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EXPRESSION OF ENDOGENOUS LECTINS IN HUMAN SMALL-CELL CARCINOMA AND UNDIFFERENTIATED CARCINOMA OF THE LUNG*

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Karl Freudenberg and the Glycoproteins. — Karl Freudenberg, after having completed much of his fundamental work on stereochemistry, in particular the stereochemistry of carbohydrates[1], took up the study of blood-group specificity and the determinant factors of blood groups A and B. It was suggested at that time that the blood groups are characterized by certain glycoproteins on the erythrocyte membrane. Nothing was known, however, about the nature of the sugar moieties involved or the sequence of sugars in the prospective oligosaccharides. It is thus remarkable that he was able to characterize the composition of the carbohydrate moiety of blood group A substance to the extent of finding that galactose and *N*-acetylglucosamine are present in these glycoproteins[2]. While much further research on glycoconjugates has been accomplished in the ensuing forty years, Alberts *et al.*[3] were recently moved to say that "the function of the oligosaccharide side chains in membrane glycolipids and glycoproteins is unclear, even today".

ABSTRACT

Endogenous carbohydrate-binding proteins (lectins) were detected in specimens of tumor tissue (undifferentiated carcinoma and xenografted small-cell carcinoma) from human lung. Fractionation of salt and detergent extracts on different sets of Sepharose columns covalently derivatized with lactose, asialofetuin, melibiose, mannan, and fucose, successive elution with a chelating agent and a specific sugar, and analysis of the eluates by gel electrophoresis, resulted in the characterization of the profiles of endogenous carbohydrate-binding proteins. All preparations were devoid of enzymatic activity. Comparison between the patterns of the two types of lung carcinoma showed significant qualitative differences, *e.g.* the presence of fucose-binding proteins of apparent molecular weights 60,000 and 80,000 in the undifferentiated carcinoma, and the presence of β-galactoside-binding proteins of apparent molecular weights 18,000 and 22,000 in the small-cell lung carcinoma. These proteins were not detectable in normal lung tissue. Such differences, documented for the first time for human lung tumors, are of potential

* Dedicated to the memory of Karl Freudenberg (1886–1983) on the centenary of his birth.

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importance as a step towards a lectin-based refinement of lung-cancer diagnosis and therapy.

INTRODUCTION

Although mortality from all forms of human cancer combined provides valuable information on general trends, detailed study of specific tumor types should help focus attention in basic cancer research. The sharp and continuing rise in deaths from lung cancer has substantially affected overall cancer mortality rates[4]. It is therefore mandatory to expand the possiblities for proper therapeutic management and accurate assessment of tumor histology, as offered by biological markers[5].

Since characteristic changes of glycoconjugates in malignant or transformed cells are widely being exploited as potential tumor markers, attention has also started being paid to their endogenous ligands, the carbohydrate-binding proteins (lectins). Biochemical analysis has so far supported the working hypothesis that the pattern of endogenous lectins is qualitatively and quantitatively different between tumors of different classes, between tumors of the same basic histological classification differing in their degree of malignancy and differentiation, and between tumors and normal, nonmalignant tissues[6-8]. The proposal that these proteins may prove to be a new class of tumor markers[9] has been further supported by analysis of the endogenous lectins of a model system of virus-transformed fibroblasts having different growth and metastatic properties[10,11], and by the observation that the asialoglycoprotein receptor, the first mammalian lectin that has been biochemically characterized, appears to be a cell-surface marker for an early hepatic preneoplastic alteration[12]. In view of these observations, we have recently started a program on vertebrate lectins, assuming that glycoproteins together with the corresponding lectins form an intercellular recognition system[9]. The present study compares the pattern of endogenous lectins in human lung tumors with propensity for early metastasis, *viz.* undifferentiated lung carcinoma and small-cell lung carcinoma.

MATERIALS AND METHODS

Reagents. — All sugars (lactose, D-mannose, L-fucose, and melibiose), fetuin, and mannan were obtained from Sigma (Munich, F.R.G.); Sepharose 4B and concanavalin A-Sepharose 4B were from Pharmacia Fine Chemicals (Freiburg, F.R.G.); plant lectins were purchased from Medac (Hamburg, F.R.G.); and cyanogen bromide and divinyl sulfone were from Merck (Darmstadt, F.R.G.). Other chemicals were commercially available analytical grade. The column supports for affinity chromatography were prepared after suitable activation and were checked for coupling efficiency as described[13,14]. *p*-Aminophenyl derivatives of lactose, α-D-mannopyranose, and α-L-fucose were prepared by catalytic reduction of the *p*-nitrophenyl derivatives (Sigma, Munich, F.R.G.).

Tumors. — Tumor material, trimmed of connective tissue and necrotic parts,

was obtained from the Cancer Center of the University of California, San Diego, and the research department of the Behring Company (Marburg, F.R.G.). Whereas the specimen of undifferentiated lung carcinoma came from autopsy, tumors of the small-cell lung carcinoma originated from the transplantation of tumor cells of the line OAT-75 (ref. 15) into nude mice.

Preparation of carbohydrate-binding proteins. — The preparations for the xenografted tumors were carried out three times with only minor quantitative differences, while the autopsy material sufficed for only one procedure. Histological examination excluded any significant contribution to the analysis by infiltrating cells. All steps were performed at 4°. Typically tumor material of each sample was blended in a Waring Blendor with cold acetone for 1 min. The homogenate was filtered under reduced pressure and processed as described[13,14]. The acetone powder was extracted twice with buffer A (20mM tris–HCl, pH 7.8, containing 0.2M NaCl, 1mM dithiothreitol, and 0.1mM phenylmethanesulfonyl fluoride). These supernatants, designated salt extract, were combined and brought to a final concentration of 0.5% Triton X-100, 25mM $CaCl_2$ and 1.25M NaCl. The insoluble pellet was subsequently extracted three times with buffer B (20mM tris–HCl, pH 7.8, containing 2% Triton X-100, 0.4M KCl, 0.1mM phenylmethanesulfonyl fluoride, and 1mM dithiothreitol). These supernatants, designated detergent extract, were combined and brought to a final concentration of 25mM $CaCl_2$. The salt and detergent extracts were applied separately to a set of five columns each (0.9 × 12 cm lactose-, asialofetuin-, melibiose-, and mannan-Sepharose 4B, 0.5 × 10 cm fucose-Sepharose 4B), equilibrated with buffer C (20mM tris–HCl, pH 7.8, containing 1.25M NaCl, 25mM $CaCl_2$, 0.05% Triton X-100, and 1mM dithiothreitol). Triton X-100 was included in the buffer in an effort to overcome the tendency of lectins to self-association. After extensive washing, the Ca^{2+}-dependent carbohydrate-binding proteins were eluted with buffer C containing 4mM EDTA instead of 25mM $CaCl_2$. The Ca^{2+}-independent carbohydrate-binding proteins were eluted by application of the specific sugars (0.5M in buffer C) from the set of columns that had been reequilibrated with buffer C. The sugar was removed by dialysis in tubing which was impermeable to molecules of molecular mass greater than 6000 Da. All samples after this affinity chromatography were concentrated by ultrafiltration (Diaflo Ultrafiltration Model 50 fitted with a YM-5 membrane). Detergent was removed by chloroform extraction.

Analytical procedures. — Gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed on 7.5% or 10% running gels with a 3% stacking gel[16]. All gels were stained by the silver-staining procedure[17]. Special care was taken to avoid contamination with skin proteins, especially keratins ranging from 65 to 68 kDa, in this highly sensitive method[18]. Standards for molecular mass estimation were: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (44 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.3 kDa) (from Sigma, Munich, F.R.G.). Protein was determined by the dye-binding assay adapted for microtiter plates, with bovine serum albumin as standard[19]. The thereby determined yields for each preparation

are given in the legends to the figures. Assays for β-galactosidase, sialyl transferase, fucosyl transferase, α-mannosidase, and α-fucosidase, to rule out contamination of the lectin preparations by enzymatic activities, were performed as described[13,14]. Cytochemical markers for ascertaining the specificity of the isolated proteins were prepared from the *p*-aminophenyl derivatives of the sugars by diazotization and reaction with bovine serum albumin, and coupling of the thereby synthesized neoglycoprotein to fluorescein isothiocyanate[20]. The proteins were spotted on nitrocellulose and the assay was performed as described[13], in the absence of inhibitors of binding of the cytochemical marker and in the presence of 2.5 mg unlabeled marker per mL, with reduction of fluorescence to the background level.

RESULTS

The five different types of affinity support, employed in this study to isolate carbohydrate-binding proteins by affinity chromatography, represented a selection of the sugar moieties commonly present in glycoconjugates. These supports were designed for the isolation of receptors for simple or more complex β-galactosides, present in lactose or asialofetuin, for α-galactosides, present in melibiose, for mannose-rich structures like mannan, and for α-fucosides. The pattern of carbohydrate-binding proteins having different sugar specificities was further divided into categories of dependence on Ca^{2+} ions for binding activity (elution by EDTA for Ca^{2+}-dependent sugar receptors and subsequent elution by the appropriate sugar for the remaining Ca^{2+}-independent sugar receptors); and into categories of dependence on salt or detergent extraction, corresponding to soluble cytoplasmic and extracellular

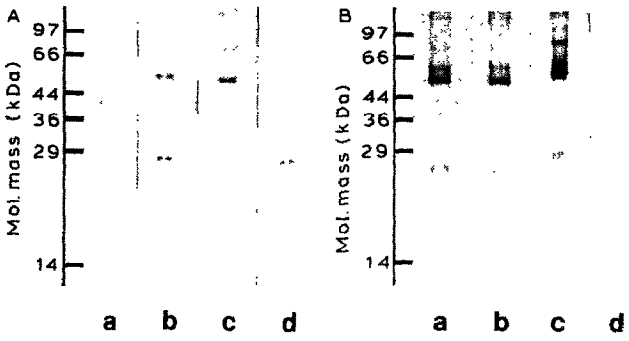

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate of endogenous carbohydrate-binding proteins of undifferentiated human lung carcinoma. The total yield in μg per 10 g tumor is given for each preparation (salt extract 588 mg; detergent extract 227 mg). A, elution with EDTA from immobilized lactose (a, 71 μg), from immobilized melibiose (b, 18 μg), and from immobilized fucose (c, 17 μg) after application of salt extract to the columns, and from immobilized lactose (d, 19 μg) after application of detergent extract to the columns; B, elution with specific sugar from immobilized lactose (a, 34 μg), immobilized melibiose (b, 20 μg), and immobilized fucose (c, 22 μg) after application of salt extract to the columns, and from immobilized lactose (d, 19 μg) after application of detergent extract to the columns.

or peripheral membrane proteins, and detergent-released membrane proteins. The conditions of affinity chromatography were chosen to avoid contamination of the preparations by enzymatic activities, and the products consistently gave negative responses to activity tests.

Analysis of the different fractions of the salt extract of acetone powder from undifferentiated lung carcinoma tissue by gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol revealed four carbohydrate-binding proteins with an apparent Ca^{2+} requirement, having apparent molecular weights of 28,000, 42,000, 54,000, and 56,000 (Fig. 1A). The β-galactoside-specific protein of apparent molecular weight 42,000 that was relatively abundant compared to other proteins, as seen from the yields, given in the legend to Fig. 1, was not detectable in the detergent extract. In general, only three galactoside-specific proteins were found in the detergent extract. The elution of each column by its specific sugar yielded proteins in similar quantity relative to EDTA elution except for a reduced amount from lactose-Sepharose after application of salt extract to the column. Two proteins of apparent molecular weights 14,000 and 30,000 were specific for β-galactosides, whereas three other proteins of apparent molecular weights 27,000, 54,000, and 58,000 were bound to immobilized α- and β-galactosides (Fig. 1B). No protein exhibited affinity to the α-mannoside structures present in mannan. The fucose-binding proteins of apparent molecular weights 56,000, 60,000, and 80,000 were accompanied by a protein band at apparent molecular weight of around 29,000. A similar faint protein band at this apparent molecular weight was observed after EDTA

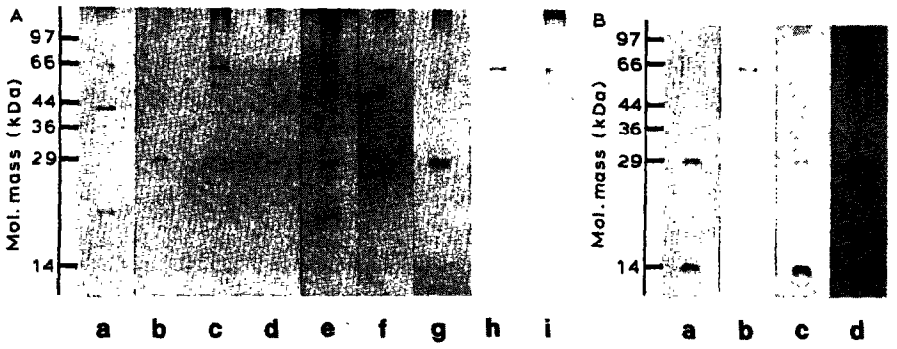

Fig. 2. Polyacrylamide-gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate of endogenous carbohydrate-binding proteins of xenografted human small-cell lung carcinomas (OAT-75). The total yield in μg per 10 g tumor is given for each preparation (salt extract 1495 mg; detergent extract 406 mg). A, elution with EDTA from immobilized lactose (a, 12 μg), from immobilized asialofetuin (b, 51 μg), from immobilized melibiose (c, 16 μg), from immobilized mannan (d, 76 μg), and from immobilized fucose (e, 7 μg) after application of salt extract to the columns, and from immobilized lactose (f, 18 μg), from immobilized asialofetuin (g, 7 μg), from immobilized mannan (h, 35 μg), and from immobilized fucose (i, 6 μg) after application of detergent extract to the columns; B, elution with specific sugar from immobilized lactose (a, 94 μg) and immobilized melibiose (b, 0.5 μg) after application of salt extract to the columns, and from immobilized lactose (c, 52 μg) and from immobilized asialofetuin (d, 6 μg) after application of detergent extract to the columns.

elution from fucose-Sepharose, indicating partial elutability by EDTA without a clear intrinsic requirement for Ca^{2+}, as already noted in other cases[8,13]. Such quantitative gradations within preparations can also be taken as an indicator of the presence of distinct lectins, not merely different subunits.

Similar gel-electrophoretic analysis of the salt and detergent extracts of small-cell lung carcinoma after affinity chromatography gave a pattern with certain differences from the foregoing. Three proteins with specificity for β-galactosides were isolated after EDTA elution. Besides the protein of apparent molecular weight 42,000, also seen in the profile of the undifferentiated carcinoma, two further proteins of apparent molecular weights 22,000 and 66,000 were present only in extracts of this tumor type (Fig. 2A). The more complex β-galactoside-terminal chains of asialofetuin bound a predominant protein of apparent molecular weight 29,000 from both extracts, and two additional minor bands of apparent molecular weights 54,000 and 56,000 only from the detergent extract. Further proteins with specificity for α-mannosides and α-fucosides were seen at apparent molecular weights of 29,000 from the salt extract and 66,000 from the detergent extract. In both cases the yield (see legend to the figure) from mannan-Sepharose was considerably higher than that from fucose-Sepharose, where the detergent extract contained a further protein of apparent molecular weight 180,000. No protein had been detectable in either eluate from mannan-Sepharose in the case of the undifferentiated carcinoma. Only galactoside-specific proteins were present after sugar elution (Fig. 2B) of the samples from small-cell carcinoma. They comprised two equally abundant proteins of apparent molecular weights 14,000 and 29,000 and a further minor band of apparent molecular weight 18,000 from lactose-Sepharose, and proteins from immobilized asialofetuin and melibiose of apparent molecular weights 14,000, 27,000, and 29,000 or 66,000, respectively, in considerably lower yield than the proteins eluted from immobilized lactose.

In a detailed comparison (Table I), significant qualitative differences corroborated the histologic differences between these types of lung carcinoma. These differences included three additional galactoside-binding proteins of apparent molecular weight 18,000, 22,000, and 66,000, and two additional fucose-specific proteins from the small-cell lung carcinoma. But by comparison with the undifferentiated lung carcinoma these tumors lacked three fucose-binding proteins and one galactoside-binding protein. The limited amount of protein obtained from each fraction did not allow detailed structural analysis in this initial study, so proteins were only counted as different in cases of significant molecular weight differences, and this may well have led to an underestimation. Some of the carbohydrate-binding proteins detected in lung tumors had not been detected in normal lung, as shown in Table I.

DISCUSSION

The main emphasis in this study was on the detection of endogenous carbo-

TABLE I

CARBOHYDRATE-BINDING PROTEINS OF NORMAL HUMAN LUNG AND PRIMARY LUNG TUMORS[a]

Tissue	*Proteins, designated by molecular weight[b], eluted from columns derivatized as shown*									
	Elution with EDTA					*Elution with specific sugar*				
	Lactose	*Asialofetuin*	*Melibiose*	*Mannan*	*Fucose*	*Lactose*	*Asialofetuin*	*Melibiose*	*Mannan*	*Fucose*
Lung[c]	28	28	28,54, 56	28	54,56, 62,180	14,31, 54,58	—	54,58	—	54,58
	34,66	28	—	—	180	14	54	54	—	—
Undifferentiated lung carcinoma	28,42	—	28,56	—	28,54	14,27, 54,58	—	27,54, 58	—	29,56, 60,80
	28	—	—	—	—	14,30	—	—	—	—
Small-cell lung carcinoma (OAT-75)	22,42 66	29	66	29	29	14,18, 29	—	66	—	—
	66	29,54, 56	—	66	66,180	14,29	14,27, 29	—	—	—

[a]The extraction and elution conditions permit a division of the pattern into salt-extractable (first line) or detergent-extractable (second line), Ca^{2+}-dependent (elution by EDTA) or Ca^{2+}-independent (elution by specific sugar) lectins. [b]Apparent molecular weight is given in thousands; [c]Unpublished.

hydrate-binding proteins (lectins) in human lung cancer and on the comparative description of lectin profiles for two different types of human lung carcinoma. Contamination of the preparations by enzymatic activities could be ruled out by negative results from activity tests. Contamination of the preparations by non-lectin sugar-binding compounds such as serum amyloid P component or antibodies could easily be ruled out by simple consideration of the molecular weights [21-23]. The patterns of endogenous lectins exhibited qualitative differences between the two types of tumors and normal long tissue. They also revealed differences from other types of human tumors studied so far in this respect, *e.g.* the presence of characteristic β-galactoside-binding proteins of apparent molecular weight 18,000 and 22,000, whereas an α-galactoside-binding protein of apparent molecular weight 66,000 had already been detected in pure embryonic carcinoma[6,7]. The presence of characteristic, individual lectins permits the conclusion that the lectin pattern is a potential aid in histodiagnosis as well as in the analysis of the histogenesis and differentiation of lung-cancer cells. A detailed comparison with such profiles for other types of cells may furthermore help to clarify histological relationships that are still a matter of controversy. This is exemplified by the suggestion that small-cell lung cancer is derived from macrophages or their precursors[24].

Furthermore, since lectins are the potential endogenous receptors for glycoconjugates, the lectins in tumor tissue may also form a functional link to the glycoconjugates of normal cells and to detected differences in cell-surface glycoconjugates in lung carcinoma, also proposed to be of value as biomarkers[25-27]. They may thereby participate in recognitive interactions with glycoconjugates in the progression of tumor growth and spread[6,7]. A description of factors contributing to the establishment of metastasis is especially important for tumors with propensity for early metastasis, and may be helpful in the design of suitable therapeutic approaches. A specific interaction between glycoconjugates and endogenous lectins in tumors, for example, allows one to envisage the use of the carrier potential of specific glycoconjugates to target drugs to tumor cells. Experimentally, the application of hybrid molecules having a defined carbohydrate part as an entry function and a cotransported drug that is released intracellularly has been successfully tested with tumor cells *in vitro*[28,29]. Therefore, lectins having restricted expression or cell-type specificity, considered as targets for drug transport, may offer a further means of compensating for the heterogeneity of expression of antigenic markers, which is commonly noted in studies of lung tumors using monoclonal antibodies[30] and which generally threatens the success of targeted drug delivery.

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