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Properties of α_1-antitrypsin secreted by human adenocarcinoma cell lines

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α_1-Antitrypsin; (α_1-AT) produced by various human carcinoma (non-hepatoma) cell lines were analyzed. Five out of eight cell lines secreted detectable amounts of α_1-AT into the conditioned media. All were adenocarcinoma cell lines. The tumor cell-derived α_1-ATs had higher molecular weights (MW) than the normal plasma form. Most of this difference was an overall reflection of altered *N*-glycosylation. As judged by binding of lectins, the glycosylation had shifted towards higher levels of triantennary oligosaccharides and higher levels of fucosylation. The conditioned media also contained lower MW α_1-AT species, possibly, proteolytically cleaved forms.

α_1-Antitrypsin; Adenocarcinoma cell; Glycosylation

1. INTRODUCTION

α_1-Antitrypsin (α_1-AT) is a glycoprotein and a major component of serum proteinase inhibitors. The mature protein is a single polypeptide containing one cysteine residue, no disulphide bridges, and three carbohydrate side chains [1]. It is classically considered to be synthesized by liver cells and, to a lesser extent, by macrophages.

Increased α_1-AT in sera or tumor tissue from various cancer patients have been described [2,3]. We have previously shown that cultured human colorectal adenocarcinoma cell lines produce functionally active α_1-AT [4], and patients with immunohistochemically α_1-AT- positive colorectal carcinomas have a worse prognosis than those with negative ones [5]. Similar results were obtained in patients with gastric carcinomas and lung adenocarcinomas [6,7]. A human breast carcinoma cell line, MCF-7, secretes an α_1-AT-like 61 kDa protein which may act as a positive growth factor under the stimulation of estradiol [8]. These lines of evidence suggest that the tumor cell-derived α_1-AT may play a significant role in tumor biology. However, little is known about the properties of carcinoma (except for hepatoma) cell-derived α_1-AT. In the present paper we describe some of the properties of α_1-AT secreted by various human adenocarcinoma cell lines.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Four human colorectal adenocarcinoma cell lines, RCM-1, RCM-2, RCM-3, and CoCM-1; a lung adenocarcinoma cell line, LC-2/ad; a squamous cell carcinoma cell line, LC-1/sq; a renal cell carcinoma cell line, MRT-1; and a urinary bladder transitional cell carcinoma cell line, UMK-1; were used. All lines have been established in our laboratory [4,9,10], and were grown in a mixture of RPMI 1640 and Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gland Island, NY, USA) and kept at 37°C in a humidified atmosphere containing 5% CO_2. To collect serum-free conditioned medium (SFCM), confluent cells were rinsed 3 times with serum-free medium and cultured in the serum-free medium. SFCM was harvested and cultures were re-fed every day for 4 days. The media collected on the first day were discarded while subsequent harvests were collected, centrifuged (2,000 × *g*, 20 min) and stored at −40°C.

2.2. *Column chromatography*

High-performance liquid chromatography (HPLC) was performed with Pharmacia's FPLC system (Uppsala, Sweden). Concanavalin A (Con A) affinity HPLC fractionation was performed on a LA-Con A column (0.45 × 15 cm, Seikagakukogyo, Tokyo, Japan) equilibrated with 50 mM Tris-HCl (pH 7.2), 200 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$. Bound protein was eluted with a linear gradient of 0–100 mM α-methyl-D-glucoside in the initial buffer. Partial purification of α_1-AT from SFCM was as described previously [4]. *Datura stramonum* agglutinin (DSA) affinity chromatography was performed on a DSA–agarose column (1 ml, Seikagakukogyo) equilibrated with 50 mM phosphate buffer (pH 7.0), 150 mM NaCl, 0.01% NaN_3. *Lotus tetragonolobus* agglutinin (LTA), affinity chromatography was performed on a LTA–agarose column (1 ml, Seikagakukogyo) equilibrated with the same buffer as DSA–agarose. Sample was applied on the lectin–agarose column and re-circulated through the column for 30 min at 4°C. After washing the columns with the buffer, bound proteins were eluted with a stepwise gradient of 2 ml each of 0.5%, 1%, and 2% of a mixture of chitooligosaccharides for the DSA column, and with 3 ml each of 20 mM and 50 mM α-L-fucose for the LTA column.

2.3. *Con A affinity electrophoresis*

Con A affinity electrophoresis was carried out at 15°C, using 0.6 mg Con A (Sigma, St. Louis, MO, USA) in a 0.9% agarose gel (LE agarose, FMC BioProducts, Rockland, MO, USA). Barbital buffer (pH 8.6), ionic strength 0.05, was used. After the electrophoresis (4.5 V/cm, 6h) the proteins were transfered onto a nitrocellulose paper (0.45 μm, Schleichen and Schuell, Dassel, Germany) by the capillary method overnight. After transfer the paper was blocked with 1.25%

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non-fat milk protein in 50 mM Tris-HCl (pH 7.6), 200 mM NaCl (TBS) for 3 h at room temperature, followed by brief washing in TBS, 0.05% Tween 20. Then the paper was incubated in 0.5% rabbit anti-human α_1-AT IgG (Dakopatts, Glostrup, Denmark) in TBS, 3% FBS, 50 mM α-methyl mannoside (α-MM) for 2 h. After 3 washes in TBS, 0.05% Tween 20, the paper was incubated in peroxidase-conjugated secondary antibody (0.2% swine anti-rabbit immunoglobulin IgG in TBS, 1% FBS, 50 mM α-MM for 1 h followed by 3 washes in TBS. 4-Chloro-1-naphthol was used as the colour reagent. Bovine α_1-AT did not cross-react under these conditions.

2.4. *Western blotting*

SDS-PAGE was performed under reducing conditions using a 10% separating gel. After electrophoresis, the proteins were transfered electrophoretically onto nitrocellulose paper [11]. Then, the paper was processed for immunostaining using rabbit anti-human α_1-AT IgG as described above. α-MM was removed from the buffer for this case.

2.5. *Enzyme immunoassay*

96-well microtiter plates were coated with sheep anti-human α_1-AT IgG (40 μg/ml, Serotec, UK) in 10 mM sodium carbonate buffer (pH 9.6). After washing with phosphate-buffered saline (pH 7.2), 0.1% Tween 20 (PBS-Tween), the wells were blocked with PBS, 3% bovine serum albumin (Fraction V, Boehringer-Mannheim, Germany), for 1 h at 37°C. Then samples (200 μl/well, diluted with PBS-Tween) were applied and incubated for 1 h at 37°C followed by 3 washes with PBS-Tween. Subsequently 200 μl of peroxidase-conjugated secondary antibody (sheep anti-human α_1-AT IgG, 10 μg/ml, Serotec) in PBS-Tween, 1% FBS, was applied, and incubated for 1 h at 37°C. After 3 washes with PBS, the colour reaction was done by using 0.5 mg/ml *O*-phenylenediamine, sodium phosphate buffer (0.1 M, adjusted to pH 5.0 with 0.1 M citric acid). Medium containing 10% FBS, or serum-free medium, was used as a control. In any case the samples and standards were diluted with PBS-Tween, 3% FBS. Bovine α_1-AT, which was present in FBS, did not cross-react in this system.

2.6. *N-Glycosidase F treatment*

N-Glycosidase F (Boehringer-Mannheim) treatment was done according to the manufacturer's instructions. Briefly, samples were boiled for 3 min in the presence of 1% SDS, then incubated overnight, at 37°C, in 50 mM phosphate buffer (pH 7.8), 1% β-mercaptoethanol, 0.5% Nonidet P-40, 20 mM EDTA, with or without 10 U/ml *N*-glycosidase F. After treatment, the samples were processed for Western blotting as described above.

2.7. *Trypsin inhibition assay and zymography*

The inhibition assay for TPCK-treated trypsin (Sigma) was described previously [4]: Bz-L-Arg-pNA (Sigma) was used as a substrate. Zymography was used for detecting α_1-AT-degrading proteinase bands separated on SDS-PAGE [12]. Gels containing 0.5 mg/ml α_1-AT were used.

3. RESULTS

3.1. *Secretion of α_1-AT by human adenocarcinoma cell lines*

Five out of eight cell lines secreted protein immunoreactive with antibodies to human α_1-AT: all were adenocarcinoma cell lines. Apparently the amount of α_1-AT in each conditioned medium was much higher in LC-2/ad, RCM-1 and MRT-1 than the other cell lines (Table 1). Treatment of RCM-1 with 10 μg/ml cyclohexamide for 24 h inhibited the secretion of α_1-AT almost completely (94%). Western blot analysis using a rabbit anti-human α_1-AT IgG identified a broad protein band of 53-70 kDa and an intense band of 59-62 kDa (Fig. 1). Immunoadsorption of the antibody with pure α_1-AT eliminated these bands (data not shown). An additional weak band of approximately 90 kDa was occasionally seen in the case of RCM-1. The size of the protein is apparently larger than normal human plasma α_1-AT which migrated to a position of 56 kDa in our gel. Since the higher molecular weight (MW) and the broad band of the tumor cell-derived α_1-AT may be an overall reflection of altered glycosylation, we examined the affin-

Table I

Secretion of α_1-AT by carcinoma cell lines

Cell line	Passage numbers	Type	Origin	α_1-AT	
				ng/ml/24 h	ng/10^6 cells/24 h
RCM-1	44	Adenocarcinoma	Rectum	20.5	13.0
RCM-1 + cyclohexamide 10 μg/ml				1.2	0.6
RCM-1	102			124.0	46.5
RCM-2	60	Adenocarcinoma	Rectum	< 1.0	–
RCM-3	58	Adenocarcinoma	Rectum	5.2	9.8
CoCM-1	37	Adenocarcinoma	Colon	4.4	1.2
MRT-1	90	Renal cell carcinoma (adenocarcinoma)	Kidney	25.5	42.4
LC-2/ad	54	Adenocarcinoma	Lung	416.0	693.3
LC-1/sq	72	Squamous cell carcinoma	Lung	< 1.0	–
UMK-1	42	Transitional cell carcinoma	Urinary bladder	< 1.0	–
UMK-1	115			< 1.0	–

α_1-AT antigen levels were determined by EIA using sub- or confluent cultured cells in 25 cm^2 flask with 5 ml growth medium for 24 h.

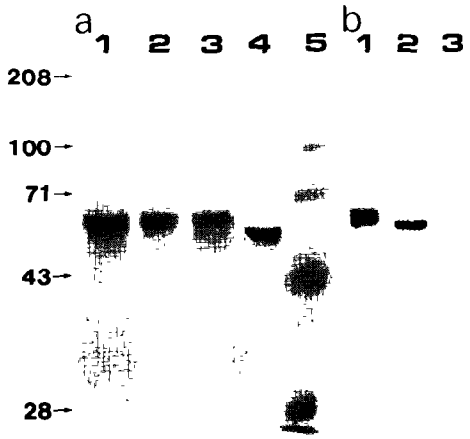

Fig. 1. Western blot analysis of α_1-AT derived from RCM-1 (a) and LC-2/ad (b). Lanes a1–3, SFCMs of RCM-1 of 102nd, 84th, 42nd passage, respectively. 30 μl each of 50 × concentrated SFCM was applied; lane b1: partially purified LC-2/ad α_1-AT, 0.6 μg; lanes a4,b2, normal plasma α_1-AT (1 and 0.4 μg, respectively); lanes a5,b3, prestained MW marker.

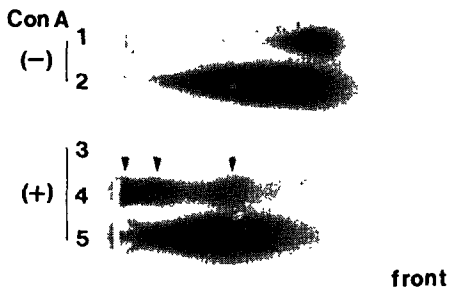

Fig. 2. Con A affinity electrophoresis of α_1-AT. Lanes 1,4, 0 3 μg of normal plasma α_1-AT; lanes 2,5, 10 μl (50 × concentrated) of SFCM of LC-2/ad; lane 3,5 μl of FBS. After electrophoresis, the proteins were transfered to nitrocellulose paper and immunostained with anti-human α_1-AT IgG. The electrophoretic pattern of normal α_1-AT was significantly altered by Con A showing 3 main bands (arrow head).

ity of the tumor α_1-AT to various lectins and the effects of *N*-glycosidase F treatment.

3.2. *Con A affinity*

When partially purified RCM-1-derived α_1-AT was analyzed with Con A HPLC, only 10% of the activities were retained in the column, whereas 78% of the activities of commercially obtained human plasma α_1-AT were retained. Con A affinity electrophoresis followed by immunoblotting using a rabbit anti-human α_1-AT IgG also revealed decreased affinity of LC-2/ad-derived α_1-AT to Con A (Fig. 2). A similar result was obtained in the case of RCM-1 (data not shown). Under these electrophoretic conditions, the tumor α_1-AT showed a considerable broad band when compared with the plasma α_1-AT. Although the electrophoresis pattern of the plasma α_1-AT was significantly affected by Con A resulting in three main bands (i.e. strong, moderate, and weak or no affinity to Con A), the pattern of the tumor α_1-AT was only slightly affected by Con A and most of the protein show weak or no affinity to Con A.

3.3. *DSA and LTA affinity*

Results of DSA–agarose column chromatography are shown in Fig. 3. Both RCM-1, and LC-2/ad-derived α_1-AT contain DSA-bound fractions. About 40% and 12% of the RCM-1 and LC-2/ad-derived α_1-AT, respectively, bound to the column. In contrast, only 2% of the normal α_1-AT bound to the column. When analyzed with LTA–agarose column chromatography, part of the RCM-1 derived α_1-AT was retained in the column and eluted with L-fucose. Most of the plasma α_1-AT was not retained in the column. Although part of the LC-2/ad-derived α_1-AT was also retained in the column, the amount was much lower than that of RCM-1-derived α_1-AT (Fig. 4). Of particular interest is the observation that the molecular sizes of the DSA- and LTA-bound tumor α_1-AT were clearly larger than the plasma α_1-AT, and even larger than the unbound tumor α_1-AT.

3.4. *Effects of* N-*glycosidase F treatment*

Fig. 5 shows the results of *N*-glycosidase F treatment. Both plasma and the tumor α_1-ATs were totally sensitive to *N*-glycosidase F. After treatment, plasma α_1-AT decreased its size from 56 kDa to 44 kDa. Treatment of the tumor α_1-AT resulted in 51, 44, 42, 39, and 37 kDa products for RCM-1; 44, 42, 39, and 37 kDa products for LC-2/ad; and a 44 kDa product for MRT-1. In any case the main products were of the same MW in size, i.e. 44 kDa. This result, together with the results of lectin affinity, clearly indicated that the higher MW species of tumor α_1-AT were derived mostly from ab-

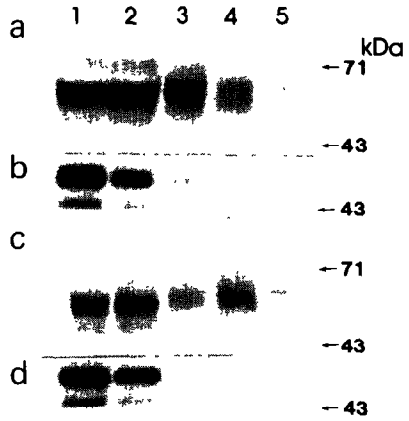

Fig. 3. Western blot analysis of α_1-AT fractionated with DSA affinity chromatography. (a) SFCM of RCM-1; (b,d) normal plasma α_1-AT; (c) SFCM of LC-2/ad. Lane 1, flow through, 3 ml; lane 2, first washing with the initial buffer, 3 ml; lanes 3–5, elution with 0.5, 1, 2% of a mixture of chitooligosaccharides in the buffer, 2 ml each. 10% of each fraction was applied on each lane.

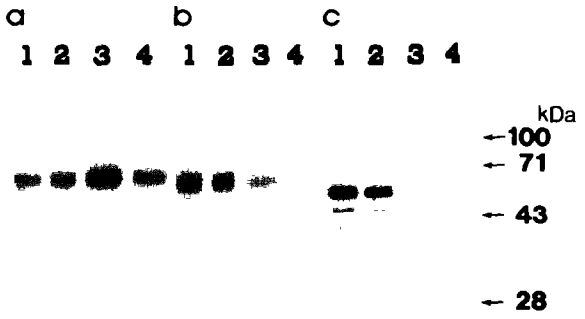

Fig. 4. Western blot analysis of α_1-AT fractionated with LTA affinity chromatography (a) SFCM of RCM-1; (b) SFCM of LC-2/ad; (c) normal plasma Aα_1-AT. Lane 1, flow through, 3 ml; lane 2, first wash with the initial buffer, 3 ml; lane 3,4, fractions eluted with 3 ml of 20 and 50 mM α-L-fucose, respectively. 10% of each unbound fraction or 30% of each eluted fraction was applied

normal glycosylation. Furthermore, the presence of varying sizes of polypeptides, mostly smaller than 44 kDa, might also contribute to formation of the broad band seen in Western blot analysis. The 51 kDa product which is present in the case of RCM-1 may be an α_1-AT–proteinase complex, since RCM-1 secreted a serine proteinase which was sensitive to the endogenous α_1-AT [13]. The presence of lower MW products in the cases of RCM-1- and LC-2/ad-derived α_1-AT suggested that the conditioned media had α_1-AT-degrading activities. Results of an α_1-AT zymogram of the conditioned media support this hypothesis. All media examined contained α_1-AT-degrading activities aside from their size and strength (Fig. 6). The main α_1-AT-degrading activity of RCM-1 was larger than 200 kDa. Similar high MW proteinase was also detected in gelatin zymography (data not shown) and by gel-filtration chromatography of SFCM of RCM-1 [13].

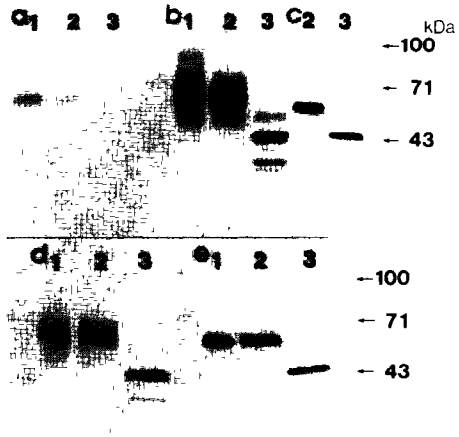

Fig. 5. Western blot analysis of α_1-AT after treatment with *N*-glycosidase F. SFCMs of (a) MRT-1; (b) RCM-1; (d) LC-2/ad; and (c,e) normal plasma α_1-AT. Lane 1, sample without any treatment; lane 2, with overnight incubation in the digestion buffer without *N*-glycosidase F; lane 3, after *N*-glycosidase F treatment.

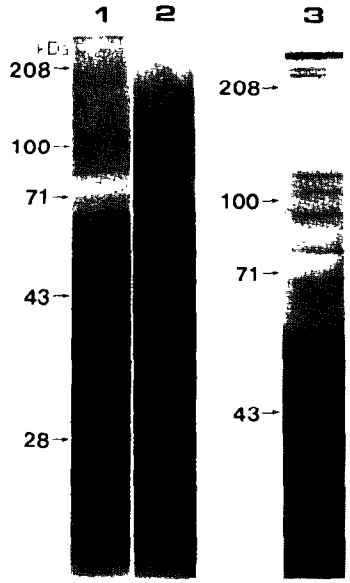

Fig. 6. α_1-AT zymography. 45 μl of 20 × concentrated SFCM were applied on a 10% SDS-polyacrylamide gel containing 0.5 mg/ml α_1-AT. (Lane 1) LC-2/ad; (lane 2) RCM-1; (lane 3) MRT-1. In lane 3, 7.5% gel was used and part of the incorporated α_1-AT was also electrophoresed.

4. DISCUSSION

There have been a few reports concerning the properties of α_1-AT derived from hepatoma cells [14] since α_1-AT is considered to be synthesized mostly by hepatocytes. In this report, we showed that besides hepatoma cells, adenocarcinoma cell lines also secrete α_1-AT. The data presented here provide further evidence for differences in the glycosylation of α_1-AT between the normal plasma form and the adenocarcinoma-derived form. According to the Con A and DSA affinity, it seems likely that the tumor-derived α_1-AT is a mixture of heterogeneously glycosylated proteins and that most of the proteins have two or three triantennary-type glycans [15] or abnormally glycosylated side chains [14], and part of the protein may possibly have triantennary oligosaccharides with C-2, 6-linked structures with or without repeating *N*-acetyllactosamine units [16]. Furthermore, the LTA affinity experiments revealed that parts of the protein, particularly in the case of RCM-1, have higher levels of fucosylation than normal plasma α_1-AT, since LTA specificity is directed towards fucosyl-D-galactose, fucosyl chitobiose and lacto-*N*-fucopentaous bearing Lex^Y [17]. Interestingly, Thompson et al. described the presence of LTA-extractable fucosylated forms of α_1-AT in sera from ovarian cancer, and that increases in LTA-extractable α_1-AT are associated with a poor response to chemotherapy [18]. Although the source of the LTA-extractable α_1-AT is uncertain, according to the present study, the tumor itself would be one of the sources. Similar changes of N-glycosylation pattern, i.e. shifts towards higher levels

of triantennary oligosaccharides and higher levels of fucosylation, have also been described in cases of tumor cell-derived human chorionic gonadotropin [19], and serum transferin of patients with hepatocellular carcinoma [20].

The role and significance of the tumor-derived α_1-AT, in particular abnormally glycosylated forms, are not known at present. Since α_1-AT can inhibit natural killer (NK) cell activity [21], and Con A non-reactive isoforms of α_1-AT inhibit the NK activity more efficiently [22], the tumor α_1-AT may modulate the host NK activity in favor of the tumor cells. By using purified tumor α_1-AT, further investigations concerning structures with altered sugar chains and their effects on tumor–host interactions would be available. SFCM of tumor cells also contained possibly proteolytically cleaved forms of α_1-AT, and α_1-AT-degrading activities, such that the biological significance of fragmented α_1-AT should also be considered. For example, proteolytically processed α_1-AT can act as a neutrophil chemoattractant [23], and the infiltrated neutrophils may enhance tissue destruction in the tumor microenvironment. Further characterization of the tumor cell-derived α_1-AT-degrading activities is now underway.

REFERENCES

[1] Heidtmann, H and Travis, J. (1986) in: Research Monographs in Cell and Tissue Physiology, vol. 12 (Barrett, A.J. and Salvesen, G. eds.) pp. 441–456, Elsevier, Amsterdam.

[2] Harris, C.C., Primack, A. and Cohen, M.H. (1974) Cancer 34, 280–281.

[3] Chawla, R.K., Lawson, D.H., Sarma, P.R., Nixson, D.W. and Travis, J. (1987) Cancer Res. 47, 1179–1184.

[4] Kataoka, H., Nabeshima, K., Komada, N. and Koono, M. (1989) Virchows Arch. B 57, 157–165.

[5] Karashima, S., Kataoka, H., Itoh, H., Maruyama, R. and Koono, M. (1990) Int. J. Cancer 45, 244–250.

[6] Tahara, E., Ito, H., Taniyama, K., Yokozaki, H. and Hata, J. (1984) Hum. Pathol. 15, 957–964.

[7] Higashiyama, M., Doi, O., Kodama, K., Yokouchi, H. and Tateishi, R. (1992) Br. J. Cancer 65, 300–302.

[8] Lykkesfeldt, A.E., Laursen, I. and Briand, P. (1989) Mol. Cell Endocrinol. 62, 287–296.

[9] Kataoka, H., Itoh, H., Suzumiya, J., Sumiyoshi, A. and Koono, M. (1989) Hum. Cell 2 (suppl.), 39.

[10] Itoh, H., Kataoka, H., Koita, H., Nabeshima, K., Inoue, T., Kangawa, K. and Koono, M. (1991) Int. J. Cancer 49, 436–443.

[11] Towbin, H., Staehelim, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.

[12] Chin, J.R., Murphy, G. and Werb, Z. (1985) J Biol. Chem. 260, 12367–12376.

[13] Kataoka, H , Maruyama, R., Ishihara, A. and Koono, M. (1989) Invasion Metastasis 9, 149–166.

[14] Carlson, J , Eriksson, S., Alm, R. and Kjellström, T. (1984) Hepatology 4, 235–241.

[15] Bayard, B., Kerckaert, J.-P., Laine, A. and Hayem, A. (1982) Eur. J. Biochem. 124, 371–376.

[16] Cummings, R.D. and Kornfeld, S. (1984) J. Biol. Chem. 259, 6253–6260.

[17] Petrynıak, J. and Goldstein, I.J. (1986) Biochemistry 25, 2829–2838.

[18] Thompson, S., Guthrie, D. and Turner, G.A. (1988) Br. J. Cancer 58, 589–593.

[19] Hard, K., Damm, J.B.L., Spruijt, M.P.N., Bergwerff, A.A., Kamerling, J.P., VanDedem, G W.K. and Vliegenthart, J.F.G. (1992) Eur. J. Biochem. 205, 785–798.

[20] Yamashita, K., Koide, N., Endo, T., Iwaki, Y. and Kobata, A. (1989) J. Biol. Chem. 264, 2415–2423.

[21] Laine, A., Leroy, A., Hachulla, E., Davril, M. and Dessaint, J.-P. (1990) Clin. Chim. Acta 190, 163–174.

[22] Lejeune, P.-J., Mallet, B., Farnarier, C. and Kaplanski, S. (1989) Biochim. Biophys. Acta 990, 122–127.

[23] Banda, M.J., Rice, A.G., Griffin, G.L. and Senior, R.M. (1988) J. Biol. Chem. 263, 4481–4484.