Hyperplastic Gastric Tumors with Spasmolytic Polypeptide–Expressing Metaplasia Caused by Tumor Necrosis Factor-α–Dependent Inflammation in Cyclooxygenase-2/Microsomal Prostaglandin E Synthase-1 Transgenic Mice

Masanobu Oshima,1,2 Hiroko Oshima,1,2 Akihiro Matsunaga,1 and Makoto Mark Taketo1

1Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto and 2Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

Abstract

We showed recently that Helicobacter infection induces expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in the mouse stomach, and that transgenic mice expressing both cyclooxygenase-2 and microsomal prostaglandin E synthase-1 (K19-C2mE mice) develop hyperplastic gastric tumors with inflammatory histopathology. To investigate possible roles of proinflammatory cytokines and acquired immunity in the gastric hyperplasia of K19-C2mE mice, we introduced knockout mutations for tumor necrosis factor-α (TNF-α; Tnf), interleukin-1 receptor-α chain (Il1r1), and Rag2 genes, respectively. Among the compound mutants, only the Tnf (−/−) K19-C2mE mice showed significant suppression of hyperplastic tumors with reduced cell proliferation. In contrast, tumorigenesis remained unaffected in either compound mutants of K19-C2mE containing Il1r1 or Rag2 mutation, indicating that neither interleukin-1β signaling nor T cell/B cell response was required for the development of hyperplastic tumors. Importantly, spasmolytic polypeptide/trefoil factor 2–expressing metaplasia (SPEM) in the K19-C2mE stomach was also suppressed in the Tnf (−/−) K19-C2mE mice, indicating that TNF-α–dependent inflammation is responsible for SPEM development. Because gastric metaplasia to the SPEM lineage is considered as a preneoplastic lesion of gastric cancer, it is possible that inhibition of TNF-α–dependent inflammation, together with eradication of Helicobacter, can be an effective prevention strategy for gastric cancer. (Cancer Res 2005; 65(20): 9147-51)

Introduction

Gastric cancer is the second most common malignancy in the world. Epidemiologically, Helicobacter pylori infection is closely linked to gastric cancer (1). Helicobacter infection induces cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) in the gastric mucosa (2, 3). Expression of COX-2 is also elevated in gastric cancer tissues (4). With simultaneous induction of both COX-2 and mPGES-1, prostaglandin E2 (PGE2) production is increased efficiently in the Helicobacter–infected gastric mucosa.

As a model for Helicobacter infection, we recently constructed a transgenic mouse mutant (K19-C2mE) that simultaneously expresses COX-2 and mPGES-1 in the gastric mucosa (3) using the cytokeratin 19 gene promoter (5). The transgenic mice develop inflammation-associated hyperplastic tumors in the proximal glandular stomach through mucosal macrophage accumulation and their activation (3). Gastric hyperplasia and mucous cell metaplasia with Brunner's gland–like morphology in the K19-C2mE mice is similar to that found in the Helicobacter–infected mice (3, 6). Importantly, similar mucous metaplasia is also found in the gastrin gene knockout mice (7) and signal transducers and activators of transcription 3 (STAT3)–activating gp130 (8, 9) mice (8). In these models, gastric glands are replaced with spasmolytic polypeptide/trefoil factor 2 (TFF2)–expressing metaplasia (SPEM), which is considered as a preneoplastic lesion of gastric cancer (9–11).

Accumulating evidence indicates that inflammatory responses play important roles in the development of some types of cancer through induction of cytokines, chemokines, and growth factors (12). Such an inflammatory microenvironment may promote tumor cell proliferation, survival, and angiogenesis. Tumor necrosis factor-α (TNF-α), one of the proinflammatory cytokines, is a key regulator of inflammatory processes that activate nuclear factor-κB (NF-κB). Recently, it has been reported that TNF-α–mediated NF-κB activation is responsible for the development of hepatocellular carcinoma (13) and colitis-associated colonic tumorigenesis (14).

Here we show that TNF-α–dependent inflammation is responsible for gastric hyperplasia in K19-C2mE transgenic mice. Moreover, TNF-α–dependent inflammation is essential for the expansion of the preneoplastic SPEM lineage cells. These results suggest that inhibition of TNF-α–dependent inflammation, together with Helicobacter eradication, can prevent gastric tumor development through suppression of SPEM formation.

Materials and Methods

Animals. Construction of K19-C2mE transgenic mice was described previously (3). Mice homozygous null for TNF-α (Tnf), interleukin-1 (IL-1) receptor α chain (Il1r1), and Rag2 were purchased from Jackson Laboratory, Bar Harbor, ME (Tnf and Il1r1) and from Taconic, Germantown, NY (Rag2). The K19-C2mE mice were crossed with respective knockout mouse strains to generate Tnf (−/−) K19-C2mE, Il1r1 (−/−) K19-C2mE, and Rag2 (−/−) K19-C2mE mice. Littermate simple K19-C2mE transgenic mice were used as controls. Compound mutants and control mice were examined histologically at 20 weeks of age (n = 10 for each genotype). For treatment with meloxicam (Daichi Pharmaceutical, Tokyo, Japan), K19-C2mE mice were dosed with 10 mg/kg/d of the compound by oral administration for 3 weeks in mice that were 78 to 80 weeks of age. All animal experiments

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Requests for reprints: Makoto Mark Taketo, Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, and Makoto Mark Taketo, Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.
were carried out according to the protocol approved by the Ethical Committee at Kyoto University.

**Histopathology and immunohistochemistry.** Tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4 μm thickness. These sections were stained with H&E. The height of the gastric mucosa at the proximal glandular stomach was determined as mucosal thickness. For immunostaining, anti-mouse K1-67 antibody (DakoCytomation, Carpinteria, CA) and rat monoclonal antibody for F4/80 (Serotec, Oxford, United Kingdom) were used as the primary antibody. Staining signals were visualized using the Vectorstain Elite Kit (Vector, Burlingame, CA).

**Reverse transcription-PCR.** Total RNA was extracted from the glandular stomach using ISOGEN (Nippon Gene, Tokyo, Japan). Extracted RNA was reverse-transcribed and PCR-amplified. RT-PCR was carried out using the following primer sets: TFF2 (5'-AGTCCAGTGGACAGCAT-3', 3'-TCGGCAGTACACTTCTAG-3'), MUC6 (5'-CAAGTAAGTGGCGTCATTAG-3', 5'-GAGGGGTTGTTACTCCGGA-3'), and TNF-α (5'-CTCTCTGTTGCTACCAGCGG-3', 3'-AAAGTAGACCTGCTCCGGAC-3'). Specific glyceraldehyde-3-phosphate dehydrogenase primers were used for the internal control.

**In situ hybridization.** Rehydrated paraffin sections were digested with 5 μg/ml of proteinase K, hybridized overnight with 500 ng/ml of riboprobe, and stringently washed in 2× SSC/50% formamide, followed by 0.1× SSC. Riboprobes were labeled using digoxigenin-labeling reagent (Roche Diagnostics, Indianapolis, IN). Sense probes were used as negative controls (data not shown). TSA Biotin system (Perkin-Elmer, Wellesley, MA) was used for signal amplification.

**Differential labeling of proliferating cells.** Mice were injected i.p. with 200 μl of bromodeoxyuridine (BrdUrd) solution (Roche) at 48 hours before euthanasia (n = 3 for each group). Tissue samples were fixed in 70% ethanol, paraffin-embedded and sectioned at 5 μm thickness. These sections were stained with anti-BrdUrd antibody (Roche) followed by Ki-67 immunostaining. The number of double-positive (BrdUrd+, Ki-67+) cells and BrdUrd single-positive cells were counted for five high-power fields from each section. For inhibition of TNF-α signaling, neutralizing anti-TNF-α antibody (R&D Systems, Minneapolis, MN) was injected i.p. at 30 μg/d from the day before BrdUrd injection until the day of sacrifice.

**Statistical analysis.** Statistical analyses were carried out by unpaired Student's t test, and P values < 0.05 were considered significant.

**Results and Discussion**

**Inflammation-dependent hyperplastic tumors of the stomach.** We previously constructed K19-C2mE transgenic mice that simultaneously express both COX-2 and mPGES-1 in the gastric mucosa, and develop gastric hyperplastic tumors with inflammatory responses (3). By 80 weeks of age, K19-C2mE mice developed large tumors in the entire surface of the glandular stomach (Fig. L1L). Histologically, these tumors consisted of hyperplasia with abnormal glandular architecture. Importantly, we found inflammatory cell infiltrations in the submucosa and lamina propria. Notably, the tumor growth was significantly suppressed by treatment with meloxicam, a nonsteroidal anti-inflammatory drug (NSAID), for 3 weeks in mice that were 78 to 80 weeks of age (Fig. L1).

**Suppression of gastric tumorigenesis by Tnfα knockout mutation.** In the glandular stomach of K19-C2mE mice, expression is elevated significantly for proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (3). Moreover, expression of TNF-α is suppressed significantly by treatment with a COX-2 inhibitor, NS-398 (3). Among these cytokines, TNF-α plays a key role in carcinogen-induced tumorigenesis in the skin and liver (15, 16). Likewise, IL-1β signaling is involved in hepatic melanoma metastasis (17). To investigate whether these cytokines were involved in the inflammation-dependent gastric hyperplasia of K19-C2mE mice, we introduced knockout mutations for the TNF-α (Tnf) and IL-1 receptor α chain (Il1r1) genes, respectively. In the control K19-C2mE mice at 20 weeks of age, hyperplastic tumors developed in the proximal glandular stomach (Fig. 2A), and the mucosal thickness increased to 2.6 times of that in the wild-type mice (Fig. 2B). However, tumor growth was suppressed significantly in the Tnf (−/−) K19-C2mE mice, with the mucosal thickness reduced to the wild-type level (Fig. 2A and B). Histologically, few inflammatory signs were evident, with only mild hyperplasia in the proximal glandular stomach (Fig. 2C). Only a minor population of the Tnf (−/−) K19-C2mE mice (2 of 18) developed focal hyperplastic tumors with inflammatory infiltrations (data not shown). In contrast, tumor growth and mucosal thickness were unaffected in Il1r1 (−/−) K19-C2mE mice (Fig. 2A and B). Histologically, their submucosa was infiltrated with inflammatory cells as in the stomach of K19-C2mE mice (Fig. 2C).

It has been reported that Th1 immune response is a critical component of gastritis and gastric hyperplasia in the Helicobacter-infected mouse model (20). To investigate such a possibility in the inflammation-associated gastric hyperplasia, we introduced a knockout mutation of Rag2 gene into the K19-C2mE mice to generate Rag2 (−/−) K19-C2mE mice that lack T and B lymphocytes. Surprisingly, similar hyperplastic tumors developed in the compound mutants to those in the control K19-C2mE mice (Fig. 2A), and the mean mucosal thickness was essentially the same (Fig. 2B). Although submucosal inflammatory infiltrations were found in the Rag2 (−/−) K19-C2mE histology, they lacked CD3ε-positive T cells (Fig. 2C). Moreover, the level of mucosal macrophage accumulation in the Rag2 (−/−) K19-C2mE mice was the same as that in the K19-C2mE mice (data not shown).

It seems that TNF-α-dependent innate immunity is sufficient for gastric epithelial hyperplasia, and that activation of acquired immunity is dispensable.

**Suppression of differentiation by tumor necrosis factor α-dependent inflammation.** To investigate cell proliferation and differentiation, we labeled K19-C2mE mice with BrdUrd and Ki-67 at different time points and examined epithelial cells. Namely, the proliferating cells were pulse-labeled first with BrdUrd at 0 hours, and immunostained at 48 hours with anti-BrdUrd and anti-Ki-67 antibodies. Only those cells proliferating at both time points were labeled for BrdUrd and Ki-67, whereas those differentiated within 48 hours were labeled only with BrdUrd. In the wild-type normal (i.e., nonhyperplastic) mucosa, most BrdUrd+ cells migrated to the differentiated zone 48 hours after BrdUrd injection (Fig. 3A). The ratio of the double-positive (BrdUrd + Ki-67+) cells to the total BrdUrd+ cells at 48 hours was 6.5 ± 1.4%, which was consistent with a previous report (21). In the hyperplastic tumor tissues, the proliferative zone labeled with Ki-67 was expanded significantly compared with that in the normal mucosa (Fig. 3A).

Importantly,
most BrdUrd+ cells were still in the proliferative zone at 48 hours. The ratio of the double-positive (BrdUrd+ Ki-67+) cells was significantly higher than in normal mucosa (14.7 ± 2.3%), indicating that differentiation and migration of the proliferating progenitor cells were suppressed in the inflamed mucosa (Fig. 3B). Notably, treating K19-C2mE mice with neutralizing anti-TNF-α antibody helped recover migration of the BrdUrd+ cells to the differentiated zone of the tumor tissue, reducing the ratio of BrdUrd+ Ki-67+ cells significantly to the basal level of 3.9 ± 2.2% (Fig. 3B). Submucosal inflammatory infiltration still remained after treatment with anti-TNF-α antibody (data not shown). These results, taken together, indicate that excess TNF-α in the K19-C2mE mouse stomach caused sustained proliferation of the progenitor cells, keeping them from differentiating and migrating to the lumen.

Figure 1. Suppression of gastric hyperplastic tumors in K19-C2mE mice by NSAID treatment. A, large gastric tumors developed in the glandular stomach at 80 weeks of age (left). Tumorigenesis was suppressed by treatment with meloxicam, a NSAID, for 3 weeks (right). Photographs (top) and H&E staining (bottom, ×200). *, inflammatory cell infiltrations in the submucosa and lamina propria, respectively. B, relative mucosal thickness determined in the proximal glandular stomach of meloxicam-treated mice compared with the mean value of the no-drug control K19-C2mE. ●, mucosal thickness of individual mice; *, P < 0.05.

Suppression of spasmolytic polypeptide–expressing metaplasia development in Tnf (−/−) K19-C2mE mice. It has been reported that SPEM is associated with gastric Helicobacter infection and adenocarcinoma in both humans and mice (9, 10), suggesting that SPEM is a preneoplastic lesion of gastric cancer (11). It is also known that the gastric glands are replaced with TFF2-expressing SPEM cells in gastrin (−/−) mice (7) and in STAT3-activating gp130757F/F mice (8), both accompanied by inflammatory responses. These results suggest that inflammatory responses themselves cause SPEM development rather than Helicobacter infection in the gastric mucosa. Because we also found mucous cell metaplasia with Brunner’s gland–like morphology in the K19-C2mE stomach (Fig. 2C; ref. 3), we examined expression of TFF2 by in situ hybridization (Fig. 4A). In the

Figure 2. Suppression of gastric hyperplastic tumors by disruption of the TNF-α gene. A, photographs of the stomach of wild-type, control K19-C2mE, and compound mutants of K19-C2mE with Tnf (−/−), Il1r1 (−/−), and Rag2 (−/−), respectively (left to right). Arrowheads, hyperplastic tumors in proximal glandular stomach. Note that tumorous growth was suppressed only in Tnf (−/−) K19-C2mE. B, relative mucosal thickness of the respective compound mutants compared with the mean value of wild-type (mean ± SD). WT, wild-type; CT, K19-C2mE; Tnf, Tnf (−/−) K19-C2mE; IL-1, Il1r1 (−/−) K19-C2mE; and Rag, Rag2 (−/−) K19-C2mE (n = 10). *, P < 0.05. C, histopathology of K19-C2mE compound mutants with Tnf (−/−), Il1r1 (−/−), and Rag2 (−/−), respectively (top to bottom, H&E; ×200). Arrows, submucosal inflammatory cell infiltrations. Arrowheads, mild hyperplastic lesion; ●, mucous metaplastic cells with Brunner’s gland–like morphology. Insets, immunostaining with anti-CD3ε antibody (boxed areas). Note that CD3ε-positive T cells are absent in the Rag2 (−/−) K19-C2mE submucosa.
wild-type mice, expression of TFF2 was limited in the neck cells as previously described (9, 11). However, we detected TFF2 in the mucous metaplastic cells of the hyperplastic tumors, which indicates that they are of SPEM lineage (Fig. 4A). Because mucous metaplasia is suppressed by treatment with a NSAID or a COX-2 inhibitor (Fig. 1A; ref. 3), TFF2 levels in the stomach should also be decreased by COX-2 inhibition. Moreover, we also found one of the other SPEM markers, MUC6, in both mucous neck cells and SPEM cells (Fig. 4A; ref. 7). Consistently, the mRNA levels for TFF2 and MUC6 were significantly elevated in the K19-C2mE tumors (Fig. 4B). Importantly, we found expression of TFF2 and MUC6 only in the gland neck of the Tnf−/− K19-C2mE mice (Fig. 4A). Histologically, these cells appeared to be normal mucous neck cells, rather than Brunner’s gland–like cells such as SPEM cells are. Consistently, the mRNA levels for TFF2 and MUC6 decreased significantly in the Tnf−/− K19-C2mE mice (Fig. 4B). These results are supported by our previous report that treatment of K19-C2mE mice with pentoxifylline, an inhibitor for proinflammatory cytokines, results in the decrease of metaplastic mucous cells in the glandular stomach (3). Therefore, TNF−/−-dependent inflammation causes expansion of SPEM cells, a possible preneoplastic lesion of gastric cancer.

It is established that inflammation is a critical component of development of some types of cancer (1, 12). The proinflammatory cytokine TNF-α plays a key role in mediating the inflammatory process through activation of NF-κB. Recently, it has been shown that epithelial NF-κB suppresses apoptosis by induction of antiapoptotic protein, such as A1/Bfl1, cIAP1, GADD45β, and Bcl-xL, whereas stromal NF-κB enhances proliferation of epithelial cells by induction of cytokines, chemokines, and growth factors, such as TNF-α, IL-1β, intercellular adhesion molecule, IL-6, macrophage inflammatory protein-2, and KC (13, 14). These results collectively indicate that inflammatory responses stimulate proliferation, and suppress the apoptosis of epithelial cells. Consistently, we have shown here that TNF−/−-dependent inflammation stimulates proliferation of the undifferentiated gastric epithelial cells, causing hyperplastic tumors. In addition to the regulation of apoptosis and proliferation, we have also shown that TNF−/−-dependent inflammation is responsible for the expansion of the SPEM lineage. Because a minor population of Tnf−/− K19-C2mE mice showed small hyperplastic foci with inflammatory reactions, it is possible that factors other than TNF-α are also induced by inflammation and play an important role for SPEM formation, although TNF-α.

Figure 4. Expression of TFF2 and MUC6 in hyperplastic tumors, which is suppressed by knockout mutation of Tnf. A, in situ hybridization of TFF2 and MUC6 in the gastric mucosa of wild-type (×200), Tnf+/+ K19-C2mE (×400), and Tnf−/− K19-C2mE (×200) mice (left to right). Arrows, TFF2-positive cells in the neck; arrowheads, MUC6-positive cells. Note that most mucous metaplastic cells express both TFF2 and MUC6. Insets, higher magnification of TFF2- (top) and MUC6- (bottom) positive cells. B, representative RT-PCR from wild-type, Tnf+/+ K19-C2mE, and Tnf−/− K19-C2mE gastric mucosa. Note that expression of TFF2 and MUC6 increased in the Tnf−/− K19-C2mE mice, but decreased significantly in the Tnf−/− K19-C2mE mice.
seems to play a major role. Gastric metaplasia with SPEM lineage expansion is considered as a preneoplastic lesion of gastric adenocarcinoma both in humans and in mice (9–11). Thus, our results suggest that inhibition of TNF-α–dependent inflammation can be an additional preventive strategy against gastric cancer development through suppression of SPEM cell metaplasia, not to mention eradication of *H. pylori*.

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### References


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