Inhibition of cancer cell growth by anti-Tn monoclonal antibody MLS128

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Summary

Tn-antigens are generally masked by covalently linked carbohydrates but are exposed in most primary and metastatic epithelial malignant tumors, providing sensitive markers for detection of carcinoma. Here, therapeutic potentials of tumor-associated carbohydrate antigen-specific antibodies were investigated. MLS128, an anti-Tn monoclonal antibody, binds to a carbohydrate epitope consisting of three consecutive Tn-antigens (GalNAcα-Ser/Thr). MLS128 treatment significantly inhibited colon and breast cancer cell growth. MLS128 bound to 110-210 kDa glycoproteins on the cell surface. MLS128 treatment caused down-regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor in LS180 colon cancer cells, suggesting that MLS128-inhibited cancer cell growth is in part mediated by down-regulation of growth factor receptors. This study provides the first insights into the potential use of this particular type of anti-Tn antigen antibodies as a cancer therapeutic.

Keywords: IGF-I receptor down-regulation, Growth inhibition, Colon cancer, Breast cancer, Tn-antigen motif, Antibody therapeutics

1. Introduction

Oncogenic transformation is often associated with dysregulation of glycosylation processes that then leads to altered patterns of carbohydrate functionalization on the surface of cancer cells. Some of these tumor-associated carbohydrate antigens are involved in metastatic processes and are associated with a poor prognosis, thus representing excellent targets for cancer intervention. Tn-antigen (GalNAcα-Ser/Thr) and T-antigen (Galβ1-3GalNAcα-Ser/Thr) are antigens associated with carcinomas and are generally masked by covalently linked terminal carbohydrate moieties in normal human tissues but are exposed in most primary and metastatic epithelial malignant tumors. These epitopes are readily detectable in about 90% of carcinomas and in their metastases, thus providing sensitive and specific markers for pre-clinical detection of carcinoma and for treatment monitoring during and after cancer therapies (1-4).

The present study investigated the therapeutic potential of anti-Tn-antigen antibodies using MLS128 that was derived from a mouse immunized with LS180 human colon carcinoma cells (5). MLS128 is IgG3 that recognizes the structure of three consecutive Tn-antigens (Tn3) (6,7). This manuscript is the first to describe the inhibition of colon and breast cancer cell growth by MLS128. MLS128 bound to 110-210 kDa glycoproteins on the cell surface. The antibody treatment of LS180 cells caused down-regulation of insulin-like growth factor-I receptor (IGFIR) and...
epidermal growth factor receptor (EGFR), both of which play a critical role in cell growth (8,9). MLS128-inhibited cancer cell growth is thus at least in part mediated by growth factor receptor down-regulation. This study provides the basis for the potential development of anti-Tn antigen antibodies as cancer therapeutics.

2. Materials and Methods

2.1. Materials

Production and characterization of MLS128 were as previously described (5,6). Cell lines used were human colon adenocarcinoma LS180 and HT-29 cells, human breast carcinoma MCF-7 and MDA-MB-231 cells, and human normal mammary epithelial MCF-10A cells, which were obtained from American Tissue Type Culture Collection. Rabbit anti-IGFIRβ antibody and anti-EGFR monoclonal antibody (mAb) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-rabbit or -mouse secondary antibody labeled with biotin was from Kirkegaard & Perry Lab. (Gaithersburg, MD, USA). Murine IgG kappa from murine myeloma was purchased from Sigma (St. Louis, MO, USA). Protein G-Sepharose was from Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-insulin receptor (IR) polyclonal antibody (10) was used for detection of both α and β subunits of IGFIR because of its cross-reactivity to IGFIR due to sequence homology (11). Human placental IGFIR was purified as previously described (12). Purified human placental IGFIR was obtained from Beckman Research Institute of the City of Hope, CA, USA.

2.2. Cell culture

LS180 cells were cultured in MEM containing 10% fetal calf serum (FBS). HT-29 cells were cultured in McCoy’s 5A (Invitrogen, Carlsbad, CA, USA) containing 10% FBS. MCF-7 cells were cultured in IMEM (GIBCO, Gland Island, NY) containing 5% FBS and 11.25 nM insulin. MDA-MB-231 cells were cultured in DME/F-12 (GIBCO, Gland Island, NY) supplemented with 5% FBS and 11.25 nM insulin. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 4.5 mg/mL d-glucose, and 110 μg/mL pyruvic acid. MCF-10A cells were cultured in DEME/F-12 (GIBCO, Gland Island, NY) supplemented with 5% FBS, 10 μg/L insulin, 20 μg/mL EGF (St. Louis, MO, USA), and 0.5 μg/mL hydrocortisone. All culture media included 50 units/mL of penicillin-streptomycin and 0.2% Fungizone.

2.3. Cancer cell growth

Effects of MLS128 treatment on colon and breast cancer cell growth were determined by counting cells. Cells (~10^4) were plated in wells of a 96-well plate and cultured in respective medium containing 10% or 5% FBS for 24 h. Cells were then cultured in the medium containing 1% FBS with PBS, 160 nM (25 μg/mL) of MLS128 or control mAb, unless otherwise stated, for 4 days. After 24, 48, 72, and 96 h of culturing, cells were collected from each well by treatment with Trypsin-EDTA (Sigma) for 15 min followed by centrifugation at 190 × g for 10 min. Cell pellets were suspended in 0.04% Trypan blue (Sigma). Live cells were counted using a hemocytometer. Triplicate wells were prepared for each data point.

2.4. Binding of MLS128 to colon and breast cancer cells

Binding of MLS128 to LS180, HT-29, and MCF-7 cells was measured with the fluorescence activated cell sorting (FACS) Calibur HG (Becton Dickinson, Franklin Lakes, NJ, USA). Cell suspensions (2 × 10^5 per tube) were prepared, washed twice with ice-cold PBS containing 1% FBS, and incubated with 200 μL of the antibody solution (5 μg/mL or 25 μg/mL in PBS) for 30 min at 4°C. After cells were washed twice with the same buffer, they were incubated with 200 μL of goat anti-mouse IgG (H+L)-RPE (Southern Biotechnology, Birmingham, AL, USA) for 30 min at 4°C. After cells were washed twice with PBS, they were suspended in 400 μL of PBS for FACS analyses.

2.5. Western blotting analyses of soluble plasma membranes

Cells (~10^4) collected by centrifugation and washed twice with ice-cold PBS were suspended in 750 μL of 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and Protease Inhibitor Cocktail (Sigma). Live cells were counted using a hemocytometer. Triplicate wells were prepared for each data point. Supernatants of cell homogenates after centrifugation at 200 × g for 10 min were subjected to centrifugation at 12,000 × g for 30 min. Pellets were solubilized in 100 μL of Buffer A containing 1% NP-40 by rotation for 1 h at 4°C. Soluble membrane fractions were recovered by centrifugation at 12,000 × g for 10 min.

Solubilized membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 6.5% gel and transferred to Immobilon-P transfer membranes (Millipore Co., Bedford, MA, USA). The membrane was blocked with 5% skim milk in TBS for 1 h at room temperature. After incubation with MLS128 (6.25 μg/mL) for 2 h, bound MLS128 was detected using biotin-labeled secondary antibodies, the Vectastain ABC-Amp® kit, and the Vector substrate kit (Vector Lab., Inc., Burlingame, CA, USA) as previously described (13).

2.6. Lysis and immunoblotting of MLS128-treated LS180 cells

Cells were washed three times with ice-cold PBS and lysed in 50 μL of TNESV lysis buffer (50 mM Tris-
HCl, pH 7.4, 1% NP40; 2 mM EDTA; 100 mM NaCl, 10 mM sodium orthovanadate, 1 mM PMSF, 20 μg/mL leupeptin, and 20 μg/mL aprotinin) per 3.5-cm dish. Lysates were clarified by centrifugation at 12,000 × g for 20 min at 4°C. Soluble cellular proteins were subjected to SDS-PAGE followed by Western blotting with anti-IGFIR or -EGFR antibodies.

3. Results

3.1. Effects of MLS128 on cancer cell growth

The growth of LS180 colon cancer cells incubated with 25 μg/mL of MLS128 for 24-96 h was inhibited while LS180 cells treated with 25 μg/mL of a control IgG3 or PBS steadily grew (Figure 1A). To examine whether or not such growth inhibition can be seen with cancer cells other than LS180, time-course experiments similar to Figure 1A were carried out. As is apparent from Figures 1B and C, the growth of HT-29 colon cancer cells and MCF-7 breast cancer cells, respectively, was also significantly inhibited. Thus, the effect MLS128 had on LS180 cells should also take place in other cancer cell lines bearing MLS128-reactive cell surface glycoproteins.

3.2. Binding of MLS128 with a variety of cancer cell lines

The binding of MLS128 to cell surface antigens of LS180 cells as well as HT-29 and MCF-7 cells was confirmed by FACS as shown in Figures 2A, B, and C, respectively. Western blotting analyses of plasma membranes prepared from various cancer cell lines revealed that MLS128 recognized proteins with ~110 kDa in various human carcinoma cell lines including LS180 and HT-29 colon cancer cells (Figure 2D, lanes 1 and 2, respectively) as well as PC-3 and Hep G2 cells (data not shown), whereas 150-200 kDa protein bands were detected in MCF-7 and MDA-MB-231 breast cancer cells as well as HT-29 cells (Figure 2D, lanes 3, 5, and 2, respectively). In contrast, bands reactive to MLS128 were not detected in a normal breast epithelial cell line, MCF-10A (Figure 2D, lane 4). The 125, 75, and 73 kDa proteins detected are endogenous biotin-containing enzymes that were unavoidably stained by the immunoblotting procedure used (14).

3.3. Down-regulation of receptors for IGF-I and EGF by MLS128 in LS180 cells

Next investigated was whether or not the expression levels of growth factor receptors such as IGFIR or
EGFR changed after MLS128 treatment of LS180 cells. IGFIR was specifically examined since a number of previous studies found that IGFIR plays a critical role in cell growth and apoptosis (15) and since the current authors have shown that anti-IGFIR antibodies inhibit breast cancer growth by down-regulation of IGFIR (16-18). Cell lysates were prepared from LS180 cells treated with MLS128 or PBS for 24, 48, and 72 h, and subjected to Western blotting with antibodies for growth factor receptors. The receptor amounts were quantitated by normalization with 73-75 kDa biotin-containing enzymes as internal controls (14), and this clearly showed that MLS128 caused down-regulation of both IGFIR and EGFR (Figures 3A and B, respectively).

The 110 kDa glycoproteins (GP) that were identified as MLS128 binding sites on the LS180 cell surface appear to have sizes from those of β (95 kDa) to IGFIR α (130 kDa) subunits. To verify that the 110 kDa GP is not either of the IGFIR subunits, LS180 cell lysates and purified human placental IGFIR were applied to a SDS-PAGE gel side-by-side in triplicate and immunoblotted with MLS128, anti-IGFIR β subunit polyclonal antibody, or anti-IR polyclonal antibody that are IGFIR-cross-reactive due to > 50% amino acid sequence identity (11) (Figures 4A, B, and C, respectively). As shown in Figure 4B, the β subunit of IGFIR in LS180 cell lysates and the purified IGFIR was immunostained with the anti-IGFIR β subunit antibody that was used throughout this study. Both α and β subunits of the purified IGFIR were distinctly stained with the IGFIR-cross-reactive anti-IR polyclonal antibody (Figure 4C, lane 2) whereas those of the LS180 lysates were barely stained (Figure 4C, lane 1), indicating that although the α and β subunits of the purified IGFIR loaded onto the gel were much more abundant than those in LS180 lysates, neither α nor β subunits of the purified IGFIR bound to MLS128 (Figure 4A, lane 2), while the 110 kDa GP in LS180 cells was immunostained with MLS128. These results clearly indicate that the 110 kDa GP is not the IGFIR subunit and that human IGFIR does not contain MLS128-reactive Tn3 epitopes.

In summary, MLS128 binding to the 110 kDa GP on LS180 cell surface induces growth inhibition at least in part through IGFIR and EGFR down-regulation via an as yet unknown mechanism (Figure 5).

4. Discussion

The present study demonstrated that MLS128 inhibits the growth of not only LS180 cancer cells but also...
HT-29 colon and MCF-7 breast cancer cells. During the last two decades, T and Tn epitopes have been extensively studied as cancer-associated carbohydrate antigens, and mAbs to these antigens have been isolated (1-7). Immunization with different cancer cells expressing abundant glycoproteins with various Tn-epitopes clearly resulted in the production of Tn3-binding mAbs. Examples of those are, in fact, MLS128 and 83D4, which were isolated from hybridomas generated by immunizing mice with LS180 and MCF-7 cells, respectively (5-7).

Avichezer et al. previously reported immunoreactivity of mAbs against T and Tn-antigens with human carcinoma cells including ovarian and breast cancer cells (3). In their study, however, reduced cell viability of cancer cells was shown after 72 h of incubation at rather high concentrations (~100 μg/mL) of mAbs with unknown epitopes. In contrast, well-characterized MLS128 was chosen for the present study as a first step in evaluating the therapeutic potential of anti-Tn-antibodies. The epitope of MLS128 has been defined as Tn3 (6, 7). The unique specificity of MLS128 may provide an advantage over other anti-Tn antibodies with a broad range of specificities as reported (3) when used in anti-cancer applications. Evaluation of MLS128 binding to a variety of cancer cells should indicate the spectrum of potential applications for future MLS128-based cancer therapy.

MLS128 appears to bind to GPs with ~110 kD on the LS180 cell surface. Immunoreactive species of similar and higher molecular weights were observed with HT-29 and MCF-7 cells. Purification and identification of these glycoproteins is now underway in order to further investigate the mechanisms by which MLS128 inhibits the growth of LS180, HT-29, and MCF-7 cancer cells. Singh et al. reported that peanut agglutinin (PNA) bound to cell surface T-antigen expressing glycoproteins of ~110-180 kDa in HT-29 cells (19). Their ~110-180 kDa GPs purified with a PNA affinity-column were identified as splice variants of CD44 (CD44v). The MLS128-reactive bands in HT-29 cells detected on the Western blot (Figure 2D, lane 2) resembled those reported as CD44v. Binding of PNA to CD44v, however, stimulates proliferation of HT-29 cells, which is the opposite effect MLS128 has on HT-29 cell growth, thus suggesting that the target glycoproteins for MLS128 on the HT-29 cell surface are not likely to be CD44v. Further study is required to determine which glycoproteins specifically bind to MLS128 and to reveal cellular mechanisms that result in either growth stimulation or inhibition in HT-29 cells.

As far as the mechanisms of growth inhibition in LS180 cells are concerned, this study has provided evidence that both IGFIR and EGFR were down-regulated in LS180 cells after > 48 h of incubation with MLS128. The down-regulation of IGFIR is particularly interesting since IGFIR plays a critical role in cell growth, counteracting apoptosis and tumor transformation (9, 15). IGFIR down-regulation by anti-IGFIR antibodies has been extensively investigated, which suggests that it is the mechanism for IGFIR antibody-induced inhibition of breast and other cancer cell growth (17, 20). Unlike previous studies, however, MLS128 in this study caused down-regulation of IGFIR without directly binding to the receptor. Regarding the effect of MLS128 on MCF-7 cells, the IGFIR level did not change after 24-72 h of treatment (data not shown), suggesting that cell growth inhibition by MLS128 is not likely to be mediated by down-regulation of IGFIR in MCF-7 cells.

The present study suggests that MLS128 may be an excellent candidate for development of an anti-cancer therapeutic in addition to its already established diagnostic potential (21, 22). While this manuscript was in preparation, Ando et al. reported that mouse-human chimeric anti-Tn IgG1 induced anti-tumor activity against Jurkat cells (23). Murine anti-Tn mAb KM3413 were isolated by immunizing mice with mucins isolated from LS180 cell culture supernatants, which is a strategy similar to isolation of MLS128. KM3413 has been shown to have affinity for both Tn3- and Tn2-biotin but this affinity is 1/10th of that of MLS128. The presumption is that KM3413 binds to > 100 kDa GPs including CD43 highly expressed in Jurkat cells. Anti-tumor activity by chimeric antibody as was demonstrated in Jurkat-inoculated mice has been shown to be due to the ADCC activity of the human IgG1, Fc domain. Thus, the mechanism of anti-tumor action of this anti-Tn antibody clearly differs from that of the MLS128 used in the present study. Ongoing experiments by the current authors also include determination of the inhibitory effects of MLS128 in vivo, production of recombinant forms of MLS128, and expression of human single-chain antibodies with affinity for Tn3 (manuscripts in preparation) for further therapeutic development of MLS128-type antibodies.

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References


