Chapter 9
Plasma Membrane-Associated Sialidase Confers Cancer Initiation, Promotion and Progression

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Introduction

An increase in sialylation is often found in cell surface glycoproteins of malignant cells (Lau and Dennis 2008), and altered sialylation of glycolipids is also observed as a ubiquitous phenotype (Hakomori 2010). Despite the number of reports describing involvement of sialic acids in cancer, the molecular mechanism and significance are not fully understood. To understand further ganglioside neoplastic alterations, we have focused on a human ganglioside-specific sialidase NEU3, which cleaves sialic acids preferentially from gangliosides. Ganglioside sialidase activity levels fluctuate consistently with cell differentiation, cell growth, and malignant transformation. Alterations of the activity levels associated with malignant transformation were described in 3T3-transformed cells (Yogeeswaran and Hakomori 1975), in BHK-transformed cells (Schengrund et al. 1973) and in mouse epidermal JB6 cells exposed to phorbol esters (Miyagi et al. 1990). However, little was known about the

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molecular mechanisms underlying such sialidase alterations until development of gene cloning studies. Mammalian sialidases so far identified (NEU1–NEU4) are encoded by different genes and differ in their major subcellular localization and enzymatic properties. Each sialidase has been found to play a unique role depending on its particular properties (Miyagi and Yamaguchi 2012) and behave in different manner in cancers (Miyagi et al. 2012). We have found that NEU3 is a key enzyme for ganglioside degradation because of its strict substrate preference to gangliosides, which co-localize with this sialidase in the surface membranes (Miyagi et al. 1999), and plays as a signal modulator (Miyagi et al. 2008). In various human cancers, NEU3 shows a remarkable upregulation. Although NEU4 can hydrolyze gangliosides as well as glycoproteins and oligosaccharides, our recent studies have exhibited its opposite behavior to NEU3 in carcinogenesis with a tendency of downregulation especially in colon cancer (Yamanami et al. 2007). We have now presented evidence of NEU3 for crucial involvement in cancer initiation and promotion in addition to progression in colon cancer through ganglioside modulation.

Upregulation of NEU3 Promotes Cancer Progression

Our previous studies demonstrated a significant upregulation of NEU3 in human various cancers including colon (Kakugawa et al. 2002), renal (Ueno et al. 2006), and prostate (Kawamura et al. 2012) cancers. The expression was increased in the surgical specimens of tumor tissues as compared to adjacent non-tumor tissues in the levels of mRNA and sialidase activity. Our attempt has been made to elucidate the significance and molecular mechanisms underlying increased NEU3 expression. In colon cancer cells, the increase caused suppression of cell differentiation and apoptosis, accompanied with increased Bcl-2 and decreased caspase3 expression. The endogenous sialidase level was downregulated in the process of differentiation and apoptosis of the cells induced by sodium butyrate. Compared to non-tumor mucosa, colon cancer tissues exhibited a marked accumulation of lactosylceramide (Lac-cer), a possible NEU3 product, and addition of Lac-cer to the culture confirmed to reduction of apoptotic cells during sodium butyrate treatment, indicating that high expression of NEU3 leads to protection against programmed cell death, probably through modulation of gangliosides (Kakugawa et al. 2002).

Further investigation of the mechanisms of NEU3-mediated cell survival revealed that its silencing caused apoptosis without specific stimuli, accompanied by decreased Bcl-xL and increased mda7 (melanoma differentiation associated gene-7, differentiation and apoptosis-inducing protein) and GM3 synthase mRNA levels in HeLa cells, whereas overexpression resulted in the opposite effects (Wada et al. 2007). Human colon and breast carcinoma cell lines, HT-29 and MCF-7 cells, appeared to be similarly affected by treatment with the NEU3 siRNA, but interestingly non-cancerous human WI-38 and NHDF fibroblasts and NHEK keratinocytes showed no significant changes. NEU3 silencing inhibited Ras activation and its overexpression to stimulate it with consequent influence on ERK and Akt. NEU3

promoted EGFR phosphorylation in response to EGF and co-immunoprecipitated with EGFR in the cells, suggesting that NEU3 suppresses apoptosis of cancer cells by promoting EGFR phosphorylation, probably through its association with EGF receptors and consequent activation of Ras cascades, especially via the Ras/ERK pathway.

NEU3-transfected colon cancer cells exhibited increased adhesion to laminins and consequent cell proliferation, but a decrease in cell adhesion to fibronectin, collagen I and IV, compared to control cells (Kato et al. 2006). On laminin-5, NEU3 clearly stimulated phosphorylation of FAK and ERK, and markedly enhanced tyrosine phosphorylation of integrin β4, with recruitment of Shc and Grb-2. These results indicate that NEU3 differentially regulate cell proliferation through integrin-mediated signaling depending on the extracellular matrix. This selective NEU3 effect may be favorable for cancer cell growth, because laminin-5 has been reported to increase and in contrast fibronectin to reduce progression of carcinoma.

NEU3 expression was found to be increased in renal cell carcinomas (RCCs) compared to adjacent non-tumor tissues, significantly correlating with elevation of interleukin (IL)-6, a pleiotropic cytokine, which has been implicated in immune responses and pathogenesis of several cancers (Ueno et al. 2006). Up-regulation of NEU3 in the tumor tissues was strongly linked to the IL-6 expression level and NEU3 in renal cancer ACHN cells was activated by IL-6 in a positive feed back manner on the cytokine function, mainly through the PI3K/Akt pathway, resulting in suppression of apoptosis and promotion of migration. Either NEU3 transfection or IL-6 treatment resulted in suppression of apoptosis and promotion of cell motility, and the combination resulted in synergistic effects. NEU3 hardly affected MAPK or IL-6-induced STAT3 activation but promoted the PI3K/Akt cascade in both IL-6 dependent and independent ways. Furthermore, IL-6 promoted Rho activation and the effect was potentiated by NEU3, leading to increased cell motility whereas NEU3 silencing resulted in decreased Akt phosphorylation and inhibition of Rho activation. Glycolipid analysis showed a decrease in ganglioside GM3 and an increase in Lac-cer after NEU3 transfection, and addition of these lipids to the culture apparently affected cell apoptosis and motility, consistent with to the observations in colon cancer cells. The results indicate that NEU3 activated by IL-6 exerts IL-6-mediated signaling largely via the PI3K/Akt cascade in a positive feedback manner and contributes to expression of a malignant phenotype in RCCs.

Up-regulation of NEU3 was also detected in prostate cancer, showing a significant correlation with malignancy as assessed by the Gleason score (Kawamura et al. 2012). In androgen-sensitive LNCaP cells, forced overexpression of NEU3 significantly induced progression-related transcription factor EGR-1, androgen receptors and prostate specific antigen, PSA, both with and without androgen, the cells becoming sensitive to low concentration of hormone. This NEU3-mediated induction was abrogated by inhibitors of PI3K and MAPK, in line with increased phosphorylation of Akt and ERK1/2 in NEU3-overexpressing cells. NEU3 silencing moreover caused a reduction in the cell growth of androgen-independent PC-3 cells in culture and of transplanted tumors in nude mice. These data strongly suggest that NEU3 regulates the progression of prostate cancer through androgen receptor signaling.

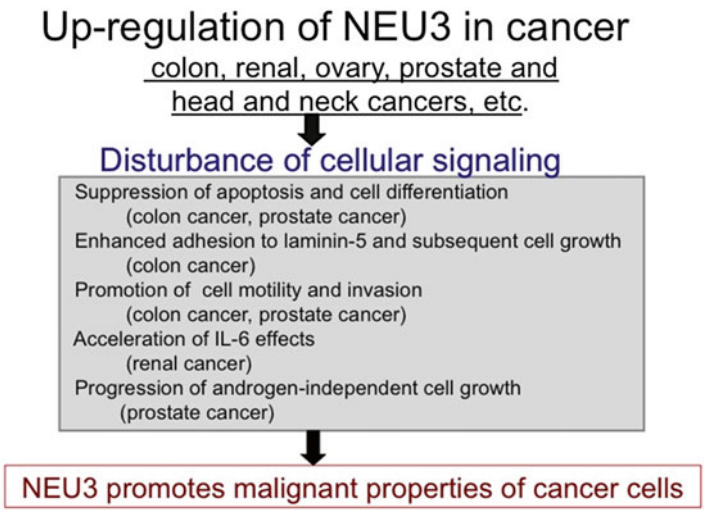

Fig. 9.1 Upregulation of NEU3 observed in various human carcinomas causes disturbance of cellular signaling, leading to augmentation of malignant properties of cancer cells

As illustrated in Fig. 9.1, all the results together display that NEU3 augments cancer progression through promoting cell survival, adhesion, growth and motility by potentiating signaling including MAPK/ERK, PI3K/AKT and FAK/ERK pathways, probably via Ras activation. To gain insights into regulation mechanisms of *NEU3* gene, we have recently determined the gene structure and assessed transcription factor involvement (Yamaguchi et al. 2010). NEU3 expression was found to be diversely regulated by Sp1/Sp3 transcription factors binding to alternative promoters. Such transcriptional control might also account for the upregulation of NEU3 in cancer, because Sp1 and Sp3 are now known to play critical roles in regulating the transcription of genes involved in cell growth control and tumorigenesis (Wierstra 2008). The expression of NEU3, in fact, exhibited good correlations with those of Sp1 or Sp3 in cancer, implying a promoting role in NEU3 gene transcription.

NEU3 Is Involved in Cancer Initiation and Promotion

To investigate whether NEU3 participates in cancer initiation and promotion as well as progression, a possible role of NEU3 in promoting tumorigenesis in vivo has been demonstrated in human NEU3 transgenic mice treated with a carcinogen, azoxymethane (AOM), for induction of precancerous colonic aberrant crypt foci (ACF) (Shiozaki et al. 2009). ACF were induced in the mice significantly more frequently than in their control wild-type counterparts. Enhanced phosphorylation of EGF receptor, Akt and ERK and up-regulation of Bcl-xL protein were observed

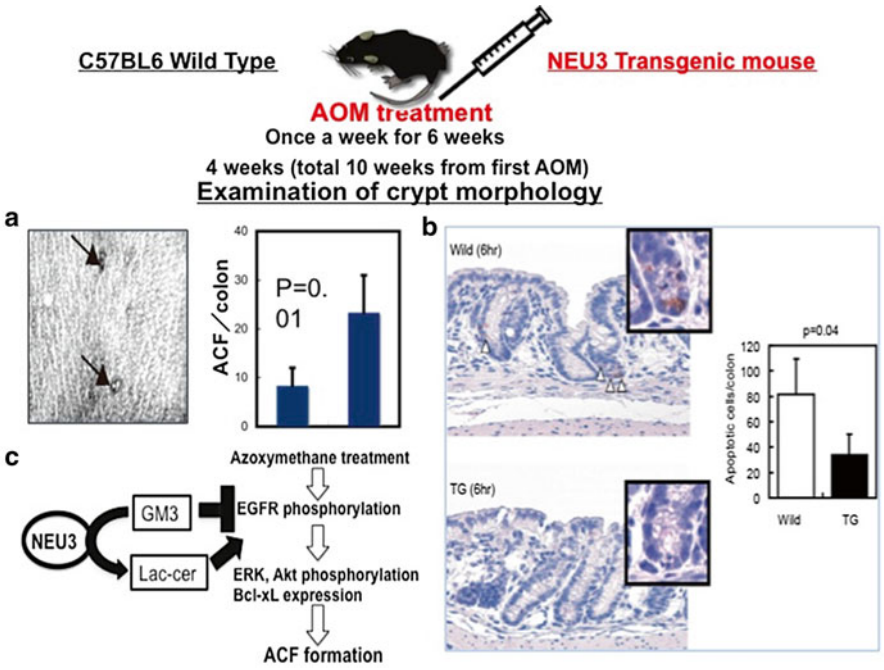

Fig. 9.2 Promotion of aberrant cript foci (ACF) formation in NEU3 transgenic mice. The susceptibility of NEU3 transgenic mice to induction of ACF was examined by treatment with azoxymethane (AOM) (Shiozaki et al. 2009). (**a**) Mice were injected with AOM (i.p., 15 mg/kg/week) for 6 weeks, and 4 weeks later ACF had formed in the NEU3 transgenic mice significantly more than in the control wild-type mice. (**b**) Numbers of apoptotic cells were stained in immuno-histochemical sections with anti-cleaved caspase 3 antibody. (**c**) Enhanced phosphorylation of EGFR, Akt and ERK and up-regulation of Bcl-xL protein were observed in the transgenic colon mucosa

in the transgenic colon mucosa, but no changes were found in cell proliferation, suggesting that the increased ACF formation was due to suppression of apoptosis, as shown in Fig. 9.2. Thus, NEU3 up-regulation may be important to the promotion stage of colorectal carcinogenesis in vivo. When *Neu3*-deficient mice were exposed to dimethylhydrazine, there were no differences in the incidence or growth of tumors from wild-type mice. On the other hand, the *Neu3*-deficient mice were less susceptible to colitis-associated colon carcinogenesis induced by AOM and dextran sodium sulfate, indicating an involvement of NEU3 in inflammation-dependent tumor development (Yamaguchi et al. 2012). In addition to the observations in genetically engineered mouse models, we have provided evidence of a close link between NEU3 expression and Wnt/β-catenin signaling in colon cancer cells by analyzing cancer stem-like characteristics and tumor initiating capability (Takahashi et al. in submission). NEU3-silencing in colon cancer cells resulted in a significant decrease in clonogenicity on soft agar and in vivo tumor growth, along with down-regulation of stemness genes. Under sphere-forming conditions, endogenous

NEU3 expression was significantly increased. Null-activity mutants of NEU3 failed to activate relevant signaling, indicating that the activation is dependent on ganglioside changes.

The available data strongly suggest participation of NEU3 in tumor initiation and promotion, since constitutive activation of Wnt/β-catenin signaling is implicated in the maintenance of cancer stem cells and initiation of the process of colon carcinogenesis (Clevers 2006). However, it is thought to be insufficient for progression without additional Ras activation (Phelps et al. 2009). In this context, NEU3 may be a pivotal molecule involved in both cancer initiation and progression, by regulating Wnt/β-catenin and Ras/MAPK signaling pathways.

Conclusion

In this review, we describe possible roles of the plasma membrane-associated sialidase NEU3 in cancer. We have so far demonstrated that NEU3 is markedly upregulated in various human cancers and the aberrant increase causes augmentation of malignant properties of cancer cells, including increased cell survival, adhesion, migration and invasion. Here, we present evidence of an involvement of NEU3 in cancer initiation and promotion as well as progression using genetically engineered mouse models and NEU3- silenced cancer cells. Treatment with NEU3 siRNA brings about reversal of some malignant properties of cancer cells in culture and tumor regression *in vivo*. Thus, NEU3 may be a prominent determinant in cancer initiation, promotion and progression, and represent an attractive target for treatment.

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