Clear cell carcinoma of the human ovary synthesizes and secretes a transferrin with microheterogeneity of lectin affinity

Kiyoshi Ohkawa[1], Koji Takada[1], Naoko Takizawa[1], Takashi Hatano[1], Yutaka Tsukada[2] and Makoto Matsuda[1]

[1]*Department of Biochemistry, Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato ku, Tokyo, 105, Japan* and [2]*Department of Biomedical Research, Special Reference Laboratory, Inc., Tokyo 192, Japan*

Received 27 June 1990

Human ovarian clear cell carcinoma cell line (transferrin (Tf)-non-producer), HAC 2, cells were adapted to grow in chemically defined synthetic medium when the cells were cultured with medium containing 10 μg/ml of insulin at least for 6 months. They synthesized and secreted constantly the 80 kDa protein immunologically similar to human serum Tf (15 ± 12 ng/ml/10^7 cells/3 days). By sensitive lectin-affinity electrophoresis followed by antibody-affinity blotting technique, a concanavalin A weakly bound or unbound, lentil lectin, a strongly reactive abnormal band, which was rarely found in human serum Tf, was detectable in the Tf synthesized by HAC 2 cells (HACTf). These findings suggest that the HACTf may act as one of the autocrine growth factors and that this heterogeneity of HACTf for lectin affinity is ascribed to differences in the carbohydrate moiety of the Tf.

Transferrin; Fucosylation; Lectin affinity; Antibody-affinity blotting

1. INTRODUCTION

Transferrin (Tf), the major iron-transporting serum glycoprotein of 80 kDa in vertebrate, is known as the growth factor required for all proliferating cells in vitro [1,2] and in vivo [3]. Tf is mainly synthesized in liver parenchymal cells but is also synthesized in other cells (myoblast [4], chorioid plexus [5], oligodendroglia [6], lymphocyte [7], yolk sac endoderm [8], and Sertoli cells [9]) as reported previously. Transformed cells in the malignant tissues described above also possessed the ability to producing the Tf at various degrees [10,11]. Additionally few reports have been documented that Tf is also synthesized in the various cancerous cells as one of the autocrine growth factors, such as lung cancer [12] and breast cancer [13]. The possibility has been also reported that the carbohydrate moieties of each Tf differed from others [6]. To characterize the Tf synthesized by Tf-non-producer cells, we reported here that another case of cancer cell, ovarian clear cell carcinoma cell line, HAC 2, was able to synthesize the 80 kDa protein (HACTf) with immunoreacting to human Tf with heterogeneity of lectin-affinity.

2. MATERIALS AND METHODS

2.1. *Cell line*

Human ovarian clear cell carcinoma cell line, HAC 2, was kindly provided by Dr. M. Nishita, Tsukuba University School of Medicine and was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Flow Lab. North Ryde, Australia). For adaptation of HAC 2 clone to serum-free chemically defined synthetic medium supplemented with Na_2SeO_3 (ISRPMI) which was reported previously [14], the cells were initially transferred to ISRPMI with addition of insulin (10 μg/ml, Sigma, St. Louis, MO, USA) and cultured over 6 months. From the 8th week of culturing time, a HAC 2 cell clone was able to replicate continuously but slowly in ISRPMI without addition of insulin. Conditioned media obtained from each culture were stocked at −80°C until use.

Correspondence address: K. Ohkawa, Department of Biochemistry, Jikei University School of Medicine, 3-25-8, Nishi-shinbashi, Minato ku, Tokyo 105, Japan

2.2. *Antibody against human Tf*

Polycloncal goat antibody against human Tf was obtained from Cappel (No. 0201-1441, Lot 32730. Organon Teknika, West Chester, PA, USA). Monospecificity of the antibody was checked by immunoelectrophoresis. Specific antibody against human Tf was purified by affinity chromatography on BrCN-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled to human Tf (Sigma). Briefly, 20 ml (20 mg/ml) antibody was passed through the affinity column at a flow rate of 10 ml/h. The column was washed with 10 mM Na-phosphate buffer, pH 7.0, 0.5 M NaCl, 0.02% NaN_3, and 1 mM PMSF (wash buffer) and eluted by 3 M $MgCl_2$ in wash buffer. The eluate was dialyzed against 10 mM Na-phosphate buffer, pH 7.0, 0.15 M NaCl, 0.02% NaN_3 and 1 mM PMSF (PBS) for 24 h at 4°C. Biotinylation of the antibody was performed by the method described by Guesdon et al. [15]. Briefly, the specific antibody was dialyzed against 0.1 M $NaHCO_3$ containing 0.5 M NaCl at 4°C overnight. One ml of the dialyzed antibody (1 mg/ml) was mixed with 100 μl of *N*-hydroxysuccinimidobiotin (Pierce Chemical, Rockford, IL, USA) dissolved in dimethylsulfoxide (DMSO, 1 mg/ml). The mixture was incubated for 4 h at room temperature and then extensively dialyzed against 20 mM Tris HCl, pH 7.5, 0.5 M NaCl, 0.01% sodium merthiolate (TBS) at 4°C overnight.

2.3. *Enzyme-linked immunosorbent assay (ELISA) for human Tf*

96 well microtiter plates (Nunc, Denmark) with 10 μg/ml immobilized specific antibody against human Tf were incubated with standard human Tf (Sigma) or conditioned media at 4°C overnight. Wells were then incubated with biotinylated anti-human Tf antibody

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

(2 μg/ml in 0.05% Tween 20 in TBS) for 5 h at room temperature followed by avidin-conjugated horseradish peroxidase (HRP, 2 μg/ml in 0.1 M $NaHCO_3$, Cappel) for 30 min at room temperature. Substrate reaction using *O*-phenylendiamine was determined at 492 nm in a microplate reader, MRP A4 (TOSOH, Tokyo, Japan).

2.4. *Purification of HACTf*

HAC 2 conditioned media were loaded onto the specific anti-human Tf IgG-coupled Sepharose 4B (Pharmacia) column. The column was washed with wash buffer and the Tf was eluted by 8 M urea in PBS. The eluate was extensively dialyzed against PBS at 4°C for 24 h. Tf from normal human serum was also prepared by this method.

2.5. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was carried out using 7.5% gel according to Laemmli [16]. The resulting gel was stained by either Coomassie blue or silver staining kit (BioRad, Richmond, CA, USA). For molecular weight markers we used protein markers (BioRad) with ovalbumin (43 kDa), bovine serum albumin (66.2 kDa) and phosphorylase b (97.4 kDa).

2.6. *Metabolic labelling and immunoprecipitation of HACTf*

HAC 2 cells cultured with ISRPMI in 75 cm^2 flasks (Falcon No. 3084, Oxnard, CA, USA) were once washed with Dulbecco's PBS and were incubated in L-leucine-free minimum essential medium (GIBCO, NY, USA) supplemented with Na_2SeO_3 and L-glutamine; after 60 min the media were discarded and [^{14}C]-L-leucine (5 μCi/ml,sp.act. 12.5 GBq/mmol, Amersham, Tokyo, Japan) in the same medium were added to the flasks and further cultivated for 24 h. After incubation, the conditioned media were stocked, dialyzed against PBS, concentrated by ultrafiltration (Amicon, Danvers, MA, USA). 20 μl (1 mg/ml) of goat anti-human Tf IgG or preimmune goat IgG was added to the concentrated conditioned media containing 25,340 cpm of radiolabeled proteins in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate. After 24 h incubation at 4°C, 50 μl of rabbit antibody against goat IgG (1 mg/ml) was added and incubation was continued for an additional 24 h at 4°C. The immunoprecipitates were collected by centrifugation at 3000 rpm for 30 min through a 1 ml cushion of 1 M sucrose in PBS and washed 4 times with 0.05% Tween 20 in TBS. The immunoprecipitates were dissolved in 40 μl of SDS sample buffer (1% SDS, 1% 2-mercaptoethanol, 4 M urea in 0.0625 M Tris-HCl, pH 6.8) by boiling for 10 min and centrifuged in Eppendorf microtubes. Aliquots were subjected to SDS-PAGE and the gels were fixed, stained by Coomassie blue. Destained gels were incubated with DMSO, treated with 22.2 (w/v) 2,5-diphenyloxazole in DMSO for 3 h, soaked in water to remove DMSO, dried and exposed to X-ray film (Fuji, Tokyo, Japan) at −70°C for fluorography [17].

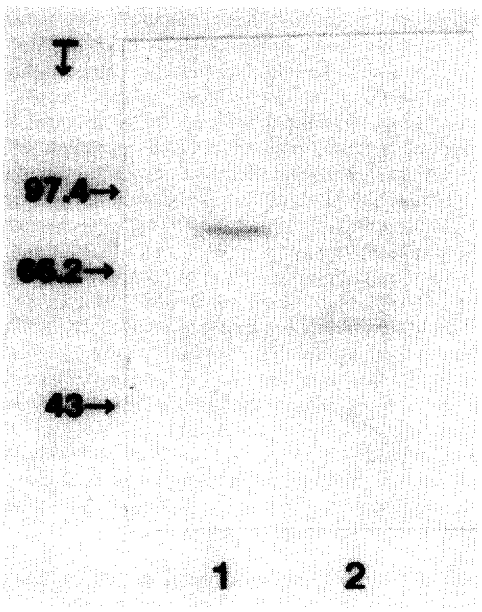

Fig. 1. Fluorograph of metabolically radiolabelled protein synthesized and secreted by HAC 2 cells. Immunoprecipitation by anti-human Tf antibody shown in lane 1 and by preimmune goat IgG shown in lane 2. Molecular weight markers in kDa.

2.7. *Lectin-affinity electrophoresis and antibody-affinity blotting analysis for fractionation of molecular species of HACTf*

HACTf was fractionated by concanavalin A (ConA, Hohnen, Tokyo, Japan) or lentil lectin (LcHA, Hohnen, Tokyo, Japan) affinity agarose electrophoresis followed by antibody affinity blotting as described previously [18]. Briefly, antibody-coated nitrocellulose papers were prepared by incubation with 100 μg/ml specific goat antibody to human Tf for 30 min, then by fixing with glutaraldehyde vapour for 30 min, neutralizing for 3 min with $NaBH_4$, followed by washing with TBS. The nitrocellulose paper was blocked with 0.5% Tween 20 in TBS for 30 min, then washed with TBS.

Electrophoresis was performed on 1.0% agarose gel plates (Litex type HSA, Denmark) in Tris/barbital buffer (pH 8.6, ionic strength 0.02) containing either 0.1 mg/cm^2 ConA or 20 μg/cm^2 LcHA. 1 μl of 100 ng/ml samples were electrophoresed on the plates at 4°C at a voltage gradient of 15 V/cm. After lectin affinity electrophoresis, HACTf was transferred by capillary blotting to nitrocellulose paper coated with antibody to human Tf. The nitrocellulose paper was then washed twice in 0.05% Tween 20 in TBS, treated with rabbit anti-human Tf IgG (Dakopatts, Glostrup, Denmark), followed by treatment with swine antibody to rabbit IgG labelled with HRP (Dakopatts). Color was developed by incubating the nitrocellulose paper with 3,3′-diaminobenzidine tetrachloride (Polyscience, Warrington, PA, USA) and H_2O_2 in TBS. After color development, the membranes were washed, dried and scanned with densitometer (Dualwavelength, Flying-spot scanner CS-9000, Shimazu, Tokyo, Japan) at 495 nm.

2.8. *Chemicals*

All other chemicals were used of reagent grade.

3. RESULTS

3.1. *Determination of HACTf in culture medium*

The secretion of Tf was not detectable by ELISA when HAC 2 cells were cultured with either FBS-

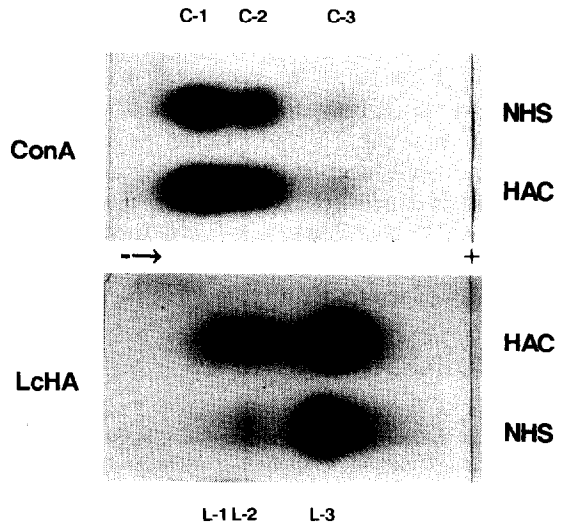

Fig. 2. SDS-PAGE of purified Tf from conditioned medium of HAC 2 (lane 1) or from normal human serum (lane 2). Molecular weight markers in kDa.

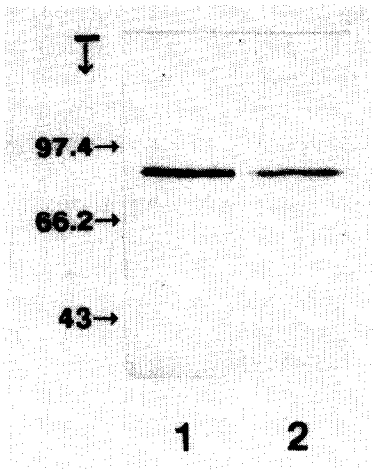

Fig. 3. Analytical Tf bands separated by ConA, or LcHA-affinity electrophoresis. Tf from HAC and from normal human serum (NHS). Each 1 μl sample was applied and electrophoresis was run until bromophenol blue migrated 9.0 cm from the origin (−). C-1–C-3 and L-1–L-3 are described in the text.

containing RPMI1640 or ISRPMI without insulin. Detection of Tf secretion into the culture media, when HAC 2 cells were cultured in ISRPMI containing insulin, indicated that the synthesis and secretion of Tf increased gradually reaching detectable levels of 15 ± 12 g/ml/10^7 cells/3 days and then remained at a constant level without addition of insulin to culture medium.

3.2. *Immunoprecipitation of HACTf*

In order to evaluate this protein synthesized, secreted from HAC 2 cells, immunoprecipitation with anti-human Tf antibody was carried out. The 80 kDa labelled protein was immunoprecipitated with antibody (Fig. 1). The electrophoretic protein profile of the conditioned media after the immunoprecipitation lacked the 80 kDa protein band (data not shown), indicating that most, if not all, of the 80 kDa protein was immunoreactive with the antibody against human Tf.

3.3. *Immunoaffinity purification of HACTf*

Tf synthesized and secreted by HAC 2 cells, separated by single step immunoaffinity column, was obtained with an average amount of 700 ng/50 ml conditioned media. This fraction gave a single band with molecular weight of 80 kDa by SDS-PAGE analysis (Fig. 2).

3.4. *Lectin-affinity electrophoresis and antibody-affinity blotting analysis of HACTf*

Lectin affinity-crossed immunoelectrophoresis which is a more quantitative technique but has a low sensitivity was not applied in this study because of the low levels of Tf in the culture media (ca 14 μg/l). Testing the affinity of antibody-coated nitrocellulose paper by dot-blot analysis of human Tf (Sigma) serially diluted with 1% bovine serum albumin in TBS, as low as 3 ng (1 μl of 3 μg/ml) Tf was detected with the dose-dependent color intensity. In contrast, using the plain nitrocellulose paper without coating of antibody showed 100-fold lower sensitivity for the detection of Tf without any dose-dependent color reaction (data not shown). By this sensitive method after ConA-affinity electrophoresis. Tf derived from normal human serum was resolved into 3 bands, i.e. strongly ConA reactive major (C-1), moderately reactive minor (C-2), and non-reactive slight but detectable (C-3) (Fig. 3) and the same

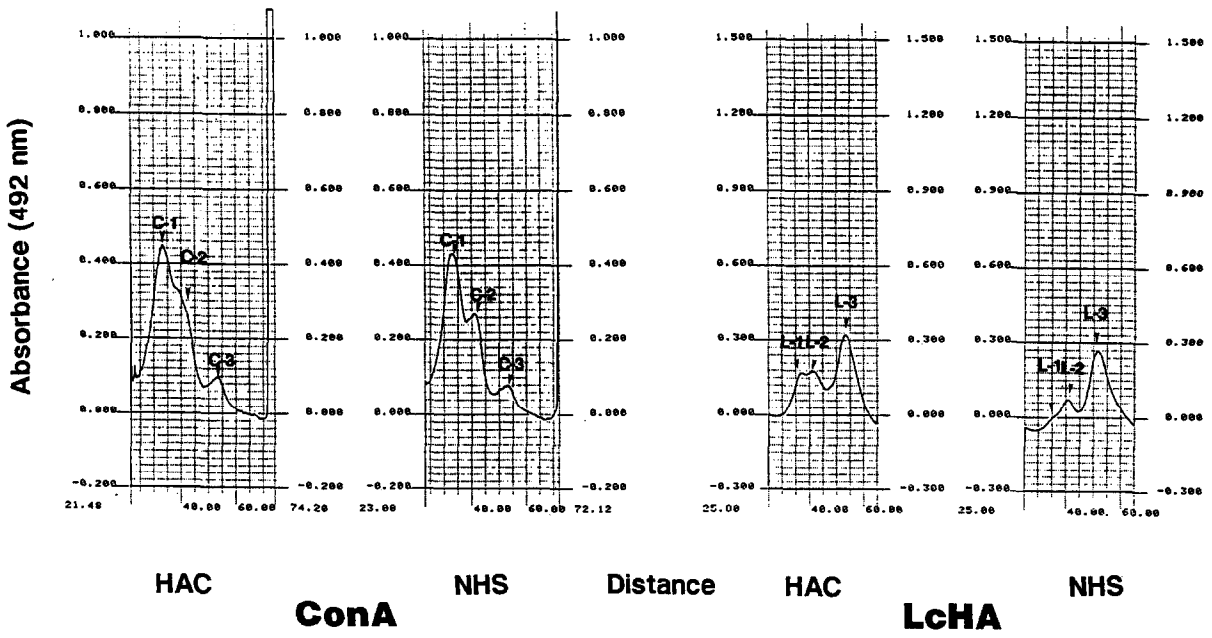

Fig. 4. Densitometric analysis was carried out according to the Materials and Methods. See Fig. 3 for other keys.

results were also obtained from the densitometric analysis (Fig. 4). This result is compatible with the profile obtained from ConA-affinity crossed immunoelectrophoresis of normal human serum Tf as previously mentioned [19]. HACTf was resolved mainly into 3 bands with significant increase of C-3 (Fig. 3). Additionally by the densitometric analyses, C-1 showed the shoulder-like peak and the increased amounts of another fraction between C-1 and C-2 was suggested (Fig. 4). More obvious difference of the electrophoretic profile between normal human serum Tf and HACTf was noted in LcHA-affinity electrophoresis. As shown in Figs 3 and 4, normal human serum Tf was separated into 3 bands of LcHA unreactive major (L-3) and weakly reactive minor (L-2) and strongly reactive but quite minor (L-1) Tf. In contrast, HACTf was also fractionated into 3 bands with significant increasing of LcHA reactive fractions. Among them, 2 bands (L-2 and L-3) probably corresponded to those of normal human serum Tf because of the same electrophoretic pattern. However, the third band (L-1) showed the property of high affinity to LcHA which was specific to HACTf. These results suggest that HACTf consists of Tf showing the heterogeneity with lectin-affinity due to differences in the carbohydrate moiety.

4. DISCUSSION

We have shown that the human ovarian clear cell carcinoma cell line, HAC 2, was able to synthesize and secrete significant amounts of 80 kDa protein into the chemically defined synthetic medium, ISRPMI. The protein shared immunoreactivity and molecular weight with purified human serum Tf. The initial addition of insulin to the medium was effective to adapt the HAC 2 cells to serum-free ISRPMI because the production of detectable amounts of Tf was induced and the synthesized Tf probably acted as a growth factor. It has been reported that the expression of Tf receptor was induced by the insulin or insulin-like growth factor(s) after the expression of Tf [12]. In our study Tf receptor had not been checked.

Reactivities to ConA as well as LcHA of HACTf were different from those of normal human serum Tf determined by the sensitive assay method of antibody-affinity blotting. Increased amounts of ConA-weakly (C-2) and -non-reactive (C-3) bands whith the appearance of another band between them was noted. Moreover, LcHA-strongly reactive band (L-1) was clearly detectable in the HACTf. These findings suggest that glycosylation of Tf in HAC2 cell differs from that in normal hepatocyte. The known carbohydrate chain specificities of ConA and LcHA are different. The oligosaccharide structure required for LcHA reactivity lies in the core fucose attached to GlcNAc [20]. In contrast, this structure of additional fucose attachment to GlcNAc does not influence the reactivity to ConA, but the increasing of tri- and tetra-antennary glycosylation of carbohydrate moieties of Tf molecules diminishes the affinity to ConA [21]. This fact concluded that the fucosylated Tf in highly branched glycans increased in HACTf molecules. The structural heterogeneity of the carbohydrate moiety of human serum Tf has been reported in some physicopathologic conditions [19,22,23] as well as in vitro hepatoma cell line [24]. Fucosylation of the carbohydrate moiety of the proteins has been demonstrated in some proteins, such as Tf [24], α-fetoprotein [25] and γ-glutamyl transpeptidase [26]. These findings show that fucosylation of the carbohydrate moiety of the glycoproteins is one of the characteristics in the expression of cancer cells. The syntheses of Tf by HAC2 cells may bestow selective growth advantages on these rapidly growing cells and Tf acting as autocrine growth factor perhaps permits tumor cell growth in metastatic lesions not well vascularized in vivo.

Acknowledgement: This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

[1] Barnes, D. and Sato, G. (1980) Anal. Biochem. 102, 255–270.
[2] Shapiro, L.E. and Wagner, N. (1989) In vitro Cell Dev. Biol. 25, 650–654.
[3] Aisen, P. and Listowsky, I. (1980) Annu. Rev. Biochem. 49, 357–393.
[4] Beach, R.L., Pipiela, H. and Feshoff, B.W. (1983) FEBS Lett. 156, 151–156.
[5] Tsutsumi, M., Skinner, M.K. and Sanders-Bush, E. (1989) J. Biol. Chem. 264, 9626–9631.
[6] Festoff, B.W., Munoz, P.A., Patel, M.K.N., Harris, M. and Beach, R.L. (1989) J. Neurosci. Res. 22, 425–438.
[7] Lum, J.B., Infante, A.J., Makker, D.M., Yang, F. and Bowman, B.A. (1986) J. Clin. Invest. 77, 841–849.
[8] Gitlin, D. and Perricelli, A. (1970) Nature (Lond.) 228, 995–997.
[9] Stallard, B.J. and Griswold, M.D. (1990) Mol. Endocrinol. 4, 393–401.
[10] Morrone, G., Gorbo, L., Turco, M.C., Pizzano, R., De Feliu, M., Bridges, S. and Venuta, S. (1988) Cancer Res. 48, 3425–3429.
[11] Shirai, T., Itoh, T., Yoshiki, T., Noro, T., Tomino, Y. and Hayasaka, T. (1976) Cancer 38, 1661–1667.
[12] Vostrejs, M., Moran, P.L. and Seligman, P.A. (1988) J. Clin. Invest. 82, 331–339.
[13] Vandewalle, B., Hornez, F., Revillion, F. and Lefebvre, J. (1989) Biochem. Biophys. Res. Commun. 163, 149–154.
[14] Nakabayashi, H., Taketa, K., Miyano, K., Yamane, T. and Sato, J. (1982) Cancer Res. 42, 3858–3863.
[15] Guesdon, J., Ternynck, T. and Abrameas, S. (1979) J. Histochem. Cytochem. 27, 1131–1139.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[18] Ohkawa, K., Abe, T., Tsukada, Y., Taketa, K., Kimura, E. and Terashima, Y. (1989) J. Clin. Chem. Clin. Biochem. 27, 337–341.
[19] Léger, D., Campion, B., Decottignies, J.P., Montreuil, J. and Spik, G. (1989) Biochem. J. 257, 231–238.

[20] Kornfeld, K., Reitman, M.L. and Kornfeld, R. (1981) J. Biol. Chem. 256, 6633–6640.
[21] Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11235–11240.
[22] Maerz, L., Hatton, M.W.C., Berry, L.R. and Regoeczi, E. (1982) Can. J. Biochem. 60, 624–630.
[23] Heegaard, N.H.H., Hagerup, M., Thomsen, A.C. and Heegaard, P.M.H. (1989) Electrophoresis 10, 836–840.
[24] Campion, B., Léger, D., Wieruszeski, J.M., Montreuil, J. and Spik, G. (1989) Eur. J. Biochem. 184, 405–413.
[25] Yoshima, H., Mizuochi, T., Ishii, M. and Kobata, A. (1980) Cancer Res. 40, 4276–4281.
[26] Yamashita, K., Hitoi, A., Taniguchi, N., Yokosawa, N., Tsukada, Y. and Kobata, A. (1983) Cancer Res. 43, 5059–5063.