Figure 1

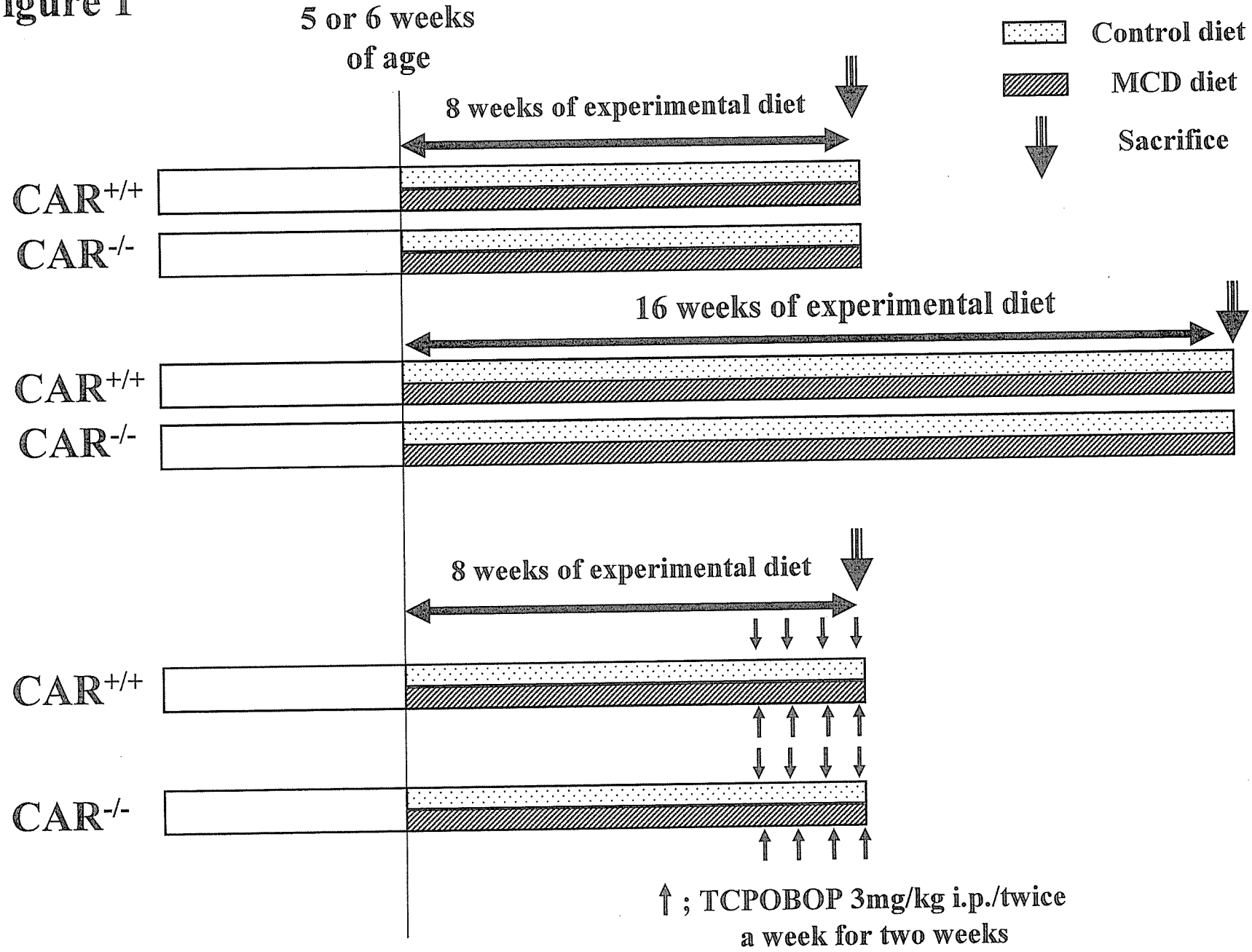
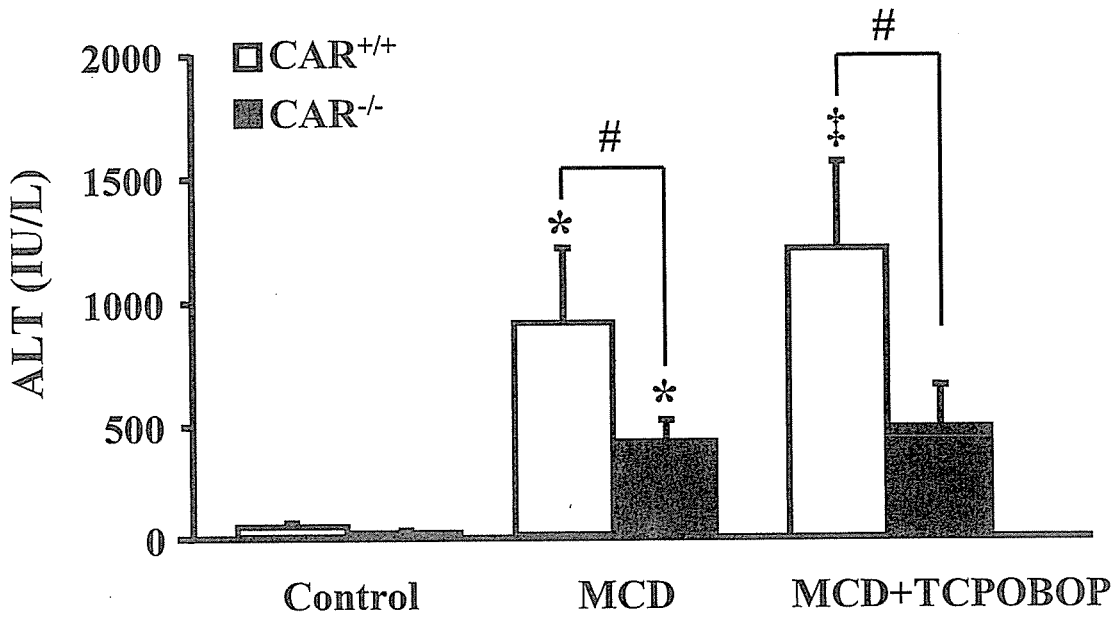

5 or 6 weeks
of age
Control diet
MCD diet
Sacrifice
8 weeks of experimental diet
CAR+/+
CAR-/-
16 weeks of experimental diet
CAR+/+
CAR-/-
8 weeks of experimental diet
CAR+/+
CAR-/-
↑ ; TCPOBOP 3mg/kg i.p./twice
a week for two weeks

Figure 2

A


2000
1500
1000
500
0
ALT (IU/L)
CAR+/+
CAR-/-
#
#
*
*
‡
Control
MCD
MCD+TCPOBOP

B

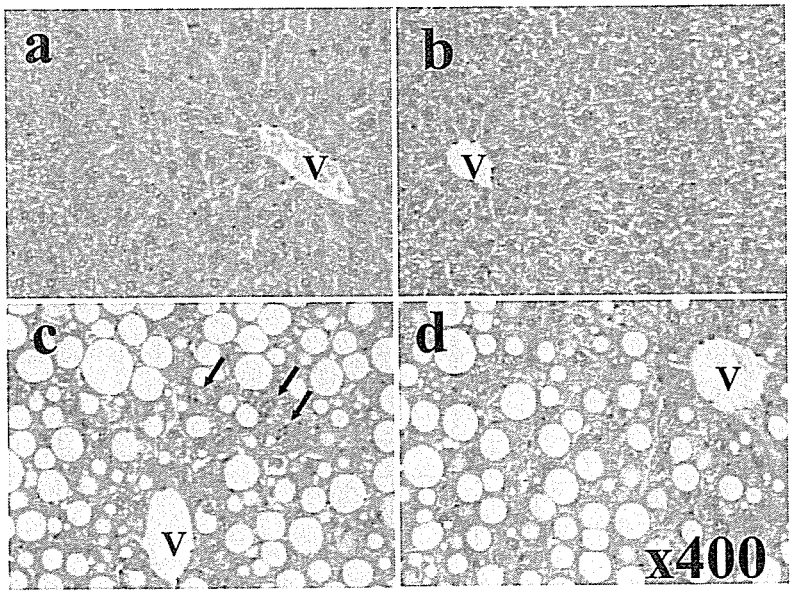
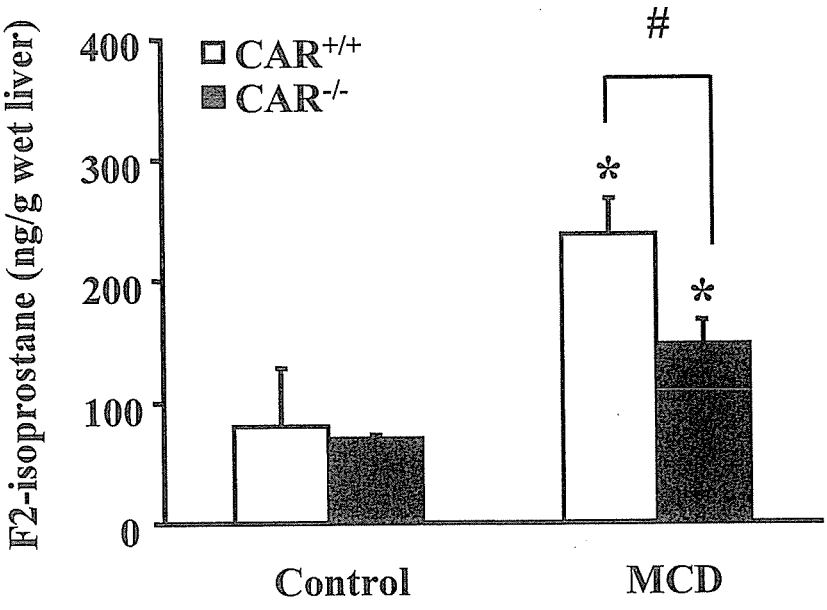

a
b
c
d
V
V
V
V
x400

Figure 3

A


F2-isoprostane (ng/g wet liver)
400
300
200
100
0
CAR+/+
CAR-/-
#
*
*
Control
MCD

B

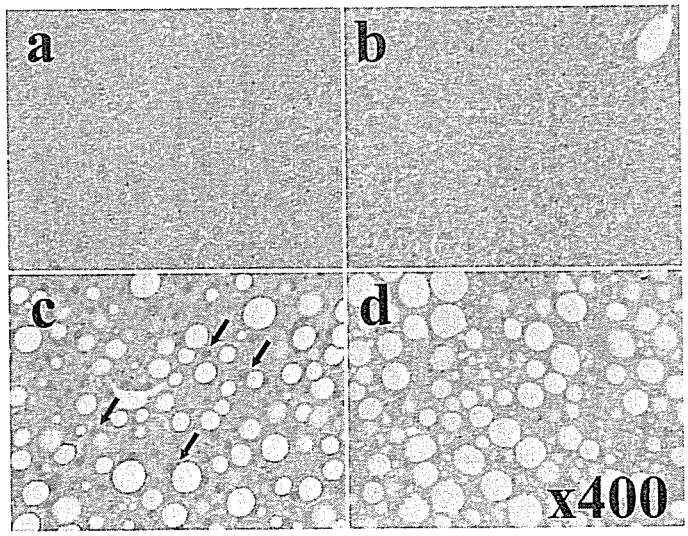
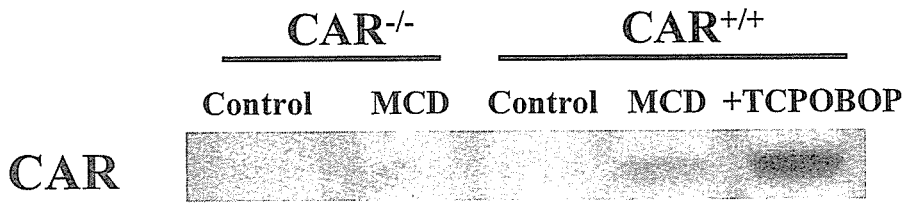

a
b
c
d
x400

Figure 4

A


CAR-/-
CAR+/+
Control
MCD
Control
MCD
+TCPOBOP
CAR

B

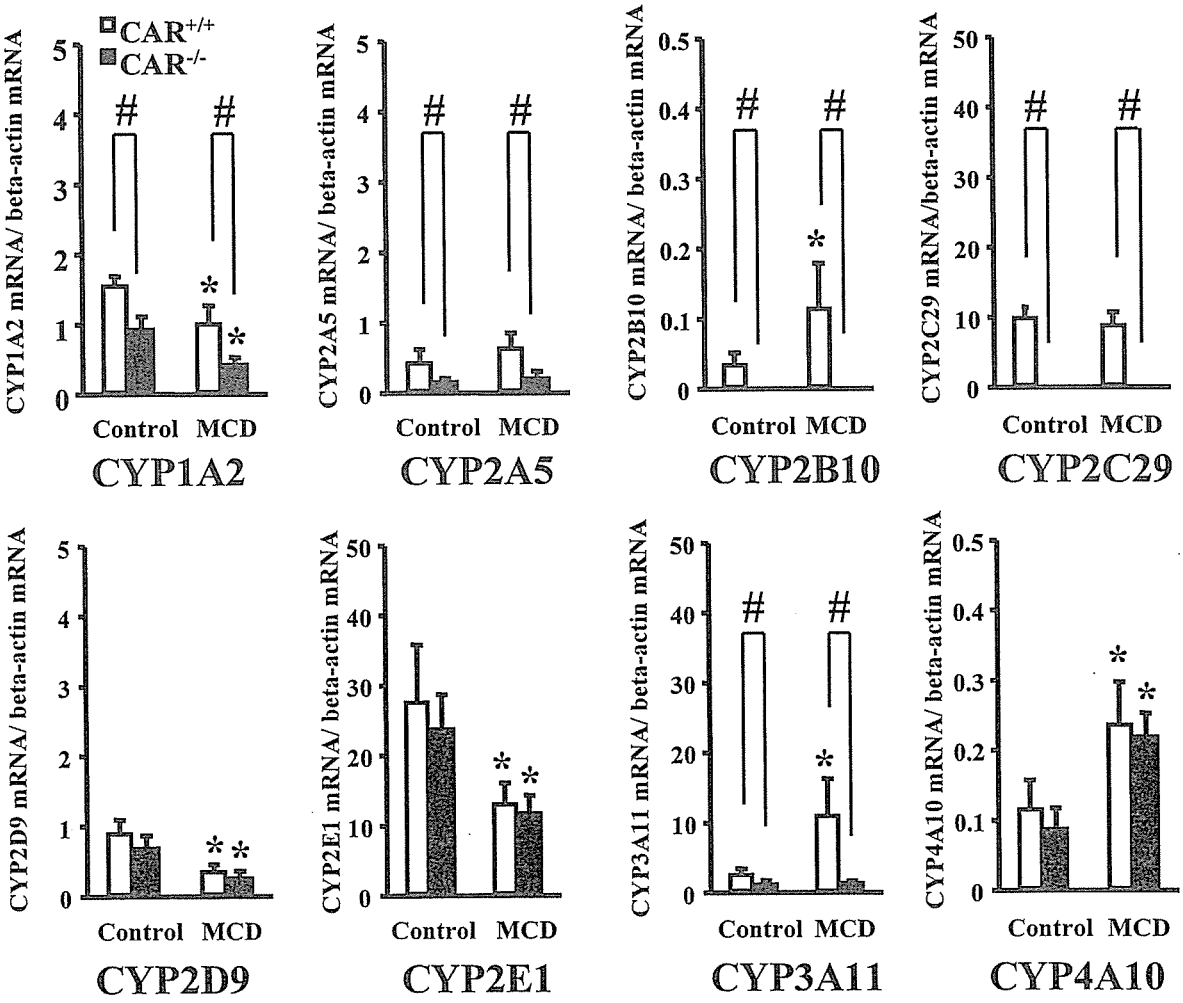

CAR+/+
CAR-/-
CYP1A2 mRNA/ beta-actin mRNA
CYP2A5 mRNA/ beta-actin mRNA
CYP2B10 mRNA/ beta-actin mRNA
CYP2C29 mRNA/beta-actin mRNA
CYP2D9 mRNA/ beta-actin mRNA
CYP2E1 mRNA/ beta-actin mRNA
CYP3A11 mRNA/ beta-actin mRNA
CYP4A10 mRNA/ beta-actin mRNA
Control MCD
CYP1A2
CYP2A5
CYP2B10
CYP2C29
CYP2D9
CYP2E1
CYP3A11
CYP4A10

Figure 4

C

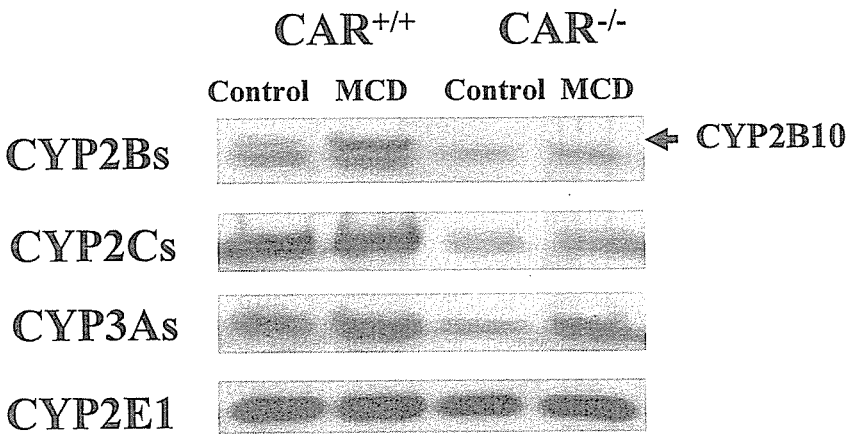
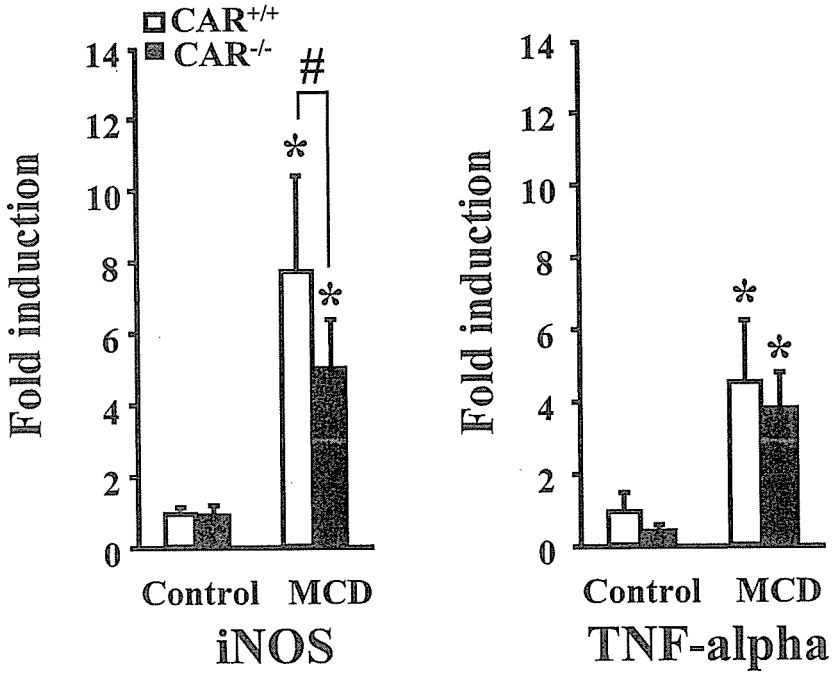
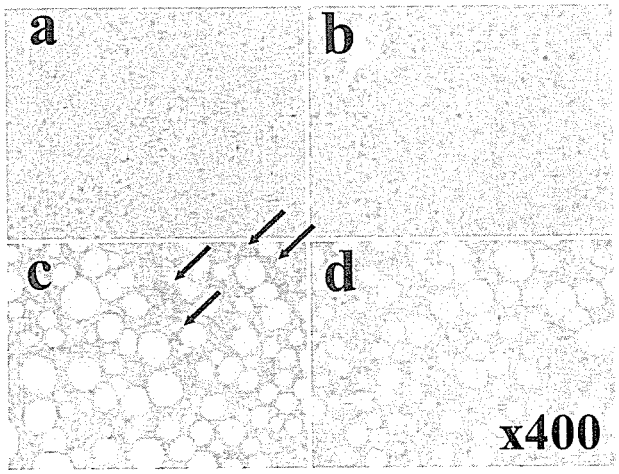

CAR+/+
CAR-/-
Control
MCD
Control
MCD
CYP2Bs
CYP2B10
CYP2Cs
CYP3As
CYP2E1

Figure 5

A


CAR+/+
CAR-/-
Fold induction
14
12
10
8
6
4
2
0
#
*
*
Control
MCD
iNOS
Fold induction
14
12
10
8
6
4
2
0
*
*
Control
MCD
TNF-alpha

B


a
b
c
d
x400

C

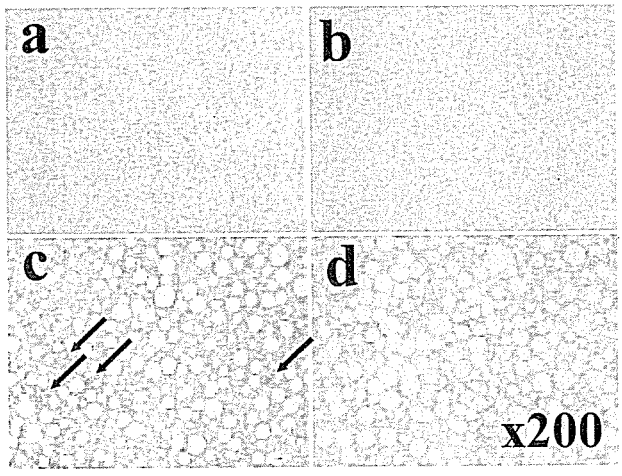

a
b
c
d
x200

Figure 6

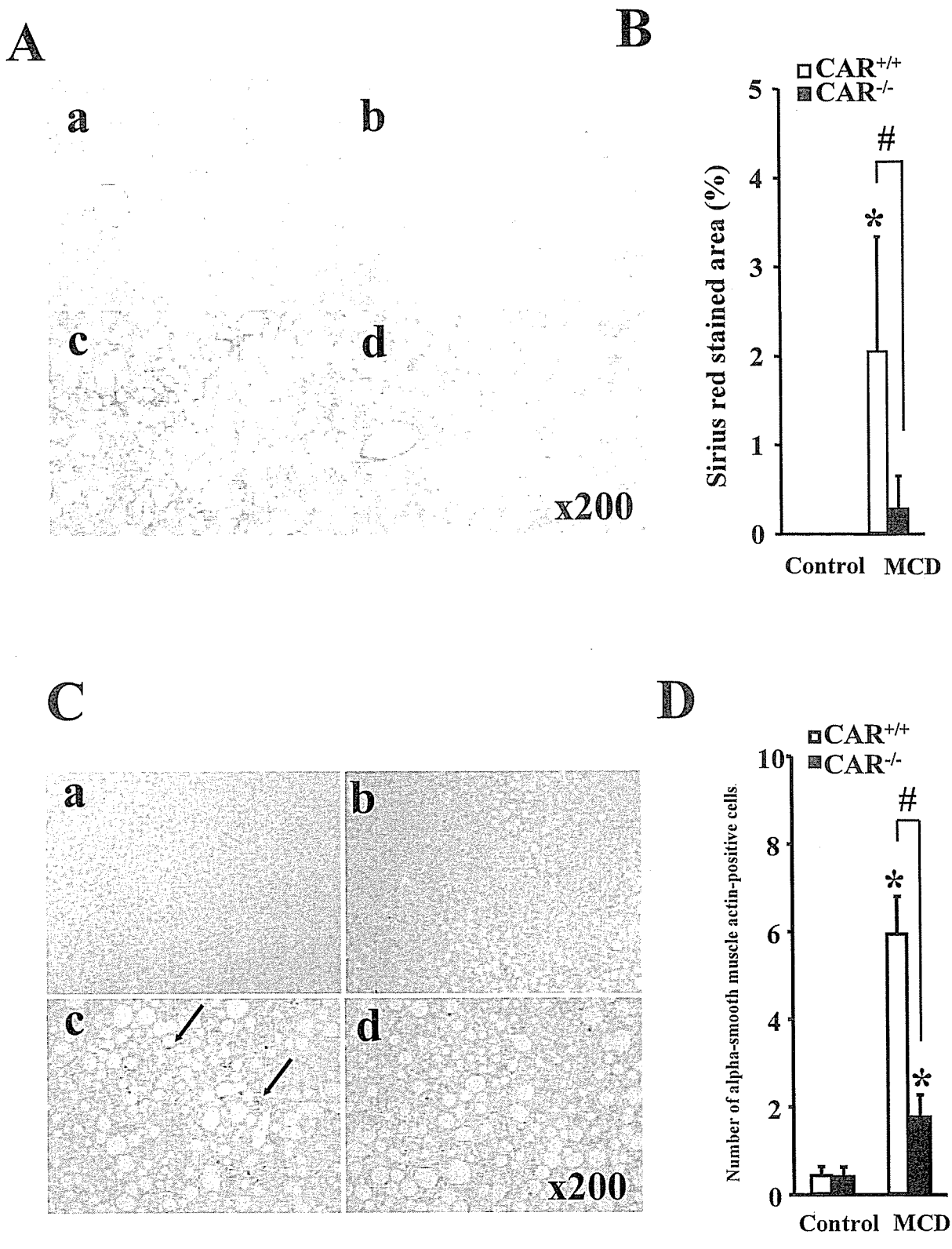
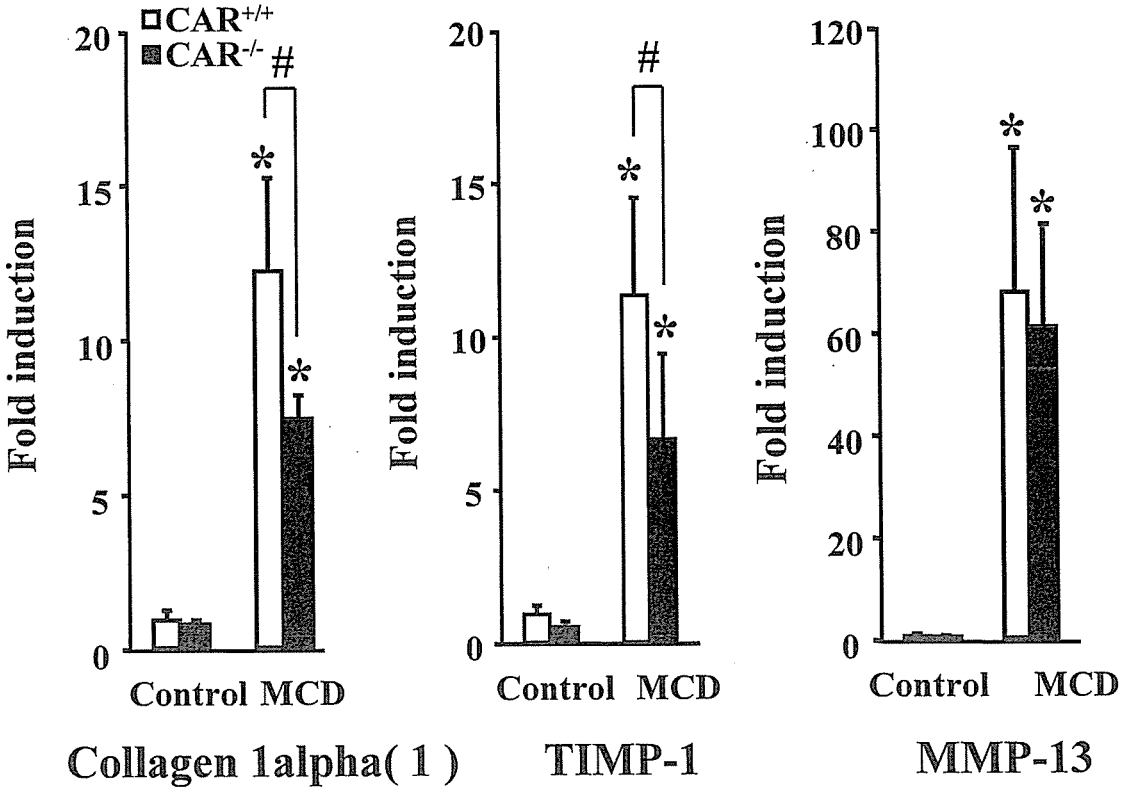

A
a
b
c
d
x200
B
CAR+/+
CAR-/-
Sirius red stained area (%)
0
1
2
3
4
5
#
*
Control
MCD
C
a
b
c
d
x200
D
CAR+/+
CAR-/-
Number of alpha-smooth muscle actin-positive cells
0
2
4
6
8
10
#
*
*
Control
MCD

Figure 7


□CAR+/+
■CAR-/-
Fold induction
20
15
10
5
0
#
*
*
Control MCD
Collagen 1alpha(1)
Fold induction
20
15
10
5
0
#
*
*
Control MCD
TIMP-1
Fold induction
120
100
80
60
40
20
0
*
*
Control MCD
MMP-13

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Integrative roles of transforming growth factor-α in the cytoprotection mechanisms of gastric mucosal injury

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Abstract

Background: Transforming growth factor α (TGFα) protects against gastric mucosal injury and facilitates wound healing. However, its overexpression is known to induce hypertrophic gastropathy resembling Menetrier's disease in transgenic (TG) mice on an FVB background, as one of the authors reported previously. We studied another TGFα-expressing mouse line on a CD1 background, whose gastric mucosa appears normal. Since this TG mouse had a strong resistance to ethanol-induced gastric injury, we considered the long-term effect of TGFα on several gastric protection mechanisms.

Methods: TGFα-expressing transgenic (TG) mouse lines bearing human TGFα cDNA under the control of the mouse metallothionein gene I promoter were generated on a CD1 mouse background, and analyzed their ethanol injury-resistant phenotypes produced by TGFα.

Results: In the TG mucosa, blood flow was well maintained after ethanol injury. Further, neural and inducible types of NO synthases were consistently and widely expressed in the TG mucosa, compared with the limited distribution of neural type NO synthase in the luminal pit region of the wild-type (WT) mucosa. COX-2 and its upstream transcription factor NfkB were constitutively elevated in the TG mucosa even before ethanol administration, whereas they were induced in the same region of the WT mucosa only after ethanol injury. Two anti-apoptotic proteins, HSP70 and Bcl-2, were upregulated in the TG mucosa even before ethanol administration, while they were not expressed in the WT mucosa before the injury. Furthermore, pro-caspase 3 activation was inhibited in the TG mucosa, while it was converted to the active form in the WT mucosa following ethanol administration.

Conclusion: We conclude that TGFα maintains the gastric mucosal defense against gastric injury by integrating other cytoprotective mechanisms.

Background

Ethanol has long been used to generate a hemorrhagic gastric mucosal injury in rodents [1]. With oral administration of absolute or hydrochloric acid-acidified ethanol, extensive hemorrhagic lesions can be produced in the gastric mucosa. This experimental gastric injury model has been frequently used for gastric mucosal protection and restitution studies. Indeed, a number of cytoprotective compounds have been tested to evaluate their potency in resisting gastric injury, including gastrointestinal hormones, growth factors, prostaglandins, bioactive amines, and mild irritants such as capsaicin [2-5]. Among these compounds, epidermal growth factor (EGF) family growth factors, notably transforming growth factor α (TGFα), have been reported as potent cytoprotective compounds against ethanol-, acetic acid-, or aspirin-induced gastric injury [2,4].

TGFα was expressed in the rat gastric mucosa after oral administration of acidified taurocholate or hydrochloric acid, suggesting its reparative role in gastric injury [6]. TGFα given intraperitoneally protected against ethanol-induced gastric injury with a significant increase in adherent gastric mucin in rats [7]. The cytoprotective function of TGFα appeared to be mediated by activation of phospholipase C-γ1, but not by prostaglandins, and was independent of the anti-secretory effect of TGFα [7]. On the other hand, prostaglandins and their synthetic enzyme, cyclooxygenase (COX), have long been proposed as cytoprotective factors against gastric injury [5,8]. EGF has been shown to express an inducible COX isoform, COX-2, in the RGM1 rat gastric mucosal cell line through an ERK MAP kinase pathway [9] and in Swiss 3T3 fibloblasts, especially by co-stimulation with gastrin [10]. Furthermore, COX-2 expression by heparin-binding EGF-like growth factor, a member of the EGF family growth factors, was blocked by a specific EGF receptor (EGF-R) inhibitor in the rat gastric mucosa [11]. Thus, the EGF-R signal appears to mediate COX-2 induction in the gastric mucosa.

The most important cytoprotective mechanisms include gastric mucosal blood flow. EGF and TGFα have been shown to exert cytoprotective effects via stimulation of capsaicin-sensitive neurons with release of calcitonin gene-related peptide (CGRP) and nitric oxide (NO) in rats exposed to orogastric ethanol administration, resulting in an increase in gastric mucosal blood flow [11,12]. In contrast, Konturek et al. minimized the role of the

CGRP-dependent increase in gastric blood flow by demonstrating that the administration of NO-releasing aspirin offered protection against absolute ethanol-induced gastric injury even in capsaicin-denervated rats, resulting in the upregulation of heat shock protein 70 (HSP70) expression and attenuation of the oxidative injury by strong upregulation of antioxidative genes such as zinc copper superoxide dismutase (SOD) and glutathione peroxidase [13].

TGFα was immunostained predominantly in GSM cells along the gastric pit in humans [14], intensively in gastric surface mucosal (GSM) cells over the proliferative zone and weakly along the glandular region under the proliferative zone in rats [15], and markedly along the mucous neck cell region in FVB/N strain mice [16]. On the other hand, EGF-R was immunostained in GSM cells lining the foveolar pit and mucous neck cell region to the lower glandular region in humans [17]. In rats, EGF-R was localized at the top-pit region and in the chief cell clusters at the bottom of the gastric glands [15]. Although TGFα is known to induce cell proliferation in primary-cultured rat gastric mucosal cells [18], and hyperplastic proliferation in the mucosa on the TGFα-expressing transgenic (TG) mouse [19,20], the distribution of TGFα and EGF-R in terminally differentiated gastric mucosal cells suggests that TGFα exerts non-proliferative functions including anti-apoptotic effects and gastric epithelial barrier formation in an autocrine/paracrine manner. For example, TGFα inhibited apoptosis of a mouse gastric mucosal cell line, GSM06, by expressing anti-apoptotic Bcl-2 family proteins through an NF-κB-dependent pathway [21]. Gastric apical and basolateral EGF-R has been shown to induce a decrease in paracellular permeability to acid for the protection of glandular cells in an acidic environment [18,22]. Thus, TGFα exerts both proliferative and non-proliferative effects on gastric mucosal functions, and these diverse TGFα effects should be re-evaluated, at least in terms of whether their results are derived from short-term treatment using culture cell lines or from long-term expression using TGFα-transgene-expressing animals.

A decade ago, one of the authors, H. Takagi, generated TGFα-expressing TG mouse lines under the control of the metallothionein gene promoter, and one of these lines (MT100) exhibited hypertrophic gastropathy resembling Menetrier's disease, as similarly shown by other investigators [19,20]. In contrast, another TG line (MT42) displayed a normal-appearing gastric mucosa, although TGFα transgene was expressed to a similar

extent in both TG lines [20]. Since the MT42 line displayed a surprising level of resistance to ethanol-induced injury to the gastric mucosa, we considered that it could be useful for exploring the non-proliferative effects of TGFα such as mucosal resistance to ethanol injury. In the present study, we investigated the long-term effects of TGFα on ethanol-induced gastric injury by analyzing changes in cytoprotective and anti-apoptotic regulatory factors, including gastric blood flow, NO synthase, COX-2, and apoptosis-associated proteins such as HSP70, Bcl-2, and caspase 3.

Methods

TGFα-expressing Transgenic Mouse and Ethanol-induced Gastric Injury

TGFα-expressing transgenic (TG) mouse lines bearing human TGFα cDNA under the control of the mouse metallothionein gene I promoter were generated on a CD1 or FVB mouse background, as described previously [20]. One of the TG lines, MT100 on an FVB mouse background, displayed hypertrophic gastropathy resembling Menetrier's disease [20], whereas the other line, MT42 on a CD1 mouse background, displayed a normal-appearing gastric mucosa. The phenotype of the TGFα-TG mouse appeared to depend on the mouse strain, because both the MT100 and MT42 lines expressed a similar level of the TGFα transgene in the stomach [20]. In the present study, we used the MT42 line of TGFα-expressing TG male mice and CD1 wild type (WT) male mice at ages 6 to 8 weeks. The animals were maintained under controlled light (7:00 AM to 7:00 PM) with food and water provided *ad libitum*.

The mice, weighing 30-35 g, were fasted for 24 h before the experiment but were allowed free access to water. All experimental procedures affecting the mice were approved by the Animal Care and Use Review Committee at the Gunma University. The mice were then given orogastric acidified ethanol (100 μl; 60% ethanol in 0.15 mol/L HCl). Three, six, and twelve hours after the acidified ethanol administration the mice were sacrificed for the evaluation of gastric injury and immunohistochemical analyses, and for RNA and protein extraction for real time PCR, Northern and Western blot analyses.

Physiological Studies

Gastric acid secretion was measured as described previously [20,23]. Briefly, WT and TG mice were fasted for 3 h and then anesthetized with ether. After the incision of abdominal wall, the pyrolus was ligated, and the incision was sutured. The gastric fluid was collected 4 h after the pyrolus ligation. For maximal acid output, acid secretion was stimulated by injecting pentagastrin subcutaneously (500 μg/kg body weight). The gastric fluid was titrated with 0.1 N NaOH to pH 7.0 using a microtitrator.

Gastric mucosal blood flow was measured with a laser Doppler flowmeter (model SFA211, Advance Co., Tokyo) by placing a probe on the surface of the gastric mucosa, as

described previously [24]. The flow signal was monitored with the MacLab recording program. The blood flow was expressed relative to the basal level.

Morphological Studies

The thickness of the gastric mucosa was measured from the highest part to the bottom of the gland with a micrometer. Similarly, the gastric injury lesions were measured (width and longitude) with a micrometer to calculate the injured area.

For immunostaining, we obtained antibodies as follows. Polyclonal antibody to H^+/K^+-ATPase was obtained by injecting rabbit parietal cell-microsomal fractions into mice [25]. Rabbit polyclonal antibody to metallothionein was a kind gift from Dr. Nagamine, Gunma University School of Health Sciences [26]. We purchased antibodies as follows: rabbit polyclonal antibody to TGFα, rabbit polyclonal antibody to EGF-R, goat polyclonal antibody to COX-2, rabbit polyclonal antibody to NFκB, and mouse monoclonal antibody to nNOS and iNOS from Santa Cruz Biotech. (Santa Cruz, CA); mouse monoclonal antibody to proliferative-cell nuclear antigen (PCNA) from Zymed Lab. (South San Francisco, CA); sheep antibody to human pepsinogen II from BioPur AB (Bubendorf, Switzerland), which also reacts to mouse pepsinogen; rabbit polyclonal antibody to HSP70 from Stressgen Biotech. (Victoria, BC, Canada); and mouse monoclonal antibody to Bcl-2 from BD Biosciences (San Diego, CA).

For horseradish peroxidase (HRP)-catalyzing immunohistochemical staining, minced stomachs were fixed in neutralized 10% formalin at 4^0C for 24 h, and was then embedded in paraffin for microtome sectioning. The deparaffinized tissue sections were rehydrated and incubated in 3% hydrogen peroxide solution to inhibit endogenous peroxidase activity. Then the sections were incubated with a first antibody described above with an appropriate dilution at room temperature for 30 min. A secondary biotinylated antibody was selected based on the first antibody-producing animal species, and was then used with HRP-conjugated streptavidin. The HRP reaction was performed with 3-amino-9-ethyl-carbazole and hydrogen peroxide according to the instructions supplied with the streptavidin-biotin staining Vectastatin Elite Kit (Vectors Laboratories, Burlingame, CA). Positive stainings appeared brown in color.

For immunofluorescent staining, minced stomachs were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 4^0C for 24 h. Small pieces of minced tissue underwent saccharose replacement and were embedded in OCT-compound in preparation for a frozen section using a microtome. For the primary immunoreaction, sliced sections were probed with a first antibody, and were then incubated either with indodicarbocyanide (Cy3)-conjugated affinity-purified donkey anti-rabbit, anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), or fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Jackson ImmunoResearch), as a secondary antibody. The secondary antibody IgG was selected based on the species on which the first antibody was raised.

In situ detection of apoptotic cells was performed using the In Situ Apoptosis Detection Kit (TAKARA BIO INC. Tokyo, Japan). The kit contained a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end-labelling (TUNEL) assay system. Stomach sections were permeabilized with proteinase K, and the 3'-OH ends of the DNA fragments were then stained following the instructions supplied with the kit. The nuclei stained dark brown were considered to be apoptotic cells.

RNA Analysis

Stomachs were trimmed and the total RNAs were extracted using TRIzol (Gibco BRL, Tokyo) according to the protocol supplied by the manufacturer. For Northern blot, the total RNA was electrophoresed on a 1.0% agarose gel and was transferred to a nylon membrane (Amersham Pharmacia Biotech, Tokyo). Hybridization was performed with a probe of the human TGFα cDNA (917 bp) labeled with [α-^{32}P] deoxy-CTP. This probe recognizes human TGFα mRNA, but does not mouse TGF mRNA.

Mouse TGFα mRNA level was measured using a real time polymerase chain reaction (PCR) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The real time PCR was carried out using the ABI Prism 7700 Sequence Detector and soft (Applied Biosystems, Foster City, CA) using the DNA fluorescent dye SYBR Green detection. Primers were designed using the Primer Express design software (Applied Biosystems). The final result for each sample was normalized by the respective GAPDH value. Primer sequences are as follows: mouse TGFα: 5'-CCTGAGCACCCGAAGAT-3'

and 5'-CCTTCCCTCATGCCTTACT-3'; GAPDH: 5'-GTCGTGGATCTGACGTGCC-3' and 5'-TGCCTGCTTCACCACCTTCT-3'.

Western Blot Analysis

Gastric tissue samples were trimmed and total protein from the gastric mucosa was homogenized in RIPA buffer containing a cocktail of protease inhibitors (phenylmethylsulphonyl fluoride, pepstatin, leupeptin, and aprotinin) (Roche Diagnostics, Mannheim, Germany). The supernatant was separated on an SDS-PAGE gel, and was then blotted to a polyvinylidene difluoride membrane. The membrane was probed with the following antibodies: antibodies to iNOS, COX-1, COX-2, HSP70, and Bcl-2, which were the same ones used for morphological studies; mouse monoclonal antibody to Bax (Santa Cruz Biotech.); rabbit polyclonal antibody to caspase 3 (Santa Cruz Biotech.); and mouse monoclonal antibody to actin filaments (Santa Cruz Biotech.). Antibody-reacted bands were detected utilizing an ECL detection system (Amersham, Buckinghamshire, UK).

Measurement of Gastrin

Serum gastrin was measured using a gastrin radioimmunoassay kit (gastrin RIA kit, Dainabot, Tokyo, Japan). The antibody is specific for gastrin with an amide moiety at its carboxyl terminus.

Measurement of Prostaglandin E_2

The gastric mucosa was homogenized at 4^0C in lysis buffer. Homogenates were centrifuged at 12000 rpm for 20 min, and the supernatants were subjected to prostaglandin E_2 assay using a Prostaglandin E_2 Monoclonal Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed by repeated measure analysis of variance (ANOVA) for the total length of the gastric lesions by unpaired t-test for gastric mucosal blood flow

decrease ratio. All values are expressed as the mean ± SE. $P < 0.05$ was accepted as statistically significant.

Results

Characterization of the TGFα-expressing TG Mouse Gastric Mucosa

The WT CD1 strain and TGFα-transgene expressing MT42 line mice grew similarly and showed no noticeable difference in their features, behaviors, or lifespans. The greatest thickness observed for the gastric wall and fundic glands were 3.50 ± 0.18 mm and 2.33 ± 0.20 mm, respectively, in the WT mice, and 3.85 ± 0.24 mm and 2.63 ± 0.16 mm, respectively, in the TG mice; there were no significant differences in the shape of the gastric mucosae and glands between the WT and TG mice. In addition to the normal appearance of the gastric mucosa, this TG mouse line also had a normal-appearing liver, pancreas, and other organs.

We then compared acid secretion capacity between the WT and TG mice. In the WT mice, maximal acid output increased significantly from basal level of 25 ± 1.4 mEq/h to 50 ± 5.8 mEq/h after the pentagastrin stimulation. By contrast, in the TG mice, maximal acid output increased to a less extent to 30 ± 5.7 mEq/h from the basal output of 23 ± 0.7 mEq/h (n=5 in WT and TG mice). The blunted acid output may reflect the reduced parietal cell mass in the TG mucosa (Figure 1e).

Expression of TGFα Transgene

TGFα-expressing cell-types were identified based on cell-type-specific immunostaining and their mucosal location. TGFα was reportedly immunostained along the GSM cells in the gastric pit in humans [14] and rats [15], and along the mucous neck cell region in the FVB strain mice [16]. In the present study, brown-colored TGFα-positive cells were distributed along the foveolar region of both the WT and TG mucosae, and along the lower glandular region of the TG mucosa (Figure 1a). The TGFα-positive cells along the foveolar region appeared to be gastric pit cells, based by their location (Figure 1b, upper panel), and those along the lower glandular region of the TG mucosa did not overlap with the green-colored H^+/K^+-ATPase-positive parietal cells (Figure 1b, lower panel), but did overlap with the pepsinogen-positive chief cells (data not shown). The antibody we used for immunostaining for TGFα could not distinguish between human and mouse TGFα, but its mRNA probe could do so for the Northern blot analysis. The human TGFα–transgene was expressed in the