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Monocyte chemoattractant protein-1 is generated via TGF-β by myofibroblasts in gastric intestinal metaplasia and carcinoma without *H. pylori* infection

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Helicobacter pylori* (*H. pylori*) stimulates secretion of monocyte chemoattractant protein 1 (MCP-1) from gastric mucosa. Monocyte chemoattractant protein-1 (MCP-1) expression and macrophage infiltration are recognized in human gastric carcinoma. We have previously generated Cdx2-transgenic mice as model mice for intestinal metaplasia. Both chronic *H. pylori*-associated gastritis and Cdx2-transgenic mouse stomach develop intestinal metaplasia and finally gastric carcinoma. In this study we have directed our attention to MCP-1 expression in the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach. Quantitative real-time PCR was performed to determine *MCP-1* and transforming growth factor-*β1* (*TGF-β1*) mRNA expression levels and single- or double-label immunohistochemistry was used to evaluate the localization of MCP-1, TGF-β type I receptor, and α-smooth muscle actin (αSMA). We determined that *MCP-1* mRNA dramatically increased in the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach, compared with normal mouse stomach. Both MCP-1 and TGF-β type I receptor were co-expressed in the αSMA-positive myofibroblasts of intestinal metaplastic mucosa and gastric carcinoma. Exogenous application of TGF-β1 increased *MCP-1* mRNA expression levels in the intestinal metaplastic tissue. Furthermore, *TGF-β1* was overexpressed and macrophage was strongly infiltrated in the gastric carcinoma. In conclusion, MCP-1 expression, which was stimulated by TGF-β1, was recognized in the TGF-β type I receptor-expressing myofibroblasts of the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach. The present results suggest that intestinal metaplasia and gastric carcinoma themselves induce MCP-1 expression independently of *H. pylori* infection. (*Cancer Sci* 2010; 101: 1783–1789)

Various inflammatory mediators have been detected in higher concentrations in *Helicobacter pylori* (*H. pylori*)-infected gastric mucosa. Furthermore, gastric carcinoma secretes a variety of chemoattractants that attract macrophages and cause them to accumulate in the tumor tissue, wherein the macrophage becomes a tumor-associated macrophage (TAM). Chemoattractant cytokines (chemokines) form a superfamily of closely related secreted proteins that specialize in mobilizing leukocytes to areas of infected tissues.[1] Monocyte chemoattractant protein-1 (MCP-1) is a CC chemokine that has functional action on monocytes and lymphocytes and plays a major role in regulating monocytes and lymphocytes migrating into tissues. Patients infected with *H. pylori* have significantly greater mRNA expression of *MCP-1* in the gastric mucosa than patients without *H. pylori* infection.[2] Chronic *H. pylori*-associated gastritis is accompanied by monocyte and lymphocyte infiltration, in addition to neutrophil infiltration.[3,4] Significant correlation is seen between *MCP-1* mRNA expression and mononuclear cell infiltration. *Helicobacter pylori* (*H. pylori*) infection of gastric epithelial cell lines also stimulates *MCP-1* expression *in vitro*.[5] In addition to *H. pylori*-infected gastric mucosa, MCP-1 expression and macrophage infiltration are recognized in gastric carcinoma and increase with the depth of tumor invasion.[6,7] These results indicate that secretion of MCP-1 may be instrumental in the monocyte infiltration of the gastric epithelium that characterizes *H. pylori* gastritis as well as gastric carcinoma.

Helicobacter pylori (*H. pylori*) induces chronic, persistent inflammatory response that accelerates remodeling of the gastric epithelium and glandular loss (gastric atrophy) followed by intestinal metaplasia, dysplasia, and progression to gastric carcinoma.[8,9] We have previously generated Cdx2-transgenic mice as model mice for intestinal metaplasia.[10,11] The Cdx2-transgenic mice specifically expressed Cdx2 in the gastric mucosa and developed intestinal metaplasia in the stomach.[10,11] Gastric carcinoma spontaneously developed from the intestinal metaplasia in all stomachs of Cdx2-transgenic mice examined.[12]

Since both chronic *H. pylori*-associated gastritis and Cdx2-transgenic mouse stomach develop intestinal metaplasia and finally gastric carcinoma, we were interested in determining whether the intestinal metaplastic mucosa and the gastric carcinoma themselves induce MCP-1 expression independently of *H. pylori* infection, using the model mouse stomach for intestinal metaplasia (Cdx2-transgenic mouse).

Material and Methods

Mice. Cdx2-transgenic mice with stomach-specific expression of Cdx2 under the control of the rat H^+/K^+-ATPase β-subunit gene promoter were used.[10] The gastric mucosa of these mice was completely changed to intestinal metaplastic mucosa.[10,11] Mice had free access to standard food and drinking water and were maintained on a 12-h light/dark cycle. All experiments in this study were performed in accordance with the Jichi Medical University Guide for Laboratory Animals. We used wild-type mice (three males and three females, 6 months old, for each experiment) and Cdx2-transgenic mice (three males and three females, 6 months old, for each experiment on intestinal metaplasia and three males and three females, 24 months old, for each experiment on gastric carcinoma).

Immunohistochemistry. Murine tissues were fixed in 4% formaldehyde in PBS overnight at room temperature, embedded in paraffin and sectioned at a thickness of 3 μm. Murine tissues were also embedded in Tissue-Tek OCT compound (Sakura Finetek, Street Torrance, CA, USA), snap frozen in liquid nitrogen, and stored at −80°C prior to use. Five-micrometer sections were cut from each frozen sample embedded in OCT compound. For histological evaluation, sections were stained with hematoxylin–eosin. The primary antibodies used for the

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immunohistochemistry were: mouse monoclonal anti-αSMA (1:400; Sigma, St. Louis, MO, USA), rat monoclonal anti-F4/80 for macrophage (1:50; AbD Serotec, Oxford, UK), rabbit polyclonal anti-TGF-β type I receptor (1:200; Novocastra Laboratories, Newcastle upon Tyne, UK), and rabbit polyclonal anti-MCP-1 (1:10; Immuno-Biological Laboratories, Gunma, Japan). To use 3,3-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries, Osaka, Japan) as the chromogenic substrate, horseradish peroxidase (HRP)-labeled polymers conjugated to secondary antibodies for primary rabbit antibody (Dako EnVision System; DakoCytomation, Carpinteria, CA, USA) were used as the secondary antibody. For immunofluorescence staining, Cy3 donkey antirabbit IgG or Alexa Fluor 488 antirat or mouse IgG (Molecular Probes, Eugene, OR, USA) was used as the secondary antibody.

The number of positive stained cells for F4/80 was counted in five microscopic fields and then the mean value was calculated.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from the gastric mucosa (normal mice), intestinal metaplastic mucosa (Cdx2-transgenic mice), and gastric carcinoma (Cdx2-transgenic mice) using the guanidinium isothiocyanate/phenol method (Isogen; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed at 37°C for 1 h in a final volume of 20 μL of reverse transcription buffer (50 mM Tris–HCl [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, and 10 mM dithiothreitol [DTT]) containing reverse transcriptase (ReverTraAce; Toyobo, Osaka, Japan), 200 pmol random primer, and 1 mM dNTPs (Sigma). One hundred nanograms of cDNA were used in each real-time PCR reaction. Expression levels for each specific gene were determined by real-time PCR using ready-to use Assay-on-Demand gene expression products (Applied Biosystems, Foster City, CA, USA): *MCP-1* Mm00432449_ml, vascular endothelial growth factor A (*VEGFa*) Mm01281449_m1, and *TGF-β1* Mm03024053_m1. Each Assay-on-Demand gene expression product contains target-specific primers and probes and a Taqman Gene Expression Master Mix containing AmpErase uracil-N-glycosylase (Applied Biosystems) to prevent re-amplification of carryover PCR products. Polymerase chain reaction (PCR) amplification and fluorescence data collection were performed with the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems), using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles for amplification (95°C for 15 s, 60°C for 1 min). All PCR reactions were performed in 96-well plates using a final volume of 20 μL and each gene was studied in triplicate. In order to normalize RNA transcript abundance for each gene, the housekeeping gene β-actin (Pre-Developed Taqman Assay Reagents; Applied Biosystems) was used to calculate the ΔC_T ($\Delta C_T = C_{T\ target}/C_{T\ actin}$). The Ct values for β-actin for the normal and Cdx2-transgenic mouse stomach tissues fell in a close range with no specific pattern of spatial or temporal variation detected (data not shown). A relative quantification approach was used in this study to describe the change in expression of the target gene in a test sample relative to a calibrator sample (reference). The relative RNA transcript abundance value was calculated as follows: first the ΔC_T for the normal and Cdx2-transgenic mouse stomach tissues was calculated. In the second step, differences between the normal and Cdx2-transgenic mouse stomach tissues were calculated as $\Delta\Delta C_T$ ($\Delta C_{T\ target}/\Delta C_{T\ reference}$). The Cdx2-transgenic mouse stomach was used as reference for MCP-1 and TGF-β1 expressions. Finally, the fold difference (relative abundance) was calculated using the formula $2^{-\Delta\Delta CT(13)}$ and was plotted as mean, $n = 6$.

***Ex vivo* effects of TGF-β1 on *MCP-1* expression in cultured gastric tissue specimens.** Normal mice and Cdx2-transgenic mice were sacrificed and stomachs were harvested. Gastric corpus was divided into two pieces. Each specimen was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM L-glutamine, 10% fetal bovine serum, and 1% antibiotic solution in the presence or absence of recombinant TGF-β1 (5 ng/mL) for 16 h at 37°C. After 16 h of cultivation, total RNA was extracted from each specimen, using the guanidinium isothiocyanate/phenol method (Isogen; Nippon Gene) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed and used for determining the *MCP-1* expression levels by quantitative real-time PCR.

Statistical analysis. Data in bar graphs represent means ± SEM. Statistical analysis was performed by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

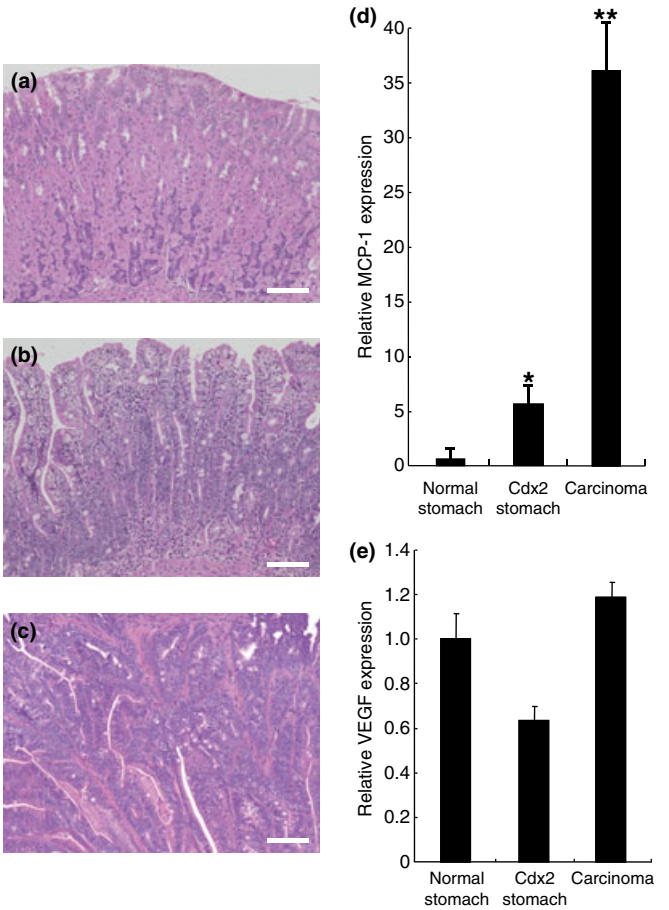

Fig. 1. Quantitative real-time PCR analysis of monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) expression. Hematoxylin–eosin staining of the normal mouse stomach (a), intestinal metaplastic mucosa (b), and gastric carcinoma (c) of Cdx2-transgenic mouse stomach. Scale bar: 100 μm. Monocyte chemoattractant protein-1 (*MCP-1*) gene expression characterised by quantitative real-time PCR (d). Monocyte chemoattractant protein-1 (*MCP-1*) mRNA levels were compared among the normal mouse stomach and the intestinal metaplasia and gastric carcinoma of Cdx2-transgenic mouse stomach. Monocyte chemoattractant protein-1 (MCP-1) exhibited a six-fold increase in intestinal metaplastic mucosa and a 37-fold increase in gastric carcinoma compared with normal mouse stomach. Each column indicates mean ± SE of six tissue samples. $*P < 0.05$ and $**P < 0.01$ *versus* control values. Vascular endothelial growth factor (*VEGF*) gene expression characterised by quantitative real-time PCR (e). Vascular endothelial growth factor (*VEGF*) mRNA levels were compared among the normal mouse stomach and the intestinal metaplasia and gastric carcinoma of Cdx2-transgenic mouse stomach.

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Results

Monocyte chemoattractant protein-1 (*MCP-1*) is expressed in intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach. Both *H. pylori* and Cdx2-transgenic mice induce intestinal metaplasia and gastric carcinoma.[8–12] Patients infected with *H. pylori* have significantly greater mRNA expression of *MCP-1* in the gastric mucosa than patients without *H. pylori* infection.[2] Furthermore, accumulating evidence indicates that inflammatory responses play important roles in the development of some types of cancer through induction of cytokines and chemokines.[14] Such an inflammatory microenvironment may promote tumor cell proliferation, survival, and angiogenesis. We were interested in whether the intestinal metaplastic mucosa and the gastric carcinoma themselves induce MCP-1 expression independently of *H. pylori* infection. We examined the mRNA level for *MCP-1* in the intestinal metaplastic mucosa (Fig. 1b) and gastric carcinoma (Fig. 1c) of Cdx2-transgenic mouse stomach, and compared it to the normal gastric mucosa (Fig. 1a) of wild-type mice. The intestinal metaplastic mucosa of Cdx2-transgenic mouse stomach exhibited a 6-fold increase and the gastric carcinoma exhibited a 37-fold increase in *MCP-1* mRNA expression compared with the normal gastric mucosa (Fig. 1d). We have reported the intestinal metaplastic mucosa of Cdx1-transgenic mouse stomach we generated.[15] The intestinal metaplastic mucosa of Cdx1-transgenic mouse stomach also exhibited a 3-fold increase in MCP-1 mRNA expression compared with the normal gastric mucosa. These results indicate that intestinal metaplastic mucosa of Cdx1- and Cdx2-transgenic mouse stomach expresses MCP-1 independently of *H. pylori* infection.

Transforming growth factor-β1 (TGF-β1) has been reported to up-regulate mRNA and protein expression of MCP-1.[16–18] Transforming growth factor-β1 (TGF-β1) has been also reported to induce VEGF synthesis.[19–22] Both TGF-β1 and VEGF play critical role in the multiple-step process of tumorigenesis of gastric carcinoma.[23,24] We examined the mRNA level for *VEGF* in the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach, and compared it to the normal gastric mucosa of wild-type mice. In contrast to MCP-1 expression, VEGF was not increased in the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach compared with the normal gastric mucosa (Fig. 1e).

Immunohistological colocalization of MCP-1 and αSMA in intestinal metaplastic mucosa and gastric carcinoma. We examined the immunohistological localization of MCP-1 in intestinal metaplastic mucosa and gastric carcinoma. Monocyte chemoattractant protein-1 (MCP-1) immunoreactivity was detected in the stromal cells of intestinal metaplastic mucosa (Fig. 2b) and gastric carcinoma (Fig. 2c) of Cdx2-transgenic mouse stomach,

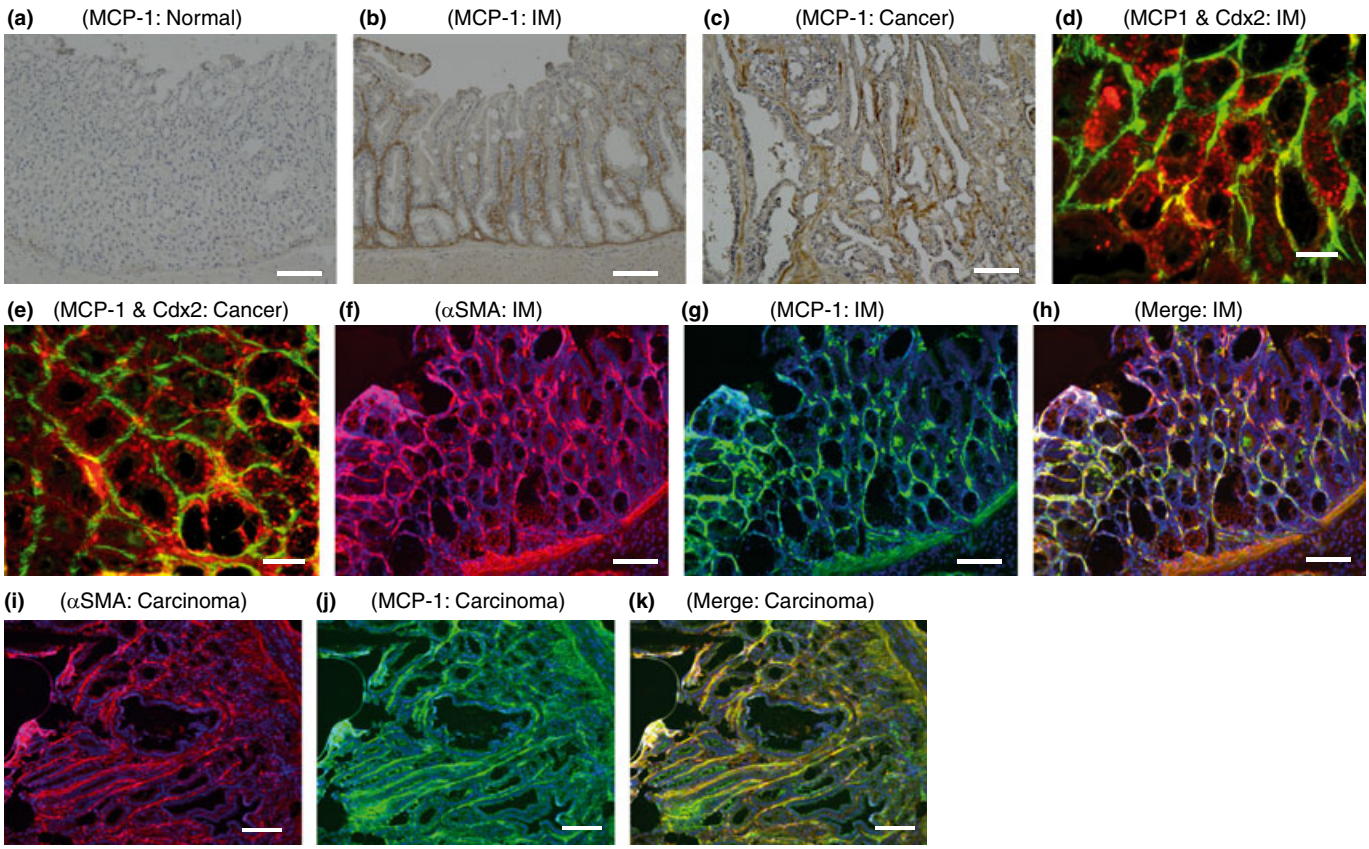

Fig. 2. Colocalization of α-smooth muscle actin (αSMA) and monocyte chemoattractant protein-1 (MCP-1) in intestinal metaplastic mucosa and gastric carcinoma by dual labeling immunofluorescence. By immunohistochemistry, we examined the expression of MCP-1 in normal mouse stomach (a), intestinal metaplastic mucosa (b), and gastric carcinoma (c) of Cdx2-transgenic mouse stomach. Intestinal metaplastic mucosa (d) and gastric carcinoma (e) of Cdx2-transgenic mouse stomach were used for analyzing the expression of Cdx2 (Cy3) and MCP-1 (Alexa 488) by dual immunofluorescence staining. Intestinal metaplastic mucosa (f,g,h) and gastric carcinoma (i,j,k) of Cdx2-transgenic mouse stomach were used for analyzing the expression of αSMA (f,i) and MCP-1 (g,j) by immunofluorescence staining (h,k; merged image). The same section was stained for αSMA (f,i: cy3) and MCP-1 (g,j: Alexa 488) by dual immunofluorescence staining. α-Smooth muscle actin (αSMA) and MCP-1 were co-localized. Panels (f–k) were counterstained with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). Scale bar: 100 μm.

while only a small amount of MCP-1 immunoreactivity was detected in the normal mouse stomach (Fig. 2a).

Next, we performed dual immunohistochemical analysis of MCP-1 and Cdx2 expression in intestinal metaplastic mucosa and gastric carcinoma. Dual fluorescence analysis showed that stromal cells around the Cdx2-positive glands exhibited MCP-1 expression in both intestinal metaplastic mucosa (Fig. 2d) and gastric carcinoma (Fig. 2e). These results indicate that MCP-1 is expressed in the stromal cells around the Cdx2-positive glands in both the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgnic mouse stomach.

Next, we performed dual immunohistochemical analysis of MCP-1 and αSMA expression in intestinal metaplastic mucosa and gastric carcinoma. Dual fluorescence analysis showed that both MCP-1 and αSMA imunoreactivities were co-localized in the same stromal cells of intestinal metaplastic mucosa (Fig. 2f, αSMA; Fig. 2g, MCP-1; Fig. 2h, merged image) and gastric carcinoma (Fig. 2i, αSMA; Fig. 2j, MCP-1; Fig. 2k, merged image). These results indicate that MCP-1 is expressed in the αSMA-positive myofibroblasts in intestinal metaplastic mucosa and gastric carcinoma induced by Cdx2 in Cdx2-transgnic mouse stomach.

Transforming growth factor-β (TGF-β) type I receptor expression. Transforming growth factor-β1 (TGF-β1) has been reported to up-regulate the mRNA and protein expression of MCP-1.[16–18] We examined the expression of TGF-β receptor. Transforming growth factor-β (TGF-β) type I receptor was expressed in the stroma of both intestinal metaplastic mucosa (Fig. 3b) and gastric carcinoma (Fig. 3c) of Cdx2-transgenic mouse stomach, but not in the normal gastric mucosa (Fig. 3a).

Next, we performed dual immunohistochemical analysis of TGF-β type I receptor and Cdx2 expression in both intestinal metaplastic mucosa and gastric carcinoma. Dual fluorescence analysis showed that stromal cells around the Cdx2-positive glands exhibited TGF-β type I receptor expression in both intestinal metaplastic mucosa (Fig. 3d) and gastric carcinoma (Fig. 3e). These results indicate that TGF-β type I receptor is expressed in the stromal cells around the Cdx2-positive glands in both intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgnic mouse stomach.

Next, we performed dual immunohistochemical analysis of TGF-β type I receptor and MCP-1 expression in intestinal metaplastic mucosa and gastric carcinoma. Dual fluorescence analysis showed that both TGF-β type I receptor and MCP-1 immunoreactivities were detected in the same stromal cells in both intestinal metaplastic mucosa (Fig. 3f, TGF-β type I receptor; Fig. 3g, MCP-1; Fig. 3h, merged image) and gastric carcinoma (Fig. 3i, TGF-β type I receptor; Fig. 3j, MCP-1; Fig. 3k, merged image). These results indicate that both TGF-β type I

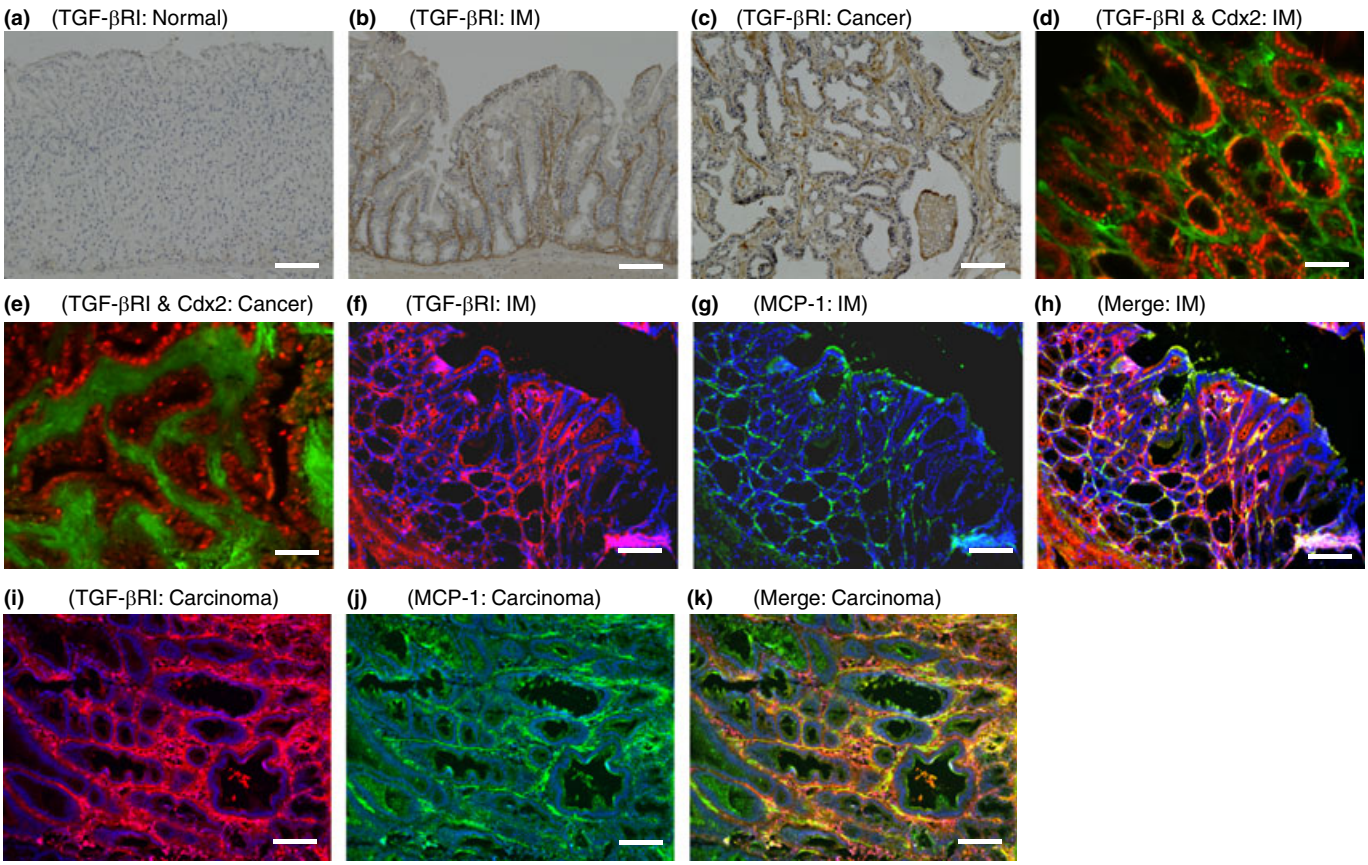

Fig. 3. Immunohistochemical staining for transforming growth factor-β (TGF-β) type I receptor and monocyte chemoattractant protein-1 (MCP-1) expression in intestinal metaplastic mucosa and gastric carcinoma. With immunohistochemistry we examined the expression of TGF-β type I receptor in normal mouse stomach (a), intestinal metaplastic mucosa (b), and gastric carcinoma (c) of Cdx2-transgenic mouse stomach. Intestinal metaplastic mucosa (d) and gastric carcinoma (e) of Cdx2-transgenic mouse stomach were used for analyzing the expression of Cdx2 (Cy3) and TGF-β type I receptor (Alexa 488) by dual immunofluorescence staining. Intestinal metaplastic mucosa (f,g,h) and gastric carcinoma (i,j,k) of Cdx2-transgenic mouse stomach were used for analyzing the expression of TGF-β type I receptor and MCP-1 by immunofluorescence staining (h,k; merged image). The same section was stained for TGF-β type I receptor (f, i: cy3) and MCP-1 (g,j: Alexa 488). Transforming growth factor-β (TGF-β) type I receptor and MCP-1 were co-localized. Panels (f–k) were counterstained with the nuclear dye DAPI. Scale bar: 100 μm.

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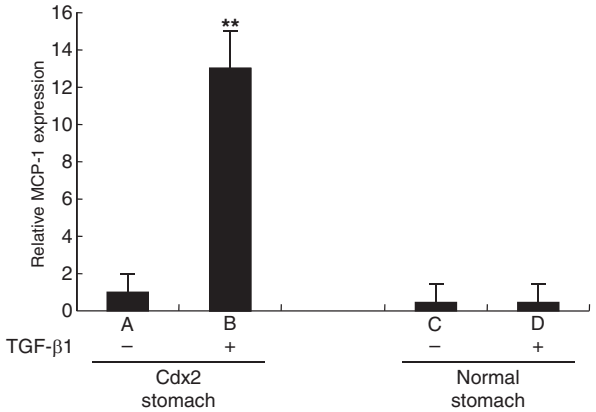

Fig. 4. Monocyte chemoattractant protein-1 (MCP-1) expression in intestinal metaplastic tissue was increased by the cultivation with transforming growth factor-β1 (TGF-β1) *ex vitro*. Monocyte chemoattractant protein-1 (*MCP-1*) mRNA in intestinal metaplastic tissue after 16 h of cultivation without TGF-β1 (A); *MCP-1* mRNA in intestinal metaplastic tissue after 16 h of cultivation with TGF-β1 (B); *MCP-1* mRNA in normal gastric tissue after 16 h of cultivation without TGF-β1 (C); *MCP-1* mRNA in normal gastric tissue after 16 h of cultivation with TGF-β1 (D). The expression levels of *MCP-1* mRNA in intestinal metaplasia significantly increased during 16 h of cultivation with TGF-β1 while the *MCP-1* level in normal gastric mucosa was not changed during 16 h of cultivation with TGF-β1 as determined by quantitative real-time PCR. Each column indicates mean ± SE of five tissue samples. $^{**}P < 0.01$ *versus* control values.

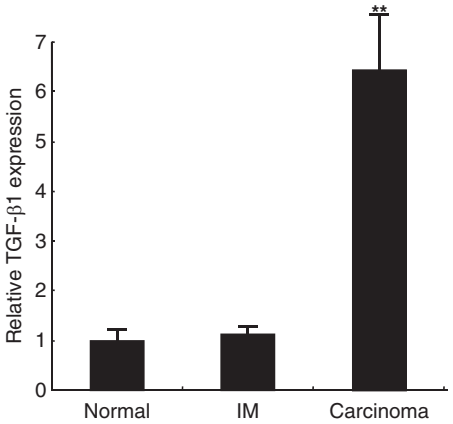

Fig. 5. Transforming growth factor-β1 (TGF-β1) expression in normal gastric mucosa, intestinal metaplastic mucosa (IM), and gastric carcinoma of Cdx2-transgenic mouse stomach. Transforming growth factor-β1 (*TGF-β1*) mRNA was compared among the normal gastric mucosa and the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach. The gastric carcinoma of Cdx2-transgenic mouse stomach exhibited a 6.5-fold increase in *TGF-β1* mRNA expression compared with the normal gastric mucosa. Each column indicates mean ± SE of six tissue samples. $^{**}P < 0.01$ *versus* control values.

receptor and MCP-1 were co-localized in the same αSMA-positive myofibroblasts.

Transforming growth factor-β1 (TGF-β1) stimulates MCP-1 expression in cultured intestinal metaplastic tissues. Intestinal metaplastic tissues and normal gastric tissues were exposed to exogenous TGF-β1 at 5 ng/mL for 16 h and MCP-1 expression levels were compared by quantitative real-time PCR. The *MCP-1* mRNA level in the intestinal metaplastic tissues exposed to TGF-β1 increased by 13-fold compared with the intestinal metaplastic tissues that were not exposed to TGF-β1 (Fig. 4). On the other hand, the expression level of *MCP-1* in the normal gastric tissues exposed to exogenous TGF-β1 was the same as that in the normal gastric tissues that were not exposed to exogenous TGF-β1 (Fig. 4).

To examine whether TGF-β1 plays a role in the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach, we carried out quantitative real-time PCR analysis to determine the expression of TGF-β1 in the normal gastric mucosa and the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach. The *TGF-β1* mRNA level in gastric carcinoma increased by 6.5-fold compared with the normal gastric mucosa and the intestinal metaplastic mucosa of Cdx2-transgenic mouse stomach (Fig. 5).

Intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach recruit macrophages. Monocyte chemoattractant protein-1 (MCP-1) is potentially involved in the migration of monocyte/macrophages. To assess whether mucosal monocyte/macrophages were involved in the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach, we performed immunohistochemical examination using rat-antimouse-F4/80 antibody to evaluate the monocyte/macrophage migration into the intestinal metaplastic mucosa and the gastric carcinoma. The number of tissue macrophages in the intestinal metaplastic mucosa increased two-fold compared with the normal gastric mucosa (Fig. 6). Macrophage in the gastric carcinoma increased six-fold compared with normal gastric mucosa (Fig. 6).

Discussion

Transforming growth factor-β1 (TGF-β1) has been reported to up-regulate mRNA and protein expression of MCP-1.[16–18] A significant up-regulation of *MCP-1* gene expression in myofibroblasts after a long-term incubation with TGF-β1 has been reported.[25] In the present study, MCP-1 and TGF-β type I receptor were co-expressed in the stromal αSMA-positive myofibroblasts, and TGF-β1 treatment resulted in a significant up-regulation of *MCP-1* gene expression. It has been reported that MCP-1 expression and macrophage infiltration are recognized in gastric carcinoma and increase with the depth of tumor invasion.[6,7] The gastric carcinoma of Cdx2-transgenic mouse stomach also expressed a robust higher *TGF-β1* and *MCP-1* mRNA, and induced intensity of macrophage infiltration.

Transforming growth factor-β (TGF-β) initiates its signals through heteromeric complexes of type II and type I serine/threonine kinase receptors. After phosphorylation by type II receptor in the receptor complex, the activated type I receptors, in turn, phosphorylate downstream receptor-regulated Smads. Transforming growth factor-β1 (TGF-β1) exerts a variety of biological effects in different cell types by mainly activating the Smad signaling pathway.[26,27] Monocyte chemoattractant protein-1 (*MCP-1*) expression is enhanced by TGF-β treatment and overexpression of its active type I receptor in human microvessel endothelial cells (HMVECs).[17] Transforming growth factor-β (TGF-β) type I receptor was expressed in the stromal myofibroblasts of both intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach. Furthermore, the myofibroblasts which express TGF-β type I receptor also expressed MCP-1 in both the intestinal metaplastic mucosa and gastric carcinoma of Cdx2-transgenic mouse stomach. Thus, robust *MCP-1* gene expression in gastric carcinoma of Cdx2-transgenic mouse stomach could be stimulated via TGF-β receptor with TGF-β1, which was overexpressed in the gastric carcinoma. In Figure 5, the expression level of TGF-β1 in the intestinal metaplastic mucosa was similar to normal tissue. However, macrophage infiltration in Figure 6 was increased in the intestinal metaplasia. In Figure 3, TGF-βRI was expressed in the intestinal metaplastic mucosa, but not in normal tissue. TGF-βRI expression in the intestinal

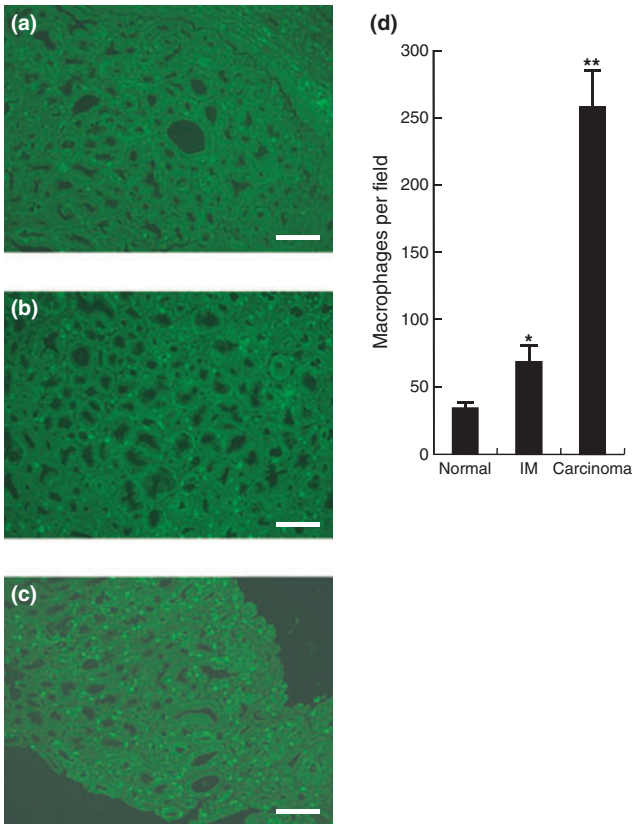

Fig. 6. Immunofluorescence staining for macrophage marker F4/80. Macrophage accumulation, which is shown by the immunostaining for macrophage marker F4/80, was recognized in the normal mouse stomach (a) and the intestinal metaplasia (IM) (b) and the gastric carcinoma (c) of the Cdx2-transgenic mouse stomach. Scale bar: 100 μm. The mean number of infiltrated macrophages per field is shown in panel (d). The number of tissue macrophages in the intestinal metaplastic mucosa and gastric carcinoma increased by two-fold and six-fold compared with the normal gastric mucosa, respectively. $*P < 0.05$ and $**P < 0.01$ *versus* control values.

metaplastic mucosa may have increased macrophage infiltration in spite of the similar expression level of TGF-β1 in both normal and intestinal metaplastic tissues.

Transforming growth factor-β1 (TGF-β1) is overexpressed in most carcinomas and has been reported as a primary inducer of myofibroblast differentiation in reactive stromal fibroblasts.[28,29] Consistent with this, the myofibroblast is a common stromal cell type in carcinoma-associated stroma in many different epithelial cancers.[29,30] Data presented here are consistent with such activated myofibroblasts. Transforming growth factor-β1 (TGF-β1) stimulated MCP-1 expression from myofibroblasts of Cdx2-transgenc mouse stomach. However, our results do not rule out the involvement of other TGF-β-regulated diverse functions for stromal cells.[27,31,32] We believe that it is highly unlikely that MCP-1 is the only pathway downstream of TGF-β in stroma promoting gastric tumor growth, but that it does partially mediate the tumorigenic action of TGF-β. It is becoming clearer that the net response to elevated TGF-β in cancer is likely to be mediated through a diverse set of downstream factors.

We have reported the pericryptal fibroblast sheath (PCFS) in intestinal metaplastic mucosa.[33] The pericryptal fibroblast sheath (PCFS) is subepithelial myofibroblasts that are located immediately subjacent to the basement membrane in intestinal metaplastic epithelial cells. These myofibroblasts are characterized immunohistochemically by positive immunoreactivity for α-SMA.[34–38] In the present study, these fibroblasts produced MCP-1. It is suggested that many inflammatory cytokines are induced in subepithelial myofibroblasts in the colonic mucosa of inflammatory bowel disease.[39,40] Subepithelial myofibroblasts might play a pivotal role in the relationship between protumorigenesis and inflammation in intestinal metaplasia as a pre-malignant condition.

There are many reports that MCP-1 plays an important role in tumor progression. However, based on several *in vitro* studies, the current belief is that MCP-1 does not promote proliferation or prosurvival characteristics in mammary tumor cells.[41] Monocyte chemoattractant protein-1 (MCP-1) is known as the most potent chemoattractant involved in macrophage migration.[42] One of the important roles of MCP-1 in tumor progression is through its involvement in macrophage recruitment relating to inflammation. It is widely accepted that a functional relationship exists between inflammation and tumor cell growth.[14,43] It is estimated that over 15% of cancer in the world is caused by infections that often induce persistent chronic inflammations in the hosts.[44] Cancer cells secrete a variety of chemoattractants that attract macrophages and cause them to accumulate in the tumor tissue, wherein the macrophage becomes a tumor-associated macrophage (TAM). Tumor-associated macrophage (TAM) is directed toward stimulating tumor growth and progression and thus has protumorigenesis activity. Tumor-associated macrophage (TAM) is derived from monocytes that have been recruited largely by chemokine MCPs.[14,43] Here we have demonstrated macrophage infiltration into the gastric stroma in both intestinal metaplasia and gastric carcinoma. Macrophage activation accelerates gastric epithelial growth and participates in gastric tumorigenesis. In gastric carcinoma, a direct association has been shown between the degree of TAM infiltration and depth of tumor invasion, nodal status, and clinical stage.[45] In the present study, the number of tissue macrophages was much greater in gastric carcinoma than in intestinal metaplastic mucosa. These results suggest that macrophages are not only related to the gastric tumorigenesis in the pre-malignant intestinal metaplastic mucosa, but they are also associated with the tumor development in gastric carcinoma in the mouse model for intestinal metaplasia (Cdx2-transgenic mice).

After eradication of *H. pylori*, inflammatory cell infiltration is reduced, while advanced intestinal metaplastic mucosa itself is usually not improved. However, well-differentiated adenocarcinoma develops from advanced intestinal metaplastic mucosa even after eradication. The present results suggest that the MCP-1 expression in intestinal metaplastic mucosa may explain the gastric carcinogenesis from intestinal metaplastic mucosa after the eradication of *H. pylori*.

In conclusion, MCP-1 expression, which was stimulated by TGF-β1, was recognized in the TGF-β type I receptor-expressing myofibroblasts of the intestinal metaplastic mucosa and the gastric carcinoma of Cdx2-transgenic mouse stomach. The present results suggest that intestinal metaplasia and gastric carcinoma themselves induce MCP-1 expression independently of *H. pylori* infection.

Disclosure Statement

The authors have no conflict of interest.

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