PDI). Jones et al. have recently reported that the steady-state redox potential of the cysteine/cystine couple in cells is considerably more oxidized than that of GSH/GSSG and thioredoxin (79). The cystine/cysteine redox couple is thus regulated independently of GSH/GSSG and is sufficiently oxidized to contribute to redox signaling and redox control on its own (79). In vitro experiments with CaCo2 cells had furthermore revealed that the extracellular redox potential defined by the ratio of extracellular cysteine to cystine is critical for cell proliferation and is independent of glutathione (77, 99). Jonas et al. (77) suggested that the proliferative effect of extracellular cystine/cysteine on CaCo2 cells might be due to an interaction with growth factor signaling eventually occurring at the cell surface. There are also suggestions that nutrients like glutamine and keratinocyte growth factor stimulate cell proliferation of CaCo2 cells through modulation of the extracellular cysteine/cystine ratio without any effect on the intracellular GSH/GSSG ratio (76).

Meanwhile it has become well accepted that intra- and extracellular redox changes have a strong impact on signaling pathways that regulate cell survival or cell death (1, 30, 77, 80, 106, 120). Schafer and Buettner in 2001 (120) proposed a model where proliferation, differentiation, apoptosis and necrosis are coupled to a given redox status. According to this model, changes in the fate of a cell are associated with or are dependent on changes in the reduction potential of various redox couples.

The ultimate challenge will be to define the molecules on the cell surface that are the targets of the cystine/cysteine cycle and have to be maintained in reduced form to allow cell survival and proliferation.

6.5 The cystine/cysteine cycle protects cells from cell death initiated in mitochondria

We finally attempted to clarify whether there is a crosstalk of the cystine/cysteine cycle to other pathways that regulate susceptibility versus resistance to apoptosis and to identify the relay stations linking the different pathways. To address this question we have studied in which subcellular compartment the cystine/cysteine cycle accomplishes its antiapoptotic action. Oxidative stress is a common element of apoptosis induced by various stimuli. We could show that in xCT-overexpressing cells the cystine/cysteine cycle suppresses the production of ROS under glutathione depletion, suggesting that control cells were dying of oxidative stress-induced cell death. The cystine/cysteine cycle thus can rescue cells from cell death, either by

scavenging intracellular ROS itself or by inducing another intracellular antioxidant/antiapoptotic system. Since mitochondria harbour the respiratory chain and are the primary targets exposed to oxygen radicals as leakage products of incomplete oxygen reduction, we assumed that oxidative stress-induced cell death is initiated at the mitochondria. We have therefore studied the role of mitochondria in the induction of cell death by BSO. Our data show that BSO induces breakdown of the mitochondrial membrane potential, caspase activation and DNA fragmentation, confirming that the mitochondrial death pathway is in fact involved. The reduction of the mitochondrial membrane potential was inhibited in xCT-overexpressing cells indicating that the cystine/cysteine cycle, presumably by secretion of cysteine, is able to counteract BSO-induced oxidative damage and to suppress dysfunction of mitochondria. Since alterations in mitochondrial function are critically involved in the induction of cell death, we wanted to determine whether caspases and if so, which type of caspases are activated during BSO-induced cell death. Using fluorogenic peptide substrates to measure caspase activity, caspases 2, 3, 8 and 9 were found to be activated after 48 hours of BSO treatment in the control cells. Activation of caspases was inhibited by xCT-overexpression. Attempts to block BSO-induced cell death in vector-transfected cells by the caspase inhibitor zVAD-fmk failed (data not shown) suggesting that redundant pathways to kill the cells have been activated by BSO. To see whether cell death induced by BSO, as defined by PI-positivity of the cells, is preceded by the appearance of Annexin V-positive apoptotic cells, kinetic experiments with low BSO concentrations were performed. These experiments revealed a substantial increase in PI-positive cells (necrosis-like cell death) without an intermediate concomitant increase in the number of Annexin V-positive apoptotic cells (data not shown). It has been proposed that apoptosis and necrosis might share common mediators such as Ca^{2+} and caspases (85) and that signaling of the Fas receptor initiates both, apoptotic and necrotic cell death (136). We conclude that in our experimental setting cell death advanced rapidly and did not allow to define an intermediate stage of Annexin-V-positive, PI-negative cells. This is compatible with the model of Schafer and Buettner (120) who proposed that, by decreasing the reducing potential of redox couples, a series of switches may be activated that drive the cells from proliferation through various stages of differentiation. When the redox environment cannot be maintained, apoptosis and finally, when the redox potential is completely breaking down, necrosis ensue. It was proposed that glutathione would control the protein thiol/disulfide equilibrium and thereby drive the cellular switches. Our data imply that under conditions of glutathion depletion the cysteine/cystine redox couple takes over the role hitherto assigned to glutathione.

Overexpression of xCT does not appear to have an impact on the expression of genes that are known to regulate apoptosis in mitochondria such as the antiapoptotic proteins Bcl-2, Bcl-x_L, and Bcl-w as well as the proapoptotic members of the Bcl-2 family, namely Bax, Bak, Bad, Bid, Bik and Bim. We cannot exclude an effect of the cystine/cysteine cycle on a gene or gene products that has not been studied. The data, however, strongly suggest that the cystine/cysteine cycle has its own impact on regulation of apoptosis by maintaining the extra- and intracellular redox balance including the intramitochondrial membrane potential rather than modulating the expression of pro- and /or antiapoptotic genes.

6.6 OUTLOOK

Several important questions have arisen from this work, but still had to remain open. Firstly, what are the target molecules (the molecular switches) that are under redox regulation by the cystine/cysteine cycle in our cellular system and that are essential for the decision whether a cell will survive or die? Secondly, what is providing the reducing equivalent under conditions of glutathione depletion inside the cells? These questions might be addressed by using toxic chemicals that selectively react with –SH groups and mark the proteins or by knocking out particular molecules (such as thioredoxin mutants) in xCT-overexpressing cells.

As the maintenance of a reducing extracellular environment is so critical for cell survival, it appears likely that molecular processes taking place at the cell surface or in the cell membrane play a decisive role. Extracellular cysteine levels may thus regulate cell survival e.g. through activation of cell surface receptors. It may also regulate certain intracellular pathways such as production and secretion of growth factors and cytokines, signal transduction mechanisms and the activity of transcription factors. Many extracellular receptors are tyrosine kinases or regulated by tyrosine kinases and phosphatases and the activity of tyrosine phosphatases is known to be inhibited by oxygen radicals. Many tyrosine kinase receptors are activating the serine-threonine kinase Akt/protein kinase B (PKB), a kinase playing a pivotal role in the regulation of cell survival and cell death thus coupling growth factor receptor signaling to the regulation of cell survival. Another serine-threonine kinase playing an important role in stress-induced signaling is apoptosis signal-regulating kinase (ASK) that is known to be regulated by thioredoxin.

Another question is whether the cystine/cysteine cycle requires basal amounts of glutathione in cells below the threshold detected in our experiments. The work presented here could not rule out the possibility that minor levels of glutathione which remain in the cell after

BSO treatment may play a role in initiating reduction of cystine into cysteine. This question can only be answered by genetic means when xCT is overexpressed in cells that are deficient in glutathione synthesis due to a defect of γ-glutamyl-cysteine-snythetase (γ-GCS) and concomitant pharmacological inhibition of γ-glutamyl-transpeptidase which may generate γ-glutamyl-cysteine from glutamine and provide a salvation pathway for the synthesis of small amounts of glutathione.

It has already been shown that during antigen exposure proliferation and activation of T helper cells is dependent on cysteine secreted from antigen-presenting cells (123). A similar cooperation has been observed in the nervous system, where neuronal cells receive cysteine through the secretion from the glia cells (109). Therefore, the cystine/cysteine cycle most likely plays an important role in maintaining the redox status in the nervous system (118). The analysis of the phenotype of xCT knock-out mice might open the possibility to study the role of the cystine/cysteine cycle in physiological and pathophysiological conditions such as under ischemic stress and infection. It is another important question to understand under which conditions the activity of the cystine/cysteine cycle may have adverse effects to the organism. As proposed by Ye et al., 1999 (151, 152), unlimited activity of the cystine/cystine cycle due to xCT overexpression in malignant glioma cells might lead to a dramatic increase in the secretion of glutamate associated with increased glutamate toxicity and neuronal death. In the hematopoetic system, the particular sensitivity of the cells to irradiation may be related to the limited uptake capacity for cystine. Limited activity of the cystine/cysteine cycle may also be necessary for an orchestrated regulation of apoptosis in the immune system. Low activity of the x_c^- system in B and T cells may be prerequisite to render the immune system highly dynamic. Generation of transgenic "gain-of-function" mice will enable us to address this question.

Drug resistance is one of the most serious problems in cancer chemotherapy. For a long time it has been known that irradiation leads to a decrease in cellular thiols and that thiols protect cells against the effects of irradiation. Drugs which are commonly used as antineoplastic drugs in the treatment of malignancies reduce glutathione levels in various cell types (83). It has been shown that the activity of system x_c^- can determine resistance to cisplatin in a human ovarian cancer cell line (102). It will be important to examine in a more systematic fashion the possible role of the cystine/cysteine cycle in drug resistance in cancer cells.

7 SUMMARY

The high susceptibility of Burkitt lymphoma (BL) cells to apoptosis at low cell density or in the absence of feeder cells is due to their low uptake capacity for cystine. The cells have an efficient uptake system for cysteine but not cystine. Availability of cystine or cysteine is the rate limiting step in the synthesis of glutathione, the major cellular antioxidant. Irradiated fibroblast feeder cells support the growth of Burkitt´s lymphoma cells because they can efficiently take up cystine and secrete it to cocultured BL cells as cysteine. Cystine, the predominant form in plasma, extracellular body fluids and in cell culture medium, is imported into the cells by the cystine-glutamate exchange transporter (x_c^- system), a heterodimeric amino acid transporter consisting of two subunits, xCT light chain and 4F2 heavy chain, that imports cystine and exports glutamate. In the absence of feeder cells and at low cell density, survival and proliferation of BL cells is dependent on thiol compounds, such as α-thioglycerol or β-mercaptoethanol, added to the medium together with scavengers of oxygen radicals, that generate mixed disufides and thus promote the uptake of cysteine and the synthesis of glutathione. To investigate the impact of system x_c^- on cell proliferation and apoptosis we transfected the BL cell line HH514 with the gene for xCT, the substrate specific subunit of system x_c^-.

In this work, for the first time a stable overexpression system for Burkitt´s lymphoma cells has been developed for xCT light chain, the subunit of system x_c^- conferring substrate specificity. The data show that overexpression of xCT is sufficient to promote cystine uptake and to rescue BL cells from oxidative –stress-induced cell death mediated by seeding the cells at low cell density. Most importantly, xCT-overexpressing cells were able to maintain cell survival and proliferation also under conditions of inhibiting glutathione synthesis by buthionine sulfoximine (BSO) ruling out glutathione as the critical determinant in the decision between cell survival and cell death. In the supernatant of xCT-overexpressing cells, extracellular cysteine levels were highly elevated as compared to vector-transfected control cells. The data support the model that the activity of system x_c^- supports cell survival and proliferation by driving an independent redox cycle, the cystine/cysteine cycle that consists of cystine uptake, intracellular reduction to cysteine, secretion of cysteine into the medium and reoxidization of cysteine to cystine by air oxygen and is able to generate the cell´s own reducing environment. xCT overexpression as well as supply of the cells with cysteine by treatment with α-thioglycerol protected HH514 cells efficiently from BSO-induced breakdown

of the mitochondrial membrane potential and the execution phase of cell death mediated by capsase activation and DNA fragmentation. The data suggest that BSO-induced cell death is initiated at the mitochondria. No difference in the expression of p53 and pro- and antiapoptotic members of the Bcl-2 family were observed when xCT-overexpressing cells and vector-transfected control cells were compared suggesting that the crucial function of the cystine/cysteine cycle is to maintain the extracellular, intracellular and intramitochondrial redox balance rather than to modulate the expression of genes that regulate susceptibility versus resistance to cell death.

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Curriculum Vitae

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Publications

1. Banjac A., Conrad M., Sato H., Bannai S., Weiss N., Kölle P., Tschoep K., Issels D. R., Peter T. Daniel and Bornkamm W. G. **The cystine/cysteine cycle: a redox cycle regulating susceptibility to apoptosis** (submitted)

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1. "Effect of cardiovascular and neurosedative drugs on the activity of membrane enzymes from rat brain", T. Orlic, G. Nikezic, T. Momic, A. Banjac, D. Kanazir, A. Horvat, XIV Congress of Yugoslavian Medical Doctors, Belgrade, Yugoslavia May 2000
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