Expression of core 2 β1,6-N-acetylgalcosaminyltransferase facilitates prostate cancer progression

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Cell surface carbohydrates expressed on epithelial cells are thought to play an important role in tumor progression. Previously, we have shown that expression of core 2 branched O-glycans is closely correlated with vessel invasion and depth of invasion in colon and lung carcinomas. In this study, we found that expression of core 2 β1,6-N-acetylgalcosaminyltransferase-I, Core2GnT, is positively correlated with the progression of prostate cancer in human patients. Statistical analysis demonstrated that Core2GnT is an independent predictor for progressed pathological stage (pT3) and for prostate-specific antigen (PSA) relapse. To determine directly the roles of Core2GnT in prostate cancer progression, we set up an experimental tumor model using the LNCaP prostate cancer cell line. Because this line does not express Core2GnT, we established an LNCaP line stably expressing Core2GnT, LNCap-Core2GnT, by transfecting cDNA encoding Core2GnT. When mock-transfected LNCaP cells and LNCaP-Core2GnT were inoculated in the prostate of nude mice, LNCaP-Core2GnT cells produced three times heavier prostate tumors than mock-transfected LNCaP cells. Furthermore, we found that LNCaP-Core2GnT cells adhered more strongly to prostate stromal cells, type IV collagen and laminin than did LNCaP-mock cells, but LNCaP and LNCaP-Core2GnT cells grew almost at the same rate on plates coated with type IV collagen or laminin. These results indicate that Core2GnT is an extremely useful prognostic marker for prostate cancer progression. The results also suggest that acquiring Core2GnT in prostate carcinoma cells facilitates adhesion to type IV collagen and laminin, and this increased adhesion may be a cause for aggressive tumor formation by prostate cancer cells expressing Core2GnT.

Key words: core 2 β1,6-N-acetylgalcosaminyltransferase/core 2-branched O-glycans/prostate cancer/prostate stromal cell/tumor metastasis

Introduction

In the United States, prostate cancer is the most common malignancy affecting men and the second leading cause of cancer death (American Cancer Society, 2003). The incidence of prostate cancer varies worldwide, with the highest rates found in the United States, Canada, and Scandinavia and the lowest rates in China and other parts of Asia (Quinn and Babb, 2002). However, increase in prostate cancer incidence has occurred in countries with relatively low incidence rates of prostate cancer (Gronberg, 2003).

Several treatments are currently available for early prostate cancer, such as watchful waiting, radical retropubic prostatectomy, laparoscopic prostatectomy, transperineal prostatectomy, extrabeam irradiation, and transperineal implantation of radioisotopes (brachytherapy). After radical prostatectomy, the disease recurs in an estimated 15–30% of patients, suggesting that undetected cancer cells may have spread beyond the prostate gland before surgery (Han et al., 2001; Roberts et al., 2001). Several clinical parameters including tumor stage, tumor grade as measured by the Gleason score (GS), and the serum level of prostate-specific antigen (PSA) are typically used to assess the risk of disease progression at the time of diagnosis (Partin et al., 1997). Kattan et al. (2001) also have developed useful nomograms to help evaluate the likelihood of disease-free survival after radical prostatectomy or brachytherapy for localized prostate cancer. However, these and other models have limitations as demonstrated by their good, but not excellent, association with outcome, as reviewed by Ross et al. (2001). It is thus imperative to develop a novel biomarker to accurately assess the risk of disease progression in patients with clinically localized prostate cancer so that appropriate treatment can be selected.

Neoplastic transformation of epithelial cells is accompanied by alterations in expression of cell surface carbohydrates (Ohyama et al., 1995; Fukuda, 1996; Watanabe et al., 2002). For example, the expression of sialyl Lewis A or sialyl Lewis X on the cell surface of colorectal cancer is positively correlated with poor patient outcome (Nakamori et al., 1993). In mucin-type glycoproteins, sialyl Lewis X and sialyl Lewis A are often present as a capping structure on core 2-branched O-glycans, and these structures have been shown to serve as selectin ligands (Lowe, 1994). Following the formation of a core 2-branched, sequential addition of sialic acid and α1,3-linked or α1,4-linked fucosidase results in formation of sialyl Lewis X or sialyl Lewis A (Lowe, 1994). It was also reported that a soluble form of N-acetylgalcosaminyltransferase-V (GnT-V) might facilitate angiogenesis (Saito et al., 2002). By contrast, forced expression of
core 3 O-glycans, which apparently down-regulates core 1 O-glycans, reduced tumor formation by human colonic carcinoma cells (Iwai et al., 2005). Core 2 β1,6-N-acetylgalactosaminyltransferase-I (Core2GnT-I) (Bierhuizen and Fukuda, 1992; Schachter and Brockhausen, 1992) is a key enzyme forming core 2-branched O-glycans (Galβ1→3GlcNAcβ1→6GalNAcα→Serine/Threonine) by catalyzing the transfer of N-acetylgalactosamine (GlcNAc) to core 1 O-glycan (Galβ1→3GalNAc→Serine/Threonine). It was reported earlier that increases in Core2GnT activity are seen in human leukemia cells and metastatic murine tumor cell lines (Brockhausen et al., 1991; Saitoh et al., 1991; Yousefi et al., 1991). Similarly, the expression of sialyl Lewis A on core 2-branched O-glycans, detected by CA19-9 antibody, is associated with the progression of colorectal carcinoma (Shimono et al., 1994).

We have cloned a cDNA encoding Core2GnT from human promyelocytic leukemia HL-60 cells by expression cloning (Bierhuizen and Fukuda, 1992) and raised antibodies against Core2GnT (Skrincosky et al., 1997). We showed that expression of core 2-branched O-glycans is closely correlated with the malignant potential of colorectal cancer and pulmonary adenocarcinoma by analyzing the expression of Core2GnT mRNA (Shimodaira et al., 1997; Machida et al., 2001). Notably, we found that the expression of Core2GnT gene is better correlated with the progression of tumors in both colon and lung cancer than expression of sialyl Lewis X or sialyl Lewis A itself (Shimodaira et al., 1997; Machida et al., 2001). These observations suggest that expression of Core2GnT and resultant core 2-branched O-glycans is highly correlated with the progression of various tumors. However, there have been no attempts to elucidate the clinicopathological significance of Core2GnT status in prostate cancer.

It has been reported that overexpression of fibroblast growth factor 8-b in human prostate cancer cell line, LNCaP cells, allowed them to evade the growth inhibitory effect of stromal cells (Song et al., 2000). It has also been shown that prostate cancer cells grew better after forming capillary-like stroma when cocultured in collagen gels and endothelial cells in fibrin (Janvier et al., 1997). Similarly, coculturing of prostate cancer cells with stromal cells facilitates angiogenesis surrounding prostate cancer cells and leads to increased size of prostate tumors (Tuxhorn et al., 2002), whereas the invasion of DU145 prostate cancer cell line was facilitated by stromal-derived hepatocyte growth factor (Nishimura et al., 1999). These results indicate the importance of prostate stromal cells in contact with prostate cancer cells in xenograft tumor formation. However, no studies have been undertaken to elucidate the roles of core 2-branched O-glycans in xenograft tumor formation by prostate cancer cells.

Here, we immunohistochemically examined Core2GnT status in prostate needle biopsy specimens using the anti-Core2GnT antibodies previously prepared (Skrincosky et al., 1997). We found that expression of Core2GnT in preoperative prostate tissue could be a highly useful predictor of the pathological stage. Moreover, we demonstrated that LNCaP prostate cancer cells form larger tumors in nude mice after LNCaP cells were transfected with a Core2GnT expression vector, and those transfected cells adhere more efficiently to prostate stromal cells than mock-transfected LNCaP cells. These results as a whole indicate that Core2GnT and core 2-branched O-glycans synthesized play a critical role in prostate cancer progression, most likely through increased interaction with prostate stromal cells.

**Results**

**Expression of Core2GnT is positively correlated with progression of prostate cancer**

To evaluate the role of Core2GnT in prostate cancer progression, we utilized antibodies specifically raised against Core2GnT protein (Skrincosky et al., 1997). Because the specimens examined in this study were embedded in paraffin, immunohistochemical detection of Core2GnT was more efficient than detection of either Core2GnT transcripts by in situ hybridization or core 2-branched O-glycans. The detection of core 2-branched O-glycans by specific antibodies is severely limited because available antibodies can detect core 2 O-glycans only when they are capped with sialyl Lewis X (Berg et al., 1991; Kumamoto et al., 1998; Kobayashi et al., 2004) or core 2-branched O-glycans attached to CD43 (leukosialin) (Piller et al., 1991). The results demonstrate that normal prostate gland barely express Core2GnT, whereas prostate cancer cells express significant levels of Core2GnT as detected by rabbit anti-Core2GnT antibodies (Figure 1A and B). Using these criteria, we then examined specimens from 69 patients exhibiting different clinical parameters. First, we found that Core2GnT expression in biopsy specimens was positively correlated with serum PSA level (Table I). Prostate cancer with a low Gleason sum (five and six) and a high Gleason sum (eight and nine) express Core2GnT in 11 and 51% of the patients, respectively. We found that 4% of Core2GnT-negative patients are at pT3 disease, whereas 53% of core 2-positive patients are with pT3 disease (Table I).

The most important clinical implication of preoperative parameter is whether it can predict PSA relapse, thus the necessity of secondary treatment. As shown in Figure 2, Core2GnT-positive patients had significantly higher risk for PSA relapse after prostatectomy. This result indicates that expression of Core2GnT alone or in combination with PSA is an excellent predictor of progression of prostate cancer.

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**Fig. 1.** Immunohistochemistry of human prostate cancer biopsy specimens. Prostate biopsy specimens were stained with anti-core 2 β1,6-N-acetylgalactosaminyltransferase-I (anti-Core2GnT) antibodies followed by horse-radish peroxidase-conjugated secondary antibodies. A normal specimen (A) and a specimen with a Gleason sum of eight (B) were examined. Counterstaining was performed using hematoxylin. Core2GnT-positive cancer cells are stained in red.
To determine the role of Core2GnT in prostate cancer progression, we stably transfected the LNCaP prostate cancer cell line with cDNA encoding Core2GnT. As shown in Figure 3A, the parent LNCaP cells do not express Core2GnT. After the transfection of Core2GnT cDNA, five clones of transfected cells expressing Core2GnT as assessed by staining with anti-Core2GnT antibodies (Figure 3A) were identified, and one was designated LNCaP-Core2GnT. Representative results using this clone are presented hereafter. The transcript (Figure 3B) and the enzymatic activity (Figure 3C) of Core2GnT were detected in LNCaP-Core2GnT but not in the mock-transfected LNCaP cells. The parent LNCaP cells were negative for HECA-452 even after transfection of Core2GnT, indicating the absence of sialyl Lewis X and sialyl Lewis A (Figure 3D).

**Table 1. Core 2 β1,6-N-acetylgalcosaminyltransferase-1 (Core2 GnT) status and clinicopathological parameters**

<table>
<thead>
<tr>
<th>Status</th>
<th>Core2GnT negative</th>
<th>Core2GnT positive</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>24</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Age (years old)</td>
<td>64.5 ± 6.6</td>
<td>68.2 ± 4.8</td>
<td>0.0102</td>
</tr>
<tr>
<td>Prostate-specific antigen (ng/mL)</td>
<td>7.4 ± 3.7</td>
<td>11.9 ± 8.9</td>
<td>0.0196</td>
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<tr>
<td>Biopsy Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>13%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>=7</td>
<td>9%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td>≥8</td>
<td>2%</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>Final pathological stage (%)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pT2</td>
<td>23 (96%)</td>
<td>21 (47%)</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>1 (4%)</td>
<td>24 (53%)</td>
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</table>

Fig. 2. Expression profile of sialyl Lewis X and core 2 β1,6-N-acetylgalcosaminyltransferase-1 (Core2GnT) on LNCaP cells before and after forced expression of Core2GnT. (A) LNCaP-mock and LNCaP-Core2GnT cells were stained with rabbit anti-Core2GnT antibodies followed by horseradish peroxidase-conjugated secondary antibody. The magnification is the same as that in Figure 1. (B) RT–PCR was performed on total RNA isolated from LNCaP-mock and LNCaP-core 2 β1,6-N-acetylgalcosaminyltransferase-1 (LNCaP-Core2GnT) cells. RT–PCR for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) is shown for positive control. Chinese hamster ovary (CHO) cells and CHO cells-expressing Core2GnT served as a negative and positive control for Core2GnT, respectively. (C) Core2GnT activity was assayed for LNCaP-mock and LNCaP-Core2GnT cells. (D) LNCaP-mock and LNCaP-Core2GnT cells were negative for sialyl Lewis X and sialyl Lewis A as assessed by FACS and HECA-452 antibody (Berg et al., 1991). CHO-PSGL-1/F7/C2 cells were used as a positive control for sialyl Lewis X expression.

Expression of Core2GnT cDNA into the LNCaP prostate cancer cell line

To determine the role of Core2GnT in prostate cancer progression, we stably transfected the LNCaP prostate cancer cell line with cDNA encoding Core2GnT. As shown in Figure 3A, the parent LNCaP cells do not express Core2GnT. After the transfection of Core2GnT cDNA, five clones of transfected cells expressing Core2GnT as assessed by staining with anti-Core2GnT antibodies (Figure 3A) were identified, and one was designated LNCaP-Core2GnT. Representative results using this clone are presented hereafter. The transcript (Figure 3B) and the enzymatic activity (Figure 3C) of Core2GnT were detected in LNCaP-Core2GnT but not in the mock-transfected LNCaP cells. The parent LNCaP cells were negative for HECA-452 even after transfection of Core2GnT, indicating the absence of sialyl Lewis X and sialyl Lewis A (Figure 3D).
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LNCaP-Core2GnT cells form larger tumors upon orthotopic inoculation into mouse prostate

To determine if expression of Core2GnT and core 2-branched O-glycans alters tumor formation in vivo, we inoculated LNCaP-Core2GnT and LNCaP-mock into the mouse prostate. Four weeks after inoculation, mice were killed, and the prostates were examined. The results shown in Figure 4A illustrate that LNCaP-Core2GnT produced much larger tumors than did LNCaP-mock cells, and the weight of prostates derived from LNCaP-Core2GnT inoculation was more than three times than that of prostate derived from mock-transfected LNCaP cells (Figure 4B). Almost identical results were obtained in a repeated experiment and the experiments using another LNCaP-Core2GnT cell line. These results indicate that expression of core 2-branched O-glycans, even in the absence of sialyl Lewis X or sialyl Lewis A on LNCaP cells, leads to significantly increased tumorigenicity.

Histological examination on tumor in nude mice

Histological examination revealed massive tumor formation of LNCaP-Core2GnT cells in the prostate of the nude mice (Figure 5A and C). The tumors formed by LNCaP-Core2GnT were positive for Core2GnT even several weeks after inoculation, whereas the mouse prostate without inoculation of LNCaP-Core2GnT cells was negative for Core2GnT (Figure 5B). Tumors formed by LNCaP-Core2GnT cells were characterized by a larger nuclei and a prominent nucleolus (Figure 5A and C). Papillary structures seen in the normal mouse prostate (Figure 5B) disappear in the tumors, further indicating the aggressiveness of these tumors.

Adhesion of LNCaP-Core2GnT cells to prostate stroma cells

We then determined whether LNCaP-Core2GnT cells adhere better to prostate stroma cells than LNCaP-mock cells. Figure 6A and B illustrates that more LNCaP-Core2GnT cells bound to stromal cells than LNCaP-mock cells with statistical significance. We then tested the adhesion of LNCaP-Core2GnT and LNCaP-mock cells to various molecules present in the extracellular matrix, which were coated on plates. As shown in Figure 6C, LNCaP-Core2GnT cells adhered more efficiently to type IV collagen and laminin than did LNCaP-mock cells. Statistical differences between two cell types were more significant for adhesion to type IV collagen and laminin than for the others. These results combined indicate that LNCaP cells expressing core 2-branched O-glycans adhere more efficiently to prostate stroma cells than do LNCaP cells lacking core 2-branched O-glycans, most likely because of the increased adhesion to type IV collagen and laminin.

We next measured the cell growth after the cells were attached to collagen IV or laminin. The initial number of cells added was adjusted so that the numbers of initially attached cells were almost the same between LNCaP-Core2GnT and LNCaP cells. The results show that the adhesion to type IV collagen or laminin did not increase the cell growth compared with the adhesion to control plates, and that there is essentially no difference in growth of LNCaP and LNCaP-Core2GnT cells (Figure 6D).

Discussion

This study demonstrates that expression of Core2GnT on human prostate cancer cells correlates positively with the aggressive potential of prostate cancer. First, expression of Core2GnT in preoperative biopsy specimens is highly correlated with advanced stages of prostate cancer and is more prevalent in patients with higher GS. Second, the expression of Core2GnT in biopsy specimen predicts advanced disease. Although all of the clinical cases examined were organ confined, the final pathological stage was pT3 for 53% of Core2GnT-positive patients, in contrast with only 4% pT3 for Core2GnT-negative patients (Table 1). Moreover, Core2GnT-positive patients exhibited a much less promising prognosis than Core2GnT-negative patients, suggesting that expression of Core2GnT is an independent predictor for recurrence of prostate cancer (Figure 2). These results indicate that expression of Core2GnT is highly correlated with tumor progression.

In this study, we also demonstrated that forced expression of Core2GnT in LNCaP prostate cancer cells led to significantly larger tumors upon inoculation to the mouse prostate. Significantly, this increase in tumor formation was achieved without expression of sialyl Lewis X or sialyl Lewis A capping structures. These results indicate that the
expression of core 2-branched O-glycans alone led to the increased tumor formation. Previously, it was shown in colon and lung carcinoma patients that expression of Core2GnT has a higher correlation with tumor progression than expression of sialyl Lewis X (Shimodaira et al., 1997; Machida et al., 2001). However, in those studies, expression of sialyl Lewis X was almost always associated with tumors, and the effect of core 2-branched oligosaccharides on tumor progression could not be separated from the effect of sialyl Lewis X capping structures on core 2-branched oligosaccharides. In this study employing LNCaP cells that lack α1,3/4-fucosyltransferase, the role of core 2-branched oligosaccharides was revealed in the absence of sialyl Lewis X and sialyl Lewis A.

Previously, it was suggested that core 2-branched O-glycans without sialyl Lewis X or sialyl Lewis A capping...
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structures can be recognized by carbohydrate-binding proteins distinct from selectins, such as galectins (Leffler and Barondes, 1986; Bourne et al., 1994; Stowell et al., 2004). Similarly, attachment of colonic carcinoma cells to liver sections was found to be dependent on core 2-branched O-glycans, but not on sialyl Lewis X (Ota et al., 2000). It has been reported also that N-acetyllactosamine–galectin interaction may be impaired in N-acetylgalactosaminyltransferase-V (GnT-V)-deficient mice and that such impairment causes a hyperimmune response in GnT-V knockout mice (Demetriou et al., 2001). On the other hand, overexpression of core 2-branched O-glycans on T-lymphocytes leads to apoptosis, probably through binding to carbohydrate-binding proteins on opposing cells (Perillo et al., 1995; Galvan et al., 2000; Priatel et al., 2000).

Based on the findings described above, it is possible to hypothesize that core 2-branched oligosaccharides on prostate epithelial cells are recognized by carbohydrate-binding proteins on stromal cells, and such binding may stimulate adhesion to stromal cells and thus tumor growth. Because galectins are shown to bind core 2-branched O-glycans, one of these carbohydrates-binding proteins is likely galectin. Indeed, the expression of galectins on the cell surface has been implicated in tumor metastasis (Raz and Lotan, 1987). As an alternative but not necessarily mutually exclusive possibility, proteins carrying core 2-branched oligosaccharides on prostate cancer cells may stimulate growth of prostate cancer cells after they acquire core 2-branched oligosaccharides. In relation to this hypothesis, it has been shown that overexpression of Core2GnT in PC12 cells results in increased phosphorylation of mitogen-activated protein kinase and c-fos promoter activation (Koya et al., 1999). A recent study showed that CD43, a major glycoprotein carrying mucin-type O-glycans, regulates interleukin-2 production through its cytoplasmic tail, suggesting that intracellular signaling is a critical factor in the activity of CD43 (Tong et al., 2004). Further studies are necessary to determine if any of these mechanisms operate in stimulating tumor formation after acquisition of Core2GnT by prostate cancer cells.

The present studies demonstrated that LNCaP cells expressing core 2-branched O-glycans adhere more efficiently to type IV collagen and laminin. However, cell growth of LNCaP cells on plates coated with collagen IV or laminin was not increased over control plates. Previously, it has been reported that the expression of the transcripts for type IV collagen α1 chain, laminin β1 chain, and S-laminin are increased in metastatic prostate cancer, compared with benign prostate glands (Pfohler et al., 1998). It has also been reported that laminin-1 and α6β1 integrin were shown to facilitate normal morphogenesis of the prostate gland, acinar formation, whereas type IV collagen rather inhibits the normal morphogenesis (Bello-DeOcampo et al., 2001). Because type IV collagen and laminin are components of basement membrane, they are likely deposited between prostate epithelial cells and stromal cells. These findings, together with the findings in this study, indicate that the adhesion to type IV collagen works more dominantly than the adhesion to laminin, preventing normal morphogenesis by prostate cancer cells. It is tempting to speculate that such effect may render those cancer cells undifferentiated, thus allowing them to be more neoplastic in nature.

Materials and methods

Patients

Between January 1994 and May 1999, 93 patients underwent staging pelvic lymphadenectomy and radical prostatectomy for clinically localized prostate cancer by a single surgeon (Y.A.) at Kurashiki Central Hospital, Kurashiki, Japan. Of these, 22 patients who had T3 disease or received neoadjuvant hormone therapy were excluded from the study, and two patients were excluded due to scarcity of biopsy specimens; the study sample thus consisted of 69 patients with clinically organ-confined (T1/T2) disease. The surgery was performed via the anatomical approach originally described by Walsh, (1992). No patients received adjuvant endocrine therapy until postoperative PSA failure. The ethical committee of Kurashiki Central Hospital approved the protocols for this study.

Preoperatively, all patients were evaluated by digital rectal examination, serum PSA, bone scan, pelvic computed tomography scan, and transrectal ultrasonography. Six to twelve prostate needle biopsy samples were obtained via ultrasound guidance by using an 18 G needle and evaluated for the presence of cancer lesion.

Pathologic diagnosis and Gleason scoring of prostate cancer

The 1997 American Joint Committee on Cancer (AJCC) staging system (Flemming et al., 1997) was used to assign the stage, and the Gleason grading system (Gleason, 1966) was used for tumor grading. According to the latter grading system, the progression of prostate cancer was divided into five stages (fifth is the most advanced stage), and the score of the most frequently observed cancer cells and that of the second most frequently observed cells are obtained, for example, 4 + 3. The Gleason scoring of each specimen was made by a single pathologist (M.E.) who was unaware of the clinical data. Staining for Core2GnT was also compared with pT (representing the stage of pathological primary lesion); pT2 is defined as the primary lesion confined to inside the prostate, whereas pT3 corresponds to the primary lesion breaking through the capsule to infiltrate outside the prostate (Inaba et al., 2003).

Measurement of PSA and follow-up of patients

Serum PSA levels were determined with IMx (Abbott Laboratories, Abbott Park, IL). Postoperative PSA values were considered elevated (PSA failure) if values of 0.1 ng/mL or greater were obtained on two consecutive visits 1 month apart. Time zero was defined as the day of surgery. Patients whose PSA level never became undetectable (<0.1 ng/mL) postoperatively were scored as failures at time zero.

Immunohistochemistry

Biopsy specimens were fixed with 10% buffered formalin for 1 h and embedded in paraffin. The samples were cut at 3 µm thickness and subjected to immunohistochemistry.
Deparaffinized specimens were incubated with rabbit anti-human Core2GnT polyclonal antibody (Skrincosky et al., 1997) followed by goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Nichirei, Tokyo, Japan). A control experiment was done by omitting the primary antibody from the staining procedure. The results of immunostaining were evaluated by persons unaware of the clinical data. Based on staining status of Golgi apparatus, specimens possessing 10% and more positive glands were judged Core2GnT positive.

Statistical analysis
The Chi-square test was used to assess the association of Core2GnT status with clinical and pathological parameters. PSA-free survival was evaluated by Kaplan-Meier curves. Differences between groups were evaluated using the log-rank test.

Stable transfectants
The human prostate cancer cell line LNCaP, purchased from the American Type Culture Collection (Rockville, MD), was maintained in RPMI 1640 medium containing 10% fetal calf serum. The cells were transfected with pcDNA3-Core2GnT using LipofectAMINE (Invitrogen, Carlsbad, CA), as described previously (Mitoma et al., 2003). After G418 selection (200 µg/mL; Invitrogen), 20 single colonies were examined for immunocytochemical detection of Core2GnT using anti-Core2GnT antibody, and five stable transfectants expressing Core2GnT were isolated (LNCaP-Core2GnT). Among the stable transfectants, two clones (LNCaP-Core2GnT-1 and -2) were used in tumor assays. Because LNCaP-Core2GnT-1 and -2 yielded identical results in the following experiments, we designated LNCaP-Core2GnT-1 cells as LNCaP-Core2GnT cells, and the results obtained by LNCaP-Core2GnT cells were shown. As a control, LNCaP cells were transfected with pcDNA3 empty vector, and the transfected cells were designated LNCaP mock.

RT-PCR of Core2GlcNAcT-I
Chinese hamster ovary (CHO) cells were transfected with mammalian expression vectors encoding P-selectin glycoprotein ligand-1 (PSGL-1), fucosyltransferase-VII (FucT-VII) and Core2GnT establishing CHO-PSGL-1/F7/C2 and CHO-PSGL-1/F7 cells, respectively (Mitoma et al., 2003; Kobayashi et al., 2004). Total RNA was prepared using the TRIZol reagent (Invitrogen). Reverse transcriptase-polymerase chain reaction (RT-PCR) of glyceraldehyde-3-phosphate dehydrogenase and Core2GnT was performed, as described previously (Mitoma et al., 2003; Kobayashi et al., 2004). HECA-452 antibody was used to detect both sialyl Lewis X and sialyl Lewis A antigen (Berg et al., 1991; Kobayashi et al., 2004).

In vitro cell proliferation
LNCaP-mock and LNCaP-Core2GnT cells were seeded onto 96-well plates at 10^5 cells/mL in RPMI 1640 containing 10% fetal calf serum and 200 µg/mL of G418 and cultured for various times. The number of living cells was measured every other day using the Cell Counting Kit (Wako Pure Chemical Industries, Tokyo, Japan). The cells (2 × 10^4 cells) were also seeded on 24-well plates coated with 10 µg of type IV collagen or laminin (Sigma, St. Louis, MO), and cell proliferation was measured as described (Mitoma et al., 1998). Triplicate cultures were used for each sample.

Orthotopic tumor cell inoculation
Balb/c nude (nu/nu) mice (6- to 8-week-old males obtained from Clea Japan [Tokyo, Japan]) were used for orthotopic tumor cell injection after anesthetizing with pentobarbital. About 2 × 10^6 cells exhibiting >90% viability as judged by trypan blue staining were suspended in 20 µL of serum-free RPMI 1640 medium and inoculated into the posterior lobe of the prostate with pentobarbital, as described previously (Inaba et al., 2003). After tumor cell inoculation, the wound was closed with surgical clips. Four weeks after inoculation, mice were killed, and prostates were removed followed by fixation with buffered formalin solution.

Adhesion assay
A prostate stromal cell line (Nishimura et al., 1999) was cultured to 50% confluence in 24-well culture plates, as described previously (Krill et al., 1997; Nishimura et al., 1999). LNCaP-mock and LNCaP-Core2GnT cells were washed three times with phosphate buffered saline (PBS), harvested, resuspended in RPMI 1640 medium containing 1% fetal calf serum, and added on prostate stromal cells at the density of 10^5 cells/mL. The cells were incubated for 10 min at room temperature with continuous rotation at 1 × g. After washing with cold PBS, bound cells were counted under a light microscope obtaining mean cell numbers in 10 different fields. Alternatively, plates were precoated with various molecules present in the basement membrane (collagen I, collagen IV, fibronectin, and laminin, purchased from Sigma), which were dissolved at 100 µg/mL of PBS. After incubation for 30 min at room temperature and washing with PBS, the wells were then treated with 0.1% bovine serum albumin solution in PBS to prevent nonspecific adhesion, before addition of LNCaP cells.

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Abbreviations
Core2GnT, core 2 β1,6-N-acetylgalactosaminyltransferase-1; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; GlcNAc, N-acetylgalactosamine; PBS, phosphate buffered saline; PSA, prostate-specific antigen; PSGL-1, P-selectin glycoprotein ligand-1; RT-PCR, reverse transcriptase-polymerase chain reaction.

References


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