The effect of cancer on the synthesis and carbohydrate composition of serum α_1-antichymotrypsin

By

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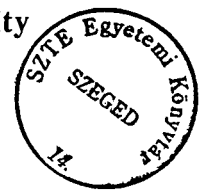

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Abstract

Protease inhibitors, have been previously used as biochemical tumour markers for the diagnosis and monitoring of cancer. In this study the level of α_1-antichymotrypsin (α_1-ACT) in serum of patients with ovarian or breast cancer was studied. A significant increase of α_1-ACT was found in both cancer groups (breast cancer; 173 ± 15% ovarian cancer; 228.6 ± 49%) as compared to healthy individuals.

As a model system, liver cell culture (Hep G2) was used for studying the mechanisms governing the alterations of α_1-ACT concentration. The addition of conditioned media from five ovarian cancer cell lines stimulated (240%) α_1-ACT synthesis in Hep G2 cells. This increase was similar to that observed in the sera from ovarian cancer patients. The α_1-ACT biosynthesis was also influenced be several cytokines (TGF-β, TGF-α and IL-6). The effect of one cytokine could be modulated by other cytokines, which supported the view that the alteration in the level of α_1-ACT was modulated by combinations of cytokines.

Quantitative changes in serum glycoproteins are often accompanied by alterations of their carbohydrate moieties. Therefore, the microheterogeneity of α_1-ACT was examined in sera from healthy individuals, ovarian and breast cancer patients using crossed immunoelectrophoresis (CIAE). The microheterogeneity of α_1-ACT did not alter in ovarian and breast cancer sera. Using lectin blotting, α_1-ACT was shown to contain NeuAc(α2,6)Gal, NeuAc(α2,3)Gal, terminal Galβ-(1,4)GlcNAc and α1,6 or α1,3 linked fucose residues. There were no differences between the normal and cancer sera in the composition of the carbohydrate structures. Neither the number of antennae on the heteroglycan structures nor the oligosaccharide content of α_1-ACT changed.

The elevated α_1-ACT level could be useful among other laboratory tests in the early diagnosis of ovarian and breast cancer.

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Abbreviations

α_1-ACT	α_1-antichymotrypsin
α_1AGP	α_1-acid glycoprotein
Asn	Asparagine
α_1-AT	α_1-antitrypsin
B	Breast cancer sera
BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CIAE	Crossed immunoaffinoelectrophoresis
Con A	Concanavalin A
Da	Dalton
D-Gal	D-galactose
D-Man	D-mannose
DMEM	Eagles medium Dulbeccos` modification
dolichol-P	Dolichol-phosphate
DSA	Datura stramonium lectin
FCS	Foetal calf serum
Fuc	Fucose
Gal	Galactose
GalNac	N-acetylgalactosamine
GlcNAc	N-acetyl-D-glucosamine
Glu	Glucose
Gp I	Glycoprotein I
Gp II	Glycoprotein II
Hep G2	Human hepatoma cell line
Hp	Haptoglobin
IL-1	Interleukin-1
IL-6	Interleukin-6
MAA	Maackia amurensis agglutinin
M	Molar
Man	Mannose
mg	Milligram
ml	Millilitre
mM	Millimolar
Mw	Molecular weight
µg	Microgram
µl	Microlitre
N	Normal or Healthy or Control sera
NBT	Nitro blue tetrazolium
NeuAc, (NANA)	N-acetylneuraminic acid (sialic acid)
NMR	Nuclear magnetic resonance spectroscopy
O	Ovarian cancer sera
Pro	Proline
RCA	Ricinus communis agglutinin
RID	Radial Immunodiffusion

SA	Serum albumin
SD	Standard deviation
Ser	Serine
Serpin	Serine-proteinase inhibitor
SNA	Sambucus nigra agglutinin
Tf	Transferrin
Thr	Threonine
TNF-α	Tumour necrosis factor-α
TGF-β	Transforming growth factor–β
v/v	Volume per unit volume
w/v	Weight per unit volume

Contents

1 Introduction

This chapter briefly introduces the structure, synthesis and role of glycoproteins and the biology and biochemistry of α_1-ACT. It also contains information about the lectins which have been used for the investigation of the carbohydrate structure of α_1-ACT.

1.1 Structural and functional aspects of glycoproteins

The vast majority of the plasma proteins circulate as mature glycoproteins. Glycoproteins are conjugated macromolecules composed of a protein moiety and a carbohydrate moiety. The protein moiety is characterised by its amino acid composition. It is often referred to as the (poly) peptide chain or backbone. The carbohydrate moiety consists of oligosaccharide chains covalently linked to the polypeptide backbone.

1.1.1 Classification of sugar chains

The carbohydrate chains of glycoproteins are classified according to the linkage between the sugar and amino acid (Kornfeld and Kornfeld, 1980). The type most commonly found in mammalian protein-bound oligosaccharides is an N-glycosidic linkage between an asparagine residue (Asn) in the polypeptide and an N-acetyl-D-glucosamine (GlcNAc) residue in the oligosaccharide (Fig. 1a). Such oligosaccharides occur in secreted glycoproteins with many diverse functions, e.g. enzymes, plasma proteins, immunoglobulins and hormones.

Three other linkages, all of them O-glycosidic type, occur in mammalian glycoproteins. O-linked oligosaccharides with N-acetyl-D-galactosamine, linked

either to serine or threonine residues, (Fig. 1b) are found primarily in mucins lining the mucous epithelia of the respiratory, genito-urinary and gastro-intestinal system. These also occur together with N-linked oligosaccharides in immunoglobulins, glycophorin and other molecules. Collagens and basal membranes contain short carbohydrate chains (glycosyl-galactosyl-hydroxylysine and galactosyl-hydroxylysine) which are not found in other glycoproteins. Many proteoglycans have been shown to carry oligosaccharides attached to polypeptide by xylosyl-serine linkage (Schachter, 1984).

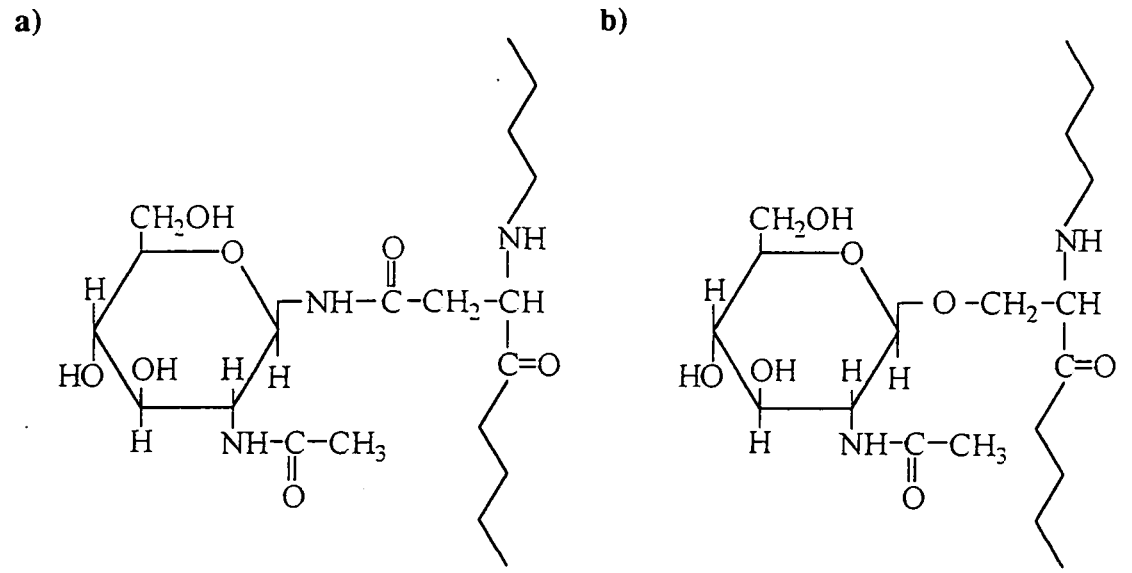


Fig. 1. The N-glycosidic (a) and O-glycosidic bonds (b). Oligosaccharides may be attached to the polypeptide core via an N-glycosidic bond involving N-acetylglucosamine and asparagine or an O-glycosidic bond involving N-acetylgalactosamine and either serine (pictured) or threonine (Martinez and Barsigian, 1987).

However, the vast majority of serum glycoproteins are exclusively N-glycosylated and for this reason only the biosynthesis of N-linked oligosaccharides will be discussed in detail.

1.1.2 N-linked oligosaccharide biosynthesis

The biosynthesis of N-linked glycoproteins is frequently described as proceeding through three sequential phases which includes: *(a)* dolichol mediated oligosaccharide assembly, *(b)* oligosaccharide linkage to the polypeptide, and *(c)* oligosaccharide processing with addition of peripheral sugars (Kornfeld and Kornfeld, 1985, Snider, 1984).

1.1.2.1 Dolichol-mediated oligosaccharide assembly

N-linked glycoproteins are not synthesised by the simple step-wise addition to a protein. Rather, individual monosaccharides are first assembled into a high mannose structure on a carrier lipid and the entire structure is then transferred in a single step to the peptide (Snider, 1984).

Attachment of the first sugar involves the transfer of GlcNAc from UDP-GlcNAc to dolichol-P, resulting in the formation of GlcNAc-P-P-dolichol. After the linkage of the first GlcNAc, a second GlcNAc is added, yielding $(GlcNAc)_2$-P-P-dolichol. Next, five individual mannose units are transferred from GDP-Man to the $(GlcNAc)_2$-P-P-dolichol. The first Man is linked in β-configuration, while the remaining four are linked in α configuration. Four additional Man are again added in α-configuration, resulting in the formation of high-mannose intermediate. These last four Man are transferred from dolichol-P-Man rather than from the nucleotide derivative. The final step in the assembly of the dolichol-oligosaccharide intermediate involves the transfer of three glucose (Glc) moieties from dolichol-P-Glc to one of the Man branches.

1.1.2.2 Oligosaccharide linkage to the polypeptide

It appears that $(Man)_5(GlcNAc)_2$-P-P-dolichol is assembled on the cytosolic surface of the rough endoplasmic reticulum, and that the more mature form of the lipid precursor $(Glc)_3(Man)_9(GlcNAc)_2$-P-P-dolichol is oriented toward the lumenal surface (Snider and Robbins, 1982). This suggests that the partially glycosylated lipid may be translocated to the lumen via a mechanism which is mediated by a transmembrane enzymatic complex (Hanover and Lennarz, 1982). Under normal conditions, $(Glc)_3(Man)_9(GlcNAc)_2$ is transferred in a single step to the growing nascent polypeptide, though other precursors with various numbers of Man can also be transferred (Chapman et al. 1979, Stoll et al. 1982).

The entire linkage process appears to depend mainly on three factors: *(a)* the presence of a fully glycosylated dolichol-intermediate, *(b)* adequate levels of the oligotransferase enzymes, and *(c)* the availability of accessible Asn-X-Ser/Thr/Cys sequences for participation in the formation of the N-glycosidic bond (Martinez and Barsigian, 1987).

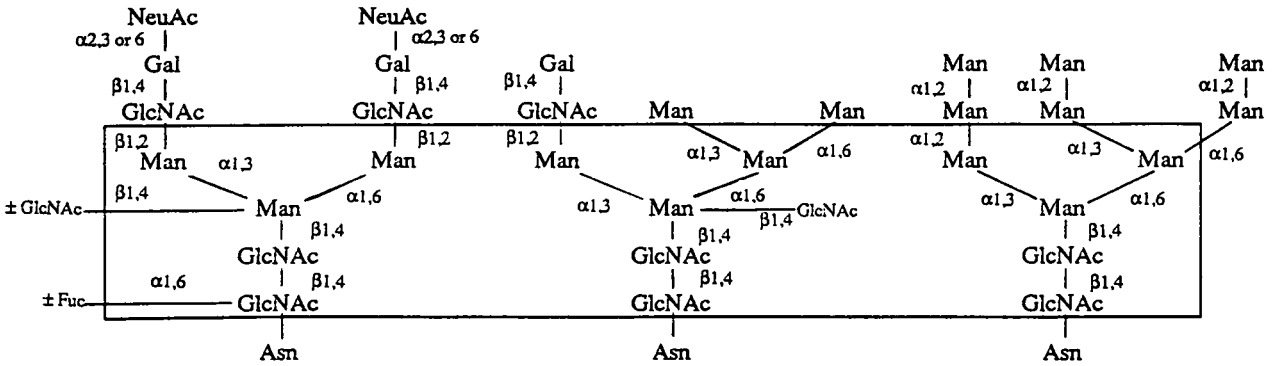
1.1.2.3 Oligosaccharide processing with addition of peripheral sugars

The trimming of monosaccharides and the addition of peripheral sugars is an orderly process that is mediated by glycosidases and transferases present in the endoplasmic reticulum and Golgi apparatus. Immediately after linkage of the high-mannose oligosaccharide $(Glc)_3(Man)_9(GlcNAc)_2$ to the polypeptide, oligosaccharide processing is initiated by the removal of the outer Glc by glucosidase I, followed by the sequential removal of the second and third Glc units by another glucosidase, glucosidase II. Processing continues during migration of the glycoprotein through the endoplasmic reticulum. The four outer α1,2-linked Man

can be cleaved by mannosidases present in the endoplasmic reticulum, or by mannosidase I which is present in the cis compartment of the Golgi.

The oligosaccharide $(Man)_5(GlcNAc)_2$ becomes the main precursor for the formation of complex carbohydrates. The first step in the addition of peripheral sugars is the linkage of one GlcNAc to outer Man of branch I. Linkage of GlcNAc is associated with activation of a mannosidase, mannosidase II, which subsequently cleaves the two remaining peripheral Man, resulting in the formation of $GlcNAc(Man)_3(GlcNAc)_2$, an oligosaccharide that can serve as a substrate for other GlcNAc transferases which are capable of initiating various patterns of branching.

The high mannose oligosaccharide $GlcNAc(Man)_5(GlcNAc)_2$ is located at a biosynthetic crossroad, in the sense that is susceptible to several alternative enzymatic modifications, which can potentially result in the formation of glycoproteins possessing either hybrid or complex carbohydrates (Fig. 2.). In general, chain elongation and processing of N-linked oligosaccharides occurs by the sequential transfer of Gal followed by sialic acid (NeuAc) or fucose (Fuc). However, for some glycoproteins, NeuAc or Fuc are not added and the Gal remains the terminal monosaccharide (Kornfeld and Kornfeld, 1985, Martinez and Barsigian, 1987, Snider, 1984).



Complex *Hybrid* *High Mannose*

Fig. 2. Structures of the major types of asparagine-linked oligosaccharides. The boxed area encloses the pentasaccharide core common to all N-linked structures (Martinez and Barsigian, 1987).

1.1.3 Microheterogeneity in glycoproteins

Microheterogeneity in glycoproteins means that carbohydrate chains differing in several structural respects are present at the same site at which a carbohydrate is attached to a protein molecule (Iwase et al. 1988).

Two types of microheterogeneity have been distinguished: *major microheterogeneity*, which reflects differences in the number of antennae on the heteroglycan structures and *minor microheterogeneity*, referring to variations in sialic acid or fucose content. Major microheterogeneity of plasma glycoproteins may be determined indirectly by CIAE employing Con A as a ligand (Mackiewicz and Kushner, 1989).

1.1.4 Roles of glycosylation

Recent studies of the function of glycoprotein glycans indicate that many glycoproteins have specific biological functions which are directly influenced by their glycosylation. A comparison of biological properties associated with the carbohydrate moiety and the polypeptide moiety of glycoproteins are shown in Table 1.

Table 1. Some of the carbohydrate- and polypeptide-associated functions of glycoproteins (West, 1986).

Carbohydrate-associated functions	Polypeptide-associated functions
Clearance of serum proteins	Catalysis
Hormone action	Hormone action
Antigenicity	Antigenicity
Immunogenicity	Immunogenicity
Cell adhesion	Cell adhesion
Signal for protein targeting	Transport
Immunosuppression	Specific binding

1.2 The biology and biochemistry of α_1-antichymotrypsin

1.2.1 α_1-ACT as a member of the serpin super family

α_1-ACT is a member of the super family of proteins denoted serpin-serine inhibitors. There are two subfamilies within the serpins; one subfamily contains proteins for which no cognate serine proteases have yet been identified and this subfamily is represented archetypally by ovalbumin, while the second subfamily contains members of which at least one serine protease can be found as the inhibitory target.

α_1-ACT belongs to the second subfamily with a relatively narrow spectrum of activity, inhibiting chymotrypsin (Laine et al. 1984), pancreatic elastase (Laine et al. 1985, Davril et al. 1987), mast-cell chymase and cathepsin G (Laine et al. 1982a, 1982b). α_1-ACT plays a role in the modulation of the immune response (Matsumoto et al. 1981), but does not modify natural killing cytotoxicity (Laine et al. 1990). α_1-ACT circulates in the plasma as a 55,000-66,000 Dalton glycoprotein with heterogeneity in the glycosylation pattern as well as in the presence or absence of two amino acid residues at the N terminus of the mature protein. There do not appear to be any biochemical differences that can be attributed to the heterogeneity.

1.2.2 Tissue localisation and regulation

α_1-ACT is synthesised predominantly in the liver and is one of the acute phase reactants; with levels rising rapidly to more than fivefold, in response to a wide variety of injuries, including surgery, acute myocardial infarctions, burns, autoimmune diseases, malignancies, infections and liver allograft rejection (Calvin et al. 1988, Hachulla et al. 1988, Shakespeare et al. 1989, Maury et al. 1988). The rise in α_1-ACT is an earlier response than that of other acute phase reactants, including α_1-AT. The mechanism of increase is related to the levels of IL-1, IL-6, TNF-α and TGF-β (Mackiewicz et al. 1991). Other studies indicate that synthesis is developmentally regulated, with mean plasma concentrations increasing from foetal levels to those found during the neonatal period, to those in young children, and finally reaching adult levels of approximately 5-7μM (Laurell et al. 1975).

In addition to synthesis in the liver, α_1-ACT is detected by immunoperoxidase staining in histiocytic cells, mast cells and endothelial cells (Papadimitriou et al. 1980), normal breast epithelial cells (Gendler et al. 1982), a breast cancer cell line MCF-7 (Laursen and Lykkesfeld, 1992), and in some tumour cells (Gaffar et al. 1980, Tahara et al. 1984, Takada et al. 1986) where it appears to

be taken up from the serum. In certain of these malignant cells (Kondo and Ohasawa, 1982), α_1-ACT has been localised by immunohystochemistry to the nucleus, a unique feature of this particular serpin.

New and intriguing information has linked α_1-ACT to the plasticity of the nervous system (Kanai et al. 1991, Mizuguchi and Kim, 1991). α_1-ACT is associated with β-amyloid deposits in Alzheimer's disease (Gollin et al. 1992), in aging brain, Down's syndrome and in the Dutch variant of hereditary cerebral haemorrhage with amyloidosis (Abraham et al. 1988, Abraham, 1992). Furthermore, it has been suggested that interaction of α_1-ACT with the β-amyloid is mediated through a linkage between serine, in position 8 of the 42 amino acid β-amyloid and the reactive loop in α_1-ACT, in manner reminiscent of its reaction with a serine protease (Potter et al. 1991).

1.2.3 Gene localisation and organisation

The gene encoding α_1-ACT is found on the distal region of the long arm of chromosome 14 along with α_1-AT. Recombination events occur five times more frequently in this region than in other regions of a comparable size, leading to the suggestion of the evolution of these two highly related genes. The intron-exon organisation of α_1-ACT is identical to that of α_1-AT inhibitor with five exons interrupted by four introns. Exons II, IV and V maintain a large number of residues that are identical in equivalent positions in α_1-AT and α_1-ACT-(51%, 44% and 46% respectively). However, exon III containing the DNA binding region, maintains only 33% identity. The reactive centre is found in exon V (Rubin et al. 1990, Rubin, 1992).

1.2.4. Protein structure

The high degree of identity of the primary sequences of α_1-ACT and α_1-AT is reflected in the similarities of the major structural motifs of the cleaved structures of the two serpins where overall organisation of β-sheets and helices is conserved between the proteins (Baumann et al. 1991). The mature, serum-derived protein has been reported to contain 398 amino acids (Mr 45,031 Dalton) starting from the tripeptide Asn-Ser-Pro at the amino terminus (Rubin et al. 1990).

1.2.5. Structure of the glycans of human-serum α_1-ACT

α_1-ACT is a glycoprotein with a carbohydrate content of 24 % (Laine et al. 1981). The complete amino acid sequence of α_1-ACT deduced from the nucleotide sequence revealed four potential glycosylation sites (in positions 8, 68, 161 and 246). In recent work, however, Rubin et al. (1990) found two other potential glycosylation sites at positions 81 and 102 in the gene nucleotide sequence.

There were no significant differences in amino acid composition of native unfractionated α_1-ACT when compared with three fractions of α_1-ACT which were obtained after separation on a Con A-affinity chromatography column. Differences existed in the carbohydrate molar ratios (Table 2), indicating that the glycans had different degrees of branching. The bound fraction contained not only biantennary glycans but also triantennary since the molar ratio D-Gal/D-Man was equal to 2.52/3 instead of 2/3. The Con A-nonreactive fraction appeared to contain glycans with more than three branches since the molar ratio D-Gal/D-Man is equal to 3.26/3 instead of 3/3 (Laine et al. 1989).

Table 2. Carbohydrate molar ratios of unfractionated α_1-ACT and of three α_1-ACT fractions obtained by Con A-affinity chromatography (calculated on the basis of 3 moles of mannose per carbohydrate chain) Laine et al. (1989).

Glycoprotein	D-Mannose	D-Galactose	N-Acetyl-glucosamine	N-Acetyl-neuraminic acid
Unfractionated	3.00	2.81	3.24	2.66
Con A-reactive fraction (Peak 3)	3.00	2.52	3.14	2.44
Con A-weakly reactive fraction (Peak 2)	3.00	2.98	3.42	2.79
Con A-nonreactive fraction (Peak 1)	3.00	3.26	3.55	2.73

Purified α_1-ACT was resolved into the four fractions in the presence of Con A in crossed immunoaffinoelectrophoresis (CIAE) and there was a good correlation between the Con A-affinity chromatography elution profile and the peaks observed in CIAE. The Con A-nonreactive fraction corresponded to peak 1 in CIAE, the Con A-weakly reactive fraction to peak 2, and the Con A-reactive fraction to peaks 3 and 4 (Hachulla et al. 1988).

The complete primary structure of the glycans was determined using high-resolution H-NMR spectroscopy (Laine et al. 1991). They demonstrated that peak 1 contained triantennary glycans, peak 2 consisted of molecules with three triantennary glycans and one biantennary glycan, and the peaks 3 and 4 consisted of α_1-ACT molecules, with on average, one triantennary glycan and three biantennary glycans. The biantennary structure was α-2,6-sialylated, the trisialylated glycan structures possessed α-2,6 and α-2,3-linked sialic acid, in the ratio 2:0.8.

1.2.6 Disease related changes in α_1-ACT

A significant increase in serum α_1-ACT is found in the initial stages of gastric and colorectal cancer groups as compared to healthy donors, and a correlation exists between the level and the cancer stage (Bernacka et al. 1988). The total concentration of α_1-ACT is also significantly increased in inflammatory sera (Hachulla et al. 1988), in sera from patients with giant-cell arthritis, polymyalgia rheumatica (Hachulla et al. 1990, Pountain et al. 1992), rheumatoid arthritis (Mackiewicz et al. 1986) and salivary adenoid cystic carcinoma (Chomette et al. 1992).

α_1-ACT is a positive acute-phase protein (whose concentration increases following a stimulus), and a number of studies have demonstrated that changes in plasma protein concentration that occur following tissue injury or infection are often accompanied by alterations in the pattern of protein glycosylation (Mackiewicz et al. 1987a, 1987b, Mackiewicz and Kushner, 1990, Pawlowski et al. 1989, Hansen et al. 1986, Hachulla et al. 1988).

The microheterogeneity of the α_1-ACT has been demonstrated using CIAE with Con A. In alcoholic cirrhosis the proportion of the Con A-nonreactive glycoforms which carried pluriantennary glycans was increased (Hachulla et al. 1992, Biou et al. 1987, 1989, Jezequel et al. 1988). This has been observed for other serum glycoproteins. In chronic inflammation associated with rheumatoid arthritis and in disseminated cancer, the pattern was similar to normal serum (Raynes et al. 1982). In acute inflammation, an increased proportion of Con A-reactive fraction was found (Hachulla et al. 1988), but it was not observed in chronic inflammation. Moreover, it was found that in chronic inflammation with acute attacks there was a decrease in this fraction.

1.3 Lectins

1.3.1 Definition and properties of lectins

Lectins are carbohydrate-binding proteins of a nonimmune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates (Liener et al. 1986).

All lectins are oligomeric proteins with several sugar binding sites, usually one site per unit. They vary in their chemical and physical properties. Many of them are glycoproteins with carbohydrate contents as high as 50 % (e.g. potato lectin) but others, like Con A are devoid of covalently bound sugars. The molecular weight of lectins range from 11, 000 to 335, 000 Daltons.

Lectins combine reversibly and noncovalently with both simple and complex carbohydrates whether they are free in solution or on cell surfaces. Such cell surface carbohydrates are referred to as lectin receptors. Binding may involve several forces, but these are mainly hydrophobic and hydrogen-bonds. Only rarely are electrostatic forces involved, since most carbohydrates are devoid of electrical charge.

Lectins occur in all classes and families of organisms, although not necessarily in every genus or species. Many lectins are found in plants, in vertebrates, in microorganisms and in viruses (Sharon and Lis, 1989). Plant lectins have been widely used for the detection, isolation, and characterisation of glycoconjugates using their characteristic binding properties. Over 100 plant lectins have already been isolated and their carbohydrate binding specificity has been, at least, partially characterised.

1.3.2 Carbohydrate specificity of lectins

The sugar specificity of lectins are usually defined in terms of the monosaccharide(s) that inhibits lectin-induced agglutination or precipitation. Such

specific inhibitors are commonly effective at concentrations in the millimolar range or lower. Lectins with similar specificity toward monosaccharides may differ in their affinity to disaccharides, oligosaccharides or glycoproteins. Although lectins are similar to antibodies in their ability to agglutinate cells, they differ, in that, lectins are not products of the immune system, their structures are diverse, and their specificity is restricted to carbohydrates (David and Ofek, 1986).

Lectins are able to bind sugars specifically, but it is known that the monosaccharide residue in a terminal non-reducing position on glycan is not the only carbohydrate moiety involved in the interaction (Debray et al. 1981). The strongest binding is to a particular sugar grouping. The binding constant of the specific free sugar with lectin may be several orders of magnitude lower than the binding constant of a glycoconjugate containing this sugar (Debray et al. 1981).

1.3.2.1 Sambucus nigra agglutinin (SNA)

SNA requires the presence of a terminal NeuAc-Gal/GalNac sequence for high affinity binding. N-acetylneuraminic acid linked by α2,6-linkages to galactose or N-acetylgalactosamine are bound with especially high affinity, whereas isomeric structures containing terminal sialic acid in the α2,3-linkage do not bind (Shibuya et al. 1987).

The marked preference of SNA for the NeuAc(α2,6)Gal sequence compared to NeuAc(α2,3)Gal can be explained in two ways. First, substitution of the C-3 hydroxyl group of the galactosyl residue by NeuAc in the 2,3-linked isomer may significantly decrease affinity. A free hydroxyl group at C-3 may play an important role in the binding of subterminal galactosyl residues to SNA. A second possible explanation may be due to differences between the three-dimensional arrangement of the sialic acid and galactosyl residues in the oligosaccharides containing 2,3- or 2,6-linkages (Shibuya et al. 1987).

1.3.2.2 Maackia amurensis agglutinin (MAA)

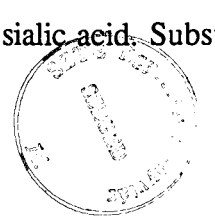
MAA interacts with high affinity with glycoconjugates containing NeuAc-linked α2,3 to galactose. Wang and Cummins (1988) observed that leukoagglutination by MAA was inhibited by low concentrations of 2,3-sialyllactose, while it was not inhibited by much higher concentrations of 2,6-sialyllactose. They also found that high concentrations of N-acetylneuraminic acid were ineffective in inhibiting leukoagglutination.

The MAA lectin binds glycoconjugates containing the terminal sequence NeuAc(α2,3)Gal, while those isomers containing sialic acid-linked α2,6 are not bound. The combined use of both the SNA and MAA in affinity chromatographic and lectin blotting techniques should permit fractionation of sialylated glycoconjugates on the basis of sialic acid linkages, and thereby greatly facilitate the isolation and structural analysis of glycoconjugates (Wang and Cummins, 1988).

1.3.2.3 Ricinus communis agglutinin I (RCA)

The best inhibitor found for RCA is a asialo biantennary oligosaccharide which presents two β1,4-linked galactose residues (Debray et al. 1981). However, the interaction of RCA with this type of oligosaccharide is strongly influenced by the linkage of the terminal galactose moieties. If the oligosaccharide unit bears two terminal galactose moieties in the linkage Gal(β1,3)GlcNAc, then the interaction with RCA is negligible. On the other hand, if the linkage between the terminal galactose and N-acetylglucosamine is β1,4, the oligosaccharide is strongly bound to the lectin. Thus, RCA discriminates between these closely related oligosaccharides which differ only in the linkage of terminal galactose.

The affinity of RCA for sialylated oligosaccharides is different, depending on the linkage between galactose and sialic acid. Substitution of galactose by α2,3-



linked sialic acid abolishes interaction of the dibranched structure with RCA, but substitution of galactose by α2,6-linked sialic acid allows some interaction. The interaction of RCA with galactose substituted at the C-6 position is thought to be due to a higher degree of rotational freedom of the α2,6 linkage (Debray et al. 1981).

1.3.2.4 Datura stramonium agglutinin (DSA)

The seeds of Datura stramonium contain a lectin (DSA) which specifically binds β1,4-linked oligomers of N-acetyl-D-Glucosamine in O-glycans. However DSA also recognises Galβ-(1,4)GlcNAc in complex and hybrid N-glycans. This lectin is a dimeric glycoprotein composed of two nonidentical subunits (Mr 40,000 and 46,000) which are joined by disulphide bonds and contain 37 % carbohydrate by weight, of which 93 % is arabinose and 7 % is galactose.

The binding site of DSA is composed of three subunits, designated (A), (B), and (C). A disaccharide can bind to the lectin in two ways, occupying subsites (A) and (B), or subsites (B) and (C). The minimal requirement for binding is an equatorial acetamido group at C-2 of the sugar which occupies subsite (B). An amino group at C-2 of the sugar greatly diminishes its binding affinity (Crowley et al. 1984).

1.3.2.5 Lotus tetragonolobus agglutinin

The specificity of Lotus agglutinin seems to be directed towards the α1,6- or the α1,3 linked fucose residue (Debray et al. 1981). It was found that the best inhibitor is the pentafucosylated glycopeptide III, which possesses four external non-reducing fucose residues α1,3-linked to the N-acetylglucosamine of the four N-

acetyllactosamine branches and one fucose residue α1,6-linked to the core N-acetylglucosamine. The L-fucose itself is less inhibitory than glycopeptide III. Addition of further sugars to Fuc(α1,6)-GlcNAc(β1)Asn sequence limits the accessibility of this structure to the lectin (Petryniak and Goldstein, 1986).

1.4 Regulation of human acute phase proteins by cytokines

Tissue injury and infection lead to a broad array of systemic and metabolic alterations, collectively termed the acute phase response (Kushner, 1982, Kushner and Mackiewicz, 1987). Among these alterations are changes in hepatic synthesis of a number of plasma proteins, referred to as acute phase proteins; synthesis of some proteins - the positive acute phase proteins - increases, while synthesis of others - the negative acute phase proteins - decreases.

The synthesis of acute phase proteins by the liver is regulated by cytokines, which can be produced by a variety of cells, including monocytes/macrophages, fibroblasts, endothelial, epithelial and tumour cells (Baumann et al. 1984, Darlington et al. 1986, Gauldie et al. 1987, Goldman and Liu, 1987). The defined cytokines which have been implicated in the acute phase response are interleukin-6 (IL-6), interleukin-1 (IL-1), tumour necrosis factor α (TNF-α), transforming growth factor α and β (TGF-α, TGF-β), interferon γ (IF-γ) and leukaemia-inhibitory factor (LIF) (Andrus et al. 1987, Darlington et al. 1986, Gauldie et al. 1987, Goldman and Liu, 1987, Perlmutter et al. 1986).

IL-6 is a cytokine with pleiotropic functions and is a key mediator of the reaction regulating the expression of the genes encoding acute-phase proteins in the liver. Only IL-6 induced essentially the same spectrum of acute phase proteins as that found in humans during inflammatory states (Castell et al. 1989, Natsuka et al. 1991). Malignantly transformed cells frequently produce transforming growth factors, originally characterised as polypeptides capable of reversibly inducing the

transformed phenotype in nonmalignant cells. Two different transforming growth factor molecules have been characterised so far. TGF-α is an epidermal growth factor (EGF)-like growth factor, whereas TGF-β is a distinct molecule and mediates its effects via specific receptors (Laiho et al. 1987).

Studies in primary hepatocyte cultures and hepatoma cell lines have shown that hepatic synthesis of human acute phase proteins can be influenced by several cytokines including IL-6, IL-1, TNF, TGF, and LIF. One cytokine can modulate the effect of other cytokines in the human model system (Mackiewicz et al. 1990).

1.5 Aims of the project

The initial aim of the project was to determine alteration in the level and carbohydrate microheterogeneity of α_1-ACT in ovarian and breast cancer sera. Variations in the pattern of carbohydrate microheterogeneity of (α_1-ACT) and of some other serum glycoproteins have been detected in various diseases (Turner, 1992). Therefore, the investigation of this glycoprotein could be used as a biochemical marker in cancer diagnosis, and to monitor the effects of cancer treatment.

The other purpose of this study was to examine a possible mechanism that could regulate the enhanced α_1-ACT synthesis observed in cancer. The changes in serum α_1-ACT concentration in cancer can be explained by the tumour cells secreting factors (possibly cytokines) which stimulate α_1-ACT synthesis in the liver. This was tested by investigating the effects of media from cultured ovarian cancer cells and the effects of cytokines on the α_1-ACT production by the Hep G2 liver cell line.

2 Experimental procedures

2.1 Materials

2.1.1 Sera investigated

Blood specimens were obtained by venepuncture from 10 healthy women (median age 56 years), from 6 patients with progressive ovarian cancer (median age 60 years) and from 4 patients with progressive breast cancer (median age 58 years). All individuals were non-smokers and their alcohol intake was very low. None of the healthy group were taking oral contraceptives or any form of medication. All cancer patients were receiving chemotherapy when the blood specimens were taken and all had detectable tumour.

Sera were separated by centrifugation at 600 g for 10 min and then stored at -20°C.

2.1.2 Chemicals

Biotinylated Lotus tetragonolobus agglutinin, nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), Concanavalin A (Con A), copper (II) sulphate pentahydrate, IL-6, TGF-α and bichinchoninic acid solution were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sambucus nigra agglutinin (SNA), Maackia amurensis agglutinin (MAA), Datura stramonium agglutinin (DSA) and Ricinus communis agglutinin (RCA)-digoxigenin-labelled, anti-digoxigenin-alkaline phosphatase conjugate, carboxypeptidase Y, transferrin, fetuin, rabbit anti-human haptoglobin, rabbit anti-human α_1-acid glycoprotein and rabbit anti-human α_1 antitrypsin were obtained from Boehringer Mannheim GmbH (Marburg, W. Germany). Sheep anti-human α_1-antichymotrypsin, anti-sheep IgG-alkaline phosphatase conjugate, human serum RID Calibrator were from The Binding Site

Ltd. (Birmingham, UK). Anti -rabbit IgG alkaline phosphatase conjugate was obtained from Seralab (Crowley Down, UK). Streptavidin-alkaline phosphatase was from Amersham International (Amersham, UK). Rabbit anti-human α_1-antichymotrypsin was obtained from Dakopatts (Glostrup, Denmark). Blue Sepharose CL-6B was from Pharmacia Fine Chemicals AB (Uppsala, Sweden). SDS-PAGE mol mass standards were from Bio-Rad Laboratories (Richmond, CA, USA). TGF-β was obtained from NIBSC (Hertfordshire, UK). The DMEM media and foetal calf serum (FCS) were obtained from NBL (Cramlington, Northumberland, UK)

2.2 Methods

2.2.1 Radial Immunodiffusion (RID)

In this technique an antigen is placed in a well, cut in agar containing the corresponding antiserum. The antigen diffuses out radially from the wells and the precipitation ring is formed at equivalence between the antigen and antibody. The diameter of the precipitation ring is proportional to the antigen concentration. By plotting (ring diameter)^2 or ring area at the equivalence point of a range of known concentrations, a calibration curve can be obtained for determining the concentration of antigen in unknown solutions. The detection sensitivity of the procedure used in this study was 2 ng protein.

Agarose (0.36 g) was dissolved at 100°C in 30 ml Barbitone buffer (pH 8.6) (25 mM Barbitone, 75 mM Tris base, 1 mM Calcium lactate, 10 mM NaCl, 10 μM $MgCl_2$). This solution was cooled to 50°C in a water bath, and 100 μl of polyclonal rabbit or sheep anti-human α_1-antichymotrypsin antibody was added. The agar was then quickly poured on to a warm plastic gel bound support between two glass plates. Into the set agar-gels, holes were punched 2 cm apart. The serum samples were diluted with Barbitone buffer (1:100 dilution), and 10 μl (standards

and control serum) or 5 µl (cancer serum) samples were pipetted into the holes, and the gel was left for 3 days at room temperature to allow the samples to diffuse.

After three days the gels were washed twice for 1 h in PBS (pH 7.4). Following drying at room temperature, the gels were stained with 0.02 %(w/v) Coomassie Brilliant Blue R250 dissolved in 25 %(v/v) ethanol, 10 % (v/v) acetic acid. The destaining was carried out in 5 %(v/v) ethanol, 10 %(v/v) acetic acid until the precipitation rings became clearly visible. The gels were then dried and the area of each ring was calculated.

2.2.2 Crossed immunoaffinoelectrophoresis (CIAE)

Affinity electrophoresis with concanavalin A (Con A) as a ligand has been widely used for the determination of microheterogeneity of acute phase proteins in serum samples (Breborowicz and Mackiewicz, 1989, Hansen et al. 1986). In this system, microheterogeneous forms with biantennary heteroglycans can be separated from forms having tri- and/or tetra-antennary units. Con A binds the unsubstituted groups of α-2-O-substituted Man residues at carbons 3,4 and 6, with at least two interacting Man molecules being required for the binding. As result, this lectin binds with bi- but not with tri- or tetra-antennary structures. For multi-heteroglycan proteins the degree of reactivity with Con A depends on the number of biantennary structures present on the molecule.

CIAE was performed by the method of Bøg-Hansen et al. (1975). Gels were prepared in a Barbitone buffer (pH 8.6) system (25 mM Barbitone, 75 mM Tris base, 1 mM Calcium lactate, 10 mM NaCl, 10 mM $MgCl_2$). The first dimension gel contained 1%(w/v) agarose and 260 µg/cm^2 (2.5 mg/ml) Con A. The gel was poured onto a 8x12 cm glass plate to a depth of 1.5 mm. Serum (10 µl) were mixed with 5 µl Bromo phenol blue, 435 µl of Barbitone buffer and 50 µl of glycerol, 350 ng of

α_1-ACT applied to 10 mm diameter slots in the gel. Electrophoresis was performed at 6-7 V/cm gel for 75 min at room temperature.

The second dimension gel contained 1 %(w/v) of agarose, 0.2 µl/cm^2 (1:500 dilution) polyclonal rabbit or sheep anti-human α_1-antichymotrypsin antibody and 5 % of methyl-α-D-glucopyranoside. The gel was poured to thickness of 1.5 mm onto a 14.5x25 cm agarose gel supporting medium (FMC Gel Bond). Electrophoresis was performed at 30 V/cm gel for 3 h at 4°C.

Gels were washed in PBS (pH 7.4) to remove the antibody, then dried at room temperature and stained with 0.02 %(w/v) Coomassie Brilliant Blue R250 dissolved in 25 %(v/v) ethanol, 10 %(v/v) acetic acid. The destaining was carried out in 5 %(v/v) ethanol, 10 %(v/v) acetic acid until the precipitation peaks become clearly visible, and then re-dried at room temperature.

Peak areas enclosed by precipitates were measured by weighing a paper tracing of each pattern. A ratio was calculated for each serum sample according to the formula:

$$\frac{\text{Proportions peak 1 + peak 2}}{\text{Proportion of peak 3}}$$

(Con-A-nonreactive fraction, peak 1; Con a-weakly reactive fraction, peak 2; Con A-reactive fraction, peak 3) and was called Rα_1-ACT (Hachulla et al. 1990).

2.2.3 Albumin-depletion of serum

Albumin, which is non-glycosylated, represents more than 50 % of the total protein in plasma and it is often the major contaminant in preparations of other plasma proteins. To minimise interference in the separation of the other serum proteins, the majority of the albumin was removed with Blue Sepharose CL-6B.

The gel was washed several times before use with PBS buffer pH 7.4 (2.68 mM KCl, 1.47 mM KH_2PO_4, 0.137 M NaCl, 8.1 mM Na_2HPO_4). After the final

wash the gel was allowed to settle, and the volume of PBS was adjusted so that the ratio of packed gel to PBS was 1:1. Thorough mixing gave a suspension from which portions were taken. Each 50 µl sample of whole serum was treated with 500 µl Blue Sepharose gel in a microcentrifuge tube. The serum and Blue Sepharose gel were mixed, and allowed to stand for 1h at room temperature. The samples were then centrifuged at 11,500 g (MSE Microcentaur, UK) for 4 min. The supernatant from each sample (albumin-depleted serum) was carefully removed and stored at -20°C until required.

To investigate protein binding to Blue Sepharose CL-6B, the pellet of gel was washed three times in PBS to remove the last traces of supernatant (Lundy and Wisdom, 1992).

2.2.4 Protein determination using bicinchoninic acid (BCA)

Bicinchoninic acid (BCA), sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ion (Cu^{+}) in an alkaline environment. This reagent forms the basis of this analytical method and is capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{2+} (biuret reaction). The colour produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations (Lowry et al. 1951, Smith et al. 1985).

Reagents A and B were freshly made up in 50:1 ratio. Reagent A consisted of an aqueous solution of 1 %(w/v) BCA-Na_2, 2 %(w/v) Na_2CO_3 . H_2O, 0.16 %(w/v) Na_2 tartarate, 0.4 %(w/v) NaOH, and 0.95 %(w/v) $NaHCO_3$. Reagent B consisted of 4 %(w/v) $CuSO_4$. $5H_2O$ in deionised water. The standard assay procedure consisted of mixing 1 vol. of sample (40 µl) (standard and unknown) with 5 vol. (200 µl) of standard working reagent (A+B) in ELISA plate. The reagent was added in triplicate to bovine serum albumin (BSA) standards in 40 µl PBS buffer pH

7.4 (2.68 mM KCl, 1.47 mM KH_2PO_4, 0.137 M NaCl, 8.1 mM Na_2HPO_4) at a range of concentrations from 2.5-20 µg/well, and also to the serum samples to be tested in 1:100 dilution with PBS (40 µl). As a blank, the reagent (200 µl) was added to PBS buffer pH 7.4 (40 µl) in triplicate.

The solutions were mixed and incubated at 37°C for 75 min. The absorbance of the solution was measured at 562 nm using a Titertek Multiskan MCC 340 spectrophotometer. A standard curve was drawn and the protein concentrations of the unknown samples were estimated.

2.2.5 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A standard SDS-PAGE system (Laemmli, 1970) was used to analyse the protein composition of human serum.

2.2.5.1 Preparation of gels

Polyacrylamide gels 8 %(w/v) were prepared as slab gels of 17 cm x 15 cm between two glass plates separated by 1.5 mm spacers. Stock solutions of acrylamide (8 ml) (30 %(w/v) acrylamide, 0.8 %(w/v) N,N'-methylene-bis-acrylamide) and running gel buffer (7.5 ml) (1.5 M Tris base, 14 mM SDS; pH 8.8) were combined with distilled water (14.2 ml) and degassed on a vacuum line. Final additions of ammonium persulphate (300 µl) and TEMED (20 µl) were made and the separating gel mixture poured between the glass plates. Before polymerisation, overlay buffer (2 ml of running gel buffer and 6 ml of deionised water) was layered on top of gel solution to create a straight edge and prevent evaporation.

Once the gel had set, the buffer layer was removed and degassed 5 %(w/v) acrylamide stacking gel which contained 1.65 ml acrylamide stock solution, 2.5 ml stacking gel buffer (0.5 M Tris base, 14 mM SDS, pH 6.8), 5.75 ml deionised water,

100 µl of ammonium persulphate and 7 µl of TEMED; was layered over the separating gel. A plastic comb was inserted into the stacking gel to create 17 x 1 cm wells. When the stacking gel had set, the comb was removed and the surface washed with electrode buffer (0.025 M Tris base, 0.2 M glycine, 3.5 mM SDS; pH 8.3).

2.2.5.2 Preparation of samples for SDS-PAGE and gel electrophoresis

Albumin-depleted sera were mixed with 50 %(v/v) of DSSB (0.125 M Tris base, 20 %(v/v) glycerol, 10 %(w/v) SDS, 20 mM EDTA, 2 %(w/v) bromophenol blue, pH 7.4), and 5 %(v/v) of 2-mercaptoethanol, boiled for 5 min, and 12-47 µl containing 0.35 µg α_1-ACT, were loaded into a gel slot. Two tracks were loaded with standard molecular weight markers: phosphorilase B (94 kDa), serum albumin (68 kDa), catalase (60 kDa), ovalbumin (45 kDa) and aldolase (40 kDa) and actin (43 kDa). Separation of the proteins was achieved by electrophoresis at a constant current of 35 mA (2197 Power supply, LKB, Bromma, UK) for 3.5 h at room temperature.

2.2.6 Silver staining

After gel electrophoresis the gel was fixed in 25 %(v/v) ethanol and 10 %(v/v) acetic acid overnight at room temperature. The gel was washed twice for 30 min with distilled water, after washing the gel was soaked in 5 mg/ml dithiothreitol for 2 hours at room temperature. The gel was then incubated in freshly prepared 0.1 %(w/v) silver nitrate for 2 hours without rinsing. The gel was rinsed rapidly twice with distilled water before incubation in developer (100 µl of 40 %(v/v) formaldehyde in 200 ml of 3 %(w/v) sodium carbonate for 10 min. The development was stopped with 10 ml of 2.3 M citric acid for 30 min. The gel was then stored in

distilled water and dried between two sheets of cellophane backing (Bio Rad Ltd.) (Thompson, 1987).

2.2.7 Lectin blotting

The samples were prepared as in 2.2.5 above. The electrophoresis was performed at 35 mA for 3.5 h at room temperature. Proteins were then transferred electrophoretically in transfer buffer (0.2 M glycine, 25 mM Tris base, 20 %(v/v) methanol) in a Transblot cell (Bio-Rad) at 0.05 A overnight and 0.2 A for 1 h onto nitrocellulose (Bio-Rad Laboratories, Richmond, U.S.A.)

The membranes were first treated for 2x30 min with 50 ml TBS-Tween 20 blocking solution (50 mM Tris-HCl, 150 mM NaCl, 0.1 %(v/v) Tween 20, pH 7.5). After being washed twice with Buffer I (1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in TBS-Tween 20, pH 7.5). The membranes were incubated for 1.5 h at room temperature with the lectin-DIG conjugate (SNA 1:2500, 0.4 ng/ml; RCA 1:1000, 1 ng/ml; MAA 1:200, 5 ng/ml) in 50 ml Buffer I. After the membranes had been washed three times for 10 min each with 50 ml TBS-Tween 20, anti-DIG AP (750 U/ml) was added in a 1:500 dilution (2 ng/ml) to 50 ml Buffer II (0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) and incubated for 1 h at room temperature.

The membranes were again washed three times for 10 min each in TBS-Tween 20 buffer. The alkaline phosphatase reaction was carried out by incubating the membrane without shaking in 10 ml of the following solution (freshly prepared): 50 ml veronal acetate buffer (0.15 M sodium acetate, 0.15 M barbitone sodium, pH 9.6), 0.1 mg/ml NBT, 4 mM $MgCl_2$, 12.5 mg/ml BCIP (50 mg/ml in dimethylformamide). The reaction was normally complete within a few minutes. The membranes were rinsed with H_2O to stop the reaction and allowed to dry (Hasebeck et al. 1990, Fischer et al. 1992a,b, 1993).

Similar blotting was performed with biotinylated Lotus tetragonolobus lectin (1:500 dilution, 2 ng/ml in PBS-Tween 20), visualised by streptavidin-alkaline phosphatase conjugate (1:1000 dilution, 1 ng/ml). The experimental procedure was performed as it is described under Immunoblotting (2.2.8).

2.2.8 Immunoblotting

Albumin-depleted serum proteins were separated by electrophoresis on 8 %(w/v) polyacrylamide gels and transferred onto nitrocellulose membranes, essentially as described previously (2.2.7).

The blots were washed for 2x1 h in 200 ml PBS-Tween 20 buffer (0.375 mM KCl, 0.7 mM KH_2PO_4, 140 mM NaCl, 8.15 mM Na_2HPO_4, 0.5 %(v/v) Tween 20, pH 7.4). All steps in the procedure were carried out at room temperature, with gentle agitation on a rotary shaker (Luckham Ltd., Sussex, UK). The blots were incubated for 3 h with different primary antibodies (polyclonal sheep anti-human α_1-ACT, 1:500 dilution, 2 ng/ml; polyclonal rabbit anti-human haptoglobin, 1:500 dilution, 2ng/ml; polyclonal rabbit anti-human α_1-AGP, 1:200 dilution, 5 ng/ml; or polyclonal rabbit anti-human α_1-AT, 1:200 dilution, 5 ng/ml).

Following three washes for 10 min each in PBS-Tween 20, the blots were incubated for 2 h with anti sheep IgG-alkaline phosphatase (1:500 dilution, 2 ng/ml), or anti rabbit IgG-alkaline phosphatase (1:5000 dilution, 0.2 ng/ml). The membranes were again washed three times for 10 min each in PBS-Tween 20 buffer.

The alkaline phosphatase reaction was carried out as described previously in section 2.2.7 (Matsudaira, 1987, Moos et al. 1988).

2.2.9 *Treatment of Hep G2 human hepatoma cells with conditioned media from ovarian cancer cells or cytokines*

Conditioned media were collected from four ovarian cancer cell lines (28M, 41M, 59M, OAW42). They were grown at 37°C for 2/3 days in Eagles medium-Dulbeccos modification (DMEM) containing 0.33 %(w/v) sodium bicarbonate, 0.1 mM sodium pyruvate, 0.02 units/ml insulin, 0.2 mM glutamine, 20 mg/ml penicillin, 20 mg/ml fungizone plus 10 %(v/v) foetal calf serum (FCS). These cell lines originated from different patients with ovarian cancer. An aliquot of the cancer cell medium was added at 1:10 dilution to the liver cell line (Hep G2) growing either in DMEM (0.33 %(w/v) sodium bicarbonate, 0.1 mM sodium pyruvate, 0.2 mM glutamine, 20 mg/ml penicillin, 20 mg/ml fungizone) plus 10 %(w/v) FCS, or in serum free medium at 37°C for 2/3 days.

For the cytokine experiment the Hep G2 cells were incubated with varying doses of the different cytokine (5 ng/ml TGF-β, 1 nM TGF-α, 50 U/ml IL-6). Cytokines were employed individually and in different combinations. Cytokines were added to a liver cell line (Hep G2) growing in either DMEM (0.33 %(w/v) sodium bicarbonate, 0.1 mM sodium pyruvate, 0.2 mM glutamine, 20 mg/ml penicillin, 20 mg/ml fungizone) plus 10 % FCS or serum free medium at 37°C for 2/3 days.

An aliquot of the appropriate non-conditioned medium was used as a control. After 2/3 days exposure to the cytokines or to the diluted cancer medium, the concentration of α_1-ACT in the liver cell medium was measured by radial immunodiffusion (Turner and Thompson, 1991).

3 Results

3.1 Measurement of α_1-ACT concentration

3.1.1 Calibration curve for α_1-ACT

The α_1-ACT concentrations were measured in human sera, therefore human serum RID calibrator was used for the standards. The correlation between the α_1-ACT concentration (1.05-16.75 ng/µl) and the area of precipitation rings was linear (Fig. 3.)

The points of each calibration curve were calculated from three, the mean and the standard deviation of three different calibration curves were determined from nine independent measurements. The standard deviation for the values of a calibration curve were less than 3.5 %. The variations between three different calibrations were less then 5 % (Table 3.).

Table 3. Comparison of the values of different calibration curve that were used for determination of α_1-ACT concentration by RID. The mean values and the standard deviations were calculated from nine independent measurements.

Concentration (ng/µl)	Area of the ring (mm^2) Calibration I	Area of the ring (mm^2) Calibration II	Area of the ring (mm^2) Calibration III	Mean ± SD of three calibrations
16.75	101 ± 1.56	109.2 ± 2.44	102.8 ± 2.21	104.3 ± 3.24
8.30	66.5 ± 2.33	75.1 ± 2.30	72.5 ± 1.56	71.4 ± 3.24
4.20	53.7 ± 0.84	54.1 ± 0.92	54.0 ± 0.72	53.9 ± 0.16
2.10	43.6 ± 0.71	43.9 ± 0.71	45.3 ± 0.51	44.3 ± 0.69
1.05	39.38 ± 1.02	41.3 ± 0.92	40.5 ± 1.03	40.5 ± 0.51

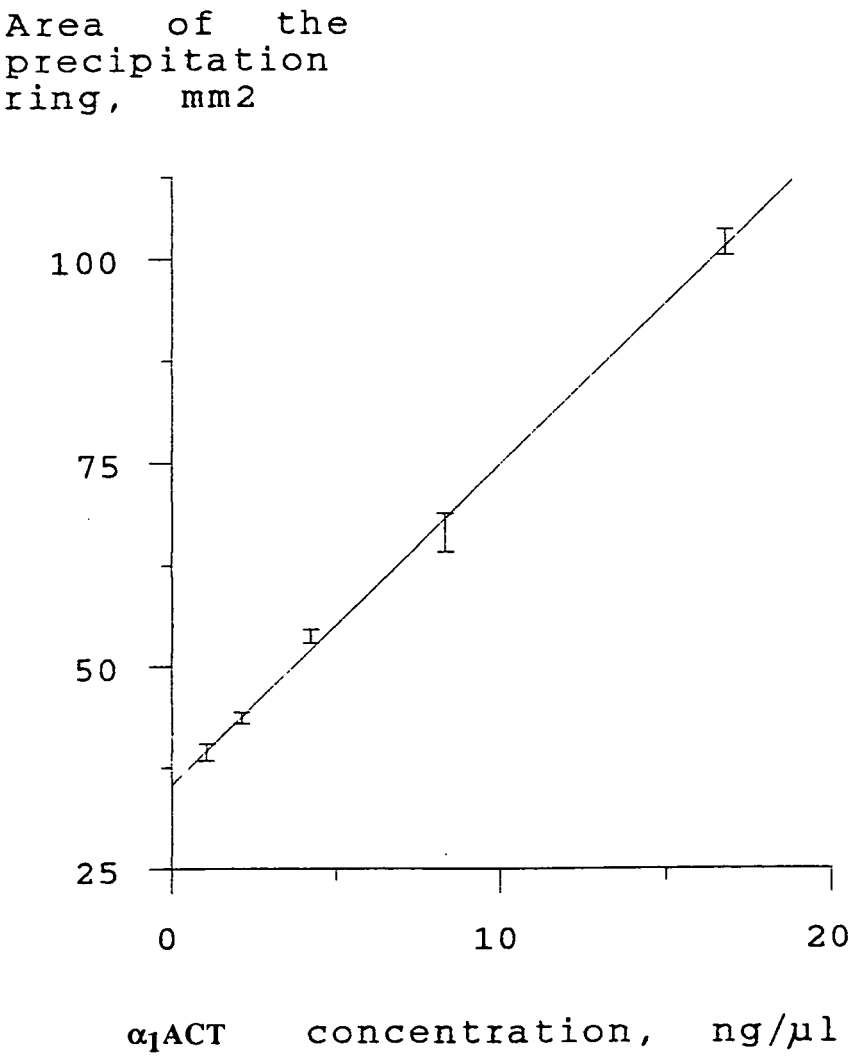


Fig. 3. Calibration curve for the determination of α_1-antichymotrypsin concentration. The concentrations were measured using RID; human serum RID calibrator was used as standards (1.05, 2.1, 4.2, 8.4 and 16.75 ng/μl). The points of the calibration curve were calculated from three independent measurements.

3.1.2 Measurement of α_1-ACT using purified protein

The concentration of purified α_1-ACT (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) was determined by RID using human serum RID calibrator standards (Binding Site, Birmingham, UK). The measured and the actual (Calbiochem) concentrations were compared (Table 4.). The measured RID concentrations were 20 % higher than the actual (Calbiochem) values. This difference can be explained by the different procedures used by the two companies to measure α_1-ACT concentration. In our measurements of α_1-ACT concentrations human serum RID calibrator was used.

Table 4. Comparison of the actual and the measured α_1-ACT concentrations using RID.

Actual concentration of purified α_1-ACT (ng/µl)	Measured concentration of purified α_1-ACT by RID using human serum calibrator (ng/µl)
12.50	15.00 ± 0.07
9.37	11.10 ± 0.07
6.25	7.40 ± 0.02

3.1.3 Investigation of α_1-ACT in `healthy` and `cancer` sera

The α_1-ACT concentrations were measured in the sera by radial immunodiffusion (RID) as described in methods (2.2.1). The area of each ring was determined and the concentrations of the α_1-ACT were calculated based on the calibration curve (Fig. 3.). The following standards were used: 16.75, 8.37, 4.20, 2.10 and 1.05 ng/µl of α_1-ACT (Human serum RID Calibrator). Each sample was

assayed in triplicate on the RID plate and for the determination of the reproducibility of this method the analysis was repeated. Therefore, the results represent the mean and the standard deviation calculated from six independent measurements (Table 5.).

Table 5. α_1-ACT levels in sera from healthy individuals and patients with ovarian or breast cancer. α_1-ACT concentrations were measured by RID, the mean values and the standard deviation were calculated from six independent measurements.

Source of serum	α_1-ACT concentration in serum (μg/μl)	Source of serum	α_1-ACT concentration in serum (μg/μl)
N_1	1.00 ± 0.05	B_1	1.03 ± 0.04
N_2	0.74 ± 0.03	B_2	1.44 ±0.09
N_3	1.07 ± 0.06	B_3	1.45 ± 0.09
N_4	0.51 ± 0.06	B_4	1.33 ± 0.02
N_5	0.67 ± 0.07	O_1	2.32 ± 0.02
N_6	0.68 ± 0.03	O_2	2.21 ± 0.07
N_7	0.95 ± 0.01	O_3	2.17 ± 0.02
N_8	0.82 ± 0.06	O_4	1.79 ± 0.07
N_9	0.66 ± 0.03	O_5	0.82 ±0.07
N_{10}	0.62 ± 0.05	O_6	1.23 ± 0.08

N, normal; B, breast cancer; O, ovarian cancer.

The mean α_1-ACT levels (mean ± SD) in the healthy individuals and patients with breast and ovarian cancer were 0.77 ± 0.15 μg/μl, 1.33 ± 0.14 μg/μl and 1.76 ± 0.49 μg/μl respectively. The concentration of α_1-ACT was increased in the breast and ovarian cancer sera, by 2 and 2-2.5 fold respectively. The mean values in both cancer groups were significantly higher than in the controls. In two of the healthy individuals (N_1, N_3) the α_1-ACT concentrations were quite high and in two of the cancer patients (B_1,O_5) the α_1-ACT was non-elevated.

3.2 *Effect of cytokines and conditioned media on the synthesis of α_1-ACT by the hepatoma cells*

3.2.1 *Effect of cytokines on α_1-ACT synthesis in Hep G2*

Cytokines (TGF-β, TGF-α and IL-6) significantly (2.4-2.8 fold) increased the synthesis of α_1-ACT in Hep G2 cells, growing in either DMEM+10 % FCS or serum free media (Table 6.). The effect of various combinations of cytokines (IL-6 and TGF-β/TGF-α) was tested on the regulation of α_1-ACT synthesis. The α_1-ACT synthesis was increased 2.1-2.3 fold in the presence of a single cytokine; whereas 2.8-4.3, 2.6-2.8 fold increase was observed after the addition of IL-6 in combination with TGF-β or TGF-α respectively. The combined effect of IL-6 with TGF-β and TGF-α was additive. The degree of cytokine stimulation was different in different media, e.g. the α_1-ACT concentration increased (TGF-β + IL-6) in DMEM + 10 % FCS media by 2.8 fold, whereas a larger increase was observed (4.3 fold) in SF media.

Table 6. Induction of synthesis of α_1-ACT in Hep G2 cells by cytokines. The level of α_1-ACT was measured by RID, the mean values and the standard deviations were calculated from three independent measurements.

Cytokine	Concentration of cytokine	α_1-ACT concentration (ng/µl) using DMEM+10 % FCS media	α_1-ACT concentration (ng/µl) using SF media	Test/ Cont. FCS	Test/ Cont. SF
Control	—	4.2 ± 0.2	1.6 ± 0.2	-	-
TGF-β	5 ng/ml	9.7 ± 0.7	4.7 ± 0.2	2.3	2.9
TGF-β + IL-6	5 ng/ml + 50 U/ml	11.6 ± 0.6	6.8 ± 0.5	2.8	4.3
TGF-α	1 nM	7.6 ± 0.4	3.5 ± 0.2	1.8	2.2
TGF-α	2 nM	9.2 ± 0.7	-	2.2	-
TGF-α + IL-6	1 nM + 50 U/ml	11.6 ± 0.5	4.2 ± 0.4	2.8	2.6
IL-6	50 U/ml	9.7 ± 0.8	3.4 ± 0.3	2.3	2.1

3.2.2 *Effect of conditioned media on α_1-ACT synthesis*

All `diluted` cancer media stimulated the synthesis of α_1-ACT in Hep G2 cells (Table 7.). The concentration of α_1-ACT increased on average by 2.4 fold. The greatest effect on α_1-ACT accumulation was observed using 59M cells (3.4-3.6 fold), but similar responses were detected with OAW42, 28M and 32M cells (2.2-2.6). The increase with 41M cells was not significant as the level of α_1-ACT in the test was only slightly higher than the control. The degree of stimulation of α_1-ACT synthesis depended on the type of the ovarian cancer cell lines, and was independent from the type of media used.

Table 7. Effect of conditioned media from ovarian tumour cells on the synthesis of α_1-ACT in liver cells . Media were added to the liver cell line (Hep G2) in 1:10 dilution (see Experimental Procedures 2.2.9). The α_1-ACT concentrations were measured by RID, the mean values and the standard deviations were calculated from three independent measurements.

Ovarian cell line	α_1-ACT concentration (ng/µl) using DMEM+10 % FCS medium	α_1-ACT concentration (ng/µl) using SF medium	Test/ Cont. FCS	Test/ Cont. SF
Control	3.6 ± 0.2	3.9 ± 0.3	-	-
OAW42	8.2 ± 0.6	8.6 ± 0.5	2.3	2.2
28M	9.2 ± 0.7	10.2 ± 0.8	2.6	2.6
32M	8.1 ± 0.5	-	2.3	-
41M	4.2 ± 0.3	4.3 ± 0.3	1.2	1.1
59M	12.4 ± 0.8	14.0 ± 0.8	3.4	3.6

The Hep G2 tissue culture supernatants were investigated by CIAE, but no patterns were obtained. This may be because the α_1-ACT concentration was too low.

3.3 Analysis of α_1-ACT in sera by CIAE

3.3.1 The reproducibility of CIAE

The precision of CIAE measurements was investigated. The within-day and day-to-day reproducibilities were checked; the variations were less than 5 %. (Table 8.).

Table 8. The relative amounts of α_1-ACT variants and Rα_1-ACT values in normal subjects and in the ovarian or breast cancer patients. Comparison of the same samples using different CIAE plates under same conditions. The measurement of peak areas and the calculation of Rα_1-ACT were the same as described under 3.3.

Source of serum	Con A-reactive fraction Peak 3 (%)	Con A-weakly reactive fraction Peak 2 (%)	Con A-nonreactive fraction Peak 1 (%)	Rα_1-ACT
N_7	41.40	34.80	23.80	1.42
N_7	42.00	34.20	23.80	1.40
B_2	35.30	35.30	29.40	1.83
B_2	36.10	37.80	26.10	1.75
O_2	41.30	33.50	25.20	1.42
O_2	40.70	33.60	25.70	1.46

N, normal; B, breast cancer; O, ovarian cancer.

3.3.2 CIAE of the different forms of α_1-ACT in sera

α_1-ACT studied by CIAE (Fig. 4.) was resolved into three immunoprecipitation peaks (Peak 1: Con A-nonreactive fraction; Peak 2: Con A-weakly reactive fraction; Peak 3: Con A-reactive fraction). This three-peak microheterogeneity of α_1-ACT was found for both healthy and cancer sera. The results are represented schematically because it was impossible to obtain satisfactory photographs

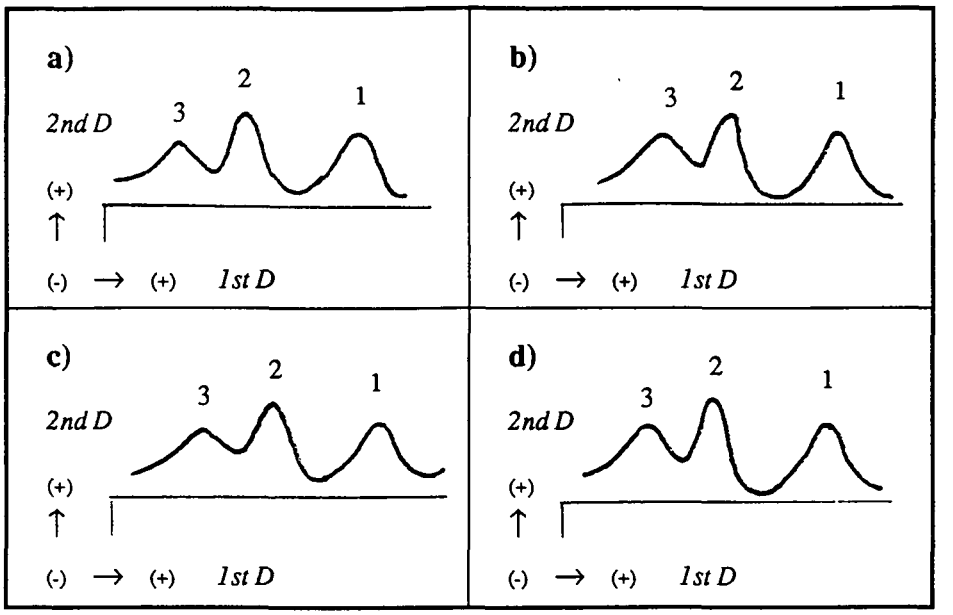


Fig. 4. CIAE of human serum against antiserum to α_1-ACT (0.2 µl/cm^2) in the second dimension. The first dimension gel contained Con A (260µg/cm^2). Typical serum samples were from (a) a normal subject, (b) a patient with ovarian cancer, (c) a patient with breast cancer, (d) same patient as (b) separated in different experiments. (1st D, first dimension; 2nd D, second dimension, (+), positive electrode; (-), negative electrode)

Analysis of peaks 1-3 for the CIAE separation of different sera are shown on Table 9. The peaks are expressed as percentage of the total immunoprecipitate and the Rα_1-ACT values were calculated by the ratio (Proportions peak 1 + peak 2)/ Proportion of peak 3. The mean Rα_1-ACT values were for healthy subjects and breast cancer patients 2.06 ± 0.40 and 2.00 ± 0.40 respectively. In patients with ovarian cancer, the mean Rα_1-ACT was 1.93 ± 0.50 slightly lower than in the controls.

The median values of Con A-reactive fractions, Con A-weakly reactive fractions and Con A-nonreactive fractions in normal subjects and in patients with ovarian or breast cancer were not significantly different (Table 9.).

Table 9. The relative areas of α_1-ACT peaks and Rα_1-ACT values in normal subjects and patients with ovarian or breast cancer. Different glycoforms of α_1-ACT were separated on CIAE. Peak areas enclosed by precipitates were measured by weighing a paper tracing of each pattern. The different peaks were delimited by vertical lines, and their relative proportions were expressed as percentages of the whole precipitate area. Rα_1-ACT was calculated for each serum sample according to the formula: (Proportions peak 1 + peak 2)/ Proportion of peak 3.

Source of serum	Con A-reactive fraction Peak 3 (%)	Con A-weakly reactive fraction Peak 2 (%)	Con A-nonreactive fraction Peak 1 (%)	Rα_1-ACT
N_1	28.8	34.4	36.5	2.46
N_2	40.3	32.5	27.2	1.48
N_3	28.8	41.0	30.2	2.47
N_7	41.4	34.8	23.8	1.42
N_8	28.2	36.6	35.2	2.55
N_9	30.6	37.5	31.9	2.27
N_{10}	36.0	36.0	28.0	1.78
N_{1-10} Average ± Standard deviation	33.44 ± 5.0	36.10 ± 1.9	30.40 ± 3.5	2.06 ± 0.43
B_1	36.5	40.6	22.9	1.74
B_2	35.3	35.3	29.4	1.83
B_3	26.7	39.7	33.6	2.75
B_4	37.5	35.3	27.2	1.67
B_{1-4} Average ± Standard deviation	34.00 ± 3.6	37.73 ± 2.4	28.28 ± 3.2	2.00 ± 0.40
O_1	28.7	39.6	31.7	2.48
O_2	41.3	33.5	25.2	1.42
O_3	35.1	42.4	22.5	1.85
O_4	26.5	36.0	37.5	2.78
O_5	37.2	39.8	23.0	1.69
O_6	42.2	40.7	17.1	1.37
O_{1-6} Average ± Standard deviation	35.20 ± 5.1	38.70 ± 2.6	26.20 ± 5.7	1.93 ± 0.50

N, normal; B, breast cancer; O, ovarian cancer.

According to these data the major microheterogeneity of α_1-ACT did not change in ovarian or breast cancer.

3.4 Investigation of albumin-depleted sera

3.4.1 Albumin depletion of sera

Treatment of serum samples with Blue Sepharose CL-6B removed the majority of the albumin (Fig. 5.). It also appeared that Blue Sepharose treatment removed a proportion of other proteins. To investigate this possibility a further experiment was carried out; the pellet of the gel was washed three times in PBS before electrophoretic analysis (Fig. 6.). It can be seen that after this treatment the pellet contains mainly albumin and that the concentrations of other proteins are small. Therefore, the results shown in Fig. 5. were obtained because of inadequate washing of the pellet.

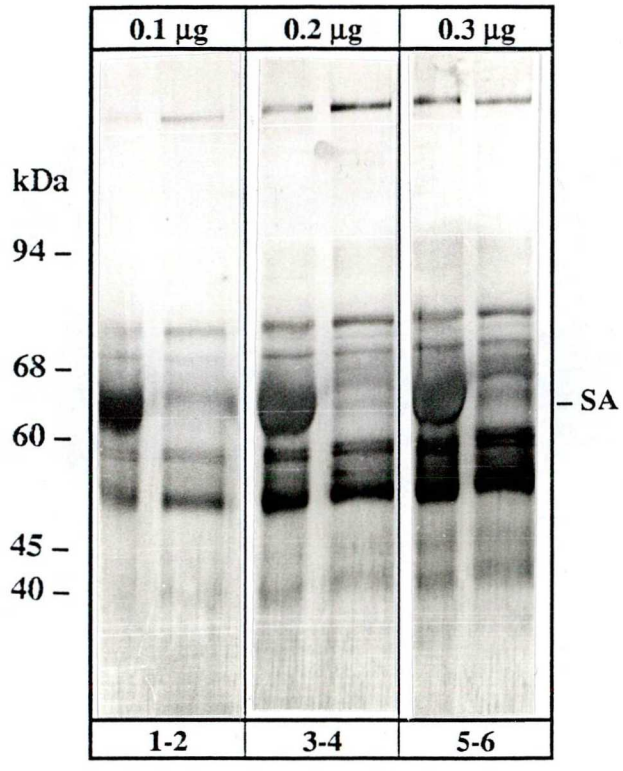


Fig. 5. A silver-stained gel of albumin-depleted sera. Samples of whole sera (50 µl) were treated with Blue Sepharose gel to remove the albumin as described under Experimental procedures section 2.3. Albumin-depleted healthy serum (10 µl) and the pellet (suspended in 0.5 ml PBS) were diluted (1:10) in 50 %(v/v) of DSSB (0.125 M Tris base, 20 % glycerol, 10 % SDS, 20 mM EDTA, 2 % bromophenol blue pH 7.4) and 5 %(v/v) 2-mercaptoethanol. Pellet containing 0.1 (lane 1), 0.2 (lane 3) and 0.3 (lane 5) µg protein, or depleted serum, containing 0.1 (lane 2), 0.2 (lane 4) and 0.3 (lane 6) µg protein were loaded onto the gel. The protein components of samples are shown after silver staining.

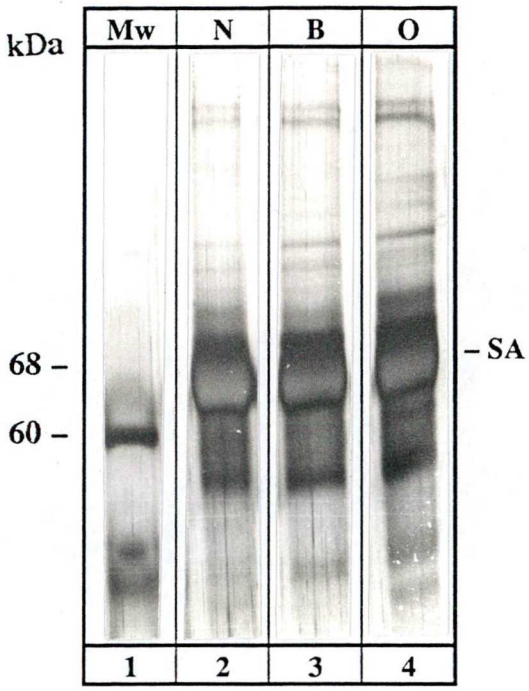
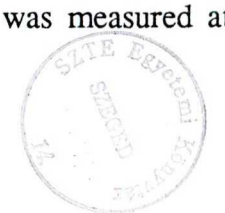


Fig. 6. Three times washed pellet on silver-stained gel. The experiment was carried out as described in Fig. 5, except that the pellet was washed three times with 0.5 ml PBS by centrifugation (11,500 g, 4 min, 4°C). The albumin containing Blue Sepharose CL-6B pellets (200 µl) were suspended in 500 µl DSSB buffer, 280 µl distilled water and 20 µl 2-mercaptoethanol. Molecular weight markers (lane 1); N_1, normal (lane 2); B_1, breast cancer (lane 3) and O_1, ovarian cancer (lane 4). The gel was photographed after silver staining.

3.4.2 Total protein concentration of albumin-depleted sera

The total protein concentration of each albumin-depleted sera was measured using bicinchoninic acid (BCA) protein assay as described in methods section 2.2.4. The absorbance of the solution was measured at 562 nm and the



protein concentrations were calculated based on the calibration curve. The following standards were used: 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 20.0 μg/well bovine serum albumin (BSA). The correlation between the BSA concentration and the absorbance was linear. The points of the calibration curve were calculated from three independent measurements. Each sample was assayed in triplicate on the ELISA plate (Table 10.).

Table 10. Total protein concentration in albumin-depleted serum. The protein concentration was determined using BCA protein assay. The mean values and the standard deviations were calculated from three independent measurements.

Source of serum	Total protein concentration (μg/μl)	Source of serum	Total protein concentration (μg/μl)	Source of serum	Total protein concentration (μg/μl)
N_1	25.65 ± 0.60	B_1	28.30 ± 1.00	O_1	37.35 ± 1.30
N_2	21.60 ± 0.80	B_2	26.10 ± 1.00	O_2	33.75 ± 1.70
N_3	25.65 ± 0.75	B_3	28.35 ± 1.10	O_3	33.75 ± 1.55
N_4	22.95 ± 0.60	B_4	24.30 ± 0.85	O_4	27.90 ± 0.95
N_5	27.90 ± 0.85			O_5	34.65 ± 1.05
				O_6	39.60 ± 1.65
N_1-N_5	24.75 ± 1.98	B_1-B_4	26.76 ± 1.56	O_1-O_6	34.5 ± 2.7

N, normal; B, breast cancer; O, ovarian cancer.

The mean total protein concentration (mean ± SD) in the healthy individuals and patients with breast and ovarian cancer were 24.75 ± 1.98 μg/μl, 26.76 ± 1.56 μg/μl and 34.5 ± 2.7 μg/μl, respectively. The total protein concentration was increased in ovarian cancer sera, by 1.4 fold; the mean value in this cancer group was significantly higher than the control. The increase of the total protein concentration in breast cancer sera was not significant, it was only slightly higher than the control.

3.4.3 The α_1-ACT concentration after albumin-depletion

After albumin-depletion the α_1-ACT concentrations were measured using RID. The concentrations before and after albumin-depletion were compared, and the percentage yield of α_1-ACT was calculated in each case (Table 11.).

Table 11. α_1-ACT concentration in sera, before and after albumin-depletion. The α_1-ACT concentration was measured by RID, and the percentage yield of α_1-ACT was calculated.

Source of serum	α_1-ACT concentration in serum before Sepharose treatment (μg/μl	α_1-ACT concentration in serum after Sepharose treatment (μg/μl)	% yield of α_1-ACT
N_1	1.00 ± 0.05	0.78 ± 0.05	78
N_2	0.74 ± 0.03	0.57 ± 0.02	76
N_3	1.07 ± 0.06	0.92 ± 0.06	86
N_4	0.51 ± 0.06	0.32 ± 0.02	64
N_5	0.67 ± 0.07	0.50 ± 0.03	75
B_1	1.03 ± 0.04	0.99 ± 0.06	95
B_2	1.44 ± 0.09	1.03 ± 0.08	71
B_3	1.45 ± 0.09	0.95 ± 0.04	66
B_4	1.33 ± 0.02	1.08 ± 0.07	82
O_1	2.32 ± 0.02	1.78 ± 0.07	77
O_2	2.21 ± 0.07	1.56 ± 0.08	71
O_3	2.17 ± 0.02	1.77 ± 0.09	82
O_4	1.79 ± 0.07	1.88 ± 0.03	87
O_5	0.82 ± 0.07	0.57 ± 0.01	70
O_6	1.23 ± 0.08	0.89 ± 0.02	70

N, normal; B, breast cancer; O, ovarian cancer.

Treatment of serum samples with Blue Sepharose CL-6B removed 23.0 ± 6.6 % of α_1-ACT. A small amount of α_1-ACT bonded to the beads and the complete removal of the supernatant was very difficult.

3.4.4 Identification of main glycoproteins by their molecular weight

The protein composition of albumin-depleted sera from healthy individuals and cancer patients were analysed by SDS-PAGE and silver staining (Fig. 7.).

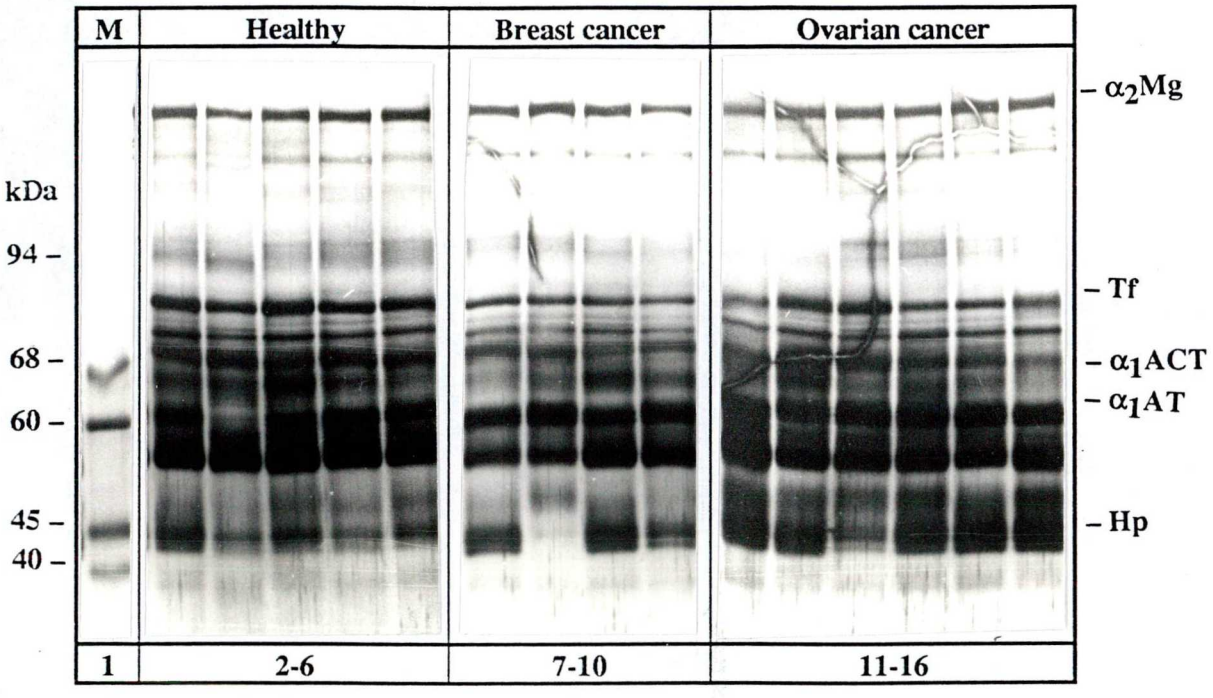


Fig. 7. Protein composition of albumin depleted sera from control, breast and ovarian cancer patients. A same amount of protein (3.5 μg) was loaded on each line. The following samples were applied: N_5, N_4, N_3, N_2, N_1 (lanes 2 to 6 respectively), B_4, B_3, B_2, B_1 (lanes 7 to 10 respectively), O_6, O_5, O_4, O_3, O_2, O_1 (lanes 11 to 16 respectively), molecular weight markers: phosphorilase B (94 kDa), serum albumin (68 kDa), catalase (60 kDa), ovalbumin (45 kDa) and aldolase (40 kDa) (lane 1).

The main glycoproteins in albumin-depleted sera could be identified by their molecular weight. The following glycoproteins were detected:

Name of protein	**Mw** (kDa)	
α_2-macroglobulin	180	(subunite size)
transferrin	76	
α_1-antichymotrypsin	65	
α_1-antitrypsin	60	
haptoglobin	45	(β chain)

The amount of α_2-macroglobulin did not show significant changes in cancer patients. The transferrin level declined in the sera from cancer patients, the IgG level decreased in the sera from breast cancer patients. The α_1-AT concentration was increased in both breast and ovarian cancer sera; the haptoglobin level increased dramatically in the sera from ovarian cancer patients.

3.4.5 Identification of main glycoproteins by the use of specific antibodies

To confirm the position of the major glycoprotein in serum and to accurately identify the position of α_1ACT, sera were investigated using specific antibodies to α_1ACT, α_1-AT, α_1-AGP and haptoglobin. The blots obtained are shown in Fig. 8. and the Mw of the major bands were calculated using molecular weight markers.

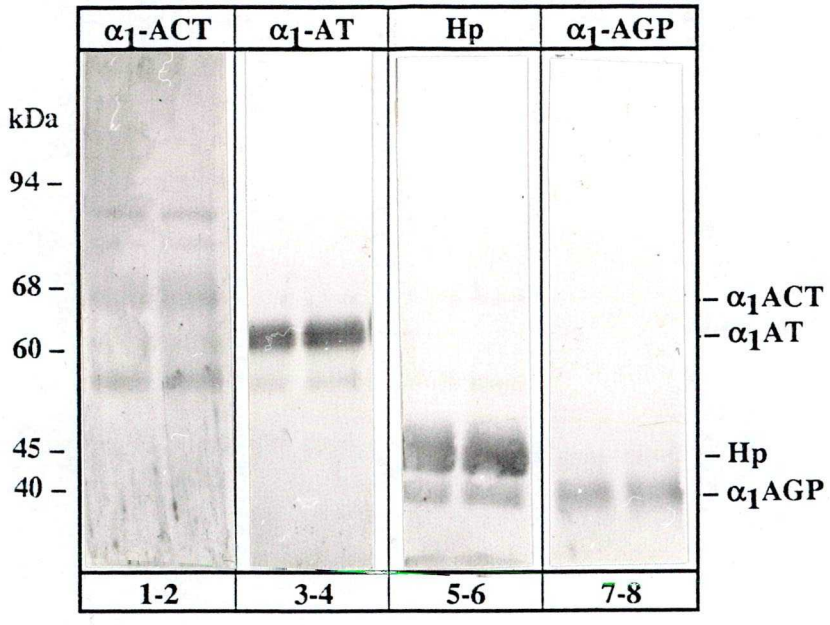


Fig. 8. Immunoblot analysis of the serum proteins detected by different antibodies. α_1-antichymotrypsin antiserum (lanes 1-2), α_1-antitrypsin antiserum (lanes 3-4), haptoglobin antiserum, (lanes 5-6), α_1-acid glycoprotein antiserum (lanes 7-8).

The following glycoproteins were identified:

Name of protein	**Mw** (kDa)	
α_1-antichymotrypsin	65	
α_1-antitrypsin	60	
haptoglobin	45	(β chain)
α_1-acid glycoprotein	40	

3.5 The carbohydrate components of α_1-ACT and other serum glycoproteins using lectins

The sera separated on SDS-gels and the carbohydrate components in α_1-ACT and other serum glycoproteins were analysed using different lectins. The intensity of the reaction indicated the presence of a particular carbohydrate structure.

3.5.1 Investigation of α2,6-linked NANA by SNA

The SNA lectin binds glycoconjugates containing the terminal sequence NeuAc(α2,6)Gal, while those isomers containing NANA-linked α2,3 are not bound. Using SNA five major lectin-binding bands were detected in all serum samples examined (Fig. 9.). These glycoproteins were identified by their molecular weights on the blots relative to the standards.

The following glycoproteins were detected with SNA: α_2-macroglobulin (180 kDa), transferrin (76 kDa), α_1-ACT (65 kDa), α_1-AT (60 kDa) and haptoglobin (45 kDa). The SNA reacted with α_1-ACT, suggesting α_1-ACT contained NeuAc(α2,6)Gal residues in sera from healthy individuals and from patients with ovarian or breast cancer. The intensity of α_1-ACT-SNA reaction suggested that the NeuAc(α2,6)Gal level decreased in some ovarian and breast cancer patients (B_3, B_4, O_1, O_2) compared to the controls, but this was not found in all cancer sera suggesting that the change was not specifically correlated with their disease states.

SNA also reacted with two other proteins (glycoprotein I, 150 kDa and glycoprotein II, 105 kDa) in the samples of O_4, O_5 and O_6 (Fig. 6.). These glycoproteins were not detected by SNA in healthy or breast cancer sera. The reaction with these glycoproteins suggested that they contained α2,6-sialylated glycans.

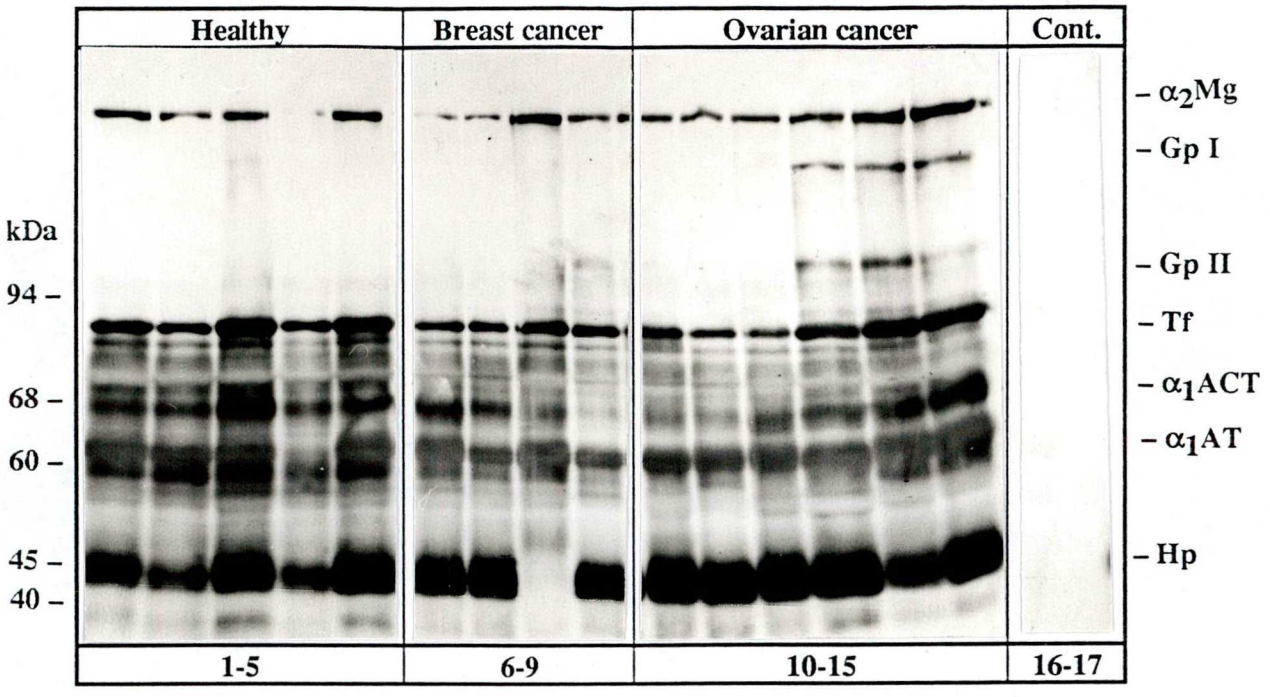


Fig. 9. Lectin blots of serum proteins detected by SNA. From each albumin-depleted serum, 3.5 μg of α_1-ACT was applied: N_1, N_2, N_3, N_4, N_5 (lanes 1 to 5 respectively) B_1, B_2, B_3, B_4 (lanes 6 to 9 respectively), O_1, O_2, O_3, O_4, O_5, O_6 (lanes 10 to 15 respectively). Transferrin and carboxypeptidase Y (2.0 μg) were used as positive and negative controls respectively (lane 16-17 respectively).

3.5.2 Analysis of α2,3-linked NANA using MAA

NANA linked by α2,3-linkages to galactose binds to MAA with particularly high affinity, whereas isomeric structures containing terminal NANA in the α2,6-linkage do not shown high reactivity. MAA reacted with; α_2-macroglobulin (180 kDa), transferrin (76 kDa), α_1-ACT (65 kDa) and IgG (Fig. 10.) on lectin blots.

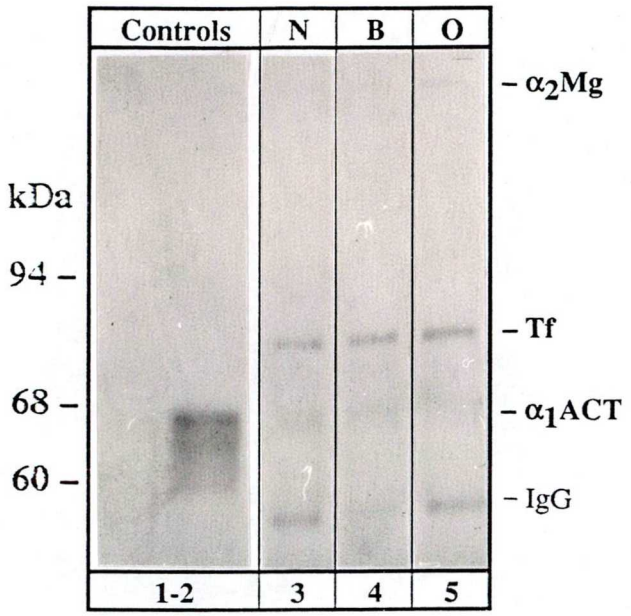


Fig. 10. Lectin blots of the serum proteins detected with MAA. Carboxypeptidase Y and fetuin (2.0 μg) were used as a negative and positive control (lanes 1-2, respectively). From each albumin-depleted serum, 3.5 μg of α_1-ACT was applied: N_1 (lane 3), B_1 (lane 4), O_1 (lane 5).

The MAA reacted weakly with α_1-ACT in all the specimens examined suggesting that this glycoprotein also contained NeuAc(α2,3)Gal residues. There was no difference between the different types of sera.

3.5.3 Investigation of β1,4-linked galactose by RCA

RCA recognises asialylated or α2,6 sialylated (but not the α2,3 sialylated) β1,4-linked galactose residues. Five major lectin-binding bands were detected in all sera investigated. From their positions relative to the molecular markers these were identified as: α_2-macroglobulin (180 kDa), transferrin (76 kDa), α_1-ACT (65 kDa), α_1-AT (60 kDa) and haptoglobin (45 kDa) (Fig. 11.).

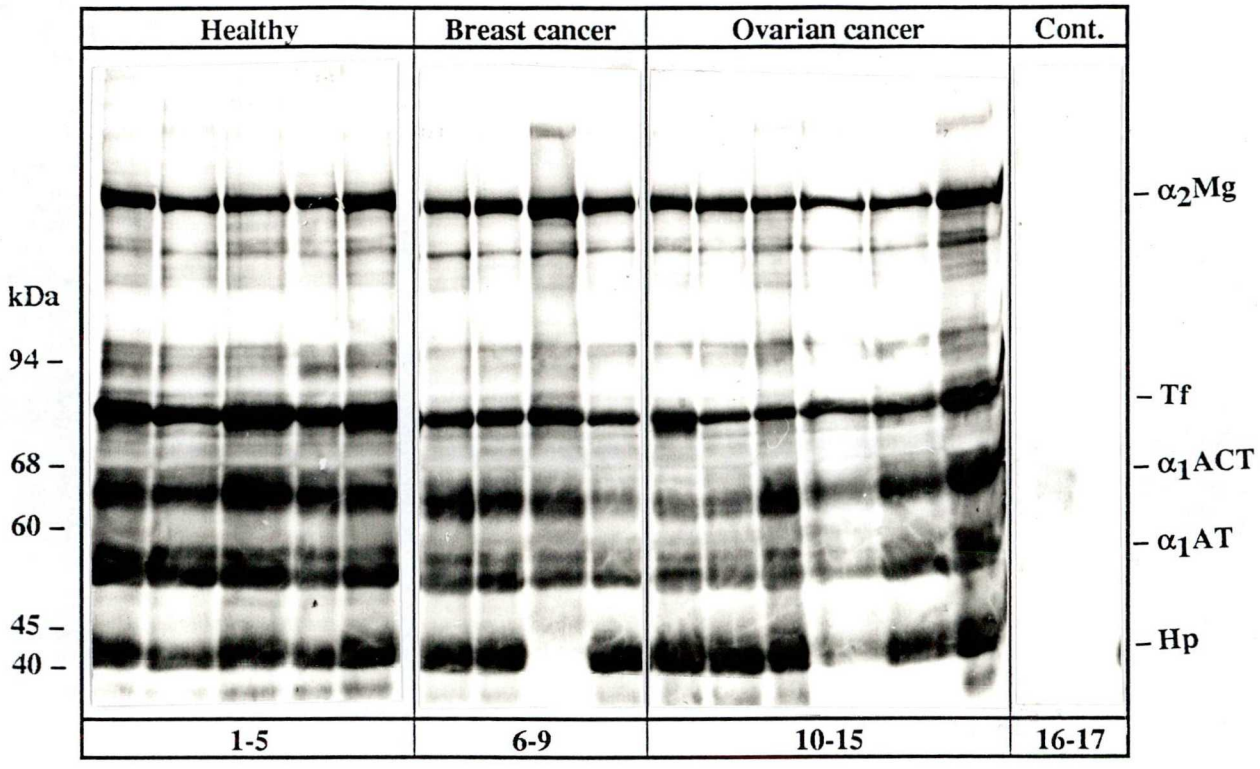


Fig. 11. Lectin blots of the serum proteins detected by RCA. From each albumin-depleted sera, 3.5 μg of α_1-ACT was applied: N_1, N_2, N_3, N_4, N_5 (lanes 1 to 5 respectively) B_1, B_2, B_3, B_4 (lanes 6 to 9 respectively), O_1, O_2, O_3, O_4, O_5, O_6 (lanes 10 to 15 respectively). Fetuin and Carboxypeptidase Y (2.0 μg) were used as positive and negative controls (lane 16-17 respectively).

The RCA reaction with α_1-ACT suggested that this glycoprotein contained either α2,6 sialylated β1,4-linked galactose residues or asialylated galactose. The intensity of α_1-ACT-RCA reaction was lower in B_4, O_1, O_2, and O_4 samples than the controls. In the SNA experiment decreased staining was observed for B_4, O_1, O_2, samples. This suggests that the NeuAc(α2,6)Gal level in α_1-ACT was the same in most normal and cancer sera, but in a few there was less sialic acid linked 2-6 to galactose.

RCA also recognised the glycoprotein I (150 kDa) and glycoprotein II (105 kDa) in all sera. The SNA and RCA reactions with these glycoproteins suggested that they contained terminal galactose residues in the healthy and breast cancer sera, whilst they were α2,6-sialylated in O_4, O_5 and O_6 sera.

3.5.4 Examination of N-acetyl-D-glucosamine by DSA

DSA recognises terminal and/or internal Galβ-(1,4)GlcNAc residues in complex and in hybrid N-glycans. Five major lectin-binding bands were detected in all sera investigated. These glycoproteins were identified by their Mr values as: α_2-macroglobulin (180 kDa), transferrin (76 kDa), α_1-ACT (65 kDa), α_1-AT (60 kDa) and haptoglobin (45 kDa) (Fig. 12.).

α_1-ACT reacted with DSA suggesting this glycoprotein contained terminal and/or internal Galβ-(1,4)GlcNAc residues in healthy and ovarian or breast cancer sera. The intensity of the DSA reaction showed that the Galβ-(1,4)GlcNAc content of α_1-ACT did not alter in cancer sera compared to controls.

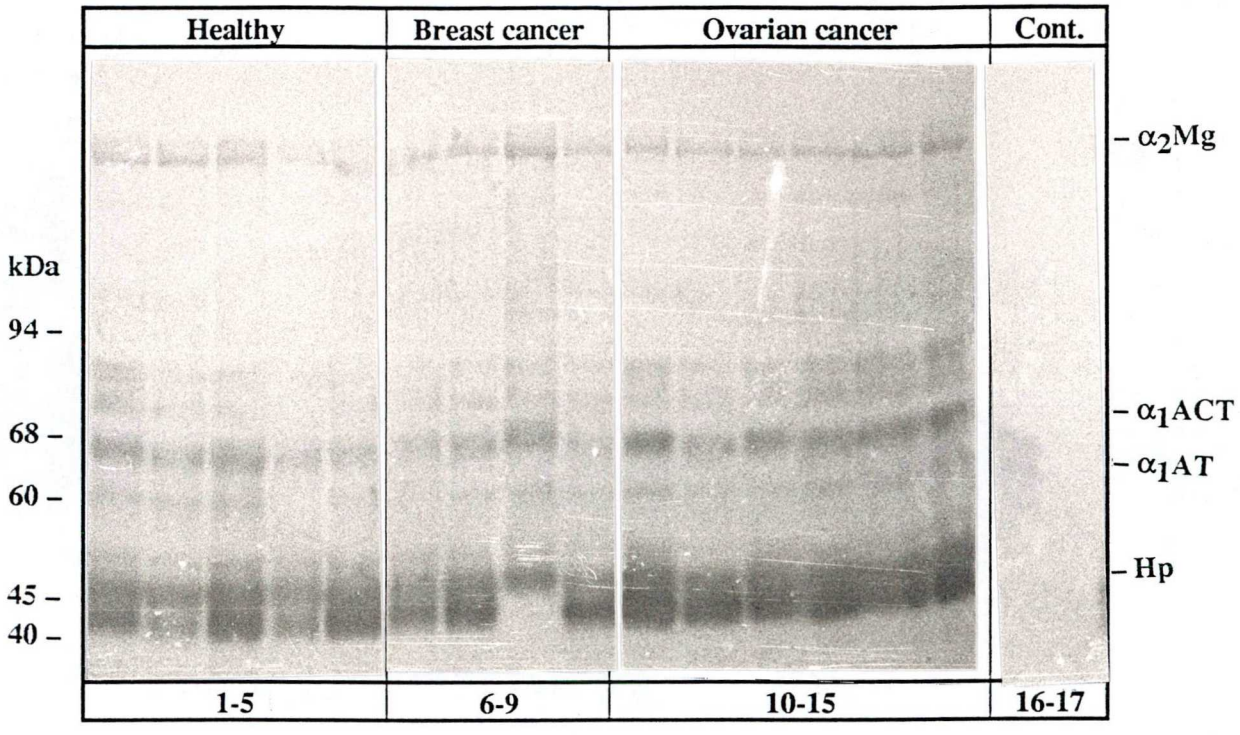


Fig. 12. Lectin blots of the serum proteins detected by DSA. From each albumin-depleted sera, 3.5 μg of α_1-ACT was applied: N_1, N_2, N_3, N_4, N_5 (lanes 1 to 5 respectively) B_1, B_2, B_3, B_4 (lanes 6 to 9 respectively), O_1, O_2, O_3, O_4, O_5, O_6 (lanes 10 to 15 respectively). Fetuin and Carboxypeptidase Y (2.0 μg) were used as positive and negative controls (lane 16-17 respectively).

3.5.5 Investigation of α1,6 and α1,3 linked fucose using Lotus agglutinin

Lotus agglutinin recognises α1,6-or α1,3 linked fucose residue of N-glycans. This lectin reacted with α_2-macroglobulin (180 kDa), transferrin (76 kDa), α_1-ACT (65 kDa) and IgG (Fig. 13.).

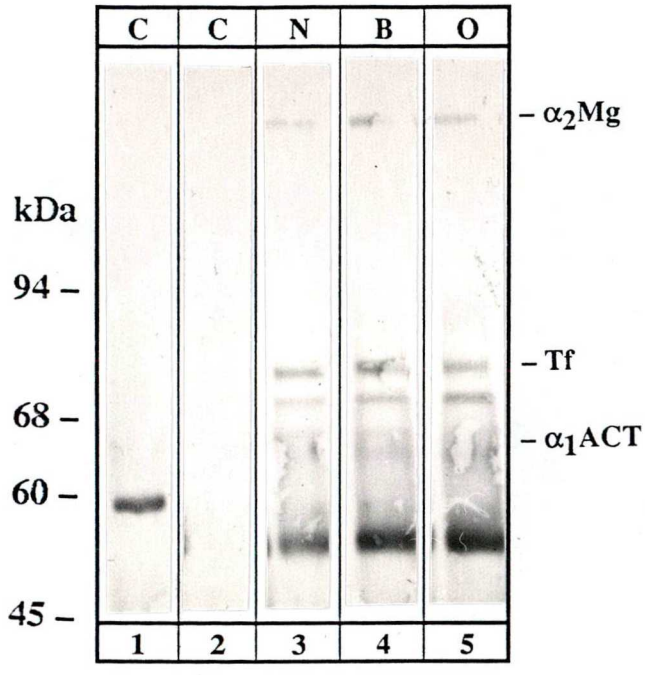


Fig. 13. Lectin blot of the serum proteins detected by Lotus agglutinin. Catalase and carboxypeptidase Y were used as positive and negative controls (lane 1-2). From each albumin-depleted serum 3.5 μg α_1-ACT was applied: N_1 (lane 3), B_1 (lane 4), O_1 (lane 5).

The Lotus lectin reacted with α_1-ACT and the intensity of this reaction did not differ in cancer sera compared to controls. It appeared that the α_1-ACT contained the same amount of fucose both in normal and in cancer sera.

4 Discussion

4.1. Changes in the serum α_1-ACT concentration in ovarian and breast cancer

α_1-ACT is an inhibitor of several serine proteolytic enzymes that play a role in blood coagulation, fibrinolysis, the metabolism of connective tissue, and inflammatory and immunological processes. Antiproteases are also partly responsible for the stabilisation of cell membranes (Travis and Salvesen, 1983).

The concentration range of α_1-ACT is 0.18-0.85 g/l in healthy serum (Table 12.).

Table 12. Comparison of α_1-ACT concentration in healthy serum in different studies.

Determination procedure	Concentration of α_1-ACT (g/l)	Reference
Electroimmunoassay	0.18-0.26	Matsumoto et al. 1982
RID	0.41 ± 0.07	Bernacka et al. 1982
RID	0.65 (0.47-0.85)	Bowen et al. 1982
Electroimmunoassay	0.4 ± 0.12	Mackiewicz et al. 1986
Electroimmunoassay	0.43 (0.34-0.47)	Hachulla et al. 1988
Electroimmunoassay	0.28 ± 0.01	Bernacka et al. 1988
RID	0.61 (0.34-0.84)	Hachulla et al. 1990
RID	0.43 (0.32-0.84)	Hachulla et al. 1992
RID	0.77 (0.51-1.07)	Váradi et al 1993a,b

In different studies either the normal concentration range or the average ± standard deviation was given. In our experiments the measured concentration of α_1-ACT (0.77 ± 0.15 g/l) in normal sera was comparable with other published results. The normal range for α_1-ACT depends mostly on the method that is used.

Elevated levels of α_1-ACT have been reported in sera of patients with inflammatory diseases (Meryn et al. 1985, Hachulla et al. 1990, 1992, Plusa and Tchorzewski, 1985); collagen diseases (Ekerot and Ohlsson, 1984); pregnancy

(Raynes, 1982); and in some malignant diseases (Bernacka et al. 1988, Laine and Hachulla, 1991). It was found in this study that the concentration of α_1-ACT was increased in the breast and ovarian cancer sera, by 2 and 2-2.5 fold respectively. The mean values of both cancer groups were significantly higher than that of the controls.

4.2 Stimulation of α-antichymotrypsin synthesis in Hep G2 cell line

Elevated levels of α_1-ACT in serum from patients with malignant tumours may be caused by enhanced synthesis in the liver. This could occur as a result of increased release of proteases by the lysosomes from tumour cells, or from the inflammatory process surrounding the tumour. According to other authors, the tumour cells themselves may be a third source of the antiproteases (Gaffar et al. 1980, Tahada et al. 1984, Takara et al. 1986). The results of this study show that ovarian tumours can directly affect the synthesis of host molecules. All diluted cancer media stimulated the synthesis of α_1-ACT in Hep G2. The concentration of α_1-ACT was increased in the conditioned media by 2.4 fold. Similar increase of α_1-ACT concentration was observed in the sera from ovarian cancer patients. Therefore the Hep G2 cells could be used as an *in vitro* model to test experimentally these interactions.

Another explanation of the elevated α_1-ACT is that tumour cells and inflammatory cells can produce factors (e.g. IL-1, IL-6, TNF, TGF) that could stimulate the synthesis and release of α_1-ACT in the liver (Laiho et al. 1987, Turner and Thompson, 1991). Cultured liver cells are an experimental system to test this hypothesis. Using the hepatoma cell line (Hep G2), growing in either DMEM+10 % FCS or serum free media, all cytokines (TGF-β, TGF-α and IL-6) increased α_1-ACT accumulation about 2.4-2.8 fold. TGF-β is a multifunctional peptide that is synthesised by a variety of cells (Assoian et al. 1983, 1987). It is a major participant in inflammatory and immunological response to injury and infection, and in the

consequent process of tissue repair (Roberts and Sporn, 1988). It appears that one of its roles is to limit proteolysis by inhibiting synthesis of proteolytic enzymes or by stimulating synthesis of antiproteases (Edwards et al. 1987, Laiho et al. 1987). It was demonstrated in this study that TGF-β was involved in regulating the synthesis of an antiprotease, α_1-ACT.

The IL-6 alone increased the α_1-ACT biosynthesis by 2.1-2.3 fold; in combination with TGF-β or TGF-α the α_1-ACT concentration increased by 2.8-4.3, 2.6-2.8 fold, respectively. The various combinations of IL-6 with TGF-β/TGF-α showed an additive effect. The effect that one cytokine could be modulated by another cytokine supports the view that the alteration in level of α_1-ACT is modulated by combinations of cytokines (Mackiewicz and Kushner, 1990).

4.3 Changes in the microheterogeneity of α-antichymotrypsin in ovarian and breast cancer patients

Quantitative changes in acute phase proteins in serum are often accompanied by qualitative alterations of their carbohydrate moieties; this is referred to as microheterogeneity. The major microheterogeneity reflects changes in the number of branches on heteroglycan antennary structures. Affinity electrophoresis with concanavalin A (Con A) as a ligand has been widely used for the determination of major microheterogeneity of acute phase proteins (Breborowicz and Mackiewicz, 1989, Hansen et al. 1986). In this system microheterogeneous forms with biantennary heteroglycans can be separated from forms having tri- and/or tetra-antennary units.

α_1-ACT studied by CIAE was resolved into three immunoprecipitation peaks (Peak 1: Con A-nonreactive fraction; Peak 2: Con A-weakly reactive fraction; Peak 3: Con A-reactive fraction). This three-peak microheterogeneity of α_1-ACT was found both for the normal and pathological sera studied. The peak distribution and

the pattern were also very similar. The major microheterogeneity (the number of branches on heteroglycan antennary structures) of α_1-ACT did not alter in ovarian and breast cancer.

Bowen et al. (1982) observed that α_1-ACT displayed three peaks on CIAE with free Con A in the first dimension. The proportions of each protein component (1, 2 and 3) were altered in disease. The changes in proportions of protein components of α_1-ACT were largest in acute diseases. In cancer, the proportions of the protein components were slightly different to those seen in acute diseases. The pattern in chronic conditions appeared more like the normal pattern but with each peak increased. It is difficult to compare the α_1-ACT microheterogeneity observed in my study with the result obtained by Bowen et al. (1982), because they did not use sugar to dissolve the affinity precipitate. However, the α_1-ACT pattern shown by Bowen et al. (1982) in cancer patients was very similar to that observed in this study.

Raynes et al. (1982) noticed that α_1-ACT displayed three peaks on CIAE, and four peaks were obtained when methyl glucose was added to the second dimension gel. There was an increase in the Con A binding form of α_1-ACT in acute inflammation, and in the chronic inflammation associated with rheumatoid arthritis. In disseminated cancer, the pattern was the same as in normal serum, despite the marked increase in the serum concentration. The α_1-ACT pattern for three peaks (1, 2 and 3) shown by Raynes et al. in cancer patients was very similar to that described in this study, the only difference being the absence of peak 4.

Hachulla et al. (1988, 1990, 1992), Laine and Hachulla (1991), Laine et al. (1989, 1991) observed that α_1-ACT was resolved into four peaks using CIAE. Only three peaks were obtained without sugar added to the second dimension, resulting in the disappearance of peak 4. An increase in the Con A-reactive fraction was found in acute inflammation, but this was not found in chronic inflammation. Moreover, there was a decrease in this fraction in chronic inflammation with acute attacks (Table 13.).

Table 13. Relative variations in the three microheterogenous forms of α_1-ACT in the various inflammatory situations as compared with healthy donors. Hachulla et al.(1988).

Inflammatory syndromes	Con A-nonreactive fraction (Peak 1)	Con A-weakly reactive fraction (Peak 2)	Con A-reactive fraction (Peak 3)
Acute inflammation			
(MI)	—	↓	↑
(SI)	↓↓	↓	↑↑
Chronic inflammation (MBC)	—	—	—
Chronic inflammation with acute attacks (CTD)	↑↑	—	↓

↑, increased; ↓, decreased; —, no relative variation; MI, myocardial infarction; SI, septic inflammation; MBC, metastatic breast cancer; CTD, connective-tissue disease.

In the current study only three peaks were obtained in CIAE using 5 methyl-α-D-glucopyranoside; it may be that the higher concentration of the specific displacer used resulted in the formation of one less peak. The concentration of Con A and anti-human α_1-antichymotrypsin antibody used were the same as used in previous studies, but the methyl-α-D-glucopyranoside concentration was higher than that used by Raynes et al (1982) and Hachulla et al. (1988). The 3 peaks of α_1-ACT pattern observed was very similar to that shown by Hachulla et al. (1988) in cancer patients, the only difference being the absence of peak 4.

Both the glycosylation and/or the clearance, of α_1-ACT might vary in different inflammatory processes. It is possible, therefore, that a stimulus which causes a change in the rate α_1-ACT synthesis may not change the glycosylation. This could occur if glycosylating enzymes were not induced as rapidly as those involved with protein synthesis. Therefore, the change in the glycosylation pattern seen in the

acute response to tissue injury compared with that in chronic inflammation, may be explained by the subsequent induction of the glycosylating enzymes.

4.4 The molecular weight of α_1-ACT

α_1-ACT is a highly glycosylated protein with an estimated molecular weight of 55-68 kDa in SDS-PAGE (Table 14.).

Table 14. Comparison of the molecular weight of serum α_1-ACT in different publications.

Molecular weight of α_1-ACT (Dalton)	Reference
64 000	Siddiquit et al. 1980
58 000	Laine and Hayem, 1981
64 000	Matsumoto et al. 1981
68 000	Bowen et al. 1982
59 000	Laine et al. 1984
68 000	Bao et al. 1987
58 000 - 64 000	Lindmark et al. 1989
55 000 - 66 000	Rubin et al. 1990
65 000 - 68 000	Váradi et al 1993a,b

In our experiments the determined Mr of α_1-ACT was comparable with other published results.

4.5 Carbohydrate content of α_1-ACT

The carbohydrate structure of α_1-ACT was also investigated using different lectins (SNA, MAA, RCA, DSA, and Lotus lectin). α_1-ACT reacted with all of these lectins suggesting the presence of terminal NeuAc(α2,6)Gal; NeuAc(α2,3)Gal;

terminal and/or internal Galβ(1,4)GlcNAc; and α1,6-or α1,3 linked fucose residues. This suggests that α_1-ACT is a complex type N-linked glycoprotein. The results with Lotus lectin are not in accordance with the observation of Laine et al. (1991) who found previously that α_1-ACT did not contain fucose. This is probably due to the different sensitivity of the determinations.

Laine et al. (1991) also reported that the biantennary structures of α_1-ACT were α-2,6-sialylated and that the trisialylated glycan structures possessed α-2,6 and α-2,3-linked NANA (Fig. 14.), in the ratio 2:0.8. These results are in accordance with observation of the current study. Laine et al. (1991) also determined the proportions of bi- and triantennary glycans in each microheterogeneous form of α_1-ACT.

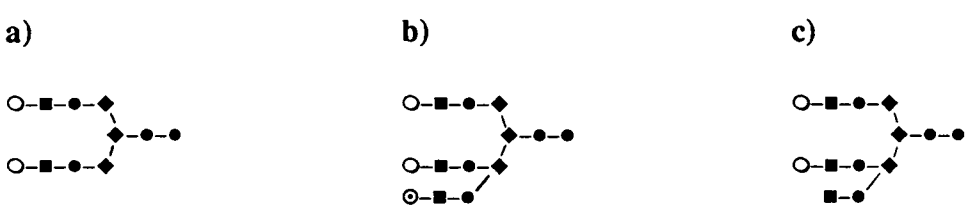


Fig. 14. The primary structures of the biantennary (a), and the triantennary (b, c) glycans form normal human serum α_1-ACT. (●) GlcNAc; (◆) Man; (■) Gal; (○) NeuAc(α2-6); (⊙) NeuAc(α2-3) (Laine et al. 1991).

The majority of breast and ovarian cancer sera contained similar amounts of NeuAc(α2,6)Gal, NeuAc(α2,3)Gal, terminal and/or internal Galβ(1,4)GlcNAc, and α1,6-or α1,3 linked fucose residues, to the levels in the normal sera.

4.6 Investigation of other proteins in human sera

The amount of α_2-macroglobulin did not show significant changes in sera from ovarian or breast cancer patients. Gordon and Koj (1985) reported no acute

phase increase for this protein in man. On the other hand, transferrin levels declined in the cancer sera while α_1-AT concentration increased in both breast and ovarian cancer sera. Transferrin is considered to be a negative acute phase protein, whereas α_1-AT is considered to be a positive acute phase protein (Gordon and Koj, 1985).

The IgG level decreased in breast cancer sera, whereas its concentration was similar in ovarian cancer sera compared to the controls. The haptoglobin level increased dramatically in the sera from ovarian cancer patients. The total protein concentration was significantly higher in ovarian cancer sera, while it was similar in breast cancer sera compared to controls. These results suggest that in ovarian and breast cancer the synthesis of some serum proteins are different.

4.7 Possible uses of serum α_1-ACT analysis in the future

The normal range of α_1-ACT was 0.18-0.85 g/l. However, false positive results were obtained in some healthy individuals and false negative results were demonstrated in a few cancer patients. To determine the exact correlation between the elevated serum α_1-ACT level, and the clinical diagnosis, a more detailed study needs to be carried out on a larger number of patients.

Above 1.0 g/l serum α_1-ACT concentration the chance of pathological alterations are high. High α_1-ACT level does not mean necessarily the presence of malignant tumour, because other diseases like myocardial infarction, rheumatoid arthritis, acute viral hepatitis can also induce the enhanced synthesis of different acute phase proteins. From the medical point of view it is very important to distinguish between malignant tumours and other diseases. For example acute inflammation is also followed by high α_1-ACT concentration but it is possible to distinguish it from malignant tumours based on the different microheterogeneity that can be determined by CIAE.

The determination of α_1-ACT concentration, combined with clinical examinations and other clinical chemistry tests, could be useful in early diagnosis of malignant tumours.

5 Conclusions

1. A protease inhibitor, α_1-ACT, was investigated in serum from ovarian and breast cancer patients. It was found that the concentration of α_1-ACT was increased in breast and ovarian cancer sera. The mean values for both cancer groups were significantly higher than that of the controls.

2. A model system for studying the mechanisms governing the alterations of α_1-ACT concentration was investigated. Addition of conditioned media from five ovarian cancer cell lines stimulated α_1-ACT synthesis in liver cells growing either in DMEM or SFM. α_1-ACT levels increased to the same degree in this *in vitro* model as observed in the sera from ovarian cancer patients. The α_1-ACT biosynthesis was also influenced by several cytokines. The effect of one cytokine could be modulated by another cytokine, which supported the view that the alteration in level of α_1-ACT was modulated by combinations of cytokines. The tumour cells may synthesise α_1-ACT directly and/or be produced by several hormones which enhance the α_1-ACT production in the liver.

3. Using CIAE, the microheterogeneity of α_1-ACT was examined in sera from healthy donors and in sera from patients with ovarian or breast cancer. α_1-ACT studied by CIAE was resolved into three peaks. This type of microheterogeneity was found both for normal and pathological sera. The CIAE patterns were similar for both normal and pathological sera. Because the microheterogeneity of α_1-ACT did not alter in ovarian and breast cancer sera, the investigation of glycoforms was not useful as a biochemical marker in diagnosis.

4. Using lectin blotting, α_1-ACT was shown to contain NeuAc(α2,6)Gal, NeuAc(α2,3)Gal, terminal Galβ-(1,4)GlcNAc and α1,6 or α1,3 linked fucose residues. All these components were found in α_1-ACT from ovarian and breast

cancer sera and in sera from healthy individuals. There were no differences in the composition of the carbohydrate structures in the normal and cancer sera. In a few cases of ovarian cancer there were changes in the glycosylation of α_1-ACT detected by SNA and RCA.

5. In ovarian and breast cancer the synthesis of some serum proteins are different. In some cases the effect of ovarian and breast cancer on the glycosylation may be different.

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