Plasma membrane-associated sialidase (NEU3) promotes formation of colonic aberrant crypt foci in azoxymethane-treated transgenic mice

Kazuhiro Shiozaki,[1,2] Kazunori Yamaguchi,[1,2] Ikuro Sato[3] and Taeko Miyagi[1,2,4]

[1]Division of Biochemistry, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-shiode, Natori, Miyagi, 981-1293, Japan; [2]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 4-1-8 Honmachi, Kawaguchi, Saitama, 332-0012; [3]Division of Pathology, Miyagi Cancer Center Research Institute, 47-1 Nodayama, Medeshima-shiode, Natori, Miyagi, 981-1293, Japan

(Received September 9, 2008/Revised November 14, 2008/Accepted December 7, 2008/Online publication February 2, 2008)

Human plasma membrane-associated sialidase (NEU3) specifically hydrolyzes gangliosides, and it is up-regulated in colon cancer and plays an essential role in the expression of malignant phenotypes. To clarify the role of NEU3 in tumorigenesis *in vivo*, we examined the susceptibility of NEU3 transgenic mice to induction of colonic aberrant crypt foci (ACF) by azoxymethane. Mice were injected with azoxymethane (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. Enhanced phosphorylation of epidermal growth factor (EGF) receptor, Akt and ERK and up-regulation of Bcl-xL protein were observed in the transgenic colon mucosa, but no changes were found in cell proliferation, suggesting that the increased ACF formation is due to suppression of apoptosis. Immunohistological analysis with anti-cleaved caspase 3 antibody showed an actual reduction in apoptotic cells in the transgenic mucosa at 6 h after the first azoxymethane injection, when apoptosis in the colonic crypt occurs. Consistent with our previous observations of human colon cancer, thin-layer chromatography of the gangliosides from the transgenic colon mucosa revealed decreased GM3 and increased lactosylceramide as compared to those from the control mucosa, probably because of catalysis of gangliosides by NEU3. The results of this study provide the first evidence that NEU3 essentially increases azoxymethane-induced ACF formation in colon mucosa by suppression of apoptosis, possibly via activation of the EGF signaling pathway, and thus indicate that up-regulation of NEU3 is important to the promotion stage of colorectal carcinogenesis *in vivo*. (*Cancer Sci* 2009; 100: 588–594)

Colorectal carcinogenesis is thought to occur in several steps that lead from normal mucosa to carcinoma. Aberrant crypt foci (ACF) with dilated irregular luminal openings and thicker epithelial linings are the earliest identifiable mucosal abnormalities in carcinogen-treated rodents and in humans harboring colonic cancer lesions. Formation of ACF is thought to be a precursor of colon cancer.[1–3] Epidermal growth factor receptor (EGFR) signaling is activated in human colon ACF, and expression of related molecules, including proliferating cell nuclear antigen (PCNA), tumor growth factor (TGF)-α, EGFR, and cyclo-oxygenase-2 (COX-2), is increased in hyperproliferative ACF.[4] Azoxymethane (AOM), a DNA-alkylating reagent, is often used to induce the formation of ACF in rodents as an experimental model of colonic tumorigenesis in the premalignant phase. AOM is metabolized to methylazoxymethanol by P450 in the liver, and bacterial flora in the intestine activates methylazoxymethanol to methyldiazonium, which exerts colonotropic mutagenicity.[5] In AOM-induced ACF, an increase of c-fos, decrease in c-myc and hexosamidase, and mutation of the *k-ras*, *APC*, and *β-catenin* genes are observed.[6]

Sialic acids have been shown to play a role in various biological processes by influencing the conformation of glycoproteins, and by recognizing and masking the biological sites of molecules and their binding sites to the cells. Aberrant sialylation is a characteristic feature of cells that have undergone malignant transformation and is closely associated with the malignant phenotype, including metastatic potential and invasiveness.[7,8] A previous study has shown changes in expression of sialic acid-related enzymes in AOM-induced tumors compared with control colon mucosa, suggesting that impaired sialic acid metabolism occurs in cancer.[9] However, neither how aberrant sialylation occurs nor the consequences of the changes are fully understood. Sialidase catalyzes the removal of sialic acids from the sugar chains of glycoproteins and glycolipids. There are four sialidases of mammalian origin. They differ in intracellular localization and substrate specificity, and their names are abbreviated as Neu1, Neu2, Neu3 and Neu4.[10,11] We previously found that these sialidases behave in different ways during carcinogenesis. The plasma membrane-associated sialidase, Neu3, is involved in cell differentiation and transmembrane signaling in neural cells,[12–14] and the human ortholog NEU3 has been found to be up-regulated in colon, renal, and ovary cancer, in comparison with adjacent tissue.[15–17] Up-regulation of NEU3 has been found to increase cancer malignancy *in vitro* by increasing cell attachment and cell motility and inhibiting apoptosis,[15,16,18,19] and knockdown of NEU3 in cancer cells induces apoptosis.[20]

Although we have demonstrated that NEU3 plays an important role in the malignancy of colon cancer cells, how NEU3 affects tumorigenesis *in vivo* is unknown. Since we previously generated transgenic (TG) mice by introducing a human NEU3 cDNA,[21] in this study we examined the occurrence of ACF induced by AOM in human-NEU3 TG mice to explore the role of NEU3 in colon carcinogenesis.

Materials and Methods

Animals. We previously generated human NEU3 TG mice (DAB/2 × C57BL/6) by introducing a human *NEU3* cDNA under the control of the β-actin promoter.[21] Genomic DNA isolation and hybridization with a dig-labeled probe were used to confirm the mouse genotypes. Genetically matched male wild-type mice and male NEU3 TG mice, 5 weeks of age, were used in all experiments. The mice were fed an autoclaved formula diet and water *ad libitum* under a 12/12 h light-dark cycle, and they were handled in accordance with the Guidelines

[4]To whom correspondence should be addressed.
E-mail: miyagi-ta173@pref.miyagi.jp

Abbreviations: APC, adenomatous polyposis coli; BSA, bovine serum albumin; CCND1, cyclin D1 gene; CUGBP2, CUG binding protein 1; ERK, extracelluter signal-regulated kinase; GM3, ganglioside, NeuAcα2→3Galβ1→4Glcβ1→Cer; HPTLC, high performance thin layer chromatography; Lac-cer, Lactosylceramide, [Correction added after publication 2 February 2009: Lactosylceramid was corrected to Lactosylceramide] Galβ1→4Glcβ1→Cer; MAPK, mitogen-activated protein kinase

doi: 10.1111/j.1349-7006.2008.01080.x

of the Animal Care Committee of the Miyagi Cancer Center. To avoid the effect of gene loci disrupted by the inserted transgene, several different lines were used in this study.

Sialidase activity assays. Protein was extracted from crypt epithelial cells by sonicating the tissue in modified ristocetin-induced platelet agglutination (RIPA) buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 1% Nonidet P40, 2 mM edetic acid [EDTA], 7.5 μg/mL aprotinin, 10 μg/mL leupeptin, 10 mM NaF, 2 mM orthovanadate, 0.25% sodium deoxycholate and 2 mM phenylmethylsulfonyl fluoride [PMSF]). Sialidase activity was measured with mixed gangliosides (Sigma) as substrate in the presence of Triton X-100.[22] The sialic acids released were converted to fluorescent compound with malononitrile and measured by reversed phase liquid chromatography.[20] Protein concentrations were measured by a dye-binding assay (Bio-Rad Laboratories). NEU3 expression in the mucosa was also confirmed by immunoblotting with anti-NEU3 antibody that we prepared previously.[23]

ACF analysis. AOM (Wako Pure Chemical Industries, Osaka, Japan) was stored in glass tubes at –20°C until use. Mice were intraperitoneally injected with AOM in sterile saline solution at a dose of 15 mg/kg body weight once a week for 6 weeks,[24] as such mice have been used as 'a spontaneous tumor progression model'.[5] Control animals were injected with saline in the same manner. The mice were killed 4 weeks after the final AOM injection, and the colon was opened longitudinally, rinsed with saline solution, placed flat mucosa-side up between sheets of wet filter paper, and fixed in 10% buffered formalin for 48 h. The fixed colon was inspected for ACF by staining with 0.2% methylene blue for 10 min, rinsing with saline, and examining the mucosa of the colon under a microscope. The crypts in the ACF were larger than the adjacent normal crypts and had a thicker epithelium and deformed luminal opening. The mucosa of 10 mice from each group were examined.

Quantitative reverse transcription-polymerase chain reaction RT-PCR analysis. Quantitative analysis of endogenous Neu3 was performed by real-time PCR using a Light Cycler rapid thermal cycler system (Roche) as described previously.[20] Neu3 primers were 5′-CTCAGTCAGAGATGAGGATGCT-3′(forward) and 5′-GTGAGACATAGTAGGCATAGGC-3′(reverse).

Western blot analysis. Mucosal lysates obtained as described above were clarified by centrifugation at 10 000 *g* for 10 min and then resolved on 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to a polyvinylidene difluoride membrane, the membrane was blocked with 1% BSA in TBST and incubated with primary antibodies. Bound antibodies were visualized by using the appropriate peroxidase-coupled secondary antibodies with ECL detection (Amersham Bioscience). Antibodies for Akt, phospho Akt (Ser^{473}), p44/42 MAPK, phospho p42/44 MAPK (Thr^{202}/Tyr^{204}), phospho EGFR (Tyr^{845}) and cleaved caspase 3 (Asp^{175}) were obtained from Cell Signaling Technology, anti-PCNA and anti-EGFR antibodies from Santa Cruz Biotechnology, and anti-Bcl-xL antibody from BD Bioscience. For the detection of Bcl-xL in the mitochondria, mitochondrial fractions of mice mucosa were prepared using the Mitochondria Isolation Kit (Sigma). Efficacy of the extraction in each sample was compensated by fluorophore JC-1 staining assay.

Immunohistochemistry. To assess cleaved caspase-3 and Ki-67 expression, fixed colon tissue was embedded in paraffin, pretreated for antigen recovery by heating in 0.01 M citrate (pH 6.0) in a microwave oven, and incubated with anti-cleaved caspase 3 antibody (Cell Signaling Technology) and anti-Ki-67 antibody (Dako Cytomation). Signal-positive cells were counted under microscope.

Thin layer chromatography. Glycolipids were extracted from colonic mucosa as described elsewhere,[16] fractionated by thin layer chromatography on HPTLC plates (Baker, Phillipsburg, NJ, US) in chloroform/methanol/H_2O (60:35:8, v/v/v), and visualized with orcinol-H_2SO_4. For the confirmation of GM3 changes, immunostaining of thin layer chromatography was performed using monoclonal anti-GM3 antibody (M2590, Nippon Biotest, Tokyo, Japan) and avidin-biotin immunoperoxidase staining kit (Vector).

Statistical analysis. Results are expressed as means ± SD. The differences between the data from the experimental groups were analyzed for statistical significance by Student's *t*-test or Welch's *t*-test.

Results

Increased ACF formation in NEU3 TG mice. In comparison with the mucosa of the wild-type mice expressing endogenous murine Neu3 sialidase, a 33-fold increase in sialidase activity was found in the NEU3 transgenic colon mucosa (Fig. 1a), when assayed with gangliosides as substrates. The sialidase activity level was comparable to those in other tissues previously reported.[21] Such an increase in sialidase activity is possible to occur actually *in vivo*, because we previously discovered NEU3 expression levels to be increased by 3- to 100-fold in human colon cancer tissues compared to adjacent non-tumor mucosa.[15] NEU3 expression was confirmed by Western blotting (Fig. 1a). The TG mice grew normally and no spontaneous tumorigenesis was detected in any of their tissues. No significant differences in mortality or behavior were observed in the wild-type mice or TG mice after the AOM injections. AOM was injected for 6 weeks, and 4 weeks after the final AOM injection, the mucosa of the entire colon was stained with methylene blue solution to evaluate ACF formation. Few ACF were observed in the wild-type mice or TG mice that were not injected with AOM. After AOM injection, most of ACF consisted of 1–3 small crypts, together with only a few lesions that contained ≥4 crypts, were detected in the mice. As shown in Fig. 1(c), the TG mice injected with AOM showed a larger number of ACF in comparison with the wild-type mice with AOM (9.0 ± 3.8 ACF/colon in the wild-type and 22.2 ± 7.9 ACF/colon in the TG mice; $P = 0.010$). The average number of crypts in the ACF, on the other hand, was almost the same in both groups (1.9 ± 0.3 in the wild-type mice and 2.0 ± 0.3 in the TG mice). Typical ACF in methylene-blue stained sections are shown in Fig. 1(b). No difference in the distribution of the ACF was observed throughout the colon. To know whether endogenous Neu3 is involved in ACF formation, Neu3 mRNA levels in the colon mucosa were estimated at 4 weeks after final AOM injection. Endogenous Neu3 levels were significantly increased in AOM-treated mucosa of wild-type mice, compared to those with saline treatment (Fig. 1d). Colons were opened 15 weeks after the final injection to examine them for tumor progression. However, there were few tumors in either the wild-type or TG mice, because of low susceptibility of C57BL6 mice to tumor induction by AOM. It is probably due to modest hepatic metabolism for chemical carcinogens, including lower expression of cytochrome P450 that converts AOM to methylazoxymethanol.[5,25]

Suppression of apoptosis in NEU3 TG colonic mucosa. To explore the mechanism of increased ACF formation in the AOM-injected NEU3 TG mice, we investigated signaling pathways involved in activation of cell survival and proliferation during AOM-induced tumorigenesis. We first examined whether NEU3 TG mice changed EGFR phosphorylation after AOM treatment, because a recent report has described EGFR signaling as one of the crucial pathways in ACF formation.[4] Western blot analysis of the colonic mucosa after the series of six AOM injections showed a marked enhancement of EGFR phosphorylation in TG as compared with wild-type colon mucosa ($P = 0.0007$, shown in Fig. 2a). When Akt and ERK, downstream of EGFR signaling, was examined, Akt phosphorylation level in the TG mice was significantly higher than that in the wild-type mice ($P = 0.002$)

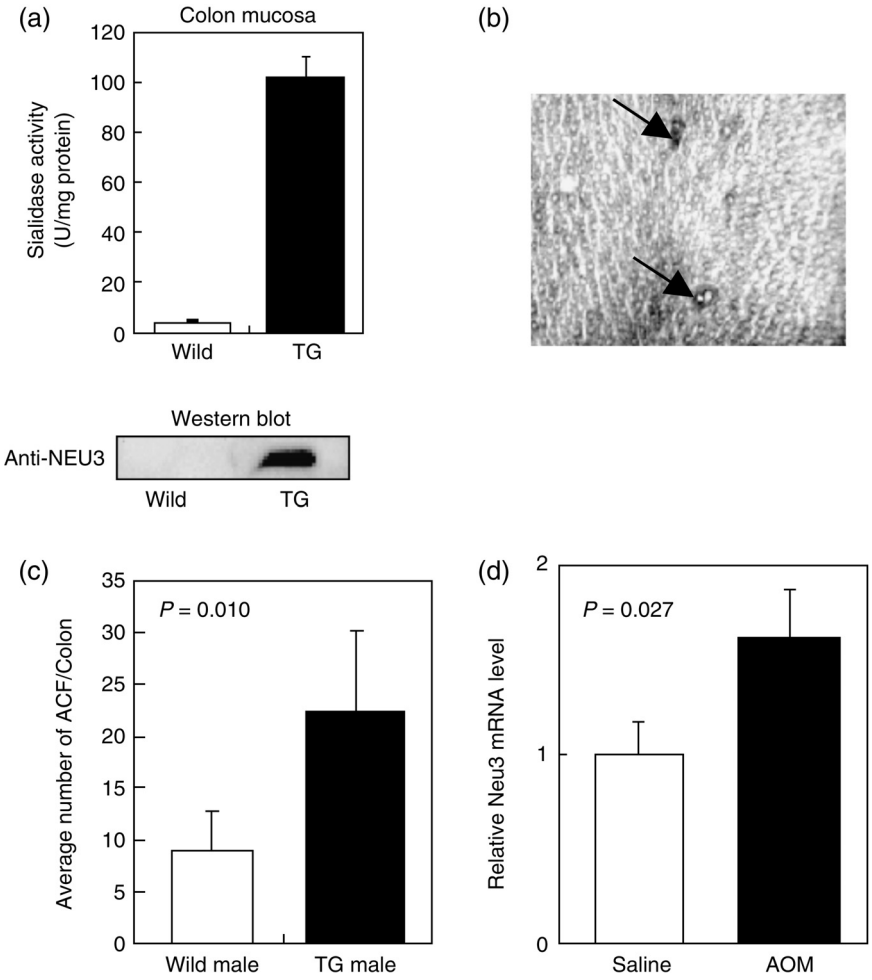

Fig. 1. Increased aberrant crypt foci (ACF) formation in colonic mucosa of azoxymethane (AOM)-injected human plasma membrane-associated sialidase (NEU3) transgenic (TG) mice. (a) NEU3 expression in colonic mucosa of NEU3 TG mice was examined for the sialidase activity and the protein level. Sialidase activity was measured with gangliosides as a substrate, and the protein level was detected by Western blot analysis with anti-NEU3 antibody in the NEU3 TG and the wild-type mice. The values are means ± SD of three independent experiments each carried out with three animals. (b) The number of ACF after AOM treatment was significantly larger in the NEU3 transgenic than in the wild-type mice. A total of 10 animals were examined for ACF formation and ACF were counted in methylene blue-stained colon. Data are means ± SD. (c) Methylene blue-stained ACFs are indicated by arrows in the colonic mucosa of NEU3 transgenic mice. (d) Endogenous Neu3 mRNA level was estimated by real-time PCR in the colon mucosa of wild-type mice with or without AOM treatment. The values are means ± SD of three independent experiments.

(Fig. 2b), and the level of phosphorylated ERK1/2 was also higher in the TG mucosa ($P = 0.04$) (Fig. 2c). However, in the saline-injected groups the phosphorylation levels of both signals were hardly changed by NEU3 overexpression. The above changes are consistent with other reports that ACF formation in AOM-treated animals is accompanied by increased phosphorylation of Akt and/or ERK1/2,[26,27] and suggest that the increased ACF formation in the TG mice is attributable to suppression of apoptosis and/or stimulation of cell proliferation via activation of EGFR and the subsequent Akt and ERK pathways. When ERK phosphorylation levels were examined also in the sites of ACF, ERK activation was clearly detected by immunohistological analysis (data not shown).

To understand further how the ACF increase arose, cell proliferation was assessed on the basis of PCNA and Ki-67 expression. After the AOM injections, the mucosal PCNA levels of the TG mice and the wild-type mice were almost similar, as measured by Western blotting (Fig. 2d), and the level of Ki-67 expression at the base of the crypts assessed by histochemical analysis was unaffected by NEU3 overexpression (data not shown). Apoptosis suppressors, including Bcl-2 or Bcl-xL are known to increase cell survival during AOM-induced tumorigenesis by preventing the release of cytochrome c.[28] We then investigated mitochondrial apoptotic signaling, and found that Bcl-xL expression was increased in the colonic mucosa of the TG mice in comparison with the wild-type mice ($P = 0.03$) (Fig. 2e), but the amounts of cleaved-Bid, a competitor for Bcl-xL and an apoptosis enhancer, were not different between the two groups (Fig. 2f). These results indicate that the suppression of apoptosis via down-regulation of mitochondrial signaling may be a cause of the increased ACF induction by AOM in the colonic mucosa of the TG mice.

Apoptosis has been reported to be induced by a single dose of AOM in rodents and to peak 6 h after injection of a single injection of AOM in mice: expression of p53-activated p21,[24] after DNA damage and p53-regulated Bax,[29] are increased in a small subpopulation of cells with apoptosis morphology at 6 h after AOM injection. To confirm the occurrence of apoptosis suppression in the TG colonic mucosa, TG mice were injected with a single 15 mg/kg body weight dose of AOM, and their colons were examined histologically at 0, 3, 6, and 12 h after the injection. Immunohistological analysis with anti-cleaved caspase 3 antibody revealed few apoptotic cells 0, 3, and 12 h after injection in both the TG and the wild-type mice (data not shown), but numerous apoptosis-positive cells were detected at 6 h (Fig. 3a), which is consistent with the reports above. At the base of crypts, apoptotic cells were observed in just a small subpopulation of colonocytes in the wild-type mice, but there was a significantly smaller number of apoptotic cells in the colonic mucosa of the TG mice than in the wild-type (Fig. 3b) ($P < 0.04$), and the decrease in apoptotic cells was accompanied by a higher level of mitochondrial Bcl-xL (Fig. 3c) and enhanced phosphorylation of Akt in the TG mice (Fig. 3d). These results indicate that the AOM-induced apoptosis was suppressed in the TG mucosa, and that the decreased apoptotic cells led to promotion of ACF formation.

We then investigated how NEU3 caused the suppression of apoptosis in colonic mucosa via alteration of signaling pathways. NEU3 specifically hydrolyzes gangliosides, and altered glycolipid

doi: 10.1111/j.1349-7006.2008.01080.x

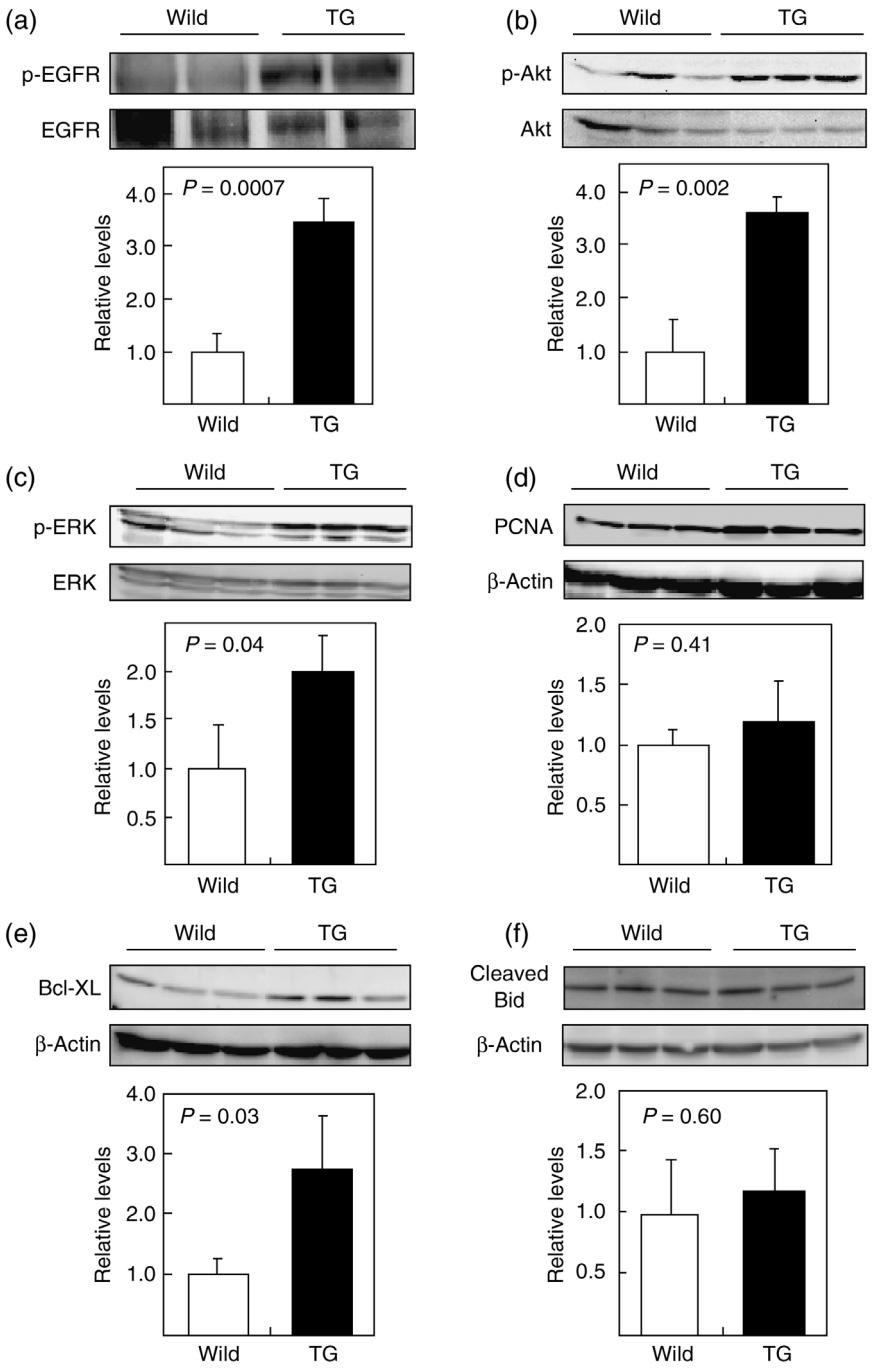

Fig. 2. Altered phosphorylation and protein levels of the molecules related to cell proliferation and apoptosis in azoxymethane (AOM)-treated human plasma membrane-associated sialidase (NEU3) transgenic (TG) mouse colonic mucosa. The phosphorylation levels of epidermal growth factor receptor (EGFR) (a), Akt (b) and ERK1/2 (c) and protein levels of proliferating cell nuclear antigen (PCNA) (d), Bcl-xL (e) and cleaved-Bid (f) were measured by Western blot analysis. Quantitative data presented are means ± SD from 5–8 experiments and the values are relative to those observed in the wild-type mice.

expression has been demonstrated to influence cell apoptosis using several types of cells. In our previous observations, lactosylceramide (Lac-cer) rescued cell survival,[15,16,20] and in contrast, ceramide,[30] or globotriaosylceramide,[20] reduces cell growth, when they are added to the cell culture. In this context, to observe how gangliosides undergo alterations by NEU3 overexpression in TG colon mucosa, glycolipids from the mucosa were analyzed by thin layer chromatography (Fig. 4a, left). The intensity of a band corresponding to GM3 was significantly decreased and a band with mobility similar to Lac-Cer was significantly increased in the TG mucosa in comparison with the wild-type mucosa (Fig. 4b, left). These changes were plausible because GM3 is a good substrate for NEU3 *in vitro* and Lac-Cer is a product of desialylation of GM3.[22] Immunostaining with anti-GM3 antibody after thin layer chromatography confirmed the decrease in GM3 in the TG mucosa (Fig. 4a, right). These results suggest that the change in GM3 and Lac-Cer content by NEU3 catalysis may be one of the causes of the increased occurrence of ACF in the TG mice.

Discussion

In the present study we demonstrated increased AOM-induced ACF formation in the colonic mucosa of NEU3 TG colon mice, possibly as a result of suppression of apoptosis via enhancement of Bcl-xL expression and phosphorylation of Akt and ERK induced by EGFR activation. This is the first study to report that NEU3 attenuates apoptosis signaling *in vivo*. We previously showed that NEU3 up-regulation suppresses cell apoptosis, and that the suppression is accompanied by Bcl-2 stimulation in

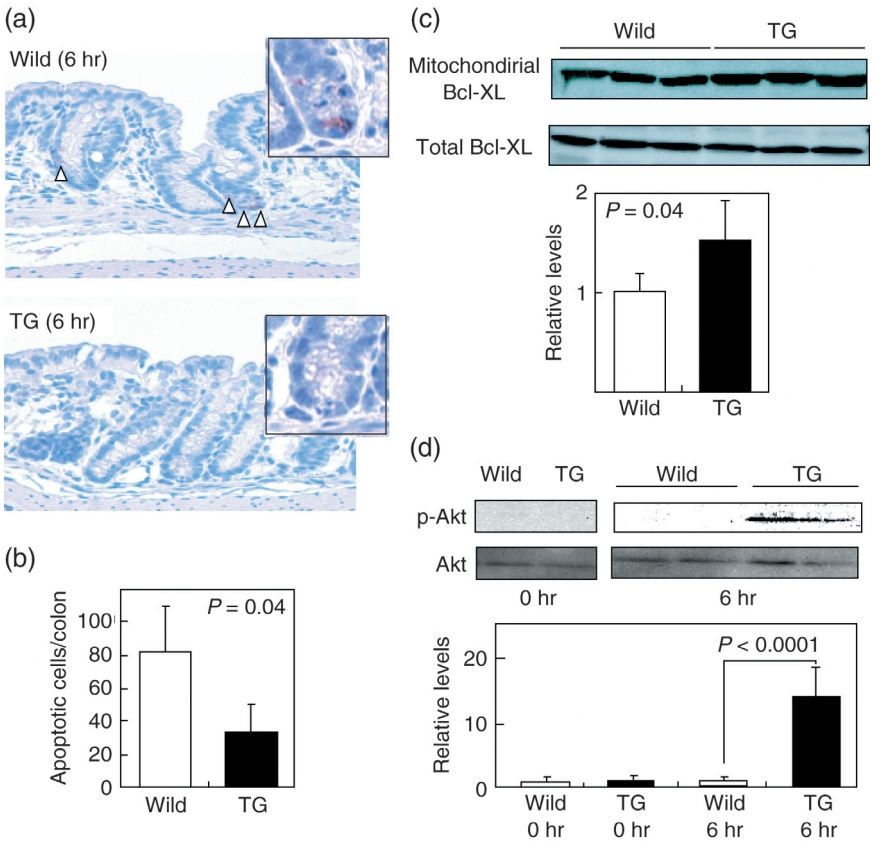

Fig. 3. Decreased azoxymethane (AOM)-induced apoptosis of colonic crypts in human plasma membrane-associated sialidase (NEU3) transgenic (TG) mice. (a) Altered expression of cleaved-caspase 3 in a subpopulation of colonocytes was observed at 6 h after a single injection of AOM in the wild (upper) and NEU3 TG (lower) colonic mucosa by immunohistochemical analysis. (b) Numbers of apoptotic cells in immunohistochemical sections were stained with anti-cleaved caspase 3 antibody and counted for colon sections from four different animals. Data are means ± SD. (c) Mitochondrial fractions were prepared from colon lysates, and evaluated mitochondrial Bcl-xL levels by Western blotting. Quantitative data presented are means ± SD from three different animals. (d) Akt phosphorylation levels were assessed in colon lysates at 0 h and 6 h after AOM injection by Western blotting. Quantitative data are means ± SD from two different animals.

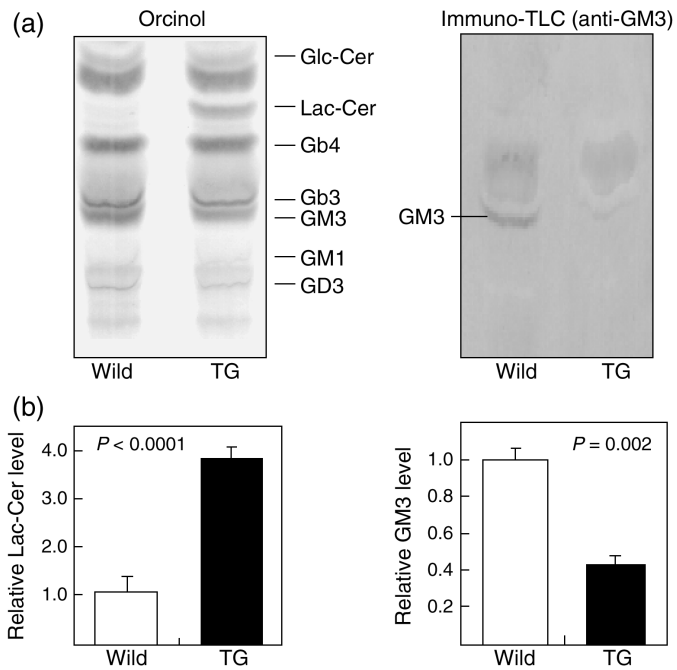

Fig. 4. Alterations of glycolipids in colonic mucosa of human plasma membrane-associated sialidase (NEU3) transgenic (TG) mice. (a) Gangliosides were extracted from colonic mucosa of three different animals, subjected to thin layer chromatography and visualized with orcinol/H_2SO_4 (left panel), as described in MATERIALS AND METHODS. GM3 changes were confirmed by immuno-staining thin layer chromatography using a monoclonal anti-GM3 antibody and an avidin-biotin staining (right panel). (b) For quantification, experiments were repeated separately three times and relative intensity of the glycolipid bands corresponding to Lac-Cer and GM3 was shown in the graph. Data are means ± SD.

several colon cancer cell lines,[15] and is associated with Akt and ERK activation in both HeLa cells,[20] and ACHN human renal carcinoma cells.[16] Our recent studies also showed that NEU3 enhanced the phosphorylation of ERK via the EGFR/Ras/ERK pathway in cancer cells.[20] With regard to EGFR signaling in colonic ACF, other investigators described that expression levels of EGFR itself and EGFR-related molecules including TGF-α, CCND1, COX-2, CUGBP2 were up-regulated in human colonic ACF,[4], and on the contrary, that pan-erb2 inhibitor EGFR related protein suppressed ACF formation together with the decrease of EGFR phosphorylation.[31] In line with these previous results, herein we document activation of EGFR and of its down-stream molecules, Akt and ERK, in the colonic mucosa of AOM-injected TG mice. Moreover, endogenous Neu3 level in the mucosa of wild-type mice was significantly increased by AOM treatment, indicating that Neu3 is involved in the process of colorectal carcinogenesis. These results suggest that enhanced EGFR phosphorylation induced by NEU3 is likely one of the crucial mechanisms in ACF formation.

Previous reports[24,29] have suggested that deregulation of apoptosis contributes to the development of AOM-induced cancer, accounting for the occurrence of apoptosis in colonic crypts 6 h after an AOM injection followed by rapid decrease of the apoptotic cells and their restriction to a small population of colonocytes, associated with stimulation of Bax expression. DNA damage by AOM is thought to cause p53-induced apoptosis followed by entailing Bax expression,[29] and that cleaved-Bid binds to Bax and the Bax then localizes to the mitochondrial membrane, resulting in cytochrome c release. On the contrary, Bcl-xL binds to Bax, competing the binding of Bax to the Bid and suppressing apoptosis. The results of the present study showed that apoptosis actually occurred at 6 h after AOM injection more evidently in the colonic crypt of wild-type mice than in that of the TG mice in which Bcl-xL expression was increased by NEU3 up-regulation, but the amount of cleaved-Bid did not

doi: 10.1111/j.1349-7006.2008.01080.x

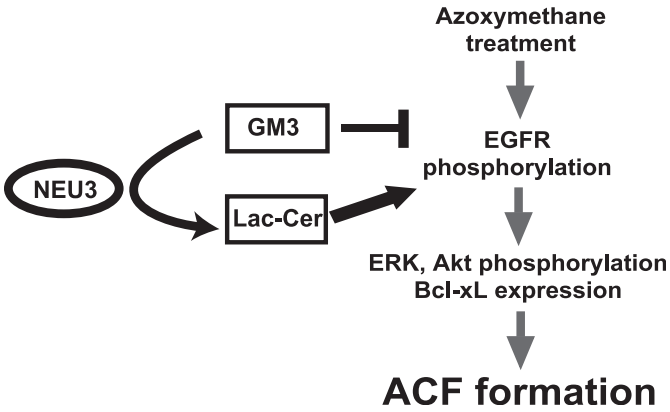

Fig. 5. A possible role of human plasma membrane-associated sialidase (NEU3) in enhancement of azoxymethane (AOM)-induced aberrant crypt foci (ACF) formation. A hydrolysis of GM3 and a production of Lac-Cer by NEU3 enhance epidermal growth factor receptor (EGFR) phosphorylation induced by AOM treatment. EGFR activation leads to stimulation of phosphorylation of Akt and ERK and up-regulation of Bcl-xL, which may prevent damaged colonocytes from apoptosis. Cells survived from apoptosis develop ACF and possibly tumor.

change. Thus, the majority of the damaged cells are eliminated by apoptosis, leading to reduction of ACF formation in the wild-type mice, whereas NEU3-mediated apoptosis suppression by the induction of anti-apoptotic molecules including Bcl-xL may cause promotion of ACF formation in TG mice. The mechanism responsible for suppression of apoptosis by NEU3 up-regulation is likely to be an accumulation of enzyme products and/or a reduction of the substrates. The level of GM3, one of the good substrates for NEU3, was reduced, and Lac-Cer, the product generated by GM3 desialylation, was increased in the TG mucosa, although the quantitative data on ganglioside composition in the site of ACF were not provided. It is likely that these changes are closely related to the suppression of apoptosis. This idea is not contradictory to our previous findings *in vitro*,[20] that GM3 synthase mRNA is up-regulated during induction of apoptosis and GM3 promotes apoptosis, whereas increased Lac-Cer causes stimulation of EGFR phosphorylation and suppresses apoptosis of human cancer cells as described earlier. A possible mechanism for the increased ACF formation induced by NEU3 overexpression is summarized in Fig. 5. This may also be supported by reports,[32–34] that GM3 directly interacts with EGFR and inhibits EGFR dimerization and phosphorylation. Therefore, desialylation of GM3 by NEU3 may be essential for ACF formation in NEU3 TG mucosa. These data indicate that NEU3 plays an important role in the development of malignancy by suppressing apoptosis via activation of EGFR, Akt and ERK *in vivo* as well as *in vitro*.

Acknowledgments

We appreciate the expert technical assistance of Ms. Momo Shiozaki and Mr Kazuaki Sato. This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas Cancer from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett* 1995; **93**: 55–71.
2 Magnuson BA, Carr I, Bird RP. Ability of aberrant crypt foci characteristics to predict colonic tumor incidence in rats fed cholic acid. *Cancer Res* 1993; **53**: 4499–504.
3 Bruce WR, Archer MC, Corpet DE *et al*. Diet, aberrant crypt foci and colorectal cancer. *Mutat Res* 1993; **290**: 111–8.
4 Cohen G, Mustafi R, Chumsangsri A *et al*. Epidermal growth factor receptor signaling is up-regulated in human colonic aberrant crypt foci. *Cancer Res* 2006; **66**: 5656–64.
5 Neufert C, Becker C, Neurath MF. An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. *Nat Protoc* 2007; **2**: 1998–2004.
6 Mori H, Hata K, Yamada Y, Kuno T, Hara A. Significance and role of early-lesions in experimental colorectal carcinogenesis. *Chem Biol Interact* 2005; **155**: 1–9.
7 Dennis JW, Granovsky M, Warren CE. Glycoprotein glycosylation and cancer progression. *Biochim Biophys Acta* 1999; **1473**: 21–34.
8 Hakomori S. Glycosylation defining cancer malignancy: new wine in an old bottle. *Proc Natl Acad Sci USA* 2002; **99**: 10231–3.
9 Corfield AP, Rainey JB, Clamp JR, Wagner SA. Rat colonic mucosal cell sialic acid metabolism in azoxymethane-induced tumors. *Biochim Biophys Acta* 1985; **840**: 264–70.
10 Miyagi T, Yamaguchi K. Sialic acids. In: Kamerling JP, Boons G, Lee YC, Suzuki A, Taniguchi N, Voragen AGJ (eds). *Biochemistry of Glycans: Sialic Acids in Comprehensive Glycoscience*. Amsterdam: Elsevier BV, 2007: 297–322.
11 Miyagi T, Wada T, Yamaguchi K. Roles of plasma membrane-associated sialidase NEU3 in human cancers. *Biochim Biophys Acta* 2008; **1780**: 532–7.
12 Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y, Miyagi T. Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation. *J Biol Chem* 2000; **275**: 8007–15.
13 Proshin S, Yamaguchi K, Wada T, Miyagi T. Modulation of neuritogenesis by ganglioside-specific sialidase (Neu3) in human neuroblastoma NB-1 cells. *Neurochem Res* 2002; **27**: 841–6.
14 Da Silva JS, Hasegawa T, Miyagi T, Dotti CG, Abad-Rodriguez J. Asymmetric membrane ganglioside sialidase activity specifies axonal fate. *Nat Neurosci* 2005; **8**: 606–15.
15 Kakugawa Y, Wada T, Yamaguchi K *et al*. Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. *Proc Natl Acad Sci USA* 2002; **99**: 10718–23.
16 Ueno S, Saito S, Wada T *et al*. Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility. *J Biol Chem* 2006; **281**: 7756–64.
17 Nomura H, Tamada Y, Miyagi T *et al*. Expression of NEU3 (plasma membrane-associated sialidase) in clear cell adenocarcinoma of the ovary: its relationship with T factor of, pTMN classification. *Oncol Res* 2006; **16**: 289–97.
18 Kato K, Shiga K, Yamaguchi K *et al*. Plasma-membrane-associated sialidase (NEU3) differentially regulates integrin-mediated cell proliferation through laminin- and fibronectin-derived signalling. *Biochem J* 2006; **394**: 647–56.
19 Yamaguchi K, Hata K, Wada T, Moriya S, Miyagi T. Epidermal growth factor-induced mobilization of a ganglioside-specific sialidase (NEU3) to membrane ruffles. *Biochem Biophys Res Commun* 2006; **346**: 484–90.
20 Wada T, Hata K, Yamaguchi K *et al*. A crucial role of plasma membrane-associated sialidase in the survival of human cancer cells. *Oncogene* 2007; **26**: 2483–90.
21 Sasaki A, Hata K, Suzuki S *et al*. Overexpression of plasma membrane-associated sialidase attenuates insulin signaling in transgenic mice. *J Biol Chem* 2003; **278**: 27896–902.
22 Miyagi T, Wada T, Iwamatsu A *et al*. Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J Biol Chem* 1999; **274**: 5004–11.
23 Wang Y, Yamaguchi K, Wada T *et al*. A close association of the ganglioside-specific sialidase Neu3 with caveolin in membrane microdomains. *J Biol Chem* 2002; **277**: 26252–9.
24 Poole AJ, Heap D, Carroll RE, Tyner AL. Tumor suppressor functions for the Cdk inhibitor p21 in the mouse colon. *Oncogene* 2004; **23**: 8128–34.
25 Suzuki R, Kohno H, Sugie S, Nakagama H, Tanaka T. Strain difference in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. *Carcinogenesis* 2006; **27**: 162–9.
26 Derdak Z, Fulop P, Sabo E *et al*. Enhanced colon tumor induction in uncoupling protein-2 deficient mice is associated with NF-kB activation and oxidative stress. *Carcinogenesis* 2006; **27**: 956–61.
27 Roy HK, Olusola BF, Clemens DL *et al*. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis* 2002; **23**: 201–5.
28 Wu B, Iwakiri R, Ootani A *et al*. Dietary corn oil promotes colon cancer by inhibiting mitochondria-dependent apoptosis in azoxymethane-treated rats. *Exp Biol Med (Maywood)* 2004; **229**: 1017–25.

29 Aizu W, Guda K, Nambiar P *et al*. p53 and its co-activator p300 are inversely regulated in the mouse colon in response to carcinogen. *Toxicol Lett* 2003; **144**: 213–24.
30 Valaperta R, Chigorno V, Basso L *et al*. Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts. *FASEB J* 2006; **20**: 1227–9.
31 Schmelz EM, Xu H, Sengupta R *et al*. Regression of early and intermediate stages of colon cancer by targeting multiple members of the EGFR family with EGFR-related protein. *Cancer Res* 2007; **67**: 5389–96.
32 Miljan EA, Meuillet EJ, Mania-Farnell B *et al*. Interaction of the extracellular domain of the epidermal growth factor receptor with gangliosides. *J Biol Chem* 2002; **277**: 10108–13.
33 Mirkin BL, Clark SH, Zhang C. Inhibition of human neuroblastoma cell proliferation and EGF receptor phosphorylation by gangliosides GM1, GM3, GD1A and GT1B. *Cell Prolif* 2002; **35**: 105–15.
34 Wang XQ, Yan Q, Sun P *et al*. Suppression of epidermal growth factor receptor signaling by protein kinase C-alpha activation requires CD82, caveolin-1, and ganglioside. *Cancer Res* 2007; **67**: 9986–95.

doi: 10.1111/j.1349-7006.2008.01080.x