In Search for Biomarkers of Aging

A Proteomics Approach

Op zoek naar biomarkers van veroudering

Een proteomics benadering

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

AD	Alzheimer disease
ANOVA	Analysis of variance
CAT	Catalase
CF	Cystic fibrosis
CFU	Colony forming units
CNBr	Cyanogen bromide
CO3	Complement C3
DBP	Vitamin D-binding protein
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Endothelial cell
ELISA	Enzyme-linked immuno sorbent assay
EPC	Bone marrow-derived endothelial progenitor cell
ER	Endoplasmic reticulum
ERAD	ER associated degradation
FDA	Food and Drug Administration
FH	Familial hypercholesterolemia
Fv	VH and VL combined
GBM	Glomerular basement membrane
GMP	Good manufacturing process
GP	Goodpasture
GPX	Glutathione peroxidase
GRAS	Generally recognized as safe
GRD	Glutathione reductase
GRP78	Glucose regulated protein 78kDa
GSH	Glutathione
GST	Glutathione S-transferase
H_2O	Water
H_2O_2	Hydrogen peroxide
HSA	Human serum albumin
HSP	Heat-shock protein
HUVEC	Human umbilical vein endothelial cell
IgG	Immunoglobulin G
IL	Interleukin

IGF	Insulin growth factor
MCS	Multiple cloning site
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
NHS	N-hydroxysuccinimide
NLS	Nuclear localization signal
O_2	Oxygen
$^{\bullet}O_2^-$	Superoxide anion radical
$^{\bullet}OH$	Hydroxyl radical
PAI-1	Plasminogen activator inhibitor type 1
PBC	Primary Biliary Cirrhosis
PDI	Protein disulfide isomerase
PRD	Peroxireductase
ROS	Reactive oxygen species
scFv	VH and VL combined with a synthetic linker
SLE	Systemic Lupus Erythmatosus
TGF	Transforming growth factor
TNFα	Tumor necrosis factor alpha
TPX	Thioredoxin peroxidase
TRD	Thioredoxin reductase
TropC	Troponin C
UPR	Unfolded protein response
VH	Variable heavy chain domain of a classical antibody
VHH	Variable heavy chain domain of a heavy chain antibody
VL	Variable light chain domain
2D-DIGE	Two-dimensional difference gel electrophoresis
2D-GE	Two-dimensional gel electrophoresis

Chapter 1

General introduction

1. Research on aging

Aging is a degenerative process that occurs with time, often after the age of reproductive ability. Although aging is an inevitable and universal phenomenon, research on age-associated diseases, such as Alzheimer disease (AD), cancer and atherosclerosis, has by far exceeded advances in our understanding of the underlying mechanisms of the aging process itself. An explanation for this observation is that in the minds of many people, we do not die from aging, but from the diseases associated with aging. However, age-associated diseases are caused by the increase of molecular disorder, which is inevitable in any biological system (Hayflick, 2000). Understanding the mechanisms of aging could therefore aid in understanding and prevention of these age-associated diseases. This recognition has led to a growth in depth, breadth and molecular detail in this exciting research field. In the future the interest in aging will probably increase even further as the number of aged people worldwide continues to grow.

In addition to mammalian cellular and organismal aging models, the use of lower eukaryotic organisms, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, has provided many new insights in aging mechanisms (Gami and Wolkow, 2006; Helfand and Rogina, 2003; Katic and Kahn, 2005). These model organisms share surprisingly many characteristics with higher eukaryotes with respect to pathways that are linked to longevity, and which influence the molecular mechanisms involved in aging that will be described below. Furthermore, these organisms have a reduced complexity, are relatively easy to manipulate, and have a shorter lifespan, which offers great advantages for research over higher eukaryotic systems.

Research on both higher and lower eukaryotic model systems have revealed that numerous aspects are involved in the course of the aging process. In the following paragraphs, the main theories of aging that have evolved over the years will be described, starting at the changes that occur on the molecular level, followed by the consequences of these alterations at the cellular and tissue level.

2 Molecular mechanisms of aging

2.1 Endogenous and environmental challenges

All living organisms have a unique property called homeostasis that is maintained by many defense and repair mechanisms. During life, homeostasis is continuously challenged by environmental and endogenous factors. The severity of these factors and the effectiveness of the organism to counteract these challenges, ultimately determine the organism's lifespan.

One of the most important theories of aging, called the free radical theory of aging, emerged almost 50 years ago and was initiated by Denham Harman (Harman, 1956). Harman based his theory on parallels between effects of aging and effects of ionizing radiation. He suggested that free radicals produced during life cause cumulative oxidative damage, resulting in aging and ultimately death. Through many decades this theory has gained much support by many individual groups and has evolved to a theory with many different facets.

It has become clear that reactive oxygen species (ROS) are produced by internal and external systems, and can oxidize cellular components such as proteins, carbohydrates, lipids and DNA (Davies, 1995; Halliwell and Gutteridge, 1990; Rice-Evans and Burdon, 1993), which can lead to loss of function of these cellular molecules. One of the main sources of ROS are the mitochondria during normal aerobic respiration (Chance et al., 1979), because of the incomplete reduction of oxygen to water. It is estimated that about 0.1% (Fridovich, 2004) or more (Chance et al., 1979) of the oxygen consumed by mitochondria is converted to superoxide anion radicals ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2). The latter can be further reduced to the highly reactive hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 1990) (Figure 1). The conversion of hydrogen peroxide to the hydroxyl radical is unlikely to occur spontaneously in an aqueous solution. However, various transition metals, such as Fe^{2+}, Ti^{3+} and Cu^{+}, are capable of reducing hydrogen peroxide to the hydroxyl radical (Halliwell and Gutteridge, 1990). Hydrogen peroxide itself is relatively neutral and can freely cross membranes. As a result, the catalytic availability of the transition metals determines the cellular localization of hydrogen peroxide transition to the hydroxyl radical. Therefore, the damaging effects of H_2O_2 are not limited to the place of origin.

Several sites in the respiratory chain are involved in ROS production, such as nicotinamide adenine dehydrogenase, ubisemiquinone and flavosemiquinone (Boveris and Chance, 1973; Finkel and Holbrook, 2000; Turrens and Boveris, 1980; Wei et al., 1981). The importance of mitochondrially produced ROS in aging is exemplified by the correlation of metabolic rate to the longevity of an organism. It was found that organisms with a higher respiratory rate had a shorter average lifespan (Livingstone and Kuehn, 1979). Moreover, food or caloric restriction has been shown to have beneficial effects on lifespan extension, which is among other things, thought to be mediated by a lowered respiratory rate (Koubova and Guarente, 2003).
Nevertheless, different species with a similar respiratory rate can have enormous differences in their average lifespan. An example of this is the little brown bat of North America, which is about one-half the size of a mouse with a high metabolic rate, but which can reach 30 years of age (Austad, 1997). Apparently, some organisms have evolved mechanisms that allow the organism to survive longer than would be expected based on their respiratory rate. This can depend on several factors, such as beneficial environmental circumstances or differences in the ability of an organism to deal with the produced damaging agents, as will be discussed later.

Although the mitochondria are the main site of ROS production, there are also other internal sources (Table 1), e.g. peroxisomes produce H_2O_2 as a byproduct during oxidative metabolism of long chain fatty acids (Adams et al., 1982). Furthermore, during the activation of the immune response, ROS can be locally produced in high concentrations by specific immune cells (Adams et al., 1982; Mackaness, 1970). Although this reaction is meant to have a protective function to annihilate the foreign entity, this also causes damage to molecules of neighboring cells.
This illustrates the importance of a well-regulated defense system for the life expectancy of an individual. If an infection results in a delayed or under-induced response, the organism is vulnerable for diseases that can lead to early death. However, an over-induced response can lead to needless cellular damage and a shortened lifespan. Consequently, a well-regulated immune response to different infections would therefore be of critical importance to obtain a long lifespan.

Strikingly, there is compelling evidence that with increasing age major changes arise of the adaptive as well as the innate immune system (Grubeck-Loebenstein and Wick, 2002), which induce a more pro-inflammatory immune state, called inflamm-aging. One of the changes concerns the alteration of important functions of members of the adaptive immune response, such as T-cells. The expression of some very important cytokines needed for T cell clonal expansion, including interleukin (IL)-2, are decreased with aging (Gillis et al., 1981), which contributes to the dysfunction of the adaptive immune response and the increased incidence of infections and several age-related diseases, such as auto-immune disorders, cancers and atherosclerosis (Castle, 2000a; Castle, 2000b; Fulop et al., 2005). Also some specific functions of the innate immune response are altered (Fulop et al., 2004; Plackett et al., 2004), possibly caused by the imbalance in the adaptive immune response. One of these changes is the production of pro-inflammatory cytokines TNFα, IL-1 and IL-6. Indeed, the increased presence of these primary pro-inflammatory cytokines in plasma has been described (Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Pedersen et al., 2000). This pro-inflammatory immune state upon aging might result in an increase in basal ROS production by the immune system.

Internal	External
Mitochondrial respiratory chain	UV radiation
Immune cells	Ionizing radiation
Peroxisomal β-oxidation	Transition metal salts
Prostaglandin synthesis	Pollutants
Cytochrome P450	

Table 1: Internal and external sources of ROS. Adapted from Boonstra and Post, 2004.

Next to the internal sources of ROS, there are also external sources (Boonstra and Post, 2004) that can induce damage, such as ultraviolet and ionizing radiation (Cerutti, 1985; Pollycove and Feinendegen, 2003), pollutants (Cosgrove et al., 1985; Stone and Pryor, 1994), transition metal salts (Samson and Nelson, 2000), and natural phenolic compounds present in plant food (Gold et al., 1992) (Table 1).

All these damaging circumstances that an organism faces during life challenge the survival of the organism. To maintain homeostasis, organisms have

evolved two levels of intervention at the molecular level. The first line of defense is focused on neutralization of the damaging agents. If this line of defense is insufficient or fails and damage is induced, there is a second line of defense that can repair the induced damage or ultimately removes the damaged compound.

2.2 Protection against ROS

With regard to the first line of defense, aging research has mainly been focused on systems that deal with ROS. Cells contain an elaborate network of mechanisms to deal with these agents, corroborating the importance of ROS defense for proper functioning of cells. The enzyme superoxide dismutase (SOD) (Stevens et al., 1975) was one of the first findings that gained credibility for the free radical theory of aging. This enzyme reduces two super oxide molecules to oxygen (O_2) and hydrogen peroxide (Figure 1). Other enzymes, catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GRD), peroxireductase (PRD), thioredoxin peroxidase (TPX) and thioredoxin reductase (TRD) were also discovered to be part of an elaborate enzymatic antioxidant defense network (Figure 1).

Support for the free radical theory of aging and the role of antioxidant enzymes herein came from over-expression experiments of enzymes, such as SOD and CAT, in model organisms like *D. melanogaster*, or SOD/catalase mimetics in *C. elegans*, which resulted in an increased lifespan (Melov et al., 2000; Sohal and Weindruch, 1996). This demonstrated that increased protection against oxidative damage could elongate organismal survival. Increased protection has also been described in caloric restricted animals in addition to the earlier described decrease in respiratory rate. Caloric restriction can lead to an increased expression of anti-oxidant enzymes, such as SOD and catalase in various organisms. This is mediated by decreased circulating levels of insulin and insulin growth factor (IGF)-1 in caloric restricted animals, which regulate the expression of these anti-oxidant genes via the insulin/IGF-1 signaling pathway (Heilbronn and Ravussin, 2003).
Interestingly, the level of protection against ROS can differ upon cellular localization. An organelle that is likely to be more sensitive to oxidative stress is the endoplasmic reticulum (ER), which is involved in proper folding of proteins that enter the cellular secretory pathway. In the ER, glutathione

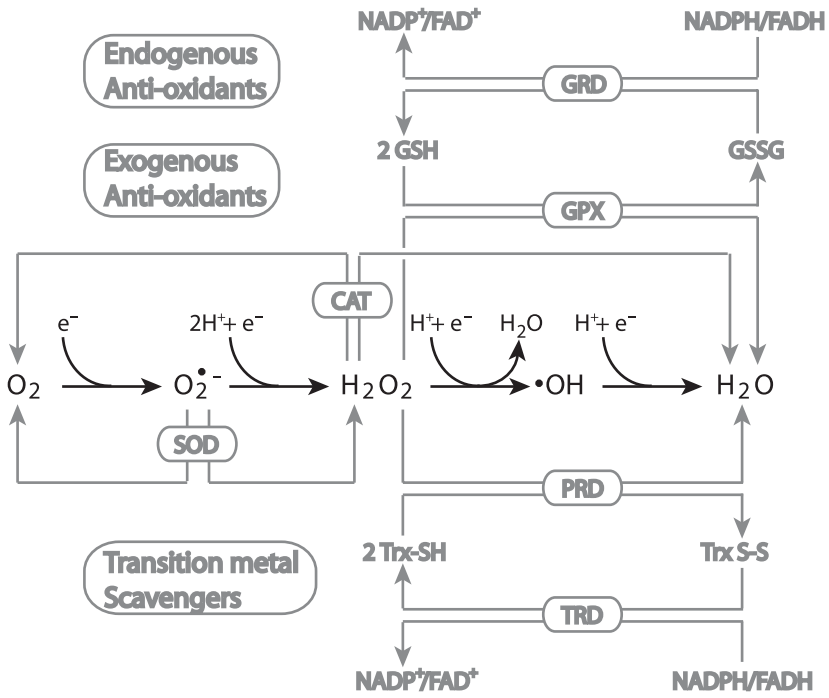

Figure 1: Formation of the different ROS, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), during the successive one-electron reductions of oxygen to water (black) and the cellular anti-oxidant defense systems that protect the cell from oxidative damage (grey). The defense mechanisms include the anti-oxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GRD), peroxireductase (PRD), thioredoxin peroxidase (TPX), and thioredoxin reductase (TRD). SOD converts superoxide to oxygen and hydrogen peroxide. The latter is subsequently converted to oxygen and water by catalase, or to water by the joint efforts of the enzymes GPX, GRD, PRD, TPX and TRD via the oxidation of NADPH/FADH. Furthermore, a cell contains endogenous and exogenous anti-oxidants and transition metal scavengers. The anti-oxidants can serve as sink for radicals, and the transition metal scavengers mainly inhibit the conversion of hydrogen peroxide to the hydroxyl radical. Scheme modified from Katic and Kahn, 2005.

(GSH), the major redox buffer of cells, is in a more oxidized state compared to the cytoplasm (Hwang et al., 1992). This creates an environment that stimulates proper disulphide bond formation, which is essential for correct folding of the proteins that enter the secretory pathway. As a result, the ER is more vulnerable for oxidative stress compared to the cytoplasm. Indeed, upon high levels of oxidative stress, the highly abundant ER resident proteins that assist in protein folding are primarily oxidized (van der Vlies et al., 2002).

In addition to anti-oxidant enzymes, an organism contains proteins that sequester transition metals (Figure 1) and thereby prevent their catalytic availability for reducing hydrogen peroxide to the hydroxyl radical. One of the most important transition metals is iron. In the circulation, iron is primarily sequestered by transferrin, but after cellular uptake, iron is stored by the intracellular protein ferritin. The importance of iron metabolism in aging is illustrated by the association of iron accumulation in brain regions that are affected in the age-associated neuro-degenerative disorders Alzheimer's and Parkinson's disease (Zecca et al., 2004).

Next to specific enzymes and proteins, cells also contain anti-oxidant molecules or scavengers that either have endogenous origin, such as urate and thioredoxin, or have exogenous origin, such as β-carotene (precursor of vitamin A), α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Figure 1). The exogenous sources of anti-oxidant molecules are primarily determined by food intake. This exemplifies the possible beneficial effects of diet intake on the survival of an organism.

Several studies have revealed the beneficial effects of endogenous and exogenous agents on the attenuation of oxidative stress or damage, and on the attenuation of functional deterioration associated with aging (McDonald et al., 2005; Seidman, 2000; Socci et al., 1995).

2.3 Molecular repair mechanisms and their implications in aging

When the first line of defense is inadequate, cellular molecules such as DNA, lipids and proteins are damaged, which challenges cellular functionality. Therefore, a cell is equipped with several mechanisms that repair or degrade the affected molecules. Below, the importance of these repair mechanisms and their implications in aging will be described.

2.3.1 Chromosomal DNA damage

Many different DNA repair mechanisms have been described, which underlines the important role of DNA in cellular integrity. Damage or mutations cannot only be inflicted by ROS, as described above, but can also be caused by chemicals and radiation or during DNA replication prior to cell division. For oxidative damage alone, more than 100 different oxidative DNA lesions have been described (Hoeijmakers, 2001), which exemplifies the complex nature of DNA repair mechanisms. These repair mechanisms are well-regulated and

repair is executed by large protein complexes that each recognize and repair a different class of DNA damage (Hoeijmakers, 2001). Like all biological systems, DNA repair systems are not perfect, which ultimately leads to accumulation of genetic errors. A classic theory of aging, called the somatic mutation theory, is based on this aspect of life. This theory states that the rate of accumulation of genetic mutations, and thus accumulation of illegal gene products, ultimately determines an organism's lifespan. Indeed, there appears to be a correlation between DNA repair rates and lifespan among mammals (Promislow, 1994). Furthermore, dysfunctioning DNA repair mechanisms are associated with pathologies that show early signs of aging and a decreased lifespan, as is found in Werner's syndrome and Cockayne syndrome patients (Bender et al., 2003; Chen et al., 2003a; Chen et al., 2003b), or an increased susceptibility for developing age-related diseases, such as cancer (de Vries et al., 1995). Interestingly, in mice it has been shown that mutation of genes involved in the functioning of a limited set of DNA repair mechanisms induce symptoms of accelerated aging (Hasty et al., 2003), while mutations concerning other DNA repair mechanisms have no obvious or an embryonic lethal effect (Engelward et al., 1997; Tebbs et al., 1999). These different effects probably depend on the frequency of certain DNA damages and the availability of other DNA repair mechanisms that can replace the dysfunctioning DNA repair mechanism. These examples clearly demonstrate the important role of several DNA repair mechanisms in maintaining chromosomal DNA integrity and thus in determining lifespan and protection against diseases (Figure 2).

2.3.2 Telomere shortening

Next to chromosomal DNA mutations, telomere shortening has been implicated in the organismal aging process (Figure 2). Telomeres are simple DNA repeat sequences at the chromosome ends that form complexes with proteins to protect the chromosomes from degradation, end-to-end fusions and activation of the p53-dependent DNA damage response pathway involved in induction of apoptosis (van Steensel et al., 1998). Shortening of the telomeres can lead to genomic instability and loss of proliferative ability, called senescence (von Zglinicki, 1998; von Zglinicki, 2000), which will be discussed in more detail later. The loss of proliferative ability is thought to be caused by disruption of the telomere structure when telomere DNA reaches a critical

length. Indeed, it has been shown that disruption of the protein structure forming the telomere complex induces premature senescence mediated via the p53 or the p16/pRB pathway (Smogorzewska and de Lange, 2002).

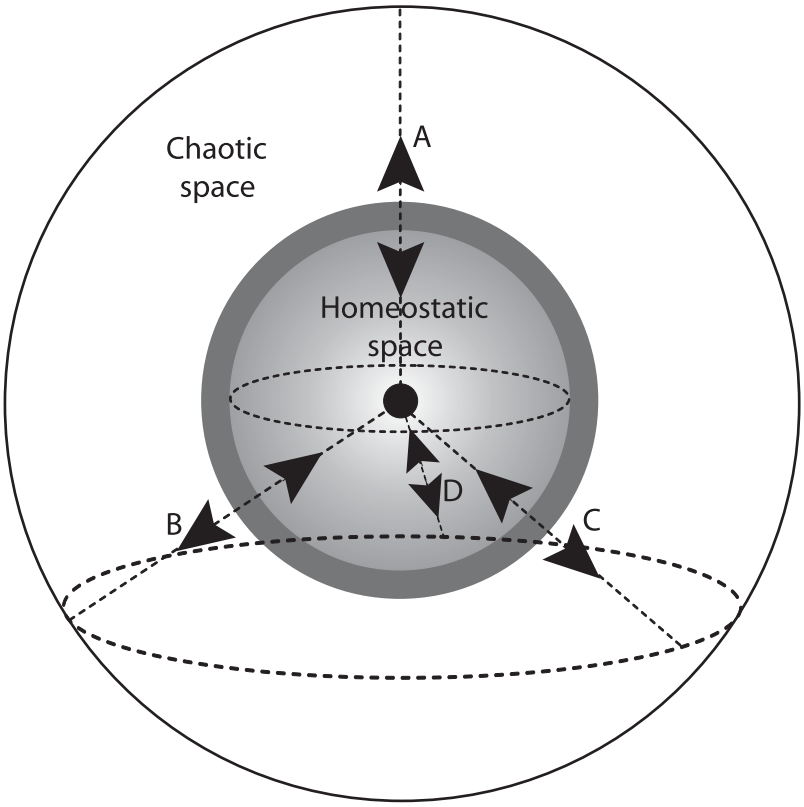

Figure 2: Model of the molecular processes that influence cellular fate during aging. Several molecular protection and repair mechanisms, such as ROS protection, DNA repair, protein quality control and others (strings A-D), are involved in maintaining cellular homeostasis (homeostatic space). Upon loss of efficiency of one or more of these molecular mechanisms during aging or upon stress, the cell will shift from the center of homeostatic space. The molecular protection and repair mechanisms will attempt to regain cellular homeostasis. These processes are also dependent on the energy levels of the cell, which decrease with increasing age. Consequently, aged cells experience larger shifts from the center of homeostatic space when challenged compared to young cells. When the molecular disorder reaches a critical level (the boundary between homeostatic and chaotic space) the cell will go into a state of replicative arrest, or go into apoptosis or necrosis.

Telomere erosion is caused by the inability of DNA polymerases to replicate the 3'-ends of chromosomes to their very end during DNA replication prior to every cell division. This is called the end-replication problem. Telomere shortening can be significantly accelerated by oxidative damage to telomeric DNA. It seems that DNA repair mechanisms are less able to repair this damage because of the tight nature of the protein complexes that form the telomeres. These damages cause stalling of DNA polymerase activity, which can lead to substantial telomere erosion (Cordeiro-Stone et al., 1999; von Zglinicki, 2000).

In germ line and cancer cells telomere length is maintained by the activity of the ribonucleoprotein telomerase that can elongate telomeric DNA (Greider and Blackburn, 1985). However, most cells normally do not possess telomerase activity. Many studies have shown that with increasing age, telomere length is shortened in a variety of tissues (Allsopp et al., 1992; Coviello-McLaughlin and Prowse, 1997; Frenck et al., 1998; Hastie et al., 1990; Op den Buijs et al., 2004). Furthermore, in birds and mammals telomere shortening has been shown to be correlated with maximum lifespan (Haussmann et al., 2003; Vleck et al., 2003). This suggests that telomere shortening is not only involved in cellular replicative lifespan, but also in organismal lifespan (Katic and Kahn, 2005).

2.3.3 Mitochondrial DNA damage

Another aspect of DNA damage and repair in aging involves mitochondrial DNA, which is included in the mitochondrial free radical theory of aging. This theory states that the ROS species produced by the mitochondria during normal aerobic respiration primarily subject these same mitochondria to a high degree of oxidative stress. Mitochondria contain double stranded closed circular DNA (mtDNA) that encodes for 13 mitochondrial proteins, of which most are involved in the electron transport chain. The induced oxidative stress can damage these genes, which could lead to dysfunctional electron transport proteins and an increased incomplete reduction of oxygen to water, resulting in increased oxidative stress (Beckman and Ames, 1998; Cottrell and Turnbull, 2000; Ozawa, 1998; Wei, 1998). Indeed, an age-related decline in mitochondrial respiration has been shown in several tissues of different species (Hsieh et al., 1994; Sugiyama et al., 1993; Yen et al., 1989) as well as an increased production of ROS (Sohal et al., 1994). This theory explains, at

least in part, the observed increased oxidative stress with increasing age and the reduced mitochondrial coupling found in aging tissues (Marcinek et al., 2005; Ritz and Berrut, 2005). Especially the reduced mitochondrial coupling could be very important for the ongoing deterioration of an aging individual, as all maintenance systems that protect and repair damaged cellular molecules require a lot of energy. A decrease in energy levels will greatly influence the efficiency of these maintenance systems and predispose a cell to accumulation of damaged molecules (Figure 2).

2.3.4 Protein damage

Next to damage to DNA, proteins are also damaged in the course of aging (Figure 2). At least two theories involving proteins have been implicated in the aging process, the altered proteins theory and the waste accumulation theory (Kirkwood, 2005). In normal functioning cells with an adequate energy supply, there is a relatively high turnover of proteins, even of undamaged proteins. The regulation of protein synthesis and degradation rates allows the cell to rapidly modify intracellular protein levels to adapt efficiently to intra- and extracellular environmental changes (Martinez-Vicente et al., 2005).
Over time, proteins are subjected to numerous damaging circumstances that can alter the protein and could lead to impairment or loss of protein function. This includes heat-induced denaturation, but especially oxidative modification, which can lead to increased levels of protein carbonyls (Levine, 2002), oxidized methionines and cysteines (Davies, 2000; Stadtman, 2001; Stadtman et al., 2003), glycation (Baynes, 2001), protein cross-linking (Squier, 2001) and aggregate formation. A cell has evolved mechanisms that either create an environment that enables the protein to regain its original composition, or target it for degradation. Both mechanisms require energy for proper functioning.

The first protein regulation mechanism is carried out by chaperones that sequester and assist in folding of denatured or newly synthesized proteins. Chaperones reside both in the ER, called ER chaperones, and in the cytoplasm, called heat-shock proteins (HSP) and ultimately determine the fate of the damaged protein. Chaperone binding of a denatured protein prevents the protein to aggregate and creates an environment for the denatured protein to refold. When a cell is exposed to high levels of stress and thus massive protein

denaturation occurs, the maximum refolding capacity of the chaperone system can be reached. This triggers a stress response, which results in the specific up-regulation of chaperone and protein degradation genes and thus in an increased refolding and degradation capacity (Riezman, 2004; Schroder and Kaufman, 2005). For the ER and the cytoplasmic protein refolding machinery, these responses are referred to as the unfolded protein response (UPR) and the heat-shock response.

The second protein regulation mechanism involves the recognition of the altered proteins and their subsequent degradation. There are several systems that are involved in protein degradation, such as the ubiquitin-proteasome system, calpains and lysosomes. Of these systems the proteasome system and the lysosomal system are responsible for most of the intracellular protein turnover.
The proteasome system has been shown to be responsible for the selective degradation of oxidized proteins in mammalian cells, via ubiquitination of the damaged protein prior to degradation (Sitte et al., 1998).
The lysosome is a cellular organelle that contains a large assortment of hydrolases capable of degrading a wide variety of macromolecules. Lysosomes do not only degrade extracellular proteins that are internalized via endocytosis, but can also degrade long-lived intracellular macromolecules or even whole organelles, in a process called autophagy, which is activated under different kinds of stress conditions (Cuervo, 2004; Shintani and Klionsky, 2004). These macromolecules and organelles are surrounded by de novo formed membranes, prior to fusion to and degradation by the lysosome (Klionsky, 2005; Shintani and Klionsky, 2004) or are directly engulfed by the lysosome (Farre and Subramani, 2004; Klionsky, 2005).

Despite the existence of these protection and repair mechanisms, oxidative protein damage is still detectable under normal physiological conditions (Agarwal and Sohal, 1994; Smith et al., 1991), which suggests that these systems are insufficient to protect against all oxidative damage even during basal levels of ROS generation. Furthermore, there is evidence of the functional decline of the chaperone and several degradation mechanisms with increasing age (Carrard et al., 2002; Colotti et al., 2005; Cuervo and Dice, 2000; Soti and Csermely, 2003). As the chaperone and degradation pathways

are also composed of proteins, the decreased functioning of these systems can also, at least in part, be caused by damage of members of these pathways, as was proposed for ER chaperones (Rabek et al., 2003). In addition, mildly oxidized proteins have been shown to be good substrates for degradation, but extensive oxidatively modified proteins are more resistant to degradation and are prone to aggregate (Grune et al., 1997). This implies that with increasing oxidative stress upon aging, proteins that are bad substrates for degradation will accumulate. Furthermore, the lysosome also reveals striking changes with increasing age that impair lysosomal function, such as decreased regulation of lysosomal pH, changes in hydrolase activities, increase in lysosomal volume and accumulation of indigestible materials as lipofuscin (Terman and Brunk, 2004). A consequence of the progressive decline of these mechanisms would be the accumulation of oxidized proteins, macromolecules and organelles during aging. Indeed, with age increased levels of oxidatively modified proteins have been found in tissues of several species (Agarwal and Sohal, 1994; Garland et al., 1988; Head et al., 2002; Smith et al., 1991; Starke-Reed and Oliver, 1989). This increase could have deleterious effects on cellular functioning and could thus be involved in the decline of organismal vitality.

2.3.5 Lipid damage

As for DNA and proteins, lipids can also be damaged by a wide variety of agents (Figure 2). Although information about these changes is currently not so widespread as for DNA and proteins, there are indications that lipid damage can play an important role in the decline of cellular functioning. Lipid oxidation results in lipid peroxide formation that has been shown to reduce membrane fluidity, can inactivate membrane-bound proteins and can be degraded into cytotoxic aldehydes, such as malondialdehyde and hydroxynonenal (Richter, 1987). Increased levels of these breakdown products have been shown in tissues of *D. melanogaster* and rat with increasing age (Odetti et al., 1994; Zheng et al., 2005). Oxidized lipids are also substrates for degradation. For instance, it has been shown that phospholipase A2 preferentially hydrolyzes fatty acids from oxidized liposomes and reveals increased activity upon oxidative stress (van den Berg et al., 1993; van Rossum et al., 2004).

3 Changes at the cellular and tissue level during aging

The described molecular changes of DNA, proteins and lipids that occur in time, will have an impact on cellular functioning, and therefore on functioning of tissues, organs and the organism itself. However, there are unique cellular mechanisms involved in aging that are influenced by the molecular changes described above.

3.1 Senescence

As mentioned above, normal cells cannot divide indefinitely and can go into a state of replicative arrest, called replicative senescence (Figure 2). This was first demonstrated in tissue culture experiments with primary human fibroblasts by Hayflick and Moorhead (Hayflick and Moorhead, 1961). It was proposed, and later shown, that the erosion of telomeres caused this loss of proliferative capacity (Harley et al., 1990; Olovnikov, 1973). Cells that go into senescence undergo morphological changes, changes in chromatin structure and gene expression and cease to respond to mitogenic stimuli (Narita et al., 2003; Serrano and Blasco, 2001; Shelton et al., 1999). They can survive in this non-dividing state for months and are less sensitive for induction of apoptosis, a process that involves programmed cell death, which will be discussed later. The physiological role of senescence is still not clear, as one would need to know how many cells *in vivo* undergo senescence and how many senescent cells need to accumulate to cause organismal aging.

However, induction of senescence is thought to protect damaged cells from unlimited cell proliferation, which could lead to cancer (Campisi, 2005). Therefore, senescence is thought to have a protective function early in life, but with increasing age accumulation of these cells could have deleterious effects on tissue functionality. In recent years it has become clear that senescence can also be induced by different kinds of stress, such as DNA damage and oxidative stress (Serrano and Blasco, 2001), which occurs within days after stress induction. Although these different signals probably elicit a common cellular response via different pathways, a distinction is made between replicative senescence, which is induced after extensive proliferation, and stress-induced senescence, which is induced by various forms of stress. It is very well possible that in the *in vivo* situation even a combination of the two may occur.

3.2 Cell turnover

Whenever a cell faces severe stress, the cell is dependent on his ability to adapt to or resist the opposed stress and to replace or repair the damaged molecules. The mechanisms that play a part in these processes have been described earlier. However, whenever the stress and consequently the damage are too severe, cellular homeostasis will be lost and the cell will eventually die (Figure 2). There are two distinct forms of cellular death, necrosis and apoptosis. Necrosis is often induced by severe forms of stress in which cellular integrity cannot be maintained, which leads to cell lysis and secretion of cellular proteins in the circulation. This can occur during a local immune response or during obstruction of circulation, which leads to local and relatively large areas of cellular death. Indeed, intracellular proteins have been detected in human blood samples (Pieper et al., 2003a), although the precise origin of these proteins is still obscure.

Apoptosis is a consequence of the induction of an internal suicide program, which can be induced by several signal transduction pathways that detect cellular stress or damage, such as DNA damage via the p53 signaling pathway (Gomez-Lazaro et al., 2004), ER stress via the activation of the UPR (Orrenius et al., 2003) or protein denaturation via the heatshock response (Finkel and Holbrook, 2000), some of which also have been shown to induce senescence. Induction of senescence or apoptosis probably depends on environmental circumstances, the level of cellular damage and on cell cycle status (Boonstra and Post, 2004). Apoptosis distinguishes itself from necrosis, in that the cell retains its membrane integrity and is thus not subjected to cell lysis. During apoptosis, chromatin is segregated in sharply circumscribed masses at the edge of the nuclear envelope. The cytoplasm is condensated and closed membrane vesicles are formed by blebbing of the plasma membrane, which are phagocytized by nearby cells. Therefore, during apoptosis there is no severe leakage of cellular proteins into the circulation.

Cells that are lost by processes as apoptosis or necrosis are normally replaced either by cell division of neighboring cells or by differentiation of stem cells (Op den Buijs et al., 2004), depending on the tissue type and environmental circumstances. As mentioned above, there is a limitation in the number of cell divisions in normal cells, which can be caused by the erosion of telomeres. Stem cells are also subjected to the aging process, which can result in exhaustion of the stem cell pool or a reduced differentiation capacity of stem

cells (Anversa et al., 2005; Kamminga and de Haan, 2006; Quarto et al., 1995). This is also caused by the molecular changes that occur in time. A consequence of these two factors is the decrease in regenerative capacity, and ultimately a decrease in functioning of tissues upon aging. This also illustrates the two-edge sword of processes such as apoptosis in aging. When apoptosis pathways have a low threshold, there is a decreased chance of developing diseases as cancer, as relatively low levels of damage will cause programmed cell death. However, it also predisposes the organism to a high cell turnover rate, which sensitizes the organism for a reduced vitality later in life. This was elegantly demonstrated in a mouse model in which a mutant form of p53 showed constitutive activation. These mice had a lower incidence of cancer, but showed faster aging (Tyner et al., 2002).

4 Understanding the interactions of aging theories

All of the above mentioned changes will, at least in part, play a role in the aging process and the associated decline in organismal survival. Although strides have been made to understand the role of these processes in the development of aging, a general consensus has not been reached on the relative importance of each of them. This has led to initiatives to develop a theory in which the different contributors that are thought to play a role in the aging process are considered together, called the network theory of aging (Kirkwood et al., 2003). This would allow the understanding of the interaction and possible synergistic mechanisms of these processes. Furthermore, understanding these relations is necessary to determine the processes that are involved in the early and the late stages of aging. Recognizing these connections could be very important in our search for optimal mechanisms of intervention in the aging process (Kirkwood, 2005). As every species invests differently in maintenance of damage prevention and repair, differences herein are expected. Even in different tissues of an organism the importance of the various aging mechanisms may differ. For instance, highly proliferative tissues will suffer more from telomere erosion and somatic mutations than post-mitotic tissues because of the requirement of DNA replication prior to every cell division. On the other hand, post-mitotic cells will suffer more from waste accumulation, such as protein aggregates, as in proliferative tissues these aggregates will be divided between daughter and mother cell (Kirkwood, 2005), which will result in dilution of the aggregates in newly synthesized cell material. Furthermore, every tissue is exposed to different environmental circumstances, which requires different levels of protection.

Mapping the differences that occur in the course of the aging process, and thereby defining so called biomarkers of aging could provide the information necessary to understand the aging process and the contributions of the different mechanisms herein. Moreover, it would enable the analysis of the effects of aging intervention studies and ultimately, it could be used to determine the biological over the chronological age of an individual. The heterogeneity of the aging process, most likely caused by life style and variation in genetic make-up (Kirkwood et al., 2005), and the difficulty of discriminating between changes caused by normal aging and age related pathologies make it difficult to obtain high accuracy in sensitivity and specificity with single markers. For that reason, multiple analytes in different

tissues, organs or bodily fluids should be investigated to really grasp the importance of aging theories in the onset and progression of aging, as with multiple independent markers one can increase the sensitivity and specificity compared to the use of a single marker (Anderson, 2005).
This approach was started with the genomics era that provided information about genetic expression differences (Lee et al., 1999; Lee et al., 2000; Zou et al., 2000), and is now extending to the field of proteomics that provides information about protein expression and modification differences, which will be the focus of this thesis.

4.1 Antibodies in proteomics

In the last few years, proteomics has become one of the fastest growing research fields. This has led to the development of numerous high throughput assays and improvement of several existing techniques that enable the analysis of several proteins in a single experiment. Despite these developments, it has become clear that this field faces us with even more challenges than the genomics era, because of some unfavorable characteristics of proteins, e.g. the presence of differently modified isoforms of a single protein, differences in protein stability, protein-protein interactions and the presence of highly abundant bulk proteins that hamper the analysis of less abundant proteins.
Affinity ligands, such as antibodies, can provide solutions for several of these problems. Antibodies can be used for detection of a single protein in a biological sample with high specificity and sensitivity. The use of a large set of different antibodies, each with different antigen specificities thus would enable the simultaneous analysis of several proteins. This principle forms the basis of the development of antibody micro-arrays, in which antibodies are immobilized on a surface, each in a separate spot, to capture their respective antigen (Angenendt, 2005). Depending on the specificity and affinity of the used antibodies, this technique provides the expression analysis of several predetermined target proteins simultaneously and with high sensitivity.
Another application for antibodies in proteomics is as an affinity ligand for purification or depletion of target proteins (Pieper et al., 2003b). The purification of a protein from a biological sample provides methods to study protein-protein interactions, as proteins that are co-purified with the target antigen can be analyzed on gel or with mass spectrometric techniques.

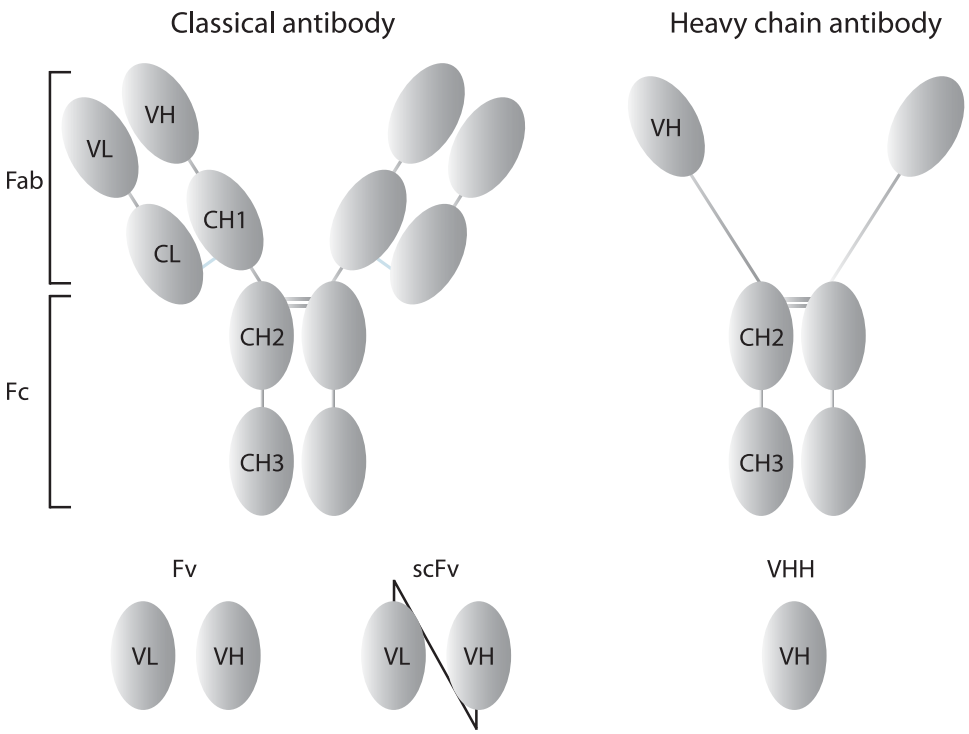

Figure 3: Schematic representation of the differences between classical and heavy chain antibodies, and their antigen binding fragments. A classical antibody consists of two identical light and heavy chains, whereas the heavy chain antibody consists of only two identical heavy chains. The smallest antigen binding domain of a classical antibody (Fv) consists of the combination of the variable light (VL) and variable heavy (VH) chain domain, which can be linked via a synthetic linker to obtain a single chain Fv fragment (scFv). The smallest antigen binding domain of a classical antibody consists of only the variable domain of the heavy chain (VHH).

Furthermore, many biological samples contain highly abundant bulk proteins that interfere with the detection of less abundant proteins. Affinity ligands can be used to deplete these abundant proteins, thereby enabling the analysis of previously non-detectable proteins (Pieper et al., 2003a).

Moreover, antibodies can be used to analyze protein expression patterns by making use of the existence of common epitopes present in several different proteins. Such an epitope enables the use of a single antibody to visualize or purify several different proteins simultaneously. This approach has been successfully applied for post-translational protein modifications, such as phosphorylation and ubiquitination (Gronborg et al., 2002; Maguire et al., 2002; Richter et al., 2005).

4.2 Generation of specific antibodies

The successful application of antibodies in proteomics requires reliable affinity molecules in respect to sensitivity as well as specificity. Antigen specific antibodies can be obtained using several different techniques.

The traditional method of obtaining antibodies is by immunization of an animal with the target antigen, followed by the isolation of the polyclonal serum a few weeks after immunization. Although the generation time for these antibodies is short and the costs are relatively low, there are several disadvantages. The polyclonal antibody pool contains many different antibodies with various affinities for the target antigen. This heterogeneity renders polyclonal antibodies relatively less specific compared to monoclonal antibodies, as each different antibody in this pool could potentially cross-react with proteins other than the target antigen. Polyclonal antibodies are therefore less suitable for use in proteomics. Furthermore, the antibody source is not infinite, requiring new immunizations and new specificity and sensitivity tests, as every new immunization will yield a different antibody pool with their own specificity and sensitivity.

Monoclonal antibodies are obtained by screening the B-cell repertoire of an immunized animal for cells that produce antigen specific antibodies. These cells are then fused to myeloma cells giving rise to an immortal hybridoma cell line (Kohler and Milstein, 1975). Once the fabrication of such a cell line is established an indefinite source of antibody is obtained. On the other hand, the generation time of such an antibody is relatively long, because of the elaborate screening protocols and therefore the costs are high compared to polyclonal antibodies.

Recombinant antibodies share all the advantages of monoclonal antibodies and moreover, have additional advantages. Most recombinant antibodies only contain the antigen binding domain of classical antibodies (Figure 3), although recombinant whole classical antibodies have been described. As they are relatively small and have a reduced complexity compared to the classical antibodies, they can be efficiently cloned and produced in microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*. An additional advantage of recombinant antibodies is that they can be genetically modified (Clackson, 1991), which enables the addition of affinity tags or the construction of multi-valent antibodies to increase the avidity of the antibody molecule.

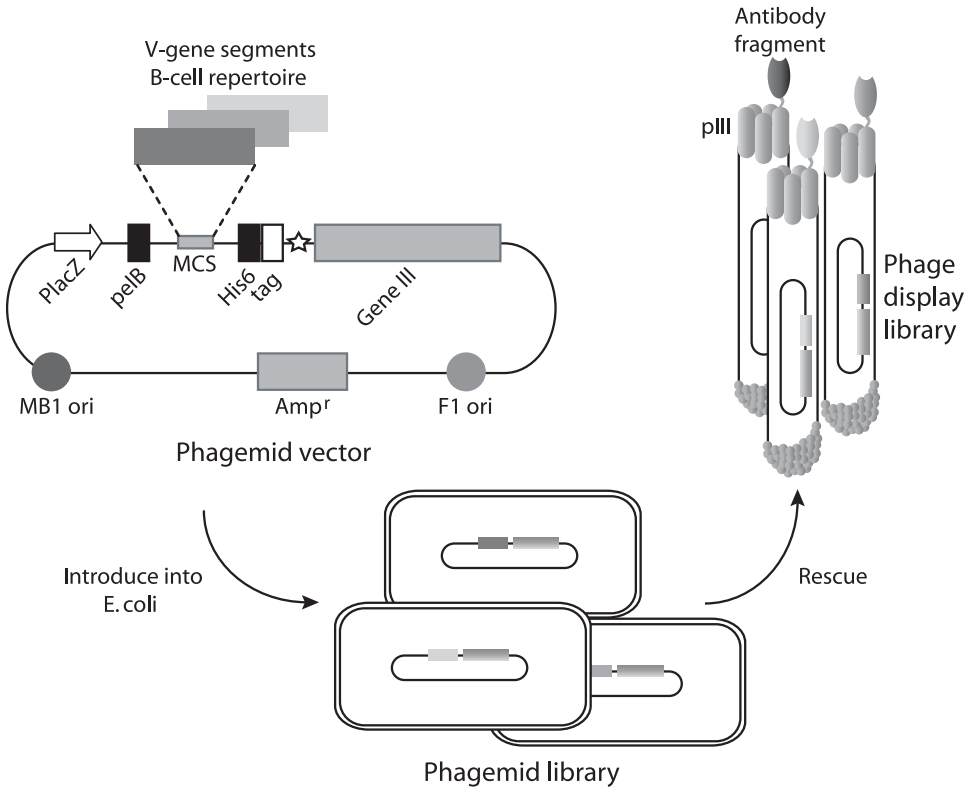

Figure 4: Schematic representation of the construction of a phage display library. V-gene segments, of B-cells from immunized or non-immunized animals, can be amplified by cDNA synthesis and PCR. The obtained genes are inserted into the multiple cloning site (MCS) of a phagemid vector, in frame with a purification (His6) and a detection tag (tag), and gene III, the gene encoding the phage coat protein pIII. A phagemid library is obtained by introduction of this vector into *E. coli*, after which phage particles, displaying the antibody fragments on their surface, can be rescued via infection with helperphage to obtain a phage display library. The lacZ promoter, pelB sequence and the amber stop codon (star) allow the production and transportation of soluble antibody fragments to the periplasmic space. These antibody fragments can be purified by means of their His6 tag.

Most importantly, these characteristics also allow the construction of highly diverse antibody display libraries from the B-cell repertoire of immunized (Clackson et al., 1991) or non-immunized animals (de Haard et al., 1999; Marks et al., 1991). These libraries offer an *in vitro* selection system that can be used to obtain antibodies with predefined characteristics (Dolk et al., 2005a; Verheesen et al., 2003). The methodology of these libraries depends on the linkage of phenotype, the antibody, and genotype, the gene that codes

for the antibody. This linkage is accomplished by the immobilization or display of the antibody on a particle that contains the gene. Display methods using yeast (Boder and Wittrup, 1997; Schreuder et al., 1996), bacteria (Francisco et al., 1993), phage (Smith, 1985; Winter et al., 1994) and even ribosomes (Hanes and Pluckthun, 1997) have been developed. Historically, phage display is the most widely used of the display libraries. The most popular phage display methodology uses phagemid vectors, which are small vectors containing a phage packaging signal and a multiple cloning site that is used to clone the gene coding for the antibody in front of geneIII of the non-lytic filamentous phage fd or M13, which codes for the phage minor coat protein pIII (Figure 4). Expression of this construct in phage infected bacteria results in the display of the antibody fused to the pIII coat protein on the newly synthesized phage particle (Garrard et al., 1991; Hoogenboom et al., 1991). Large phage display libraries can be used to enrich for antibodies that bind a specific antigen. This is achieved by phage binding to the target antigen, washing to remove non-specific phage, followed by elution of the bound phage (Figure 5). Multiple rounds of selection can be performed sequentially.

The strength of phage display is that the selection protocols can be adapted to enrich antibodies suitable for a specific application, which significantly reduces the need for elaborate screening protocols, as is needed for hybridoma technology.

Furthermore, the use of phage display offers an additional advantage. Large parts of the selection and screening protocols can be robotized and provide the basis for high throughput selection of antibodies. This can reduce the production cost of an antibody significantly.

However, it should be noted that the successful selection of specific antibodies depends on the selection strategy used. As phage display drives the selection to clones that have advantages over other clones, the use of a sub-optimal selection strategy can result in selection of antibodies with unwanted characteristics, such as clones with high growth rates or clones that bind to a dominantly exposed epitope. It is therefore essential that much attention must be paid to the selection strategy used and to the analysis of the selection output after every cycle.

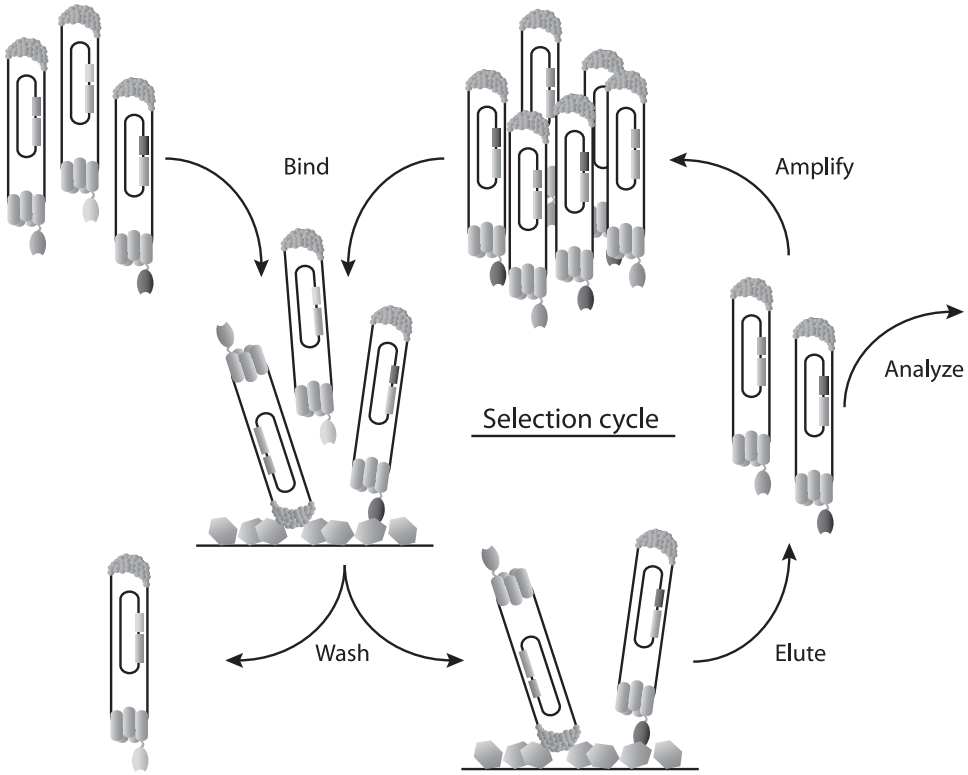

Figure 5: Schematic representation of the *in vitro* phage display selection cycle. Enrichment of antigen specific antibodies from large phage display libraries that can contain billions of different antibodies is accomplished by successive selection cycles. Each cycle consists of a binding step, where the phage display library is incubated with immobilized antigen, followed by a washing step to dispose of non-bound phage, and an elution step to obtain the bound phage. These phage can be amplified to start a new selection cycle, via infection of *E. coli* cells with the eluted phage and subsequent phage rescue using helperphage, or can be analyzed for antigen specificity and sensitivity in several different applications.

Antibodies derived from animals belonging to the species of *Camelidae* have additional advantages over other recombinant antibodies. Besides classical antibodies, these species possess antibodies that lack the light chain (Hamers-Casterman et al., 1993) (Figure 3). As a result, the antigen-binding domain (VHH) of these antibodies consists of only one domain, which offers several advantages over conventional recombinant antibodies. They represent the smallest antigen binding domains derived from antibodies (Muyldermans, 2001), and they are more stable than conventional antibodies or their

derivatives (Dolk et al., 2005b; van der Linden et al., 1999), making them extremely suitable for affinity chromatography (Verheesen et al., 2003). Furthermore, the single domain structure enables easy cloning to make highly diverse libraries and enables high production in *Escherichia coli* and *Saccharomyces cerevisiae*, which makes them economically attractive (Frenken et al., 2000).

5 Outline of this thesis

As brought forward, identification of protein changes that occur in time could provide new insights in the aging process. Moreover, it would enable the analysis of the effects of aging intervention studies and ultimately, it could be used to determine the biological over the chronological age of an individual.
The use of proteomic techniques could provide the identification of multiple changes that occur upon aging. Antibodies are an important tool in the development or improvement of proteomic techniques. As described above VHHs are antibody fragments with several advantages over classical antibodies and their derivatives. In this thesis, the use of these antibodies is explored in the development of new proteomics tools that can be used in the analysis of protein expression differences in aging-related biological samples.

One of the most important and most easy accessible human clinical biological samples is blood plasma, which is typically used for assessment of health status. Changes that occur during aging, such as increased cellular death and decreased tissue and organ functioning, should at least in part be reflected in the blood plasma protein composition. However, proteomic analysis is hampered by the presence of several highly abundant bulk proteins. In chapter 2, the selection of highly specific single domain Llama antibody fragments (VHH) for affinity chromatography purposes is described, which can be used to remove the highly abundant human plasma proteins, human serum albumin (HSA) and immunoglobulin G (IgG). This removal resulted in the visualization of previously masked protein spots and an increased resolution on 2D-gel. Intriguingly, these affinity ligands have superior characteristics compared to presently available affinity ligands, and use of these ligands can be expected for research as well as therapeutic purposes.
In chapter 3, the use of these affinity ligands, combined with two-dimensional difference gel electrophoresis (2D-DIGE), is shown in a human blood plasma proteomics study to reveal protein expression differences between young and old individuals. This revealed the importance of studying not only total protein expression levels, but also the different isoforms of single proteins. Furthermore, this approach demonstrated that upon aging a slightly increased pro-coagulant and pro-inflammatory state is induced. In addition, some protein expression differences indicated increased cellular damage upon aging,

manifested by an up-regulation of proteins involved in scavenging harmful cellular molecules.

The increase of oxidative stress observed during aging can damage various different cellular molecules. An organelle that seems especially vulnerable for oxidative stress is the ER. Increased oxidative stress might interfere with protein folding and lead to differences in expression of the protein involved in protein folding, the ER-resident proteins. These proteins contain a C-terminal signal sequence, which determines their ER localization. In chapter 4, the selection of VHHs specific for the C-terminal KDEL sequence present on several ER resident proteins is described that can be used to study protein expression patterns. These antibodies were used to analyze ER-resident protein expression differences upon different kinds of ER stress, to show their applicability. This clearly demonstrated the feasibility of selecting and using an antibody that recognizes a common amino acid sequence epitope for studying protein expression patterns.

Chapter 5 provides preliminary data on the expression differences of the membrane protein endoglin and several ER resident proteins found in a human umbilical vein endothelial cell (HUVEC) senescence model comparing young and senescent cells. The obtained results are used to discuss the challenges that are encountered when analyzing protein expression differences in this and other aging models.

Chapter 2

Improved anti-IgG and HSA affinity ligands: Clinical application of VHH antibody technology

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Abstract

Large scale, highly specific purification of valuable proteins from blood and removal of undesirable components promise to have wide therapeutic applications. Moreover, depletion of bulk proteins from blood is a prerequisite for clinical proteomics. Here we describe the development of specific, high affinity Camelid antibody fragments (VHH) derived from immune libraries for purification and depletion of the bulk proteins HSA and IgG from human serum and plasma for therapeutic and research purposes. The anti-IgG VHH improved depletion of IgGs from blood substantially over the classical used method based on proteinA. To demonstrate the better performance of the VHH based IgG depletions, we analyzed the presence of auto-antibodies in human plasma before and after depletion from two groups of autoimmune disease patients, notably Goodpasture (GP) and Systemic Lupus Erythmatosus (SLE). VHHs can be produced efficiently and cost effectively in *S. cerevisiae*, a GRAS organism. A good manufacturing process (GMP) for purification of these VHHs has been developed as well. Moreover, as VHHs are single protein chains, they can be coupled relatively easy to solid matrices. These three factors are important for developing affinity purification medications.

Introduction

Affinity chromatography has revolutionized the development of new techniques in pharmaceutical science and biotechnology, where it is applied as an improved method to purify valuable substances for the more common liquid chromatography separation techniques, as size-exclusion and ion-exchange chromatography (Hage, 1999). Also clinical and research laboratories have gained much interest in this separation technique. Besides methods such as direct analyte detection and removal of highly abundant proteins that obscure the analysis of less abundant proteins, affinity chromatography can also be used to purify certain highly valuable blood components or alternatively remove harmful components involved in diseases from the blood of patients. Some damaging substances, like endotoxins and drugs, are usually removed by hemodialysis although this method is often not very efficient (Kragh-Hansen et al., 2002; Otagiri, 2005). Clearly, more specific and efficient methods to remove particular damaging substances from blood for treatment of specific diseases would be advantageous. Procedures to improve the efficiency of hemodialysis in treatment of drug overdoses, or the removal of cytokines and endotoxins, such as lipopolysaccharides, are for a large extend focused on the use of the binding characteristics of human serum albumin (HSA) (Kragh-Hansen et al., 2002; Otagiri, 2005; Zimmermann et al., 1999). Therefore, pure and large amounts of HSA are needed for which affinity chromatography offers an ideal solution. Columns that have been developed for research purposes to purify or deplete HSA from blood, such as dye or antibody based columns (Gianazza and Arnaud, 1982; Pieper et al., 2003b; Steel et al., 2003; Travis and Pannell, 1973), are not suitable for this purpose because of the low specificity, or are very expensive in the case of antibody columns.

The use of proteinA based IgG affinity chromatography for the treatment of autoimmune diseases has recently been approvedby the Food and Drug Administration (FDA). Although proteinA is a well-known and frequently used protein for IgG purification, there are several unfavorable characteristics, such as high handling costs and safety aspects (Fassina et al., 2001), which makes it less suitable for clinical applications. Furthermore, it lacks specificity for all IgG subclasses, especially IgG3 (Eliasson et al., 1988). This prevents total auto-antibody depletion in some auto-immune diseases that are known for the presence of IgG3 specific auto-antibodies, such as Systemic Lupus

Erythematosus (SLE), Dilated Cardiomyopathy (DCM) and Primary Biliary Cirrhosis (PBC) (Amoura et al., 2000; Rigopoulou et al., 2005; Staudt et al., 2002). Consequently, there is a high interest in novel methods that enable the efficient, cost-effective and reliable purification of this important group of blood proteins.

Antibodies derived from animals belonging to the species of *Camelidae* are a very promising tool to be applied in this field. Besides classical antibodies, these species possess antibodies that lack the light chain (Hamers-Casterman et al., 1993). Therefore, the antigen binding domain (VHH) of these antibodies consists of only one domain, which offers several advantages over conventional antibodies, such as easy cloning to make highly diverse libraries and high production in *Escherichia coli* and *Saccharomyces cerevisiae* (Frenken et al., 2000), which makes them economically attractive. Furthermore, they represent the smallest antigen binding domains derived from antibodies (Muyldermans, 2001), and they are more stable than conventional antibodies (Dolk et al., 2005b; van der Linden et al., 1999) or their derivatives, making them extremely suitable for affinity chromatography (Verheesen et al., 2003). As these VHHs can be produced efficiently in *S. cerevisiae*, a GRAS organism, a wide range of therapeutic applications for these antibody fragments can be envisaged.

Here we describe the development of specific, high affinity VHHs against the bulk protein HSA and IgG, which can be used.for purification and depletion of the bulk proteins HSA and IgG from human serum and plasma, for therapeutic and research purposes. In a study with blood samples of GP and SLE auto-immune disease patients, the performance of the VHH column was compared to a proteinA based affinity column. In GP patients the depletion of IgG with the VHH affinity column performed at least equally well as the proteinA based column. In SLE patients, an auto-immune disease with a prevalence of IgG3 subtype specific auto-antibodies (Amoura et al., 2000), the IgG depletion with the VHH affinity column resulted in total depletion of reactive auto-antibodies whereas this was not accomplished in each individual patient sample when the ProteinA based column was used. Thus, VHH based affinity chromatography offers a technology that can be used in clinical laboratories for the development of specific and cost-effective affinity ligands for removal or purification of specific substances and can therefore greatly facilitate the progress in research and medicine. This could pave the road for improved

treatment of autoimmune disease patients and can also be used to remove microbial toxins and other harmful substances from blood, as we demonstrated recently for sepsis (ElKhattabi et al., (Submitted)).

Materials and methods

Library construction and selection of HSA and IgG specific VHHs

The anti-HSA specific VHHs were selected out of a phage display library constructed from B-lymphocytes of Llamas immunized with muscle extract using two consecutive rounds of panning with purified HSA (Sigma, Zwijndrecht, The Netherlands). The anti-IgG specific VHHs were selected out of a library constructed of Llamas immunized with total IgG. Selection was performed via panning on different purified subclasses of human IgG. Individual clones were subsequently screened in ELISA format as described before (Marks et al., 1991). Anti-IgG clones were screened for binding of all four IgG subclasses and the anti-HSA clones were screened on purified HSA (Sigma, Zwijndrecht, The Netherlands). Of each clone a DNA fingerprint was performed using the restriction enzyme Hin*F*I (van Koningsbruggen et al., 2003). The DNA of clones with a unique restriction pattern were sequenced (Sanger et al., 1977).

VHH production of selected clones

The best performing VHHs were cloned into a yeast expression vector and subsequently produced in *S. cerevisiae* as described before (Frenken et al., 2000; Thomassen et al., 2005). Secreted VHHs were purified from the fermentation medium on an ion exchange column.

Coupling of VHH to solid support

Purified VHH was coupled to a matrix using NHS (N-hydroxysuccinimide) coupling chemistry. Ligands containing primary amino groups couple directly to the active ester of NHS to form a chemically very stable amide linkage (Hermanson, 1993). As base matrix Sepharose (GE healthcare, Chalfont St. Giles, United Kingdom) was used, because it is known for its low non-specific binding. After purification, the antibody fragments were dialyzed to NHS coupling buffer (0.1 M Hepes pH 8.0). Prior to coupling of the VHHs to NHS, the matrix was washed with cold demineralized water acidified with acetic acid to pH3. Then the matrix was washed twice with NHS-coupling buffer. The washed matrix was mixed with the antibody solution and incubated overnight at 4°C head over head or 1 hour at room temperature. Subsequently, the gel material was filtered over a sintered glass filter and the non-reacted groups of the gel material were blocked with NHS block buffer (0.1M Tris pH 8.0) for 1

hour at room temperature. The coupled medium was washed using alternate low and high pH (3x10 column volumes PBS pH 2 and 3x10 column volumes PBS pH 7.4). The coupling efficiency was determined on SDS-PAGE with samples before and after coupling.

Dynamic capacity measurement

The dynamic capacity of the affinity matrices was determined on an AKTA explorer 100 (Amersham Biosciences, Roosendaal, The Netherlands). Column volume that was used for these tests was 400µl. The column was equilibrated with PBS pH 7.4 at a flow of 150 cm/hr. As sample for these experiments purified HSA and purified human IgG (Sigma, Zwijndrecht, The Netherlands) was used. Bound ligand was eluted with elution buffer (PBS pH adjusted to 2.1). The eluted fractions were immediately neutralized with one-twentieth volume 2M Tris buffer pH 8.0. The dynamic capacity was determined using peak integration of the elution peak.

BIAcore analysis

The affinities of the anti-IgG and anti-HSA VHH were determined using a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) in combination with a CM5 sensor chip (BIAcore, Uppsala, Sweden). Different concentrations of VHH (5, 25, 50, 250, and 500nM) were run over a low antigen density surface of around 1000 RU at a flow rate of 30µl/min. For the anti-HSA VHH purified HSA (Sigma, Zwijndrecht, The Netherlands), and for the anti-IgG VHH purified total IgG and purified IgG subclasses was used. Association and dissociation were measured for respectively 3 and 15 minutes. Regeneration was achieved by washing with 10mM HCl for 3 min. KD values were calculated with the BIAevaluation software.

Depletion of ligands from human plasma

This experiment was performed with the same settings as described for the dynamic capacity measurement. Column volume for these tests was 5ml. One ml Human serum (Sigma, Zwijndrecht, The Netherlands) was diluted 1:100 in PBS and run on the anti-IgG VHH column (capacity 10mg/ml). The non-bound fraction was subsequently run on the anti-HSA VHH column (capacity 12mg/ml). Bound protein was eluted with elution buffer (PBS pH2.1). To determine the efficiency of depletion, samples were evaluated on SDS-PAGE.

Furthermore, Western blots were performed using a monoclonal anti-HSA antibody (Sigma, Zwijndrecht, The Netherlands) and a polyclonal anti-human IgG antibody (Jackson Immunoresearch laboratories, West Grove, USA).
To compare the performance of the anti-IgG column to the most commonly used technique to remove and purify IgG in biotechnology and medicine, the same procedure was also performed on a proteinA column (HiTrap) (Amersham Biosciences, Roosendaal, The Netherlands). For Western blot analysis, IgG subclass specific antibodies were used (Sanquin, Amsterdam, The Netherlands).

2D-gel electrophoresis

To remove salts and lipids, samples were treated with the Clean-up kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Protein pellets were dissolved in 450µl rehydration solution (7M urea; 2M thiourea; 4% CHAPS; trace bromophenol blue; 0.5% (w/v) DTT; 0.5% (v/v) ampholytes pH3-10 non linear; 1.2% (v/v) destreak). Immobiline Dry strips pH3-10 of 24cm were rehydrated overnight with the protein samples and overlaid with 2ml Coverfluid in an Immobiline Drystrip reswelling tray. The first dimension of isoelectric focusing was run on an IPGphor system (Amersham Biosciences, Roosendaal, The Netherlands). Optimal protein focusing was achieved by starting at 500V for 1 hr, followed by 1500V for another hour. Subsequently, a constant voltage of 8000V was applied until a total of 48kV. Thereafter, the strips were equilibrated in equilibration buffer (50mM Tris/HCl pH8.8; 6M urea; 30% glycerol (v/v); 2% SDS (w/v)) containing 65mM DTT for 15 minutes followed by an incubation with 135mM iodoacetamide for 15 minutes. Second dimension was performed with lab-cast 24cm 12.5% polyacrylamide gels. Strips were loaded onto the gels and sealed with a solution of 1% agarose (w/v) containing a trace of Bromophenol Blue. The gels were run over night on the Ettan DALT Twelve system (Amersham Biosciences, Roosendaal, The Netherlands) at 1W/gel till the Bromophenol Blue dye front reached the bottom of the gel.
Gels were silver stained according to the Shevchenko protocol (Shevchenko et al., 1996).

IgG depletion and analysis of autoimmune disease plasma

Plasma of GP or SLE patients was diluted 100 times in PBS or HRP sample diluent (INOVA Diagnostics, San Diego, U.S.A.), respectively. Threehundred µl diluted plasma was incubated with 60µl protA sepharose or 60µl VHH anti-IgG sepharose (capacity 10mg/ml) at 4°C for 1.5 hours. The non-bound fraction of both columns and the diluted plasma input were analyzed for the presence of auto-antibodies with ELISA. The GP plasma samples were tested on coated glomerular basement membrane (GBM) and SLE plasma samples were tested with a Quanta LiteTM Chromatin kit (INOVA Diagnostics, San Diego, U.S.A.) according to the instructions of the manufacturer. Furthermore, the SLE samples were analyzed on SDS-page and on Western blot with IgG1, IgG3 (Sanquin, Amsterdam, The Netherlands) and total IgG (Sigma, Zwijndrecht, The Netherlands) specific antibodies. The whole procedure was performed *in triplo*.

Results

Selection and characterization of anti-HSA and -IgG specific VHHs

Our approach uses VHH antibody fragments to develop improved methods to remove or purify proteins from blood for therapeutic and research applications. To prove the value of these antibody fragments, HSA and IgG specific VHH antibody fragments were selected using phage display techniques via two consecutive rounds of panning.

To obtain VHHs that recognize all four subclasses of human IgG, the selection strategy was to switch IgG subclass in consecutive selection rounds, to direct the selection to clones specific for conserved epitopes between these subclasses. Furthermore, elution of the bound clones was performed by low pH shock, as this is preferably used for elution of bound material in affinity chromatography. The output of the selections was screened for binders by ELISA, and a Hin*F*I DNA fingerprint was performed to identify unique clones. The selection output of both selections was screened in ELISA for clones that recognized their target antigen.

VHH	Antigen	ka (1/Ms)	kd (1/s)	KA (1/M)	KD (M)
Anti-IgG	IgG1	4.76E+05	1.86E-03	2.55E+08	3.92E-09
	IgG2	7.26E+05	2.41E-03	3.01E+08	3.32E-09
	IgG3	6.64E+05	4.39E-03	1.51E+08	6.61E-09
	IgG4	5.01E+05	2.85E-03	1.76E+08	5.68E-09
	IgG	7.27E+05	1.62E-03	4.40E+08	2.27E-09
Anti-HSA	HSA	4.55E+05	4.89E-03	9.29E+07	1.08E-08

Table 1: Affinity data of the anti-IgG and anti-HSA VHHs for their respective antigen obtained with the BIAcore.

For the anti-HSA VHHs, twelve different antibodies purified from 50ml *E. coli* cultures were screened on columns using diluted human serum to identify the best performing VHHs for this specific application. For the anti-IgG VHHs, twenty different clones were obtained, of which two recognized all four IgG subclasses. These two anti-IgG clones and the four best performing anti-HSA clones were cloned into a yeast expression vector and subsequent production in *S. cerevisiae* resulted in VHHs, without any tag, secreted in the growth medium. The produced VHHs were purified from the medium on an ion exchange column (Frenken et al., 2000) and subsequently immobilized on a solid support via NHS coupling chemistry. After coupling, the dynamic capacity of the affinity matrices was determined on an AKTA explorer 100 with pure

antigen. The clones that performed best in this experiment were further evaluated. The dynamic capacity of the best performing anti-HSA affinity column typically fell in the range of 8-10mg HSA. For the best performing anti-IgG column this was in the range of 12-15mg human IgG per ml affinity matrix in a settled matrix bed. The affinity of the anti-HSA and -IgG clone for their respective antigen was determined with surface plasma resonance. The anti-HSA and anti-IgG clone recognized their respective antigen with nanomolar affinity (Table 1), which is comparable to classical antibody affinities. The affinity of the anti-IgG clone was also tested separately on all four purified human IgG subclasses, which revealed that the VHH had a broad cross reactivity for all IgG subclasses. Furthermore, species specificity of the clones was also assessed. The anti-HSA clone recognized mouse serum albumin but not from rabbit and rat (data not shown). The anti-IgG VHH did not recognize total IgG from mouse, goat and rabbit (data not shown).

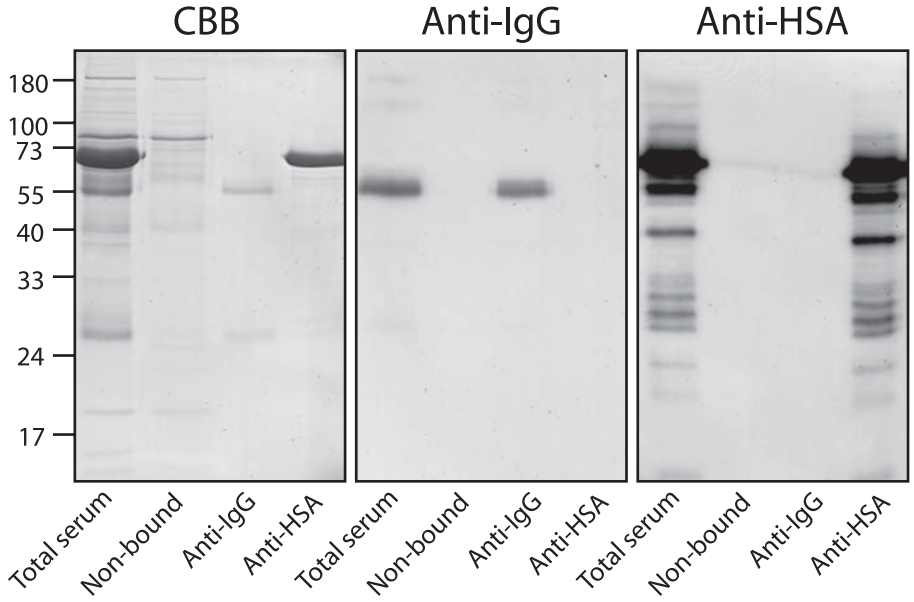


Figure 1: Assessment of the depletion of IgG and HSA from human serum. Total serum (lane 1), the non-bound fraction (lane 2) and the bound fractions of the VHH anti-IgG (lane 3) and anti-HSA (lane 4) columns were analyzed on a coomassie (CBB) stained SDS-page and on immuno blot with a commercial monoclonal antibody against IgG and HSA.

Depletion of antigens from human serum by affinity chromatography

The assessment of the obtained anti-HSA and anti-IgG clones showed a high affinity and specificity for their antigen, and functionality of the antibody fragments when immobilized onto a solid surface via primary amino groups. These matrices were used to deplete and purify HSA and IgG from human serum. First, the anti-IgG column was used to remove all IgG subclasses. Subsequently,the non-bound fraction was run over the anti-HSA column to remove HSA. Bound material was eluted by a pH shock. The whole procedure was followed on the AKTA (data not shown). This resulted in a non-bound, an IgG and a HSA fraction. These samples were analysed on SDS-page and Western blot with commercially available anti-HSA and anti-IgG specific antibodies (Figure 1). This clearly showed that no HSA or IgG was detected in the non-bound fraction. Interestingly, the anti-HSA blot of total serum showed numerous additional bands that ran primarily below the molecular weight of HSA. These proteins were also removed from the serum with our anti-HSA column. As serum contains numerous HSA fragments and modifications of HSA (Steel et al., 2003), this result indicates that this column, which contains a single monoclonal antibody, recognizes and depletes all these HSA products.

2D analysis of the obtained samples

The preceding results clearly demonstrate the depletion efficiency of our columns. To further assess the specificity of the VHH columns, the samples were further evaluated with 2D gel electrophoresis followed by MS analysis of relevant protein spots. First, the purified IgG and HSA fractions were evaluated for impurities. About 15μg of the purified IgG fraction and 50μg of the purified HSA fraction was used for this evaluation. In the IgG fraction, the IgG heavy and light chain could clearly be distinguished and no additional protein spots were observed (Figure 2). This clearly shows the specificity of the anti-IgG column. In contrast, in the HSA purified fraction several spots could be seen below and above the molecular weight of HSA (Figure 3). The most prominent protein spots were analyzed with MS and all these spots were identified as HSA. This is in agreement with the extra bands seen on Western blot with the commercial anti-HSA antibody, which are all depleted by the VHH anti-HSA column (Figure 1). The data confirm that these bands are fragments or modified forms of HSA, and furthermore illustrate the specificity and

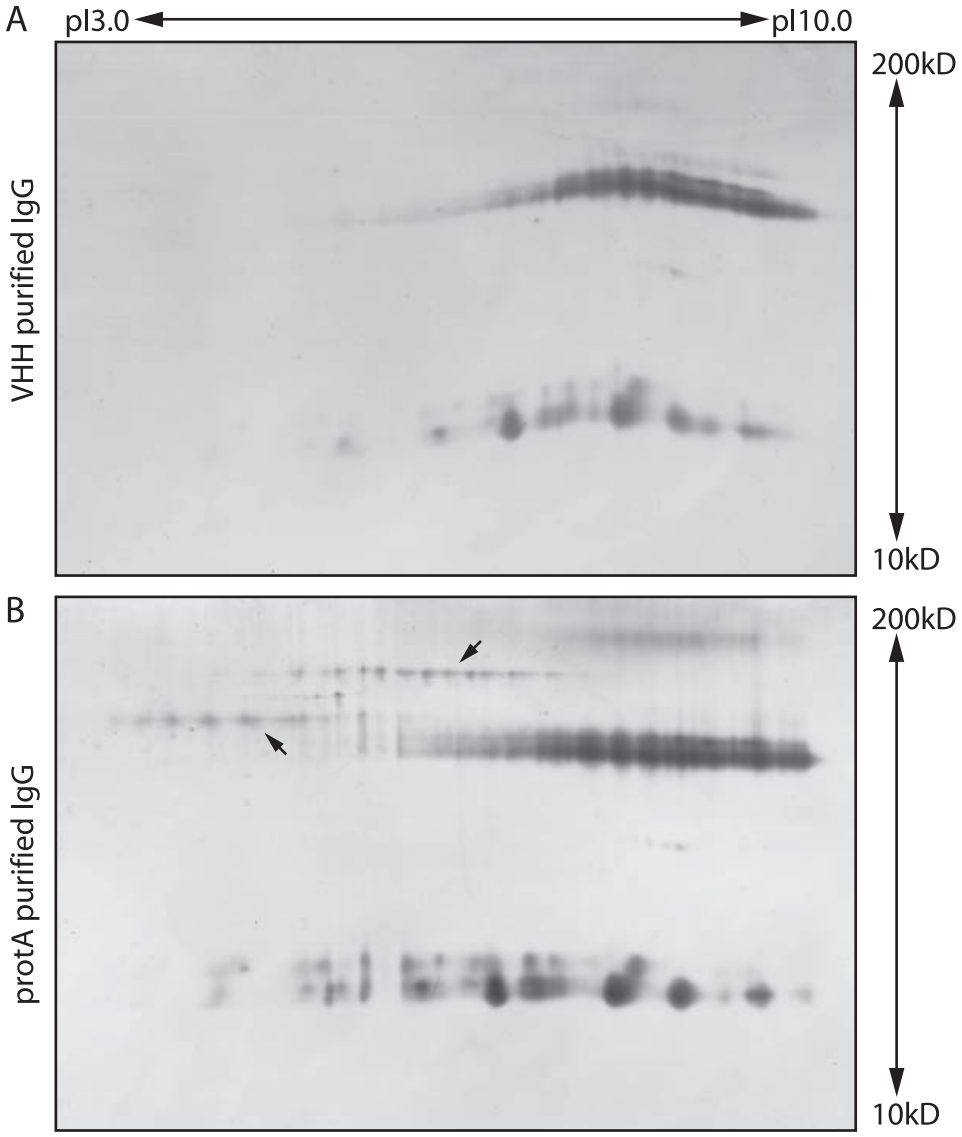


Figure 2: IgG from human serum purified with a VHH based affinity column (A) and a proteinA based column (B). Arrows indicate additional protein spots in the IgG sample purified with the proteinA affinity column.

efficiency of the VHH anti-HSA column to remove HSA, its fragments and modified products from serum.

The specific removal of abundant proteins from serum should increase the amount of spots that can be detected on a 2D gel. To evaluate this effect, the non-bound fraction was compared with the total serum sample. One hundred μg of both samples was analyzed on a 2D gel (Figure 4). These gels confirmed the data shown in figure 1. The use of the VHH affinity columns efficiently

depleted HSA and the IgG heavy chain from serum. In the depleted serum sample, a clear increase was observed in the amount of spots that could be detected. Removal of HSA and IgG revealed spots originally masked by these bulk proteins, especially in the case of HSA. Furthermore, the total amount of protein per spot increased. At the molecular weight of the light chain no total depletion was seen. This result can be explained by the presence of non-heavy chain bound light chain in blood (Abe et al., 1998; Hannam-Harris and Smith, 1981).

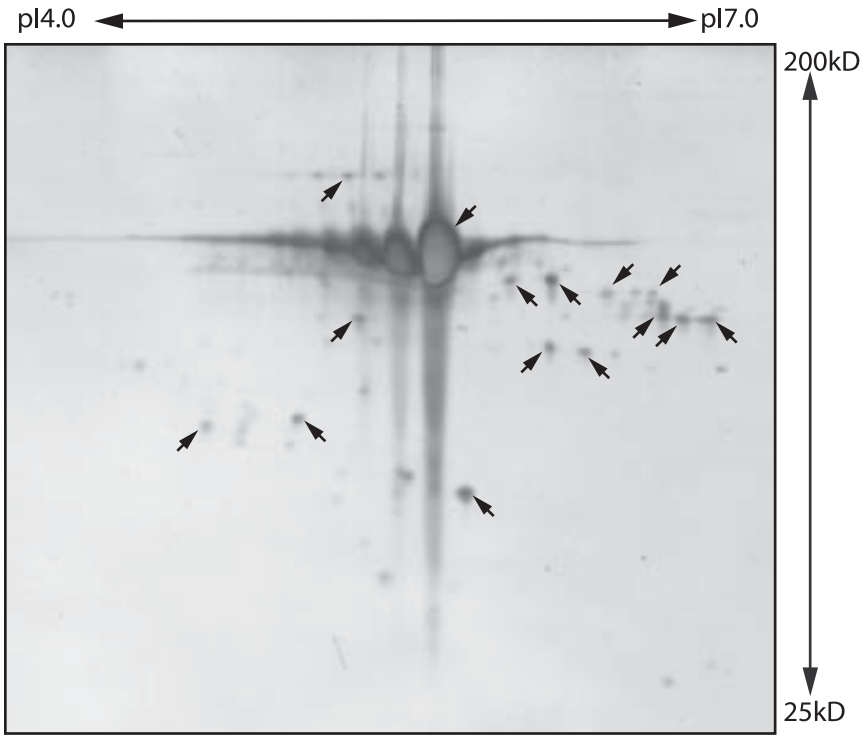


Figure 3: 2D gel of purified HSA from human serum eluted from the VHH anti-HSA column. Arrows indicate spots that were chosen, based on the relative spot abundance, for MS. All spots were positively identified as HSA.

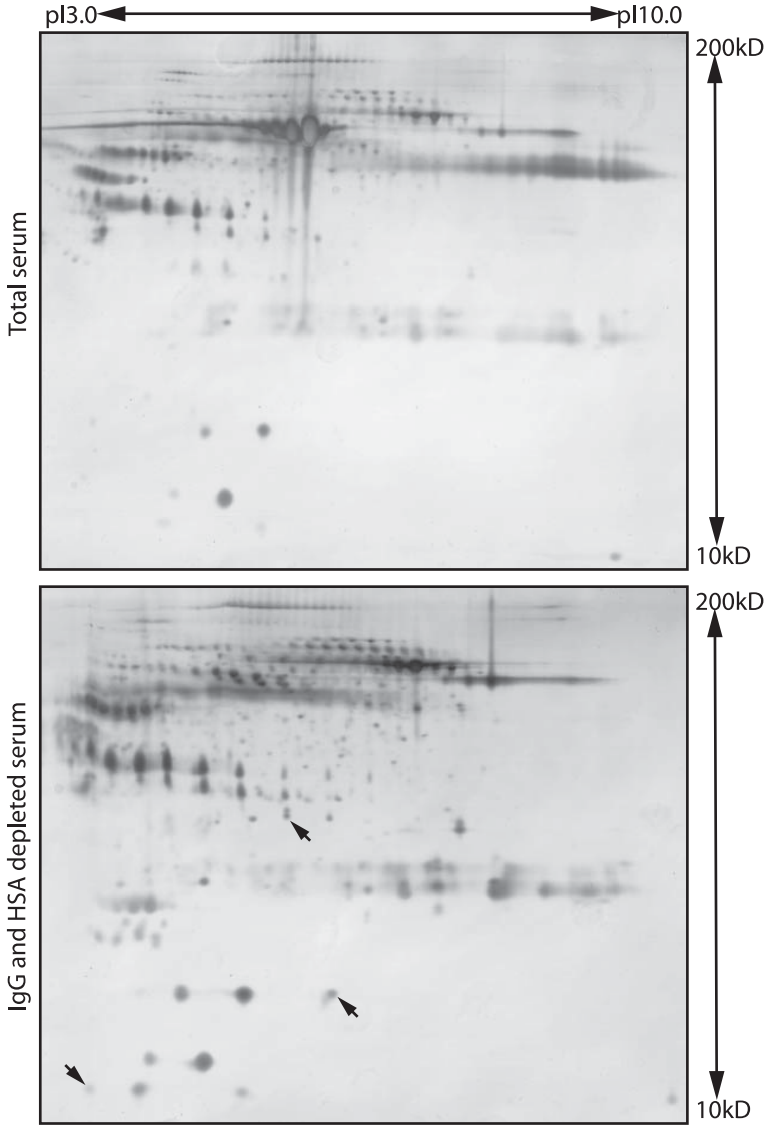


Figure 4: 2D gels of 150μg total serum and 150μg IgG and HSA depleted serum. The gels clearly show an increase in resolution and detection of proteins in the depleted human serum fraction. Arrows indicate examples of spots not seen in the total serum sample.

Performance of the anti-IgG VHH column compared to a proteinA based column

The most widely used method in affinity chromatography to isolate immunoglobulin G from biological samples is the application of proteinA from *Staphylococcus aureus* as the ligand. However, it is well known that this protein does not bind all IgG subclasses equally well (Eliasson et al., 1988). To show the potential of the VHH based anti-IgG column, its performance was compared to the proteinA method. Therefore, affinity columns of both ligands were used on the AKTA explorer 100 and non-bound and elution fractions were obtained as described before. Analysis of these samples on Western blot with total IgG and subclass specific IgG commercial antibodies (Figure 5) clearly showed that the anti-IgG VHH column efficiently depleted all IgG subclasses whereas the proteinA column removed only a small fraction of IgG3 and not all IgG4. Furthermore, analysis on 2D gel of the purified IgG fraction obtained with the proteinA column showed that this sample contained additional proteins (Figure 2B), which were not seen with the VHH column (Figure 2A).

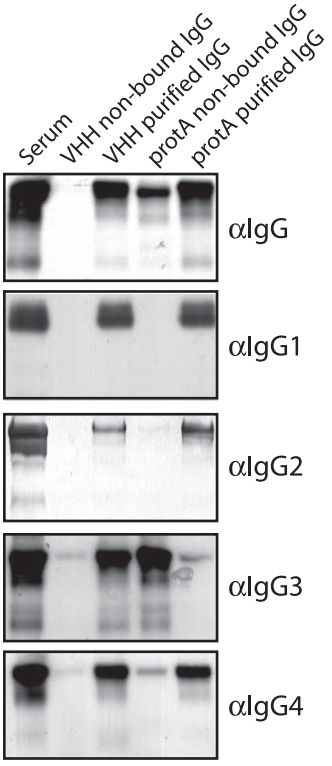


Figure 5: Increased performance of the anti-IgG VHH affinity column compared to a proteinA affinity column. The total serum sample was run on a SDS-page next to the non-bound fraction of the anti-IgG VHH and the proteinA column and the purified fractions of both columns. The depletion of IgG was evaluated on western blot with an antibody against human IgG and subclass specific antibodies. The VHH affinity column depleted all IgG subclasses from human serum, whereas the proteinA column was not able to bind IgG3 and was furthermore less efficient in depleting IgG4.

Application of the VHH anti-IgG affinity column in treatment of autoimmune diseases

Next to treatment of autoimmune diseases with plasmaphoresis and immuno suppressive therapy, specific methods to remove the self-reactive antibodies would be of great advantage. To show the possible applicability of our VHH affinity columns in therapeutic applications and to compare it with an accepted standard, GP and SLE autoimmune disease plasma were used in a set of proof of principle studies.

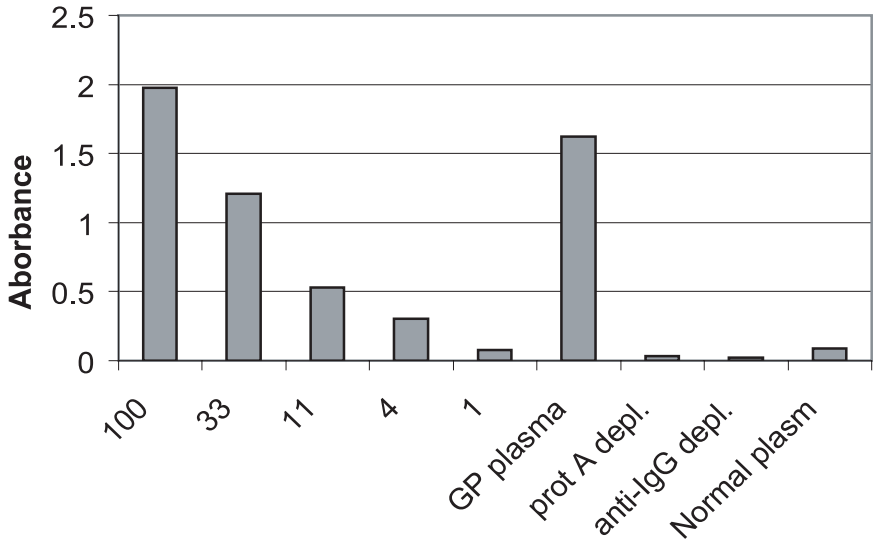
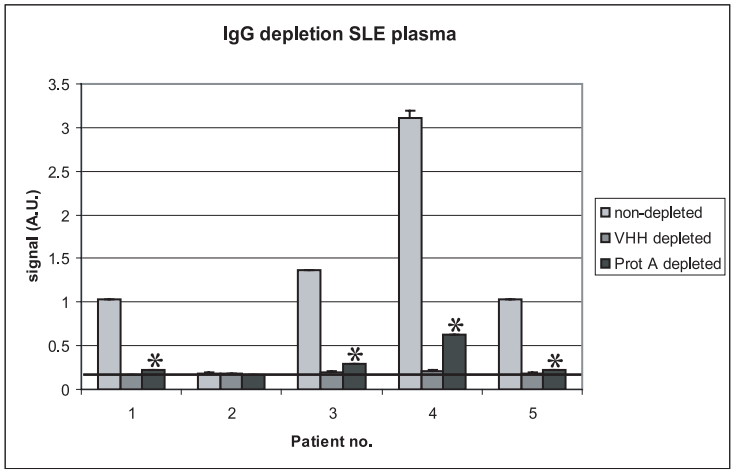


Figure 6: ELISA for measurement of auto-antibodies present in plasma of GP patients against the GBM. Depletion of IgG from plasma of Goodpasture patients with the VHH anti-IgG affinity column reduces the obtained reactivity signal to a minimum and performs at least comparable to the proteinA affinity column.

From plasma of patients suffering from these diseases IgG was removed with proteinA based or VHH based affinity ligands. The presence of self-reactive antibodies in the plasma was tested before and after depletion with dedicated ELISA kits. This showed that the VHH column performed at least equally well as the proteinA based column (Figure 6) with a sample of a GP patient. The VHH and protA column treated GP plasma showed a reduction of antibody reactivity till levels obtained with control plasma.

Remarkably, the plasma samples of SLE patients treated with the VHH based affinity ligand contained less SLE related auto-antibodies compared to the samples depleted with the proteinA affinity ligand (Figure 7), as the proteinA based depletion always gave higher signals than those obtained with the VHH based depletion.

A



B

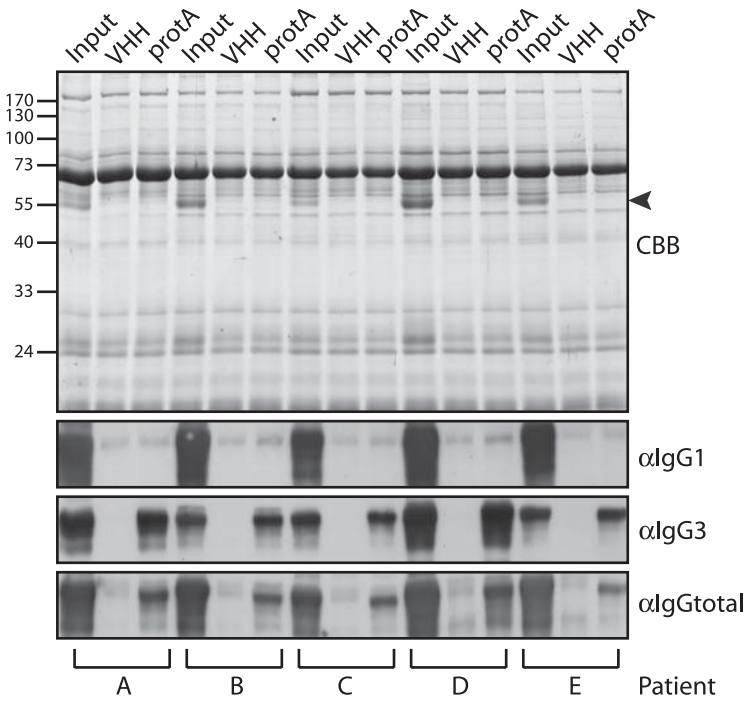


Figure 7: Representative picture of the superior IgG depletion from SLE plasma patients with the VHH anti-IgG affinity column compared to a proteinA affinity column. The ELISA for measurement of auto-antibodies against chromatin with the Quanta Lite Chromatin kit (A) shows the reduction of auto-antibodies present in plasma of SLE patients when treated with the VHH anti-IgG and proteinA affinity columns. The VHH affinity column performs better in all depicted patients with a positive ELISA signal in the non-depleted sample (p-value students-t-test ≤ 0.003) and reduces the obtained signal to background levels (black line). The depletion is analyzed on SDS-page (arrowhead) and Western blot (B) with antibodies against IgG1, IgG3 and total IgG. The increased performance of the VHH affinity column is due to the total depletion of all IgG subclasses, whereas proteinA does not deplete IgG3.

The performance of the IgG depletion was subsequently evaluated on a CBB stained gel and Western blot with IgG specific antibodies. No difference in non-specific protein loss was seen with both columns on the CBB stained gel. Furthermore, both columns depleted IgG1 with comparable efficiency, indicating that the maximal capacity of both columns was sufficient. However, again there was an enormous difference between the columns in the depletion of IgG3. Intriguingly, the SLE plasma sample that contained most IgG3 showed the highest difference between the two columns in the presence of auto-antibodies after depletion. This clearly shows the beneficial properties of the VHH based anti-IgG columns over the proteinA based affinity ligand in treatment of auto-immune diseases.

Discussion

HSA and IgG specific VHHs were selected out of two phage display libraries in two consecutive rounds of panning. The selected VHHs were tested for antigen specificity and affinity and subsequently immobilized on a solid surface and evaluated for their ability to purify and deplete their respective antigens from human serum. The performance was evaluated using 1D- and 2D-gel electrophoresis, immuno blot and BIAcore. Furthermore, the performance of the anti-IgG affinity column was compared to protein A and the potential of the anti-IgG column for medical applications was shown in a pilot experiment.

HSA and IgG specific VHHs with nanomolar affinity (Table 1) could be selected out of a phagemid immune library in a fast and directed manner. Phage display allows the selection of specific antibodies that are suitable for predetermined applications, simply by adjusting the selection and screening protocols (Dolk et al., 2005a; Verheesen et al., 2003). To obtain antibodies capable of binding all four subclasses of IgG the selection protocol was designed to drive the selection towards conserved epitopes between these subclasses. Therefore, the antigen used in sequential selection rounds was switched from one subclass to another. Furthermore, the selection outputs were screened with purified fractions of all subclasses of IgG. Before the affinity of the selected VHHs was determined, their performance was first tested in the application that they were selected for. This strategy was chosen because the affinity, although important, is not the only prerequisite for obtaining a good affinity column. For instance Chemical coupling of antibodies can cause loss of antigen binding capacity.

The obtained antibodies were very efficient in the depletion of their target antigens from human serum (Figure 1). Furthermore, the obtained antigens HSA and IgG could easily be eluted from the support. Analysis of the purified protein samples showed no proteins that were not related to the target antigen. The absence of protein contaminations in the purified antigen samples further illustrates the specificity of this method (Figure 2 and 3). Although total depletion of the IgG heavy chain was observed with the VHH anti-IgG column, a portion of proteins running at the molecular weight of the light chain remained visible. This indicates that the selected antibody binds to the heavy chain of IgG. The presence of soluble IgG light chain in human plasma can at

least in part explain this result. Furthermore, the light chains of IgA and IgM could be responsible for this observation.

The depleted serum sample resulted in 2D gels with more detectable protein spots (Figure 4). This is comparable to results obtained by other groups with conventional antibodies and proteinA (Pieper et al., 2003b; Steel et al., 2003). However, because of the relatively low production costs of the antibody fragments and their high stability, the use of VHH based affinity columns is especially attractive for clinical applications. This potential of the anti-IgG affinity column is further illustrated by the removal of self-reactive antibodies from GP plasma (Figure 6) with comparable results as obtained with a proteinA based column, and from SLE plasma with improved auto-antibody removal as obtained with proteinA (Figure 7).

Although proteinA recognizes IgG molecules within several species, this shows that the anti-IgG VHH affinity ligand has great potential to replace proteinA for large-scale purification and depletion of IgG molecules in humans. The broad cross reactivity for the different IgG subclasses (Figure 5) could enable treatment of auto-immune patients caused by any IgG subclass, and is expected to outperform proteinA based columns in treatment of diseases caused by subclass IgG3 (Amoura et al., 2000; Rigopoulou et al., 2005; Staudt et al., 2002). An indication that IgG3 removal in auto-antibody diseases might be of high importance is the association of the increase of IgG3 auto-antibodies in SLE with active nephritis (Amoura et al., 2000) and the finding that particularly IgG3 auto-antibodies in PBC causes more severe biochemical and histological disease (Rigopoulou et al., 2005). Ultimately, autoimmune patients would be helped best with a method that can specifically remove the IgGs that cause the disease. The combination of the superior characteristics of VHH antibodies in affinity chromatography, together with phage display to select antibodies that can recognize only one subtype of IgG or even anti-idiotypic antibodies would enable this. Selection of anti-idiotypic antibodies for SLE is currently in progress. By modification of the selection and screening protocol such specific affinity ligands could be obtained, not only for autoimmune diseases, but also for other diseases caused by harmful substances in the blood such as sepsis (ElKhattabi et al., (Submitted)).

Acknowledgements

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Chapter 3

Differential protein profiles during aging in human plasma: Changes in protein expression and processing

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Abstract

With increasing age, an individual is subjected to numerous changes at the molecular, cellular and tissue level. As blood is distributed throughout the body, disturbances in an organism should, at least in part, be reflected in the protein composition of the blood. To understand the mechanisms of aging, several studies have focused on identifying these changes, mainly by hypothesis-driven research. Although some aging-related changes in protein expression have been identified, these studies have failed to acknowledge the presence of numerous isoforms and protein fragments of a single gene product in this complex proteome. Here, we describe the proteomics analysis of pooled human plasma samples of young, middle aged and old individuals depleted of human serum albumin and IgG with 2D-DIGE and mass spectrometry, which enables accurate differential protein expression analysis. This resulted in the identification of a set of differentially expressed protein spots. Several were in line with data from others and confirmed a slight pro-inflammatory and pro-coagulant status upon aging. Intriguingly, in several occasions multiple differentially expressed protein spots were identified to originate from a single gene product. For some of these proteins the different isoforms exhibited divergent expression patterns. The most prominent of these proteins was complement C3 (CO3), which showed a clear increase in the presence of the CO3 fragment C3dg with increasing age. This study clearly indicates the importance of protein isoforms analysis in addition to total protein levels, and results in a set of proteins that might be used to define biological age.

Introduction

The aging process is one of the most common events in modern life and is accompanied by a decline in functionality. This imposes a huge impact on society, both socially and economically. Despite increasing efforts little is known about the precise mechanisms responsible for this phenomenon. Currently, it is generally accepted that aging is associated with a progressive malfunctioning of several biological mechanisms. Over time, an organism is subjected to numerous damaging agents that either have an endogenous origin, like the generation of reactive oxygen species during oxidative phosphorylation in the mitochondria or during an immune response (Adams et al., 1982; Chance et al., 1979; Mackaness, 1970), or have an environmental origin, like radiation and chemicals (Cerutti, 1985; Cosgrove et al., 1985; Pollycove and Feinendegen, 2003; Stone and Pryor, 1994). Although an organism has several mechanisms to prevent and to repair damage caused by these agents, over time damage will accumulate. This ultimately leads to a decline in the ability of cells to respond to disturbances in cellular homeostasis, which has major consequences for the vitality of the organism.

Due to these events, changes in RNA as well as protein levels occur. Insight in these changes could aid in understanding the mechanisms underlying the aging process. Furthermore, it could provide a possibility to determine the biological age of an organism, rather than its chronological age, and it could facilitate in monitoring the effects of intervention studies to slow down the aging process.

The goal of the present study is to use advanced proteomics techniques to identify multiple independent biomarkers in human blood related to aging.

One of the most important and most accessible human clinical biological materials is blood plasma. For several plasma proteins, differences in expression during aging that have been described in literature involve mostly cytokines and other proteins involved in the immune response (Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Pedersen et al., 2000; Ritchie et al., 1998). Most of these results were obtained with immunological assays, which are usually very specific, sensitive and accurate in providing quantitative information. However, this approach neglects the presence of differences in posttranslational modification or the occurrence of protein fragments of a single gene product, which are very common for plasma proteins (Anderson, 2005; Misek et al., 2005).

Two-dimensional gel electrophoresis (2D-GE) is a well-established technology, which allows the separation of hundreds of proteins simultaneously. Moreover, 2D-GE is a highly suitable technique to detect protein isoforms. Accordingly, it is the most frequently used method despite the limitations towards sensitivity and reproducibility compared to immunological assays. Recent developments in 2D-GE analysis have paved the road to detect small expression differences with high accuracy. First of all, removal of highly abundant bulk proteins has improved the resolution of 2D gels and has increased the detection of low abundant proteins (Klooster et al., (Submitted)-a; Pieper et al., 2003a; Steel et al., 2003). Furthermore, advanced protein labeling techniques have become available, which enable the detection of small expression differences with a high confidence level. In Two-dimensional Difference Gel Electrophoresis (2D-DIGE) experimental variation is reduced due to the combination of multiple sample analysis in a single gel, and internal standard correction (Alban et al., 2003; Chen et al., 2005; Lilley and Friedman, 2004).

Here, we describe the differential expression analysis with 2D-DIGE of plasma samples of young (17-28 years old), middle aged (45-55 years old) and old (85 years old) individuals. Eighty-nine protein spots were considered as differentially expressed during aging of which 48 were positively identified with mass spectrometry. Several of the identified spots were in line with data described in literature, such as the differential expression of several acute phase proteins. In addition, remarkable differential expression of protein isoforms, originating from single gene products were detected upon aging, among which the differential expression of several complement C3 (CO3) fragments were the most striking. This illustrates the importance of developing methods to discriminate and analyze modified forms of a single protein. The differential expression of several of the identified proteins and their implications in the aging process will be discussed.

Materials and methods

Sample preparation

Citrate plasma samples were obtained from 45 individuals that were divided in three different age groups, 17-28 years, 45-55 years and an 85 years old group, of 15 individuals each. Samples of each group were pooled and depleted of human serum albumin (HSA) and immunoglobulin G (IgG) before 2D-DIGE analysis.

HSA and IgG depletion

To obtain better resolution on 2D-gel human serum albumin (HSA) and IgG were removed from the plasma samples with affinity resin based on Llama heavy chain antibody (VHH) technology as described before (Klooster et al., (Submitted)-a). The VHH anti-IgG resin (dynamic antigen capacity 10mg/ml) and anti-HSA resin (dynamic antigen capacity 12mg/ml) were mixed in a 1:2 ratio. The mixed affinity resin was washed extensively prior to use in ten times column volumes of Tris buffer (0.1M Tris/HCl pH 8.5; 0.5M NaCl) for three times followed by three washes with acetate buffer (0.1M sodiumacetate pH3.0; 0.5M NaCl). Next, the affinity resin was washed three times in citrate buffer to neutralize the pH. Plasma samples were diluted 100 times in ice-cold citrate buffer and incubated at 4°C for 30 minutes with 50µl bedvolume per 100µl diluted sample. After incubation, the non-bound fraction was taken for differential protein analysis. The protein content of these samples was determined with a BCA protein assay (Pierce, Rockford, U.S.A.) according to the instructions of the manufacturer.

Protein labeling

In order to obtain the optimal reaction conditions, 50µg of each protein sample was treated with the Clean-up kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Protein pellets were dissolved in 15µl buffer (30mM Tris/HCl pH8.5, 7M urea, 4% CHAPS). Cy5, Cy3 and Cy2 labelling was performed according to the instructions of the manufacturer (Amersham Biosciences, Roosendaal, The Netherlands). The labelled samples were diluted in rehydration solution (7M urea; 2M thiourea; 4% CHAPS; trace bromophenol blue; 0.5% (w/v) DTT; 0.5% (v/v) ampholytes pH 4-7; 1.2% (v/v) Destreak (Amersham Biosciences, Roosendaal, The Netherlands)) to a final volume of 450µl.

Two-dimensional gel electrophoresis

Immobiline Dry strips pH 4-7 of 24cm (Amersham Biosciences, Roosendaal, The Netherlands) were rehydrated overnight with the labeled protein samples and overlaid with 2ml Coverfluid in an Immobiline Drystrip reswelling tray. The first dimension of isoelectric focusing was run on an IPGphor system. Optimal protein focusing was achieved by starting at 500V for 1 hr, followed by 1500V for another hour. Subsequently, a constant voltage of 8000V was applied until a total of 48kV. Thereafter, the strips were equilibrated in equilibration buffer (50mM Tris/HCl pH8.8; 6M urea; 30% glycerol (v/v); 2% SDS (w/v)) containing 65mM DTT for 15 minutes followed by an incubation with 135mM iodoacetamide for 15 minutes. The second dimension was performed with lab-cast 24cm 12.5% polyacrylamide gels. Strips were loaded onto the gels and sealed with a solution of 1% agarose (w/v) containing a trace of Bromophenol Blue. The gels were run over night on the Ettan DALT Twelve system (Amersham Biosciences, Roosendaal, The Netherlands) at 1W/gel till the Bromophenol Blue dye front reached the bottom of the gel.

Gel imaging

All gels were scanned in between low fluorescent glass plates at 100µm pixel resolution with the Typhoon image scanner 9400 (Amersham biosciences, Roosendaal, The Netherlands). The Cy5 images were scanned using a 633nm laser and a 670nm BP30 emission filter. Cy3 images were scanned with a 532 laser in combination with a 580nm BP30 emission filter. Cy2 images were scanned using a 488nm laser and an emission filter of 520nm BP40. The photomultiplier tube was set to achieve maximum sensitivity without pixel saturation. Silver stained images were analyzed with a GS-710 Calibrated Imaging Densitometer (Biorad, Hercules CA, USA).

Differential analysis

Prior to differential analysis, images were cropped with ImageQuant (Amersham Biosciences, Roosendaal, The Netherlands) to remove insignificant parts of the gel. Subsequently, images were subjected to median filtering in Image Quant Tools (Amersham Biosciences, Roosendaal, The Netherlands) to remove dust-related pixels. Differential analysis was performed with the Decyder V 5.01 (Amersham Biosciences, Roosendaal, The Netherlands)

software package. Analysis of variance ANOVA was applied to extract relevant expression profiles.

Spot picking and in-gel digestion

Spot picking was performed with post-stained silver images of CyDye labeled gels to minimize the risk of mismatched spot picking. Silver staining was performed according to Shevchenko (Shevchenko et al., 1996). Spots of interest were subjected to in-gel tryptic digestion as described previously (Kolkman et al., 2005).

Nano LC-MS/MS

Nano LC-MS/MS was performed with an Agilent 1100 series LC system (Agilent, Palo Alto, U.S.A.) coupled with a Thermo Finnigan LTQ or LTQ-FT (Thermo electron Company, Waltham, MA, U.S.A.) as described previously in literature (Kolkman et al., 2005). Briefly, peptide extracts were acidified with 0.1M acetic acid and injected on a trap column (Aqua^{TM} C18 RP (Phenomenex, Torrance, U.S.A.), 20 mm x 100 µm ID) at 5µl/min. Subsequently, the peptides were transferred with a split-reduced flow rate of 100nl/min on the analytical column Aqua^{TM} C18 RP (Phenomenex, Torrance, U.S.A.), 20 cm x 50 µm ID. Elution of the peptides was achieved with a linear gradient from 0-50% B (0.1M acetic acid in 80% (v/v) acetonitrile) in 60 minutes. The column effluent was directly introduced into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives, Woburn, MA, U.S.A.). The mass spectrometer was operated in the positive ion mode and parent ions were selected for fragmentation in data-dependent mode.

Protein identification

The obtained mass spectra were subjected to a Mascot search engine (Perkins et al., 1999). Probability-based protein identification was performed by searching sequence databases using mass spectrometry data with 0.8 Da peptide tolerance, two miss-cleavages, carbamidomethylation and methionine oxidation as variable modifications. Protein annotation was performed by using the SwissProt database.

Western blot analysis

Equal amounts of plasma were size-separated on a reducing or non-reducing 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer (2.5% protifar plus (w/v) in PBS), the membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or coomassie brilliant blue (CBB) [0.1% (w/v) coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with a polyclonal anti-CO3 antibody (Abcam, Cambridge, United Kingdom) diluted 2000 times for one hour and subsequently washed 5 times in PBST (0.05% Tween-20 in PBS). Thereafter, membranes were incubated with HRP conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, Westgrove, U.S.A.) for one hour and the wash steps were repeated. Two additional washes were performed with PBS and bound antibodies were visualized by enhanced chemoluminescence (PerkinElmer, Boston, U.S.A.).

Results

Proteomic analysis

To analyze age related proteome changes, plasma samples of three different groups were compared. These groups comprised a young (Y) (17-28 years old), a middle aged (MA) (45-55 years old) and an old (O) (85 years old) group, each consisting of 15 individuals. The plasma samples within each group were pooled and subsequently, HSA and IgG were removed with VHH antibody technology (Klooster et al., (Submitted)-a) to increase 2D-gel resolution and sensitivity. Depleted protein extracts were subjected to the Ettan DIGE system and generated a plasma proteome map of about 750 protein spots. Analysis of variance was used to compare protein expression differences between the three different age groups. Expression profiles with an ANOVA p-value of ≤ 0.05 were considered as significant and resulted in 89 protein spots which exhibited age related expression differences. Of these spots, 48 were positively identified *in duplo* and linked to their molecular weight, and expression dynamics (Figure 1 and Table 1). The majority of this subset showed an increased abundance towards aging, ranging from 1.2-10 (Figure 2A), and nineteen spots showed a decreased expression at old age (Figure 2B). Analyzing protein expression dynamics with 2D gel electrophoresis offers the possibility to visualize or evaluate the expression dynamics of protein isoforms. Plasma proteins are known for their variety in isoform abundance (Anderson, 2005; Misek et al., 2005). Protein identification of the spots of interest indicated that the majority of the abundance differences could be assigned to various protein isoforms or fragments of a restricted number of parent proteins (Figure 1 and Table 1). Twenty unique proteins were identified, among which proteins that are involved in coagulation, such as fibrinogen, the immune respons, such as CO3 and several acute phase proteins, as well as proteins that neutralize damaging compounds in the blood, such as gelsolin and vitamin D binding protein (DBP). The majority of the abundance differences were caused by haptoglobin (spots 13A-13I), fibrinogen (spots 10A-10G), transferrin (spots 17A-17D), alpha-1-antitrypsin (spots 1A-1C) and CO3 (spots 8A-8F). For these proteins, the trend of regulation of all isoforms originating from a single gene product was in general the same (Figure 3A-E). However, there were exceptions for some alpha-1-antitrypsin (Figure 3A), complement C3 (Figure 3B) and haptoglobin (Figure 3D) isoforms.

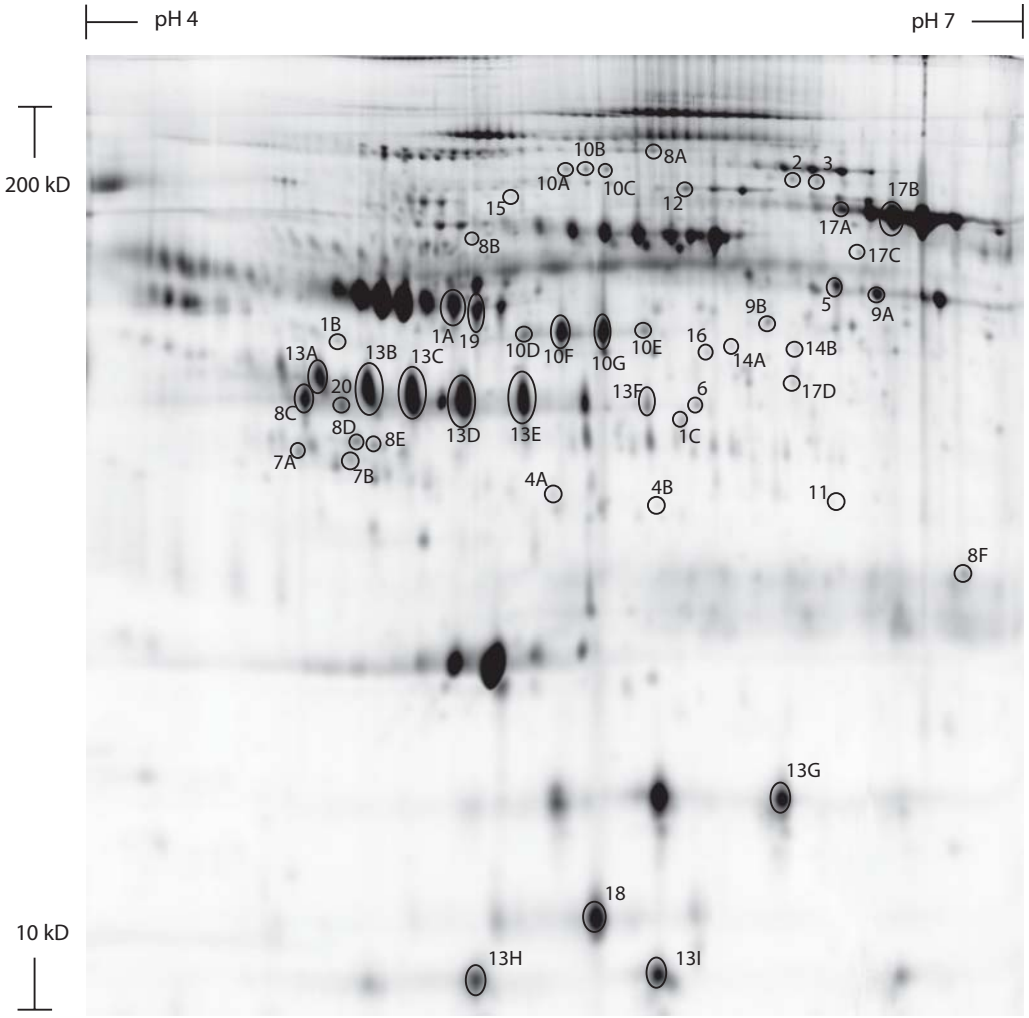


Figure 1: 2D-DIGE analysis of HSA and IgG depleted pooled plasma samples of young, middle aged and old individuals revealed differential expression between the young and the old group of 89 different protein spots of which 48 were positively identified *in duplo*. The numbers refer to the identified differentially expressed proteins and their isoforms, as shown in table 1.

Spot		Protein	Acc. nr.	Mw	Reg. O/Y	ANOVA	SC%	pept.
1	A	Alpha-1-anti-trypsin	P01009	46737	0.8	7.9E-04	62	23
	B				0.6	3.8E-05	23	11
	C				4.2	2.2E-03	23	12
2		Alpha-2-macroglobulin	P01023	163278	0.7	1.7E-04	9	16
3		CFAB/alpha-2-macroglobulin	P00751	86902	0.7	1.6E-03	8	13
4	A	Apo-E	P02649	36268	1.3	1.8E-03	54	22
	B				0.6	3.1E-04	58	23
5		Apolipoprotein H	P02749	39610	1.2	1.7E-02	52	19
6		Apolipoprotein-L1	O14791	43927	0.7	1.1E-03	24	23
7	A	Clusterin	P10909	53065	0.9	3.1E-02	32	14
	B				1.3	2.0E-02	28	14
8	A	Complement C3	P01024	188704	0.8	7.5E-04	27	48
	B				0.3	3.3E-03	17	39
	C				5.1	6.1E-04	16	32
	D				6.4	7.9E-06	7	14
	E				9.6	3.3E-07	7	14
	F				4.4	5.1E-07	3	8
9	A	Fibrinogen Beta chain	P02675	56613	1.4	5.5E-03	60	33
	B				1.8	1.1E-04	22	12
10	A	Fibrinogen gamma chain , alpha	P02679	52139	1.9	3.0E-04	39	20
	B				1.9	6.7E-05	35	18
	C				1.9	2.1E-03	38	19
	D				2.0	2.0E-04	50	26
	E				1.7	1.2E-04	37	20
	F				1.6	2.4E-05	52	32
	G				1.5	4.2E-05	62	35
11		Ficolin	O75636	33381	0.6	1.2E-03	17	6
12		Gelsolin	P06396	85698	1.3	2.5E-03	39	29
13	A	Haptoglobin	P00738	66058	1.2	1.4E-06	37	26
	B				1.3	7.6E-05	40	36
	C				1.3	9.8E-05	37	23
	D				1.3	7.4E-05	45	25
	E				1.3	3.2E-05	37	22
	F				0.7	1.2E-04	37	18
	G				0.8	8.3E-04	23	12
	H				1.7	2.5E-08	14	9
	I				2.5	7.0E-08	11	8
14	A	IgM	P01871	50242	1.5	3.6E-06	27	12
	B				0.7	2.0E-04	17	9
15		Inter-alpha-trypsin inh. heavy ch. H4	Q14624	103358	1.3	3.8E-04	16	14
16		Pigment epithelium-derived factor	P36955	46513	1.3	4.5E-04	36	15
17	A	Transferrin	P02787	79332	0.7	1.4E-06	46	48
	B				0.8	2.2E-02	70	82
	C				0.7	4.0E-02	45	41
	D				0.7	5.0E-02	20	15
18		Transthyretin	P02766	16001	0.8	2.5E-04	68	10
19		Vitamin D-binding protein	P02774	54561	0.7	9.9E-04	67	34
20		Zinc alpha 2 glycoprotein	P25311	33872	1.3	8.5E-04	54	22

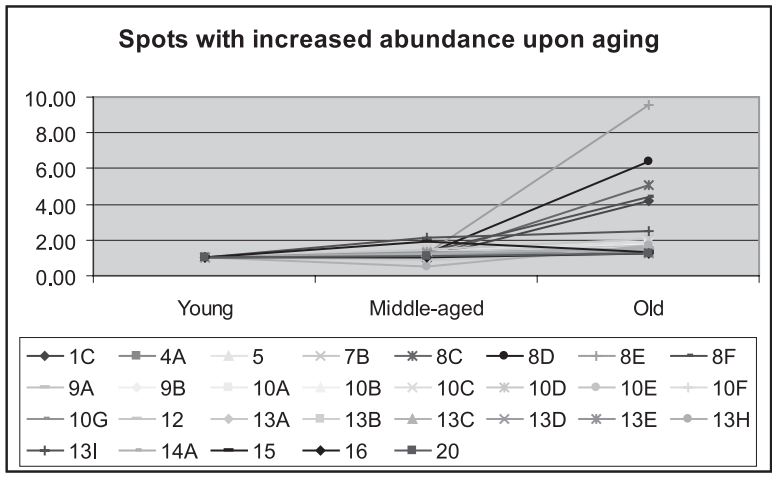
Table 1: *In duplo* identified differentially expressed protein spots between the young and old group in human plasma. The differential expression of the old group is normalized to the expression in the young group. The accession number (Acc. Nr.), molecular weight of the unprocessed precursor (Mw), the statistical significance (ANOVA), and of the protein identification percentage of sequence coverage (SC%) and number of peptides (pept.) are indicated .

For alpha-1-antitrypsin, three protein spots were identified. Two showed a small decrease in expression upon aging (spots 1A and 1B) and one showed a marked increase (spot 1C)(Figure 3A). Of these spots, spot 1A was the most abundant, while the other two spots were hardly visible.

Haptoglobin, an acute phase protein that has many isoforms mainly caused by its glycosylation pattern, showed mostly patterns that gradually increased

upon aging (Figure 3D). In contrast, a minor part of the differential expressed haptoglobin isoforms showed a divergent pattern (spots 13F-13I). All these isoforms were less abundant compared to the isoforms that showed a minor increase in expression upon aging (spots 13A-13D). Of the haptoglobin spots that showed a divergent expression pattern, two decreased slightly upon aging (spots 13F and 13G), one showed a marked decrease in the middle aged population and a marked increase in the old population (spot 13H), while the last spot showed an increased abundance in both the middle aged and the old population (spot 13I).

A



B

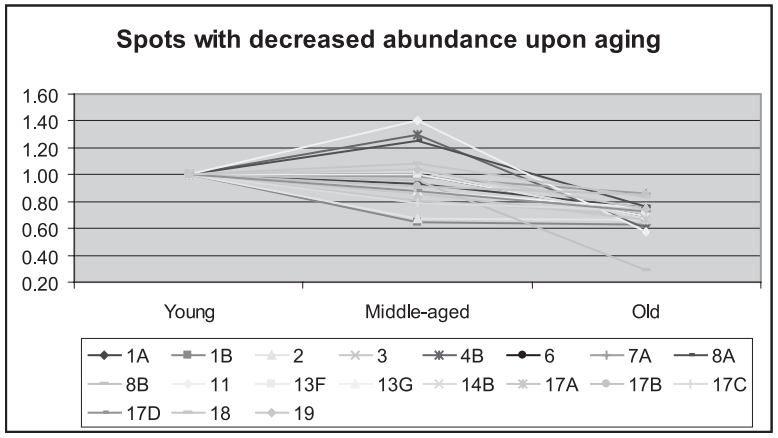


Figure 2: Differentially expressed protein spots with a significant increased (A) and decreased abundance (B) upon aging.

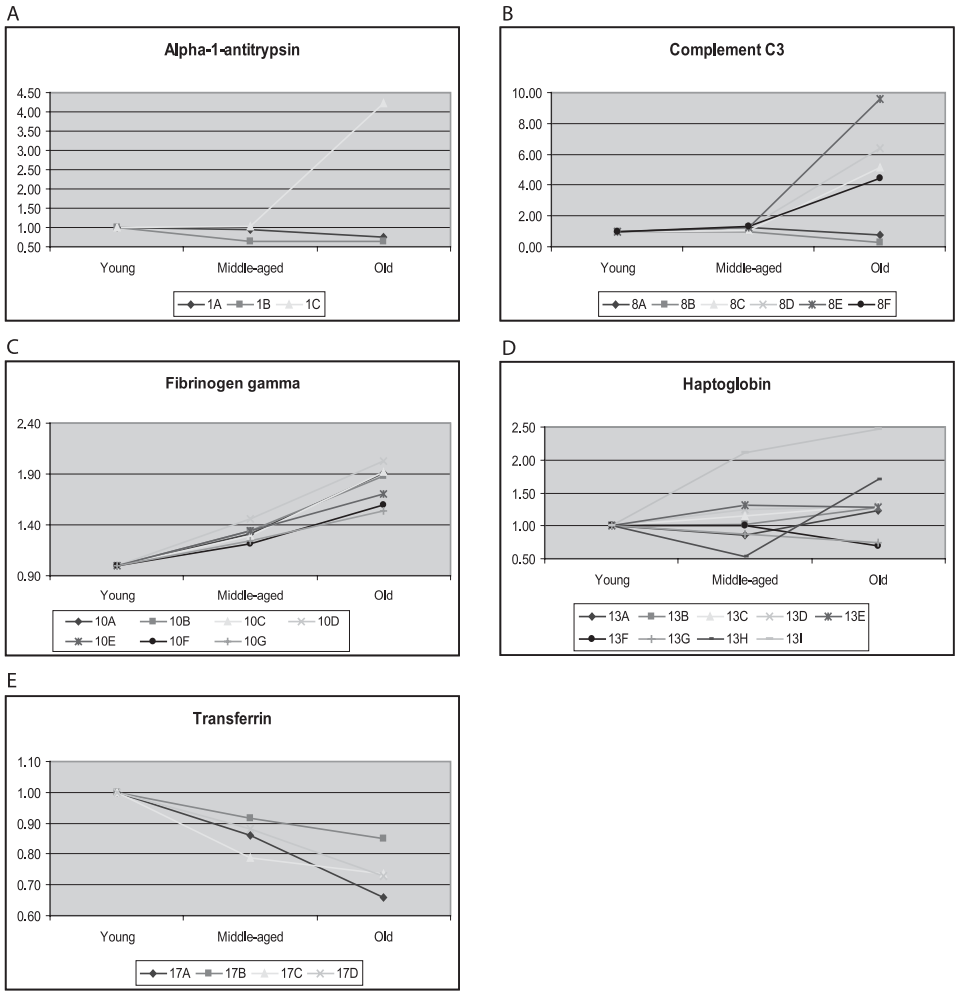


Figure 3: The expression patterns in the different age groups of several different isoforms of alpha-1-antitrypsin (A), CO3 (B), fibrinogen gamma (C), haptoglobin (D) and transferrin (E). Not all isoforms of a single gene product show a similar expression pattern. Some isoforms of alpha-1-antitrypsin (spot 1C), complement C3 (spots 8A and 8B) and haptoglobin (spots 13F, 13G and 13H), show an expression pattern that is not consistent with the majority of the isoforms.

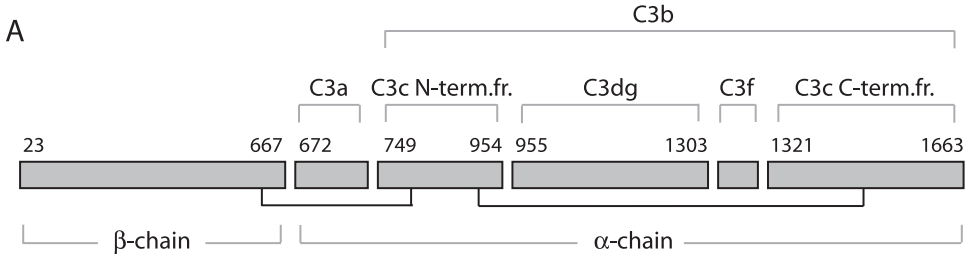
These data show that in addition to total protein levels, levels of protein isoforms could be important determinants with respect to aging.

Strikingly, the most substantial differences between the young and old population were found in six spots identified as CO3, of which four gave an increase in expression above a factor four between the young and old group (spots 8C-8F), while two showed a marked decrease in expression (spots 8A and 8B)(Figure 3B).

Differential expression of CO3 protein fragments

CO3 plays a key role in the activation of the immune system. Its functionality is regulated by specific proteases that cleave the mature protein (Figure 4A) and thereby induce conformational changes, which leads to several different active components, each with different specific functions. During these cleavages, soluble polypeptide chains are released from the mature protein while others remain attached via disulfide bonds.

A



B

Spot	Regulation O/Y	CO3 fragment	Seq. (Th)*	SC (%)*	Mw kD(th.)	pI(Th)*
8A	0.8	C3 alpha chain	672-1663	58	113	5.55
8B	0.3	C3 alpha chain (no C3c/C3a)	955-1663	32	80	4.97
8C	4.4	C3c C-terminal fragment	1321-1663	80	39	4.79
8D	6.4	C3dg	955-1303	32	39	5.00
8E	9.6	C3dg	955-1303	32	39	5.00
8F	4.4	C3c N-terminal fragment	749-954	37	34	6.89

Figure 4: Identification of complement C3 cleavage products that are differentially expressed between the young and old group. During activation, complement C3 undergoes several conformational stages induced by specific proteases that cleave the mature protein (A). The mature protein is first cleaved by the enzyme complex C3 convertase into C3a and C3b. C3a is a mediator of local inflammatory processes, while C3b can bind covalently to target surfaces via a reactive thioester. Furthermore, C3b is involved in amplification of complement activity by formation of the C3 convertase C3bBb. Additional cleavages of C3b by factor I first releases C3f, which inactivates C3b into iC3b. Further cleavage by factor I releases C3dg, which is thought to be a key regulator in linking innate and adaptive immune responses. For each CO3 spot found in our study, the precise splice product was identified by matching the recovered peptides from the FTMS to the amino acid sequence of CO3 (B). The sequence coverage (SC), theoretical molecular weight (Mw) and pI for each splice product are indicated.

In this study we have identified several differentially expressed CO3 spots, at different molecular weights on the 2D-gel, indicating that these spots were protein fragments of the mature CO3 protein. To confirm this, the sequences of the CO3 spots identified by the LTQ-FT were examined further, which

enabled the annotation of the different CO3 fragments (Figure 4A and 4B). Spots that decreased upon aging were identified as the alpha chain of CO3 (spot 8A) and the alpha chain without the C3a fragment and the N-terminal C3c fragment (spot 8B). The spots that increased upon aging were assigned to both C3c fragments of the alpha chain (spots 8C and 8F) and C3dg (spots 8D and 8E). These results indicated that on average with increasing age, there is an increase in the cleavage of the CO3 alpha chain, which leads to an increased presence of C3dg and C3c in the circulation.

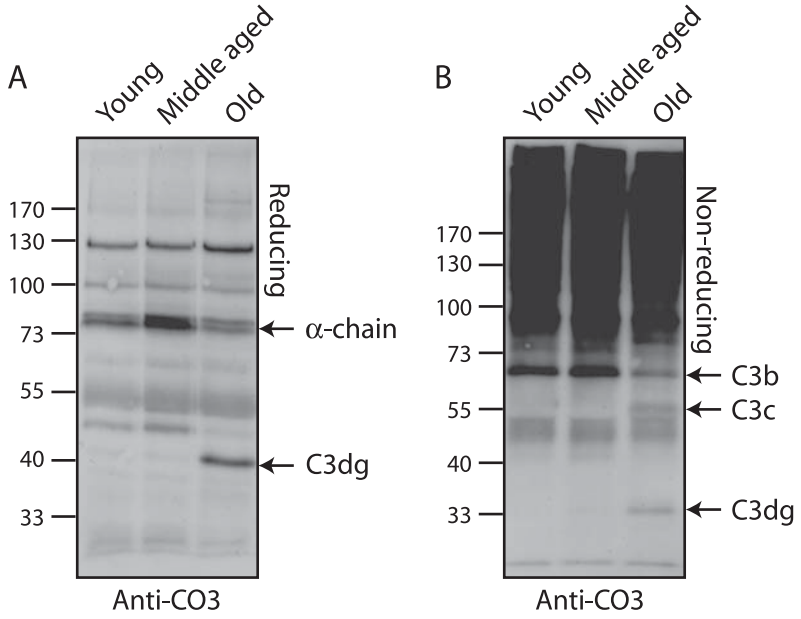


Figure 5: Verification of differential expression of CO3 splice products during aging. The pooled plasma samples of the different groups were analyzed on Western blot with a polyclonal anti-CO3 antibody. Samples were analyzed on blots that were obtained by running the samples on a reducing and a non-reducing gel. On both blots there was a clear difference in expression of several protein bands (arrows).

To verify these results, the pooled plasma samples were further analyzed on Western blot with a polyclonal anti-CO3 antibody. First, samples were run on a reducing gel which showed a protein band running at ~40kDa that showed an apparent increase in expression in the old group compared to the young and middle aged group (Figure 5A). Furthermore, there was an inverse correlation with protein bands running at ~45 and ~80kDa, suggesting that the 40kDa product was a protein fragment originating from the 45 or 80kDa proteins.

Experiments with non-reducing gels showed a similar pattern (Figure 5B). Two bands were observed at ~33kDa and ~55kDa that showed a clear increase in the old group compared to the young. Again an inverse correlation was seen with a protein band running at ~70kDa. These data were in line with the degradation of the alpha chain of CO3 into the components C3dg and C3c with increasing age.

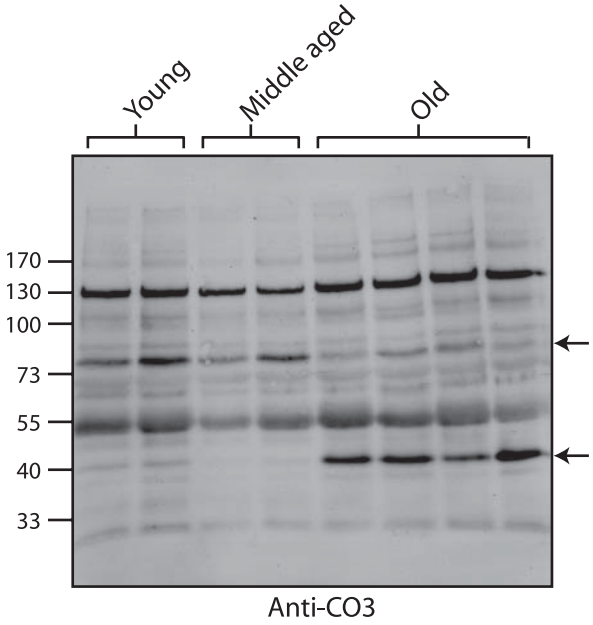


Figure 6: Confirmation of differential CO3 splice product expression with increasing age. All individual plasma samples were analyzed on Western blot with a polyclonal anti-CO3 antibody. Although there is individual variability within each group, there is a clear difference between the individuals in the old and the young and middle aged groups (arrows).

Because this observed difference could be caused by the presence of a few individuals with a pro-inflammatory immune state, the original citrate samples of all individuals (Figure 6), as well as heparin plasma samples of young and old individuals that were not included in the 2D-DIGE analysis (data not shown), were analyzed on Western blot. Although variability between individuals was observed, there was a clear difference between the individuals from the old and the young group that corresponded to the results obtained with the pooled samples. Especially the protein band running at ~40kDa was clearly up-regulated in all samples from the old individuals.

Discussion

The aim of this study was to search for age-related protein changes in human plasma. Proper depletion of HSA and IgG, and subsequent analysis with 2D-DIGE resulted in a set of proteins, which showed expression profiles with a significant ANOVA p-value ≤ 0.05. Some of the identified differentially expressed proteins were either protein fragments or post-translational modified forms of single gene products.

To our knowledge, the present study is the first to study age-related protein expression in blood with a 2D-DIGE approach, resulting in a relatively large panel of differentially expressed proteins. Several of the identified proteins agreed with results that have been described by others in studies focusing on one or a few blood components during aging. This included IgM (Ritchie et al., 1998), clusterin (Ishikawa et al., 1998; Mackness et al., 1997; Trougakos and Gonos, 2002; Witte et al., 1993), alpha-2-macroglobulin (Ritchie et al., 2004a) and several acute phase proteins, which are involved in the maintenance of blood homeostasis, such as haptoglobin (Ritchie et al., 2000), fibrinogen (Aillaud et al., 1986; Ernst and Resch, 1993; Tofler et al., 2005), transferrin and transthyretin (Ritchie et al., 1999). Next to this, our approach resulted in several other proteins, which have not been described earlier as being differentially expressed during aging. These included the proteins gelsolin, DBP, ficolin, pigment epithelium-derived factor, inter-alpha trypsin inhibitor and zinc alpha-2-glycoprotein. Some of these expression differences are in line with the accepted view of increased cellular damage and an increased pro-coagulant (Tofler et al., 2005) and pro-inflammatory (Gomez et al., 2005) state with increasing age.

The identification of plasma gelsolin and DBP are intriguing with respect to tissue damage, as they are thought to act in concert with each other to neutralize the potential damaging effects of cellular actin released in the blood. Plasma gelsolin is an actin severing molecule that caps actin released in the blood by tissue or cell damage. Thereby, it prevents the formation of actin filaments that can obstruct the microvasculature. The up-regulation found in the present study could reflect an attempt of the system to cope with increased cell damage with increasing age and thereby prevent further tissue damage induced by microvascular obstruction. Monomeric G-actin molecules that dissociate from the pointed filament ends of the actin polymer bound by gelsolin are sequestered by DBP (Vasconcellos and Lind, 1993). This could

induce an increased uptake and degradation of DBP when bound to actin, as has been shown in rats (Dueland et al., 1990) and would be in line with reports that describe decreased DBP blood levels upon acute tissue damage (Dahl et al., 1998; Schiodt et al., 1997).

The many different up-regulated fibrinogen spots are indicative of a pro-coagulant state with increasing age. This is in line with data from others (Aillaud et al., 1986; Ernst and Resch, 1993; Tofler et al., 2005) who have described up-regulation of fibrinogen in aging subjects, and which has also been shown to be a risk factor for developing cardiovascular diseases like thrombosis. This pro-coagulant state has also been shown to correlate to increased levels of other pro-coagulant factors, such as von Willebrand factor, PAI-1 and t-PA antigen (Haverkate et al., 1995; Thompson et al., 1995), which were not observed in the present study, and which is thought to be caused by a low-grade inflammatory state associated with aging.

Indeed, many of the identified proteins in our study are related to the immune response and are also indicative of a pro-inflammatory immune state. Some differentially expressed proteins belong to the acute phase proteins, such as fibrinogen, haptoglobin, alpha-1-antitrypsin, transthyretin and transferrin, and are critical for maintaining or re-establishing homeostasis. The hepatic synthesis of these proteins is regulated by the primary cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNFα) (Ruminy et al., 2001). Upon infection, injury or other physiological conditions that distort homeostasis, an acute phase response is triggered in which the positive acute phase proteins, including haptoglobin, fibrinogen and alpha-1-antitrypsin are up-regulated and the negative acute phase proteins, including transferrin and transthyretin, are down-regulated (Ruminy et al., 2001). Although there is some variation in differential expression between different protein isoforms, in general the overall average expression of the identified positive acute phase proteins show a slight increase and all negative acute phase proteins show a slight decrease during aging. Although no individual parameters are determined for these proteins in our experiments, these results are in line with data from others (Aillaud et al., 1986; Ernst and Resch, 1993; Ishikawa et al., 1998; Mackness et al., 1997; Ritchie et al., 1998; Ritchie et al., 1999; Ritchie et al., 2000; Ritchie et al., 2004a; Tofler et al., 2005; Trougakos and Gonos, 2002; Witte et al., 1993). Interestingly, alpha-1-antitrypsin forms an exception to this observation. The most abundant differentially expressed

alpha-1-antitrypsin spot (spot 1A) shows a very slight decrease in expression, while the other two alpha-1-antitrypsin spots, which are less prominent show a decreased (spot 1B) and a marked increased expression (spot 1C) (Figure 3A). Data from others using immuno-assays (Ritchie et al., 2000) show no or at most a very slight increase in alpha-1-antitrypsin expression after the age of 55. An explanation for this apparent contradiction is the use of different detection techniques in the different experiments. Immuno-assays provide information on total protein level expression, while 2D-DIGE provides information about separate protein isoforms that reveal differential expression. The differential expression of several acute phase proteins, suggests that during aging a minor acute phase response is triggered. There are several indications that with increasing age circumstances arise that stimulate this more pro-inflammatory immune state, known as inflamm-Aging. Several changes in the adaptive and innate immune system arise with increasing age, which contribute to this disturbed immune state (Grubeck-Loebenstein and Wick, 2002). One of the changes concerns the alteration of important functions of members of the adaptive immune response, such as T-cells. The expression of some very important cytokines needed for T cell clonal expansion, including IL-2, are decreased with aging (Gillis et al., 1981). This contributes to the dysfunction of the adaptive immune response and the increased incidence of infections and several age-related diseases, such as auto-immune disorders, cancers and atherosclerosis (Castle, 2000a; Castle, 2000b; Fulop et al., 2005; Wick, 2000). Also some specific functions of the innate immune response are altered (Fulop et al., 2004), possibly caused by the imbalance in the adaptive immune response. One of these changes is the production of pro-inflammatory cytokines TNFα, IL-1 and IL-6. Indeed, the increased presence of these primary pro-inflammatory cytokines in plasma has been described(Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Ferrucci et al., 2005; Pedersen et al., 2000) and could be an explanation for the differential expression of the acute phase proteins found in the present study as these cytokines are known to induce an acute phase response.Furthermore, during life LPS and other bacterial breakdown products that for a large extent seem to originate from the normal bacterial flora of the gut might accumulate in the organism (Winchurch et al., 1982). Moreover, different protein modifications, such as carbonylation and glycation of plasma proteins, and endothelial and blood cell proteins, have been shown to

accumulate during aging (Goswami and Koner, 2002; Jana et al., 2002; Wautier and Schmidt, 2004). With an increase in pathological events upon aging, such as atherosclerotic plaque formation and an increase in the amount of cell mortality and thus the release of intracellular proteins in the circulation (Ji et al., 2002; Seifert et al., 1990), these, among others, could induce this slightly increased and constitutive activation of the immune response.

Another indication for the disturbed function of the innate and adaptive immune response are the observed differences in expression of several CO3 fragments, especially of C3dg and C3c (Figures 3B and 4). This protein is a positive acute phase protein and plays a central role in the activation of the complement system, which is part of the innate immune response. Furthermore, it is widely appreciated that it plays key roles in several steps of the adaptive immune response, and thus is considered to be the link between these separate immune response systems (Sahu and Lambris, 2001). The expression of CO3 is controlled by several different factors. During infection of an individual, CO3 can form complexes (e.g. with immunoglobulins), which are removed from circulation. Furthermore, cytokines, IL-1 and IL-6, produced during inflammation induce the hepatic production of CO3. The overall ratio between production and removal of CO3 determines the overall expression levels of CO3 in the blood. This ratio could even result in normal circulating levels of CO3 during an infection (Ritchie et al., 2004b). From literature, there are conflicting data concerning the expression levels of this protein during aging. CO3 levels were either reported to be unchanged (Bellavia et al., 1999; Oyeyinka and Salimonu, 1999; Ritchie et al., 2004b) or increased (Nagaki et al., 1980) during aging. This study clearly shows that not total CO3 levels, but CO3 protein fragments could be important determinants. The increased presence of the CO3 fragments C3dg and C3c are indicative of an increased activation of the complement system at old age, which could be caused by accumulation of illegal post-translationally modified proteins during aging, such as glycation or by increased atherosclerotic plaque formation.

As most immunological based assays that are used for measuring CO3 levels are not suitable for detecting differences in protein fragments or post-translational modified forms of CO3, this could explain the conflicting results found by others.

To increase the information that can be extracted from a single gene product, methods have to be developed that enable the accurate analysis of protein

isoforms. This could be accomplished by isoform specific monoclonal antibodies or the application of mass spectrometric techniques.

Ultimately, the described expression differences with aging could be used for developing methods for determining the biological age of an individual over the chronological age, which could facilitate in monitoring the effects of intervention studies to slow down the aging process. Therefore, distinction must be made between differences induced by pathologies or infections and aging or age-associated pathologies. In combination with the detection of proteins known to be highly induced upon an infection, but mildly induced with increasing age, such as C-reactive protein and several cytokines (Ferrucci et al., 2005), the above described differences together could be used for the development of such an approach.

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Chapter 4

Selection and characterization of KDEL-specific VHH antibody fragments and their application in studying ER resident protein expression during ER stress

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Abstract

Several diseases are caused by defects in the protein secretory pathway, particularly in the endoplasmic reticulum (ER). These defects are manifested by the activation of the unfolded protein response (UPR) that involves the transcriptional up-regulation of several ER resident proteins, the down-regulation of protein translation and up-regulation of ER associated degradation (ERAD). Although this transcriptional up-regulation of ER resident proteins during ER stress has been described extensively, data on the differential protein expression levels of these same proteins are hardly available. Tools that would enable the simultaneous analysis of these proteins would be of high importance. Here, we describe the successful selection and characterization of VHH antibody fragments from a non-immune phage display library that recognize a conserved epitope present in several of these ER resident proteins, i.e. the C-terminal KDEL sequence, to study the differences in protein expression that occur during ER stress. In an ER stress model, involving treatment of HeLa cells with H_2O_2, DTT and tunicamycin, we show that the ER resident proteins endoplasmin and especially GRP78 are up-regulated on the protein level. In addition, our data show that there is a marked difference in the expression profile of KDEL-containing proteins after treatment with different stress inducers, which are probably related to the extent of ER stress.

Introduction

The ER is of critical importance for proper functioning of cells. Several diseases, such as familial hypercholesterolemia (FH) and cystic fibrosis (CF), are caused by problems that occur in the cellular secretory pathway (Hobbs et al., 1992; Kim and Arvan, 1998; Lukacs et al., 1994; Rutishauser and Spiess, 2002). Furthermore, there are indications that malfunctioning of the ER might even play a role in the aging process (Li and Holbrook, 2004; Rabek et al., 2003; van der Vlies et al., 2002). The protein secretion pathway involves several quality assurance and control mechanisms that ensure the correct folding and modification of secreted proteins, either membrane bound or soluble. The endoplasmic reticulum (ER) is a very important organelle in this process. Here, the newly translated proteins enter the protein secretion pathway where specialized proteins aid in the folding of the polypeptide chains into their correct conformation: Folding chaperones enable the correct folding of the amino acid chain, while other proteins are involved in the formation of disulfide bonds and other post translational modifications of the newly synthesized proteins. The importance of these processes is revealed during events that interfere with the correct folding or modifications of proteins in the ER (Schroder and Kaufman, 2005), as can be provoked by specific drugs (Pakula et al., 2003) and, as mentioned before, is reflected by the fact that several diseases are caused by malfunctioning of the ER (Kim and Arvan, 1998; Rutishauser and Spiess, 2002). When the protein folding capacity of the ER does not meet the demands, the ER responds by what is known as the unfolded protein response (UPR). The UPR consists of the activation of unique signal transduction routes via special sensors in the ER membrane in which the ER resident protein GRP78 plays an important role (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002). The UPR involves expansion of the ER, the transcriptional up-regulation of several ER resident proteins, the down-regulation of protein transcription and translation (Cox et al., 1997; Harding et al., 1999; Martinez and Chrispeels, 2003; Pakula et al., 2003), and up-regulation of ER associated degradation (ERAD) (Friedlander et al., 2000; Travers et al., 2000). When these processes cannot overcome the folding problems and the ER stress persists, the cells can eventually go into apoptosis (Orrenius et al., 2003; Yoneda et al., 2001).

The differential transcription of ER resident protein genes has been demonstrated in several diseases and in several model systems for ER stress

(Arvas et al., 2006; Kozutsumi et al., 1988; Martinez and Chrispeels, 2003). Although differential protein expression upon ER stress has been shown for some ER resident proteins (Hoozemans et al., 2005; Vattemi et al., 2004), it is less well described. To gain further insight in the processes that occur during ER stress, an antibody that has wide species specificity and that can recognize several ER resident proteins would be of high importance.

Many of the ER resident proteins contain special amino acid sequences that cause their specific retention in the ER. One of these ER-retention mechanisms involves the KDEL receptor, present in the *cis*-golgi that recognizes the amino acid sequence KDEL or a closely related tetra-peptide present at the C-terminus of several ER resident proteins (Munro and Pelham, 1987; Scheel and Pelham, 1996). Binding of a protein that contains this sequence to the KDEL receptor causes the uptake of the protein-receptor complex in COPI coated vesicles. COPI mediated retrograde transport of these vesicles to the ER and the subsequent release of the KDEL containing protein by the receptor, ensures the specific retention of the protein in the ER (Majoul et al., 1998; Wilson et al., 1993).

This ER retention signal enables the selection of antibodies with the before-mentioned characteristics, as this signal is conserved in several ER resident proteins and among many different organisms. In this study, we specifically selected and isolated single variable domain antibody fragments of heavy chain antibodies (VHH) from a large Llama-derived non-immune library that recognize the C-terminal amino acid sequence KDEL. The single domain structure of these antibody fragments simplifies the construction of a non-immune phage display library with wide antibody variability and enables high production yields in microorganisms (Frenken et al., 2000). A specific selection protocol was devised to drive the selection to the four amino acid KDEL epitope. To show the applicability of this antibody in the study of ER resident protein expression levels, KDEL containing proteins in HeLa cells were monitored during ER stress induced by H_2O_2, DTT or tunicamycin. The ER resident proteins endoplasmin and especially GRP78 show an up-regulation on the protein level in this ER stress model. Interestingly, there is a marked difference in the expression profile of KDEL-containing proteins after treatment with these different stressors, which is probably related to the extent of ER stress. The results illustrate that the obtained antibodies are a valuable tool in studying ER resident protein expression in ER stress models and ER-related

diseases. Furthermore, these results demonstrate the power of phage display in combination with single domain antibody fragments, as the obtained antibody fragments perform better than a commercially available anti-KDEL antibody obtained by hybridoma technology.

Materials and methods

Cloning and expression of recombinant proteins

The cDNA encoding Troponin C (TropC) was PCR amplified from a total human muscle cDNA preparation, with primers TropCforward 5'-CGGGATCCGATGACATCTACAAGGCTGCGG-3' and TropCreverse 5'-CCCAAGCTTCTCCACACCCTTCATGAACTCC-3'. For PCR, an initial denaturation step of 5 minutes at 95°C was followed by 25 cycles of 95°C for 1 minute, 62°C for 1 minute and 72°C for 2 minutes. The use of these primers introduced a 5'-*Bam*HI and a 3'-*Hin*dIII restriction site that allowed directed in-frame cloning in the pET28a expression vector (EMD Biosciences, Novagen Brand, Madison, U.S.A.). To introduce the amino acid sequence KDEL at the 3'-end of the Troponin C (TropC) gene, primers TropCKDELforward 5'-AGCTTAAAGATGAACTCTAAC-3' and TropCKDELreverse 5'-TCGAGTTAGAGTTCATCTTTA-3' were annealed and cloned behind the TropC gene with the restriction enzymes *Hin*dIII and *Xho*I. The obtained constructs were sequence verified (Sanger et al., 1977) and produced in *E. coli* strain BL21(DE3)-RIL (Stratagene, La Jolla, U.S.A.) according to standard procedures**.**

To construct a C-terminally KDEL-tagged version of glutathione S-transferase (GST), the pRP261 vector was used. This vector is a derivative of vector pGEX-3X (Amersham Biosciences, Roosendaal, The Netherlands). The primers GSTKDELforward 5'-GATCAAAGATGAGCTCTA-3' and GSTKDELreverse 5'-AGCTTAGAGCTCATCTTT-3' were annealed and cloned into pRP261 with the restriction enzymes *Bam*HI and *Hin*dIII. This cloning strategy resulted in a C-terminally KDEL-tagged GST in which the KDEL sequence was separated from the GST-encoding sequence by a factor Xa protease cleavage site. The expression construct was sequence verified and the KDEL-tagged GST was produced in *E. coli* strain DH5α according to standard protocols.

Purification of recombinant proteins

TropC with and without the C-terminal KDEL sequence was purified by means of its N-terminal His_6-tag using immobilized metal ion affinity chromatography (IMAC) according to the instructions of the manufacturer (Clontech laboratories, Mountain View, U.S.A.). The recombinant GST protein carrying the C-terminal protease factor Xa site and the KDEL sequence was purified with a VHH anti-GST column. VHH anti-GST was coupled to cyanogen bromide

(CNBr) activated sepharose 4B fastflow beads (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Prior to use, the column was washed extensively four consecutive times with PBS of low (pH 2.0) and neutral pH (pH 7.4). Thereafter, a lysate of DH5α bacteria, induced to express the KDEL-tagged GST construct, was applied and the flow through was reloaded twice. Next, the column was washed twice with ten bed volumes of PBS pH 7.4. Bound protein was eluted by applying a total of three bed volumes PBS pH 2. The eluted fraction was neutralized with 1M Tris pH 7.5 and extensively dialyzed to PBS.

Selection of KDEL specific VHHs

Selection of VHHs specific for the C-terminal KDEL sequence was performed using a large Llama-derived (VHH) non-immune library, which was constructed from eight non-immunized Llamas and had a clonal diversity of about $5x10^9$. This library was kindly provided by Unilever Research Vlaardingen, The Netherlands.

To obtain KDEL specific VHHs, two consecutive rounds of phage panning were performed. For the first round, 1ug of GST-Xa-KDEL was coated overnight at 4°C in a well of a maxisorp plate (Nunc, Roskilde, Denmark). The following day, the well was washed three times with PBS containing 0.05% Tween20 (PBST) and blocked shaking at room temperature for one hour with 2.5% (w/v) Marvell (skimmed milk powder) in PBST.

Thereafter, the blocked well was incubated for two hours at room temperature with $3{\cdot}10^{12}$ colony forming units (CFU) of library phage, pre-incubated for 30 minutes in a solution containing 120μg/ml GST (Sigma-Aldrich, Steinheim, Germany) and 1.5% (w/v) Marvell in PBST. Wells were washed 20 times with 200μl PBST and two times with PBS. Phage bound to the KDEL sequence were specifically eluted by incubation with two units of factor Xa (Amersham Biosciences, Roosendaal, The Netherlands) in incubation buffer (50mM Tris/HCl pH 8.0; 150mM NaCl; 1mM $CaCl_2$) for one hour. Output phage were rescued essentially as described before.

For the second round of selection, 1μg of TropC-KDEL antigen was coated overnight at 4°C. The selection procedure was comparable to the first round of selection with the following adjustments; $1{\cdot}10^{10}$ CFU of rescued phage of the first selection round were used and no competition was performed during the incubation. After washing, bound phage were eluted with 100mM triethylamine

(TEA) for 10 minutes and the eluted fraction was neutralized with half a volume of 1M Tris/HCl pH 7.5. Output phage were used to infect exponentially growing *E. coli* TG1 cells and plated on LB agar plates containing 2% D-glucose and 100μg/ml ampicillin.

Primary evaluation of selected clones

Screening for KDEL-specific clones was performed by phage ELISA (Marks et al., 1991). Clonal phage were produced in 96 wells microtiter plates as described before and tested for their ability to bind 1μg GST-Xa-KDEL or TropC-KDEL coated on a maxisorp 96 wells plate (Nunc, Roskilde, Denmark). As negative controls, their KDEL lacking counterparts were used as antigen. Furthermore, a *Hin*FI DNA fingerprint was performed as described before (van Koningsbruggen et al., 2003). Antigen-reactive clones having a different *Hin*FI fingerprint pattern were sequenced (ServiceXs, Leiden, The Netherlands).

Re-cloning of anti-KDEL VHH

Monovalent VHH was obtained by transformation of the *E. coli* strain BL21(DE3)-RIL (Stratagene, La Jolla, U.S.A.) with the phagemid vector containing the VHH gene. This allowed expression of a monovalent VHH containing a C-terminal Myc- and His_6-tag, but without the bacteriophage gene3 protein fused to it.

Bivalent VHH were obtained by cloning the antibody fragment twice into the expression vector pUU-11 via two consecutive cloning steps. First, a *Pst*I restriction site was introduced in the framework 1 (FR1) region of the VHH-encoding genes by means of PCR with primers RK1 5'-GTGCAGCTGCAGGAGTCTGGGGGA-3' and MPE25WB 5'-TTTCTGTATGGGGTTTTGCTA-3'. The PCR product was cloned into the vector at the 3'-end of a triple alanine linker as a *Pst*I-*Bst*EII fragment. A second antibody fragment was cloned from the phagemid vector in front of this linker as a *Sfi*I-*Not*I fragment. The obtained construct was sequence verified. This allowed expression of a bivalent VHH with a triple alanine linker and a C-terminal Myc- and His_6-tag.

To obtain an antibody fragment that could specifically be immobilised on a solid support, VHH encoding genes were re-cloned into the expression vector pUR5850 (De Haard et al., 2005). This vector allows expression of C-terminal Myc and His_6-tagged protein in the periplasmic space of *E. coli* and it adds a

biotinylation sequence (LRSIFEAQKMEW) between these tags. The bivalent VHH was cloned into this vector as a *Sfi*I-*Bst*EII fragment via partial digestion from pUU-11. Upon expression of the construct in the *E. coli* strain AVB101 (Avidity, Denver, U.S.A.), which expresses the *BirA* gene under control of an IPTG-inducible promotor, the lysine in the biotinylation tag is biotinylated. Purification of the antibody fragments was carried out by means of their His_6-tag using IMAC as described above.

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen corporation, Breda, The Netherlands) containing 7.5% fetal calf serum, 100U/ml penicillin and 100μg/ml streptomycin, in a humidified atmosphere containing 5% CO_2 at 37°C.

Western blot analysis

For Western blot analysis, cells were grown in 10cm∅ dishes till 90% confluency, washed twice in PBS and lysed in 700μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein quantification assay (Pierce, Rockford, U.S.A.). Equal amounts of protein were size-separated on a 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer [2.5% (w/v) protifar plus (Nutricia, Zoetermeer, The Netherlands) in PBS], membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or coomassie brilliant blue (CBB) [0.1% (w/v) coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with the appropriate antibodies in block buffer. After every incubation with antibody, membranes were washed 5 times in PBST (0.05% Tween20 in PBS). After the last antibody incubation, membranes were washed two additional times with PBS. Bound antibodies were visualized by enhanced chemo luminescence (PerkinElmer, Boston, U.S.A.)

Immunoprecipitation (IP)

HeLa cells were grown till 90% confluency in 10cm∅ petri dishes, washed twice with PBS and lysed in 700μl IP buffer [10% (v/v) glycerol; 1% (v/v) NP40; 100mM NaCl; 50mM Tris/pH7.4; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein assay.

For IP with commercially available antibodies [αPDI (Benham et al., 2000), anti-KDEL (Stressgen, Victoria, Canada) or anti-Calreticulin (Stressgen, Victoria, Canada)], 500μg of HeLa cell lysate was incubated for two hours at 4°C with 3μg of antibody. Next, 20μl of a mix of proteinA and G beads were added to the cell lysate and incubated for an additional hour at 4°C.

For IP with the VHH antibody, 25μl streptavidin beads (Interchim, Montiuçon, France) were incubated for two hours with 5μg biotinylated bivalent VHH. Hereafter, beads were washed three times with twenty bed volumes of IP buffer and subsequently incubated with 1mg HeLa cell lysate for 4 hours at 4°C.

All beads were washed four times in twenty bed volumes of IP buffer. Finally, 50μl 2xSB [16% (v/v) glycerol; 0.15M DTT; 3.3% (v/v) SDS; 0.01% (w/v) bromphenol blue; 20mM Tris/HCl pH6.8] was added to the beads and beads were subsequently boiled for five minutes before Western blot analysis.

Protein identification

Immunoprecipitated proteins were loaded on a 12% in-house prepared SDS gel of 20 cm by using a Hoeffer gel system (Amersham biosciences, Roosendaal, The Netherlands). Proteins were visualized with Coomassie Brilliant Blue and bands of interest were sliced out of the gel and subjected to tryptic digestion (Shevchenko et al., 1996). Peptide extracts were identified with an Agilent 1100 LC system (Agilent, Palo Alto, USA) coupled to a Thermo Finnigan LTQ (Thermo electron Company, Waltham, MA, USA) as described previously in literature (Kolkman et al., 2005).

Immunofluorescence (IF)

HeLa cells were grown on 15mm∅ cover slips till 70% confluency. Cells were washed twice with PBS at 37°C and subsequently fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cover slips were washed twice

with PBS for 5 minutes and cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The wash steps were repeated and non-reacted aldehyde groups were quenched with 50mM glycine in PBS for 10 min. Cells were washed twice with 1% BSA in PBS (blocking buffer) and subsequently blocked in the same buffer for 30 minutes. Thereafter, cover slips were incubated with the appropriate antibodies diluted in blocking buffer. The VHH anti-KDEL antibody was detected with a monoclonal anti-Myc antibody (9E10), followed by incubation with GAM-ALEXA555. The anti-PDI antibody was detected with GAR-ALEXA488. Each incubation with antibody was performed for one hour, subsequently followed by four wash steps with blocking buffer for 5 minutes. Finally, cells were washed twice with PBS, cover slips were mounted onto glass slides with Mowiol-PPD, air-dried and examined using a fluorescence microscope.

Induction of ER-stress

For stress resistance assessment, HeLa cells were grown in a 12 wells plate (Corning, U.S.A.) till 70% confluency and treated with decreasing amounts of dithio-threitol (DTT), hydrogen peroxide (H_2O_2) or tunicamycin (TM). The survival of the cells was followed in time. The highest concentration of the stress inducing agents where minimal cell death was observed was then chosen for further experiments. Cells were grown in 6cm∅ dishes till 70% confluency and treated for the indicated time points with DTT (1.5mM), H_2O_2 (50μM) or tunicamycin (10μg/ml). Treated cells were washed twice in PBS and lysed in 200μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein assay (Pierce, Rockford, U.S.A.). Seven μg of each sample was analysed by Western blot as described above.

Results

Selection of KDEL-specific VHHs

Phage display was used to obtain VHH antibody fragments specific for the C-terminal amino acid sequence KDEL found in several mammalian ER resident proteins. Such antibody fragments could be used to monitor ER resident protein expression in biological samples. To this aim, a large Llama-derived (VHH) non-immune library was used with a clonal diversity of about $5x10^9$. The selection strategy used to obtain KDEL-specific VHH fragments, consisted of a combination of three steps during two selection rounds to specifically drive the selection towards this four amino acid epitope. (i) During the first round of selection, GST competition was performed during incubation of the phage library with immobilized GST-Xa-KDEL antigen. (ii) In addition, KDEL-bound phage were eluted by site-specific proteolysis with factor Xa, which cleaved off the C-terminal part (GIKDEL) of the coated protein. Both steps reduce the selection of GST specific antibody fragments and drive the selection towards enrichment of KDEL-specific VHHs. (iii) Furthermore, another KDEL-tagged antigen (TropC-KDEL) was used in the second round of selection. Only phage recognizing identical epitopes present on both antigens, i.e. the C-terminal KDEL sequence, should therefore be selected.

A

	Colony count 1st round	Colony count 2nd round
Input	$3•10^{12}$	$1•10^{10}$
Output KDEL	$2•10^{5}$	$6•10^{5}$
Output controle	$2•10^{4}$	$2•10^{4}$

B

	Output/input ratio	Signal/noise ratio
1st round	$6.6•10^{-8}$	10
2nd round	$6.0•10^{-5}$	30
Enrichment	909	

Table 1: Results of the selection against the C-terminal four amino acid epitope KDEL with a non-immune Llama phage display library. The amount of input and output phages of the first and second round are depicted (A) as well as the enrichment in output/input ratio and the increase in signal to noise ratio between the first and second round (B).

The titers of selected phage obtained in the first and second round of selection compared to background binding (signal/noise ratio), as well as the enrichment in output/input ratio, are depicted in table 1. Especially the latter indicated successful selection of KDEL-specific VHH fragments. Therefore, individual clones from this selection output were screened for antigen specificity. Phage of individual clones were tested in ELISA for binding to coated GST-Xa-KDEL and TropC-KDEL. About 50% of the tested clones gave

ELISA signals of at least four times the background on the KDEL-tagged antigens and signals comparable to background on the non-tagged proteins, and were therefore classified as positive. Next to this ELISA, a *Hin*FI DNA fingerprint was performed to test the diversity of the selected clones. All positive clones showed a similar *Hin*FI restriction pattern. This pointed towards a high degree of similarity between the selected clones, as was evidenced by sequence analysis of 11 clones. Three clones (1, 5 and 11), showed differences in their complementarity determining regions (CDRs) (Figure 1) and were therefore selected for further analysis.

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                  FR1                 CDR1       FR2             CDR2
Cl 1   AVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 7   AVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 4   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 9   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 2   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 6   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 3   DVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 8   DVQLVESGGGLVQAGDSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 10  DVQLVESGGGLVQAGDSLRLSCAASGRTFS SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 5   EVQLVESGGGLVQAGDSLRLSCADSGRTFS QYTMG WFRRAPGKEREFVA TISTLGGMTYYADSIKG
Cl 11  QVKLEESGGGLVQAGGSLRLSCAASGRTFS TYTMG WFRQAPGKERELVA AISWGGSRYYADSVEG

                  FR3                          CDR3          FR4
Cl 1   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 7   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 4   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 9   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTRVTVSS
Cl 2   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 6   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVST
Cl 3   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGIQVTVSS
Cl 8   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 10  RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVST
Cl 5   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGIQVTVSS
Cl 11  RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA KPPG-GIVTDTRKYDY WGQGTQVTVST
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Figure 1: Aligned amino acid sequence of the 11 KDEL-specific clones that were sequenced. Only clones 5 and 11 show differences in the complementarity determining regions (CDR) compared to the other clones.

Characterization of selected anti-KDEL VHH antibody fragments

To further evaluate these three clones, the performance of the different VHHs was tested in ELISA. A positive signal was only obtained at relatively high concentrations of antibody (higher than 150nM), which suggested a low affinity of the antibodies for the KDEL sequence. To increase the apparent affinity of the monoclonal VHHs, bivalent constructs were made and tested in ELISA. The bivalent clones (EC_{50} 15nM) performed approximately 100 times better than their monovalent (EC50 1.5μM) counterparts as is shown for clone 1 (Figure 2).

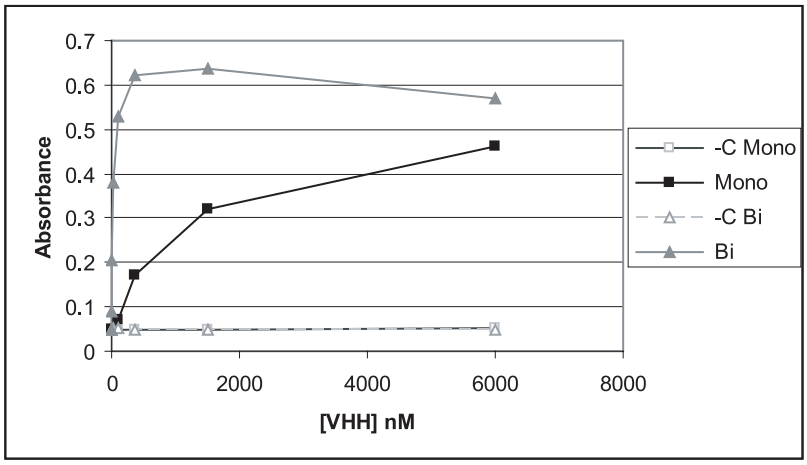

Figure 2: Effect of bivalency on the performance of the anti-KDEL VHH antibody fragments. An ELISA was performed on coated TropC-KDEL, detected with decreasing amounts of monovalent (EC_{50} 1.5μM) and bivalent (EC_{50} 15nM) clone 1 anti-KDEL. As negative control, a non-specific VHH was used.

To test the bivalent VHHs for their binding to ER resident KDEL-containing proteins in cellular extracts, they were first tested on Western blot of a HeLa cell lysate. Separate lanes of the blot were incubated with decreasing concentrations of each clone (Figure 3). All VHH clones detected several proteins in the HeLa extract and the observed band pattern at the highest concentration of antibody (1500nM) showed many similarities between the different clones, indicating that the different VHHs recognized the same proteins.

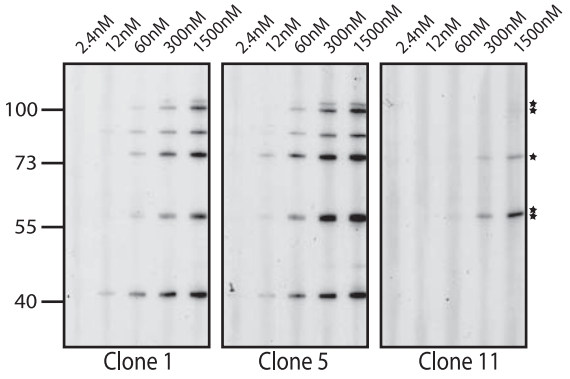

Figure 3: Comparison of the performance of the bivalent anti-KDEL clone 1, 5 and 11 on Western blot containing a HeLa cell lysate. The molar concentrations of the VHHs used for detection are depicted. Proteins detected by all three clones are indicated (asterix).

Protein	Acc.nr.	Mw	Function
Endoplasmin	P14625	92kD	Molecular chaperone
Putative alpha-Mannosidase C1 orf22	Q9BZQ6	100kD	
GRP78/BiP	P11021	72kD	Molecular chaperone
Calreticulin	P27797	48kD	Molecular chaperone
PDI A1	P07237	57kD	Catalyzes S-S bond rearrangement
PDI A6	Q15084	48kD	Catalyzes S-S bond rearrangement
ERp46	Q8NBS9	47kD	Possesses thioredoxin activity

Table 2: ER-resident proteins containing a C-terminal KDEL sequence. SWISSprot accession numbers (Acc.nr.), molecular weight of the unprocessed precursor (Mw) and function of the proteins are indicated.

Some of the detected proteins had a molecular mass corresponding to that of known ER resident proteins containing a C-terminal KDEL sequence, such as endoplasmin, putative alpha-mannosidase C1 orf22, GRP78, calreticulin, PDI A1, PDI A6, and ERp46 (Table 2). However, with decreasing amounts of antibody, a clear difference in the performance of the different VHH clones was observed (Figure 3). The bivalent construct of clone 11 did not detect the same number of proteins as clone 1 and 5. Furthermore, higher antibody concentrations were needed to obtain an equivalent signal with clone 11 compared to the other clones, indicating a lower affinity for the KDEL epitope. Based on these results, clone 5 was designated as the clone that performed best in this application and was therefore used for further experiments.

Proof of antigen specificity

To show that clone 5 recognized the KDEL sequence when it is present at the C-terminus of a protein, a competition experiment was performed. Bivalent VHH clone 5 was used to stain a Western blot of a HeLa cell lysate in the presence of the peptides KDEL, LEDK and KDELG (synthesized by Pepscan, Lelystad, The Netherlands) (Figure 4). With increasing amounts of the KDEL peptide, a clear reduction in the signal was observed. In contrast, when the peptides LEDK or KDELG were added, no competition was observed. The latter observation indicated that the VHH only recognized the KDEL sequence when present at the C-terminus of a peptide or protein. This was supported by ELISA experiments using proteins and peptides containing internal KDEL sequences, in which no positive signal was obtained with any of these antigens (data not shown).

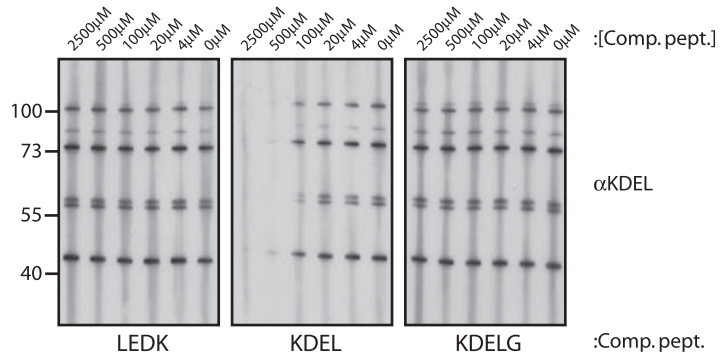

Figure 4: Specificity of the selected VHH clone 5 for the C-terminal KDEL sequence. During incubation of the anti-KDEL antibody at a 500nM concentration, increasing amounts of soluble peptide (LEDK, KDEL or KDELG) was added to different lanes to compete for the antigens present on blot.

To further confirm the specificity of the anti-KDEL VHH clone 5, immunoprecipitation (IP) experiments were performed with commercially available antibodies against known KDEL-containing proteins, i.e. anti-calreticulin, anti-PDI and anti-KDEL. The latter antibody has been shown to detect only the KDEL containing proteins endoplasmin and GRP78. Precipitated proteins were detected with the bivalent anti-KDEL VHH clone 5 (Figure 5). These data show that the proteins detected by the anti-KDEL VHH in a HeLa cell lysate indeed are the ER resident proteins endoplasmin, GRP78, protein disulfide isomerase (PDI) and Calreticulin.

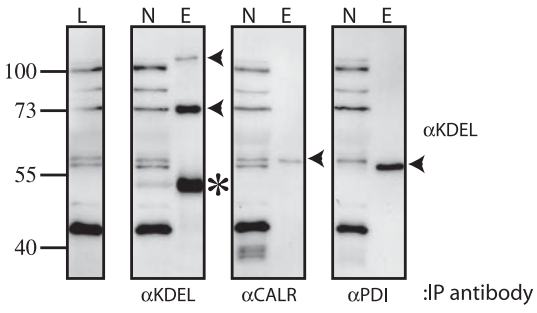

Figure 5: The anti-KDEL VHH recognizes the ER resident proteins endoplasmin, GRP78, PDI and Calreticulin. Commercially available antibodies against these known KDEL containing ER resident proteins were used to precipitate proteins from a HeLa cell lysate. The lysate (L), non-bound (N) and bound fraction (E) were analyzed on immuno blot with the bivalent anti-KDEL clone 5 VHH, after immuno-precipitation with the respective antibodies. The arrowheads indicate the precipitated proteins and the asterix indicates the heavy chain of the antibody used for the IP.

To identify the four additional proteins detected on Western blot, biotinylated bivalent clone 5 was used to purify the proteins from a HeLa cell lysate. The precipitated product was analyzed on SDS-page and on Western blot with the bivalent anti-KDEL clone 5 (Figure 6). The protein bands that corresponded to the molecular weight of the bands obtained on Western blot were excised from gel, trypsin digested and used for mass spectrometry (MS). The proteins endoplasmin (ionscore 7929), GRP78 (ionscore 5495) and PDI A1 (ionscore 2266) were positively identified, corroborating the results obtained with the commercial antibodies directed to known KDEL-containing proteins. Furthermore, the second lowest band consisted of the two KDEL proteins PDI A6 (ionscore 3830) and thioredoxin domain-containing protein 5 (ERp46) (ionscore 508), whereas the lowest band contained two proteins with a RDEL and not a KDEL retention signal. The most prominent protein found in the latter was collagen-binding protein 2 (colligin 2)(ionscore 1385), but also thioredoxin domain-containing protein 4 (Erp44) was found (ionscore 222). The results for the two remaining bands were inconclusive. However, judging from its molecular weight, one of the bands could be the putative alpha manosidase (table 2). From these data, it was concluded that the anti-KDEL VHH recognizes both KDEL and RDEL containing proteins.

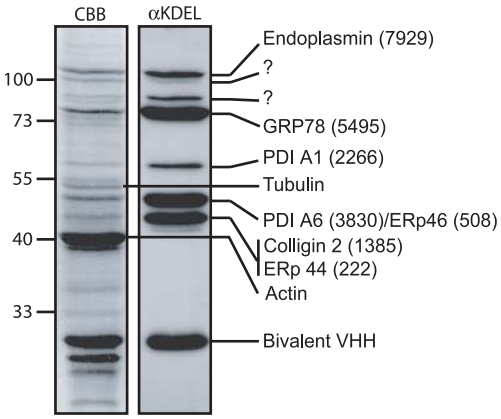

Figure 6: Characterization of the antigens recognized by the anti-KDEL clone 5 VHH. Target proteins were precipitated with the biotinylated bivalent VHH and analyzed on a coomassie (CBB) stained PVDF membrane. The CBB stained protein bands that corresponded to the signal obtained with the anti-KDEL antibody were identified by MS. Ionscores for the KDEL containing proteins are indicated between brackets.

Next, immuno fluorescence (IF) experiments were performed to further demonstrate that the VHH clone 5 recognized proteins in the ER. Labelling of HeLa cells with the bivalent anti-KDEL VHH showed almost identical labelling compared to that obtained with an antibody against the well-known ER marker PDI (Figure 7). This further proves that this antibody recognises specific proteins in the ER and illustrates its use in IF.

Finally, the anti-KDEL VHH was tested on lysates of cells from different mammalian species to investigate whether this antibody can be used to detect ER resident proteins in a wide variety of species (Figure 8). In all species tested (man, mouse, chimp and hamster) the band pattern of recognized proteins was similar, with the exception of one protein that was only detected in COS-1 and HeLa having a MW of approximately 90kDa. A comparison of the VHH antibody with a commercially available monoclonal anti-KDEL antibody (Stressgen, Victoria, Canada) showed that the VHH antibody recognized more proteins on Western blot (Figure 8).

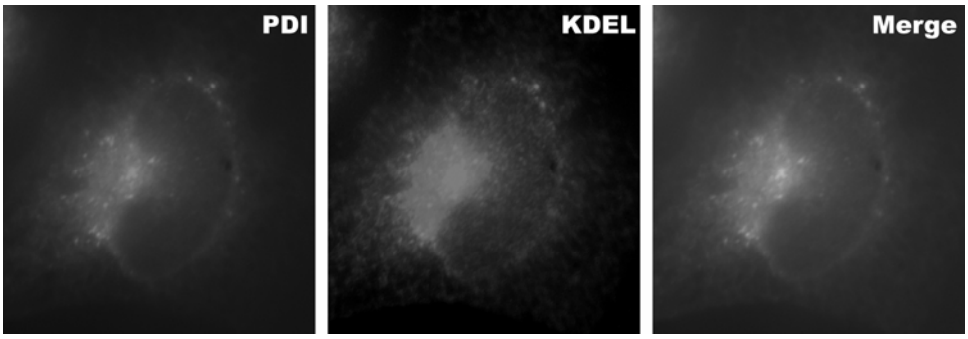

Figure 7: Immuno fluorescence staining of HeLa cells labeled with an anti-PDI antibody and the bivalent anti-KDEL clone 5 VHH. The merged picture clearly shows that both antibodies recognize antigens that co-localize in the ER.

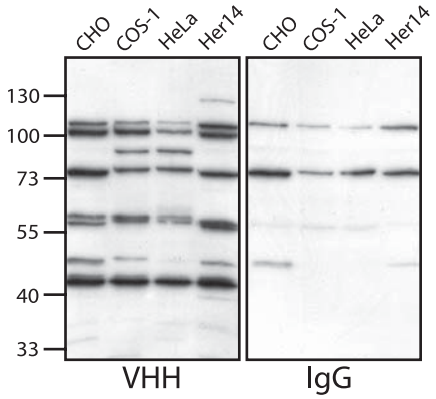

Figure 8: The anti-KDEL clone 5 VHH has wide species specificity. The performance of the bivalent VHH clone 5 (VHH) was analyzed and compared to a commercial anti-KDEL antibody (IgG) on a blot containing lysates of hamster (CHO), chimpanzee (COS-1), human (HeLa) and murine (Her14) cells.

Monitoring the ER-stress response with the KDEL-specific VHH

To show the applicability of this antibody in studies of ER stress, the ER response of HeLa cells was monitored after induction of stress by treatment with several different chemical agents. These agents, dithio-threitol (DTT), hydrogen peroxide (H_2O_2) and tunicamycin (TM) are known to cause an

unfolded protein response (UPR) at the level of transcription (Pakula et al., 2003). First, the viability of HeLa cells was estimated after incubation with different concentrations of these stress-inducing agents for 24 hours. The highest concentration of stressor that resulted in minimal cell loss after 24 hours was then used for subsequent experiments, which was 1.5mM DTT, 50μM H_2O_2 or 10μg/ml tunicamycin.

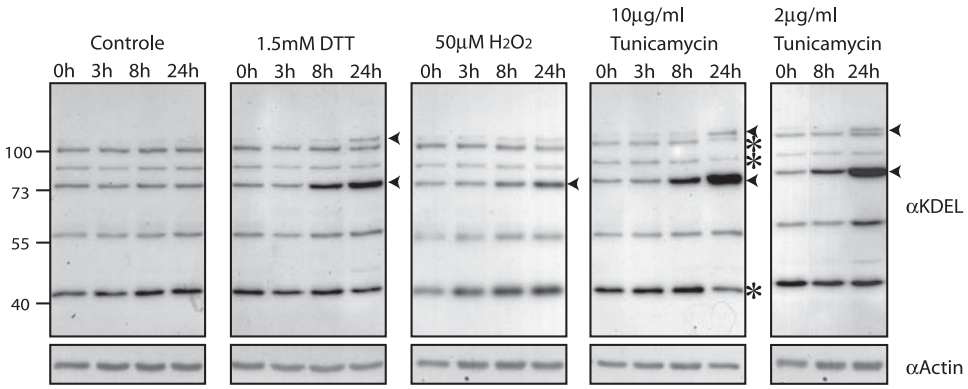

Figure 9: Differential ER-resident protein expression during ER stress induced with several different chemical agents and visualized by the anti-KDEL clone 5 VHH. Immuno blot with bivalent VHH clone 5 anti-KDEL. Cells were grown for 24 hours and subjected to the different stress inducers DTT (1.5mM), H_2O_2 (50μM) and tunicamycin (10μg/ml and 2μg/ml). Samples were taken after the indicated time periods. Proteins that are up- (arrowheads) or down-regulated (asterix) are indicated.

Cells were stressed for 24 hours with the determined concentrations of stress inducing agents and samples were taken at time points 0, 3, 8 and 24 hours. Cell lysates were prepared and samples were analyzed by Western blot with the bivalent anti-KDEL VHH clone 5 (Figure 9). This clearly showed that during treatment with the different stress inducing agents, especially GRP78 was up-regulated at the protein level. Furthermore, endoplasmin showed an up-regulation during treatment of the cells with DTT and Tunicamycin, but hardly after treatment with H_2O_2. The bands corresponding to the proteins PDI A1 and calreticulin showed a slight up-regulation with all three stress inducing agents. A striking observation was that some proteins also recognized by clone 5 anti-KDEL showed an apparent down-regulation after 24 hours treatment with tunicamycin. These results clearly show that upon ER stress, especially GRP78 shows a clear up-regulation at the protein level, whereas other ER

resident proteins show hardly any up-regulation or even a down-regulation. Furthermore, there is a clear difference in response upon treatment with different stressors, wherein tunicamycin treatment results in the most severe response after 24 hours. These differences were less apparent when tunicamycin concentrations were reduced to 2μg/ml, resulting in comparable differences as observed after the DTT treatment (Figure 9), which suggests that the variation between the effects of the different stressors is caused by the extent of ER stress

Discussion

To obtain KDEL-specific VHH antibody fragments that would be useful in the study of differential ER-resident protein expression, a large Llama-derived non-immune VHH library was used. Two *E. coli* expressed heterologous proteins, tagged with the KDEL sequence at their C-terminus, were used in two consecutive selection rounds. Of three different selected VHHs, the VHH that performed best in Western blot was subsequently thoroughly tested for its specificity and use in several biomolecular applications such as ELISA, Western blot, immuno fluorescence and immuno precipitation. Furthermore, the successful application of this antibody was shown in an ER stress model.

After selection and screening for anti-KDEL VHHs, the clones that were positive in phage ELISA were further evaluated. ELISA with soluble antibody did not result in comparable signals obtained by phage ELISA, in which the signal is amplified by the HRP-conjugated anti-M13 (anti-pVIII coat protein) antibody. Only very high concentrations of antibody yielded positive signals, which could be due to low antibody affinity. To increase the apparent affinity of the VHHs, bivalent constructs were made, which performed much better in ELISA than the monovalent constructs (Figure 2). This shows that, although selection using a non-immune phage display library may result in antibodies with relatively low affinity, the single domain character of the VHH allows the easy and quick modulation of the apparent affinity by means of simple cloning steps.

The high sequence similarity between the selected KDEL specific clones 1-10 indicated that these clones had advantageous characteristics over clone 11 which differed in all CDR regions from the other clones. The superior performance of clone 1 and 5 in Western blot, where a clear difference in performance was observed with clone 11, suggests that this was caused by a higher affinity of these clones for the KDEL epitope (Figure 3). Competition experiments with the peptides KDELG and KDEL (Figure 4) suggested that, at least clone 5, recognized only the KDEL sequence when present at the C-terminus of a protein. This had to be an essential feature of this antibody, as numerous non-ER resident proteins contain an internal KDEL sequence. The C-terminal KDEL specificity was confirmed by identification of the bands obtained on Western blot with clone 5 in two separate experiments; an IP experiment

with commercial antibodies against known KDEL containing proteins (Figure 5), and MS of precipitated proteins in an IP sample obtained with clone 5 (Figure 6). All these identified proteins were confirmed to be ER resident proteins containing a C-terminal KDEL or RDEL sequence by searching the SWISSprot database. The latter finding may be explained by the high degree of similarity between the charge of the amino acids arginine (R) and lysine (K). Indeed, the selected VHHs could also bind to C-terminal HDEL containing proteins in phage ELISA, although the signal was very low, suggesting a very low affinity for this epitope (data not shown). In the IP fraction obtained with clone 5, several other proteins were seen that did not correspond to the signal obtained on western blot. One band was especially prone and was identified as actin. Also another cytoskeleton protein was identified, tubulin (Figure 6). As ER resident proteins can bind a wide range of different proteins, e.g. via hydrophobic patches, co-immuno precipitation of proteins that are normally not exposed to the proteins that reside in the ER could be expected, since these proteins come into contact with each other during the IP protocol.

The selection of an antibody from a non-immune phage display library that specifically recognizes a four amino acid epitope, only when present at the C-terminus of a protein, is remarkable. Especially, since the average epitope recognized by an antibody is composed of more amino acids. Comparison of the bivalent version of this antibody with a commercially available anti-KDEL antibody, which was obtained with classical hybridoma technology, showed the superior performance of the selected antibody fragment (Figure 8).

In various ER stress models, using HeLa cells, we showed the successful applicability of the clone 5 VHH in cellular studies. We showed a clear increase of the proteins GRP78 and endoplasmin after treatment with the thiol reducing agent DTT and the N-glycosylation inhibitor tunicamycin (Figure 9). The up-regulation of GRP78 was less apparent upon induction of oxidative stress with H_2O_2 and there was no up-regulation observed of endoplasmin after this treatment. This clearly shows a different reaction of HeLa cells to the different forms of ER stress. There are two obvious explanations for these differences: It could either be that stress induced by different stress inducers results in different stress specific responses of the ER in regulating ER resident protein expression levels or the observed differences are caused by differences in the

extent of ER stress. The results obtained after treatment of cells with lower concentrations of tunicamycin (Figure 9) support the latter, since this resulted in comparable protein expression differences as observed after DTT treatment. This suggests that the up-regulation of the ER resident proteins endoplasmin and especially GRP78 at the protein level may be a measure for the severity of ER stress. In our experiments, H_2O_2 is less effective in inducing ER stress than tunicamycin at the concentrations tested. This is probably caused by different specificities of the used stress inducers. As H_2O_2 damages a wide variety of cellular components (i.e. DNA, lipids and proteins), treatment of cells with this agent results in cell death before a severe ER stress response is induced. In contrast, tunicamycin primarily influences mechanisms important for ER functionality (i.e. N-glycosylation), which allows treatment of cells with concentrations that are more effective in inducing an ER stress response.
Another intriguing aspect of these results is that some KDEL containing proteins are not affected in terms of protein expression while others show an apparent decrease in expression during severe ER stress (Figure 9). In contrast, it has been shown that several of these proteins are up-regulated at the transcriptional level during an UPR (Arvas et al., 2006; Kozutsumi et al., 1988; Martinez and Chrispeels, 2003). This illustrates the complex nature of the UPR, where the balance between transcription, translation and degradation determines the overall expression levels of a protein. Apparently, during expansion of the ER upon induction of stress, the ratio between different ER resident proteins shifts. The impressive up-regulation of GRP78 exemplifies its important role in maintaining ER homeostasis and its role in the control of the UPR (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002). This up-regulation could be a negative feedback mechanism for the UPR and inhibit the induction of apoptosis while trying to compensate for the decreased folding capacity of the ER. The anti-KDEL antibody will allow the analysis to what extend the observed differential protein expression is conserved within different cell types and upon treatment with other stress inducers like thapsigargin, which induces ER Ca^{2+} depletion. It has been suggested that ER stress caused by ER Ca^{2+} depletion does not necessarily trigger the same signalling pathways as non-glycosylated proteins do (McCormick et al., 1997).

Our results clearly show that it is possible to select an antibody against a conserved epitope of only four amino acids and that this antibody can be

successfully applied in studies of ER homeostasis regulation. The application could be further extended to other ER stress studies as in ER storage diseases. The existence of several other biological mechanisms that utilize conserved epitopes for a specific function, such as the nuclear localization sequence (NLS) or the peroxisomal targeting sequence, could offer a target for similar selection experiments as has been done for protein modifications as phosphorylation, and glycation (Gronborg et al., 2002; Maguire et al., 2002; Richter et al., 2005). This would offer a useful tool in examining protein expression levels of specialized protein groups in a cell. The application of VHH antibody fragments combined with phage display techniques offer useful tools to be applied in this field.

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We would like to thank Unilever Research Vlaardingen (The Netherlands) for providing us with the non-immune phage display library. We thank the lab of Prof. I. Braakman for providing us with the polyclonal anti-PDI antibody. We thank Dr. P. Verheesen for his technical assistance during the selection of the antibody fragments. We thank the Netherlands Proteomics Centre for identification of the precipitated KDEL containing proteins. We thank members of the Verrips lab for helpful discussions. This work was in part supported by Unilever Research Colworth (UK).

Chapter 5

Differential protein expression in senescent HUVECs

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Abstract

Senescence is a cellular process that is manifested by the loss of replicative ability and loss of cellular function. It is thought to be involved in the degenerative process of aging and in several diseases that are associated with aging. Senescence can be induced by various mechanisms, such as telomere erosion and oxidative stress, but the precise mechanisms and more importantly, the occurrence and significance of this phenomenon *in vivo* still remains to be fully elucidated. To resolve these questions, identification of the changes that occur prior to and during induction of senescence are essential. Here we describe preliminary results on the differential expression of several ER-resident proteins, involved in proper folding of proteins that enter the secretory pathway, and of the transmembrane protein endoglin, involved in the regulation of endothelial cell proliferation, upon induction of replicative senescence in human umbilical vein endothelial cells. The possible implications and consequences of the observed expression differences and the challenges of cellular senescence research will be discussed.

Introduction

Aging is accompanied by an increase in the prevalence of age-associated diseases. Of these diseases, atherosclerosis has one of the highest incidences in the aged population in western society and consequently causes a high stress on the medical system. The pathology is initiated by the accumulation and oxidation of LDL particles in the vessel wall and the subsequent intrusion of monocytes (Glass and Witztum, 2001). The disturbed integrity and functioning of the endothelial monolayer in blood vessels upon aging is thought to play a key role in the initiation of this disease. The endothelial cells (ECs) have an important regulatory barrier function on the interface of the blood and the rest of the vasculature. It is involved in the transport of macromolecules to and from the surrounding tissues, it regulates coagulant activity and it regulates the intrusion of white blood cells from the circulation. Endothelial integrity is normally maintained by several repair mechanisms. Damaged endothelial cells are either replaced by division of surrounding endothelial cells or by bone marrow-derived endothelial progenitor cells (EPCs) that circulate in the blood. Thus, both mechanisms are involved in maintaining endothelial monolayer integrity (Op den Buijs et al., 2004). As many other cell types, endothelial cells cannot divide indefinitely and eventually they will enter a state of irreversible growth arrest called senescence (Hayflick and Moorhead, 1961). This state can be caused by several mechanisms, like telomere shortening, induction of oncogenic proteins and intracellular oxidative stress (Campisi, 2000; Chiu and Harley, 1997; Serrano and Blasco, 2001), and is manifested by an enlarged and flattened cell morphology, increased granularity and vacuolization. Furthermore, proteins involved in cell cycle inhibition, such as p21, are expressed (Antropova et al., 2002; Chen et al., 2006; Vaziri and Benchimol, 1996). Although these cells are viable and metabolically active, they have altered gene expression (Shelton et al., 1999), which is reflected by altered protein expression (Eman et al., 2006). Although senescence is a process that is thought to have a protective function against the development of diseases, such as cancer, early in life, accumulation of these cells at specific sites could lead to loss of endothelial function and consequently a disturbed barrier function where cells and molecules can enter the rest of the vessel wall freely. Under normal circumstances, endothelial cells have a low average turnover rate of approximately once every 3 years (Foreman and Tang, 2003). However, at sites of injury, bifurcations and

branching points, there is a dramatic increase in cellular replication (Caplan and Schwartz, 1973). This can ultimately lead to induction of senescence and consequently to an increased risk in endothelial dysfunction.

Indications that senescent ECs are relevant *in vivo* and could be important in the development of pathological processes are the identification of cells with increased shortened telomeres at sites susceptible to atherosclerosis (Chang and Harley, 1995; Okuda et al., 2000) and the accumulation of senescent ECs on the surface of atherosclerotic plaques (Minamino et al., 2004; Vasile et al., 2001). Despite these findings, the occurrence and significance of this phenomenon *in vivo* still remain a matter of debate (Cristofalo et al., 2004).

Although several potential biomarkers for senescence have been described (Eman et al., 2006; Shelton et al., 1999), identification of additional changes that occur is needed to further unravel the mechanisms and consequences of EC senescence.

As there is an increase of oxidative stress with increasing age, several cellular processes are affected. An organelle that is likely to be more sensitive for this increased oxidative stress is the endoplasmic reticulum (ER), as glutathione (GSH), the major redox buffer of cells, is in a more oxidized state in the ER as compared to the cytoplasm (Hwang et al., 1992). Indeed it has been shown that upon severe oxidative stress primarily several ER-resident proteins are oxidized, which are responsible for proper folding of proteins that enter the secretory pathway (van der Vlies et al., 2002). Furthermore, carbonylation of these ER resident proteins has been shown to occur upon aging, which is thought to affect proper functioning of these proteins (Rabek et al., 2003). As the expression of several of these proteins is affected by various forms of ER stress via activation of the unfolded protein response (UPR) (Pakula et al., 2003; Schroder and Kaufman, 2005), we hypothesized that changes in expression of these proteins might occur during replicative senescence.

In addition, we hypothesized that as senescent cells lose the ability to replicate, the expression of proteins involved in the control of endothelial cell proliferation might change. A clear candidate is endoglin (CD105), a transmembrane protein involved in transforming growth factor (TGF)-β signaling, which is needed for cell proliferation (Lebrin et al., 2004). Endoglin modulates the balance between ALK1 and ALK5 signalling pathways, which are activated by binding of the TGF-β receptor to its ligand, but they have opposite effects. TGF-β/ALK5 signaling is a negative regulator of EC migration

and angiogenesis via induction of plasminogen activator inhibitor type 1 (PAI-1) expression (Stefansson and Lawrence, 1996; Stefansson et al., 2001), while TGF-β/ALK1 signaling promotes EC proliferation and migration. Endoglin is a positive regulator of the ALK1 pathway and a negative regulator of the ALK5 pathway. Consequently, endoglin protein levels determine the growth capacity of ECs (Lebrin et al., 2005). Here we describe a decrease in cellular endoglin expression and differential expression of several ER resident proteins in replicative senescent human umbilical vein endothelial cells (HUVEC). The decreased expression of endoglin was found in only part of the senescent population. Furthermore, the differential expression of the ER resident proteins differed slightly between different HUVEC isolations. The differences and possible consequences of differential expression of these proteins upon replicative senescence will be discussed. Additionally, the observed differences are used to reveal the challenges of aging research.

Materials and methods

HUVEC isolation and culturing

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from umbilical veins according to the method of Jaffe (Jaffe et al., 1973). Culturing of HUVECs was performed in fibronectin coated culture flasks in EGM-2 (Cambrex, New Jersey, U.S.A.) at 37ºC in 95% rel. humidity and 5% CO2. Medium was changed every two or three days and cells were passaged at 90% confluency. Cells were counted at the moment of seeding and passaging, to allow calculation of population doubling after each passage. When the cells ceased proliferation they were kept in culture for two weeks with continued medium replenishment. Prior to every experiment, a senescence-associated β-galactosidase assay (Cell Signalling Technology, Beverly, MA, U.S.A.) was performed according to the instructions of the manufacturer to determine the presence of senescent cells, together with microscopical inspection of cell morphology.

Western blot

For Western blot analysis, cells were grown in 10cm∅ dishes till 90% confluency, washed twice in PBS and lysed in 700μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; 0.1% SDS; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein quantification assay (Pierce, Rockford, U.S.A.). Equal amounts of protein were size-separated on a 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer [2.5% (w/v) protifar plus (Nutricia, Zoetermeer, The Netherlands) in PBS], membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or coomassie brilliant blue (CBB) [0.1% (w/v) coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with the appropriate antibodies in block buffer. After every incubation with antibody, membranes were washed 5 times in PBST (0.05% Tween20 in PBS). After the last antibody incubation, membranes were washed two additional times with PBS. Bound antibodies were visualized by enhanced chemo luminescence (PerkinElmer, Boston, U.S.A.)

Biotinylation of cell surface proteins

Cultured HUVECs were placed on ice, washed twice with ice-cold PBS, and subsequently labeled for 20 minutes with 100µg/ml sulfo-NHS-LC-LC-biotin (Pierce, Rockford, U.S.A.) in PBS. After labeling, cells were washed twice with 10mM lysine in PBS and subsequently incubated for 5 minutes in the same solution to block all remaining reactive NHS groups. Excess liquid was removed and cell lysates were prepared.

Immuno fluorescence

HeLa cells were grown on 15mm∅ cover slips till 70% confluency. Cells were washed twice with PBS at 37°C and subsequently fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cover slips were washed twice with PBS for 5 minutes and cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The wash steps were repeated and non-reacted aldehyde groups were quenched with 50mM glycine in PBS for 10 min. Cells were washed twice with 1% BSA in PBS (blocking buffer) and subsequently blocked in the same buffer for 30 minutes. Thereafter, cover slips were incubated with an anti-endoglin antibody, which was detected with GAM-ALEXA555. Each incubation with antibody was performed for one hour, subsequently followed by four wash steps with blocking buffer for 5 minutes. Finally, cells were washed twice with PBS, cover slips were mounted onto glass slides with Mowiol-PPD, air-dried and examined using a fluorescence and confocal microscope.

Results

Confirmation of replicative senescence

To confirm the induction of replicative senescence *in vitro*, a senescence-associated β-galactosidase assay was performed. Furthermore, changes in cell morphology were monitored (Figure 1). The senescent cells were enlarged, had a clear flattened cell morphology and increased granularity compared to the young cells. Furthermore, with increasing passages the number of positive cells in the senescence-associated β-galactosidase assay increased significantly (Figure 1 and 2).

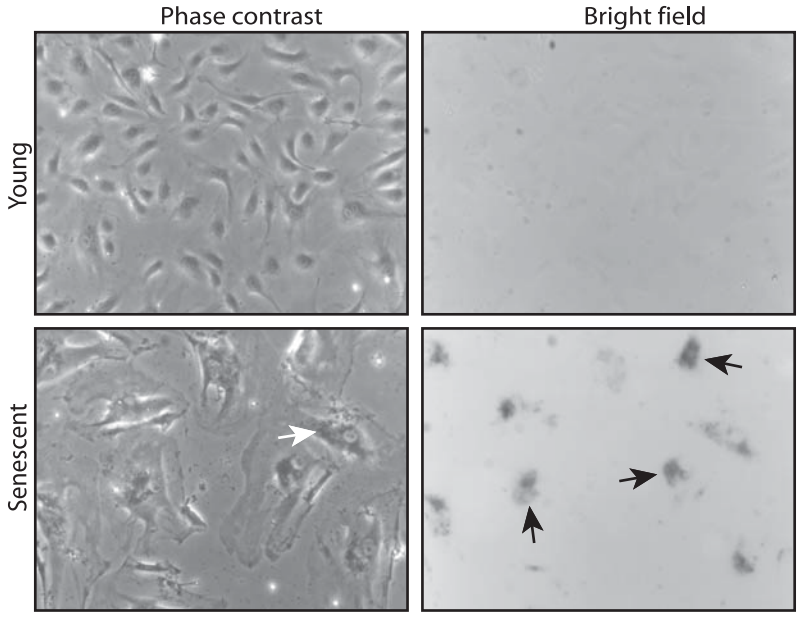

Figure 1: Apparent changes in cell morphology and an increase of β-galactosidase activity in senescent HUVEC cells. The morphology of late passage HUVEC cells is clearly enlarged and flattened with increased granularity (white arrow) compared to early passage cells (phase contrast). Furthermore, the late passage cell population reveals a clear increase in senescence-associated β-galactosidase assay positive cells (black arrows).

One feature of senescent cells described in literature is the expression of p21(Cip) with increasing passages (Antropova et al., 2002; Chen et al., 2006; Vaziri and Benchimol, 1996). This increased expression was also found in cell lysates of late passage cells in our senescent HUVEC model, which further confirmed induction of senescence (Figure 3A).

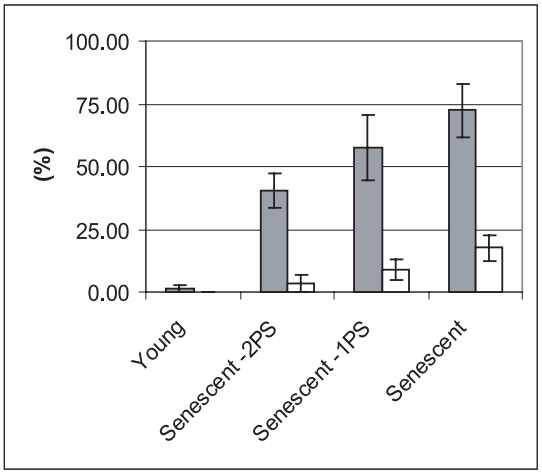

Figure 2: There is an increased prevalence in the late passage HUVECs (two passages prior to senescence (-PS and -2PS) and the senescent population) of β-galactosidase positive cells (grey bars), followed by an increased prevalence of cells that stain negative for endoglin (white bars). HUVECs were labeled with the senescence-associated β-galactosidase assay, followed by IF with a monoclonal anti-endoglin antibody. The percentage of β-galactosidase positive and endoglin negative cells were determined in five randomly chosen microscopic fields.

Changes in expression pattern of ER-resident proteins in senescent HUVECs

To evaluate whether ER functionality is affected upon replicative senescence, the expression of several ER-resident proteins was evaluated with increasing passages. Cell lysates from early passage and the last three passages of cultured HUVECs were evaluated on Western blot with an anti-KDEL antibody (Klooster et al., (Submitted)-b) that recognized the proteins endoplasmin, glucose regulated protein 78kDa (GRP78), calreticulin, PDI A1, PDI A6, ERp46, collagen binding protein 2 and thioredoxin domain-containing protein 4 (Figure 3B). A clear difference in expression pattern was observed between the early passage and senescent cell population (Figure 3B). The expression of the protein bands corresponding to endoplasmin and collagen binding protein2/thioredoxin domain-containing protein 4 appeared to decrease upon induction of replicative senescence. In contrast, the expression of calreticulin and especially PDI A1 appeared to increase, while the expression of the remaining detected ER-resident proteins remained relatively constant. These expression differences were also observed with two additional HUVEC isolations (data not shown), although some exceptions were observed. PDI A1 was not up-regulated in one of the HUVEC isolations, which was linked to a decreased expression of the protein band corresponding to the proteins PDI A6 and ERp46.

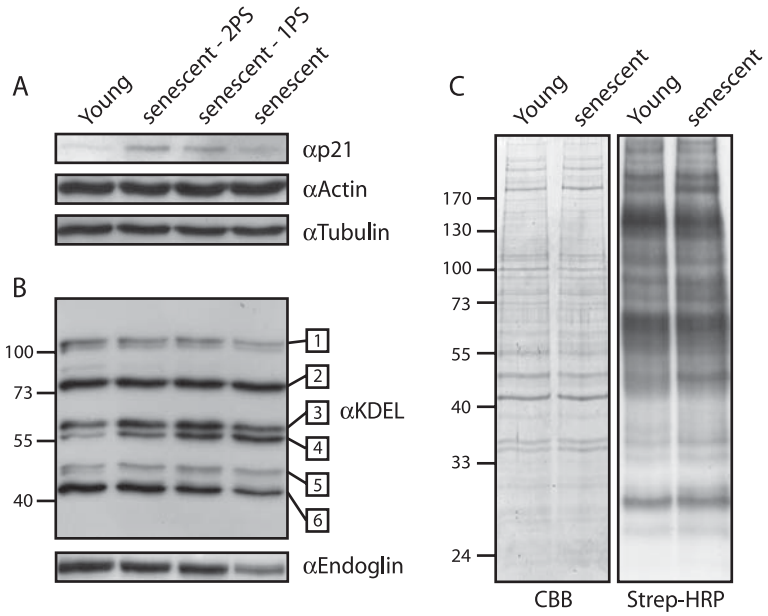

Figure 3: Upon induction of replicative senescence, HUVEC cells revealed differential expression of several intracellular and membrane bound proteins. Cell lysates were obtained from early passage and the last three passages of isolated HUVECs and subsequently probed with protein specific antibodies on Western blot. (A) The up-regulation of p21(Cip) with increasing passages, which is described in literature, confirmed induction of replicative senescence. Detection of tubulin and actin confirmed that equal amounts of protein were loaded. (B) The expression of several ER-resident proteins detected with an anti-KDEL antibody changed with increasing passages. The protein bands corresponding to endoplasmin (1) and collagen binding protein2/thioredoxin domain-containing protein4 (6) showed an apparent decrease in expression, while the protein bands corresponding to the proteins GRP78 (2) and PDI A6/ ERp46 (5) appeared to remain relatively constant. In contrast, the expression of calreticulin (3) and especially PDI A1 (4) appeared to increase. Detection of endoglin expression showed a clear decrease in the senescent cell population, while in the passages prior to senescence there is no apparent decrease in expression compared to the early passage cells. (C) Labeling of membrane bound proteins with biotin and subsequent analysis on Western blot with HRP conjugated streptavidin revealed that the cell morphological changes of senescent cells induced no apparent ratio difference between total (CBB) and surface protein (Strep-HRP) levels.

Decreased endoglin expression in senescent HUVECs

To evaluate whether the loss of proliferative ability of senescent cells correlated to the expression of endoglin, a membrane protein needed for cell proliferation, cell lysates of HUVECs cells were evaluated on Western blot with an endoglin specific antibody (Figure 3B). This revealed that endoglin expression was clearly decreased in the senescent HUVEC culture compared to

the early passage cells, while almost no difference was found in the two passages before senescence. As cell morphology changes during replicative senescence, the ratio between membrane and intracellular protein levels might change, resulting in the observed expression differences on Western blot. Therefore, surface proteins of young and senescent HUVEC cells were biotinylated and subsequently detected on Western blot to evaluate whether the ratio between surface and total protein levels changed or were constant (Figure 3C). No apparent differences in signal intensities were observed, indicating that the decreased expression of endoglin in the senescent population was not caused by cell morphological changes. These same results were obtained with two additional HUVEC isolations (data not shown).

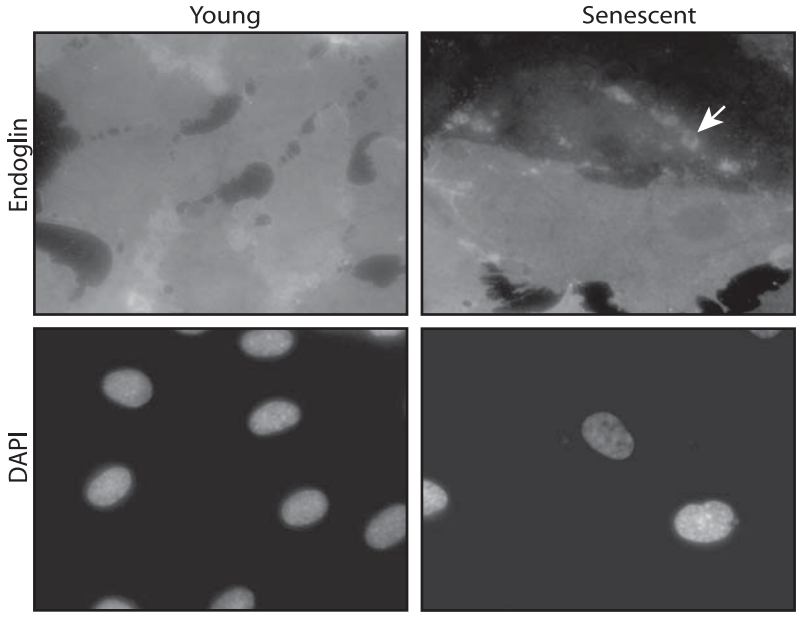

Figure 4: Decreased endoglin labeling in part of the senescent HUVEC population. Cultured cells were labeled with DAPI to visualize the nuclei and a monoclonal anti-endoglin antibody. While all young cells revealed an even and equal membrane staining, there was a clear decreased expression in some of the late passage cells. In some of the endoglin negative cells clear aggregate-like structures were observed that revealed autofluorescence (arrow).

Heterogenous endoglin expression in the senescent HUVEC population

Immuno fluorescence (IF) experiments were performed, to evaluate whether all or part of the HUVECs in the senescent population showed a decrease in endoglin expression. This analysis clearly showed an even and equal membrane staining of endoglin in all early passage cells, while in the late

passage cells the membrane staining was very heterogenous (Figure 4). A large portion of the late passage cells showed an expression that was comparable to the young cells, whereas others showed an apparent decrease, or no detectable endoglin expression. The appearance of these cells increased significantly in the last three passages (Figure 2).

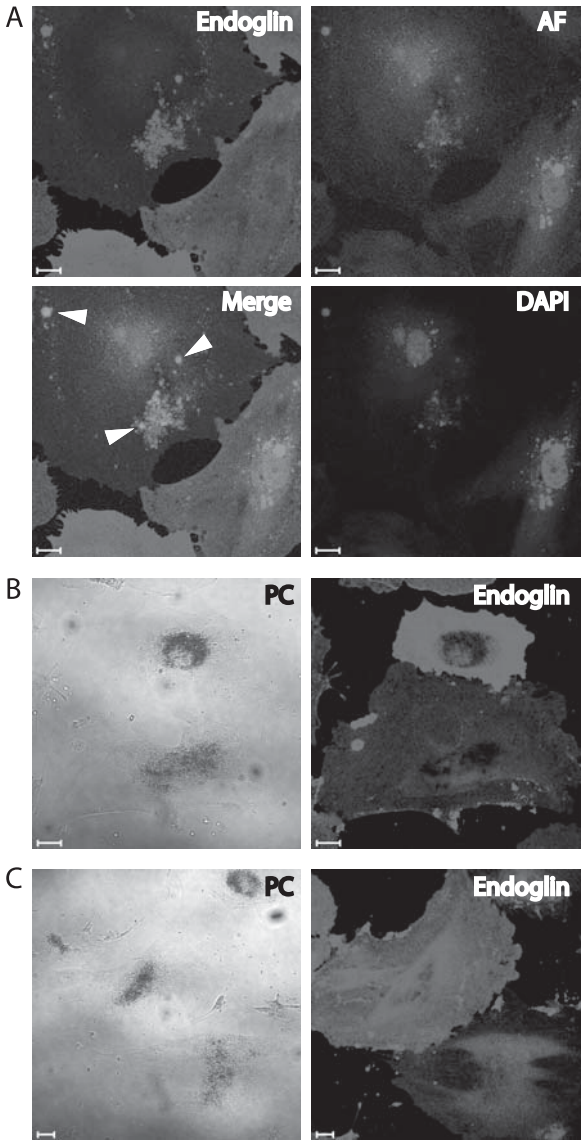

Figure 5: Heterogenous endoglin labeling in senescent HUVECs. HUVECs cells were stained with the β-galactosidase activity assay kit and subsequently labeled with a monoclonal anti-endoglin antibody. Several endoglin negative cells revealed clear aggregate-like structures that showed auto-fluorescence (AF). This suggested that these structures were protein aggregates containing lipofuscin (Merge, white arrowheads). The extent of aggregate formation differed per cell (A). Phase contrast pictures (PC) revealed that several β-galactosidase positive cells were also positive when labeled with the monoclonal anti-endoglin antibody. However, all endoglin negative cells stained positive in the senescence-associated β-galactosidase assay (Phase contrast). Even cells with reduced levels of endoglin were β-galactosidase positive (B, C). Bar represents 20μm.

Intriguingly, in part of the late passage cells staining was observed in aggregate like structures (Figure 4). These structures revealed auto-fluorescence suggesting that these were protein aggregates containing

lipofuscin (Figure 5A), which are known to accumulate in post-mitotic and aging cells (Seehafer and Pearce, 2006; Terman and Brunk, 2004).

Detection combining the senescence-associated β-galactosidase assay and the monoclonal anti-endoglin antibody revealed that several β-galactosidase positive cells did not show a decreased expression of endoglin (Figure 5B). However, all HUVEC cells with a decreased expression or a negative staining pattern for endoglin were positive in the senescence-associated β-galactosidase assay (Figure 5B and 5C).

Discussion

Here, we show that induction of replicative senescence in HUVECs is associated with changes in expression of several ER-resident proteins and with a decreased expression of the transmembrane protein endoglin.

Endoglin, a TGF-β type III receptor, fulfills a regulating role in TGF-β signal transduction. Endoglin is a negative regulator of TGF-β/ALK5 signaling. This pathway is a negative regulator of EC migration and angiogenesis via induction of plasminogen activator inhibitor type 1 (PAI-1) expression (Stefansson and Lawrence, 1996; Stefansson et al., 2001). In addition, endoglin is a positive regulator of TGF-β/ALK1 signaling, which promotes EC proliferation and migration. Thus, endoglin is thought to be a modulator of the balance between the ALK1 and ALK5 signaling pathways and consequently endoglin protein levels determine the growth capacity of ECs (Lebrin et al., 2005).

The observation that the majority of the cells in the senescent population were still endoglin positive (Figure 4) suggests that the decreased expression of endoglin is a secondary effect during replicative senescence induction. This is further supported by the increased expression of p21(Cip), which is already observed in the two passages prior to senescence (Figure 1A). However, the differential endoglin expression could have an enormous impact on endothelial functioning. As it regulates ALK1 and ALK5 signaling pathways, expression of the fibrinolysis inhibitor PAI-1 (Stefansson and Lawrence, 1996; Stefansson et al., 2001) and the tight junction component claudin 5 (Watabe et al., 2003) might be affected by this decreased endoglin expression. An increased expression of PAI-1 *in vivo* could induce a local more pro-coagulant environment, which could lead to an increased risk of developing a blockage of the vasculature. Furthermore, the disturbed regulation of claudin 5 could affect the barrier function of the endothelial cell, causing enhanced exchange of blood components, such as LDL, with the rest of the vascular wall, which could increase the chance of developing atherosclerosis.

In addition, the expression of several ER-resident proteins was changed with increasing passages. These expression differences did not resemble the expression differences observed during acute ER stress, when the UPR is activated, e.g. up-regulation of GRP78 and endoplasmin (Klooster et al., (Submitted)-b). However, the changed expression pattern does indicate that the functionality of the ER changes upon induction of senescence. Especially

the increased expression of PDI A1 is intriguing, as increased expression was already observed two passages prior to senescence, while the changes in expression of the other detected ER-resident proteins was, for the most extent, visible only in the senescent population. As PDI A1 is involved in proper disulphide bond formation, this increased expression indicates that with increasing passages the capacity for proper disulphide bond formation is increased, which might indicate that this process is disturbed in senescent cells.
As with the decreased expression of endoglin, the precise mechanisms that cause these changes in ER-resident protein expression are unclear, as protein expression is controlled on several levels and could therefore be caused by changes in gene expression, RNA processing, translational control and protein degradation. Consequently, to understand the processes that contribute to induction of senescence, identification of both gene and protein expression differences are essential.

Although the differential expression of several ER-resident proteins was observed in three different HUVEC isolations, exceptions were observed. These differences could be caused by differences in growth conditions, but also by the different genetic makeup of the donors. The genetic makeup of an organism ultimately determines the efficiency to adapt to different environmental conditions, e.g. some individuals are better equipped than others to withstand oxidative stress. This heterogeneity is also illustrated by the huge variations in maximal passage number between different HUVEC isolations, which can vary from 10 to almost 25 passages. As senescence can be induced by different mechanisms, variation in differential protein expression between different isolations might depend on which mechanisms are primarily involved in senescence induction. Furthermore, heterogeneity is also observed in the senescent HUVEC culture itself, illustrated by the differences in cell morphology, β-galactosidase staining, amount of aggregate formation and endoglin labeling between cells in a single senescent HUVEC culture. These differences are most likely caused by the random and multi-factorial nature of the mechanisms that cause replicative senescence. The heterogeneity illustrates one of the challenges that are faced upon analysis of EC senescence or aging phenomena per definition. Recognition of this

challenge underscores the need for mapping the different RNA and protein expression differences in biological samples of different individuals.
However, this heterogeneity does pose us with an experimental sensitivity issue, as differential expression of specific proteins caused by senescence in one cell is diluted in a pool of cells that might reveal different expression patterns. This could explain the small protein expression differences found in a proteomics study that compared protein expression levels in young and senescent HUVECs (Eman et al., 2006). Therefore, if we really want to understand the changes and consequences of cellular senescence, analysis of single cell expression levels with regard to time and localization will be crucial. In addition, potential senescence membrane markers, such as endoglin, could be exploited to fractionate senescent cells in populations that have similar expression profiles.

Acknowledgements

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Chapter 6

General discussion

General discussion

The initiation and progression of the aging process depends both on the damaging effects of internal and external environmental conditions and on the efficiency of an organism to neutralize the imposed damage. The random and multi-factorial nature of the mechanisms that are involved in the aging process results in large variations in changes on the molecular and cellular level, even in genetically identical populations (Kirkwood et al., 2005). To understand the interactions and possible synergistic mechanisms of the aging process, and identify the processes that are involved in the early and late stages of aging, the differences that occur in time, so called aging biomarkers, need to be identified. As it is unlikely that one biomarker offers enough information to obtain a high level of specificity and sensitivity, multiple markers are needed (Anderson, 2005). These biomarkers of aging could be used to monitor the effects of aging intervention studies or to determine the biological over the chronological age of an individual.

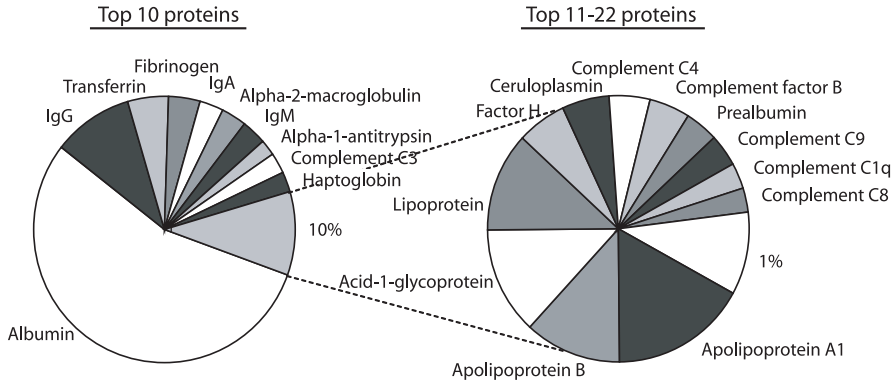

Figure 1: Twenty-two abundant proteins together constitute 99% of the total protein content of blood plasma. Graph depicts the top 10 human plasma proteins that constitute 90% of the total protein content of plasma, followed by the top 11-22 that constitute 9% of the total protein content of plasma. Removal of these proteins would allow the differential analysis of the remaining 1% of the plasma proteins.

Proteomics techniques that have been developed and improved in recent years, allows the simultaneous identification of multiple changes that occur on the protein level. In spite of these efforts, such analyses are still hampered by some unfavorable characteristics of proteins in research. The application of antibodies or their derivatives could provide solutions for some of these challenges. Some favorable characteristics of VHH antibody fragments (Dolk et

al., 2005b; Frenken et al., 2000; Muyldermans, 2001; van der Linden et al., 1999) make them highly suitable for application in this field, especially in combination with phage display technology, which allows selection of VHHs with predefined characteristics (Dolk et al., 2005a; Verheesen et al., 2003), even in an automated setup.

In this thesis, the development of VHH based proteomics techniques is described for identification of protein expression differences in biological samples related to aging.
Studies started with the selection of VHHs against the highly abundant bulk proteins HSA and IgG in human serum. The subsequent application of these antibodies to deplete these proteins from pooled plasma samples of young and old individuals and the comparison of the depleted plasma samples with 2D-DIGE technology resulted in the identification of several differentially expressed proteins. These results indicated an increased pro-coagulant and pro-inflammatory state with increasing age and further suggested an increase in tissue damage with aging. Even though this is in line with previously described data (Aillaud et al., 1986; Ernst and Resch, 1993; Ishikawa et al., 1998; Mackness et al., 1997; Ritchie et al., 1998; Ritchie et al., 1999; Ritchie et al., 2000; Ritchie et al., 2004a; Tofler et al., 2005; Trougakos and Gonos, 2002; Witte et al., 1993), these data do not represent the full nature of the differences that occur in human blood upon aging. Most of the identified differentially expressed proteins are members of the top 22 abundant proteins present in blood, which together represent 99% of the total protein content of plasma (Figure 1). Therefore, data on the differential expression of proteins that are less abundant are missing. This illustrates the limitations of present 2D-technology. Although the reproducibility is increased by the introduction of 2D-DIGE technology and more reliable results are obtained, the sensitivity of this technique is still an issue of concern. Especially the analysis of the plasma proteome offers great challenges in this respect, as it is estimated that the dynamic range, the ratio between the highest and lowest abundant protein, of the plasma proteome is at least greater than 10^{10}. This number is simply based on concentrations of the highest and lowest abundant plasma proteins presently known, such as HSA (~50mg/ml) and the cytokine IL-6 (~1-5pg/ml) respectively (Anderson, 2005). With 2D-technology, protein spots can be

detected with a maximal dynamic range of 10^2-10^3 (Patton, 2002). The sensitivity of spot identification is even lower.

For cellular proteomes this situation is less extreme, with the most abundant proteins only constituting around 5% of the total protein content as is described for yeast (Futcher et al., 1999). However, irrespective of the dynamic range, the sheer number of different proteins complicates the analysis of any proteome, considering the total number of genes, differential RNA splicing, differential protein translation initiation and numerous different post-translational modifications (Anderson, 2005). Among other things, such as the heterogeneity in an aging population as discussed in chapter 5, this greatly limits the analysis of the full spectrum of protein changes that occur during aging.

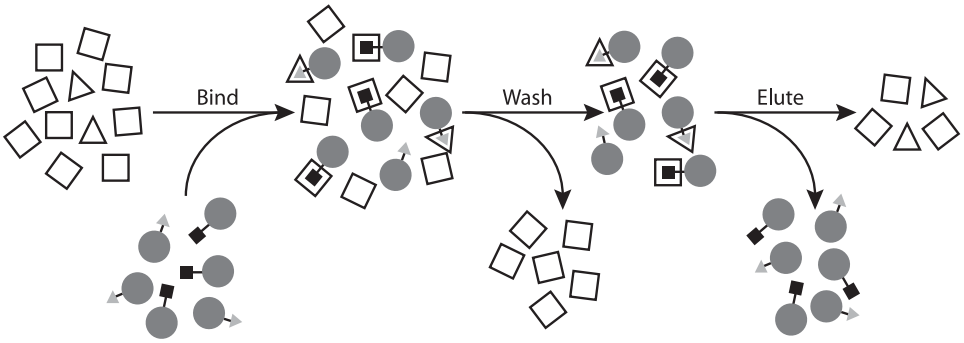

Figure 2: Schematic representation of protein equalizer beads (Ciphergen). Random peptide libraries are immobilized on beads, which are added to a biological sample containing high (square) and low abundant proteins (triangle). In theory, an equal amount of each peptide is present in this library. Depending on the amount of beads used, an under-capacity of peptides, which bind to the high abundant proteins, and an over-capacity of peptides, which bind to the low abundant proteins can be obtained. The high abundant proteins that are not bound will be washed away, thereby concentrating the low abundant proteins. Ultimately, the ratio between the amount of beads used and the sample volume determines the concentration of the low abundant over the high abundant proteins.

In proteomics research, this recognition has led to further development of affinity chromatography based techniques that reduce the complexity of proteomes and enable the analysis of low abundant proteins. For the plasma proteome, one of these strategies involves an extension to the in this thesis described depletion of the high abundant proteins HSA and IgG. At present, techniques are available that enable the depletion of the 22 most abundant proteins in human plasma (Schuchard et al., 2005) (Figure 1), which offers

the potential analysis of the remaining 1% total protein content in plasma. Furthermore, techniques have been developed for the enrichment of minor abundant proteins, such as the use of protein equalizer beads (Righetti et al., 2005) (Figure 2). This approach is based on random peptide libraries in which each peptide has a certain affinity for a specific protein. In theory, this library contains comparable amounts of each peptide. Consequently, depending on the amount of beads used, low abundant proteins can be enriched while the bulk of high abundant proteins will be lost during the washing steps (Figure 2). Both approaches would be beneficial in respect to the enormous dynamic range found in the plasma proteome. However, the beneficial aspects of these approaches have to be verified in proteomics studies that are aimed at identification of protein expression differences, such as the reproducibility of these techniques. Furthermore, large amounts of biological samples are needed to analyze the minor abundant proteins.

Fractionation or analyzing a confined set of proteins is another popular approach for reducing the complexity of a proteome. This can be achieved by separating proteins based on their charge, hydrophobicity, solubilization and size (Fountoulakis et al., 1999; Pieper et al., 2003a; Pieper et al., 2003b; Rothemund et al., 2003), or by fractionation of cellular organelles (Snape et al., 1990). In addition, fractionation can also be achieved by using antibodies that recognize conserved amino acid epitopes, as described for the KDEL sequence in chapter 4, or that recognize post-translational modifications, as has been shown for phosphorylation (Gronborg et al., 2002; Maguire et al., 2002). However, the separation potential of these techniques often lacks in specificity as several proteins are usually found in multiple fractions and because of unwanted protein-protein interactions during sample preparation. This hampers the interpretation and reduces the reproducibility of these techniques.

Most of the above described techniques are usually combined with 2D-gelelectrophoresis and/or mass spectrometry. This enables the discovery of novel biologically relevant target proteins. However, because of the labor-intensive protocols, the use of these techniques seems less suitable for use in clinical laboratories. Furthermore, it should be noted that results obtained with all of these proteomics studies should be verified with an additional technique as observed differences can be caused by the variety of different steps that are applied during sample preparation. Furthermore, sample handling is

another issue that should be treated with care as it has been shown that prolonged storage of samples may result in the modification of some proteins, which can lead to misleading results and conclusions (Franzen et al., 1993). This further necessitates the confirmation of the obtained results in additional experiments with preferably fresh biological samples.

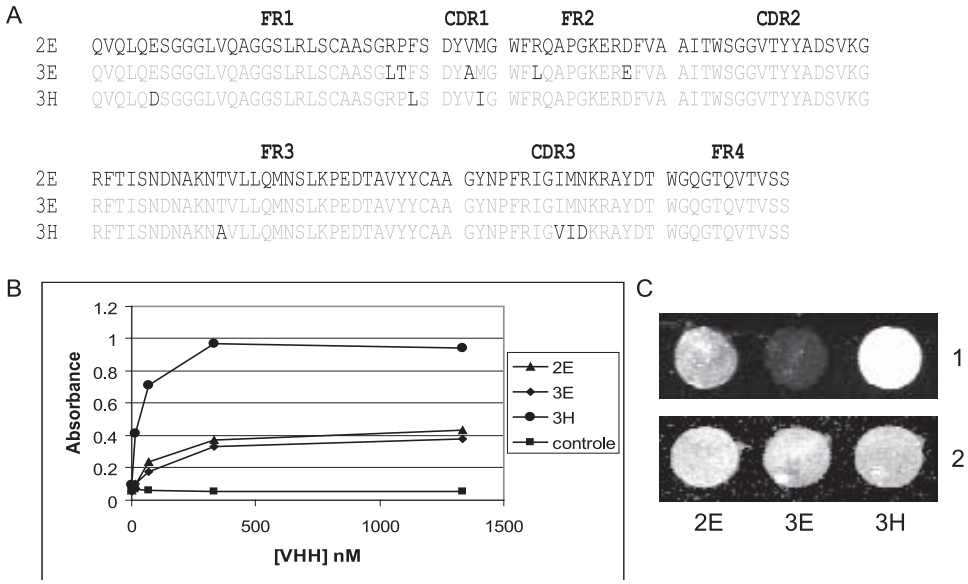

Figure 3: Non-specific covalent immobilization of antibody fragments can decrease antibody activity. (A) Three VHH antibody fragments, clone 2E, 3E and 3H, selected against the same antigen revealed highly homologues amino acid sequences. (B) Differences in binding characteristics were revealed in an ELISA with 500ng/ml coated antigen, detected with different concentrations of purified VHH. Clone 2E and 3E showed similar binding characteristics, indicating comparable binding affinities, while clone 3H showed superior binding characteristics over the other clones, indicating a higher binding affinity. These differences in binding characteristics are most likely caused by differences in the CDR regions, especially CDR3. (C) Upon non-specific covalent immobilization on an aldehyde glass-slide and subsequent incubation with Cy3 labeled antigen, the highest signals were obtained with clone 3H (panel 1), which was in agreement with the ELISA. In contrast to the results obtained with ELISA, clone 3E revealed significantly lower signals compared to clone 2E when immobilized on the aldehyde glass-slides, while similar amounts of VHH were immobilized (panel 2). These differences were therefore most likely caused by differences in preferential coupling of specific amino acid residues between clone 2E and 3E.

A proteomics technique that could enable the expression analysis of low abundant proteins, without high abundant protein depletion or sample fractionation, is the application of antibody micro-arrays, in which antibodies

are immobilized on a surface, each in a separate spot, to capture their respective antigen (Haab et al., 2001). Furthermore, antibody micro-arrays offer several additional advantages, such as the small amount of biological sample needed, and the fully automated manner in which these experiments can be performed, allowing the analysis of large numbers of samples.

Although this technique provides the simultaneous analysis of several target proteins with high sensitivity, several obstacles still have to be overcome. This approach requires the generation of numerous monoclonal antibodies that have to be tested extensively on antigen specificity and sensitivity, which makes it a far from straight forward approach. To enable the generation of such antibodies, fully automated selection and screening protocols are required, which cannot be realized for hybridoma technology as it depends on animal immunization and cell culture maintenance. The application of recombinant antibody fragments in combination with phage display technology could offer a solution for generation of a wide variety of antibodies with different specificities (Amstutz et al., 2001). However, the issue of specificity and sensitivity of the selected antibodies is still a matter of concern.

In addition to antibody generation, antibody activity has to be maintained after immobilization on the array surface. A large portion of classical antibodies has been shown to lose their functionality upon immobilization (Haab et al., 2001), which can also occur with recombinant antibodies. VHHs with highly homologues amino acid sequences can reveal large differences in antigen binding when covalently immobilized on an array surface (Figure 3). This is most likely caused by the preferential coupling of specific amino acid residues in or close to the binding domain of the antibody. Improvement of antibody functionality can be obtained by specific immobilization via the C- or N-terminus of the antibody molecule. Especially recombinant antibodies are suitable for such an approach, as they can be genetically modified with specific tags fused to their C- or N-terminus designed for directed immobilization, which can significantly increase their activity (Figure 4). Ideally, a tag should be used that enables specific covalent immobilization on an array surface, which has low background characteristics, without losing antibody functionality.

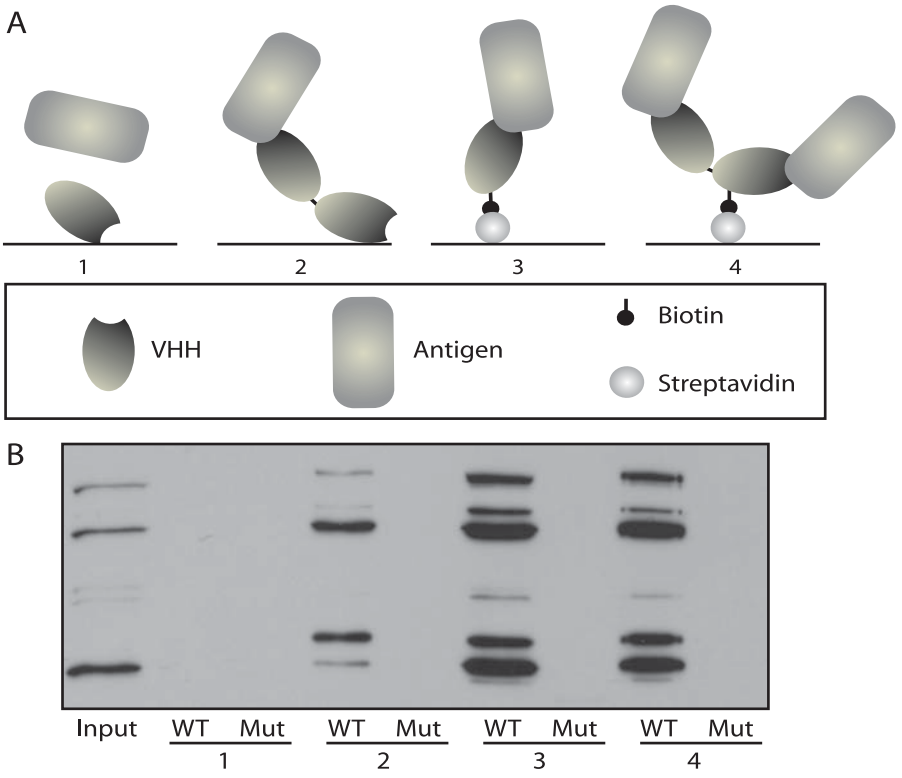

Figure 4: Directed immobilization of antibody fragments or application of antibody fragments with two binding domains can increase the binding activity significantly compared to non-specific covalently immobilized antibody fragments. (A) Schematic representation of non-specific covalent immobilization of monovalent (panel 1) and bivalent (panel 2) VHH antibody fragments, and directed immobilization on immobilized streptavidin via a biotin label attached to a biotinylation sequence at the C-terminus of monovalent (panel 3) and bivalent (panel 4) VHHs. (B) Immuno precipitation of ER-resident proteins from a HeLa cell lysate with the anti-KDEL VHH clone 5 (WT) and a binding-negative mutant of this antibody fragment. Non-specific covalent immobilization of the monovalent VHH on CNBr-sepharose did not result in antigen binding (1). Binding activity was improved upon use of bivalent VHHs (2). The best binding activity was observed upon immobilization of monovalent (3) or bivalent (4) VHHs to streptavidin via a biotin tag at the C-terminus.

Despite these challenges, the application of antibody micro-arrays seems to offer great potential for analyzing protein expression differences because of the high specificity and sensitivity that can be obtained. As it is quite unlikely that the first generation of antibody arrays will contain antibodies to recognize all the proteins from higher eukaryotic organisms, arrays should be constructed to study a predefined set of proteins in a biologically relevant

setting. Indeed, such arrays have been developed to study differences in expression of several cytokines (http://www.raybiotech.com).

Arrays containing antibodies directed against the potential aging biomarkers described in chapter 3 and 5 could be exploited to develop an antibody micro-array for aging research. Such an array could be used to study the effect of aging intervention studies or to determine the biological over the chronological age of an individual. This requires the selection of antibodies that specifically recognize unique epitopes on a specific isoform of a single gene product, which is one of the challenges that affinity ligand based proteomics is confronted with.

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Summary

Understanding the aging process

Research on both higher and lower eukaryotic model systems has revealed that numerous aspects are involved in the course of the aging process. During life, an organism is continuously challenged by environmental and endogenous factors. The severity of these factors and the effectiveness of the organism to counteract these challenges, ultimately determine the organism's lifespan. One of the damaging factors that has received much attention in aging research is reactive oxygen species (ROS) that can have both an internal and external cellular origin. A cell contains many defence mechanisms that attempt to neutralize these damaging agents. However, when this level of defence is insufficient or fails and damage is induced, a cell contains additional molecular mechanisms that attempt to repair or degrade the damaged cellular molecules.

Despite these molecular defence mechanisms, damage accumulates in time, which can affect cellular functioning. There are unique cellular mechanisms that are influenced by the molecular changes that occur in time. When a certain threshold of damage is reached, a cell can die or go into a state of irreversible cell cycle arrest, called senescence. Ultimately, these cellular mechanisms can hamper the regenerative capacity of tissues, and could therefore have an effect on organismal functioning.

Although strides have been made to understand the role of the above described molecular and cellular processes in the aging process, a general consensus has not been reached on the relative importance of each of them. Mapping the changes that occur in the course of the aging process, and thereby define so called biomarkers of aging, could provide the information necessary to understand the aging process and the contributions of the different mechanisms herein. This approach was started with genomics that provides information about genetic expression differences, and is now extending to the field of proteomics that provides information about protein expression and modification differences, which is the focus of this thesis.

Antibodies in proteomics

The large interest in proteomics in the last few years has led to the development of numerous high throughput assays and improvement of several existing techniques that enable the analysis of several proteins in a single experiment. Affinity ligands, such as antibodies, are a useful tool to be applied

in this field, as they can be used for purification and detection of proteins. The successful application of antibodies in proteomics requires reliable affinity molecules in respect to sensitivity as well as specificity. The traditional method of obtaining antibodies is by immunization of an animal with the target antigen, followed by the isolation of the required antibodies from the blood of the immunized animal. This results in either polyclonal or monoclonal antibodies. A polyclonal antibody obtained from the serum of the immunized animal is a pool of different antibodies, each with their own specificity and sensitivity for the target antigen. Because of the heterogeneity of polyclonal antibodies, they are usually less specific than monoclonal antibodies and are therefore less suitable for use in proteomics. Monoclonal antibodies are obtained by isolation of the B-cell repertoire of an immunized animal and subsequent screening of this repertoire for cells that produce antibodies with the required characteristics. Fusion of the obtained B-cells with myeloma cells gives rise to an immortal hybridoma cell line, which results in an indefinite source of antibody of which each has the same specificity and sensitivity for the respective antigen. This makes monoclonal antibodies highly suitable for use in proteomics. Conversely, generation of these antibodies is relatively expensive.

Application of recombinant antibodies offers a less expensive alternative as they allow the construction of highly diverse antibody display libraries from the B-cell repertoire of immunized or non-immunized animals. These libraries offer an *in vitro* selection system that can be used to obtain antibodies with predefined characteristics. As this process can be fully automated, production costs of a monoclonal antibody can be reduced significantly.

Recombinant antibodies derived from animals belonging to the species of *Camelidae* have additional advantages over other recombinant antibodies. Besides classical antibodies that contain two light and two heavy chains, these species possess antibodies that lack the light chain. As a result, the antigen-binding domain of these antibodies (VHH) consists of only one domain, which makes them easy to clone. Furthermore, they represent the smallest antigen binding domains derived from antibodies, they are more stable than conventional antibodies or their derivatives, and they can be produced efficiently in *Escherichia coli* and *Saccharomyces cerevisiae*.

Depletion of bulk proteins from human plasma

Changes that occur during human aging, such as increased cellular death and decreased tissue and organ functioning, could be reflected in the blood plasma protein composition. However, proteomic analysis of blood plasma is hampered by the presence of several highly abundant bulk proteins. In chapter 2 of this thesis, the selection of highly specific VHHs against the abundant plasma proteins human serum albumin (HSA) and immunoglobulin G (IgG) is described for affinity chromatography purposes. These affinity ligands were used to remove these abundant proteins from plasma. This removal resulted in the visualization of previously masked protein spots and an increased visibility of previously non-detectable protein spots on 2D-gel. Furthermore, the potential clinical application of the VHH based anti-IgG affinity ligand was demonstrated in a study using plasma of Good Pasture (GP) and Systemic Lupus Erythematosus (SLE) auto-immune disease patients. In SLE patients, an auto-immune disease with a prevalence of IgG3 subtype specific antibodies, the VHH based anti-IgG column performed better than a proteinA based affinity column. It was demonstrated that this was caused by the IgG subclass specificity of proteinA. Protein A was not able to deplete IgG3 from plasma, whereas the VHH based anti-IgG affinity ligand was able to deplete all four IgG subclasses.

Changes in protein expression and processing in plasma during aging

In chapter 3, the use of these affinity ligands, combined with two-dimensional difference gel electrophoresis (2D-DIGE), is shown in a human blood plasma proteomics study to reveal protein expression differences between young and old individuals. 89 protein spots were considered as differentially expressed during aging of which 48 were positively identified with mass spectrometry. The differential expression of several of the identified proteins was in line with data described by others, and suggested that upon aging a slightly increased pro-coagulant and pro-inflammatory state is induced. Furthermore, remarkable differential expression of protein isoforms, originating from single gene products, were detected upon aging, which demonstrated the importance of studying levels of protein isoforms next to total protein expression levels.

Among the differences, the differential expression of several complement C3 (CO3) fragments was the most striking, with the highest difference in the old population (ten times up-regulation). These results suggested that upon aging this important regulator of both the innate and the adaptive immune response

is activated. From literature, there are conflicting data concerning the expression levels of CO3 during aging, which were all obtained with immunological assays. CO3 levels were either reported to be unchanged or slightly increased during aging. As these assays are not suitable for detecting differences in protein fragments or post-translational modified forms of CO3, this further demonstrates the importance of analyzing levels of protein isoforms, as important information might be missed by analyzing only total protein expression levels.

Application of common epitope specific antibodies

Another strategy for using antibodies in proteomics is selecting antibodies that recognize epitopes present in several different proteins, so called common epitopes. Such an epitope would allow the use of a single antibody to visualize or purify several different proteins simultaneously. Common epitopes comprise post-translational modifications, such as ubiquitination and phosphorylation, but also conserved amino acid sequences that are used for specific cellular functions, such as cellular localization.

In chapter 4, the successful selection is described of VHHs that recognize a common epitope present on several endoplasmic reticulum (ER)-resident proteins. This C-terminal KDEL amino acid sequence determines the ER localization of these proteins. The application of the KDEL specific VHHs for Western blot analysis of cellular lysates, resulted in a band pattern of eight different proteins.

In an ER stress model, the value of these VHHs was demonstrated. Upon induction of ER stress with hydrogen peroxide, tunicamycin or dithiothreitol, clear upregulation of two ER-resident proteins was revealed, while the other proteins showed no, or a slight decrease in expression.

The existence of several other biological mechanisms that utilize conserved epitopes for a specific function, such as the nulear localization sequence (NLS) or the peroxisomal targeting sequence, could offer a target for similar selection experiments. This could offer a straightforward procedure to simultaneously analyze multiple protein expression differences in a biological sample.

Differential protein expression in senescent endothelial cells

Endothelial cells (EC) have an important barrier function on the interface of the blood and the rest of the vasculature. Damaged ECs are either replaced by division of surrounding endothelial cells or by bone marrow-derived endothelial progenitor cells (EPC) that circulate in the blood. As many other cell types, ECs cannot divide indefinitely and eventually they will enter a state of irreversible growth arrest called senescence. Although these senescent cells are viable and metabolically active, they have altered gene expression, which is reflected by altered protein expression. As these changes suggest altered cellular functioning, accumulation of these cells at specific sites could lead to loss of endothelial function and consequently a disturbed barrier function. This could sensitize these sites for development of pathological processes, such as atherosclerosis. However, the significance of this phenomenon *in vivo* still remains a matter of debate. Mapping the differences that occur upon induction of senescence is needed to further unravel the mechanisms and consequences of EC senescence.

In chapter 5, the expression of several different proteins is analyzed upon induction of senescence based on two hypotheses. (i) During aging, an increase in oxidative stress is observed. As the ER seems especially vulnerable for oxidative stress and expression of ER-resident proteins has been shown to occur during such an event, changes in expression of these proteins can be expected. With one of the VHH anti-KDEL antibodies, the expression of several ER-resident proteins was analyzed. Intriguingly, clear changes were observed. However, these changes did not resemble the expression differences observed during severe ER stress in chapter 4. They do suggest that ER functionality changes upon induction of senescence.

(ii) As senescent cells lose the ability to replicate, the expression of proteins involved in the control of endothelial cell proliferation might change. Therefore, the expression of the membrane protein endoglin, which is needed for cell proliferation, was analyzed during induction of senescence. The expression was clearly decreased in the senescent population, although this decreased expression was only observed in part of the cells in the senescent population. This decreased expression could have an enormous impact on endothelial functioning, as endoglin regulates the expression of the fibrinolysis inhibitor plasminogen activator inhibitor type 1 (PAI-I) and the tight junction component claudin 5.

These data clearly show that during replicative senescence changes occur at the protein level that could influence cellular and consequently tissue functionality, which could provide an environment for the development of age-associated diseases like atherosclerosis.

The future of affinity ligands in proteomics

Although differences in protein expression have been described in this thesis, these data do not represent the full nature of the differences that occur during aging. Most of the identified differentially expressed proteins in chapter 3 are members of the top 22 abundant proteins present in blood. Therefore, data on the differential expression of proteins that are less abundant are missing. In the proteomics field, this recognition has led to further development of affinity chromatography based techniques that reduce the complexity of proteomes and enable the analysis of low abundant proteins. Strategies are, the removal of high abundant proteins, the enrichment of minor abundant proteins or fractionation of protein samples based on charge, hydrophobicity, solubilization or size of the proteins. However, it should be noted that results obtained with all of these proteomic studies, should be verified with an additional technique, as observed differences can be caused by the variety of different steps that are applied during sample preparation. Furthermore, sample handling is another issue that should be treated with care as it has been shown that prolonged storage of samples may result in modification of some proteins, which can lead to misleading results. This further necessitates the confirmation of the obtained results in additional experiments with preferably fresh biological samples.

A proteomics technique that could enable the expression analysis of low abundant proteins without the need for high abundant protein depletion or sample fractionation, is the application of antibody micro-arrays. In antibody micro-arrays antibodies are immobilized on a surface, each antibody in a separate spot, to capture their respective antigen.

However, there are several obstacles that still have to be overcome. This approach requires the generation of numerous monoclonal antibodies that have to be tested extensively on antigen specificity and sensitivity, which makes it a far from straightforward approach. Furthermore, antibody activity has to be maintained after immobilization on the array surface.

Despite these challenges, the application of antibody micro-arrays offers great potentials for analyzing protein expression differences because of the high specificity and sensitivity that can be obtained. As it is unlikely that the first generation of antibody arrays will contain antibodies that recognize all the proteins from higher eukaryotic organisms, arrays should be constructed to study a predefined set of proteins in a biologically relevant setting. Arrays containing antibodies directed against some of the proteins described in chapter 3 and 5, could be exploited to develop an antibody micro-array for aging research. However, this would require the selection of antibodies that specifically recognize unique epitopes on a specific isoform of a single gene product, which is one of the challenges that affinity ligand based proteomics is confronted with.

Samenvatting

Het begrijpen van het verouderingsproces

Onderzoek aan zowel hoge als lage eukaryotische modelsystemen heeft laten zien dat verschillende processen bij het verouderingsproces betrokken zijn. Gedurende het leven wordt een organisme continu blootgesteld aan schadelijke omgevings- en endogene factoren. De ernst van deze schadelijke factoren en de effectiviteit waarmee een organisme hiertegen kan optreden bepalen uiteindelijk de levensduur van het organisme. Een van de meest onderzochte schadelijke factoren die een rol spelen tijdens veroudering zijn reactieve zuurstof radicalen, die zowel een interne als externe cellulaire oorsprong kunnen hebben. Een cel bevat verschillende verdedigingsmechanismen, die deze schadelijke factoren proberen te neutraliseren. Wanneer dit niet afdoende gebeurt, worden er cellulaire moleculen beschadigd, wat een negatieve invloed kan hebben op het functioneren van de cel. Gelukkig bevat een cel ook mechanismen die de beschadigde moleculen kunnen herstellen of opruimen om zo het evenwicht in de cel weer te herstellen. Ondanks deze verdedigingsmechanismen neemt het aantal beschadigde cellulaire moleculen in de tijd toe. Aangezien dit het normaal functioneren van de cel onder druk zet, bevat een cel ook cellulaire mechanismen die in werking treden als een bepaalde drempelwaarde van schade is bereikt. Een cel kan dan doodgaan of in een onomkeerbare staat terechtkomen waarbij deling van de cel niet meer mogelijk is. Uiteindelijk kan dit leiden tot een verlies van functionele cellen en een gebrek aan regeneratieve capaciteit van weefsel, wat ook het functioneren van het organisme negatief kan beïnvloeden.

Ondanks het feit dat al deze moleculaire en cellulaire processen een rol lijken te spelen bij veroudering, is er nog geen overeenstemming bereikt over het belang van elk van deze processen hierbij. Het in kaart brengen van de veranderingen die ontstaan gedurende veroudering, zogenaamde biomarkers van veroudering, kunnen belangrijk zijn voor het begrijpen van het verouderingsproces en de mate waarin de verschillende processen hierin een rol spelen. Dit proces werd gestart met genomics, dat informatie geeft over verschillen in gen-expressie, en is nu uitgebreid met proteomics, dat informatie geeft over de expressie en modificatie van de uiteindelijke genproducten, de eiwitten. Het ontwikkelen van nieuwe proteomicstechnieken en het identificeren van eiwitexpressieverschillen tijdens veroudering vormen de focus van dit proefschrift.

Antilichamen in proteomics

De grote interesse in proteomics in de laatste jaren heeft geleid tot de ontwikkeling van verschillende 'high throughput'-assays en verbetering van bestaande technieken die het analyseren van eiwitexpressieverschillen in een enkel experiment mogelijk maken. Moleculen die eiwitten kunnen binden, zogenaamde affiniteitsliganden, zoals antilichamen, bieden een goede mogelijkheid voor toepassing in dit onderzoeksveld, aangezien ze gebruikt kunnen worden voor het zuiveren en aantonen van eiwitten. Voor een succesvol gebruik van deze antilichamen moet men er zeker van zijn dat het antilichaam een hoge affiniteit heeft voor het eiwit dat hij bindt en dat het antilichaam geen andere eiwitten bindt (dus specifiek is). De traditionele methode voor het verkrijgen van antilichamen is door dieren te immuniseren met het targeteiwit, gevolgd door het isoleren van de geproduceerde antilichamen uit het bloed van het geïmmuniseerde dier. Dit kan resulteren in polyklonale of monoklonale antilichamen. Polyclonale antilichamen zijn vaak niet zo specifiek als monoklonale antilichamen en zijn daardoor minder geschikt voor het gebruik in proteomics. Monoclonale antilichamen zijn daar vaak wel geschikt voor, maar zijn relatief duur om te maken.

Het gebruik van recombinante antilichamen biedt hiervoor een goedkoop alternatief. Dit zijn vaak alleen de eiwitbindende delen van een normaal antilichaam. Met behulp van een ingewikkelde selectietechniek kunnen recombinante antilichamen worden verkregen met vooraf bepaalde eigenschappen. Doordat dit proces geautomatiseerd kan worden, kunnen de productiekosten extreem worden verlaagd.

Recombinante antilichamen verkregen van de soort *Camelidae* (VHH) hebben additionele voordelen. Deze zijn stabieler dan andere antilichamen, kunnen makkelijker worden geproduceerd en ze zijn de kleinste antilichaamfragmenten die kunnen worden verkregen uit antilichamen. Het gebruik van deze antilichamen voor de ontwikkeling van proteomicstechnieken staat beschreven in dit proefschrift.

Verwijderen van bulkeiwitten uit humaan plasma

Veranderingen die optreden tijdens veroudering, zoals verhoogde celdood en verlaagd functioneren van weefsel en organen, kunnen leiden tot veranderingen in de expressie van eiwitten in bloed. Het analyseren van deze verschillen wordt bemoeilijkt door het bestaan van verschillende veel

voorkomende eiwitten in het bloed. In hoofdstuk 2 van dit proefschrift wordt beschreven hoe VHHs worden verkregen, die de bulkeiwitten HSA en IgG kunnen binden. Deze VHHs zijn vervolgens gebruikt om deze bulkeiwitten uit bloed te verwijderen, wat een betere analyse van de minder voorkomende eiwitten mogelijk maakt. Verder is aangetoond dat deze VHHs ook gebruikt zouden kunnen worden voor klinische toepassingen als beter alternatief voor bestaande technieken bij de behandeling van auto-immuunpatienten.

Eiwitexpressie veranderingen in bloedplasma gedurende veroudering

In hoofdstuk 3 wordt met behulp van de hierboven beschreven VHHs en een geavanceerde proteomicstechniek (2D-gelelectroforese) gezocht naar verschillen in expressie van bloedeiwitten tussen jonge en oude mensen. Uiteindelijk zijn er verschillende eiwitten gevonden die een verandering in expressie lieten zien tussen de jonge en oude groep. Enkele van deze verschillen kwamen overeen met resultaten van anderen en suggereren dat veroudering gepaard gaat met een licht verhoogde pro-coagulante en pro-inflammatoire staat. Verder werden er van sommige eiwitten verschillende isovormen gevonden. De diversiteit in expressieverschillen van isovormen uit een enkel genproduct liet zien dat het belangrijk is om afzonderlijke isovormen van hetzelfde genproduct te bestuderen.
Verder werden er vooral verschillen gevonden in de expressie van delen van het eiwit complement C3 (CO3). Dit eiwit is betrokken bij de activatie van de immuunrespons. De verhoogde aanwezigheid van enkele fragmenten van dit eiwit in het bloed van de oude groep, suggereerde dat de activiteit van dit eiwit in oude mensen is verhoogd. Dit verschil is nog niet eerder aangetoond wat verklaard kan worden doordat de technieken die door anderen zijn gebruikt, niet geschikt zijn voor het analyseren van eiwitfragmenten die zijn ontstaan uit een enkel genproduct.

Het gebruik van antilichamen die meerdere eiwitten herkennen

Een monoklonaal antilichaam herkent een bepaalde plek op een eiwit, het epitoop. Het komt in de natuur geregeld voor dat een epitoop op verschillende eiwitten voorkomt. Een antilichaam dat zo'n epitoop kan binden, kan dus gebruikt worden voor het aantonen en zuiveren van meerdere verschillende eiwitten. In hoofdstuk 4 wordt het verkrijgen en testen van zo'n antilichaam beschreven. Dit antilichaam herkent een epitoop dat voorkomt op eiwitten die

zich bevinden in het endoplasmatisch reticulum (ER), een organel dat betrokken is bij de vouwing van eiwitten die worden gesecreteerd door de cel. In een cellulair model wordt met het gebruik van dit antilichaam aangetoond dat de expressie van verschillende van deze ER-residente eiwitten verandert als de cel wordt blootgesteld aan verschillende soorten stress. Dit soort antilichamen biedt de mogelijkheid om op een snelle manier de expressie van verschillende eiwitten tegelijk te analyseren.

Verschillen in expressie in senescent endotheelcellen

Endotheelcellen vormen de barrière tussen het bloed en het omliggende weefsel. Wanneer ze beschadigd worden, worden ze vervangen door deling van naburige cellen of door speciale cellen die circuleren in het bloed. Zoals vele andere cellen kunnen deze cellen niet ongelimiteerd delen. Op een zeker moment zullen ze in een onomkeerbare staat terecht komen waarbij celdeling niet meer mogelijk is. Dit heet senescence. Senescent cellen leven nog en zijn metabolisch actief, maar er treden wel veranderingen op in de expressie van hun genen. Aangezien dit een verandering van cellulaire functie suggereert, kan accumulatie van deze cellen leiden tot een verstoorde barrièrefunctie van het endotheelweefsel, wat het ontstaan van pathologische processen, zoals atherosclerosis, in de hand kan werken. Of dit proces ook daadwerkelijk een rol speelt in levend weefsel is nog steeds niet duidelijk. Het in kaart brengen van de verschillen die ontstaan tijdens het senescent worden van cellen, zou hierbij een oplossing kunnen bieden. In hoofdstuk 5 wordt gekeken naar de verschillen in eiwitexpressie van een vooraf bepaalde set van eiwitten tussen jonge en senescent cellen.

Deze eiwitten zijn gekozen op basis van twee verschillende hypothesen. (i) Tijdens veroudering vindt er een graduele verhoging plaats van stress veroorzaakt door reactieve zuurstof radicalen. Er zijn aanwijzingen dat het ER extra gevoelig is voor deze stress. Bovendien is bekend dat ER stress leidt tot expressieverschillen van enkele ER-residente eiwitten. Met de VHH beschreven in hoofdstuk 4 is de expressie van enkele van deze ER-residente eiwitten geanalyseerd. Een duidelijk verschil in expressie is zichtbaar, maar deze verschillen komen niet overeen met de verschillen die te zien zijn tijdens ER stress. Desondanks laat het wel zien dat tijdens het senescent worden van deze cellen de functie van het ER lijkt te veranderen. (ii) Aangezien senescent cellen niet meer kunnen delen, kan de expressie van eiwitten die betrokken

zijn bij celdeling veranderen. Het membraaneiwit endogline, dat voor celdeling nodig is, is daarom een interessant eiwit. De expressie van dit eiwit wordt duidelijk verlaagd in de senescent celpopulatie, maar deze verlaging vond niet plaats bij alle cellen in deze populatie. Aangezien dit eiwit ook betrokken is bij verschillende andere cellulaire processen die belangrijk zijn voor het functioneren van de endotheelcel, zou deze verlaagde expressie nadelige gevolgen kunnen hebben voor het functioneren van het endotheel.

Toekomst van affiniteitsliganden in proteomics

Ondanks het feit dat in dit proefschrift verschillende veranderingen zijn gevonden in de expressie van eiwitten gerelateerd aan veroudering, geven ze bij lange na niet alle veranderingen weer, die een rol spelen bij processen van veroudering. De meeste in hoofdstuk 3 gevonden eiwitten die expressieveranderingen vertonen, zijn redelijk veelvoorkomende eiwitten. Informatie over eiwitten die slechts in lage mate voorkomen in bloed, is dus niet gevonden. Om analyse van deze eiwitten toch mogelijk te maken zijn verschillende groepen begonnen met het ontwikkelen van aanvullende technieken, die de complexiteit van biologische samples reduceren. Hierbij wordt het sample opgedeeld in verschillende fracties, die dan afzonderlijk worden geanalyseerd. Resultaten die uit deze studies voortkomen zullen echter met andere technieken geverifieerd moeten worden, aangezien elke stap gedurende sample-opwerking verschillen kan induceren.

Een techniek die het mogelijk zou kunnen maken om biologische samples te analyseren zonder deze eerst te moeten fractioneren is het gebruik van antilichaam micro-arrays. Hierbij worden verschillende antilichamen geïmmobiliseerd op een oppervlak, elk op een verschillende plaats. Deze antilichamen zullen hun antigen binden als het biologische sample wordt toegevoegd, waarna het gebonden antigen op verschillende manieren kan worden aangetoond. Alhoewel er een grote potentie is voor deze techniek, zijn er nog enkele obstakels die overwonnen moeten worden, zoals het genereren en testen van specifieke en hoog affine antilichamen.

Verder zal het een uitdaging worden om antilichamen te genereren, die een afzonderlijke isovorm van een genproduct kunnen herkennen. Dit zou het mogelijk maken om de eiwitexpressieverschillen gevonden in hoofdstuk 3 verder te analyseren.

Dankwoord

Dankwoord

Dit is het laatste gedeelte van mijn proefschrift. Echt fantastisch dat het zover is gekomen. Er zijn heel veel mensen die aan dit proefschrift hebben bijgedragen, zowel direct ("Je kunt die proef ook op deze manier doen") als indirect ("Zullen we even een satétje nemen bij de Basket?").

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Curriculum vitae

Rinse Klooster werd geboren op 14 maart 1977 te Heerlen. Na het behalen van zijn VWO-diploma aan het Stella Maris College te Meerssen in 1995, begon hij datzelfde jaar aan de studie Fundamentele Biomedische Wetenschappen aan de Universiteit Utrecht. De studie werd afgesloten met twee stageperiodes. Het eerste stageonderzoek werd uitgevoerd bij de vakgroep Moleculaire Celbiologie aan de Universiteit Utrecht onder leiding van Drs. G.S. van Rossum en Prof. Dr. J. Boonstra. Dit onderzoek was gericht op het doorgronden van de signaaltransductie-routes, die betrokken zijn bij de regulatie van de activiteit van het enzym cPLA2. De tweede stage werd uitgevoerd bij Unilever Research Colworth in Groot-Brittannië onder leiding van Dr. Steven Grant en Dr. Paul van der Logt. Gedurende deze stage werd Llama-antilichaamtechnologie gebruikt voor het ontwikkelen van nieuwe producten voor Unilever. In mei 2001 behaalde Rinse Klooster het doctoraal-diploma Fundamentele Biomedische Wetenschappen, waarna hij direct zijn promotieonderzoek startte onder leiding van Prof. Dr. Ir. C.T. Verrips en Dr. J.A. Post, hetgeen resulteerde in dit proefschrift. Per 1 januari 2006 is Rinse Klooster werkzaam als postdoc aan het Leids Universitair Medisch Centrum (LUMC) in Leiden.

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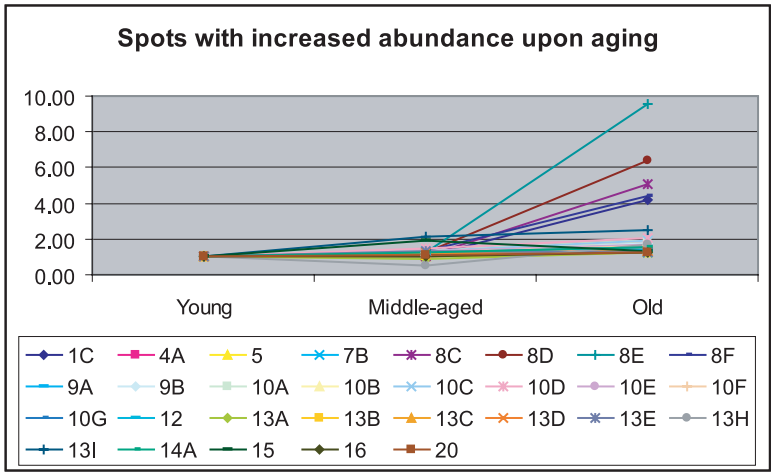
A



B

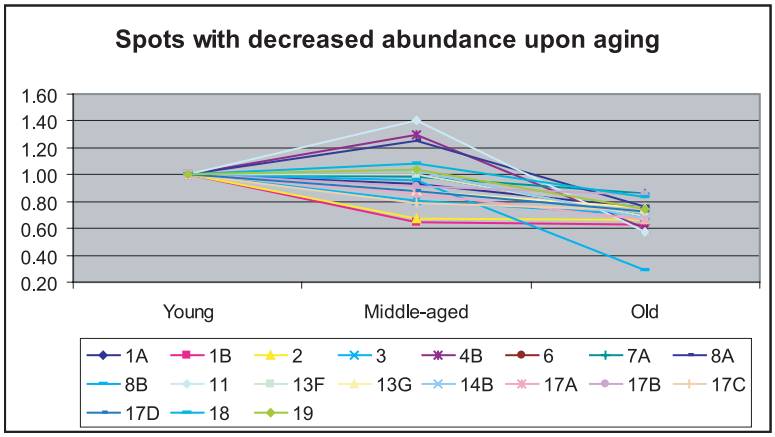


Figure 2: Differentially expressed protein spots with a significant increased (A) and decreased abundance (B) upon aging.

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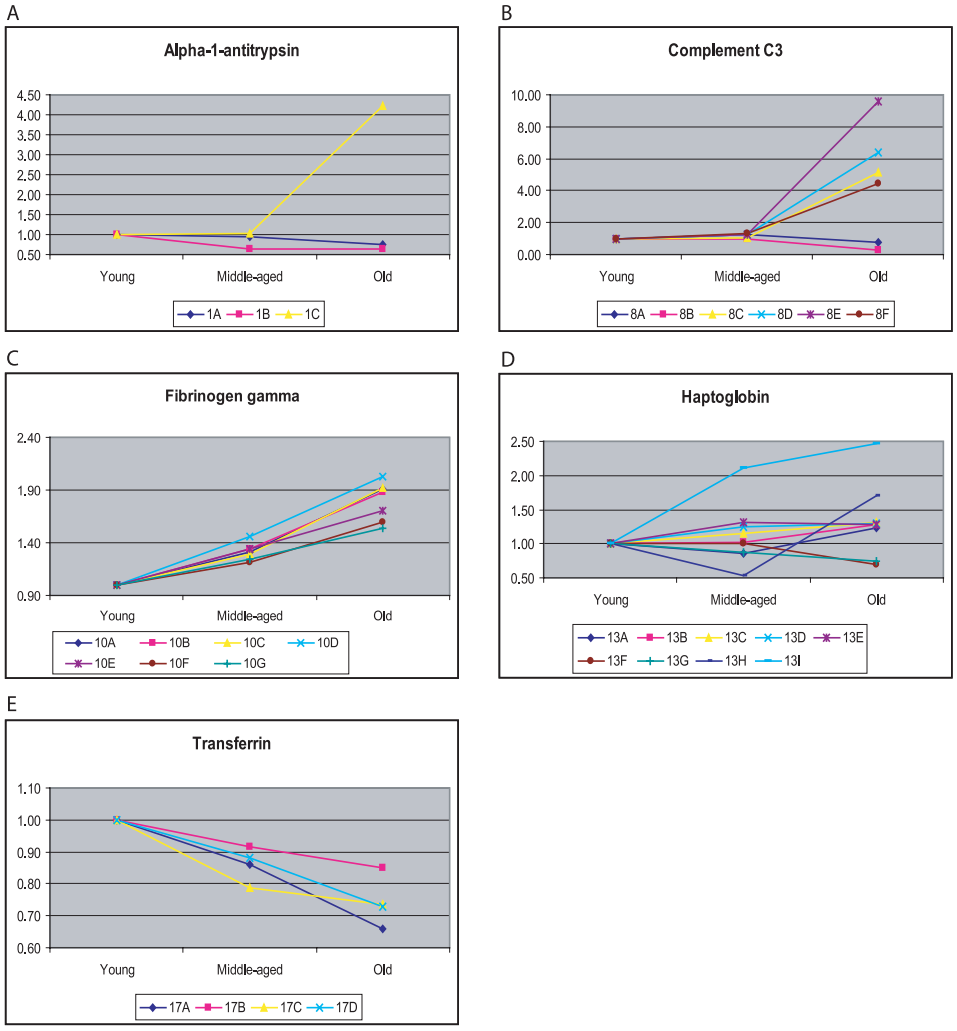

Figure 3: The expression patterns in the different age groups of several different isoforms of alpha-1-antitrypsin (A), CO3 (B), fibrinogen gamma (C), haptoglobin (D) and transferrin (E). Not all isoforms of a single gene product show a similar expression pattern. Some isoforms of alpha-1-antitrypsin (spot 1C), complement C3 (spots 8A and 8B) and haptoglobin (spots 13F, 13G and 13H), show an expression pattern that is not consistent with the majority of the isoforms.

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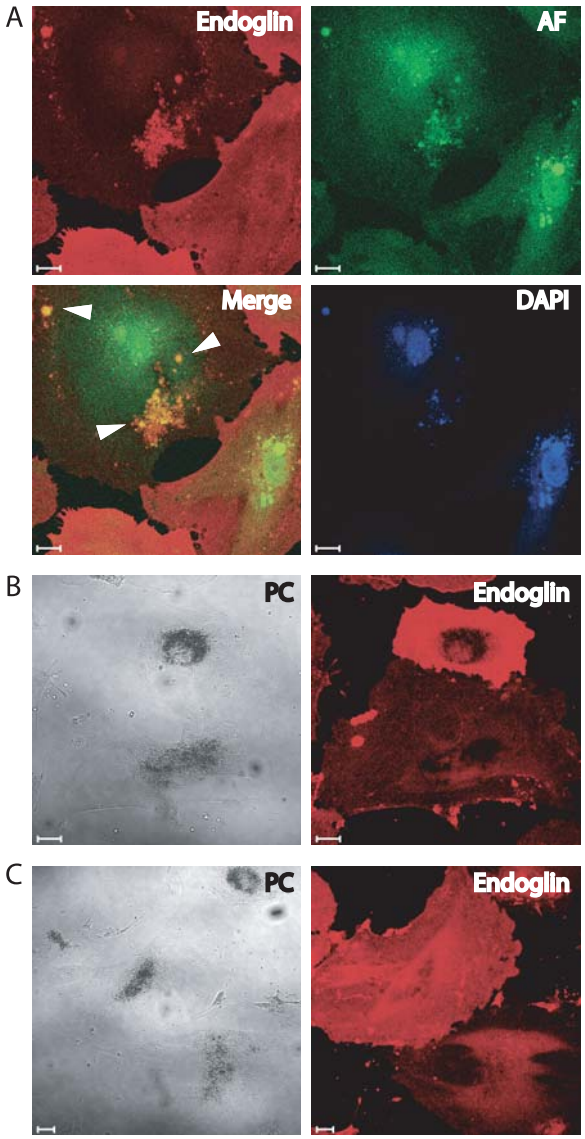

Figure 5: Heterogenous endoglin labeling in senescent HUVECs. HUVECs cells were stained with the β-galactosidase activity assay kit and subsequently labeled with a monoclonal anti-endoglin antibody. Several endoglin negative cells revealed clear aggregate-like structures that showed auto-fluorescence (AF). This suggested that these structures were protein aggregates containing lipofuscin (Merge, white arrowheads). The extent of aggregate formation differed per cell (A). Phase contrast pictures (PC) revealed that several β-galactosidase positive cells were also positive when labeled with the monoclonal anti-endoglin antibody. However, all endoglin negative cells stained positive in the senescence-associated β-galactosidase assay (Phase contrast). Even cells with reduced levels of endoglin were β-galactosidase positive (B, C). Bar represents 20μm.