Modification of the erythrocyte surface in rats bearing Yoshida ascites sarcoma is brought about by a tumour variant of α_2 -macroglobulin

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Erythrocytes from the circulation of rats bearing Yoshida ascites sarcoma exhibit higher concanavalin A (ConA)-mediated agglutinability than those from normal animals. A tetrameric glycoprotein of subunit molecular mass 170 kDa, purified from the cell-free ascites fluid, was found to confer higher ConA-mediated agglutinability on erythrocytes *in vitro*. An antiserum to this tumour-derived protein failed to detect any cross-reactive component in normal rat plasma or in any of the normal tissues examined. An immunoreactive protein was, however, detected in blood plasma when the acute-phase reaction was stimulated by injection of turpentine. The cross-reactive acute-phase protein was purified by ConA-affinity, gel-filtration and ion-exchange chromatography, and identified as α_2 -macroglobulin. The acute-phase protein and the protein obtained from the ascites fluid have identical or very similar native and subunit molecular masses, subunit arrangement and pI. They both are able to inhibit trypsin and, as a consequence, acquire greater mobility in native PAGE. In addition, the two proteins bind to rat erythrocytes non-specifically, and in similar amounts. However,

despite these similarities, the acute-phase protein is unable to enhance the agglutinability of erythrocytes. The two proteins differ in their carbohydrate content, but this differential glycosylation is not the cause of the difference in their surface modification activity. The chemically deglycosylated proteins show a small but consistent difference in the size of their polypeptides. Their tryptic peptide maps, although largely similar, show some differences, as do their amino acid compositions. It is probable that the proteins are independent members of the same (α -macroglobulin) family. The rat embryo is also found to express a soluble protein consisting of a 170 kDa polypeptide that cross-reacts with the antibody to the tumour-derived protein. The purified embryo protein is able to alter the ConA-mediated agglutinability of erythrocytes *in vitro*, and also yields a tryptic peptide map that is identical to that of the tumour-derived protein. The modification of the host cell surface in the tumour-bearing rats is thus caused by what appears to be a tumour (oncofetal?) variant of α_2 -macroglobulin.

INTRODUCTION

Using an animal model system, we are investigating the mechanisms of paraneoplastic syndromes, which arise due to structural and functional modifications of host cells that are not in contact with the tumour [1,2]. Yoshida ascites sarcoma cells growing in the rat peritoneal cavity enhance the concanavalin A (ConA)-mediated agglutinability of erythrocytes in the circulation [3]. Three distinct fractions isolated from cell-free ascites fluid were able to modify erythrocyte agglutinability *in vitro*, and at least one of these was found to diffuse into the circulation from the peritoneal cavity [4]. From this *in vitro* active fraction, a tetrameric glycoprotein of subunit molecular mass 170 kDa was purified. The protein was found to bind to erythrocytes in a non-saturable manner at a few thousand copies per erythrocyte [4], and to reduce the surface charge of the cells [5]. It was shown to be present in the tumour cell lysate and plasma of tumour-bearing animals, but not in normal rat plasma [5].

We report here that none of the tissues examined from normal rats showed the presence of the protein, but that a structurally and functionally closely related protein appeared in the plasma under acute-phase conditions. This protein was purified and identified as α_2 -macroglobulin. However, neither the acute-phase plasma (APP) nor the purified protein was able to modify erythrocyte ConA-mediated agglutinability. An immunoreactive protein was also found in the rat embryo. The purified embryonic protein was able to enhance the agglutinability of

erythrocytes *in vitro* in a manner similar to that of the tumour-derived protein, and appears to be identical to it.

MATERIALS AND METHODS

Materials

The sources of most of the chemicals used have been described previously [4]. Azoalbumin, tunicamycin and chelating Sepharose were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The deglycosylation kit was purchased from Oxford Glyco-System, Abingdon, Oxon., U.K. Banana lectin was purified from a local variety of the fruit as described by Koshte et al. [6], and was conjugated to Sepharose by the procedure of March et al. [7]. The lectin was bound at a concentration of 1 mg/ml of settled gel. Purification of the ascites fluid 170 kDa-subunit protein and the production of antibodies against it have been described [4].

Ascites fluid, erythrocytes and rat tissues

The propagation of Yoshida ascites sarcoma in inbred Wistar rats, and the collection and processing of ascites fluid, were essentially as described [3].

Normal rat tissues and embryos (irrespective of age) were collected and immediately placed in cold Tris-buffered saline (TBS; 0.01 M Tris/HCl, pH 7.4, containing 0.15 M NaCl). After about 5 min, the tissue/embryo was freed of any extraneous

Abbreviations used: APP, acute-phase plasma; ConA, concanavalin A; MeMan, methyl- α -D-mannopyranoside; TFMS, trifluoromethane sulphonic acid; Tos-Phe-CHCl₂, tosylphenylalanylchloromethane ('TPCK').

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matter and homogenized in fresh TBS (2 ml/g tissue wt.) containing 5 mM EDTA, 1 mM PMSF and 10 μ g/ml each of aprotinin, leupeptin and pepstatin. Four 10 s bursts were applied at intervals of 30 s in a Polytron-type homogenizer, with the vessel held on ice. The homogenate was then centrifuged at 100 000 *g* for 1 h. The pellet was homogenized again in the same medium containing, in addition, 1% Triton X-100, and re-centrifuged.

The acute-phase response was stimulated in rats by subcutaneous injection of 0.2 ml of oil of turpentine per 100 g body weight. Blood was collected 2 days later by cardiac puncture under anaesthesia. Plasma and erythrocytes were separated from EDTA-anticoagulated blood, and the erythrocytes were washed as described previously [3,4].

Purification of the APP protein

A 10 ml sample of APP was passed over a 1.6 cm $\times$ 20 cm column of ConA-Sepharose. After removing the unbound protein by washing with TBS, the bound protein was eluted initially with 40 mM glucose, and then with 200 mM methyl- α -D-mannopyranoside (MeMan), both in TBS. The latter fraction (50–60 mg of protein) was dialysed and then loaded on a 2.15 cm $\times$ 130 cm column of Sepharose 4B, and eluted with TBS. Protein in the first peak was concentrated and applied (6–7 mg) on a DEAE-Sepharose column (1 cm $\times$ 5 cm), also in TBS. The unbound fraction contained pure 170 kDa polypeptide by SDS/PAGE. This procedure is similar to that used for the purification of 170 kDa-subunit protein from ascites fluid [4], except that the pH of the buffer during DEAE-Sephadex chromatography was 7.4 rather than 8.0.

Authentic α_2 -macroglobulin was purified by the procedure of Schaeufele and Koo [8].

Purification of the embryo protein

The aqueous extract of pooled embryos was passed over ConA-Sepharose, and the bound protein was eluted with 200 mM MeMan. The eluate was dialysed, concentrated and applied on a banana-lectin-Sepharose column. The bound protein was eluted with 500 mM MeMan. This yielded a homogeneous 170 kDa polypeptide, as confirmed by SDS/PAGE.

Inhibition of trypsin activity

Samples of 10 μ g each of the ascites fluid protein, the APP protein and BSA were incubated with tosylphenylalanyl-chloromethane (Tos-Phe-CHCl₂; 'TPCK')-treated trypsin in molar ratios (protein/enzyme) of 0, 1:1, 5:1 and 10:1. After 1 h at 37 °C, 2 vol. of 2% (w/v) azocasein in 0.01 M PBS, pH 7.4, was added, mixed and incubated at 37 °C for 10 min. An equal volume of 5% (w/v) trichloroacetic acid was added, and the mixture was left at room temperature for 10 min and then centrifuged at 16 000 *g* for 3 min. Aliquots of the supernatant were removed and mixed with an equal volume of 0.5 M NaOH, and the absorbance was read at 405 nm [9]. The percentage inhibition was calculated from a standard curve of trypsin activity done in parallel.

Electrophoresis and immunoblotting

Native PAGE and SDS/PAGE were carried out as described by Van Leuven et al. [10] and Laemmli [11] respectively. Immunoblots were prepared and developed as described previously [4].

Peptide mapping

Dried salt-free protein was solubilized in 8 M urea in 0.4 M ammonium bicarbonate, pH 8.0. The protein was reduced by the addition of 4 mM dithiothreitol and incubation at 50 °C for 15 min. After cooling to room temperature, 8 mM iodoacetamide was added, followed 15 min later by the dilution of urea in the protein sample to 2 M with distilled water, and the addition of Tos-Phe-CHCl₂-treated trypsin. The ratio of the carboxy-methylated protein to trypsin was 1:25 (w/w). After incubation at 37 °C for 24 h, the tryptic digest was applied on a C-18 column. The peptides were fractionated by HPLC using a gradient of 0.06% trifluoroacetic acid, and 80% acetonitrile containing 0.056% trifluoroacetic acid [12]. The value of the blank, consisting of only trypsin incubated under identical conditions, was subtracted from the chromatograms.

Other methods

ConA-mediated agglutination of erythrocytes, radioiodination of proteins and binding of the proteins to erythrocytes were

Figure 1 Detection of the 170 polypeptide in APP and in rat embryos

An aqueous extract of pooled rat embryos was prepared as described in the Materials and methods section. The equivalent of 60 μ g protein from the extract and the APP was subjected to SDS/PAGE (7% acrylamide) under reducing conditions, and transblotted. The blot was probed using the antibody against the ascites fluid protein. Lanes: A, ascites fluid protein; B, APP protein; C, embryo protein.

Figure 2 Purification of the APP 170 kDa-subunit protein, and its immunological identity with the ascites fluid protein and α_2 -macroglobulin

The 170 kDa-subunit protein was purified from rat APP as described in the Materials and methods section. Authentic α_2 -macroglobulin was isolated by the method of Schaeufele and Koo [8]. The ascites fluid protein was purified as described in [4]. Upper panel: the APP total protein (lane A; 25 μ g of protein), the ConA-bound protein (lane B; 13 μ g of protein), the Sepharose 4B peak I (lane C; 10 μ g of protein) and the DEAE-Sepharose unbound protein (lane D; 2 μ g of protein) fractions obtained from APP were subjected to SDS/PAGE (7% gel, reducing conditions). The gel was stained with silver. Lower panel: 5 μ g of the ascites fluid protein (lane A) and 10 μ g each of the purified APP protein (lane B) and authentic α_2 -macroglobulin (lane C) were electrophoresed, transblotted and probed using the antibody to the ascites fluid 170 kDa-subunit protein, as described in the Materials and methods section.

Figure 3 Trypsin-inhibitory activity of the 170 kDa-subunit proteins from ascites fluid and APP, and their conversion from slow to fast forms

Upper panel: 10 μ g samples of the ascites fluid (■) and APP (▲) proteins and of BSA (as a control; ●) were incubated with Tos-Phe-CHCl₂-treated trypsin in molar ratios (protein/trypsin) of 0, 1:1, 5:1 and 10:1 for 1 h at 37 °C. The activity of trypsin in the incubated samples was assessed using azocasein. Results shown are means of two experiments. Lower panel: 10 μ g samples of the ascites fluid (lanes A and C) and APP (lanes B and D) proteins were incubated with Tos-Phe-CHCl₂-treated trypsin in molar ratios of 0 (lanes A and B) and 10:1 (lanes C and D) as above. Native PAGE was carried out [10] on a 5% separating gel, pH 5.7. The electrode buffer was Tris/borate, pH 8.6.

performed as detailed in [4]. The methods for the determination of native and subunit molecular masses, the isoelectric point and the amino acid and monosaccharide compositions of the glycoproteins have also been described [4].

Data were analysed by Student's *t* test. Statistically significant results ($P < 0.05$) are indicated with asterisks in Figures.

RESULTS

Presence of the 170 kDa polypeptide in rat tissues

Samples of 60 μ g of protein from the aqueous and Triton X-100 extracts of rat adrenal glands, brain, breast, granulocytes, heart, intestine, kidney, liver, lymphocytes, (peritoneal) macrophages, muscle, ovary, pancreas, skin, spleen, testis and uterus were examined for the presence of the 170 kDa-subunit protein by immunoblotting. None of these tissues/cells revealed the presence of the polypeptide (results not shown). Next, the APP and the aqueous extract of rat embryos were examined, both of which showed the presence of the polypeptide (Figure 1). The Triton extract of the embryo did not contain the polypeptide in more than trace amounts (results not shown).

Purification of the APP protein and its identification as α_2 -macroglobulin

The protein consisting of the 170 kDa polypeptide was purified from APP (Figure 2, upper panel) essentially using the procedure employed for the purification of the ascites fluid protein [4]. The acute-phase protein was found to show a native molecular mass of 687 kDa and a subunit molecular mass of 302 kDa and 170 kDa under non-reducing and reducing conditions respectively. Evidently the protein is a tetramer consisting of two dimers, each composed of disulphide-bonded monomers. The characteristics of the protein correspond to those of α_2 -macroglobulin [13], a major acute-phase protein of rat plasma and a broad-spectrum proteinase inhibitor. To confirm that the purified acute-phase protein was indeed α_2 -macroglobulin, the latter was isolated by a procedure described in the literature [8]. Both of the purified proteins cross-reacted with the antibody to the ascites fluid protein (Figure 2, lower panel), confirming their relationship with each other and with the tumour-derived protein.

Figure 4 Ability of the purified APP protein to modify the ConA-mediated agglutinability of erythrocytes

Washed rat erythrocytes were incubated for 2 h at 37 °C with purified APP (◆) and ascites fluid (■) proteins at 500 μ g/ml. The cells were washed, incubated with the indicated concentrations of ConA, and 250 μ g/ml ConA plus 0.05 M MeMan. ●, Agglutination of cells incubated with TBS alone. Results are means $\pm$ S.E.M. of three experiments; *significant difference from control ($P < 0.05$).

Table 1 Carbohydrate composition of the ascites fluid and APP 170 kDa-subunit proteins

Monosaccharide	Composition (% by wt.)	
	Ascites fluid protein	APP protein
Mannose	6.90	2.40
Galactose	1.90	1.30
N-Acetylglucosamine	2.65	3.09
N-Acetylgalactosamine	0.00	0.00
Sialic acid	0.66	0.80
Fucose	0.00	0.00
Total	12.11	7.59

Figure 5 Effect of deglycosylation of the ascites fluid-derived protein on the ConA-mediated agglutinability of erythrocytes

The purified ascites fluid 170 kDa-subunit protein (500 µg/ml) was deglycosylated with TFMS. The solution was dialysed exhaustively to remove TFMS, during which a portion of the protein was precipitated. The protein remaining in solution (200–270 µg/ml in different experiments) was tested for its ability to modify the ConA-mediated agglutinability of erythrocytes. The native (undeglycosylated) protein was also used at the same concentrations. Cells were incubated with TBS alone (●), the native protein (■) and the TFMS-deglycosylated protein (◆). Results are means $\pm$ S.E.M. of five experiments; *significant difference from control ($P < 0.05$).

The isoelectric point of the APP protein ranged between 5.30 and 5.35. The pI of the ascites fluid-derived protein was 5.3 [4].

α_2 -Macroglobulin inhibits proteinases by a trapping mechanism [14]. During this process it undergoes a conformational change and acquires greater mobility on native PAGE [10]. The ascites fluid and APP proteins were found to inhibit the activity of trypsin (Figure 3, upper panel) and were also converted from slow into fast forms (Figure 3, lower panel). This indicates that the two proteins are also functionally related.

Binding to erythrocytes

The 125 I-labelled proteins from the ascites fluid and the APP bound to erythrocytes in similar manner. The binding was not saturated up to 500 µg/ml, the highest protein concentration used (results not shown). About 12000 and 10990 molecules of the ascites fluid and APP proteins respectively were bound per cell.

Erythrocyte surface modification activity

Although the ascites fluid- and APP-derived proteins have similar molecular characteristics and are immunologically and functionally related, the protein from the APP was unable to enhance the ConA-mediated agglutinability of erythrocytes (Figure 4). Incubation of erythrocytes in APP also did not modify the agglutinability of the cells; nor did erythrocytes from the blood of animals undergoing the acute-phase response exhibit higher agglutinability with ConA (results not shown).

Carbohydrate moiety and modification activity

Despite its structural, functional and immunological similarities to the ascites fluid-derived protein, the acute-phase protein was unable to modify the agglutinability of erythrocytes. It was

Figure 6 Purity and activity of the rat embryonic protein

The ConA-binding protein from the aqueous extract of rat embryos was loaded on a banana-lectin–Sepharose column. The bound protein was eluted with 500 mM MeMan. Upper panel: purified ascites fluid protein (lane A) and purified embryo protein (lane B); 5 µg of protein per lane. The gel was silver stained. Lower panel: ConA-mediated agglutinability of erythrocytes treated with 500 µg/ml ascites fluid protein (■) or rat embryo protein (◆). ●, TBS-incubated cells. Results are means $\pm$ S.E.M. of four experiments; *significant difference from control ($P < 0.05$).

possible that the two proteins possessed identical polypeptide chains but differed in their abilities to modify the ConA-mediated agglutinability of erythrocytes due to differences in their post-translational modification, i.e. glycosylation. The total sugar content and monosaccharide composition of the proteins did indicate differences in their carbohydrate moieties (Table 1). The characteristics of binding of the proteins to a panel of lectins indicated that the ascites fluid protein may predominantly carry hybrid-type, and the APP protein largely biantennary-complex-type, oligosaccharides (results not shown). However, the trifluoromethane sulphonic acid (TFMS)-deglycosylated ascites fluid protein was found to be as active as the native protein (Figure 5). (The deglycosylated protein contained 0.12% mannose, 0.01% galactose and 0.31% *N*-acetylglucosamine on a weight basis, corresponding to only 1.75%, 0.52% and 11.7% of the respective sugars in the native protein.) Also, the unglycosylated protein synthesized in culture in the presence of tunicamycin by Yoshida sarcoma cells (and which now appeared in the non-ConA-binding fraction) was active (results not shown). The differences in glycosylation are evidently not responsible for the differences in the erythrocyte surface modification activity of the two proteins.

Purification of the embryonic 170 kDa-subunit protein and measurement of its modification activity

The protein purified from the embryo extract by ConA- and banana-lectin-Sepharose chromatography yielded a homogeneous 170 kDa-subunit protein (Figure 6, upper panel). (The banana lectin binds to carbohydrate structures carrying more than six mannose units [6].) This protein was able to modify the ConA-mediated agglutinability of erythrocytes in a similar manner to the tumour-derived protein (Figure 6, lower panel).

Polypeptides of ascites fluid, APP and embryonic proteins

Of the three structurally and immunologically similar proteins, only the ascites fluid and embryo proteins were able to alter erythrocyte agglutinability. Since glycosylation is not responsible

for this activity, the polypeptide chains of the two proteins may possibly be identical and differ from that of the inactive acute-phase protein. Analysis of the mobilities of the TFMS-deglycosylated proteins on SDS/PAGE showed that the APP polypeptide is slightly larger than the tumour- and embryo-derived polypeptides (Figure 7, upper panel). The amino acid compositions (results not shown) of the three proteins indicated a general similarity; however, greater similarity was seen between the ascites fluid and embryo proteins. These two proteins showed differences compared with the APP protein, especially in the contents of glycine, phenylalanine, serine and valine.

Tryptic peptide analysis using a C-18 column revealed approx. 65 peptides from each of the three proteins. Qualitative identity between the peptides of the ascites fluid and embryo proteins was observed (Figure 7, lower panel). The two proteins contained 15

Figure 7 SDS/PAGE of TFMS-deglycosylated polypeptides, and tryptic peptide mapping

Upper panel: purified ascites fluid, APP and rat embryo proteins were deglycosylated by treatment with TFMS. The proteins were subjected to SDS/PAGE on a 7% gel under reducing conditions. Lanes A and F, ascites fluid native protein; lane B, rat embryo deglycosylated protein; lanes C and E, ascites fluid deglycosylated protein; lane D, APP deglycosylated protein. Lanes A and F contained 10 μ g of protein, lanes B, C, and D approx. 5 μ g of protein and lane E 20 μ g of protein. Lower panel: the proteins purified from ascites fluid (AF), embryo extract and APP were treated with trypsin, and the peptides were separated on a C-18 column by HPLC as described in the Materials and methods section. The 'trypsin blank' has been subtracted from the runs. The numbers above the peaks represents the retention time (min). ▼, Peptides not found in the APP protein digest; ●, peptides found only in the APP protein digest.

peptides (▼ in Figure 7) that were not found in the APP protein digest. Also, 11 peptides (marked ● in the figure) occurring in the latter were absent from the ascites fluid- and embryo-derived proteins. The remaining peptides were common to all three proteins, suggesting their close similarity. The ascites fluid and embryo proteins are probably identical to each other.

DISCUSSION

We reported previously that a 170 kDa-subunit protein in Yoshida sarcoma ascites fluid is responsible for the modification of ConA-mediated agglutinability of erythrocytes in tumour-bearing rats [4]. This protein diffuses from the site of tumour growth into the circulation, binds non-specifically to erythrocytes and enhances their ConA-mediated agglutinability. It was found to reduce the charge on the cell surface [5], providing a possible explanation for the increase in agglutinability [15]. The protein was not detectable in normal rat plasma by Western blotting. In the present work, we also failed to detect it in other tissues of the normal adult rat.

Specific proteins appear in the plasma during the acute-phase reaction, which occurs in most diseases [16]. Also, embryo-specific proteins are not expressed in adult life. APP and the rat embryo were both found by immunoblotting to contain the 170 kDa polypeptide. The APP protein, identified as α_2 -macroglobulin, possesses physicochemical and trypsin-inhibitory properties almost identical to those of the ascites fluid protein. Also, both proteins bind to erythrocytes in a similar manner and at similar amounts. However, the APP protein is unable to modify the agglutinability of erythrocytes. The two proteins must obviously differ from each other in some respect, and the difference must pertain to a characteristic(s) other than the carbohydrate moiety, since the latter is dispensable for modification activity.

The embryonic protein, unlike the acute-phase protein, is able to enhance the agglutinability of erythrocytes *in vitro*. The deglycosylated polypeptides of the tumour protein and the embryo protein have a slightly smaller size than the APP polypeptide (Figure 7). The amino acid compositions of the three proteins showed overall similarity, but greater similarity was seen between the ascites fluid and embryo proteins. The majority of about 65 tryptic peptides obtained from the three proteins occurred in all of them (Figure 7). However, 15 peptides found reproducibly in the tumour- and embryo-derived proteins were

not found in the acute-phase protein, while the latter yielded 11 peptides that were unique to it. In view of the large similarities, and some differences, the tumour and embryo proteins appear to be distinct variants of α_2 -macroglobulin. Their primary sequences are being obtained by reverse transcription-PCR using primers based on the sequence of the cDNA of the APP protein [17].

The tryptic peptide maps of the tumour-derived and embryonic proteins showed qualitative identity. It is thus probable that the two proteins are identical. Tumours are known to express embryo-specific proteins [18], carcino-embryonic antigen and α -fetoprotein being two well known examples. The tumour-derived 170 kDa-subunit protein would thus appear to be an oncofetal variant of α_2 -macroglobulin. The present results show that its appearance in the adult organism causes modification of the properties of at least some host cells.

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