Volume 308, number 1, 46–49 FEBS 11394 August 1992

The metastatic potential of rat prostate tumor variant R3327-MatLyLu is correlated with an increased activity of *N*-acetylglucosaminyl transferase III and V

Ellen W. Easton[a], Irene Blokland[a], Albert A. Geldof[b], B. Ramanath Rao[b] and Dirk H. van den Eijnden[a]

[a]*Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands* and [b]*Department of Endocrinology, Academisch Ziekenhuis Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands*

Received 8 June 1992; revised version received 22 June 1992

Enzyme activities of *N*-acetylglucosaminyltransferase (GlcNAc-Tase) I–V involved in *N*-linked complex-type carbohydrate synthesis were determined in a non-metastatic hormone-dependent rat prostate tumor (R3327-H) and a related, hormone-independent variant metastasizing to lymph nodes and lungs (R3327-MatLyLu). In the metastasizing variant a significantly increased activity of both GlcNAc-Tase III and GlcNAc-Tase V was observed, whereas the activities of GlcNAc-Tase I and II were essentially unchanged. The increase in activity of GlcNAc-Tase III is particularly noteworthy since it indicates that elevated expression of this enzyme cannot be considered as an exclusive marker of hepatic malignancy.

N-Acetylglucosaminyltransferase; Metastasis; Rat prostate tumor; R3327-MatLyLu

1. INTRODUCTION

Prostate cancer is one if the major lethal malignancies in males, and death is always caused by metastasis to lungs, skeleton or lymph nodes [1]. The R3327 prostate tumor in the Copenhagen rat is often used to investigate the process of prostate tumor cell metastasis. Several R3327 tumor variants have become available, with differences in hormone sensitivity, metastatic potential and target organ specificity [2]. We have used two of these variants, the R3227-H variant (hormone-sensitive and non-metastasizing) and the R3327-MatLyLu variant (hormone-insensitive and metastasizing to lymph nodes and lungs), to investigate the possible role of cell surface carbohydrates in the metastatic process.

Malignant transformation of cells has often been found to be associated with an increase in the average molecular weight of *N*-linked complex-type cell surface carbohydrates [3–10]. This increased molecular weight has been attributed to increased branching on the trimannosyl core caused by an elevated *N*-acetylglucosaminyltransferase (GlcNAc-Tase) V activity [11,12], to increased polyactosaminoglycan formation [8,10,13] or to increased sialylation [6,9]. Furthermore, a correlation between GlcNAc-Tase V activity and metastatic behavior of cells was observed in murine MDAY-D2 cells [14] and in oncogene-transfected rat 2 fibroblasts [15]. In NIH 3T3 cells expressing the N-*ras* proto-oncogene, an invasive potential was correlated with an increased GlcNAc-Tase III and V expression [10].

We have determined the activities of GlcNAc-Tase I–V, enzymes that are involved in the synthesis on *N*-linked complex-type di-, tri- and tetra-antennary glycans, in the R3327-H and the R3327-MatLyLu prostate tumor variants to investigate whether there is a correlation between altered glycosylation as indicated by changed glycosyltransferase levels and metastatic potential. We found that in the metastasizing variant the activity of two of the branching GlcNAc-Tases was significantly increased.

Correspondence address: D.H. van den Eijnden, Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, NL-1081 BT Amsterdam, The Netherlands. Fax: (31) (20) 642 8555.

2. MATERIALS AND METHODS

2.1. *Materials*

Oligosaccharide Manα1→3(Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc(Man$_5$GlcNAc$_2$) was isolated from the urine of loco-intoxicated sheep [16], which was kindly supplied by Dr. C.D. Warren (Massachusetts General Hospital, Boston, MA). GlcNAcβ1→2Manα1→3(Manα1→6)Manβ-O-(CH$_2$)$_8$COOCH$_3$ (GA-3139A) was a kind gift of Dr. O. Hinsgaul (University of Alberta, Edmonton, Alta.). Oligosaccharides GlcNAcβ1→2Manα1→3(GlcNAcβ1→2Manα1→6)-Manβ1→4GlcNAc (di), GlcNAcβ1→2(GlcNAcβ1→4)Manα1→3(GlcNAcβ1→2Manα1→6)-Manβ1→4GlcNAc (tri), GlcNAcβ1→2Manα1→3(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→6)-Manβ1→4GlcNAc (bis) and GlcNAcβ1→2Manα1→3(GlcNAcβ1→2(GlcNAcβ1→6)Manα1→6)-Manβ1→4GlcNAc (tri') were obtained by β-galactosidase (jack bean) digestion [17] of the corresponding β4-galactosylated compounds, which were kindly donated by Dr. G. Strecker (Université de Lille, Villeneuve d'Ascq, France). Amberlite IRA-743 was obtained from Sigma (St. Louis, MO).

UDP-[^{14}C]GlcNAc (302 Ci/mol) was obtained from Amersham International (Amersham, UK), and UDP-[^{3}H]GlcNAc (18.5 Ci/mol)

Published by Elsevier Science Publishers B.V.

from Dupont-New England Nuclear (Boston, MA). Unlabeled UDP-GlcNAc was purchased from Sigma (St. Louis, MO). The unlabeled sugar nucleotide was used to dilute the radioactive compounds to the desired specific radioactivity. All other chemicals were of the best quality available from commercial sources.

2.2. *Tumors*

Copenhagen rats, originally obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, MD, were bred (brother × sister) and housed in our animal facilities according to the institutional animal welfare regulations. The R3327-MatLyLu tumor (hormone-insensitive and metastasizing to lymph nodes and lungs) and R3327-H tumor (hormone-sensitive and non-metastasizing) were originally obtained from Drs. D.S. Coffey and J.T. Isaacs (Johns Hopkins School of Medicine, Baltimore, MD). They have been maintained by subcutaneous transplantation in castrated and intact male Copenhagen rats, respectively [18]. Of each tumor variant, specimens from three different rats were analyzed.

2.3. *Enzyme preparations*

Tumors were dissected from the animals and cut into small pieces and homogenized in 10 mM sodium cacodylate, pH 7.0, using a Polytron homogenizer. The homogenate contained 35–90 mg protein per ml and was stored at −20°C until use. Under this condition the enzyme preparations retained full activity for at least 6 months. The protein concentration was determined according to Peterson [19].

2.4. *Glycosyltransferase assays*

The methods for assaying the activity of GlcNAc-Tase I and II were modified from Harpaz and Schachter [20]. Incubation mixtures consisted of 50 nmol $Man_3GlcNAc_2$ (GlcNAc-Tase I) or 100 nmol oligosaccharide GA-3139A (GlcNAc-Tase II), 70 nmol UDP-[^{3}H]GlcNAc (specific radioactivity 1 Ci/mol), 5 μmol 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 6.3, 1 μmol $MnCl_2$, 0.1 μl Triton X-100 and cell homogenate (0.35–0.9 mg of protein) in a total volume of 50 μl. Incubations were conducted for 1–2 h at 37°C. In the case of GlcNAc-Tase I the reaction was stopped by the addition of 450 μl of H_2O and the mixture was passed through a 1 ml column of Dowex 1X8 (100–200 mesh), Cl^--form. The eluate and 3 washes of 0.5 ml of H_2O each were collected and radioactivity incorporated into the acceptor oligosaccharide was counted by liquid scintillation. In the assay of GlcNAc-Tase II the reaction was stopped by the addition of 5 ml of H_2O. Separation of the labelled oligosaccharide product from the radioactive sugar-donor substrate was carried out using Sep-Pak C-18 reverse-phase cartridges (Water, Milford, MA) according to Palcic et al. [21]. The activities of GlcNAc-TASE III, IV and V were determined simultaneously using oligosaccharide di as a common acceptor substrate. Incubations were conducted for 16 h at 37°C as described by Koenderman et al. [17]. The reaction was stopped by adding 0.5 ml H_2O and pasing the mixture through a 1 ml column of Dowex 1-X8, Cl^--form. The column was washed twice with 0.7 ml H_2O. To the combined eluates 150 μl 2.7 M $NaBH_4$/0.1 M NaOH was added and the mixture was left at room temperature for 1 h. Excess of $NaBH_4$ was destroyed by the addition of 4 M acetic acid. Cations were removed by passing the mixture through a column of 0.5 ml Dowex 50W-X16, H^+ form and borate was removed by passage over a 2 ml column of Amberlite IRA-743 column. The columns were washed with 4.0 ml H_2O, and the eluate was lyophilized and dissolved in 100 μl H_2O. Separation of the different products was accomplished by HPLC [17].

In all assays, control incubations were carried out without acceptor to correct for endogenous acceptor activity. Assays were done for each rat tumor homogenate at least in duplicate. In the HPLC assays radiolabeled references were added to allow correct product identification.

3. RESULTS AND DISCUSSION

In this study we investigated the possible correlation between aberrant *N*-glycosylation and tumor cell metastasis. The activity of GlcNAc-Tase I–V was assayed in two rat prostate tumor variants, and for each enzyme the activity in the R3327-MatLyLu tumor relative to that in the R3327-H tumor was taken as a measure for the change in expression in the metastasizing cells. GlcNAc-Tase I and II each catalyze a committed step in the synthesis of *N*-linked complex-type di-antennary glycans [20], while the other enzymes are involved in the formation of bisected (GlcNAc-Tase III) [22] and tri- and tetra-antennary (GlcNAc-Tase IV and V) [23,24] structures. The R3327-H tumor is hormone-dependent and grows well in intact animals but not in castrated rats, while the hormone-independent R3327-MatLyLu tumor grows equally well in intact and castrated animals [18]. Therefore the differences observed in the activities of the enzymes between the two variants are most likely due to an acquired genetic disposition rather than to hormonal influences.

No significant difference between the two variants was observed in the activity of GlcNAc-Tase I and GlcNAc-Tase II (Table I). This indicates that the potential to synthesize *N*-linked complex-type glycans is the same in both tumors. However, the activity of GlcNAc-Tase III was highly increased in the metastasizing variant, while the activity of GlcNAc-Tase V was elevated to a lesser extent (Table I, Fig. 1). GlcNAc-Tase IV activity was below detection in either variant, although we cannot rule out the possibility that some GlcNAc-Tase IV activity is present in the H-variant (Fig. 1).

An increased activity of GlcNAc-Tase V has been correlated with malignant transformation in several cases [10–12,14,15]. In a number of these cases elevation in the expression of this enzyme was associated with metastasis or invasive potential.

Increased activity of GlcNAc-Tase III, on the other hand, has been suggested to be a marker specific of hepatic malignancy, as high activities of this enzyme have been reported in rat [17,25] and human [26]

Table 1

N-Acetylglucosaminyltransferase (GlcNAc-Tase) activities (nmol·mg^{-1} protein·h^{-1}) in the non-metastatic, hormone-dependent R3327-H and the metastatic, hormone-independent R3327-MatLyLu rat prostate tumor variants

GlcNA-Tase	Linkage formed	Activity in		Activity ratio (R3327-MatLyLu R3327-H)
		R3327-H	R3327-MatLyLu	
I	GlcNAcβ1→2Manα1→3	4.7	5.0	1.1
II	GlcNAcβ1→2Manα1→6	10.8	14.2	1.3
III	GlcNAcβ1→4Manβ1→4	0.02	0.13	6.5
IV	GlcNAcβ1→4Manα1→3	<0.005	<0.005	–
V	GlcNAcβ1→6Manα1→6	0.005	0.013	2.6

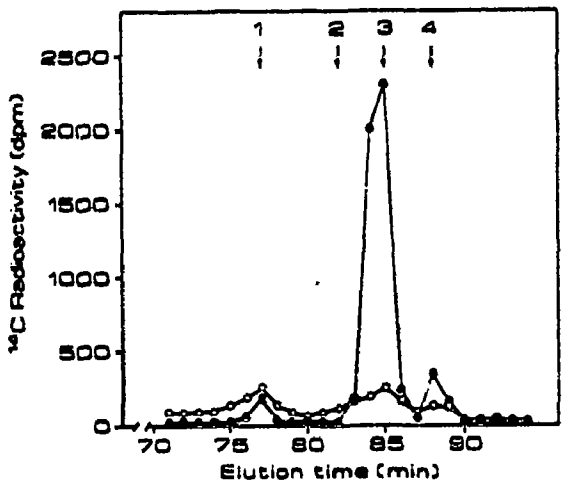

Fig. 1. Assay of GlcNAc-Tase III, IV and V activities by HPLC. Transferase activities were measured in homogenates of the R3327-H (○) and the R3327-MatLyLu (●) prostate tumor variants using oligosaccharide di as the acceptor substrate. After incubation the products of glycosyltransferase action were isolated and reduced as described in section 2. Analysis of the resulting material was performed on a Lichrosorb-NH_2 column. Elution was carried out at a flow rate of 2 ml·min^{-1} using a linear gradient of acetonitrile/15 mM potassium phosphate, pH 5.2, starting at 4:1 (v/v) and decreasing acetonitrile by 0.2%·min^{-1} [17]. The arrows indicate the elution positon of the reduced forms of (1) the substrate (di), and the products of (2) GlcNAc-Tase IV (tri), (3) GlcNAc-Tase III (bis) and (4) GlcNAc-Tase V (tri').

hepatomas, preneoplastic hepatic nodules [27], and in human liver cirrhosis (a condition that may result in the development of a primary hepatoma) [26]. In rat hepatomas the increased GlcNAc-Tase III activity was shown to correlate with the presence of bisected structures on glycoproteins of hepatoma origin while these structures were absent on glycoproteins produced by normal liver [28,29]. Recently we have reported an increased activity of GlcNAc-Tase III to occur in mouse NIH 3T3 fibroblasts expressing the human N-*ras* protooncogene [10]. This result correlated well with lectin binding studies of surface glycopeptides of NIH 3T3 cells transfected with *ras*-oncogenes, which showed an increased affinity for E-PHA [4], a lectin specific for bisected structures [30]. By analogy, based on the results obtained in the present study, we predict that an increased amount of bisected structures will also be present on the R3327-MatLyLu cells. Elevated levels of GlcNAc-Tase III and bisected structures therefore are not an exclusive characteristic of neoplasia of the liver.

Moreover, it should be noted that the presence of bisected structures at the cell surface or on circulating glycoproteins, as such is not necessarily indicative of a malignant status. Apart from the cases mentioned above, bisected structures have also been found on a number of normal glycoproteins, including ovalbumin [31], ovotransferrin [32], and ovomucoid [33] from hen oviduct; human red blood cell band 3 glycoprotein [34] and glycophorin [35]; and γ-glutamyltranspeptidase from bovine [36], rat [37] and human kidney [38]. However, although the presence of bisected structures is a normal characteristic of some glycoproteins, an aberrantly increased expression of GlcNAc-Tase III and its products may well be correlated with malignant transformation and a metastatic potential.

Although recently some lectin-binding studies have been performed with R-3327 tumor variants none of them have provided information about the amount of bisected and tri'-antennary structures [39–41]. However, based on our results we expect an increased amount of *N*-linked tri'-antennary structures to occur on the R3327-MatLyLu cells, in addition to the above mentioned increase in bisected structures. Since tumor cell adhesion is thought to play an important role in the establishment of site-specific tumor metastasis [42], and carbohydrate structures may act as specific ligands in cell–cell adhesion processes [43] it is tempting to speculate about a role of the increased amount of bisected and tri'-antennary structures in the R3327-MatLyLu variant in its metastasis to lymph nodes and lungs.

Acknowledgements: We would like to thank Dr. D.H. Joziasse for his helpful comments on this manuscript.

REFERENCES

[1] Geldof, A.A. and Rao, B.R. (1990) Anticancer Res. 10, 1303–1306.
[2] Isaacs, J.T. and Coffey, D.S. (1983) Clin. Oncol. 2, 479–498.
[3] Yamashita, K., Ohkura, T., Tachibana, Y., Takasaki, S. and Kobata, A. (1984) J. Biol. Chem. 259, 10834–10840.
[4] Santer, U.V., Gilbert, F. and Glick, M.C. (1984) Cancer Res. 44, 3730–3735.
[5] Bolscher, J.G.M., Van der Bijl, M.M.W., Neefjes, J.J., Hall, A., Smets, L.A. and Ploegh, H.L. (1988) EMBO J. 7, 3361–3369.
[6] Van Beek, W.P., Smets, L.A. and Emmelot, P. (1973) Cancer Res. 33, 2913–2922.
[7] Pierce, M. and Arango, J. (1986) J. Biol. Chem. 261, 10772–10777.
[8] Hubbard, S.C. (1987) J. Biol. Chem. 262, 16403–16411.
[9] Warren, L., Fuhrer, J.P. and Buck, C.A. (1972) Proc. Natl. Acad. Sci. USA 69, 1838–1842.
[10] Easton, E.W., Bolscher, J.G.M. and Van den Eijnden, D.H. (1991) J. Biol. Chem. 266, 21674–21680.
[11] Yamashita, K., Tachibana, Y., Ohkura, T. and Kobata, A. (1985) J. Biol. Chem. 260, 3963–3969.
[12] Arango, J. and Pierce, M. (1988) J. Cell. Biochem. 37, 225–231.
[13] Van den Eijnden, D.H., Koenderman, A.H.L. and Schiphorst, W.E.C.M. (1988) J. Biol. Chem., 263, 12461–12471.
[14] Dennis, J.W., Laferté, S., Waghorne, C., Breitman, M.L. and Kerbel, R.S. (1987) Science 236, 582–585.
[15] Dennis, J.W., Kosh, K., Bryce, D.-M. and Breitman, M.L. (1989) Oncogene 4, 853–860.
[16] Sadeh, S., Warren, C.D., Daniel, P.F., Bugge, B., James, L.F. and Jeanloz, R.W. (1983) FEBS Lett. 163, 104–109.
[17] Koenderman, A.H.L., Koppen, P.L., Koeleman, C.A.M. and Van den Eijnden, D.H. (1989) Eur. J. Biochem. 181, 651–655.
[18] Geldof, A.A. and Rao, B.R. (1990) Prostate 16, 279–290.
[19] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[20] Harpaz, N. and Schachter, H. (1980) J. Biol. Chem. 255, 4885–4893.
[21] Palcic, M.M., Heerze, L.D., Pierce, M. and Hindsgaul, O. (1988) Glycoconjugate J. 5, 49–63.

[22] Narasimhan, S. (1982) J. Biol. Chem. 257, 10235–10242.
[23] Gleeson, P.A. and Schachter, H. (1983) J. Biol. Chem. 258, 6162–6173.
[24] Cummings, R.D., Trowbridge, I.S. and Kornfeld, S. (1982) J. Biol. Chem. 257, 13421–13427.
[25] Nishikawa, A., Fujii, S., Sugiyama, T., Hayashi, N. and Taniguchi, N. (1988) Biochem. Biophys. Res. Commun. 152, 107–112.
[26] Ishibashi, K., Nishikawa, A., Hayashi, N., Kasahara, A., Sato, N., Fujii, S., Kamada, T. and Taniguchi, N. (1989) Clin. Chim. Acta 185, 325–332.
[27] Narasimhan, S., Schachter, H. and Rajalakshmi, S. (1988) J. Biol. Chem. 263, 1273–1281.
[28] Kobata, A. and Yamashita, K. (1984) Pure Appl. Chem. 56, 821–832.
[29] Yamashita, K., Hitoi, A., Tanaguchi, N., Yokosawa, N., Tsukada, Y. and Kobata, A. (1983) Cancer Res. 17, 1010–1027.
[30] Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230–11234.
[31] Yamashita, K., Tachibana, Y. and Kobata, A. (1978) J. Biol. Chem. 253, 3862–3869.
[32] Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Spik, G., Fournet, B. and Montreuil, J. (1979) Eur. J. Biochem. 100, 569–574.
[33] Yamashita, K., Kamerling, J.P. and Kobata, A. (1982) J. Biol. Chem. 257, 12809–12814.
[34] Fukuda, M., Dell, A. and Fukuda, M.N. (1984) J. Biol. Chem. 259, 4782–4791.
[35] Yoshima, H., Furthmayr, H. and Kobata, A. (1980) J. Biol. Chem. 255, 9713–9718.
[36] Yamashita, K., Tachibana, Y., Shichi, H. and Kobata, A. (1983) J. Biochem. (Tokyo) 93, 135–147.
[37] Yamashita, K., Hitoi, A., Matsuda, T., Tsuji, A., Katunuma, N. and Kobata, A. (1983) J. Biol. Chem. 258, 1098–1107.
[38] Yamashita, K., Hitoi, A., Matsuda, Y., Miura, T., Katunuma, N. and Kobata, A. (1986) J. Biochem. (Tokyo) 99, 55–62.
[39] Abel, P.D., Foster, C.S., Tebbutt, S. and Williams, G. (1990) J. Urol. 144, 760–765.
[40] Bashir, I., Sikora, K. and Foster, C.S. (1990) Biochem. Soc. Trans. 18, 968–969.
[41] Palmeiro, E., Sikora, K. and Foster, C.S. (1990) Biochem. Soc. Trans. 18, 1285–1287.
[42] Flier, J.S., Underhill, L.H. and Zetter, B.R. (1990) N. Engl. J. Med. 322, 605–612.
[43] Springer, T.A. and Lasky, L.A. (1991) Nature 349, 196–197.