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IMPACT OF LYSOSOMAL FUNCTION IN CANCER AND APOPTOSIS

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Front cover photograph: DAPI stained nuclei of healthy U937 cells Back cover photograph: DAPI stained, fragmented nuclei of apoptotic U937 cells

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Hur svårt kan det va?...

ABSTRACT

Lysosomes, the recycling units of the cell, participate in the signaling pathway to apoptosis, which has stimulated the search for anti-cancer drugs targeting the lysosomal compartment. Lysosomes are, however, often altered in cancer cells. The aim of this thesis was to investigate the involvement of lysosomes during apoptosis in normal and cancer cells. We developed and used flow cytometric methods to measure cytosolic and lysosomal pH in cells. The cytosolic pH of U937 cells decreased, in a caspase-independent way, by 1.4 pH-units during apoptosis. Concomitantly, the lysosomal pH increased from 4.3 to 5.2, suggesting that proton release from lysosomes might be responsible for cytosolic acidification. When studying the lysosomal pH of head and neck squamous cell carcinoma (HNSCC) cell lines and normal oral keratinocytes (NOKs), the pH was significantly increased in three of five HNSCC cell lines, as compared to NOKs. Moreover, high lysosomal pH correlated to low expression of the B subunit of the vacuolar V_0/V_1 -ATPase, a necessary component of the proton pump responsible for lysosomal acidification, and to reduced intrinsic cisplatin sensitivity. Cisplatin-induced apoptosis was, at least partly, dependent on lysosomal cathepsins. When investigating the colony formation ability of the two HNSCC cell lines LK0412 and SqCC/Y1, both were found to give rise to holoclones, indicating the presence of cells with cancer stem cell properties. Holoclone cells from the LK0412 cell line were less sensitive to cisplatin compared to more differentiated paraclone cells. Moreover, we detected differences in intracellular localization of the lysosomal compartment and expression of cathepsins between holo- and paraclone cells.

This thesis shows that changes found in the lysosomal compartment of cancer cells, such as alteration of lysosomal pH, might influence the outcome of a drug treatment. In addition, differences in drug sensitivity between subpopulations of tumor cells may affect the outcome of an anticancer therapy.

SAMMANFATTNING

Programmerad celldöd eller apoptos är en viktig mekanism för att upprätthålla balans mellan kroppens celler. Vid exempelvis cancer fungerar inte styrningen av denna process, vilket leder till att för få celler dör och en tumör kan växa ohämmat. Denna avhandling fokuserar på lysosomen, en mycket sur organell i cellen som är ansvarig för nedbrytning av cellmaterial. Hos cancerceller är lysosomerna ofta förändrade. Vi har undersökt lysosomernas roll under apoptos hos normala celler och hos cancerceller. För att kunna undersöka pH-förändringar under apoptos har vi utvecklat metoder att mäta cytosoliskt och lysosomalt pH med hjälp av en teknik som kallas flödescytometri. I apoptotiska celler ser vi att det cytosoliska pH:t sjunker med 1.4 pH-enheter till pH 5.7 samtidigt som det lysosomala pH:t ökar från 4.3 till 5.5. Detta tyder på att läckage av vätejoner från lysosomerna kan orsaka en försurning av cytosolen under apoptos. Genom att studera normala orala keratinocyter och jämföra dessa mot fem olika cellinjer etablerade från skivepitelcancer från munhåla har vi också funnit ett samband mellan det lysosomala pH:t och känsligheten för cellgiftet cisplatin. Cisplatinbehandling leder till apoptos hos alla celler men en högre dos krävs hos celler som har ett högt lysosomalt pH. Tumörer tros innehålla ett litet antal sk cancerstamceller, som har förmåga att kontinuerligt kopiera sig själva utan att åldras. Överlevnad av dessa celler tros vara orsaken till att en tumör återkommer efter en behandling. Vi visar i denna avhandling att cellinjer från skivepitelcancer innehåller celler som har cancerstamcellsegenskaper, och att dessa celler kan ha en lägre känslighet mot cisplatin jämfört med mer utvecklade cancerceller.

Lysosomerna utgör ett intressant framtida mål för nya cancerläkemedel. I denna avhandling visar vi att förändringar i det lysosomala systemet kan påverka effekten av ett läkemedel och att skillnader mellan olika sub-populationer av celler från samma tumör kan påverka resultatet av en behandling.

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. Cathrine Nilsson, Katarina Kågedal, Uno Johansson and Karin Öllinger (2003) Analysis of cytosolic and lysosomal pH in apoptotic cells by flow cytometry. Methods in Cell Science 25: 185-94
- II. Cathrine Nilsson, Uno Johansson, Ann-Charlotte Johansson, Katarina Kågedal and Karin Öllinger (2006) Cytosolic acidification and lysosomal alkalinization during TNF-α induced apoptosis in U937 cells. Apoptosis 11: 1149-59
- III. Cathrine Nilsson, Karin Roberg, Roland C Grafström and Karin Öllinger (2008) Intrinsic differences in cisplatin sensitivity of head and neck cancer cell lines correlates to lysosomal pH. International Journal of Cancer, submitted
- IV. Cathrine Nilsson, Karin Roberg, Roland C Grafström and Karin Öllinger (2008) Radiation and cisplatin sensitivity in head and neck cancer cells with stem cell properties. Manuscript

ABBREVIATIONS

BH	Bcl-2 homologue
BP	bandpass
CIC	chloride channel
CSC	cancer stem cell
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas ligand
FSC	forward scatter
HBSS	Hank's buffered salt solution
HNSCC	head and neck squamous cell carcinoma
IAP	inhibitor of apoptosis proteins
I-κB	NF-κB inhibitory protein
LAMP	lysosome associated membrane protein
LBPA	lysobisphosphatic acid
LP	longpass
MOMP	mitochondrial outer membrane permeabilization
MPR	mannose phosphate receptor
MSDH	O-methyl-serine dodecylamide hydrochloride
NF-κB	Nuclear factor-ĸB
NHE	Na ⁺ /H ⁺ exchanger

NOK	normal oral keratinocyte
PI3K	phosphatidylinositol 3'-kinase
PS	phophatidyl serine
RIP	receptor-interacting kinase
ROS	reactive oxygen species
SP	side population
SSC	side scatter
TIC	tumor initiating cell
TGN	trans-Golgi network
TNF	tumor necrosis factor
TNFR	TNF receptor
TRADD	TNF-R associated death domain
TRAF	TNF-R associated factor
TRAIL	TNF-related apoptosis-inducing ligand

INTRODUCTION

Lysosomes

Lysosomes are cytoplasmic organelles that were originally discovered by de Duve, who named them after the Greek word for 'digestive body' (de Duve *et al.*, 1955; de Duve, 1959; de Duve, 2005). Lysosomes contain soluble and membrane-associated hydrolases with acidic pH optima responsible for intracellular degradation.

Lysosomes are ~0.5 μ m in diameter, concentrated near microtubule organizing centers, and typically constitute 0.5-5 % of the cellular volume (Luzio *et al.*, 2003). They are morphologically heterogeneous and resemble other organelles of the endocytic and secretory pathways. The lysosomal limiting membrane is a 7-10 nm thick single bilayer, of which the lipid composition is not fully known (Winchester, 2001). However, lysosomes, as well as late endosomes, are reported to contain the unique lysobisphosphatic acid (LBPA), which is highly hydrophobic (Kobayashi *et al.*, 1998; Winchester, 2001). Lysosomes contain luminal membrane vesicles, visible in electron microscopy (Luzio *et al.*, 2003). LBPA is restricted to these internal membranes and is believed to contribute to its tubular/vesicular organization and to take part in sorting proteins into the luminal membrane enabling their degradation (Kobayashi *et al.*, 1998; Luzio *et al.*, 2003).

Lysosomes are enriched in integral transmembrane glycoproteins called lysosome associated membrane proteins (LAMPs), lysosome integral membrane proteins (LIMPs) and lysosome glycoproteins (lgps) in the membrane (Winchester, 2001; Luzio *et al.*, 2003). Most abundant are LAMP-1 and LAMP-2 constituting ~50% of all proteins in the membrane (Chang *et al.*, 2002; Eskelinen, 2006). The specific functions of these proteins are largely unknown, but it is now clear that they fulfill functions far beyond their initially suggested roles in maintaining the structural

integrity of the lysosomal membrane (Chang *et al.*, 2002; Eskelinen, 2006). In addition to these major lysosomal proteins there are also less abundant ones that are expressed in a cell type- or tissue-specific manner.

Lysosomal degradation

Lysosomes constitute the major and terminal degradative compartments of eukaryotic cells (Dell'Angelica *et al.*, 2000; Luzio *et al.*, 2003). The static view of lysosomes as garbage disposal units has changed in recent years, as it has been shown that lysosomes can interact with other compartments of the endosomal/lysosomal system. There are three well-described routes for delivery of macromolecules to lysosomes: endocytosis, phagocytosis and autophagy.

In general the endocytic pathway is well understood (Kobayashi et al., 1998; Luzio et al., 2003) (Figure 1). Cell surface proteins and lipids are endocytosed and delivered to early endosomes. The majority is rapidly recycled back to the cell surface for reutilization by so-called recycling endosomes. This is the case for many receptors, for example the transferrin receptor, which are returned to the cell surface for repeated binding. Some components, such as the activated EGFR receptor, are instead selectively and efficiently transported to late endosomes and finally to lysosomes to be degraded. The mechanism of delivery from late endosomes to lysosomes is still debated and several hypotheses exist (Luzio et al., 2003). Maturation of endosomes to lysosomes is no longer regarded as the most likely route of delivery, and there is little evidence for vesicular traffic between late endosomes and lysosomes. The "kiss and run" hypothesis was proposed by Storrie and Desjardins and is described as transient fusion and fission processes between the two organelles (Storrie and Desjardins, 1996). The occurrence of complete fusion events between endosomes and lysosomes, possibly developed from an initial kiss, was demonstrated by Mullock et al (Mullock et al., 1998). The fusion would result in a hybrid organelle that acts as the major site for degradation. Lysosomes would then be reformed

from this hybrid organelle by a maturation process which includes condensation of content and removal of some membrane proteins and soluble content by vesicular carriers. Phagosomes are transient vesicles that sequester extracellular materials for their controlled degradation (Bagshaw *et al.*, 2005).



Figure 1: Two proposed routes for delivery of macromolecules to lysosomes: endocytosis and autophagy. Endocytosed material may either be returned from early endosomes to the plasma membrane by recycling endosomes or transported to late endosomes. Delivery to lysosomes may be through transient fusions and fissions (kisses) between the late endosomes and lysosomes, or by complete fusions creating a hybrid organelle. Lysosomes are reformed from the hybrid by condensation and removal of content by vesicular carriers. For autophagic degradation, cytoplasm and organelles are sequestered into an autophagosome, which fuses with a lysosome to create an autolysosome responsible for breakdown. In the trans-Golgi network, macromolecules are modified, sorted and packaged for either secretion or for transport to other cell compartments such as early and late endosomes. In order to maintain cellular homeostasis, some proteins are also retrieved from Luzio *et al* 2003 and Levine 2007.)

Autophagy occurs in response to the need to remodel membranes and remove organelles and/or long-lived proteins during steady state. In addition, during starvation the capacity to degrade endogenous cytoplasmic content is important for survival (Yoshimori, 2004; Levine, 2007). During the process, a single membrane structure called isolation membrane surrounds portions of the cytoplasm and organelles (Yoshimori, 2004) (Figure 1). Fusion of the tips of the isolation membrane forms an autophagosome surrounded by a double membrane. Fusion of autophagosomes with lysosomes creates autolysosomes in which degradation occurs.

Lysosomal hydrolases

By definition, lysosomes contain mature acid-dependent hydrolases (Dell'Angelica et al., 2000), that are responsible for degradation of varying macromolecules including both exogenous and endogenous lipids, glucoconjugates, nucleic acids, and proteins (Johnson et al., 1996; Mason, 1996; Pisoni, 1996; Winchester, 1996). The required hydrolases are synthesized, N-glycosylated, and folded in the endoplasmic reticulum (ER) (Luzio et al., 2003; Bagshaw et al., 2005). In the cis-Golgi, they are recognized and tagged with mannose 6-phosphate moieties, which are recognized by mannose 6-phosphate receptors (MPRs) in the trans-Golgi network (TGN). The bound hydrolases are first delivered to endosomes, where they dissociate from the receptor as a result of the acidic luminal pH, allowing the receptors to recycle back to the TGN (Figure 1). Fusion events between endosomes and pre-existing lysosomes may then be the major route for delivery to lysosomes. The lack of mannose 6-phosphate receptors is an important hallmark of lysosomes separating them from late endosomes.

Cathepsins

Before their intracellular localization was known, the first lysosomal proteases were discovered in 1941 by Fruton and colleagues, who named

them cathepsins A, B, C and D (Gutman and Fruton, 1948). The lysosomal cathepsins are small proteins of approximately 20-40 kDa and have an optimum activity at acidic pH (Bond and Butler, 1987; Mason, 1996). Lysosomal proteases are synthesized as prepro enzymes containing a signal peptide (pre sequence) directing them to the ER (Mason, 1996). The propeptide keeps the enzymes inactive and cleavage during, or shortly after, packaging into lysosomes renders the enzymes active. Cathepsins are divided into aspartic, cysteine and serine proteases according to the amino acid present in the active site. Cathepsins D and E are the only proteases with aspartic acid as the catalytic residue, while the cysteine cathepsins are many (B, C, F, H, K, L, O, S, V, W, X) (Mason, 1996; Turk *et al.*, 2001). Cathepsins A and G are both serine proteases. The best characterized cathepsins are B, H, L and D.

Cathepsin B has an approximate pH optimum of 5 and the instability of the protease increases above pH 6 (Mason, 1996). Depending on substrate and vesicular pH it has both endopeptidase- and exopeptidase activity. Cathepsin B is found ubiquitously in all mammalian cells and tissues and is believed to have a general role in protein turnover. Cathepsin H is primarily an exopeptidase but show weak endopeptidase activity as well. Cathepsin L is one of the most powerful endopeptidases in cells and appears to have a very broad specificity (Mason, 1996). The level of expression of the enzyme is very high in many cell types. It is active in the pH range 4-8, but due to its instability at neutral pH, it appears to degrade most proteins optimally below pH 6. Extracellular and cytosolic endogenous inhibitors of the cystatin family exist to inhibit cysteine cathepsins (Turk *et al.*, 2001). These inhibitors control inappropriate action of the cathepsins after accidental escape from the lysosomes.

Cathepsin D is an endopeptidase with preference for hydrophobic amino acids (Mason, 1996; Godbold *et al.*, 1998). It has limited activity against native proteins but high activity against denatured proteins at pH

3.5-5. Most data has indicated that the enzyme is only active below pH 6.0 but contradictory data exist (Kenessey *et al.*, 1997; Johansson *et al.*, 2008).

The ability of the lysosomal cathepsins to hydrolyze proteins, and to spare others, is determined by the structure of the substrate. This in turn depends on the lysosomal environment; the low pH causes at least partial denaturation of many proteins and is a factor of outmost importance. The lysosomal enzymes are stabile at low pH and become more unstable at the neutral cytosolic pH.



Figure 2: Structure and function of the vacuolar V_0V_1 -ATPase. (Modified from Futai *et al* 2000.)

The lysosomal V₀/V₁-ATPase

Transport into and out of the endo/lysosome is often facilitated or dependent on the highly acidic milieu of the organelle. A vacuolar H⁺-ATPase-proton pump (V₀/V₁-ATPase) located in the membrane is responsible for acidification of the lysosomal lumen. V₀/V₁-ATPases are ubiquitous components of eukaryotic organisms and are in many cells only expressed in vacuolar membranes of the endocytic and secretory pathways where they maintain an acidic interior by pumping protons from the cytoplasm into the vacuole lumen. However, in some specialized cells (renal intercalated cells, macrophages, osteoclasts and tumor cells) V₀/V₁-ATPases can also be found at the plasma membrane (Forgac, 1999). The V₀/V₁-ATPase has a ball-and-stalk structure with membrane extrinsic and intrinsic domains, termed V₁ and V₀, respectively (Forgac, 1999; Futai *et al.*, 2000) (Figure 2). The V₁ domain is a 570 kDa complex (subunits A-H) responsible for hydrolysis of ATP. The V₀ domain is a 260 kDa integral complex (subunits a, b, c, c'and c'') that is responsible for translocation of protons across the membrane. The mechanism for proton translocation is not clearly determined but it is believed to be via a rotary mechanism (Forgac, 1999). In the presence of ATP, H⁺ is pumped over the membrane without using a counter ion and the pumping activity is therefore said to be electrogenic (Rudnick, 1986; Forgac, 1999; Jentsch, 2007). This creates an electric potential ($\Delta\Psi$) across the membrane; the interior becomes positively charged and more acidic. However, H⁺ will also leak from the lumen back into the cytoplasm and in the absence of a neutralizing current, the luminal positive charge would soon inhibit further acidification.

To achieve efficient acidification, Cl⁻ is required to generate HCl in the lysosomal lumen. Several different chloride channels have been shown to reside predominantly on intracellular vesicles. Endocytic and secretory organelles have an internal pH ranging from 4.5 to 6.4 and the vesicles are progressively acidified (Futai et al., 2000; Grabe and Oster, 2001) (Figure 3). pH variations in different compartments might depend on presence of different kinds of Cl⁻ channels; ClC-7 is proposed to be present in late endosomal and lysosomal membranes whereas ClC-3 and ClC-6 probably are expressed predominantly on late endosomes (Jentsch, 2007). ClC-5 is found in the early vacuoles of the endocytic pathway. Regulation of the activity of the V_0/V_1 -ATPase itself may also participate in creating the varying pH in different compartments (Forgac, 1999). One level of regulation is reversible assembly and disassembly of the protein complex, where the dissociated domains of the V_0/V_1 -ATPase are reutilized. Another mechanism of regulation may be reversible formation of inhibitory disulfide bonds between cysteine residues at the catalytic site in response to the redox state of the cytoplasm. Data implying that there is equilibrium between reduced (active) and oxidized (inactive) states of the V₀/V₁-ATPase in vivo has been presented (Forgac, 1999). Thirdly, a change in the coupling efficiency of the V_0/V_1 -ATPase is also proposed as a mechanism to regulate vacuolar acidification. In addition, V₀/V₁-ATPase activity is

regulated by the interaction with activating and inhibiting proteins, though these mechanisms remain largely unknown. Changes in pump density have been shown to control proton transport in the plasma membrane but a role in controlling vacuolar pH has not been established. Moreover, the regulation of organelle pH is not only dependent on changes in the activity of the V₀/V₁-ATPase itself but other factors such as passive proton leakage, the buffering capacity of the luminal matrix, and chloride, potassium and sodium ion concentrations influence the $\Delta \Psi$ (Grabe and Oster, 2001).



Figure 3: Localization of V_0V_1 -ATPase in intracellular compartments. pH of different compart-ments is regulated by inflow of Cl⁻ and the H⁺ pumping ability of the ATPase protein complex. EE: early endosome; ER: endoplasmic reticulum; G: Golgi; LE: late endosome; L: lysosome; SG: secretory granule. (Modified from Grabe and Oster 2001.)

Different forms of cell death

Cell death is an essential part of the normal development and is critically important for tissue homeostasis. It is also the response of living cells to xenobiotic agents, inflammation, and modulations in the environment, such as changes in oxygen supply. The death of cells may occur through different mechanisms leading to distinct morphologies. Historically, three types of cell death have been distinguished in mammalian cells by morphological criteria. Type I cell death is more commonly known as apoptosis, a word coined in 1972 by Kerr *et al* to describe the morphology of this type of cell death (Kerr *et al.*, 1972) (Figure 4). Apoptosis is characterized by rounding up of the cell, reduction of cellular volume, condensation of the chromatin (pyknosis), fragmentation of the nucleus (karyorrhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing and formation of apoptotic bodies that contain nuclear or cytoplasmic material. The plasma membrane remains intact until late stages of the process.



Figure 4: Morphological changes during necrotic (type III) and apoptotic (type I) cell death.

Type II cell death is characterized by accumulation of autophagic vacuoles in the cytoplasm and is more often referred to as autophagic cell death (Kroemer *et al.*, 2005). This term is most often used just to indicate the presence of autophagic vacuoles during cell death and does not necessarily mean that the cell death is dependent on the vacuolization. The presence of autophagic vacuoles in dying cells could indicate either that the cells have activated autophagy as an attempt to survive, or that autophagy is part of the death process. Recent results have shown that induction of autophagic cell death is regulated by expression of autophagy proteins (Atg protein) and that this kind of cell death is an alternative death pathway when apoptosis cannot occur (Eskelinen, 2005). Alternatively, formation of autophagic vacuoles may be necessary for initiation of cell death while

the final cellular breakdown requires signaling to the apoptotic program (Eskelinen, 2005).

Type III cell death is better known as necrosis. Initially, the term necrosis was used to describe irreversible tissue damage, which apparently occurs after the involved cells have already died (Majno and Joris, 1995). Nowadays the term is used to describe a type of cell death that is accidental and acute. The morphology of cells dying by necrosis can be quite diverse but a unifying criterion is an early permeabilization of the plasma membrane, leading to release of cellular contents that trigger an inflammatory response (Golstein and Kroemer, 2007) (Figure 4). Necrosis is associated with early signs of mitochondrial dysfunction, such as production of reactive oxygen species (ROS), swelling and ATP depletion.

The mode of cell death, and how it presents itself morphologically, depends on various factors such as the cell type, the energy level and the signaling pathway. Furthermore, the stimulus and the intensity of the same, as well as other environmental changes are important determinants. All three types of cell death can be found both during development and adult life, and there also exist various intermediate forms displaying both apoptotic and necrotic morphological characteristics. Many names of these types of cell death have also been coined, such as apoptosis-like programmed cell death (Leist and Jäättelä, 2001) and necrosis-like programmed cell death (Kitanaka and Kuchino, 1999; Leist and Jäättelä, 2001). Cell death is thus defined according to morphological criteria and the biochemistry behind these variants of cell death is presently being revealed.

Apoptosis – the role of caspases

The biochemical signaling responsible for the apoptotic morphology is yet quite well known. Our understanding of programmed cell death is based on studies by Horvitz and his colleagues in *Caenorhabtidis elegans* (Metzstein *et al.*, 1998). They identified the *ced-3* and *ced-4* genes of *C. elegans* which were found to be required for somatic cell death, whereas another gene, *ced-9*, was required to prevent cell death. The product of a fourth gene, *egl-1*, served as an upstream initiator of cell death (Conradt and Horvitz, 1998). These four gene products constitute the core of the death machinery in *C. elegans* and are conserved between species. The protease encoded by the *ced-3* gene has several mammalian homologues called caspases (*cysteine aspartate-specific proteases*) (Fuentes-Prior and Salvesen, 2004). There are 11 caspases in humans, some implicated in apoptosis (caspase-2, -3, -6, -7, -8, -9 and -10) and others in activation of pro-inflammatory cytokines (caspase-1, -4 and -5) or in keratinocyte differentiation (caspase-14) (Timmer and Salvesen, 2007).

The caspases involved in apoptosis can be divided into initiator caspases (-2, -8, -9 and -10) and executioner caspases (-3, -6 and -7) (Fuentes-Prior and Salvesen, 2004). In cells, all caspases are present as inactive zymogens (procaspases) which become active after dimerization (Boatright and Salvesen, 2003). Each dimer contains two identical catalytic units composed of one large and one small subunit that are formed from the procaspases by an internal cleavage. However, recent studies have shown that cleavage is neither required nor sufficient for activation of the initiator caspases. Initiator caspases are dimerized and activated in multiprotein complexes via two major pathways known as the intrinsic and extrinsic pathway (described below).

Executioner caspases exist in the cytosol as inactive dimers and are activated by proteolytic cleavage by initiator caspases (Boatright and Salvesen, 2003). The executioner caspases then degrade a number of target proteins. Approximately 300 substrates have been identified in different models of apoptosis (Fischer *et al.*, 2003). Most substrates lose their function due to the proteolytic processing but some gain a function and become active. Proteins cleaved by caspases include, among others, those

involved in DNA synthesis, DNA repair, and cell cycle regulation, cytoskeletal proteins and adhesion proteins (Earnshaw *et al.*, 1999; Nicholson, 1999; Fischer *et al.*, 2003; Van Damme *et al.*, 2005; Timmer and Salvesen, 2007). A classical example is the degradation of ICAD (Inhibitor of caspase-activated DNase). CAD released from its inhibitory protein will cause internucleosomal fragmentation of DNA. Caspase-mediated protein degradation causes loss of cell structure and vital functions. The characteristic morphological features that define apoptosis are thus largely dependent on caspases. Noteworthy, 'apoptosis' has developed into a term that can apply to cell death also without caspase activation (Kroemer *et al.*, 2005).

Caspase activation and activity can be regulated by interactions with inhibitor of apoptosis proteins (IAPs) (Earnshaw *et al.*, 1999). The mammalian inhibitors cIAP-1, cIAP-2, XIAP, NAIP and survivin can inhibit apoptosis induced by a variety of stimuli. XIAP, cIAP-1, and cIAP-2 bind to and inhibit caspase-3 and -7. They also bind to procaspase-9 and prevent its activation. In addition, cIAP-1 and -2 have been observed to bind to TRAF-1 and -2 raising the possibility that the cIAPs might exert additional effects by inhibiting death receptor-initiated caspase activation. Expression of mammalian IAPs varies widely among different cell types.

The intrinsic pathway to apoptosis

The intrinsic pathway is activated in response to cellular stress, such as ionizing radiation, chemotherapeutic drugs, removal of growth factors, and mitochondrial damage (Boatright and Salvesen, 2003). A crucial event in the intrinsic pathway is the mitochondrial outer membrane permeabilization (MOMP) leading to release of proteins located in the space between the outer and inner membrane into the cytosol (Borner, 2003; Donovan and Cotter, 2004; Green and Kroemer, 2004). Cytochrome c is released and becomes part of a complex called the apoptosome which also consists of the CED-4 homolog Apaf-1 (apoptotic protease activating

factor 1) that recruits caspase-9 (Borner, 2003; Green and Kroemer, 2004). The complex also contains ATP. Within the apoptosome, several monomeric caspase-9 molecules are dimerized and activated.

In addition to cytochrome c, other proteins such as AIF (Apoptosis inducing factor), Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with a low pI) and Htr2A/Omi are released during MOMP (Green and Kroemer, 2004; Turk and Stoka, 2007). Of these proteins, Smac/DIABLO and Htr2A/Omi have important functions by binding to IAPs neutralizing their function as caspase inhibitors. The mechanism of MOMP remains controversial but two main models have been suggested (Green and Kroemer, 2004): (i) a so called permeability transition (PT) pore opens in the inner membrane, leading to loss of the mitochondrial membrane potential, and swelling of the matrix with subsequent breakage of the outer membrane as a result, or (ii) formation of pores by pro-apoptotic proteins from the Bcl-2 family.

The Bcl-2 family of proteins

The Bcl-2 proteins are central regulators of apoptosis. The family is subdivided into anti-apoptotic members including Bcl-2 and Bcl-X_L, which protect cells from apoptosis, and pro-apoptotic members such as Bax and Bak and the many BH3-only proteins, which trigger or sensitize cells to apoptosis (Borner, 2003). The anti-apoptotic members are homologs of the *C. elegans* CED-9 protein and contain three to four so-called Bcl-2 homology domains (BH1-BH4). Bax, Bak and Bok are multidomain (BH1-BH3) pro-apoptotic members, which have no known *C. elegans* homologs, while the EGL-1 protein is represented by the large group of BH3-only proteins including Bik, Bim, Bad, Bid, Noxa and PUMA among others.

It has been suggested that anti-apoptotic Bcl-2 proteins act as scavengers for BH3-containing members while the multidomain proapoptotic members are believed to mediate release of mitochondrial proteins by forming channels in the outer mitochondrial membrane (Borner, 2003). The balance between anti-apoptotic and pro-apoptotic members determine the probability of mitochondrial permeabilization. The knowledge of how the multidomain pro-apoptotic proteins become active at the membrane is still inconclusive. It has been proposed that the BH3-only proteins function as 'sensors' and 'mediators' of apoptotic responses (Borner, 2003). Different BH3-only members may sense, and become 'activated' by various apoptotic stimuli. Such activation may be by transcriptional induction (PUMA, Noxa in a p53 dependent way), posttranslational phosphorylation (Bad, Bik), proteolysis (Bid) or release from sequestration with macromolecular structures (Bim and Bmf from the cytoskeleton). Once 'active' the BH3-only proteins may interact with anti-apoptotic Bcl-2 family members to trigger activation of pro-apoptotic proteins and release of pro-apoptotic factors.

The extrinsic pathway to apoptosis

The extrinsic pathway leading to initiator caspase activation is dependent on the binding of death ligands to death receptors of the Tumor Necrosis Factor (TNF)-receptor family (Boatright and Salvesen, 2003). This pathway is mainly utilized to eliminate unwanted cells during development and for immune cells to remove infected cells. The most well-known members of the TNF super family are Fas-Ligand (FasL) and TNF-a. All death receptors contain a protein-protein interaction domain called death domain (DD), which can recruit other DD-containing proteins acting as adaptors for binding of procaspase-8 and -10 via their death effector domains (DED) (Boatright and Salvesen, 2003). The complex formed is called DISC (death-inducing signaling complex). When the initiator monomers are dimerized in the DISC, a weak activity inherent in the procaspases allows cleavage of caspase dimers. This cleavage appears not to be required for formation of an active site but is thought to stabilize the dimer in the DISC complex. The N-terminal DED, through which the caspase is connected to the adaptor proteins, is removed and the active

protease is released to the cytosol. In some cell types, called type I, active caspase-8 then cleaves and robustly activates caspase-3 (Barnhart *et al.*, 2003; Wajant *et al.*, 2003). However, in several other cell types, called type II, the activation of caspase-3 is inefficient and there is need for amplification of the death signal via activation of the mitochondrial pathway. Small amounts of active caspase-8 can cleave the pro-apoptotic Bcl-2 family member Bid generating an active truncated form (tBid) that translocates to mitochondria and stimulates cytochrome c release. By this mechanism the extrinsic and intrinsic pathways are interconnected.

TNF-alpha signaling in apoptosis

While the prominent function of Fas receptor activation is induction of apoptosis, binding of TNF- α to TNFR-1 may induce either cell proliferation or apoptosis (Wajant *et al.*, 2003) (Figure 5). TNF- α , which is a major pro-inflammatory mediator, is mainly produced by macrophages but also by a variety of other tissues. The pro-inflammatory effect of TNF- α is mediated by activation of the transcription factor nuclear factor- κB (NF-KB). In vitro stimulation of TNFR-1 often leads to strong activation of apoptosis only when protein synthesis is reduced or the NF-KB pathway is inhibited. Binding of TNF- α to TNFR-1 causes trimerization of receptors and leads to recruitment of the DD-containing protein TRADD (TNF receptor associated death domain). This protein serves as a platform for binding of FADD (Fas-associated death domain), TRAF2 (TNF receptorassociated factor 2), and RIP (receptor-interacting kinase). FADD is responsible for binding to caspase-8 and -10 by its DED while TRAF 2 and RIP recruit and activate the IKK (I-kB kinase) complex that is responsible for marking the I-KB (NF-KB inhibitory protein) for proteasomal degradation. Once released from IkB, NF-kB can act as a transcription factor for a number of inflammatory related genes as well as for several anti-apoptotic proteins. TNF- α also induces the activation of kinases of the SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase) group that translocate into the nucleus and enhance the activity of transcription

factors important for proliferation, differentiation, and apoptosis (Wajant *et al.*, 2003).

In addition to transcriptional activation of NF- κ B, TNFR-1 activation might lead to signaling through sphingolipids (Figure 5). FAN (factor associated with neutral sphingomyelinase activation) binds to the membrane-proximal region of TNFR-1 and results in activation of neutral sphingomyelinase (nSMase) with subsequent production of ceramide (Wajant *et al.*, 2003). A pro-apoptotic function of nSMase has been described (Segui *et al.*, 2001). Several studies have also implicated FADD-dependent acid SMase (aSMase) activation and ceramide production in TNFR-1 induced cell death (Dressler *et al.*, 1992; Obeid *et al.*, 1993; Wiegmann *et al.*, 1994; Schwandner *et al.*, 1998; Wiegmann *et al.*, 1999). Thus, the outcome of TNF- α binding to the TNFR-1 may be faceted and hard to predict.



Figure 5: Intracellular signaling after binding of TNF- α to TNF Receptor 1 (TNFR-1). Receptor activation leads either to survival through transcription of pro-inflammatory and anti-apoptotic genes or to activation of programmed cell death.

Changes in cytosolic pH during apoptosis

The intracellular H^+ concentration is affected during apoptosis and there are reports showing both cytosolic acidification and alkalinization. Intracellular alkalinization during apoptosis has been observed during cytokine deprivation (Khaled *et al.*, 1999), gamma radiation (Dai *et al.*, 1998), ceramide application (Belaud-Rotureau *et al.*, 2000) and exposure to staurosporine (Fujita *et al.*, 1999) or drugs targeting the proteasome (Kim *et al.*, 2003). This alkalinization is thus encountered during apoptosis involving the mitochondrial pathway and seems to be a caspaseindependent early transient event that may, or may not, be followed by a cytosolic acidification (Khaled *et al.*, 1999; Belaud-Rotureau *et al.*, 2000; Lecureur *et al.*, 2002; Huc *et al.*, 2004). Data indicate that this alkalinization origin either via pH_i-regulating transporters in the plasma membrane or the mitochondria.

A considerably higher number of studies have reported intracellular acidification during apoptosis. Since the first report on acidification during apoptosis in mammalian cells in 1992 (Barry and Eastman, 1992), several reports support the finding that this is a general mechanism. Cytosolic acidification has been detected in response to stimuli such diverse as overexpression of Bax (Matsuyama et al., 2000), UV irradiation (Gottlieb et al., 1996; Matsuyama et al., 2000), staurosporine (Ishaque and Al-Rubeai, 1998; Matsuyama et al., 2000), etoposide (Barry et al., 1993), anti-Fas antibodies (Gottlieb et al., 1996), growth factor deprivation (Li and Eastman, 1995; Rebollo et al., 1995; Gottlieb et al., 1996; Furlong et al., 1997), and somatostatin (Thangaraju et al., 1999). Cytosolic acidification has thus been observed both in death receptor-mediated and mitochondriadependent apoptosis and it has been shown to be either caspase-dependent or -independent. Acidification induced by death receptor ligation has been shown to occur downstream caspase activation (Gottlieb et al., 1996; Szabo et al., 1998; Liu et al., 2000; Gendron et al., 2001; Waibel et al.,

2007). As source of this late cytosolic acidification, both mitochondrial dysfunction and alterations of plasma membrane pH_i-regulation, including alteration of the Na^+/H^+ exchanger (NHE), has been implicated (Lang et al., 2000; Gendron et al., 2001). In contrast to the acidification seen after death receptor stimulation, the decrease in pH_i elicited by mitochondriadependent stimuli appears to be caspase-independent (Furlong et al., 1997; Zanke et al., 1998; Matsuyama et al., 2000). It has been proposed that mitochondria are responsible for the acidification by trapping of organic bases in the mitochondrial matrix, ROS production and by reverse operation of the F₀F₁-ATPase (Matsuyama et al., 2000; Matsuyama and Reed, 2000; Lagadic-Gossmann et al., 2004). The latter scenario has been disputed by reports considering it to be thermodynamically unlikely since the presence of the proton motive force that creates a great H+ gradient in the mitochondrial matrix would hamper the reversal of the H+ pump (Pervaiz and Clement, 2002). A reverse operation of the pump, causing consumption of ATP, outward pumping of protons, and a raise in mitochondrial membrane potential, can be explained by a change in the ADP/ATP ratio or an impairment of the exchange of ATP for ADP between mitochondrial matrix and cytosol (Matsuyama et al., 2000; Matsuyama and Reed, 2000). However, how these changes in ADP/ATP are created is not clear. Decreases in NHE activity has also been suggested as an alternative mechanism of this early cytosolic acidification (Lagadic-Gossmann *et al.*, 2004).

The exact role and the importance of the cytosolic acidification are still debated. Recent reports indicate that it has great contributions by affecting the kinetics of the process but may not be essential for apoptosis to occur. Several endonucleases with low pH-optimum are activated during apoptosis and cellular degradation would be facilitated by acidification of cytosol (Lagadic-Gossmann *et al.*, 2004). In addition, several reports demonstrate that the activation of caspase-9 and -3 is enhanced by cytosolic acidification. The complete maturation of the apoptosome and

activation of caspase-9, is dependent on cytosolic acidification (Beem *et al.*, 2004). Caspase-3 is kept inactive in the cytosol by a 'safety catch' mechanism consisting of an Asp-Asp-Asp tripeptide (Roy *et al.*, 2001). This tripeptide is removed upon acidification and this enhances both autocatalytic maturation and increases the vulnerability to proteolytic activation by initiator caspases. Moreover, acidification may indirectly modify the activity of caspases by modulating the activity of endogenous caspase inhibitors, such as IAPs. Changes in pH_i may also affect the functions of Bcl-2 family proteins. *In vitro* dimerization, as well as channel formation in synthetic membranes, is enhanced by a low pH (Matsuyama and Reed, 2000).

Lysosomes in apoptosis

The role of lysosomes in apoptosis was for a long time believed to be limited to the digestion of engulfed apoptotic bodies. However, partial lysosomal permeabilization with release of proteolytic enzymes into the cytosol, where they actively contribute to apoptosis signaling, is now a well described phenomenon recognized as the 'lysosomal pathway of apoptosis' (Guicciardi et al., 2004). The first report suggesting the implication of cathepsins in apoptosis came in 1996 when Deiss et al showed that cathepsin D mediates cell death induced by interferon- γ , Fas, and TNF- α (Deiss *et al.*, 1996). This report was soon followed by data on the importance of cysteine cathepsins in apoptosis (Guicciardi *et al.*, 2000; Stoka et al., 2001). Cathepsin D has since then been implicated in apoptosis induced by, for example, staurosporine (Bidere et al., 2003; Johansson et al., 2003), TNF-a (Demoz et al., 2002), oxidative stress (Roberg and Öllinger, 1998; Roberg et al., 1999; Öllinger, 2000; Kågedal et al., 2001a), sphingosine (Kågedal et al., 2001b), p53 (Wu et al., 1998), TRAIL (TNF-related apoptosis-inducing ligand) (Nagaraj et al., 2007), and hypoxia (Nagaraj et al., 2007). Cathepsin B has been shown to be essential in different models of apoptosis including bile acid-induced hepatocyte

apoptosis (Roberts *et al.*, 1997; Jones *et al.*, 1998; Faubion *et al.*, 1999; Canbay *et al.*, 2003) and TNF- α -induced apoptosis of primary hepatocytes and tumor cells (Guicciardi *et al.*, 2000; Foghsgaard *et al.*, 2001; Guicciardi *et al.*, 2001). Likewise, cell death induced by TRAIL and hypoxia exposure in oral squamous cell carcinoma cells (Nagaraj *et al.*, 2006; Nagaraj *et al.*, 2007), serum deprivation in PC12 cells (Shibata *et al.*, 1998), and cell death after brain ischemia, was cathepsin B dependent (Yamashima *et al.*, 1998; Tsuchiya *et al.*, 1999). Cathepsin L has been shown to be an important regulator of UV-induced apoptosis in keratinocytes (Tobin *et al.*, 2002) and etoposide-induced apoptosis of p39 cells (Hishita *et al.*, 2001).

The pro-apoptotic function of the cathepsins requires release from lysosomes into the cytosol by lysosomal membrane permeabilization. A massive rupture of lysosomes has been found to cause necrotic cell death while a partial permeabilization results in induction of apoptosis (Li et al., 2000; Bursch, 2001). Several mechanisms for lysosomal membrane permeabilization have been suggested. Accumulation of sphingosine, which possesses detergent properties, has been shown to destabilize lysosomes (Kågedal *et al.*, 2001b) and TNF- α signaling has been shown to generate sphingosine in several studies (Schütze et al., 1999; Werneburg et al., 2002; Werneburg et al., 2004). Lipid peroxidation of the lysosomal membrane may be induced by ROS production during for example oxidative stress induced apoptosis (Zdolsek et al., 1993; Roberg and Öllinger, 1998; Antunes et al., 2001; Dare et al., 2001; Persson et al., 2003). It has also been suggested that low concentrations of H_2O_2 may induce lysosomal destabilization indirectly by activation of phospholipase A2. Such activation, leading to degradation of membrane phospholipids, has been detected during TNF- α and oxidative stress induced apoptosis (Jäättelä et al., 1995; Suzuki et al., 1997; Zhao et al., 2001). In TNF-a induced apoptosis, lysosomal membrane permeabilization has been shown to require the presence of cathepsin B, caspase-8, and Bid (Werneburg et *al.*, 2002; Werneburg *et al.*, 2004; Guicciardi *et al.*, 2005). Recent reports have presented new data on the importance of Bcl-2 family proteins in lysosomal destabilization suggesting that pro-apoptotic Bax can permeabilize lysosomes in a manner similar to mitochondria (Kågedal *et al.*, 2005; Feldstein *et al.*, 2006; Werneburg *et al.*, 2007).



Figure 6: The extrinsic and intrinsic pathways of apoptosis and the involvement of lysosomal cathepsins. (Modified from Turk and Stoka 2007.)

Lysosomal permeabilization is often an early event preceding mitochondrial membrane permeabilization and caspase activation (Roberg and Öllinger, 1998; Guicciardi *et al.*, 2000; Li *et al.*, 2000; Bidere *et al.*, 2003; Boya *et al.*, 2003b; Boya *et al.*, 2003c; Liu *et al.*, 2003). The mechanism by which cytosolic cathepsins promote apoptosis is not fully understood. Direct cleavage and activation of caspases does not seem to be the main function, since many procaspases are poor substrates for cathepsins *in vitro* (Vancompernolle *et al.*, 1998; Stoka *et al.*, 2001). Several reports indicate that the cathepsins may act on mitochondria to

induce release of proapoptotic factors (Guicciardi et al., 2000; Stoka et al., 2001; Bidere et al., 2003; Boya et al., 2003b; Boya et al., 2003c; Zhao et al., 2003; Cirman et al., 2004; Johansson et al., 2008) (Figure 6). One link between cathepsins and mitochondria may be through the Bcl-2 family protein Bid, which is cleaved and translocated to the mitochondria following lysosomal permeabilization using lysosomotropic agents (Cirman et al., 2004; Johansson et al., 2008). By test tube experiments, Bid has been shown to be cleaved and activated by several cathepsins including cathepsin B, D, H, L, K and S (Cirman et al., 2004; Heinrich et al., 2004; Johansson et al., 2008). However, in some models of apoptosis, the action of cathepsins might be other then Bid cleavage (Boya et al., 2003a; Houseweart et al., 2003). Cathepsin D has been shown to activate Bax in a Bid-independent manner leading to mitochondrial release of AIF and to apoptosis (Bidere et al., 2003). The importance of Bax and Bak for mitochondrial permeabilization has also been shown after treatment with lysosomotropic agents (Boya et al., 2003b; Boya et al., 2003c). It appears thus, that cathepsins mediate apoptosis via multiple pathways and different cathepsins might be engaged depending on the apoptotic stimuli and cell type.

Cancer

Cancer is characterized by genetic alterations leading to activation of oncogenes and silencing of tumor suppressor genes (Hanahan and Weinberg, 2000). Tumorigenesis is a multistep process in which a series of genetic changes, each conferring some type of growth advantage, leads to the progressive conversion of normal cells into cancer cells. Six essentially acquired alterations necessary and common to most types of human cancers have been suggested by Hanahan and Weinberg (Hanahan and Weinberg, 2000). These hallmarks are (a) self-sufficiency in growth signals; (b) insensitivity to growth-inhibitory signals; (c) evasion of programmed cell death (apoptosis); (d) limitless replicative potential; (e) sustained angiogenesis; and (f) tissue invasion and metastasis. An increased mutability acquired by changes in the systems guarding the genome is a prerequisite for the multiple numbers of individual mutations necessary for tumor development to occur. A growing number of genes involved in sensing and repairing DNA damage are found to be lost in the majority of cancers, allowing genome instability and variability. The most well-known and most common change is loss of the p53 tumor suppressor protein, which should, in response to DNA damage, elicit either cell cycle arrest or apoptosis.

Head and neck squamous cell carcinomas

The vast majority of the malignancies in the head and neck region are squamous cell carcinomas. Head and neck cancer is the sixth most common type of cancer world wide, representing about 6% of all cases (Argiris *et al.*, 2008). The most important risk factors for head and neck squamous cell carcinomas (HNSCCs) are tobacco and alcohol consumption and human papilloma virus (HPV) infection. About two-thirds of the patients with HNSCCs present with advanced stage disease. For all disease stages combined, the 5-year survival is about 60 %.

A large number of genetic and epigenetic alterations govern the development of HNSCCs (Argiris *et al.*, 2008). Telomerase, involved in telomere maintenance, is reactivated in 90% of all HNSCCs and in premalignant lesions. The loss of chromosome band 9p21 is also a very common genetic aberration seen in 70-80% of HNSCCs. Overexpression of EGFR (epidermal growth factor receptor), a member of the ErbB growth factor receptor tyrosine kinase family, is found in 90 % or more of all HNSCCs. Ligation of EGFR leads to activation of downstream pathways regulating proliferation, apoptosis, metastatic potential, and angiogenesis.

Surgery and radiotherapy have long been the major treatment approaches but systemic chemotherapeutic agents may also be included to improve the clinical outcome (Argiris *et al.*, 2008). Various classes of agents, including platinum compounds, such as cisplatin, antimetabolites, and taxanes have shown activity against HNSCCs. Cisplatin is regarded as a standard agent in combination with radiation or with other substances. Lately, EGFR inhibitors have emerged as a treatment strategy.

The anti-cancer agent cisplatin – cytotoxicity and resistance

Platinating agents, including cisplatin, carboplatin, and oxaliplatin, have been used clinically for almost 30 years in the treatment of testicular, ovarian, cervical, lung, colorectal and head and neck cancer (Siddik, 2003). Cisplatin (*cis*-diammine-dichloroplatinum; *cis*- $[PtCl_2(NH_3)_2]$) is a rather small uncharged molecule with electrophilic/oxidizing properties (Jamieson and Lippard, 1999). In water solution, cisplatin is hydrolyzed and a chloride ion is exchanged to a water molecule forming a monohydrated complex (Figure 7). In its protonated, positively charged form, this monohydrated complex is very reactive. Consequently, in plasma where the chloride concentration is relatively high (100 mM), cisplatin is the dominant species whereas formation of the monohydrated complex is promoted intracellularly where the chloride concentration is low (~20 mM). The positive charge of the monohydrated complex may cause an electrostatic attraction to negatively charged cell components, including the target considered most important; DNA. The platinum atom of cisplatin forms covalent bonds to purine bases and causes intra- or interstrand cross-links (Siddik, 2003). This DNA damage will either be repaired or the apoptotic program is activated.



Figure 7: The chemical structure of the neutral chemotherapeutic drug cisplatin and its hydrolyzed form. Intracellularly, where the chloride concentration is low, a chloride ion is exchanged to a water molecule forming a reactive monohydrated complex.
Although cisplatin is a very potent inducer of apoptosis, treatment resistance is a well-recognized clinical problem. The resistance can be acquired through chronic drug exposure or it may be an intrinsic phenomenon of the tumor cell (Siddik, 2003). Resistance can be a consequence of intracellular changes that either prevent cisplatin from interacting with DNA, interfere with DNA damage signals from activating apoptosis, or both. Reduced DNA damage may be caused by decreased drug accumulation, increases in the amounts of intracellular thiol capable of inactivating the drug, and/or an enhanced rate of DNA adduct repair. In general, several mechanisms are encountered simultaneously and a high resistance is a net effect of several unrelated mechanisms. Evidence indicate that reduced drug accumulation, due to inhibited drug uptake or increased efflux, is a significant mechanism of cisplatin resistance (Siddik, 2003). The mechanisms behind cisplatin resistance have mainly been studied using highly cisplatin-resistant cell lines, generated by repeated exposures of a sensitive parental cell line to increasing concentrations of the drug. These models are argued to clinically correspond to resistance developed by chronic drug exposure. In contrast, mechanisms behind variations in intrinsic sensitivity are less well-studied.

Cancer stem cells

The prevailing idea has long been that the initiating event of carcinogenesis is the immortalization of a normal cell, an idea lately challenged by the stem cell theory of cancer (Figure 8). Stem cells are characterized by their self-renewal capacity. They can divide both symmetrically, producing two new stem cells or two progenitor cells that becomes terminally differentiated, or asymmetrically into one stem cell and one progenitor cell. Trosko *et al.* argue for a role of these normally immortal stem cells and their early progenitor cells as the targets for initiation of tumorigenesis (Trosko *et al.*, 2004). The first initiating event would then be to prevent the

terminal differentiation of the stem cell by blocking or decreasing the number of asymmetrical divisions.



The existence of a subpopulation of malignant stem cells that drives tumor growth is not a new concept (Hamburger and Salmon, 1977). However, due to technical difficulties, the existence and importance of cancer stem cells (CSCs) has been controversial. It is well known that only a minority of the tumor cells found in haematopoetic malignancies or solid cancers have the ability to form clones in cell culture or new tumors when injected into immunodeficient mice (Al-Hajj and Clarke, 2004; Neuzil et al., 2007). Such 'tumor initiating cells' (TICs) have been shown to share stem cell characteristics. Because the origin of the TIC is not yet fully established, the question whether the TICs truly represent cancer stem cells, or not, is still unclear (Figure 8). If the TICs are derived from progenitor cells, oncogenic mutations must occur to reactivate self-renewal pathways to lend the tumor stem cell properties and ability to dedifferentiate. Alternatively, the TIC may be derived from an initiated normal stem cell with self-renewing capacity.(Cobaleda et al., 1998; Trosko *et al.*, 2004). Evidence suggest that both scenarios could take place. However, in certain tissues where the stem cells are the only long-lived cells, such as many epithelial tissues, the second model may be the most plausible (Al-Hajj and Clarke, 2004). If cancer stem cells are truly derived from normal stem cells, they probably share many common properties. In normal tissue, stem cells and amplifying cells differ in their patterns of division, apoptotic sensitivity and in their expression of several genes, including those of multi-drug resistance transporters (Al-Hajj and Clarke, 2004; Locke *et al.*, 2005). The escape of resistant cancer stem cells from being killed during chemo- or radiotherapy has been suggested as an explanation to formation of secondary tumors.

Earlier observations have indicated that also cancer cell lines contain cells with stem cell properties (Locke *et al.*, 2005; Costea *et al.*, 2006; Harper *et al.*, 2007). The parallels existing between normal somatic stem cells and CSCs, such as the shared organogenic capacity, suggest that the principles of normal stem cell biology may be applied to identify

CSCs. Normal keratinocytes grown at clonal density form three types of colonies; (i) holoclones with small tightly packed cells having high proliferative potential correspond to stem cells, (ii) meroclones with an irregular shape containing fewer cells with proliferative capacity, represent early transit amplifying cells, and (iii) paraclones containing large flattened cells with low proliferation correspond to late transit amplifying and differentiated cells (Figure 8) (Barrandon and Green, 1985; Barrandon and Green, 1987). These morphological and clonogenic properties have been shown to persist in carcinoma derived epithelial cell lines after in vitro propagation (Costea et al., 2006; Harper et al., 2007; Locke et al., 2005). Also cell surface markers useful for isolation of somatic stem cells have proven valuable in cancer biology, first in identification of leukemic stem cells and later in the search for CSCs in solid tumors (Cho and Clarke, 2008). High expression of CD44 at the cell surface has been used both in breast cancer and HNSCCs to identify subpopulations of cells with tumorigenic potential (Al-Hajj et al., 2003; Prince et al., 2007; Cho and Clarke, 2008). Few reliable stem cell markers have been found for normal oral epithelium but it has been shown, using HNSCC-derived cell lines, that holoclones show consistently higher expression of stem cell-related molecules such as β 1-integrin, E-cadherin, β -catenin and epithelial specific antigen (Costea et al., 2006).

Evasion of apoptosis in cancer cells

In normal cells, un-repairable DNA damage or excessive mitogenic signaling leads to stabilization of the p53 protein and induction of apoptosis. Loss or inactivation of p53 is found in half of all human tumors and is therefore considered as one of the most important defects in the defense against malignant transformation. In addition, several other anti-apoptotic changes have been found in tumor cells, for example decreased expression of the Fas receptor in hepatomas (Strand *et al.*, 1996). Alternatively, tumor cells overexpress decoy receptors such as DcR3, a

secreted polypeptide that bind to FasL and inhibits its ability to induce Fasmediated apoptosis (Pitti *et al.*, 1998; Bai *et al.*, 2000). Downregulation of procaspase-8 has been found in small-cell lung cancer and neuroblastomas (Joseph *et al.*, 1999; Teitz *et al.*, 2000). Thus, inactivation of the death receptor pathway by these mechanisms may give tumor cells reduced susceptibility to killing by cytotoxic lymphocytes and also against suicidal signals triggered by suboptimal growth conditions (Kaufmann and Gores, 2000).

Bcl-2 is the first example of an oncogene that acts by inhibiting cell turnover rather than enhancing cell proliferation. Bcl-2 is overexpressed in a wide variety of human cancers and often associated with a poor prognosis (Reed, 1998; Kaufmann and Gores, 2000). Downregulation of the pro-apoptotic protein Bax has also been reported in various neoplasms (Reed, 1998). Aberrant expression of IAPs might also play a role in carcinogenesis, for example overexpression of survivin has been observed in different cancers (Ambrosini *et al.*, 1997; Kawasaki *et al.*, 1998; Monzo *et al.*, 1999).

The phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway is activated by many cellular stimuli and regulate proliferation as well as apoptosis. Cell surface receptor tyrosine kinases signal through Ras to the lipid kinase PI3K and then to 3-phosphoinositide-dependent protein kinases that phosphorylate and activate Akt (Kaufmann and Gores, 2000). Akt, in turn, can inhibit cytochrome c release, as well as activation of the death receptor pathway (Gibson *et al.*, 1999; Kennedy *et al.*, 1999). The PI3K/Akt pathway can be altered at several steps in tumor cells, including enhancement of signaling from tyrosine kinase receptors, constitutively active Ras isoforms, and amplification of the genes for PI3K or Akt (Kaufmann and Gores, 2000). Clearly, many of the steps of the apoptotic process can be disabled in cancer.

Cancer cells are nonetheless able to undergo programmed cell death. Particularly during early stages of tumorigenesis, cells are sensitized to death stimuli and often undergo spontaneous cell death, possibly due to activation of oncogenes such as *myc* or *ras* (Hanahan and Weinberg, 2000). Interestingly, an initial sensitization to cell death induced by lysosomal pathways is found during immortalization and oncogene-driven transformation of cells (Fehrenbacher *et al.*, 2004; Fehrenbacher and Jäättelä, 2005). Spontaneous immortalization of murine embryonic fibroblasts resulted in a cathepsin B dependent sensitization to TNF-induced cell death (Fehrenbacher *et al.*, 2004). The lysosomal death pathway, mediating caspase- and mitochondrion-independent programmed cell death, has also been suggested to remain functional in advanced tumor cells and has therefore become an interesting target for new therapeutic intervention (Fehrenbacher and Jäättelä, 2005).

Lysosomal changes in cancer

Lysosomal functions might be altered in cancer cells. Increases in expression of cysteine cathepsins often occur already in pre-malignant or early lesions (Mohamed and Sloane, 2006). In addition, studies have demonstrated that transformation of cells can increase the expression of cathepsins B and L (Tardy et al., 2006). An increase in the expression and/or activity of lysosomal cathepsins, including cathepsins B, D and L, has been demonstrated in human tumors, such as breast, lung and brain (Nomura and Katunuma, 2005; Mohamed and Sloane, 2006; Tardy et al., 2006). Moreover, overexpression of cathepsins has been correlated to aggressiveness and bad prognosis. In primary breast cancer. overexpression of cathepsin D is well studied and the concentration correlates to development of metastasis (Garcia et al., 1996; Rochefort and Liaudet-Coopman, 1999; Berchem et al., 2002).

Active as well as inactive precursor forms of cysteine cathepsins are secreted from both transformed cells and various tumors such as breast, lung and brain (Nomura and Katunuma, 2005; Mohamed and Sloane, 2006; Tardy *et al.*, 2006). Transformation of cells has been demonstrated to affect the processing and localization of cathepsins B and D (Tardy *et al.*, 2006). Extracellular roles for secreted cysteine cathepsins may be cleavage of extracellular matrix proteins, cell-adhesion proteins, and activation of pro-enzymes (Mohamed and Sloane, 2006). Cathepsin B has been shown to contribute to tumor angiogenesis possibly by degrading the extracellular matrix and inactivating tissue inhibitors of the matrix metalloproteinases, TIMP-1 and -2 (Tardy *et al.*, 2006). Cathepsin D has been demonstrated to promote tumor progression and angiogenesis when secreted in its catalytically inactive form (Berchem *et al.*, 2002; Liaudet-Coopman *et al.*, 2006). This finding suggests that it may act as a mitogenic factor on cancer cells, fibroblasts, and endothelial cells by stimulating a still unidentified receptor.

In cancer cells, secretion of cathepsins to the extracellular space is facilitated by altered trafficking of lysosomes, which results in a shift of localization from perinuclear to peripheral. Active Ras seems to be important for the changes in lysosomal trafficking and size, possibly by acting through the small GTPases RhoA, ROCK (Rho-associated coiled-coil containing protein kinase), and LIMK1 (LIM-domain kinase 1) (Nishimura *et al.*, 2002; Nishimura *et al.*, 2003; Nishimura *et al.*, 2004). *Ras* or *Src* transformation of NIH3T3 fibroblasts has been shown to change the distribution, density and ultrastructure of the endo/lysosomal compartment (Fehrenbacher *et al.*, 2008). Moreover, the downstream Ras effector PI3K, which regulates the maturation, size, and content of the lysosomal compartment, displays increased activity in many cancers (Brown *et al.*, 1995; Mousavi *et al.*, 2003).

Alterations of the lysosomes of cancer cells may have effects on their susceptibility to lysosomal membrane permeabilization. *ras* or *src* transformation of fibroblasts causes an upregulation of cysteine cathepsin expression and activity, leading to a reduction in the levels of LAMP proteins (Fehrenbacher et al., 2008). This reduction sensitizes the transformed cells to cell death induced by various anticancer drugs including cisplatin, etoposide, doxorubicin and siramesine. Interestingly, expression of the active ras or erbb2 oncogene causes reduction of LAMP levels in human colon carcinoma and breast cancer cells, respectively (Fehrenbacher et al., 2008). In the process of cancer progression, upregulation of the transcription of *lamp* mRNAs may be one way to compensate for cathepsin-mediated increase in LAMP turnover. Accordingly, increased levels of mRNAs for *lamps* have been reported for various cancers (Ozaki et al., 1998; Furuta et al., 2001). An additional way to compensate for increased susceptibility to membrane destabilization is translocation of HSP70, the major stress-inducible member of the heat shock protein 70 family, to the inner leaflet of the endo-lysosomal membranes. Such translocation has, in cancer cells, been found to stabilize the lysosomes against membrane permeabilization induced by TNF, etoposide, γ -irradiation, hydrogen peroxide, or photolysis (Nylandsted *et* al., 2004). Changes in lipid composition of the lysosomal membrane may also be present in cancer cells thereby influencing the membrane fluidity, stability and permeability (Olsson et al., 1991). Cholesterol, for example, induces rigidity and decreases both fluidity and permeability of membranes (Block, 1985). In human hepatocellular carcinomas, total levels of cholesterol were increased (Eggens et al., 1989), however, no reports on increased levels in lysosomal membranes specifically have been found.

AIMS OF THE THESIS

The general objective of this thesis was to investigate the participation of the lysosomal compartment during apoptosis signaling in normal cells and cancer cells.

More specifically the aims were to:

- develop a flow cytometric method for analysis of lysosomal pH
- characterize cytosolic and lysosomal pH during TNF-α-induced apoptosis
- investigate if proton release from lysosomes may cause cytosolic acidification during apoptosis
- study lysosomal pH of normal oral keratinocytes and head and neck squamous cell carcinoma cells (HNSCC) in relation to cisplatin toxicity and cellular uptake
- investigate the involvement of cathepsins in cisplatin-induced apoptosis
- isolate and characterize cancer stem cells in selected HNSCC cell lines (LK0412 and SqCC/Y1)
- use selected HNSCC cell lines to study sensitivity to cisplatin and radiation in isolated cells with stem cell characteristics and more differentiated cells
- characterize the lysosomal compartment in cells with stem cell characteristics and more differentiated cells

MATERIALS AND METHODS

Cells

U937 cells

Papers I and II are based on studies using the human histiocytic lymphoma cell line U937. This is a cell type that is sensitive to TNF- α without inhibition of the NF κ B pathway or of protein synthesis. The cells were grown in suspension in RPMI 1640 media supplemented with 2 mM glutamine, 10% fetal bovine serum, 50 IU/ml penicillin-G, and 50 µg/ml streptomycin at 37°C in humidified air with 5% CO₂. The cell density was kept at 1-2 × 10⁶ cells/ml by sub-cultivation twice a week.

Normal oral keratinocytes

In paper III, NOKs were used as control cells. NOKs were obtained from biopsies, containing non-keratinized squamous cell epithelium, harvested during benign surgery in the oral cavity. Primary keratinocyte cultures were derived from trypsin-digested tissue (Grafström, 2002). NOKs were grown in serum free keratinocyte media (Keratinocyte-SFM containing Bovine Pituitary Extract and recombinant Epidermal Growth factor) supplemented with penicillin 100 U/ml and streptomycin 100 μ g/ml. The cells were incubated in humidified air containing 5% CO₂ at 37°C and cultures in passages 2 and 3 were used for experiments. The experiment with NOKs was approved by the Ethical Committee of Linköping University.

Oral squamous cell carcinoma cells

In paper III and IV, oral squamous cell carcinoma cell lines (LK0412, LK0626, SqCC/Y1, UT-SCC-9, and UT-SCC-33) were used. These oral squamous cell carcinoma cell lines were grown in serum free keratinocyte media supplemented with penicillin 100 U/ml and streptomycin 100 μ g/ml. The cells were incubated in humidified air containing 5% CO₂ at 37°C and

subcultured once a week. The LK0412 cell line was established from a moderately differentiated squamous cell carcinoma from the tongue by dispase/collagenase digestion in the above described growth medium (Roberg *et al.*, 2008). LK0626, from gingiva, was established by the explant outgrowth technique using Keratinocyte-SFM supplemented with 10% of serum and was after 5 passages gradually transferred to the above described serum-free growth medium. Establishment of the LK cell lines was approved by the Ethical Committee of Linköping University. The SqCC/Y1 cell line is a well studied buccal carcinoma line that has been adapted to serum free conditions (Sundqvist *et al.*, 1991). UT-SCC-9 is from glottic larynx and UT-SCC-33 from gingiva (Pekkola-Heino *et al.*, 1995). The cell lines were adapted to serum free growth by sequential transfer of the cells to media with lower and lower concentration of serum.

Induction of cell death and inhibition of cell growth

TNF-α

In papers I and II, 1×10^6 U937 cells were exposed to 10 ng/ml of recombinant TNF- α to induce apoptosis via the death receptor pathway. The cells were incubated for varying time periods at 37°C in humidified air with 5% CO₂.

MSDH

In paper I, lysosomal permeabilization was induced by incubation of 1×10^6 U937 cells with 20 µM of the synthetic lysosomotropic detergent Omethyl-serine dodecylamide hydrochloride (MSDH) for 2 hours. MSDH is an amine with a long hydrophobic chain that is accumulated inside lysosomes and upon protonation acquires detergent properties, which cause disruption of the lysosomal membrane (Dubowchik *et al.*, 1995). MSDH has earlier been shown to induce apoptosis in macrophage-like cells, HeLa cells and fibroblasts (Li *et al.*, 2000; Terman *et al.*, 2002; Zhao *et al.*, 2003).

Cisplatin

In papers III and IV, cell death or cell growth inhibition was induced by exposure of cells to varying concentrations (1.25-10 μ g/ml) of the chemotherapeutic drug cisplatin.

Radiation

In paper IV, cells were irradiated with photons from a 4 MV linear accelerator delivering 2 Gy/min. In total, cells were exposed to 4 Gy. The field size was $30x30 \text{ cm}^2$, the source surface distance was 100 cm, and 3-cm polymethyl methacrylate was placed above and 10 cm below the cells. After exposure to irradiation, new cell culture medium was added.

Inhibitors

ATPase inhibitors

The mitochondrial F_0F_1 -ATPase was in paper II inhibited by incubation with 5 µM oligomycin, an inhibitor produced by *Streptomyces diastatochromogenes* (Slater, 1967; Robinson and Flashner, 1979). Oligomycin inhibits the H⁺ transporting ATP synthase and the Na⁺/K⁺ transporting ATPase. The antibiotic bafilomycin A1, produced by *Streptomyces griseus*, is considered to be a relatively specific and potent inhibitor of vacuolar ATPases (Bowman *et al.*, 1988). The lysosomal V₀V₁-ATPase was in paper II inhibited using 100 nM of bafilomycin A1 and in paper III using 20 nM of bafilomycin A1 leading to increase in lysosomal pH.

Caspase inhibitors

The structures of synthetic caspase inhibitors are based on the peptide sequence preceding the cleavage site of known cellular caspase substrates (Callus and Vaux, 2007). The peptides are conjugated to different chemical groups that improve cell permeability, stability and efficacy. Peptides linked to aldehydes, nitriles or ketones are reversible inhibitors that

compete with the substrate for enzyme binding. Peptides linked to a leaving group, such as fluoromethyl ketone (FMK), will chemically alter the enzyme and are irreversible inhibitors. In paper II, the synthetic tripeptide inhibitor Boc-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-FMK, 10 μ M) was used to inhibit caspases. This substance has a broad specificity and inhibits all caspases, although caspase-2 very weakly.

Cathepsin inhibitors

To study the involvement of lysosomal cathepsins in the cell death induced by cisplatin in paper III, cathepsin inhibitors were employed. Cathepsin D was inhibited by 50 μ M of pepstatin A, a pentapeptide produced and secreted by *streptomyces* species. Pepstatin A contains the amino acid statin which reacts with the catalytic site residues of aspartic proteases. Beside cathepsin D, pepstatin A inhibits renin, pepsin and cathepsin E. However, renin and pepsin are extracellular proteases and cathepsin E is expressed mainly in leukocytes and macrophages. Pepstatin A is, therefore, considered to be a specific inhibitor of cathepsin D in our experimental system. Pepstatin A is not cell permeable and is believed to reach lysosomes by endocytosis. The activity of cysteine cathepsins (mainly cathepsin B and L) was inhibited by 25 μ M of the synthetic inhibitor z-Phe-Ala-FMK (zFA-FMK), which is irreversible due to its FMK group.

Detection of cell death/cell growth inhibition

Morphological studies using light microscopy

The morphological changes characteristic of apoptosis, such as reduction of the cell volume, membrane blebbing and, later on, formation of apoptotic bodies, are readily observed in a standard light microscope. The cell remnants and apoptotic bodies are normally phagocytosed by macrophages or neighboring cells. However, in cell culture models there are no phagocytosing cells available and apoptotic cells may eventually lose the plasma membrane integrity and undergo so-called post-apoptotic necrosis. In all experiments throughout this thesis, inspection in light microscopy was regularly performed to examine cellular morphology. In paper I, morphology was judged in order to quantitatively determine the percentage of apoptotic and necrotic cells after TNF- α exposure. In order to be able to distinguish the apoptotic cells from cells which have lost plasma membrane integrity (necrotic and post-apoptotic necrotic), the cells were stained using trypan blue (a dye that binds to negative cell structures, mainly DNA, described below) that only enters cells with a permeable plasma membrane.

Assessment of caspase activity

Caspase activity is determined by several methods. To study the occurrence of active caspases in live cells, cells are incubated with a fluorescently tagged cell permeable pan-caspase inhibitor; FITC-VAD-FMK, as performed in paper I and II. This inhibitor will bind to several different active caspases in the cell. The fluorescent apoptotic cells can then be studied by fluorescence microscopy or flow cytometry.

The total activity of a certain caspase in a cell population can be studied using a fluorescently conjugated substrate molecule, and such technique is represented in papers I-III. The substrate consists of four amino acids, always ending with an aspartic acid, recognized by the caspase of interest. Cleavage results in release of a formerly quenched fluorescent molecule. For caspase-3, the substrate Ac-DEVD-7-amino-4-methylcoumarin (AMC) is incubated with lysed cells and liberated fluorescent AMC is detected. To detect the initiator caspase-8, the substrate contains the amino acids IETD that is specifically recognized by this caspase. Using free AMC, a standard curve can be prepared and the concentration of liberated AMC calculated. The concentration is then correlated to the total amount of protein in the sample (as detected by the method of Lowry, (Lowry *et al.*, 1951)) and caspase activity expressed as pmol AMC/mg protein/h.

Assessment of phosphatidyl serine exposure

Externalization of phosphatidyl serine (PS) on the outer leaflet of the plasma membrane is one characteristic change during apoptosis that signals to phagocytosing cells. PS is normally found on the inner leaflet of the membrane and is only during short periods exposed to the outside. The enzymes regulating the flipping of PS between the inner and outer leaflet are inactivated by caspase cleavage, leading to constant exposure of the phospholipid on the outside. PS can be detected using the fluorescently tagged Ca²⁺-dependent phospholipid-binding protein Annexin V that has a high affinity for this phospholipid. However, since necrotic cells loose the membrane integrity, Annexin V can bind to PS found on the inner leaflet of necrotic cells. To separate the necrotic cells from apoptotic ones, the sample is incubated with both the Fluos-tagged Annexin V (green fluorescence) and propidium iodide (red fluorescence). Propidium iodide can only pass permeable membranes and binds to DNA in necrotic cells. Necrotic cells will thus show both green and red fluorescence while apoptotic cells only present green fluorescence. The cells can either be observed in a fluorescence microscope or analyzed using flow cytometry. In paper II, U937 cells exposed to TNF-a were stained with Annexin V-FLUOS and propidium iodide, and analyzed by flow cytometry, to confirm the identity of the apoptotic cell population.

Assessment of mitochondrial membrane potential

An important parameter of mitochondrial function is the membrane potential. JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'-tetraethyl-benzimisdazolylcarbocyanine iodide) is a lipophilic cationic dye that in its monomeric form exhibits green fluorescence. Upon potential-dependent accumulation in mitochondria, an emission shift from green to red will take place due to concentration-dependent formation of red fluorescent J-aggregates (Reers *et al.*, 1991; Smiley *et al.*, 1991). Mitochondrial depolarization, associated with apoptosis, is thus indicated by a decrease in the red/green fluorescence intensity ratio. JC-1 stained cells may be studied by fluorescence microscopy using different optical filter configurations. In paper II the mitochondrial function of cells considered healthy, preapoptotic, and apoptotic was investigated using JC-1. Here the probe was excited in the flow cytometer by an argon laser and green and red fluorescence were detected by using a bandpass (BP) 530±28 nm and a long pass (LP) 670 nm filter, respectively.

Assessment of MTT reducing potential

The MTT assay, which is a widely used tool for assessment of cell viability and proliferation, was used in paper III. In cells with functionally active metabolism, the yellow tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide; MTT) is reduced to insoluble purple formazan crystals. This reduction takes place in the mitochondria and requires active reductase enzymes and therefore conversion into crystals is directly related to the number of viable cells. By addition of a solvent (usually dimethyl sulfoxide), the crystals are solubilized and the absorbance of the colored solution is quantified by spectrophotometry.

Assessment of colony-forming efficiency

To study the effect of different drug treatments or irradiation on tumor cells, a colony-forming efficiency assay is often used. This assay examines cell proliferation at low cell densities and is especially useful to evaluate the effect of clinically relevant doses of drugs or radiation. In paper IV, the cell lines were seeded at low density (100 cells/cm²) and incubated for 24 hours to allow the cells to attach and start to proliferate. Individual cells can then multiply into clones and form colonies. A seeding density that gives at least 100 but preferably less than 500 colonies per dish should be used. This will generate a sufficient number of colonies to give reliable data and reduce the risk of having several colonies growing together. The cells were after drug or radiation exposure incubated in fresh media for seven days before they were fixed in 4% formalin and stained with 2%

Giemsa. Colonies containing 32 cells or more were counted. The colonyforming efficiency of untreated cells (control) was set to 100%.

DNA-binding dyes

There are several DNA-binding dyes available. Trypan blue and the intercalating dye propidium iodide, only enter cells with a permeable plasma membrane, and are often used to distinguish necrotic cells from apoptotic ones. Using light microscopy and flow cytometry, respectively, these dyes were used in paper I for determination of the fraction of apoptotic and necrotic cells. DAPI (4,6-diamidino-2-phenylindol) is mostly used for microscopical studies of nuclear morphology. DAPI, which is excited by UV light, requires fixation to effectively stain DNA where it binds to the minor grooves of the molecule. In paper II, DAPI staining was used to confirm the identity of cells which were flow cytometrically sorted as either healthy, pre-apoptotic or apoptotic. Hoechst 33342 is a cell permeable DNA-binding dye which binds to the minor groove, and may be used for vital staining and flow cytometry. Hoechst 33342 is excited by UV and the emission may be detected using either a BP 450 ± 50 nm and/or a LP 750 nm filter as in paper IV. Several normal tissues, as well as human cancers, have been found to contain cells with the ability to efflux Hoechst 33342. These cells show low Hoechst 33342 fluorescence and are called side population (SP) cells.

Flow cytometry and cell sorting

Flow cytometry is a technique used for simultaneous study of several parameters such as size, scattering of light (granularity), DNA content and/or the occurrence of one or several proteins, of a single cell passing through a measurement chamber (Haynes, 1988). Usually 500-4000 cells per second can be measured, depending on the equipment, and the technique is therefore ideal for rapid analysis of large quantities of cells. In a flow cytometer, a single cell is hydrodynamically focused to the center of

an orifice by a surrounding sheath of particle-free fluid and is then illuminated by a laser (Haynes, 1988). The scattered light and emitted fluorescence passes through a filter system into separate detectors. The size of the cell is determined by a detector placed right in front of the laser (forward scatter; FSC) and the larger the cell, the less light will reach the detector. A detector placed at 90 degrees angle from the laser will register light reflected from the cell (side scatter; SSC). The higher granularity of the cell, the more light will be reflected. In order to study cell death, the FSC and SSC parameters can be used to define apoptotic and necrotic cells, as performed in paper I. Necrotic cells have an increased size (high FSC) while apoptotic cells are smaller (low FSC) and have a high granularity (high SSC). In addition, changes in certain apoptosis-related proteins or in the amount of DNA can be detected using fluorescent dyes or antibodies. Several fluorochromes can be excited by the laser and will then emit light with varying wavelengths that are filtered onto different detectors. Two or more fluorochromes can be used simultaneously, but only if they have emission wavelengths that separate well. Fluorochromebased flow cytometry was used in all papers of this thesis.

Specialized flow cytometers, so called cell sorters, can be used to separate and collect cells with different characteristics. This sorting can be based on the size and granularity, as in paper II. It can also be performed after fluorescent staining of cells and based on the expression of certain proteins, as in paper IV. Cells in the flow chamber have a constant laminar flow until reaching a vibrating nozzle. This vibration will create droplets containing single cells. The droplets pass through a laser and light or fluorescent emission is detected. Cells that have the desired size, granularity or fluorescence are gated and the instrument is instructed to give droplets containing such cells a certain charge. By using plates with different charges the wanted droplets can be diverted into separate tubes and collected.

pH measurements

In paper I we develop and use flow cytometric methods for analysis of lysosomal and cytosolic pH in healthy and apoptotic cells using fluorescent probes. These methods are thereafter used in the following papers. The fluorescent probes used are dual ratiometric pH indicators, which mean that after excitation at one wavelength, emission is analyzed at two different wavelengths. The fluorescence intensity at the maximum emission wavelength (FL1, Figure 9) increases with pH, while the fluorescence emission detected close to the point where the curves converge (FL2, Figure 9) does not vary with pH. The ratio of the emission detected at these two points will vary with pH but will not be affected by the amount of probe available.



Figure 9: The fluorescence emission spectra of BCECF and FITC at different pH showing the position of the respective FL1 and FL2 filters used for analyzing cytosolic and lysosomal pH.

Cytosolic pH

Cytosolic pH was determined by staining cells with the acetoxymethyl (AM) ester of the fluorescein derivative BCECF (2',7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein) for 30 minutes before washing in Hank's buffered salt solution (HBSS). A BP 530±28 nm (FL1 channel) and a 600 nm LP filter (FL2 channel) were used for detection of BCECF (Figure 9). The cells were suspended in HBSS before analysis and measurements were performed on 10 000 cells. To obtain pH-values from the FL1/FL2 ratios a standard curve was constructed by incubating cells in potassium phosphate buffers (ranging from pH 5.5 to 7.5) together with the ionophore nigericin for 1 minute.

Lysosomal pH

Lysosomal pH was determined by allowing the cells to endocytose FITCconjugated dextran for 3 days. Dextran is actively taken up by the cells and ends up in lysosomes. After a 4-hour chase in fresh medium all dextran was considered to have reached the lysosomes and the cells were washed in HBSS. A BP 530±28 nm (FL1 channel) and a BP 610±20 nm filter (FL2 channel) were used for detection of the FITC fluorescence (Figure 9). The cells were suspended in HBSS and measurements were performed on 10 000 cells. To obtain pH-values from the FL1/FL2 ratios a standard curve was constructed by incubating cells in modified Britton-Robinson buffers (ranging from pH 4.0 to 6.0) containing sodium azide, 2deoxyglucose, and the ionophore nigericin for 10 minutes in order to equilibrate the lysosomal pH with the pH of the buffer before analysis.

Immunofluorescence

Immunocytochemistry is a widely used antibody-based staining method utilized to investigate the occurrence and distribution of proteins in cells or tissues. Antibodies directed against the protein of interest may be directly

conjugated to a fluorescent molecule. Alternatively, a primary unconjugated antibody directed against the antigen is first applied followed by a secondary fluorescently tagged secondary antibody, directed against the primary antibody. This two-step method is more sensitive than using direct conjugated antibodies because several secondary antibodies can bind to different epitopes on the primary antibody. Before detection of intracellular proteins the cell is fixed in 4% paraformaldehyde, a crosslinking fixative that preserves the cell morphology. However, fixation decreases the availability of the antibody to the antigen and therefore the specimens also need to be permeabilized by the addition of a detergent, such as saponin, before incubation with the antibodies. The two-step immunofluorescence technique has been used in papers II-IV. The immunostained cells were studied by fluorescence microscope or laser scanning confocal microscope to improve the resolution and reduce the blurring. In paper IV, direct conjugated CD44 antibodies were used in flow cytometry and microscopy.

Western blot analysis

The expression of proteins in cells is determined by Western blot. The proteins are denatured by heating to 95°C in a buffer containing the anionic detergent sodium dodecyl sulphate (SDS), that binds quantitatively to proteins and makes them negatively charged, and a reducing agent such as dithiothreitol (DTT). Cell lysates are loaded onto a polyacrylamide gel and are separated electrophoretically by applying an electric field, which causes the proteins to separate according to molecular weight. The proteins are then transferred (blotted) to nitrocellulose membranes by the use of an electric voltage. The proteins of interest are detected using primary antibodies followed by horse-radish peroxidase (HRP)-conjugated secondary antibodies. This enzyme catalyzes the conversion of the chemiluminescent substrate luminol into a sensitized reagent which on further oxidation by hydrogen peroxide produces an excited carbonyl

which emits light when it decays to the singlet carbonyl. By using autoradiographic film, the proteins are visualized as dark bands on a positive film. This method is considered semi-quantitative and can be applied, as in paper III and IV, to compare the amounts of protein found in different cell types or after different stimuli.

ICP-MS analysis

Inductively coupled plasma mass spectrometry (ICP-MS) is a type of mass spectrometry that is highly sensitive and can determine a range of metals at very low concentrations (Brouwers *et al.*, 2008). The plasma consists of a gas that contains a sufficient concentration of ions and electrons to make the gas electrically conductive. When a sample enters a high temperature argon plasma it evaporates and then breaks down into atoms. At the temperatures prevailing in the plasma a significant proportion of the atoms of many chemical elements are ionized and extracted through a series of cones into a mass spectrometer and are separated on the basis of their mass-to-charge ratio. A detector receives an ion signal proportional to the concentration.

The sample preparation is relatively simple and quick; after freezedrying and mineralization in 65% nitric acid to complete dryness, the material is totally dissolved in 2% nitric acid containing an internal standard, for example rhodium, and thereafter analyzed. The concentration of the metal of interest in the sample is then corrected with respect to the standard. ICP-MS was used in paper III to determine the platinum concentrations in cell lysates after cisplatin exposure.

Statistical analysis

Data were statistically evaluated using the Mann-Whitney U-test (paper IV) and the Kruskal-Wallis multiple comparison test (papers II-III).

Correlation analysis was performed using the Spearman test (paper III). P-values ≤ 0.05 were considered significant.

RESULTS

Papers I and II

Cytosolic acidification appears to be a general mechanism during apoptosis (Lagadic-Gossmann *et al.*, 2004) and there is an increasing number of reports showing an early loss of the lysosomal proton gradient and the membrane stability during apoptosis (Brunk and Svensson, 1999; Guicciardi *et al.*, 2000; Zhao *et al.*, 2000). However, the relationship between lysosomal pH and cytosolic acidification during apoptosis has not been investigated. Flow cytometric methods, employing pH-sensitive fluorescent probes, for analysis of cytosolic pH are well established. In contrast, good methods that reliably measure lysosomal pH are lacking. In paper I, we developed a flow cytometric method for analysis of lysosomal pH in apoptotic cells and in Paper II we investigated the relationship between lysosomal and cytosolic pH during apoptosis.

For analysis of lysosomal pH, cells were loaded with FITC-dextran. A linear standard curve was obtained by using modified Britton-Robinson buffers ranging from pH 4 to 6. An efficient equilibration of the lysosomal pH with the buffer pH was achieved by addition of sodium azide, 2-deoxyglucose, and the ionophore nigericin to the buffers before incubation for 10 minutes. Cytosolic pH was measured after staining with BCECF. Using potassium phosphate buffers ranging in pH from 5.5 to 7.5, and equilibrating the cytosolic pH with the buffer by incubation with nigericin for 1 minute, a linear standard curve for cytosolic pH was obtained.

The aim of paper I was to estimate the cytosolic and lysosomal pH of apoptotic U937 cells exposed to TNF-α. The apoptotic population was, therefore, first identified in an independent sample by using the cell-permeable FITC-conjugated caspase-inhibitor FITC-VAD-FMK and the localization of these cells in a FSC/SSC plot was determined (Figure 10).

This information was used to gate the apoptotic population in the FSC/SSC plot of a sample stained for pH measurements.



Figure 10: Caspase positive cells (red) with high FITC-fluorescence detected in the FL1 channel after 4 hours exposure to TNF- α and their localization in a forward (FSC)/side (SCC) scatter plot. Calculation of the cytosolic and lysosomal pH of apoptotic (red) and healthy (black) cells using standard curves.

Apoptotic and necrotic cells were found to co-localize. However, the apoptotic population was found to increase in number (from approximately 5% to 15%) after TNF- α exposure while the number of necrotic cells was found to be 3-6% in both control and TNF- α exposed samples. The cytosolic pH of cells considered to be healthy was estimated to 7.2±0.1 while in apoptotic cells the pH was decreased to 5.8±0.1. The lysosomal pH of healthy and apoptotic cells was 4.3±0.4 and 5.2±0.3, respectively. The obtained cytosolic and lysosomal pH of normal cells is in good agreement with earlier reports, thus indicating that our methods are reliable. When analyzing the FSC/SSC plots obtained after TNF- α exposure at various time points, an early decrease in the size and granularity of U937 cells were found. After longer exposure times, the apoptotic population, characterized as granulated and FITC-VAD-FMK positive cells, increased. In paper II, healthy cells (H population) and apoptotic cells (A population), as well as the cells with an early decrease in size and granularity, were analyzed more thoroughly. Small cells with low granularity were found not to contain active caspases or to express phosphatidyl serine on the cell surface and were, thus, not considered to be apoptotic. This population was termed pre-apoptotic (P population). The cytosolic pH of the H population was again estimated to 7.2±0.1, the pH of the A population to 5.7±0.0, while the P population was in between these two, showing a cytosolic pH of 6.8±0.1. For lysosomal pH the corresponding values were 4.3±0.3, 5.5±0.3, and 4.8±0.3, respectively.

In order to investigate the mechanism underlying acidification of the cytosol, caspases were inhibited using the pan-caspase inhibitor z-VAD-FMK. Cytosolic acidification was found to be independent of caspases. Moreover, the acidification was not caused by proton release via the mitochondrial F_0F_1 -ATPase, as determined by using oligomycin, an inhibitor of this ATPase. To further study the lysosomal stability, flow cytometric cell sorting of the three identified populations was performed. In contrast to cells of the H population, in which cathepsin D was completely confined to lysosomes, cells of the P population displayed limited release of cathepsin D to the cytosol. In cells of the A population, which also displayed fragmented nuclei, there was a substantial release of the Enforced cathepsin D to cytosol. lysosomal membrane permeabilization using MSDH also resulted in translocation of cathepsin D to the cytosol and in a decrease in the cytosolic pH to 5.1 ± 0.3 . The concomitant alkalinization of the lysosomes and early release of lysosomal cathepsins supports the hypothesis that proton release from lysosomes is the mechanism behind the cytosolic acidification.

Paper III

Although cancer cells have been extensively studied and characterized for many years, very few studies use normal cells for comparisons. In a cooperation with the division of Otorhinolaryngology we gained access to NOKs and several HNSCC cell lines, and aimed to evaluate differences in the lysosomal compartment of normal cells and cancer cells. Resistance against cisplatin, which is part of the treatment regimen for advanced HNSCCs, is a well-established clinical problem. The cell lines are derived from different patients and should, thus, display varying intrinsic sensitivity towards cisplatin. In paper III we investigated if intrinsic differences in cisplatin sensitivity are correlated to alterations in the lysosomal compartment.

	NOK	LK0412	LK0626	SqCC/Y1	UT-SCC -9	UT-SCC- 33
Cisplatin LD50 (µg/ml)	4	7.6	2.2	5	3	3.6
Lysosomal pH	4.3±0.3	4.9±0.3*	4.4±0.1	4.7±0.2*	4.8±0.1*	4.6±0.1
Expression						
Cat B		-	-	-	-	0
Cat D		+	0	+	0	+
Cat L		0	+	0	0	+
Secretion						
Pro cat B		-	-	0	-	-
Pro cat D		+	+	+	0	+
Pro cat L		+	+	+	0	+

Table 1: Cisplatin LD50 concentrations for normal oral keratinocytes (NOKs) and five head and neck squamous cell carcinoma (HNSCC) cell lines after exposure to cisplatin for 48 hours. Lysosomal pH of NOKs and each cell line. Statistically significant difference compared to NOKs is denoted by *, p≤0.05. Expression of cathepsins and secretion of cathepsin proforms by HNSCC cell lines is presented as; - reduced, 0 unchanged, + increased as compared to NOKs.

The sensitivity towards cisplatin was investigated by analysis of viability using the MTT assay to determine the LD50 concentration (Table 1). In addition, measurement of caspase-3 activity showed that cisplatin induced apoptotic cell death. The involvement of lysosomal cathepsins in

cell death induced by cisplatin was investigated in NOKs and two of the HNSCC cell lines; LK0412 and LK0626 cells, representing the least and most sensitive cell line, respectively. Using immunofluorescence, cathepsin D was found in discrete granular structures resembling lysosomes in control cells, while in cells exposed to cisplatin for 24 hours, cathepsin D was more diffusely distributed suggesting relocalization from lysosomes to the cytosol. By employing inhibitors of cysteine cathepsins and cathepsin D, cisplatin-induced cell death was reduced.

As an increase in the expression and secretion of various lysosomal cathepsins, including cathepsins B, D, and L, has been demonstrated in human tumors, we investigated these parameters, by immuno blotting, in our five cancer cell lines compared to NOKs (Table 1). Furthermore, alterations in lysosomal pH were investigated using flow cytometry (Table 1). By performing a Spearman test, a significant correlation of 0.502 was found between cisplatin sensitivity and lysosomal pH.

Differences in cisplatin sensitivity have earlier been suggested to be due to differences in cisplatin accumulation. To investigate this possibility, all cell lines were exposed to 1 µg/ml cisplatin for 24 hours before platinum content was analyzed by ICP-MS. However, no significant differences in platinum accumulation were found. Extraction of mRNA data from gene expression profiling of NOKs, LK0412, and SqCC/Y1 cells indicated that the mRNA levels of ATP6V1B2, a gene encoding for the B2 subunit of the V₀V₁-ATPase, was significantly decreased in LK0412 and SqCC/Y1 cells as compared to NOKs. Since all V₀V₁-ATPase subunits are required for a functional pump, a reduction in the amount of the B2 subunit may lead to a less efficient acidification of the lysosomal lumen. By immuno blotting, we next verified that the expression of ATP6V1B2 was decreased in all cell lines having a higher lysosomal pH than that observed in NOKs. Interestingly, LK0626 cells which had a normal lysosomal pH exhibited an ATP6V1B2 expression similar to, or higher than, that of NOKs.

Paper IV

Earlier observations have indicated that cancer cell lines contain a portion of cells with stem cell properties (Locke et al., 2005; Costea et al., 2006; Harper et al., 2007). The morphological and clonogenic properties of NOKs have been shown to persist in carcinoma-derived epithelial cell lines after in vitro propagation. Therefore, we sought to verify the existence of cells with stem cell characteristics in the recently established LK0412 and the highly propagated SqCC/Y1 cell line, with the intent to investigate possible differences in cisplatin and radiation sensitivity. Moreover, we aimed to investigate if the lysosomal compartment differed between cells with CSC properties and more differentiated cells. After seeding of LK0412 and SqCC/Y1 at clonal density we identified colonies corresponding to holo-, mero-, and paraclones. Holoclones were, by direct immunofluorescent staining, found to contain a larger number of cells with bright CD44 staining at the cell periphery as compared to paraclones. Using flow cytometry, cells with high CD44 staining (CD44 bright cells, approximately 1.5 %) were identified in both cell lines.

In order to further elucidate if there are differences between holoand paraclone cells, colonies were isolated using cloning rings and cells cultured until confluency before seeded at identical densities (Figure 11). Cells originating from paraclones were found to have a lower proliferation rate as compared to cells from holoclones. In the LK0412 cell line, cells from holoclones were found to form all types of colonies including many compact holoclones, while cells isolated from paraclones mainly formed new mero- and paraclones (Figure 11). For SqCC/Y1, this difference in colony formation was only evident after a second repetition of the procedure to seed the isolated holoclone and paraclone cells at identical densities.



SqCC/Y1



Figure 11: Holo- and paraclones were isolated using cloning rings and cultured until confluency before seeded at identical densities (A). In the LK0412 cell line, cells isolated from holoclones formed many new holoclones, as well as mero- and paraclones, while cells isolated from paraclones mainly formed new mero- and paraclones. For SqCC/Y1 no morphological differences were evident. After growing for 7 days, cells were again seeded at identical densities (B). Cells isolated from LK0412 holoclones were now found to form all types of colonies and the number of holoclones was decreased compared to the first passage. Isolated paraclone cells only formed very sparse new paraclones. In the SqCC/Y1 cell line, a difference between isolated holo- and paraclone cells was now found; holoclone cells still formed all types of colonies while paraclone cells only formed new paraclones.

Next, we sought to investigate if cells considered to possess stem cell characteristics, i.e. holoclone cells and cells with bright CD44 staining,

display different sensitivity towards cisplatin and radiation as compared to more differentiated cells. Holoclones and paraclones were isolated using cloning rings and CD44 bright cells and cells displaying an intermediate CD44 staining (CD44 dim cells) were isolated using a flow cytometer. Isolated cells were after 24 hours exposed to 2 μ g/ml of cisplatin or 4 Gy of radiation and were thereafter allowed to form colonies for seven days. When evaluating the survival fraction of LK0412 and SqCC/Y1 cells, CD44 bright and -dim cells were equally sensitive to both cisplatin and radiation. Comparison of isolated holo- and paraclone cells revealed that LK0412 holoclone cells were more resistant to cisplatin. In addition, a tendency towards lower radiation sensitivity in LK0412 holoclone cells was found. No difference in sensitivity to cisplatin and radiation was found in isolated holo- and paraclone cells from the SqCC/Y1 cell line.

Earlier we have identified alterations in the lysosomal compartment of the two HNSCC cell lines, as compared to NOKs (paper III). We next investigated if differences concerning lysosomal properties could be found between subpopulations of the cells. Using immunofluorescent staining, the intracellular distribution of the two lysosomal proteins cathepsin D and LAMP-2 was found to differ between holo- and paraclone cells, as well as between CD44 bright and -dim cells. Holoclone cells and CD44 bright cells showed lysosomes gathered at one side of the nucleus while paraclone cells and CD44 dim cells displayed a more dispersed distribution. Western blot analysis of isolated holo- and paraclone cells showed a smaller amount of active cathepsin B in isolated holoclones from the LK0412 cell line. In contrast, no such difference was found in the SqCC/Y1 cell line. Furthermore, no differences in the amounts of cathepsin D and LAMP-2 were found between holo- and paraclone cells. A difference in lysosomal pH between SqCC/Y1 CD44 bright and -dim cells was observed; pH 4.8 ± 0.1 and 5.0 ± 0.1 , respectively. In contrast, no difference in lysosomal pH was found between LK0412 CD44 bright and dim cells (pH 5.0 ± 0.2 and 5.0 ± 0.1 , respectively).

DISCUSSION

For many years, release of lysosomal content to the cytosol was believed to inevitably lead to necrosis. After the first report in 1996 implicating lysosomal cathepsins in apoptosis many proofs for the importance of these hydrolytic enzymes in the apoptosis process have been presented (Deiss et al., 1996; Guicciardi et al., 2004). In 1998, Roberg and Öllinger showed, using immuno electron microscopy, that cathepsin D was released to the cytosol during apoptosis (Roberg and Öllinger, 1998). However, cathepsins are enzymes with low pH optima and it has been questioned if these enzymes are active after release to the cytosol, which is characterized by a considerably higher pH than lysosomes. In case of accidental escape of proteases from lysosomes, endogenous inhibitors, such as cystatins which block the activity of cysteine cathepsins, are present in the cytosol (Turk et al., 2002). In addition, the cysteine cathepsins B and L are denatured and inactivated at neutral or alkaline pH, unless bound to substrate or endogenous inhibitors that stabilize their active conformation (Turk et al., 1993; Song et al., 2000). However, such inactivation is not very rapid and cathepsin B has been shown to be active for up to an hour at neutral pH (Turk et al., 1994). Notably, cathepsin L which is considered to be the least stable cysteine cathepsin can remain active for a few minutes at neutral pH (Turk et al., 1993). In contrast to cysteine cathepsins, no known endogenous inhibitor of cathepsin D is currently known but cathepsin D is believed to be inactive at neutral pH. The action of cathepsin D in the cytosol has, therefore, been suggested to be non-proteolytic (Beaujouin et al., 2006; Beaujouin and Liaudet-Coopman, 2008). Nevertheless, several reports do indicate that cathepsins, including cathepsin D, have an important proteolytic function in the cytosol during apoptosis (Stoka et al., 2001; Cirman et al., 2004; Heinrich et al., 2004; Johansson et al., 2008).

Although it has not attracted much attention so far, a quite common intracellular change during apoptosis is acidification of the cytosol.

Acidification has been shown to stimulate the pore-forming capability of Bcl-2 proteins (Schendel *et al.*, 1997; Schlesinger *et al.*, 1997; Matsuyama *et al.*, 1998; Xie *et al.*, 1998; Schendel *et al.*, 1999) and the activation of caspases (Roy *et al.*, 2001; Segal and Beem, 2001; Beem *et al.*, 2004). Moreover, acidification would stabilize cathepsins located in the cytosol. A decrease in cytosolic pH ranging from 0.3 to 0.8 pH units have been reported (Barry *et al.*, 1993; Li and Eastman, 1995; Rebollo *et al.*, 1995; Gottlieb *et al.*, 1996; Furlong *et al.*, 1997; Ishaque and Al-Rubeai, 1998; Thangaraju *et al.*, 1999; Matsuyama *et al.*, 2000). In order to investigate if the magnitude of cytosolic acidification is sufficient to maintain cathepsins in an active state, methods for analysis of cytosolic and lysosomal pH in apoptotic cells were improved in paper I.

The ideal way to identify the apoptotic cells for pH measurements would be to simultaneously stain samples with a marker for apoptosis and the pH-sensitive probe. However, we have not been able to identify such a marker. Therefore, the number and location of apoptotic cells was determined in an independent sample. Small granulated cells were found to be caspase positive and had significantly lower cytosolic pH than healthy cells. The change in pH from 7.2 to 5.7, as observed in paper II, is larger than the acidification reported earlier (Barry et al., 1993; Li and Eastman, 1995; Rebollo et al., 1995; Gottlieb et al., 1996; Furlong et al., 1997; Ishaque and Al-Rubeai, 1998; Thangaraju et al., 1999; Matsuyama et al., 2000). In contrast, using a ratio imaging technique we have recently found a cytosolic acidification (change of approximately 0.8 pH units) after exposure of human fibroblasts to staurosporine (Johansson et al., 2008). Noteworthy, in test tube experiments we show cathepsin D-mediated Bidcleavage at a pH value corresponding to the pH value determined in apoptotic fibroblasts (Johansson et al., 2008). Variations in the magnitude of acidification might be explained by cell specific differences and by differences in the methods of identifying apoptotic cells. However, considering the data showing optimal degradative capacity of most cathepsins below pH 6 (Mason, 1996), a decrease in pH of 1.5 pH units in the cytosol, as found after TNF- α exposure of U937 cells, will probably maintain the activity of cathepsins.

Several data indicate that the acidification is an early event; already in the pre-apoptotic population, containing small cells with no caspase activity, the cytosolic pH was decreased (pH 6.8). In contrast to some earlier reports (Meisenholder et al., 1996; Liu et al., 2000; Matsuyama et al., 2000), the acidification was found to be caspase-independent and also independent of the action of the mitochondrial F_0F_1 -ATPase. Concomitant to the cytosolic acidification observed after TNF- α exposure, there was an increase in the lysosomal pH. FITC-dextran has earlier, by several groups, been used for measurement of lysosomal pH using varying buffer systems to create standard curves. Attempts by us, and other groups (Bach *et al.*, 1999), to use potassium phosphate buffers for lysosomal pH resulted in exponential standard curves due to lack of buffering capacity at pH 4.5. In contrast to the citrate-potassium phosphate buffers used by Myers et al (Myers et al., 1995), Britton-Robinson buffers that were modified for potassium concentration and osmolarity, were found to yield linear standard curves. The estimated pH value of healthy cells (pH 4.3 ± 0.3) correlate well with that observed in earlier reports and the method has a high reproducibility. A significant increase in lysosomal pH in both preapoptotic (pH 4.8 ± 0.3) and apoptotic cells (pH 5.5 ± 0.3) could thus be determined after exposure to TNF- α .

Importantly, using immunofluorescence, FITC-dextran was demonstrated to remain in lysosomes after TNF- α exposure, excluding the possibility that the estimated lysosomal pH of apoptotic cells represented the pH of an acidified cytosol. Studies by Bidére *et al* of size-selective release from lysosomes using FITC-dextran molecules of different molecular weight have shown that 10- and 40-kDa FITC-dextran could be released after staurosporine exposure, while 70- and 250-kDa FITC-

dextran remained inside lysosomes (Bidere *et al.*, 2003). Therefore, in order to confirm our results, pH-experiments using 70-kDa FITC-dextran was performed, resulting in pH values comparable to those earlier obtained. In papers III and IV, the 70-kDa FITC-dextran was used for analysis of lysosomal pH.

Acidification during apoptosis has earlier been suggested to be due to mitochondrial dysfunction and alterations in membrane pH_i-regulating mechanisms (Lang et al., 2000; Matsuyama et al., 2000; Matsuyama and Reed, 2000; Gendron et al., 2001; Lagadic-Gossmann et al., 2004). However, the most acidic compartment of the cell is the lysosome and lysosomes have been shown to lose their proton gradient and their membrane stability early during apoptosis (Brunk and Svensson, 1999; Guicciardi et al., 2000; Zhao et al., 2000). It might, thus, be speculated that lysosomal proton release can cause an early cytosolic acidification. A complete permeabilization of all lysosomes would result in necrosis (Kågedal et al., 2001b) while leakage from a fraction of the lysosomes would lead to apoptosis. Lysosomes constitute approximately 2.5 % of the total cell volume. If one hypothesizes that half of all lysosomes are permeabilized or lose their proton gradient, a highly approximative calculation on the effect on cytosolic pH can be performed. Such a calculation shows that the amount of protons required to change the cytosolic pH from 7.2 to 5.7 is of the same magnitude as the amount of protons that needs to be lost in order to increase the pH in lysosomes from 4.5 to 5.5. This supports our hypothesis that release of protons from lysosomes causes the cytosolic acidification. In order to confirm this hypothesis, cells were treated with the V_0V_1 -ATPase inhibitor bafilomycin A_1 to increase the lysosomal pH, and theoretically inhibit cytosolic acidification during apoptosis. However, bafilomycin A1 treatment caused cytosolic acidification in control cells by itself and could, therefore, not be used as intended. The use of NH₄Cl, for the same purpose, was hampered by an insufficient effect on lysosomal pH. Unfortunately, we could
therefore, not with certainty prove that cytosolic acidification was caused by lysosomal proton release.

Cancer cells often display several changes in the lysosomal system such as redistribution of lysosomes from a perinuclear to a peripheral location and an increase in the expression, secretion and/or activity of various lysosomal cathepsins (Mohamed and Sloane, 2006). Changes in the lysosomal compartment have, in several studies, been shown to influence the effect of anti-cancer agents. Increased endosomal/lysosomal pH of resistant cells has been suggested to reduce the uptake and the cellular accumulation of cisplatin (Chauhan et al., 2003; Liang et al., 2003; Safaei et al., 2005a; Safaei et al., 2005b). Very few studies are, however, performed using both normal and cancer cells. Moreover, comparison of the intrinsic cisplatin sensitivity of different tumor cell lines is a concept seldom used. Experiments where NOKs and five HNSCC cell lines were exposed to increasing concentrations of cisplatin showed a 3-fold difference in cisplatin LD50 concentration between the cell lines, which correlates well with the clinical situation (Siddik, 2003). By using cisplatin to induce cell death, several aspects on lysosomal function were investigated. Firstly, if lysosomal pH had any impact on cisplatin toxicity, and secondly, if cisplatin-induced cell death was mediated through release of cathepsins from the lysosomes.

Cisplatin is known to cause DNA adducts, which lead to stabilization of p53, activation of pro-apoptotic target genes, and activation of caspases via the mitochondrial pathway (Gonzalez *et al.*, 2001; Siddik, 2003). Accordingly, both NOKs and all five HNSCC cell lines showed caspase activity after exposure to the drug. The involvement of lysosomal membrane permeabilization in cisplatin-induced cell death is not well studied. In a recent report, siRNA-mediated downregulation of cathepsin D was shown to protect from cisplatin-induced apoptosis (Emert-Sedlak *et al.*, 2005). Moreover, pharmacological cysteine cathepsin inhibitors have

been shown to effectively inhibit cisplatin-induced cell death of immortalized and transformed fibroblasts (Fehrenbacher *et al.*, 2008). In accordance, our results using cathepsin inhibitors indicate that cell death induced by cisplatin is, at least partly, dependent on both cathepsin D and cysteine cathepsins. These cathepsins seem to be released to the cytosol from lysosomes after cisplatin exposure. How cisplatin causes lysosomal leakage, and if leakage occurs up- or downstream damage to nuclear DNA or mitochondria, still remains to be clarified.

The mechanism behind cellular uptake of cisplatin is still not completely understood. It was long presumed to occur by passive diffusion, but recent data also suggest that transmembrane channels, active ATP-dependent transporters, or metal receptors may be involved (Rabik and Dolan, 2007). Studies using fluorescently labeled cisplatin show that the compound becomes sequestered in lysosomes and is thereafter found in vesicles of the Golgi and in vesicles belonging to the secretory pathway (Safaei et al., 2005a). It is still not clarified if cisplatin found within lysosomes ever reaches the cytosol. It is, however, not unlikely that transporters capable of exporting cisplatin is present in the lysosome, since other metal transporters, such as the iron transporter NRAMP2, is found in this compartment (Tabuchi et al., 2000). In addition, cisplatin located in the lysosomal compartment could possibly have a direct damaging effect on the lysosomal membrane causing leakage of cisplatin. Supporting this theory, we found release of cathepsins during cisplatin exposure which is likely accompanied by release of cisplatin as well. By pre-treatment of cells with bafilomycin A₁ in order to increase the lysosomal pH before cisplatin exposure, cell death was reduced. Since the protective effect was not due to reduced uptake or accumulation of platinum after bafilomycin A₁ pre-treatment, as shown using ICP-MS, we suggest that the increased pH resulted in less active and functional cathepsins. An alternative explanation may also be that cisplatin-induced apoptosis caused cytosolic acidification due to lysosomal proton release, as shown in paper II. An

alkalinization of the lysosomes using bafilomycin A_1 would then reduce this cytosolic acidification and thereby reduce the activity of cathepsins released to the cytosol which would have an inhibitory effect on cell death.

Most cell lines having high lysosomal pH showed a lower sensitivity to cisplatin. However, the UT-SCC-9 cell line exhibited a high lysosomal pH although it was still sensitive to cisplatin. This fact demonstrates that also other factors influence the cellular sensitivity to cisplatin. However, the strength of our study is the fact that we can show a correlation between an intrinsic low sensitivity towards cisplatin and the pH of the lysosomes, a correlation previously only shown for cell lines with a high acquired resistance against the drug. In cells with acquired resistance, this correlation has been explained by reduced uptake and accumulation of cisplatin due to a high endosomal and lysosomal pH (Chauhan *et al.*, 2003; Liang *et al.*, 2003; Safaei *et al.*, 2005a; Safaei *et al.*, 2005b). In paper III, no difference in platinum accumulation was found between the investigated cell lines using ICP-MS. This indicates that lysosomal pH alone is not a determining factor for drug uptake and accumulation.

Despite the many reports showing altered lysosomal pH in cancer cells (Altan *et al.*, 1998; Chauhan *et al.*, 2003; Liang *et al.*, 2003; Safaei *et al.*, 2005a; Safaei *et al.*, 2005b), the mechanisms behind the change are less well-known. Interestingly, transformation of fibroblasts with *ras*, known to be responsible for many tumor-associated lysosomal changes, has been shown to increase the lysosomal pH by approximately 1 pH unit (Jiang *et al.*, 1990). Expression of the vacuolar V_0/V_1 -ATPase, responsible for acidification of the lysosomal lumen, has previously not been investigated in cancer cells in relation to lysosomal pH. The B subunit isoform 2 of the vacuolar V_0/V_1 -ATPase is a nucleotide-binding subunit of the V1 catalytic domain (Finbow and Harrison, 1997). In most cells where V_0/V_1 -ATPases are found only in the membranes of acidic intracellular

organelles they function as housekeeping proteins and no control of their expression has been identified. In contrast, the amount of V_0/V_1 -ATPase is increased by specific control of its expression during differentiation of monocyte to macrophage (Merzendorfer *et al.*, 1997). Interestingly, particularly the B2 subunit was transcriptionally amplified during monocyte differentiation supporting the important role of this subunit for the activity of the V_0/V_1 -ATPase. Studies have shown that inactivation of any of the genes encoding V_0/V_1 -ATPase subunits leads to loss of function (Finbow and Harrison, 1997). Therefore, the reduction in the amounts of ATP6V1B2 seen in four of the HNSCC cell lines may explain the decreased acidification of their lysosomes. It would be interesting to investigate the amounts of all V_0/V_1 -ATPase subunits in a larger number of cell lines in relation to their lysosomal pH and sensitivity to cisplatin.

In contrast to our results showing a reduced sensitivity to cisplatin in cells with a high lysosomal pH, resistance against weakly basic substances, such as anthracyclines, has been correlated to a low lysosomal pH. Accumulation of the basic drugs in acidic lysosomes hinders them to reach the cytosol and the nuclear targets (Altan *et al.*, 1998). An increase in the lysosomal pH of tumor cells may, therefore, also have positive effects, by lending drug sensitivity to the tumor cells while normal cells, having a low lysosomal pH, are less affected by the drug. It is, thus, shown that changes in the lysosomal compartment affect the outcome of a drug treatment and that the change may either have positive or negative effects depending on the drug used. It also indicates that knowledge about the lysosomal pH of the tumor cells may be useful in the clinic, in order to predict the outcome and choose a treatment that will attack tumor cells and spare normal cells.

Only a minority of the tumor cells found in haematopoetic malignancies or solid cancers have the ability to form clones in cell culture or new tumors when injected into immunodeficient mice (Al-Hajj and Clarke, 2004; Neuzil et al., 2007). These TICs have been shown to have stem cell characteristics. Also HNSCCs have been shown to contain a subpopulation of TICs, which apparently correspond to malignant stem cells (Prince et al., 2007). Recent reports have also shown that cell lines established from HNSCCs contain sub-populations of cells with stem cell characteristics (Burkert et al., 2006; Harper et al., 2007). In paper IV, we identified cells with CSC properties in two of the HNSCC cell lines used in paper III; the recently established LK0412 cell line and the highly propagated SqCC/Y1 cell line. Cells growing as holoclones, meroclones and paraclones, believed to correspond to stem cells, amplifying cells and differentiated cells, respectively, were found in both cell lines. In order to evaluate differences in the growth pattern of holo- and paraclone cells, colonies were isolated and grown for two passages. The clonal characteristics were maintained in LK0412 cells during the first and second passage after isolation whereas the distinction between SqCC/Y1 holoclone and paraclone cells was detected only after a second passage. This might indicate intrinsic differences in the original tumors from which the LK0412 and SqCC/Y1 were derived. Alternatively, differences between the two HNSCC lines may be explained by cellular alterations induced by *in vitro* culturing. The SqCC/Y1 cell line was originally established in medium containing serum and later transferred into serum free growth (Sundqvist et al., 1991), while LK0412 was established under serum free conditions (Roberg et al., 2008). Moreover, SqCC/Y1 is an extensively propagated buccal cell line whereas LK0412 was established in 2004 and used in low passages.

Expression of CD44 has proven valuable for the isolation of breast cancer cells with tumorigenic capacity, as well as in HNSCC (Al-Hajj *et al.*, 2003; Prince *et al.*, 2007). A minor population of CD44+ cancer cells, derived from primary HNSCC samples, gave rise to new tumors in vivo (Prince *et al.*, 2007). Using flow cytometry, we found that all cells expressed CD44, however, the expression was graded. For SqCC/Y1, a

population of cells (approximately 1.5%) with high CD44 expression could easily be identified, while LK0412 cells showed a more continuous expression and gating was, therefore, more arbitrary. By using direct immunofluorescent staining of unfixed cells, holoclone cells were found to have a higher expression of CD44 at the cell surface as compared to paraclones. Especially the SqCC/Y1 cell line was found to contain highly CD44 positive cells, however, such cells were in this cell line also more often found in mero/paraclones compared to in the LK0412 cell line. Thus, our results indicate that expression of CD44 cannot be used as a single marker to identify and isolate CSCs. Very few reliable stem cell markers have been identified for normal oral epithelium. However, using HNSCC cell lines it has been found that holoclones have a higher expression of molecules such as β 1-integrin, E-cadherin, β -catenin and epithelial specific antigen (Costea et al., 2006). This indicates that combinations of CD44 with other markers may be necessary to identify CSCs in LK0412 and SqCC/Y1. Such markers might be found by microarray analysis of cultured enriched holo- and paraclones. This is an attractive approach to identify cell surface markers that separate holo- and paraclone cells, which may later on be used to isolate cells by flow cytometry.

If malignant stem cells are derived from their normal counterparts, differences found between normal stem cells and amplifying cells may also be present between CSCs and their progeny. Studies have shown that both normal stem cells and cancer stem cells express high levels of members of the ATP-binding cassette (ABC) family of proteins, lending cells a high resistance against cytotoxic drugs (Hirschmann-Jax *et al.*, 2004; Burkert *et al.*, 2006; Wang *et al.*, 2007). In addition, cells expressing high levels of these proteins have the ability to efflux the vital dye Hoechst 33342, making identification and isolation of such cells from tissues and cell lines possible. However, the Hoechst 33342 negative cells (side population, SP) identified in the LK0412 and the SqCC/Y1 cell lines were not found to correspond to CD44 bright cells. Moreover, in recent studies, SP cells have

been found to lack stem cell properties (Terunuma et al., 2003; Triel et al., 2004). In normal tissues, stem cells are slowly cycling, and this may result in resistance towards chemotherapy and radiation (Maitland and Collins, 2005), and differences in apoptotic sensitivity have been found (Al-Hajj et al., 2004; Tsai, 2004). Most of the anti-cancer treatments used today do not account for differences in drug sensitivity between CSCs and their progeny. In order to investigate differences in cisplatin and radiation sensitivity, cell colonies were isolated using cloning rings. Since the distinction between mero- and paraclones is slightly arbitrary, comparisons were made between isolated holoclones and clones considered to be paraclones or possibly late meroclones. These mero- and paraclone cells could be considered to correspond to late amplifying and differentiated cells. We demonstrate a significant difference in cisplatin sensitivity between isolated LK0412 holo- and paraclones. In addition, a tendency towards lower radiation sensitivity in holoclone cells was detected. However, no such difference could be found in the SqCC/Y1 cell line.

The differences in the lysosomal compartment of LK0412 and SqCC/Y1 compared to NOKs, found in paper III, made us investigate if differences also could be found between sub-populations of these cells. In both holoclone cells and CD44 bright cells, lysosomes had perinuclear localization while in paraclone and CD44 dim cells the distribution was more dispersed. In addition, holoclone cells from the LK0412 cell line had lower levels of cathepsin B compared to paraclone cells. However, no difference was found in the levels of cathepsin D and LAMP2, which might indicate that there is no general increase in the size of the lysosomal compartment. In order to analyze the lysosomal pH, cells were loaded with FITC-dextran and stained using an APC-conjugated CD44 antibody. Lysosomal pH was analyzed in CD44 dim and CD44 bright cells separately. No difference in pH was found between CD44 bright and -dim LK0412 cells, while CD44 dim SqCC/Y1 cells had a slightly, but significantly, higher lysosomal pH than CD44 bright cells. However, it is

important to note that the limited number of cells gated as CD44 bright (~1.5%) increases the uncertainty of the results. In addition, the difficulties in defining a clear population of CD44 bright LK0412 cells may affect the result. It would have been interesting to measure lysosomal pH in holoand paraclones using the ratio imaging technique used for adherent cells. Future improvements of this technique, and the use of flow cytometric measurements on isolated and enriched holo- and paraclone cells, are possible approaches to investigate the possibility that a difference in cisplatin sensitivity between holo- and paraclones is due to differences in lysosomal pH. However, the data obtained so far do not indicate that the difference in cisplatin sensitivity is correlated to differences in the lysosomal pH.

The value of the information derived from cancer cell lines has been questioned. It is possible that selection for growth under artificial culture conditions has affected the distinction between tumorigenic and nontumorigenic cells. A recent study on subpopulations of cells from a murine ovarian cancer cell line showed that both the CSC-like cells and the non-CSC-like cells formed tumors in nude mice (Szotek et al., 2006). The surface proteins used for identifying CSCs are not necessary or sufficient for conferring stem cell properties, but merely represent identification markers. It is, therefore, important to show that a population isolated using certain surface markers also has the functional characteristics of CSCs and it would be valuable to evaluate the tumorigenic potential of isolated LK0412 and SqCC/Y1 holo- and paraclone cells. Although the use of cell lines for CSC research has been questioned, the results from our study, and several others, indicate that some kind of stem cell pattern persists in malignant cell lines even after extensive propagation. Therefore, combined with studies on primary tumors, cancer cell lines can still be considered as valuable tools in the search for effective anti-cancer treatments targeting CSCs.

CONCLUSIONS

From results presented in this thesis the following conclusions can be drawn:

- The flow cytometric methods used for analysis of cytosolic and lysosomal pH in live cells have good reproducibility and can be applied on different cell types including both suspension- and adherent cells.
- During TNF- α -induced apoptosis, there is an early, caspaseindependent acidification of the cytosol, which is accompanied by a lysosomal alkalinization, indicating that lysosomal proton release may cause the acidification.
- Several HNSCC cell lines have a high lysosomal pH as compared to NOKs, possibly due to their low amounts of the B subunit isoform 2 of the vacuolar V_0/V_1 -ATPase.
- An increase in lysosomal pH correlates to a low intrinsic sensitivity towards cisplatin, but is not related to a decrease in cisplatin accumulation.
- Cells with CSC characteristics are found in both recently established and highly propagated HNSCC cell lines.
- LK0412 cells that show colony forming patterns characteristic of CSCs are less sensitive to cisplatin as compared to more differentiated cells of the same cell line.
- There are differences in the localization of lysosomes and/or in the amount of cathepsin B when comparing cancer cells with stem cell characteristics and more differentiated cells.

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