

Review

Sulfomucins in the Human Body*

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Introduction

Mucins are large glycoproteins consisting of O-linked oligosaccharides for at least 50% of their mass. Today, two classes of mucins have been defined: epithelium associated and endothelial-leukocyte associated mucins (van Klinken et al., 1995a). The secretory epithelial mucins constitute the larger part of the protective viscous mucus that covers and protects epithelial tissues of the human oral cavity, and the tracheobronchial, gastrointestinal and reproductive tracts against potential hostile environments (Allen et al., 1993; Tabak, 1995; Nieuw Amerongen et al., 1995). With the exception of the low molecular weight salivary mucin MG2 (MUC7), secretory mucins are high molecular weight molecules (M_r , 10^6 – 10^7), disulfide linked multimeric proteins (Strous and Dekker, 1992). Besides secretory epithelial mucins, also membrane-associated epithelial mucins exist, which are associated with the epithelial cell membrane. The endothelium-leukocyte associated mucins are molecules that function as ligands for the selectin family of carbohydrate binding proteins, and as such are implicated in recognition processes, like the trafficking of leukocytes in inflammation (Rosen and Bertozzi, 1994). The structure, genetics, functions and properties of secretory mucins have been discussed in a series of comprehensive reviews (Van Klinken et al., 1995 a, b; Nieuw Amerongen et al., 1995; Gendler and Spicer, 1995; Bansil et al., 1995). The presence of sulfate as an integral part of mucin molecules has been recognized already in the early sixties (Kent and Marsden, 1963), but until now few studies have dealt with the biology and function of sulfate. Recent studies demonstrating the involvement of sulfated mucins in selectin mediated recognition processes (Lasky et al., 1992; Imai et al., 1993; Rosen and Bertozzi, 1994) have lead to a renewed interest in the specific role of sulfate. The aim of this review is to give a survey of the current knowledge of sulfated mucins in the human body. The term sulfomucins, used throughout this review, originated from histochemical studies to describe mucin that can be visualised with specific staining methods, including high iron diamine and Alcian Blue. Biochemically, there are no clear criteria, e.g. sulfate content, to qualify a mucin as sulfomucin. Furthermore it should be noted that there is not a clear-cut separation between sulfomucins and sialomucins, since one mucin molecule often contains both residues, in some instances even as constituents of the same oligosaccharide side-chain.

Mucins are widely distributed in mucous secretion fluids or are associated with plasma membranes. Up to now 9 genes of epithelial mucins have been identified, distributed over five chromosomes. Superposed on the genetic diversity, each type of mucin displays heterogeneity in oligosaccharide composition, including the terminal sugar residues. On top of that there is variation between individuals brought about by blood group antigens. Heterogeneity is further incited by the degree of sulfation. This tremendous structural heterogeneity endows mucin molecules with properties suggestive for a multifunctional role. The major biological function assigned to mucins is still the protection of tissues covered by the mucous gel. Current knowledge on the specific biological functions of the sulfate residues is fragmentary and peripheristic. Glycosylation including sulfation appears to be subject to modification under pathological conditions. There is evidence that sulfation rate-limits bacterial degradation of mucins. Moreover, accumulating data focus towards their involvement in recognition phenomena. Sulfate residues on blood group related structures provoke specific epitopes for selective interaction with microorganisms e.g. *Helicobacter pylori*. A distinct class of mucins acts as ligands for selectins, crucial in cellular recognition processes like cellular homing of lymphocytes. Whereas in earlier days mucins were only seen as water-binding molecules, protecting the underlying mucosa against harmful agents, the current picture of these molecules is characterized by the selective interaction with their environment, including epithelial-, and endothelial cells and microorganisms, thereby regulating a great number of biological processes. However, the specific role of sulfate remains to be further elucidated.

Key words: Glycosylation / Mucins / Selectins / Sialic acid / Sulfation.

Table 1 Expression of Mucins in Tissue^a.

Tissues	MUC1	MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6	MUC7	MUC8
Salivary glands	+	-	-	-	-	++	-	++	NT
Eye	+	-	-	+	NT	++	-	-	NT
Stomach	+	NT	+	+	++	+	++	-	NT
Small intestine	+	++	++	+	±	+	±	NT	-
Colon	+	++	+	+	±	+	±	NT	NT
Gall bladder	+	±	++	NT	+	+	++	NT	NT
Cervix	+	±	-	++	+	+	+	-	NT
Prostate	+	±	±	NT	NT	NT	NT	-	NT
Breast	+	-	-	+	-	-	±	NT	NT
Respiratory tract	+	+	-	++	++	++	±	NT	++

^a Composed from Gendler and Spicer, 1995; Van Klinken et al., 1995a; Shankar et al., 1997.

- negative; ± trace amount; + positive; ++ strongly positive; NT not tested.

Epithelial Mucins: Peptide and Oligosaccharide Diversity

In humans so far nine epithelial mucin genes (*MUC* genes), localized on chromosomes # 1,3,4,7,11 (Porchet et al., 1995) have been identified and partially characterized (Table 1). There is great variety in the size of mature mucins, varying between 120 and 3000 kDa. Further heterogeneity is found at the level of the mRNA (Gendler and Spicer, 1995). For instance, the *MUC1* gene product, epilysin, comprises a family of glycoproteins with a varying number of tandem repeats in the polypeptide backbone (Ligtenberg et al., 1990). A common feature of all mucin genes thus far sequenced, is that a major portion consists of tandem repeats, in which serine and threonine, potential *O*-glycosylation sites (Gooley and Williams, 1994), make up a high percentage of the amino acids. In addition, relatively few potential *N*-glycosylation sites are present which are mainly located outside the tandem repeat regions. Recently, it has been reported that in mouse submandibular mucin a *N*-glycosylation site is present within the tandem repeats (Denny et al., 1996). The presence of relatively few *N*-linked chains, in addition to *O*-linked chains, has already been reported in 1976 (Roukema et al., 1976; Nieuw Amerongen et al., 1983, 1987). Structure analysis of carbohydrate side chains of mucins isolated from different sites of the human body indicate a multitude of different glycan chains, varying in length from one to more than twenty sugar residues. Neutral and acidic oligosaccharide chains carrying one or more negatively charged sialic acids and/or sulfated sugar residues have been identified in several mucin species, including respiratory, gastric, salivary and intestinal mucins (Hanisch et al., 1993; Klein et al., 1992, 1993; Lamblin et al., 1991b; Lo-Guidice et al., 1994, 1997; Mawhinney et al., 1987, 1992a, b; Sangadala et al., 1992, 1993; Slomiany et al., 1984; Van Halbeek et al., 1982, 1994). Besides the intrinsic heterogeneity, inter-individual differences in carbohydrate make-up of a given mucin species are present which are related to blood group and secretor status. A number of blood group related antigens, that have been demon-

Table 2 A Number of Blood Group Related Antigens Found in Mucins^a.

Name	Structure
H	Fuc α 1-2Gal β 1-R
A	GalNAc α 1-3[Fuc α 1-2]Gal β 1-R
B	Gal α 1-3[Fuc α 1-2]Gal β 1-R
Le x	Gal β 1-4[Fuc α 1-3]GlcNAc-R
Le y	Fuc α 1-2Gal β 1-4[Fuc α 1-3]GlcNAc-R
Le a	Gal β 1-3[Fuc α 1-4]GlcNAc-R
Le b	Fuc α 1-2Gal β 1-3[Fuc α 1-4]GlcNAc-R
sialyl-Le a	Neu5Ac α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc-R
sialyl-Le x	Neu5Ac α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc-R
sulfo-Le a	SO $_3$ -3Gal β 1-3[Fuc α 1-4]GlcNAc-R
sulfoLe x	SO $_3$ -3Gal β 1-4[Fuc α 1-3]GlcNAc-R

^a Data based on Prakobphol et al., 1993; Lo-Guidice et al., 1997.

strated as part of oligosaccharide chains on mucins (Prakobpho et al., 1993; Lo-Guidice et al., 1997), are given in Table 2, but more complicated structures, viz. ALe b are likely to be present as well. Blood group related antigens have been indicated as ligands for bacterial lectins (Borèn et al., 1993; Borèn and Falk, 1994; Ligtenberg et al., 1990a, b, 1992; Steuer et al., 1995).

Synthesis of Epithelial Mucins

A number of studies have elucidated on the molecular level some of the steps involved in the synthesis and secretion of mucins. Like other secretory glycoproteins, the secretory mucins are synthesized on ER-associated ribosomes, and during synthesis are directly delivered into the cisternae of the endoplasmic reticulum. Here mucins become oligomerized by disulfide bonds, a process probably facilitated by co-translational *N*-glycosylation (Dekker and Strous, 1990; Klomp et al., 1994). *O*-glycosylation starts with the addition of GalNAc to either serine or threonine in the ER or transitional regions of the ER or the cis-cisternae of the Golgi-complex (Pathak et al., 1988; Piller et al., 1990; Roth et al., 1994). Elongation of the oligosaccha-

rides takes place in the Golgi-apparatus and trans-Golgi network (TGN) by an orchestrated machinery of glycosyl-transferases. Sulfate is transferred to oligosaccharide acceptors by sulfotransferases that utilize 3'-phosphoadenosine-5'-phosphosulfate as the high-energy sulfate donor. Sulfate addition is a late synthetic event that occurs within the lumen of the Golgi. The latter can be inferred from studies using Brefeldin A, a fungal metabolite causing desintegration of the Golgi-apparatus with a concomitant redistribution of Golgi-enzymes, but not of the TGN-enzymes, to the ER. Incubation with Brefeldin A prevented the appearance of mature sulfated mucins in gastric mucus secreting cells (Dekker and Strous, 1990) and the incorporation of sulfate in the endothelial-associated ligand for L-selectin, GlyCAM-1 (Crommie and Rosen, 1995). As Golgi processing reaches completion, condensing granules bud off from the Golgi-apparatus. It is assumed that the high Ca^{2+} content of the mature granules aids condensation by shielding the negative charges of sialic acid and sulfated carbohydrate residues on the mucin molecules (Verdugo et al., 1987; Verdugo, 1990).

Sulfated Oligosaccharides

A number of different sulfated oligosaccharides structures on epithelial mucins have been reported (Table 3). Using chemical methods for structure determination, Mawhinney et al. (1992a, b) and Sangadala et al. (1993) have described structures of sulfated oligosaccharides in mucins from cystic fibrosis patients. Sulfate was found either at C-6 of *N*-acetylglucosamine or at C-6 or C-4 of galactose. In contrast, using $^1\text{H-NMR}$ for structure analysis, Lamblin et al. (1991b) and Lo-Guidice et al. (1994, 1997) found sulfate only at position 3 of galactose and on position 6 of *N*-acetylglucosamine. The latter structure has also been demonstrated in porcine small intestine mucins (Karlsson et al., 1996) and in rat stomach mucins (Goso and Hotta, 1989). The occurrence of Gal-3-sulfate and GlcNAc-6-sulfate is corroborated by the demonstration of the corresponding sulfotransferases in the mucin producing tissues; in rat gastric mucosa, for example, a mucin sulfotransferase possessing GlcNAc-6-sulfotransferase activity has been detected (Slomiany et al., 1987; Carter et al., 1988). The enzyme preparation was activated by 30 mM NaF and the anti-ulcer agent sofalcone and inhibited by sucralfate, ethanol and aspirin. Kuhns et al. (1995) have

identified a novel sulfotransferase catalyzing the addition of sulfate to the 3-position of galactose of mucin O-glycan core 1. Processing of core 1 by core 2 β GlcNAc-transferase is inhibited by 3-sulfation of the galactose residue. Enzymatic activity towards core 1 substrate Gal β 1-3GalNAc- progressively decreased upon transformation of colonic cells to adenocarcinoma, which supports histochemical and biochemical data demonstrating that in developing cancerous colonic tissue a shift towards a less sulfated mucin population occurs. Lo-Guidice et al. (1995) have demonstrated sulfotransferase activity in microsomal fractions prepared from human bronchial mucosa, transferring sulfate groups to C-3 of terminal galactose residues of neutral carbohydrate chains isolated from human respiratory mucins. These authors also found that stimulation of sulfotransferase activity by NaF, AMP and ATP was due to the inhibition of hydrolases degrading the sulfate donor used, rather than that the sulfotransferase itself was activated. A sulfotransferase capable of recognizing and transferring sulfate to position 4 of GalNAc β 1-4GlcNAc on O-glycans as well as N-glycans has been purified from bovine submandibular glands. This enzyme has been implicated in the sulfation of carbonic anhydrase (Hooper et al., 1995 a). The GalNAc-4-sulfotransferase is expressed in a wide variety of tissues, but mostly in salivary (submandibular) glands, followed by lacrimal glands and pituitary and kidney tissues (Hooper et al., 1995b). In the parotid glands, a non-mucous gland, no expression of enzymatic activity could be demonstrated. GalNAc residues carrying sulfate on the C-3 or C-6 positions have been identified in porcine small intestine mucins (Karlsson et al., 1996). However, as yet no structural data have been obtained demonstrating that SO₃-4GalNAc residues occur in salivary mucins.

Sulfate and Rheological Properties of Mucins

The biological significance of the diversity in the oligosaccharide side chains of secretory mucins is not really understood. Obviously, general properties of mucin-bound oligosaccharides include protease resistance, large water-holding capacity and high charge density caused by sialic acid and sulfate residues (Wu et al., 1994). Furthermore, it is generally accepted that the numerous oligosaccharide side chains maintain the extended random coil conformation of the molecule, which together with the

Table 3 Sulfated Residues in Mucins.

Sulfate linkage	Mucin origin	Reference
SO ₃ -3Gal, SO ₃ -6GlcNAc	Human tracheobronchial mucins	Lamblin et al., 1991b; Lo-Guidice et al., 1994, 1997.
SO ₃ -4Gal, SO ₃ -6Gal		Mawhinney et al., 1992a, b; Sangadala et al., 1993.
SO ₃ -6Gal	Glycam-1	Bertozzi et al., 1995; Hemmerich et al., 1994b, 1995;
SO ₃ -6GlcNAc		Hemmerich and Rosen, 1994; Rosen and Bertozzi, 1994.
SO ₃ -6Gal β 1-4[SO ₃]-6GlcNAc		
SO ₃ -tyrosine	Protein-chain, PSGL-1	Pouyani and Seed, 1995; De Luca et al., 1995; Sako et al., 1995; Wilkins et al., 1995.

high molecular mass is responsible for the characteristic viscoelastic properties of mucins (Jentoft, 1990; Van der Reijden et al., 1993; Bansil et al., 1996). Although it is often stated that particularly charged residues as sulfate and sialic acid are important in this respect, there is no firm experimental evidence favouring such a role. For instance light scattering and circular dichroism studies on ovine submandibular mucins indicate that steric interactions of the O-linked GalNAc residue with the peptide core are primarily responsible for the expanded mucin structure, and that the contribution of sialic acid residues is less important (Rose et al., 1984; Gerken and Dearborn, 1984; Gerken et al., 1989; Shogren et al., 1989; Bansil et al., 1995). Although sulfate-rich mucins have never been subjected to these types of analysis, on basis of the minor contributions of sialic acid residues a specific role for sulfate in this respect seems neither likely. Similarly, a prevailing role of negatively charged residues in determining the viscosity of mucins has not been firmly established. In an early study a distinct effect of desialylation on the rheological properties of ovine submandibular mucins was demonstrated (Gottschalk and Thomas, 1961). In this particular type of mucins, however, sialic acid comprises more than 30% of the total molecular mass (Yamamoto and Yosizawa, 1978). It is conceivable that removal of this carbohydrate will result in a profound decrease in the hydrodynamic volume. In a recent study also a decrease in the viscosity of mucins was reported upon desialylation (Raju and Davidson, 1994), but a number of other studies have indicated that sialic acid residues have no effect, beyond that due to increase of the hydrodynamic volume (Meyer et al., 1975; Litt et al., 1977; Veerman et al., 1989a; McCullagh et al., 1992). No studies have determined the contribution of specifically the sulfate residues to the visco-elastic properties of mucins. Considering the data obtained for sialic acid, a specific role of sulfated carbohydrate residues in maintaining the visco-elastic properties of mucins does not seem likely.

Role of Sulfate in Microbial Attachment to Mucins

Because oligosaccharides often function as attachment sites for bacteria and viruses (see e.g. Karlsson, 1995), it has been proposed that the diverse mucin oligosaccharides represent a mosaic of potential receptors, designed to trap a broad diversity of microorganisms in the mucous layer, thereby impeding infection of the underlying epithelia (Lamblin et al., 1991a). In this context a prominent role can be envisaged for the terminal residues of the glycan chains, including sialic acid and galactose, which are the primary targets for interaction with microorganisms (Lamblin et al., 1991a; Levine et al., 1978; Reddy et al., 1996; Groenink et al., 1996). Sulfate residues on mucins have been implicated in binding of mucins to *H. pylori* (Piotrowski et al., 1991; Veerman et al., 1997b) and *S. aureus* (Thomas et al., 1993). It is worthwhile to note that

binding to single oligosaccharide side chains generally occurs with low to moderate affinity, and that tight binding requires multivalent interaction between microbial receptors and patches of appropriate oligosaccharide ligands (Crottet et al., 1996). Interestingly, the low molecular weight salivary mucin MG2, which binds to a wide variety of different microorganisms (Jones et al., 1987; Reddy et al., 1993; Murray et al., 1992; Hoffman and Haidaris, 1993; Groenink et al., 1996), contains a relatively homogeneous set of oligosaccharides, mainly sialylated di- and trisaccharides (Reddy et al., 1985). On the other hand, the high molecular weight salivary mucin MG1, despite its heterogeneous glycans, binds to a limited set of different microorganisms (Murray et al., 1992; Veerman et al., 1995, 1997a). Thus, it has been proposed that the diverse glycan moieties hamper formation of multiple identical receptor-ligand pairs and endow the MG1 molecules with anti-adhesive properties (Veerman et al., 1995; Nieuw Amerongen et al., 1995).

Sulfate and Protection of Mucins against Degradation

The presence of large numbers of microorganisms is usually associated with the secretion of sulfated mucin species, e.g. in the oral cavity, colon, the ears and in the respiratory tract (Table 4). The converse is not true, since sulfated mucin species are secreted by tissues such as gallbladder that are not colonized by bacteria. It has been proposed that sulfated mucins are particularly involved in protection against bacterial attacks, because sulfation confers resistance to enzymatic degradation of the mucus barrier by bacterial glycosidases or by host proteases, e.g. pepsin (Mikuni-Takagaki and Hotta, 1979; Roberton and Wright, 1997). Activity of other proteases, e.g. leukocyte elastase, is also diminished in the presence of mucins, probably because of formation of mucin-protease complexes. Also sialic acid residues have been implicated in the protease inhibition by mucins (Nadziejko and Finkelstein, 1994). Sulfate-esters are biochemically more stable than sialic acid glycosidic linkages, which are easily hydrolyzed by neuraminidase from the host or invading bacteria. Yet, a number of bacterial sulfatases have been discovered, originating in particular from colonic and oral microflora (Roberton and Wright, 1997). Enzymatic desulfation of colonic mucin by faecal bacterial sulfatases is likely to rate-limit the enzymatic degradation of secreted colonic mucin, since it greatly increases the susceptibility to degradation by faecal glycosidases (Tsai et al., 1992, 1995). In patients with active ulcerative colitis an increased activity of mucin sulfatase was observed. In six out of seven patients fluctuations in faecal sulfatase activity corresponded to clinical disease activity. Thus, it was suggested that the increased faecal sulfatase activity contributed to the perpetuation of the disease. On the other hand, in Crohn's disease faecal mucin sulfatase activity was not significantly increased. The mucin sulfatase activ-

Table 4 Ranges in Sialic Acid and Sulfate Percentage of Human Epithelial Mucins (w/w).

Tissue	Sialic acid	Sulfate	References
Salivary glands	4–10	1–7	Bolscher <i>et al.</i> , 1995; Loomis <i>et al.</i> , 1987; Oemrawsingh and Roukema, 1974;
Palatal	6	12	Roukema and Nieuw Amerongen, 1979
Submandibular	5–20	1	
Sublingual	11	2	
Stomach	4–6.5	1.5–5	Slomiany <i>et al.</i> , 1992, 1993
Lung	8–21	4–8	Chace <i>et al.</i> , 1989; Padhye <i>et al.</i> , 1991
Small intestine	low	low	Carlstedt <i>et al.</i> , 1993
Colon	10–15	6–9	Irimura <i>et al.</i> , 1991; Turck <i>et al.</i> , 1993
Cervix	15		Carlstedt <i>et al.</i> , 1983
Ear	6–13	5–30	Brown <i>et al.</i> , 1985
Eye	10–22		Chao <i>et al.</i> , 1988; Moore and Tiffany <i>et al.</i> , 1981

ity can be inhibited by bismuth subcitrate and bismuth subsalicylate. In the etiology of stomach ulcers a reduction in sulfated gastric mucins has been reported, which was ascribed to the action of a glycosulfatase released by *Helicobacter pylori* (Slomiany *et al.*, 1992).

Sulfomucins in Human Tissues and Secretions

Tissue-Dependent Glycosylation and Sulfation of Mucins

From data reported thus far (cf. Table 4) it is obvious that differences in sulfate content are present between mucin species derived from different organs or tissues. For example, low sulfated mucins are typically found in the stomach and the small intestine (Wesley *et al.*, 1983), while in colon and saliva highly sulfated mucins predominate (Levine *et al.*, 1987; Bolscher *et al.*, 1995). Several factors may account for these differences, including tissue-specific expression and/or regulation of sulfotransferase activity, and the presence of other transferases competing for the same carbohydrate acceptor, e.g. sialyltransferase. Besides, particularly in carcinoma tissues, aberrations in the biosynthetic apparatus, like a shift in enzyme compartmentalization and/or altered transport rates between cis, medial- and trans-Golgi have to be taken into account (Hanski *et al.*, 1992, 1993).

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Histochemical and Biochemical Data

Much of the knowledge about the tissue distribution of sulfated glycoconjugates including sulfomucins, has emerged from histochemical studies using histological staining methods. High Iron Diamine, in combination with Alcian Blue staining distinguishes between sulfomucins, staining brown, and sialomucins, staining blue (Spicer, 1965; Spicer and Schulte, 1992). Although this method has often been used as an investigative tool, it cannot provide an absolute classification into sulfomucins and sialomucins (Jass and Roberton, 1994). Notwithstanding this precaution, the histochemically determined tissue distribution of sulfated mucins is reasonably in line with immunohistochemical data using anti-sulfomucin antibodies (Matsushita *et al.*, 1995; Veerman *et al.*, 1997b) (Table 5). Figures 1 and 2 show examples of immunohistochemistry on human tissues obtained with mAb F2, a monoclonal antibody recognizing the sulfated Le^a antigen (Veerman *et*

Table 5 Tissue Distribution of Sulfomucins.

Tissue	Sulfo-mucins ^a	mAb F2 staining ^b	Cellular localization of F2 epitope ^b
Submandibular glands	+	++	Only in mucous acini
Sublingual glands	+	++	Subset of mucous acini
Palatal glands	+++	+++	All mucous acini
Lung tissue	++	++	Submucosal glands
Oesophagus	?	++	Mucous cells
Stomach	+	-	Not detectable
Small intestine	+	±	Luminal lining
Colon	+++	+++	Homogeneous in epithelium
Uterus	?	++	Cervical epithelium
		-	myometrium
		-	endometrium
Mammary glands	?	-	Negative
Prostate	?	+	Mucous cells
Pancreas	-	-	Negative

^a + 1–2% sulfate; ++ 2–8% sulfate; +++ > 8% sulfate.

^b mAb F2 recognizes the SO₃-3Galβ1-3GlcNAc moiety of sulfo-Lewis^a (Veerman *et al.*, 1997b).

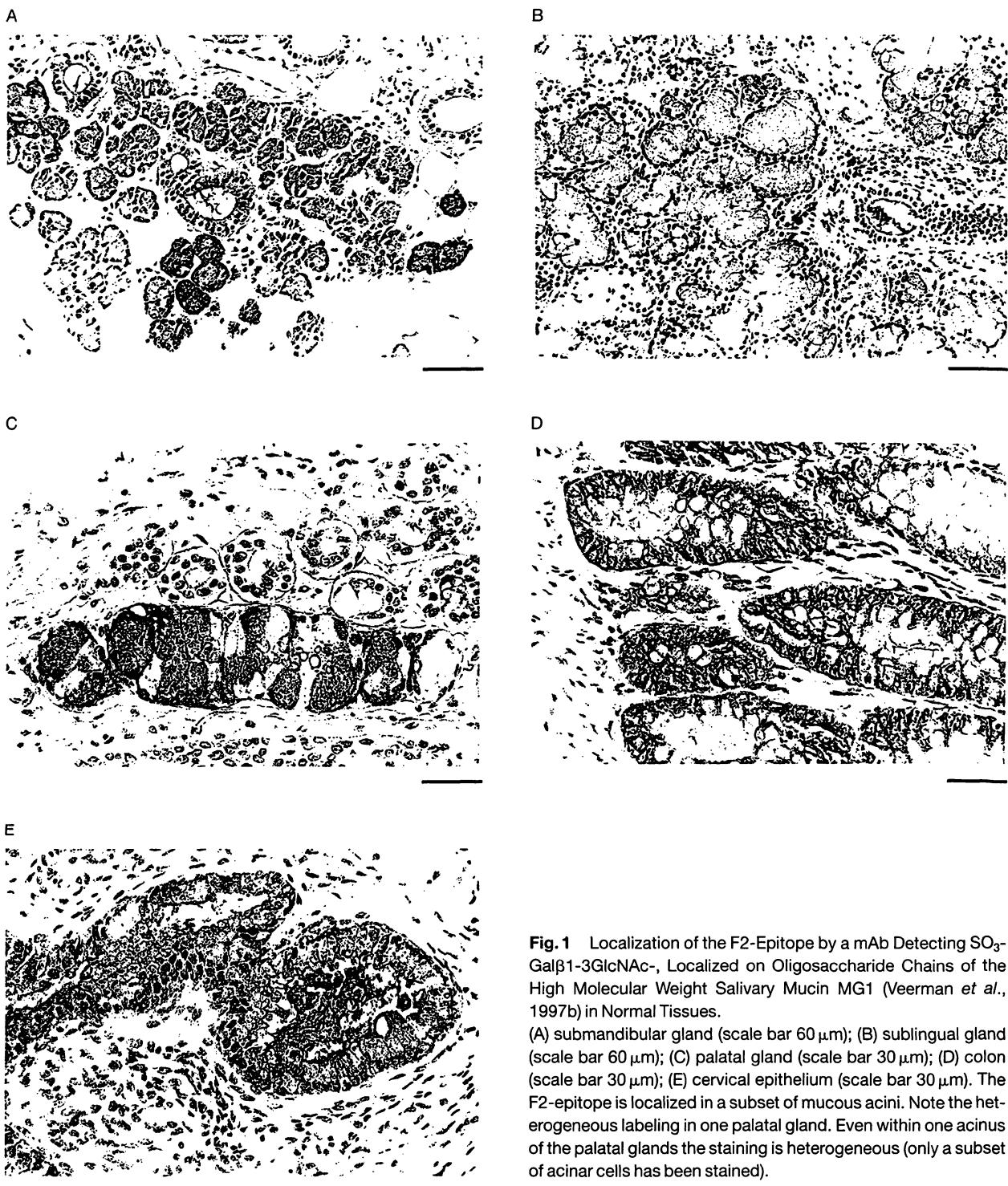


Fig. 1 Localization of the F2-Epitope by a mAb Detecting $\text{SO}_3\text{-Gal}\beta 1\text{-}3\text{GlcNAc}-$, Localized on Oligosaccharide Chains of the High Molecular Weight Salivary Mucin MG1 (Veerman *et al.*, 1997b) in Normal Tissues.

(A) submandibular gland (scale bar 60 μm); (B) sublingual gland (scale bar 60 μm); (C) palatal gland (scale bar 30 μm); (D) colon (scale bar 30 μm); (E) cervical epithelium (scale bar 30 μm). The F2-epitope is localized in a subset of mucous acini. Note the heterogeneous labeling in one palatal gland. Even within one acinus of the palatal glands the staining is heterogeneous (only a subset of acinar cells has been stained).

al., 1997b). This antibody labels sulfomucins present in seromucous salivary glands as well as in colon and cervical epithelium (Figure 1). In healthy gastric tissues no labeling occurred, but gastric intestinal metaplasia were labeled by these types of antibodies (Figure 2B; Matsushita *et al.*, 1995). Furthermore, while healthy breast tissues are negative for the F2 epitope, it is occasionally expressed in adenocarcinoma of the mammary gland (Figure 2D), exemplifying aberrant sulfation occurring in malignancy. Additional information on tissue distribution

of sulfomucins can be gathered from biochemical analysis of isolated mucin species (Table 5). This approach has also practical limitations, as can be inferred from the broad range in sulfation of the same mucin species reported by different authors (Table 4). Several reasons may cause these variations, including inaccuracy of the sulfate assays used, as well as biological variations in the source of mucin. With regard to the latter it has to be noted that in many cases secreted mucins can only be obtained from patient materials, thus essentially reflect a pathological

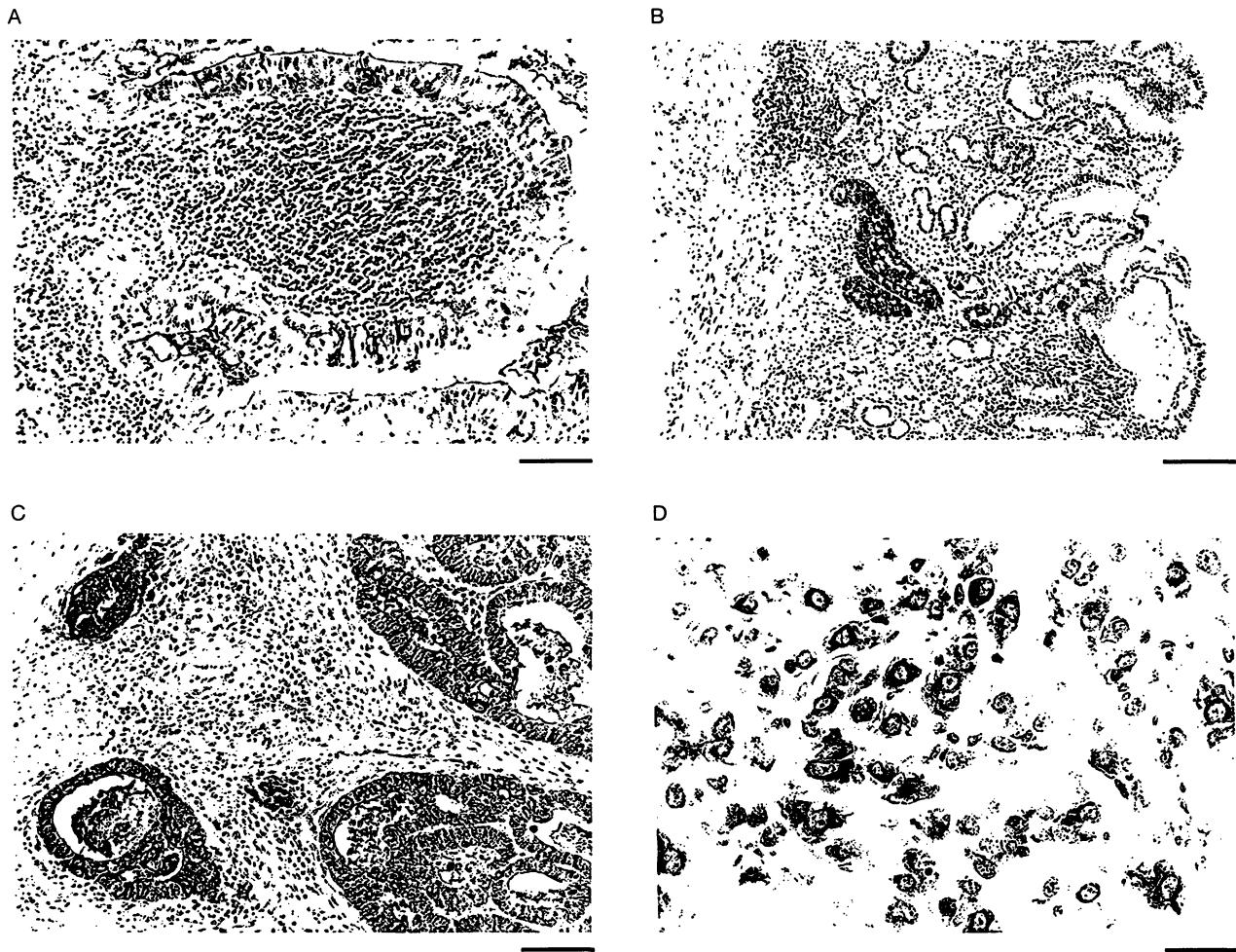


Fig. 2 Localization of the F2-Epitope in Pathological Tissues.

(A) Whartin tumor in parotid gland (scale bar 60 μm); (B) Intestinal metaplasia in stomach (scale bar 120 μm); (C) Colonic adenocarcinoma (scale bar 60 μm); (D) Adenocarcinoma of the mammary gland (scale bar 30 μm). Note that in the parotid and mammary tissue only transformed cells express the F2-epitope.

condition. As was demonstrated more than twenty years ago for mucins from cystic fibrosis patients (Roussel et al., 1975), the disease state may lead to large qualitative and quantitative variations in the mucin secretion. Besides, different glands or cells in one tissue may secrete differently sulfated mucin-glycoforms. For instance in the oral cavity, different salivary glands secrete differently glycosylated and sulfated mucin species (Veerman et al., 1989b, 1991, 1992; Bolscher et al., 1995). Similarly, tracheobronchial submucosal and epithelial glands secrete distinct mucin species (Sheehan et al., 1991; Thornton et al., 1997; Hovenberg et al., 1996b). Differently glycosylated and sulfated forms have been demonstrated in mucins isolated from various sources, including the stomach (Slomiany et al., 1987; Van Beurden-Lamers et al., 1989; Goso and Hotta, 1989), colon (Gold et al., 1981; Podolski, 1985, 1989; Podolski and Fournier, 1988), respiratory tract (Aubert et al., 1991; Bhattacharyya et al., 1990; Thornton et al., 1991a, b, 1994, 1997; Culp et al., 1995), and saliva (Veerman et al., 1992; Ramasubbu et al., 1991).

The Presence of Sulfated Mucin in Different Tissues and Secretions

Saliva In saliva at least two genetically distinct mucin species are present: the high molecular weight mucin MG1, encoded by *MUC5B* (Nielsen et al., 1997) and the low molecular weight mucins MG2 (*MUC7*, Bobek et al., 1993). While MG2 contains little sulfate (Loomis et al., 1987), the sulfate content of MG1 varies between 1 and 12%, depending on the glandular source (Bolscher et al., 1995). Using density gradient electrophoresis it was demonstrated that even one type of salivary gland can secrete several MG1 glycoforms with different acidity (Bolscher et al., 1995). Figures 1 and 2 illustrate the immunohistochemical localization of sulfomucins in salivary glands, obtained with anti-sulfomucin mAb F2 (Rathman et al., 1990; Veerman et al., 1991, 1992, 1997b). In the submandibular, sublingual and palatal glands, acinar and ductal cells were labeled by mAb F2, while the serous parotid gland, not secreting mucins, was negative (Figure 2A).

Submandibular, sublingual and palatal tissues were heterogeneously labeled, showing both positively and negatively staining mucous acini. In the palatal gland within one acinus both positive and negative staining cells were seen, suggesting that even one single secretory unit may produce differently sulfated mucin forms (Figure 1C). A specific role for sulfated mucins in the oral cavity can as yet not be assigned. *H. pylori* binds preferentially to highly sulfated salivary mucin species (Veerman et al., 1997a) but the physiological significance of this finding is not clear. *In vitro* investigations indicate that attachment, spreading and locomotion of human gingival fibroblasts on cementum is inhibited by sulfated mucins, suggesting a role in periodontal wound healing (Zentner and Heany, 1995). As has been suggested above, the presence of sulfated residues in oral mucins may protect the mucin peptide backbone against hydrolytic enzymes. A number of oral microorganisms release sulfatases with the highest levels found in all strains tested of *Streptococcus salivarius*, *S. mitis* and *S. gordonii* and in about 50% of the strains of *S. mutans* (Smalley et al., 1994). On the other hand, *S. oralis*, *S. constellatus* and *S. anginosus* display no sulfatase activity. It has been suggested that streptococcal sulfatases may contribute to the destruction of salivary and other mucins and mitigate their rescueing functions in the oral cavity. The finding that simple mucin-type Tn and sialyl-Tn carbohydrate antigens are present in salivary gland carcinomas, while they usually are cryptic in normal tissue (Therkildsen et al., 1993; Fonseca et al., 1994), suggest that modified glycosylation occurs under pathological conditions. Furthermore, Carnoy et al. (1993), have observed increased sulfation and sialylation of salivary mucin glycopeptides from CF patients. Aberrant sulfation of salivary mucins occurring under pathological conditions is also suggested by the fact that pleomorphic adenomas in the submandibular and palatal glands were negative for the sulfo-epitope recognized by mAb F2 (Veerman et al., unpublished). Strikingly, while healthy parotid tissue is negative, the F2 epitope is strongly expressed in Whartin tumour of the parotid glands (Figure 2A). It is not known, however, whether this signals the presence of mucin molecules.

Respiratory Mucins At least five *MUC* gene products are present in tracheobronchial secretions (Table 1, Guyonnet-Duperat et al., 1995; Hovenberg, 1996a; Thornton, 1997). It appears that different mucins are produced by the epithelial and the submucosal glands, the MUC5AC mucins being the prominent species produced by surface goblet cells (Hovenberg et al., 1996b). These findings confirm previous studies indicating that the mucin-like materials produced by goblet cells and submucosal gland cells differ in their relative glycosylation and sulfation levels (Lamblin et al., 1991b, 1992; Taylor et al., 1993). The mixed origin of tracheobronchial mucins may therefore account for the differences in sialic acid and sulfate contents reported for respiratory mucin preparations (Chace et al., 1989; Bhattacharyya et al., 1990; Padhye et al., 1991). A

number of studies dealing with the influence of diseases on the structure and function of tracheobronchial mucins has shown that hypersulfation occurs in mucins from cystic fibrosis patients (Roberts, 1974; Roussel et al., 1975; Boat and Cheng, 1978, 1989; Chace et al., 1989; Zhang et al., 1995; Mawhinney et al., 1992a, b; Sangadala et al., 1993; Thornton et al., 1991a; Desai et al., 1993). Structure analyses showed that more than 200 types of oligosaccharide chains were present in the cystic fibrotic sputum mucin (Lamblin et al., 1991b, 1992; Van Halbeek et al., 1982, 1994), and it has been suggested that some of these were highly sulfated, extremely long oligosaccharides (> 100 sugar residues, Sangadala et al., 1992, 1993). Mawhinney et al. (1992b) and Chance and Mawhinney (1996) showed that the major part of the oligosaccharide chains of bronchial mucins of cystic fibrosis patients are acidic, containing either one sialic acid plus one sulfate, or two or three sialic acid residues, or two sulfate residues without sialic acid. Airway irritation does not seem to drastically influence the structural properties of respiratory mucins. It has been reported that mucins from bronchitic sputum and 'normal' secretions are similar in their macromolecular properties, but differ slightly in charge density (Davies et al., 1996). The mechanism underlying the hypersulfation in CF mucins is not known. Zhang et al. (1995) have used a human bronchial xenograft model, in which pathological differences between CF and non-CF mucins can be studied in the absence of secondary disease effects such as goblet cell hyperplasia. Cumulative results of xenografts from a number of independent cystic fibrosis tissue samples demonstrated a higher level of sulfation as compared to healthy controls. Studies by Barasch et al. (1991) and Barasch and al-Awqati (1993) suggest that a pH-shift in the Golgi compartment of cystic fibrosis epithelia causes a decreased activity of modifying enzymes such as sialyltransferase.

Gastric Mucins In healthy gastric epithelium high levels of MUC1, MUC5AC and MUC6 mRNAs are present but not of MUC2, MUC3 and MUC4 (Ho et al., 1995). Characteristic for human gastric mucins is the low content of both sialic acid (4%) and sulfate (1.5%) (Slomiany et al., 1993). Gastric mucins can be fractionated in a neutral and an acidic subpopulation, the latter containing 6.5% of sialic acid and 5% of sulfate (Slomiany et al., 1992). Gastric mucins are associated with lipids (Slomiany et al., 1986), endowing the mucus layer with hydrophobic properties which are crucial for their barrier function (Lichtenberger, 1995). Although sulfate residues on stomach mucins increase their resistance against proteolytic degradation by pepsin (Mikuni-Takagaki and Hotta, 1979), it is not likely that this will be a major function here, taking into account the low level of sulfation. Human gastric mucins are able to bind to *H. pylori*, a process in which sialic acid and sulfate residues have been implicated (Tzouvelekis et al., 1991; Piotrowski et al., 1991). Interestingly, gastric metaplastic tissue secreting sulfated mucins (see Figure 2B) are no longer colonized by *H. pylori*. The development of intesti-

nal metaplasia upon chronic infection by *H. pylori* has been viewed as a mechanism by which the stomach eliminates this pathogen, and one can speculate on a role for sulfomucins here. Increased expression of sulfated mucin species, which are able to bind to the microorganism, may hamper a further migration of the pathogen through the mucus gel secreted by intestinal metaplastic tissue.

Colon Mucins The major secreted mucin of colon appears to be MUC2 (Tytgat et al., 1996). The *MUC2* gene is expressed only in goblet cells, whereas MUC3 is expressed in both goblet cells and absorptive cells, particularly in the upper parts of the crypts in the colon (Gum, 1995). In rectosigmoid villous adenomas an aberrant expression of MUC5AC has been found, in addition to an overexpression of MUC2 (Buisine et al., 1996). Tytgat et al. (1994) reported for colon mucins a sialic acid content in molar ratio nearly two times that of galactosamine, indicating that a large number of the O-glycosidic chains carries more than one sialic acid residue. In mammals and man the colon mucins contain considerable amounts of both sialic acid (up to 7%) and sulfate (up to 9%) (Irimura et al., 1991). Studies in animals suggest that the content of sulfated mucins in colon is influenced by diet and/or microflora. In pigs, sulfation of colon mucin was enhanced by an artificial diet compared to mother milk, but the sulfation decreased with age (Turck et al., 1993). Also in rat dietary changes, together with the microbial flora, influenced the amount and proportion of the mucins in the small and large intestine (Sharma and Schumacher, 1995). The colon is a major producer of mucins and in this tissue malignancy-associated changes in the carbohydrate structure of mucins were first characterized. Already in 1974 Filipe and Branfoot reported that in colon tumors the sialomucins were increased, with a concomitant decrease in the content of sulfomucins, which in healthy colon tissue predominate (Gold and Miller, 1978; Filipe, 1979, see also Figure 2C). It has been suggested that a decrease in sulfomucins

can be an indicator for early carcinogenesis in colon (Irimura et al., 1991). The sulfation of colon mucin is also diminished in ulcerative colitis (Raouf et al., 1992). Yamamori et al. (1987; 1989; Table 6) reported a decrease in sulfated colonic mucins in primary colon carcinoma. This shift in sulfation has also been observed in polyposis cell lines during progression to cancer (Vavasseur et al., 1994). Boland and Deshmukh (1990) showed that the increase of sialylation and decrease of sulfation in colon mucins is accompanied by a reduction in length of the carbohydrate chains. Furthermore, Price et al. (1993) found a reduced glycosylation of MUC2 in colon tumors. In line with these findings is the observation of Yang et al. (1994) that sulfotransferase activity towards O-glycan core 1 is reduced in colon cancer, while on the other hand, sialyltransferase and fucosyltransferase activities are increased. It has been demonstrated that enhanced sialylation of mucin-associated carbohydrate structures in human colon cancer metastasis resulted from selective metastasis of cells that produce sialomucins (Breslauer et al., 1996). The total changes in activity of the different sulfo- and glycosyltransferases resulted in increased sialylation (Gold and Miller, 1978; Corfield et al., 1990), shorter oligosaccharides (Shimamoto et al., 1989; Capon et al., 1992) and increased exposure of peptide regions of mucins (Girling et al., 1989). Similar to colon, in the small intestine MUC2 is the predominant mucin. In addition, MUC3 is expressed in this tissue (Toribara et al., 1991). Most data on the occurrence of sulfomucins in human small intestine have been gathered using histochemical techniques. It appears that goblet cells in normal small intestine contain neutral and sialomucins but no sulfated material (Filipe and Fenger, 1979). Furthermore, mucin composition changes from duodenum to ileum, particularly in the proportions of sialic acid types and in the presence of traces of sulfomucins in the ileal mucosa close to the ileo-caecal valve, suggesting a gradual transition through the small intestine to the colon (Filipe and

Table 6 Pathological Alterations in the Glycosylation of Mucins.

Tissue	Alteration	Reference
Salivary glands		
Pleomorphic adenomas	Decrease of F2 epitope ^a	Veerman et al., 1997b
Warthin's tumor	Appearance of F2 epitope	see Figure 2A
Cystic fibrosis	Increased sulfation and sialylation	Carnoy et al., 1993
Stomach		
Tumors	Decrease of MUC5 and 6	Ho et al., 1995
Intestinal metaplasia	Appearance of F2 epitope	see Figure 2B
Lung		
Cystic fibrosis	Decrease of sialic acid Increase of sulfate	Sangadala et al., 1993 Roussel et al., 1975
Colon		
Colitis	Decrease of sulfate	Raouf et al., 1992
Crohn's disease	Decrease of sulfate	Price et al., 1993
Carcinoma	Decreased glycosylation Appearance Si-Le ^x Decrease of sulfation	Hanski et al., 1993 Vavasseur et al., 1994

^a mAb F2 recognizes the SO₃-3Galβ1-3GlcNAc moiety of sulfo-Le^a (Veerman et al., 1997b).

Fenger, 1979). These findings are in line with immunohistochemical data indicating that the small intestine was hardly labeled by anti-sulfomucin mAb F2 (Veerman et al., 1997 b).

Gall Bladder Mucins A major mucin gene product expressed in gall bladder is MUC5B (Keates et al., 1997; Van Klinken et al., 1997). Carbohydrate analysis indicate that bile mucins have a relatively low content of GalNAc, suggesting that these mucin species contain long polygalactosamine chains of >30 residues long (Baeckström et al., 1994). Several lines of evidence suggest that bile mucins play an important role in the onset of gallstone formation (Qiu et al., 1995). Human gall bladder mucins promote cholesterol nucleation *in vitro* in model biles (Levy et al., 1984). Compositional analysis showed that mucins from patients with cholesterol gallstones were more extensively sulfated than were mucins from control subjects, but no difference in the efficiency with which the mucin promoted cholesterol nucleation was observed (Nagashima et al., 1974; Lee and Nicholls, 1986).

Cervical Mucins In cervical mucus particularly MUC4 and MUC5B, and to a lesser extent MUC2, MUC5AC and MUC6 mucins are present (Audie et al., 1993, 1995; Gipson et al., 1997). Chemical data obtained with pooled midcycle mucins indicate a sulfate content of approximately 1% (Yurewicz and Moghissi, 1981). The presence of sulfated mucin species has been demonstrated immunochemically in isolated cervical mucin preparations and in cervical epithelia (Figure 1E). The rheological properties of cervical mucus vary during the menstrual cyclus, depending on the hormonal status (Wolf et al., 1977). Furthermore, histochemical techniques have indicated increased amounts of O-sulfated esters during the secretory phase, compared with the proliferative phase (Gilks et al., 1989). Also for saliva it has been shown that the mucin-bound sialic acid is doubled 5 days before luteinizing-hormone-preovulation surge (Calamera et al., 1986). Although it is tempting to explain the changed rheological properties of cervical mucus by the observed variations in sialylation and sulfation, there are no data confirming such a causal relationship.

Ocular Mucins Ocular mucins form the major component of secretion from goblet cells in the conjunctiva. Using *in situ* hybridization, MUC4 mRNA was detected in the cells of the stratified conjunctival epithelium, whereas MUC5 mRNA expression was limited to goblet cells. MUC4 and MUC5 probes did not hybridize to sections of corneal epithelium. MUC2, -3,-6 and -7 could not be detected (Inatomi et al., 1996). The reported sialic acid content of ocular mucins varies from 10.5% (Chao et al., 1988) to 22% (Moore and Tiffany, 1981). It has been reported that long-term use of contact lenses resulted in a decreased glycosylation of the mucins, and increased sulfatation (Fleiszig et al., 1994), resulting in reduced binding of *Pseudomonas aeruginosa* to the corneal surface.

Ear Mucins Brown et al. (1985) characterized biochemically the mucins in middle ear effusions of patients with

secretory otitis media. They found that the carbohydrate content varied from 31 to 69% of dry weight. The sialic acid content of ear mucins ranged from 6 to 12% and that of sulfate from 5 to even 30% (Table 4). These changes have been correlated with rheological activity; with higher degrees of glycosylation, gelation occurred at lower mucin concentration. However, no correlation between sialic acid content and rheological properties could be demonstrated.

Sulfomucins as Ligands for Selectins

The identification of a family of carbohydrate-binding proteins, termed selectins, have emphasized another class of mucins, whose members act as ligands for the selectins in cell adhesion processes (Shimizu and Shaw, 1993; Elangbam et al., 1997). The three known selectins have an N-terminal C-type lectin domain that binds specifically carbohydrates in a calcium-dependent way. L-selectin is expressed constitutively on leukocytes, whereas E-selectin is expressed upon activation of endothelial cells, and P-selectin is expressed by activated platelets and endothelial cells. Interaction of the mucins with selectins initiate weak tethering to the endothelium, which is followed by strong shear-resistant adhesion mediated by integrins (Springer, 1994). The participation of the selectins in inflammatory diseases (Rosen and Bertozzi, 1994; Carlos and Harlan, 1994; Lasky, 1995; Tedder et al., 1995) has stimulated interest in the nature of their carbohydrate ligands as leads for the development of anti-inflammatory agents. Three high endothelial venule (HEV) associated mucins have been identified as ligands for L-selectin: glycosylation-dependent cell-adhesion molecule 1 (GlyCAM-1) (Lasky et al., 1992), sialomucin CD 34 (Baumhüter et al., 1993), and mucosal-vascular-addressin cell-adhesion molecule 1 (MAdCAM-1) (Briskin et al., 1993). The ligand for P-selectin, referred to as P-selectin glycoprotein ligand 1 (PSGL-1) (Norgard et al., 1993; Sako et al., 1993) appears to be a dimeric glycoprotein, with large mucin domains. A 150 kDa glycoprotein E-selectin ligand 1 (ESL-1) on myeloid cells is believed to be the major ligand for E-selectin (Patel et al., 1994; Steegmaier et al., 1995). Structure-function studies showed that *in vitro* all three selectins recognize oligosaccharide structures related to the sialyl-Le^x antigen, and bind efficiently to an oligosaccharide with a sulfate replacement for the sialic acid (Brandley et al., 1993; Yuen et al., 1992, 1994). However, while all three selectins can recognize sialyl-Le^x, each displays distinct carbohydrate ligand specificity, e.g., colon cancer cells adhere differently to L-, E-, and P-selectin (Mannori et al., 1995).

L-Selectin Ligands

GlyCAM-1 is one of the two sulfated, sialylated and fucosylated mucins on endothelial cells of the HEV that act as ligands for L-selectin (Imai and Rosen, 1993; Imai et al.,

1993; Hemmerich *et al.*, 1994a). Structural analysis of the HEV-derived ligand GlyCAM-1 indicated a strict requirement for oligosaccharide sulfation. Sialic acid and fucose residues are also critical to the recognition determinants on GlyCAM-1. The sulfated mono- and disaccharides are identified as $\text{SO}_3\text{-}6\text{Gal}$, $\text{SO}_3\text{-}6\text{GlcNAc}$, $\text{SO}_3\text{-}6\text{Gal}\beta 1\text{-}4\text{-GlcNAc}$, $\text{Gal}\beta 1\text{-}4[\text{SO}_3\text{-}6]\text{GlcNAc}$ (Hemmerich *et al.*, 1994b). Further structural studies revealed that 6'-sulfated sialyl-Le^x and 6-sulfo-sialyl-Le^x are major capping groups of GlyCAM-1, and that 6',6-disulfo-sialyl-Le^x is present on GlyCAM-1 as well (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994b, 1995; Bertozzi *et al.*, 1995). Indeed, 6-sulfo-sialyl-Le^x shows enhanced *in vitro* L-selectin binding activity compared to sialyl-Le^x (Scudder *et al.*, 1994; Sanders *et al.*, 1996). By incorporating sulfate esters on the disaccharide lactose, on positions analogous to those of the sulfated sialyl-Le^x, it appeared possible to generate a simple molecule (6',6-disulfo-lactose) with greater inhibitory potency for L-selectin than sialyl-Le^x (Bertozzi *et al.*, 1995). Also a variety of non-mucin ligands with different characteristics has been described to be recognized by L-selectin, which includes fucoidan, a homopolymer of sulfated fucose, polyphosphomannan (PPME), sulfatides and heparan sulfate-containing proteoglycans (Ley *et al.*, 1993; Norgard-Sumnicht *et al.*, 1993; Norgard-Sumnicht and Varki, 1995; Shimaoka *et al.*, 1996). The importance of sulfate was further indicated by studies with L-selectin-Ig chimeras showing that binding to the sulfated sugar ligands is position-specific and depends on the number of the sulfate groups, i.e., $(\text{SO}_3)_3\text{-}3,4,6\text{Gal}- > (\text{SO}_3)_2\text{-}3,6\text{Gal}- > \text{SO}_3\text{-}3\text{Gal}- > \text{SO}_3\text{-}3\text{Glc}- > \text{SO}_3\text{-}6\text{Gal}- > \text{SO}_3\text{-}2\text{Gal}-$ (Suzuki *et al.*, 1993). Binding by L-selectin seems to prefer certain clustering and adequate spatial arrangements of the anionic groups (Varki, 1994; Yoshida *et al.*, 1994a, b; Crottet *et al.*, 1996).

E-Selectin Ligands

Chemically synthesized sulfated Le^a and Le^x oligosaccharides have been investigated for their ability to bind E-selectin, and for their ability to inhibit E-selectin binding to immobilized sialyl-Le^a, sialyl-Le^x, or sulfated Le^a pentasaccharides. From these studies, the sulfated Le^a oligosaccharides emerge as the most potent E-selectin ligands (Yuen *et al.*, 1994). Nevertheless, physiological binding to E-selectin of myelogenous leukemia cells and normal leukocytes was provided by monosialogangliosides, i.e. the so-called myeloglycan, unbranched terminally $\alpha(2\text{-}3)$ -sialylated polygalactosamine of at least 10 monosaccharide residues, with internal multiple fucosylation at GlcNAc (Stroud *et al.*, 1996a, b) and thus without a requirement for sulfation. The principal regulation step of E-selectin ligand synthesis in human T-cells seems to be fucosylation (Knibbs *et al.*, 1996).

P-Selectin Ligands

P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes functions as a high affinity ligand for P-selectin. Independ-

ently, different research groups demonstrated that three sulfated tyrosines on top of the mucin domains of PSGL-1 in conjunction with one sialylated and fucosylated glycan are essential to mediate high affinity binding to P-selectin (Wilkins *et al.*, 1995; Sako *et al.*, 1995; De Luca *et al.*, 1995; Pouyani and Seed, 1995). This glycan chain shows a strikingly low, if any, heterogeneity, while the other oligosaccharide chains present at the consensus repeats of the mucin domain of PSGL-1 do show the glycan heterogeneity characteristic for mucins. In this case these latter ones most probably function in keeping the molecule in an extended formation, while only one chain is a part of the binding site (Li *et al.*, 1996). PSGL-1 synthesized in the presence of sulfation inhibitors binds P-selectin only weakly (Pouyani and Seed, 1995). Rolling of HL-60 cells on P-selectin-coated coverslips is strongly attenuated by treatment of cells with an inhibitor of sulfation. E-selectin can also bind to PSGL-1, albeit with a lower affinity compared to P-selectin. This recognition is independent from the tyrosine sulfation and only requires sialic acid and fucose on O-glycans (Li *et al.*, 1996).

Concluding Remarks

Besides that sulfate residues on epithelial mucins provide protection against enzymatic degradation, no other specific function can be clearly assigned on basis of the presently known data. Nevertheless, there are numerous examples of modification of the degree of sulfation occurring under pathological conditions, e.g. in cystic fibrosis and in colon carcinomas. It has been suggested that this may lead to alterations in protective properties of the mucus layer against microbial invasion. The higher levels of sulfation reflect the upregulation of sulfated mucin species, which may be due to a phenotypic change of the mucin secreting tissue. This is the case in gastric metaplastic tissues where a change from a gastric to an intestinal cellular phenotype is accompanied by a change in expression of gastric mucins (scarcely sulfated) towards more colon mucins (highly sulfated). For a better understanding of such changes, the identification of the critical processes regulating the levels of sulfation in mucins is needed. It more and more becomes clear that mucous secretions constitute a mixture of genetically different mucin species (Table 1), but it is not known whether these intrinsically differ in their level of sulfation. Analysis of the sulfate contents of a single MUC species isolated from different parts of the body will yield clues to the question whether there is an intrinsic preference in sulfation of specific MUC species (e.g. MUC5B), or whether other processes, e.g. cell-specific expression of an appropriate sulfotransferase machinery, are in control. Antisera recognizing specific peptide domains on the basis of known DNA sequences of various MUC-genes are currently being developed and such tools will be of great value for isolation and characterization of single MUC species with respect to levels and patterns of sulfated oligosaccharides.

Sulfated carbohydrates on Glycam-1 appear to play an essential role in the recognition by L-selectin. Although *in vitro* both P- and E-selectin bind to sulfated oligosaccharides, there is no experimental data supporting an *in vivo* role for sulfated residues in recognition by selectins. By studying the localization of sulfated oligosaccharides using specific antibodies, indications can be obtained about the involvement of such structures in (other) recognition processes *in vivo*.

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Review

Small Stress Proteins: Chaperones that Act as Regulators of Intracellular Redox State and Programmed Cell Death

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Small stress proteins (sHsp) are molecular chaperones whose expression was shown to enhance the survival of mammalian cells exposed to numerous types of injuries that lead to death, including heat shock, oxidative stress as well as treatments with anti-cancerous and apoptosis-inducing agents. Here, a summary of the most recent results concerning the protective activity of this family of proteins against programmed cell death is presented. (1) sHsp enhance the survival of cells exposed to oxidative stress, a phenomenon which is linked to the ability of these proteins to decrease the intracellular level of reactive oxygen species in a glutathione dependent way. (2) sHsp protect against apoptosis mediated by different agents including staurosporine, etoposide and the Fas ligand. (3) An interesting and particular aspect of sHsp concerns their transient expression during the cell division to differentiation transition. In this context, sHsp expression was shown to be essential for preventing differentiating cells from undergoing apoptosis. Small stress proteins appear therefore as novel regulators that interfere with programmed cell death induced by different pathways.

Key words: Apoptosis / Chaperone / Differentiation / Glutathione / Hsp27 / Necrosis / ROS / Small stress proteins.

Introduction

Investigations of the cellular response to heat shock and other type of physiological stresses have allowed the identification of families of proteins (the heat shock or stress proteins, Hsp). Among them, the Hsp90, Hsp70 and Hsp60 families contain proteins that display *in vitro* chaperone function(s) (Georgopoulos and Welch, 1993; Morimoto *et al.*, 1994). Another group of proteins concerns the small heat shock (or stress) proteins (sHsp) which are characterized by an *in vitro* ATP-independent chaperone activity (Jakob *et al.*, 1993; Jakob and Buchner,

1994). The family of sHsp encompasses a large number of related protein species, several of which are phosphoproteins which are characterized by a domain of homology to α A, B-crystallin proteins from the vertebrate eye (reviewed in Arrigo and Landry, 1994) (see Figure 1). Actually, sHsp generate renewed interest because these proteins may not only be involved in cellular protection against aggression, but also in essential physiological processes in unstressed cells. For example, sHsp are expressed during the cell division to differentiation transition and at specific stages during development (reviewed in Arrigo and Tanguay, 1991; de Jong *et al.*, 1993; Arrigo and Mehlen, 1994; Arrigo, 1995). Moreover, sHsp expression was found to correlate with the oncogenic status of the cell (Tétu *et al.*, 1992).

An interesting biochemical property of sHsp, which is also shared by α -crystallin, concerns the ability of these proteins to form large oligomers (200 to 800 kDa). This is a dynamic process which depends on the physiology of the cells, whether cells are exposed to stress, and probably also on the phosphorylation status of these proteins (Siezen *et al.*, 1978; Arrigo, 1987; Arrigo and Welch, 1987; Arrigo *et al.*, 1988; Mehlen and Arrigo, 1994; Kato *et al.*, 1994; Lavoie *et al.*, 1995; Mehlen *et al.*, 1995b). Studies performed with cells overexpressing sHsp have revealed the enhanced resistance of these cells to heat shock (Landry *et al.*, 1989). This resistance may result from the ability of sHsp to accelerate the recovery from the heat-induced shut-off of RNA and protein synthesis (Carper *et al.*, 1997) and nuclear protein aggregation (Kampinga *et al.*, 1994). Other reports have pointed out that sHsp may

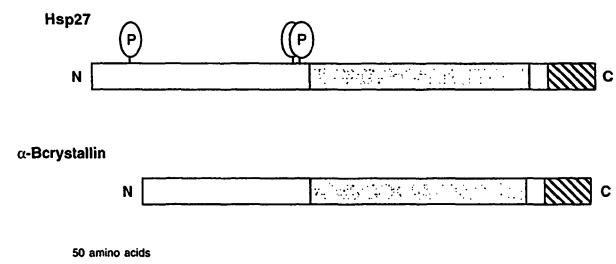


Fig. 1 Schematic illustration of Human Hsp27 and α -Bcrystallin Proteins.

The gray box represents the conserved crystallin domain and the hatched box represents the C-terminal flexible extension. Phosphorylatable sites of Hsp27 (serines 15, 78 and 82) are indicated. Based on the sequences published by Ingolia and Craig, (1982); Hickey *et al.* (1986), Carver *et al.* (1992, 1995).

also counteract the stress-induced disruption of the actin microfilament network (Miron *et al.*, 1991; Lavoie *et al.*, 1993; Huot *et al.*, 1996) and that α -crystallin could act as a molecular chaperone toward intermediate filaments (Nicholl and Quinlan, 1994). More recent studies have pointed out that the molecular mechanism by which sHsp protect against heat shock probably implies the interaction of non-native proteins with the large sHsp oligomers that accumulate during heat shock (Ehrnsperger *et al.*, 1997; Lee *et al.*, 1997). This phenomenon is thought to create a reservoir of folding intermediates that prevents further aggregation of non-native proteins and enhances their refolding by other chaperones, such as Hsp70. sHsp also confer protection against a variety of toxic chemicals used in cancer chemotherapy, i.e. cisplatin and doxorubicin, (Landry *et al.*, 1989; Huot *et al.*, 1991; Oesterreich *et al.*, 1993; Richards *et al.*, 1996; Garrido *et al.*, 1996; 1997), and recently we and others have reported that these proteins act as novel negative regulators of programmed cell death (Mehlen *et al.*, 1996a, b; Samali and Cotter, 1996; Mehlen *et al.*, 1997a, b).

The present review summarizes the most recent observations concerning the protective activity of sHsp against programmed cell death and discusses some important points in consideration of its physiological role.

Small Stress Proteins Protect against TNF α and Other Inducers of Oxidative Stress

Tumor necrosis factor (TNF α) is a pro-inflammatory cytokine which, *in vivo*, allows the elimination of many potentially harmful cancerous cells by inducing their suicide. TNF α is also cytotoxic for several transformed cells grown *in vitro*. Several research groups have reported that one of the early cellular events following the binding of TNF α to its receptor is the phosphorylation of 27 kDa polypeptides (Robaye *et al.*, 1989; Schutze *et al.*, 1989; Kaur and Saklatvala, 1988; Kaur *et al.*, 1989) which have been identified as phospho-isoforms of the mammalian small stress protein Hsp27 (Arrigo, 1990). It was first speculated that this protein might play a role in TNF α signal transduction. However, further analysis suggested that Hsp27 phosphorylation reflects some kind of cellular response to the cytotoxic action mediated by this cytokine. Indeed, expression of human Hsp27 in murine L929 fibrosarcoma or NIH 3T3-ras fibroblasts, which are highly TNF α -sensitive and devoid of endogenous sHsp expression in absence of heat shock, interfered with the cellular death induced by this cytokine (Mehlen *et al.*, 1995a, 1995b). A similar protective effect was observed in cells treated with TNF α in the presence of actinomycin D, suggesting that the protective activity of Hsp27 was not an indirect effect mediated by the transcription of specific genes. A protective activity of Hsp27 against TNF α was also reported by Wang *et al.* (1996) who also showed that Hsp27-expressing L929 clones released less arachidonic acid in response to this cytokine. The finding that other sHsp such as *Drosophila*

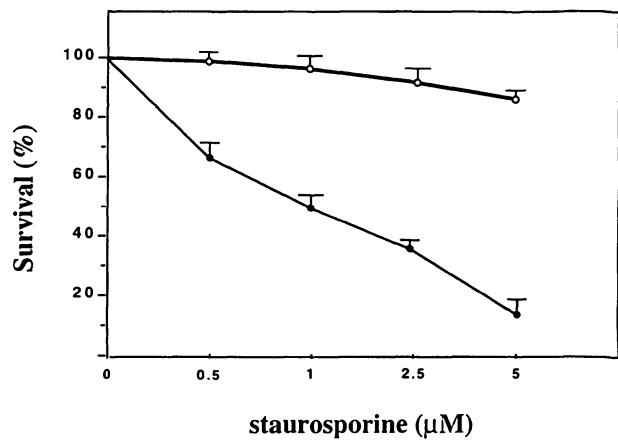


Fig. 2 Constitutive Expression of Human Hsp27 Interferes with Staurosporine-Mediated Apoptotic Death of Murine L929 Cells. Control and human Hsp27 expressing murine L929 cells were either kept untreated or treated for 24 h with increasing concentrations of staurosporine (0.5–5 μ M). Cell survival was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The values were normalized to 100% using the respective cells not treated with staurosporine. Dark circles: control cells, open circles: human Hsp27-expressing cells. Note the strong protection against apoptosis mediated by Hsp27 expression.

DHsp27, murine Hsp25/27 or even human α B-crystallin were also active allowed us to conclude that the protective function of sHsp against TNF α is localized in the conserved 'crystallin domain' shared by these proteins (Mehlen *et al.*, 1995a, 1995b) (Figure 1).

Stimulation of TNF α -receptors rapidly rises the intracellular level of reactive oxygen species (ROS), presumably because of mitochondria dysfunction (Yamauchi *et al.*, 1990; Schulze-Osthoff *et al.*, 1992, 1993; Goossens *et al.*, 1995; Mehlen *et al.*, 1995c). At low concentration, ROS can modulate gene expression (Schreck *et al.*, 1991; Schmidt *et al.*, 1995; Kretz-Remy *et al.*, 1996); however, when their concentration is too high, as is the case in TNF α treated L929 cells, they directly cause oxidative injuries that can lead to cell death (Wong *et al.*, 1989; Yamauchi *et al.*, 1990; Matsuda *et al.*, 1991; Schulze-Osthoff *et al.*, 1992; Hirose *et al.*, 1993; Mayer and Noble, 1994; Buttke and Sandstrom, 1994; Goossens *et al.*, 1995). Consequently, anti-oxidant drugs or the overexpression of detoxifiant enzymes inhibit TNF α cytotoxicity (Wong *et al.*, 1989; Mayer and Noble, 1994; Buttke and Sandstrom, 1994; Schulze-Osthoff *et al.*, 1992, 1993; Schmidt *et al.*, 1995; Goossens *et al.*, 1995). The finding that sHsp expression enhances the cellular resistance to hydrogen peroxide or menadione (Huot *et al.*, 1991; Mehlen *et al.*, 1993; Huot *et al.*, 1996; Mehlen *et al.*, 1996a) led to the hypothesis that these proteins protect against TNF α by inhibiting ROS action. However, no *in vitro* ROS detoxifiant activity were found associated with sHsp (Mehlen *et al.*, 1996a).

sHsp Modulate Intracellular Redox

Studying the molecular mechanisms responsible for the protective activity of sHsp against TNF α , we have observed that in untreated murine L929 and NIH 3T3 cells, the expression of these proteins decreased the intracellular level of ROS. Moreover, sHsp expression inhibited the burst of these reactive species in response to TNF α . Consequently, several down-stream effects induced by ROS were no more observed in TNF α -treated sHsp-expressing L929 cells, such as lipid peroxidation, protein oxidation and the activation of the transcription factor NF- κ B (Mehlen et al., 1996a). The decline in the level of ROS and the protection against TNF α mediated by sHsp were found to depend on the detoxificant molecule glutathione (Mehlen et al., 1996a). This tripeptide, which is the major source of cellular thiol (Meister and Anderson, 1983), protects cells from oxidative injuries (Yamauchi et al., 1990). As a consequence, glutathione depleting drugs, such as buthionine SL sulfoximine (BSO), a specific and essentially irreversible inhibitor of γ -glutamyl-cysteine synthetase, and diethyl mallate (DEM), a compound that binds the free sulfhydryl groups of glutathione, lead to cell death (Kane et al., 1993) or render cells more susceptible to oxidative stress-induced cell death (Zhong et al., 1993). Interestingly, sHsp expression did not protect against the oxidative stress mediated by BSO or DEM (Mehlen et al., 1996a), suggesting that glutathione is essential for sHsp protective function. Another intriguing observation was that sHsp expression increased the cellular content of glutathione until a plateau value was reached (Mehlen et al., 1996a). This particular increase in glutathione was only detected in cells that are normally devoid of constitutively expressed sHsp, as for example murine L929 or NIH 3T3 cells. In other cell types which already contain high levels of endogenous sHsp, the glutathione level was not altered by sHsp overexpression, probably because the maximum level of glutathione tolerated by cells was already reached. In unstressed L929 cells, sHsp expression was not found to modify the reduced – to – oxidized ratio of glutathione (Mehlen et al., 1996a). However, preliminary data suggest that these stress proteins induce glutathione to remain in its reduced form during or immediately after oxidative stress. How sHsp expression decreases ROS levels and stimulates glutathione to remain in its reduced form, hence stimulating the reducing power of the cell, is not yet known. Since, *in vitro*, sHsp behave as protein chaperones, it is possible that *in vivo* they act as specialized chaperones toward enzymes involved in the ROS-glutathione pathway, such as glutathione reductase, Se-glutathione peroxidase, glutathione transferase or glucose 6-phosphate dehydrogenase. In this respect, it is interesting to mention that the small stress protein α B-crystallin protects glucose-6-phosphate dehydrogenase and 6 phosphogluconate against inactivation by glycation or carbonylation (Gaena and Harding, 1995, 1996). Another possible mode of action of sHsp may be through the stimulation of the degradation of proteins that are oxidized by ROS.

Some information concerning the mode of action of sHsp have emerged from the analysis of the structural organization of mammalian Hsp27. It was observed that during the first two hours of TNF α treatment, a transient and drastic increase in the native molecular mass of most Hsp27 molecules (up to 800 kDa) occurred. Then, by four hours of treatment, the native size of this stress protein drastically regressed (< 200 kDa). During this dynamic phenomenon the phosphorylated isoforms of Hsp27 remained concentrated in the small or medium-sized oligomers (< 300 kDa) of this protein (Mehlen et al., 1995b). This transient shift toward large oligomers was not observed in cells overexpressing the detoxificant enzyme glutathione peroxidase, suggesting that the intracellular burst of ROS generated by TNF α may play an important role in this phenomenon (Mehlen et al., 1995c). Using Hsp27 non-phosphorylatable mutants or SB203580, an inhibitor of P38 kinase, we recently observed that the large aggregates of Hsp27 represent the active form of the protein which protects against TNF α . It was also found that phosphorylation, by inducing Hsp27 to concentrate in the form of small oligomers, leads to the inactivation of Hsp27 protective activity. These observations correlate with the fact that the large oligomers of Hsp27 are those which modulate ROS and glutathione levels (Mehlen et al., 1997b; X. Preville, M., Gaestel, and A.-P. Arrigo, manuscript submitted) and display *in vitro* chaperone activity (Jacob and Buchner, 1994).

sHsp Protect against Apoptosis

There exist essentially two cellular death processes:

- (i) Necrosis or death by explosion of cells following a toxic stimulus, and
- (ii) apoptosis, which corresponds to a more physiologic elimination of cells following a defined program.

Apoptosis, which is ubiquitous and drastically controlled during ontogenesis, plays a fundamental role in the maintenance of the integrity of organisms (Raff, 1992; White, 1996; Vaux and Strasser, 1996; Martins and Earnshaw, 1997). Apoptosis can also be induced *in vitro* in tissue – cultured cells by inducers, such as the Fas/APO-1/CD95 ligand, the protein kinase inhibitor, staurosporine, or cellular conditions, such as growth factor deprivation. Having shown that sHsp interfered with the cellular death induced by oxidative stress inducers, i.e. TNF α or hydrogen peroxide, which, in our experimental system, was essentially linked to a necrotic process, we have studied sHsp in the context of apoptosis generated by the stimulation of the Fas/APO-1/CD95 membrane receptors or by exposing cells to staurosporine. Fas/APO-1 receptors are related to TNF α receptors but the pathways and the type of death induced by their stimulation are different (Schulze-Osthoff et al., 1994). Moreover, *in vivo*, the ligands have different functions. TNF α is very efficient in the necrotic eradication of tumor cells, while Fas/APO-1 eliminates specific and non-pathological cells, for example in the maturation and amplification of the immune system.

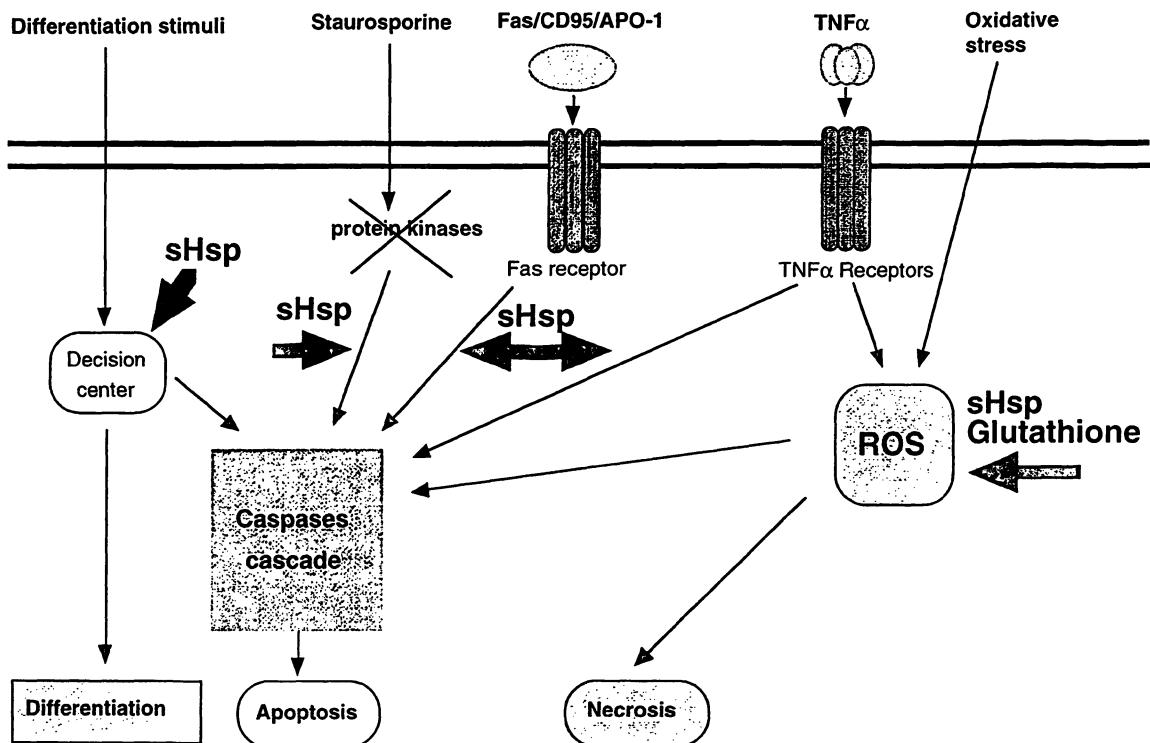


Fig. 3 Schematic Representation of the Different Pathways Influenced by the Expression of Small Stress Proteins. sHsp expression interferes, through a glutathione-dependent pathway, with the intracellular burst of ROS induced by oxidative stress or TNF α . This phenomenon, which may result from the chaperone activity of sHsp, inhibits ROS-triggered necrosis or apoptosis. sHsp also protects against apoptotic phenomena that are induced independently of ROS, i.e. apoptosis induced by Fas/APO-1/CD95 ligand or staurosporine. The molecular mechanism that underlies sHsp protective activity against apoptosis is unknown but probably occurs upstream of the caspase cascade. sHsp also interferes with apoptosis during early differentiation. In this case, sHsp probably act at the level of the decision center which controls the cell division to differentiation transition.

In L929 clones that constitutively express the Fas receptor, we have shown that the expression of human Hsp27 inhibited the apoptotic process mediated by the stimulation of the Fas receptor (Mehlen et al., 1996b). Hsp27 expression interfered also with the apoptotic death mediated by staurosporine (Mehlen et al., 1996b; see Figure 2). A protective effect mediated by sHsp against apoptosis was also observed by Samali and Cotter (1996) in U937 and Wehi-s cells exposed to actinomycin D, camptothecin and etoposide. The molecular mechanism that underlies the protective activity of sHsp against apoptosis is unknown, although preliminary results indicate that Hsp27 may act upstream of the caspase cascade (Figure 3). Moreover, it is not yet known whether sHsp act solely or in concert with other chaperones since the major heat shock (or stress) protein Hsp70 has recently been reported to protect against different types of stress-induced apoptosis (Mosser et al., 1997).

An interesting aspect of sHsp concerns their accumulation during the early phase of several processes of differentiation and at specific stages of development (Pauli et al., 1990; Spector et al., 1993, 1994; and reviewed in Arrigo and Mehlen, 1994; Arrigo, 1995). This phenomenon, which appears ubiquitously in eukaryotic cells, is characterized by a transient expression of sHsp during the cell division to differentiation transition. Recently, anti-sense cDNA technology, that resulted in a partial or almost

total inhibition of the expression of Hsp27, was used to investigate the biological significance of this physiological sHsp expression. It was first observed that a partial inhibition of the accumulation of Hsp27 interfered with the granulocytic differentiation of human promyelocytic HL-60 cells (Chaufour et al., 1996). Moreover, an almost complete inhibition of Hsp27 expression was found to abort the differentiation of murine embryonic stem (ES) cells through an exacerbation of the apoptotic process which is inherent to this phenomenon (Mehlen et al., 1997a). Hence, Hsp27 represents one of the first known proteins that control the division to differentiation transition of mammalian cells by modulating the apoptotic process.

Several reports have pointed out the importance of the redox modulator glutathione during apoptosis. For example, apoptosis provokes a powerful efflux of reduced glutathione to the exterior of the cell (van den Dobbelsteen et al., 1996) and/or induces its oxidation independently of ROS production (Slater et al., 1995; Ghibelli et al., 1995; Beaver and Waring, 1995). The apoptotic process can therefore be delayed by an artificial increase in the cellular content of reduced glutathione (van den Dobbelsteen et al., 1996). During ES cell differentiation, we have observed that the inhibition of Hsp27 accumulation attenuated the transient increase in glutathione which occurs during early differentiation and which is time correlated to Hsp27 expression (Mehlen et al., 1997a). However, the relationship

that may exist between Hsp27 and glutathione during apoptosis is unknown.

sHsp now emerge as novel negative regulators of programmed cell death. These proteins differ from other anti-apoptotic proteins such as those belonging to the IAP (inhibitor of apoptosis) (Clem and Duckett, 1997) or Bcl-2 (Hockenberry et al., 1990; Reed, 1997) families of proteins. However, in spite of the fact that both sHsp and Bcl-2 modulate ROS and glutathione levels (Kane et al., 1993; Mehlen et al., 1996a), their modes of action are not similar. Indeed, no chaperone activity has been described for Bcl-2, which, in contrast to Hsp27 (Mehlen et al., 1996a), can protect against BSO-mediated cell death (Kane et al., 1993). Moreover, Hsp27 is not a mitochondria-associated protein (Arrigo and Landry, 1994).

The anti-cell death function of sHsp suggests an implication of these proteins in the regulation of the integrity of the organism. Indeed, a variation in the level of expression of Hsp27 could entail a modulation of the reaction of cells in the face of cell death stimuli. An interesting example concerns the important cellular death process which occurs during the maturation of B and T lymphocytes. This phenomenon allows the elimination of undesirable cells via mechanisms involving, among others, the Fas/APO-1 signal. Interestingly, a transient accumulation of Hsp27 takes place during the maturation of B or T cells (Spector et al., 1992; Hanash et al., 1993), suggesting that, *in vivo*, this protein may allow some of these cells to escape apoptosis, giving rise, for example, to memory cells. Similarly, it is interesting to observe that organs where the Fas receptor/ligand is abundantly present (gonads, nervous system) also contain high levels of constitutively expressed sHsp. In addition, since *in vivo* TNF α provokes the destruction of some cancerous cell types, the expression of Hsp27 could be envisioned either as an element allowing healthy cells not to be destroyed by TNF α or, on the contrary, as an element allowing some cancerous cells to escape the immunosurveillance mediated by this cytokine. In this respect, it has recently been reported that sHsp expression modulates the growth of tumors that originated from L929 fibrosarcoma implanted in nude mice (Blackburn et al., 1997). In collaboration with C. Garrido and B. Chauffert (INSERM, Dijon, France) we have also undertaken an *in vivo* approach which reveals that, following implantation in syngeneic rats, rat colon carcinoma cells that constitutively express high levels of human Hsp27 formed more aggressive tumors that were protected against apoptosis than cells which do not contain this protein. Therefore, *in vivo* sHsp also appear to act as negative regulators of apoptosis; a property which suggests that these proteins are important modulators of tumorigenesis.

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