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Ceramide triggers caspase activation during γ-radiation-induced apoptosis of human glioma cells lacking functional p53

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Abstract. We have previously shown that treatment of human glioma U87-MG cells expressing wild-type p53 with a DNA topoisomerase II inhibitor, etoposide resulted in ceramide-dependent apoptotic cell death. However, U87-W E6 cells lacking functional p53 due to the expression of human papilloma virus type 16 (HPV-16) E6 oncoprotein were resistant to etoposide. In order to gain insight into the roles of p53 and ceramide in γ-radiation-induced glioma cell death, we used U87-W E6 and vector-infected U87-LXSN cells. U87-LXSN glioma cells expressing wild-type p53 were relatively resistant to γ-radiation. U87-W E6 cells, which lost functional p53, became susceptible to radiation-induced apoptosis. Activation of caspase-3, and formation of ceramide by acid sphingomyelinase, but not by neutral sphingomyelinase, were associated with p53-independent apoptosis. Radiation-induced caspase activation and apoptotic death in U87-W E6 cells were modified by the agents which affected ceramide metabolism. SR33557, an inhibitor of acid sphingomyelinase, suppressed radiation-induced caspase activation and then apoptotic cell death. In contrast, *N*-oleoylethanolamine (OE) and D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), which inhibit ceramidase and UDP-glucose:ceramide glucosyltransferase-1, respectively, and then augment ceramide formation, enhanced radiation-induced caspase activation. These results indicate that glioma cells with functional p53 were relatively resistant to γ-radiation, and that ceramide may play an important role in caspase activation during γ-radiation-induced apoptosis of glioma cells lacking functional p53.

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Introduction

The median survival for human patients with glioblastoma, the most malignant form of glial tumor in the central nervous system, is in the range of 1 year, even with aggressive multi-modality treatment. Involved-field radiotherapy is the most important therapeutic approach and has been shown to prolong median survival for 6-8 months (1,2). Treatment of tumors with the DNA-damaging agents elicits apoptotic death and is initially an effective means to arrest malignancy (3). However, following initial success to prevent tumor progression, resistance to further treatment often occurs (4). The poor clinical outcome of patients with glioma is associated with a characteristic *in vivo* and *in vitro* radio-resistance of these brain tumors compared to other human tumors. However, the detailed molecular mechanisms of radiation resistance as well as radiation-induced apoptotic death of glioma cells are poorly understood.

During the past decade the pathways and components involving apoptotic death were extensively studied. Several molecules are emerging as regulators of apoptosis. For example, caspases are a family of cysteine proteases, which play a central role in apoptotic cascade (5). Elevation of intracellular ceramide levels occurs in response to a variety of apoptotic stimuli. Ceramide, usually a product of sphingomyelinase (SMase)-catalyzed hydrolysis of sphingomyelin (SM), is regarded as a mediator of apoptosis (6). However, the actual contribution of ceramide to the apoptotic response remains to be clarified. p53 protein is prerequisite to arrest cell cycle for the repair of damage DNA, or alternatively to promote apoptosis, when DNA damage is too extreme (7). p53 stimulates transcriptional activation to regulate the gene products of proapoptotic proteins (8). In fact, cells with functional p53 are usually more sensitive to chemotherapeutic agents, such as etoposide, than those with mutant p53 (9,10).

We have previously shown p53-dependent ceramide formation in human glioma cells in response to etoposide using U87-MG cells expressing wild-type p53 and U87-W E6 cells lacking functional p53 (11) due to the expression of human papillomavirus type 16 (HPV-16) E6 oncoprotein (12). However, recent reports indicated the possibility that glioma cells with p53 mutations were more sensitive to γ-radiation

than those with wild-type (functional) p53 (13,14). In the present study, we have investigated the roles of p53 and ceramide in γ-radiation-induced apoptosis using these cell lines. Our data indicate that functional p53 rather protects glioma cells from γ-radiation-induced apoptosis. In glioma cells lacking functional p53, ceramide plays an important role in caspase activation.

Materials and methods

Materials. Hoechst 33258 (bisbenzimide) staining dye was obtained from Wako (Osaka, Japan). *N*-(cis-9-octadecenoyl) ethanolamine (*N*-oleoylethanolamine) (OE) was from Sigma (St. Louis, MO, USA). Immobilon-P membranes were from Millipore (Bedford, MA, USA). D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of UDP-glucose:ceramide glucosyltransferase-1 (GlcT-1), was purchased from Matreya (Chalfont, PA, USA). ECL Western blotting detection reagents were from Amersham (Buckinghamshire, UK). [1-^{14}C]Palmitic acid (55.0 mCi/mmol) was from ICN (Irvine, CA, USA). [*choline-methyl*-^{14}C]SM (52.0 mCi/mmol) was from NEN Life Science Products (Boston, MA, USA). CPP32/caspase-3 Fluorometric Protease Assay kit was obtained from BioDynamics Laboratory Inc. (Tokyo, Japan). Mch6/caspase-9 Fluorometric Protease Assay kit was obtained from BioVision Reserch Products (Mountain View, CA, USA). A broad-spectrum caspase inhibitor, z-VAD·FMK and a caspase 3 inhibitor, z-DEVD·FMK were from Enzyme Systems Products (Dubin, CA, USA). Anti-poly(ADP-ribose) polymerase (PARP) polyclonal antibody was from (Santa Cruz Biotechnology, CA, USA). (2-isopropyl-1-(4-[3-*N*-methyl-*N*-(3,4-dimethoxybphenethyl) amino] propyloxy)benzene-sulfonyl)indolizine (SR33557) was kindly supplied from Dr Jean-Marc Herbert (Sanofi-Synthelabo Recherche, France). BCA protein assay reagents were obtained from Pierce (Rockford, IL, USA). Other chemicals were of the highest quality available.

Cell culture and stimulation. The human U87-MG glioblastoma cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Functional p53 can be inactivated by the expression of type 16 human papilloma virus (HPV-16) E6 oncoprotein, which binds p53 and accelerates its proteolytic degradation through the ubiquitin pathway (12). U87-LXSN and U87-W E6 cell lines were obtained by infecting U87-MG cells with LXSN and LXSN-16E6SD retroviruses, respectively, followed by drug selection with 800 μg/ml G418 for 2 weeks (11). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin (FBS/DMEM) in a humidified atmosphere containing 5% CO_2 at 37°C. Prior to γ-irradiation, the cells were plated at a density of 1x10^4/ml and cultured for 3 days. Cells were irradiated using a ^{137}Cs source (IBL 437C Irradiator, CIS BioInternational, Gif-sur-Yvette, France) at a dose rate of 6.0 Gy/min.

Determination of apoptosis. Apoptotic cells stained with Hoechst 33258 were quantified by fluorescent microscopic analysis (15). Briefly, cells were fixed in 1% glutaraldehyde for 30 min. The cells were then stained with 10 μM Hoechst 33258 for 10 min. Nuclear morphology was observed under a fluorescent microscope (Olympus BX60, Tokyo, Japan).

Activity of caspase proteases. The DEVD- and LEHD-cleaving activities were measured by fluorometric protease assay kits according to the manufacturer's instructions (16). Cleavage of the fluorogenic peptide substrates was monitored by 7-amino-4-methylcoumarin (AMC) liberation in a Fluoroscan Ascent 1.6 plate reader (Labsystems, Stockholm, Sweden) with excitation at 380 nm and emission at 460 nm. One unit was defined as the amount of enzyme required to release 0.22 nmol AMC/min at 37°C.

Western blot analysis. Cells were solubilized with ice-cold lysis buffer (17,18). Extracted proteins (60 μg/well) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, and were electrophoretically transferred onto Immobilon-P membrane. The membranes were probed with anti-PARP antibody. Detection was performed with ECL system.

SMase assay. Membrane fraction was prepared as described previously (15,18). The activities of both N- and A-SMases were determined using a mixed micelle assay system (18). For measuring N-SMase activity, the membrane fractions (20 μg protein) were mixed with [*choline-methyl*-^{14}C]SM (40000 c.p.m. in 1 nmol of bovine brain SM in 0.25 % Triton X-100 solubilized by sonication) in 0.1 M Tris/HCl buffer (pH 7.4) containing 6 mM $MgCl_2$ and the reaction mixture was incubated for 30 min at 37°C. A-SMase activity in membrane was measured as above except that the Tris/HCl buffer was replaced with 0.1 M sodium acetate buffer (pH 5.5) containing 5 mM EDTA.

Measurement of cellular ceramide. To radio-label sphingolipids, cells were labeled with [^{14}C]palmitic acid (1 μCi/ml) for 24 h. Lipids extracted from cells were first treated in 0.1 M KOH in chloroform:methanol (1:2, v/v) at 37°C for 1 h (18). After separation of lipids by thin-layer chromatography, the radioactivity of [^{14}C]ceramide was counted in a scintillation counter (Beckman LS-6500). The changes in [^{14}C]ceramide were normalized based on 100 μg of protein.

Statistical analysis. Data are expressed as means ± SD Significance was assessed by two-way ANOVA, followed by Scheffe's pos-hoc test. $P<0.01$ was considered as significant.

Results

Sensitivity to γ-radiation according to p53 status of human glioma cells. In the previous study (11), we have shown that human glioma U87-MG cells expressing wild-type p53 underwent ceramide-dependent apoptotic cell death in response to a DNA-damaging drug, etoposide. In contrast, U87-W E6 cells lacking functional p53 due to the expression of HPV-16 E6 oncoprotein (12) were resistant to this DNA-damaging agent. Against our expectation, however, U87-W E6 cells were more sensitive to γ-radiation than U87-LXSN cells as shown in Table I. U87-LXSN cells expressing functional p53

Table I. γ-radiation-induced apoptosis in human glioma cells with or without functional p53.

Dose (Gy)	Apoptosis (% of total) U87-LXSN	U87-Wild E6
0	0.8±0.2	0.2±0.1
5	1.7±0.9	4.3±1.4
10	1.9±0.5	11.0±0.4
25	2.5±0.7	19.8±1.8

Radiation-induced apoptosis of human glioma cells. U87-LXSN and U87-W E6 cells were exposed to the indicated doses of γ-radiation and incubated for 3 days. The cells with fragmented and condensed nuclei were counted in over 1000 cells under a fluorescent microscope. Data are means ± SD from three independent experiments, each performed in triplicate.

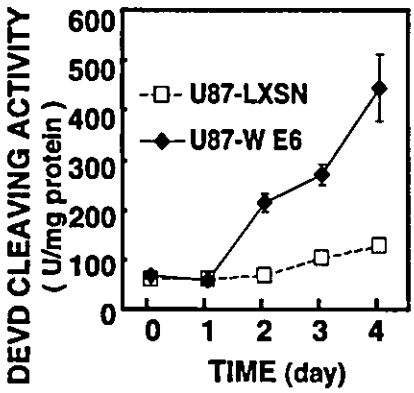

Figure 1. Activation of caspase-3(-like) proteases by γ-irradiation. U87-LXSN and U87-W E6 cells were irradiated at 25 Gy and cultured for the indicated periods. The cellular extracts (50 μg of protein) were incubated with 50 μM Ac-DEVD-MCA, a substrate for caspase-3(-like) protease at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin were measured using a spectro-fluorometer. Data are means ± SD from three independent experiments, each performed in triplicate.

were almost resistant to γ-radiation. Marked morphological changes were observed extensively in U87-W E6 cells, but scarcely in U87-LXSN cells, at 72 h after 25 Gy irradiation under a phase contrast microscopy (data not shown). Cellular processes were retracted and the rounded cells were detached from culture flasks. The nuclear staining of U87-W E6 cells with Hoechst 33258 revealed typical apoptotic changes, condensation and fragmentation of the nuclei. These results indicate that apoptotic pathway in γ-irradiated glioma cells is p53-independent.

Caspase activation by γ-radiation. The caspase family has been suggested to play a pivotal role in apoptosis (5). The DEVD-cleaving activity continuously increased from days 2 to 4 after γ-radiation in U87-W E6 cells (Fig. 1), indicating activation of caspase-3(-like) protease. Processing of caspase-3 was also confirmed by the time-dependent degradation of

Table II. γ-radiation-induced ceramide formation correlated with apoptotic cell death.

Treatments	Ceramide % of control	Formation (^{14}C dpm)	Apoptosis (% of total)
None	0	8612	0
25 Gy γ-radiation	100	26831	23
+ SR33557	35	14994	13
+ OE	224	49439	54
+ PDMP	210	46954	56
+ z-VAD·FMK	101	27099	13
+ z-DEVD·FMK	98	26483	14

U87-W E6 cells were preincubated with 5 μM SR33557, 25 μM OE, 100 μM PDMP, 100 μM z-VAD·FMK or z-DEVD·FMK, and then incubated for three days following 25 Gy irradiation. The radioactivity of [^{14}C]ceramide in non-irradiated control cells labeled with [^{14}C]palmitic acid was designated as 0% and that of cells treated with 25 Gy γ-radiation was designated as 100%. The cells with fragmented and condensed nuclei were counted under a fluorescent microscope. Data are means from two independent experiments, each performed in duplicate.

PARP, a well-known substrate of caspase-3 (data not shown). In addition, the LEHD-cleaving activity began to increase from day 1 and its gradually continuous increase was observed for 4 days after γ-irradiation (data not shown), indicating activation of caspase-9 protease. In contrast, in irradiated U87-LXSN cells, caspase activities were not significantly increased.

Formation of ceramide through activation of A-SMase. The changes of intracellular ceramide levels following γ-radiation were measured by the metabolic labeling of glioma cells with [^{14}C]palmitic acid. In response to 25 Gy γ-radiation, [^{14}C]ceramide level was significantly increased (Table II). γ-radiation gave rise to an increase in A-SMase activity at day 1 and its activity reached a peak at day 3 in U87-W E6 cells (Fig. 2). On the other hand, significant changes were not observed in N-SMase activity. In radio-resistant U87-LXSN cells, formation of ceramide and activation of A-SMase were hardly observed (data not shown).

Ceramide content affects γ-radiation-induced activation of caspase-3 and apoptosis. To further examine the role of ceramide during γ-radiation-mediated apoptosis in human glioma cells, ceramide levels were modulated by the agents, which inhibited the enzymes implicated in ceramide metabolism. SR33557, a potent inhibitor of A-SMase (19,20), suppressed [^{14}C]ceramide formation in [^{14}C]palmitic acid-labeled U87-W E6 cells (Table II). This A-SMase inhibitor blocked the γ-irradiation-induced increase of DEVD cleaving activity (Fig. 3).

OE, a ceramidase inhibitor (21), and PDMP, a GlcT-1 inhibitor (15,22,23), accelerated [^{14}C]ceramide formation

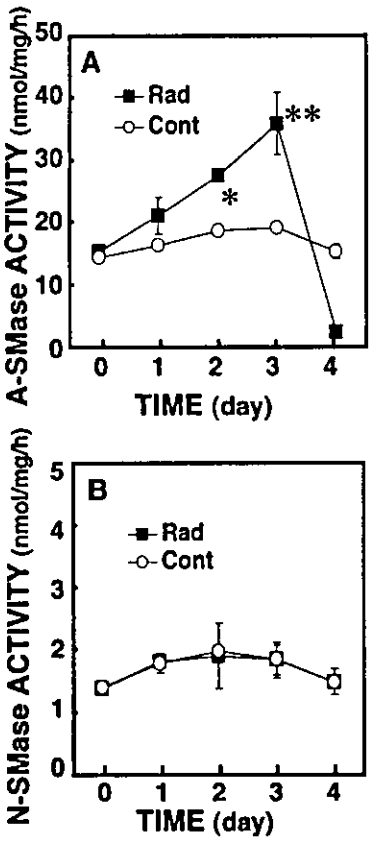

Figure 2. Changes in A- and N-SMase activities in U87-W E6 cells exposed to γ-radiation. The cells were irradiated at 25 Gy and cultured for the indicated periods. The activities of A-SMase (A) and N-SMase (B) were determined using a mixed micelle assay system with [*methyl*-^{14}C]SM at pH 7.5 and 5.5, respectively. Data are means ± SD from two independent experiments, each performed in triplicate. *P<0.01, **P<0.05 versus non-irradiated control: two-way ANOVA followed by Scheffe's post-hoc test.

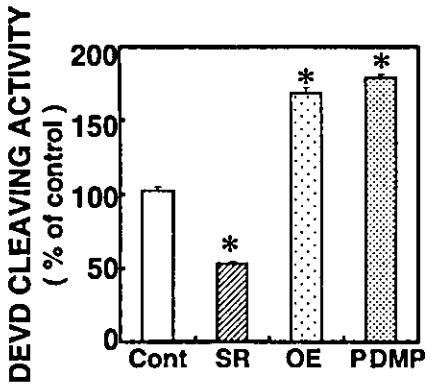

Figure 3. Activation of caspase-3(-like) protease in the absence or presence of SR33557, OE or PDMP in U87-W E6 cells. Caspase-3(-like) activity was measured fluorometrically. Data are means ± SD from three independent experiments, each performed in triplicate. *P<0.01 versus γ-irradiation alone: two-way ANOVA followed by Scheffe's post-hoc test.

in [^{14}C]palmitic acid-labeled U87-W E6 cells. At day 3, [^{14}C]ceramide level was almost doubled in the presence of 25 μM OE or 100 μM PDMP (Table II). Under the same circumstances, the number of apoptotic cells and DEVD-cleaving activity increased to >200% (Table II) and to nearly 180% (Fig. 3), respectively, compared to the cells treated with γ-radiation alone.

The increase in the number of apoptotic U87-W E6 cells induced by γ-irradiation was suppressed by z-VAD·FMK, a broad spectrum caspase inhibitor, and of z-DEVD·FMK, a selective caspase 3 inhibitor. The number of apoptotic cells at day 3 was decreased by almost a half in the presence of 100 μM z-VAD·FMK or z-DEVD·FMK (Table II). However, the γ-radiation-induced formation of [^{14}C]ceramide was not affected by these caspase inhibitors.

Discussion

In the previous study (11), we have demonstrated that p53 triggers ceramide-dependent apoptotic cascade in human glioma cells treated with etoposide. However, glioma cells with functional p53 were resistant to γ-radiation, as previously reported (13,14). Depletion of p53 by HPV-16 E6 protein resulted in increased cellular susceptibility to radiation-induced apoptosis. Therefore, in response to γ-radiation, functional p53 may drive cell cycle arrest at G1 phase rather than apoptosis, as proposed by Haas-Kogan *et al* (13).

Ceramide has been shown to exert potent proapoptotic effect in a variety of cell types (6). Defective ceramide generation has been associated with resistance to radiation-induced apoptosis in several types of cells (24,25). For example, in radiation-sensitive Jurkat T lymphoid cells and SCC61 squamous carcinoma cells, ceramide is produced following γ-radiation and thereafter induces activation of caspase cascade, whereas activation of this sequence is absent in radio-resistant SQ20B squamous cell carcinomas (25) and LNCaP prostate cancer cells (26). The formation of ceramide and activation of caspases were observed in U87-W E6 cells, but not in parent U87-LXSN cells, in response to γ-radiation. Inhibition of γ-radiation-induced ceramide formation in U87-W E6 cells by an inhibitor of A-SMase, SR33557 resulted in suppression of caspase-3 activation and apoptosis. In contrast, enhancement of ceramide formation by OE or PDMP potentiated caspase activation and then apoptotic cell death. The caspase inhibitors rescued U87-W E6 cells from γ-radiation-induced apoptosis but failed to inhibit ceramide formation. These results collectively suggest the possibility that ceramide triggers caspase activation during radiation-induced apoptosis of glioma cells.

Ceramide is mainly produced from SM by the action of N-SMase and/or A-SMase during apoptosis. N-SMase has been implicated in apoptosis caused by serum starvation (27), hypoxia (18), nitric oxide (28) and some chemotherapeutic agents (15,29). In glioma cells treated with etoposide, activation of N-SMase occurred at downstream of p53-mediated ROS formation (11). In contrast, A-SMase has been suggested to be activated in cells exposed to radiation (24), Fas (30), and TNF-α (31). These two types of SMases differ in subcellular location, pH optimum, and role in cell regulation. In γ-radiated U87-W E6 cells, the activity of A-SMase, but not N-SMase, was increased. Accordingly, SR33557, an A-SMase inhibitor, blocked ceramide formation and apoptotic cell death. Therefore, ceramide is produced by A-SMase in glioma cells during p53-independent radiation-induced apoptosis. A requirement for A-SMase in radiation-induced ceramide

formation is also demonstrated in cells from A-SMase deficient Niemann-Pick patients and from A-SMase knock out mice (24).

In summary, the data obtained in the present study indicate that p53 protects glioma cells from γ-radiation-induced apoptosis. In glioma cells lacking functional p53, ceramide produced through activation of A-SMase, but not N-SMase, triggers caspase cascade. Information and the identification of an upstream regulator of A-SMase and downstream component(s) of ceramide, will lead to better understanding of the molecular mechanism of apoptosis, and will give us a novel approach for the treatment of malignant gliomas, which are often resistant to γ-radiation.

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Production of prostaglandinE2 via bile acid is enhanced by trypsin and acid in normal human esophageal epithelial cells

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Abstract

Several reports suggest that duodenogastroesophageal reflux may produce esophagitis, Barrett's esophagus and esophageal carcinoma. And it is well known that the incidence of adenocarcinoma arising from Barrett's esophagus has been increasing during the past decade. On the other hand, cyclooxygenase-2 and prostaglandins, produced by the catalytic reaction of cyclooxygenase-2, are considered to relate to carcinogenesis of the digestive tract and other malignant tumors. Recent reports suggest that cyclooxygenase-2 is induced in Barrett's esophagus and esophageal carcinoma. The purpose of this study is to investigate the reaction of cyclooxygenase-2 expression and prostaglandinE2 production on normal human esophageal epithelial cells cultured with gastroduodenal components. Normal human esophageal epithelial cells were cultured with chenodeoxycholic acid, trypsin and in acidic condition, individually and with different combinations of these three factors. After culturing, cyclooxygenase-2 expression in the cells and amount of prostglandinE2 in culture media was evaluated by immunoblotting and enzyme-immunoassay, respectively after culturing the cells. Cyclooxygenase-2 expression was up-regulated by bile acid and prostaglandinE2 production was enhanced by bile acid with trypsin, acidic condition or both of these components, without a synergistic effect on cyclooxygenase-2 expression. Production of prostaglandinE2 via these factors was suppressed by the cyclooxygenase-2 selective inhibitor JTE-522.The results suggest that duodenogastroesophageal reflux may induce cyclooxygenase-2 expression and prostaglandinE2 production in esophageal epithelial cells, cyclooxygenase-2 specific inhibitors may have a chemopreventive effect on esophageal carcinoma.

Keywords: Cyclooxygenase-2; ProstaglandinE2; Duodenogastroesophageal reflux; Bile acid; Trypsin

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Introduction

Duodenogastroesophageal reflux causes various esophageal diseases. Barrett's epithelium, in which columnar epithelium replaces the normal squamous epithelium of the lower esophagus in response to chronic injury by reflux esophagitis (Spechler and Goyal, 1996). Most adenocarcinoma arises in Barrett's epithelium (Pera et al., 1993). The potential contribution of gastroduodenal components to the development of Barrett's esophagus remains speculative and controversial. Although some authors reported that bile reflux is a major factor in Barrett's esophagus and esophageal adenocarcinoma, others attribute only a synergistic role to bile and a major role to acidity of the refluxate (Gillen et al., 1988; Nehra et al., 1999; Vaezi and Richter, 1996). It is also well known that the incidence of adenocarcinoma of the esophagus and esophagogastric junction has been increasing during the past decade in Western countries (Cameron et al., 1995). In addition to Barrett's esophagus and adenocarcinoma, squamous cell carcinoma and adenosquamous carcinoma have been suggested to be correlated with reflux esophagitis (Kuylenstierna and Munck-Wikland, 1985; Ribet and Mensier, 1992; Rubio and Aberg, 1991). On the other hand, epidemiological studies indicated that nonsteroidal anti-inflammatory drugs (NSAIDs) decreased the relative risk of esophageal, gastric, and colorectal carcinomas (Thunn et al., 1993). The chemopreventive mechanisms of NSAIDs have yet remain to be elucidated in detail, but have been considered to relate to their effect of inhibiting cyclooxygenase-2 (COX-2) activity. Two isoformes of COX have been identified: cyclooxygenase-1 (COX-1) is constitutively expressed in most tissues and is involved in the physiological production of prostaglandins for maintaining normal physiological functions; COX-2 is involved in inflammation and has been shown to be induced by mitogens, cytokines, hormones and growth factors (DuBois et al., 1994; Hamasaki et al., 1993; Jones et al., 1993; Miller et al., 1997). It has also been reported that COX-2 mRNA and protein, but not COX-1, are markedly elevated in human gastrointestinal cancers compared with normal mucosa and chemically-induced rat colon cancer (Dubois et al., 1996; Kargman et al., 1995; Ristimäki et al., 1997; Sano et al., 1995). Prostaglandins (PG), produced by the catalytic reaction of COX-2, affect cell proliferation and inhibit immune surveillance (Goodwin and Ceuppens, 1983; Honn et al., 1981; Marnett, 1992). And, prostaglandinE2 (PGE2) is over-produced in malignant tissues expressing COX-2. Over-expression of COX-2 itself inhibits apoptosis, increases the invasiveness of malignant cells and up-regulates angiogenic factors (Tsujii and DuBois, 1995; Tsujii et al., 1997, 1998). From the perspective of duodenogastroesophageal reflux and COX-2 induction, dihydroxy bile acids activate the transcription of COX-2 and cause increases in the spontaneous production of PGE2 in a cell line established from adenocarcinoma arising in Barrett's esophagus (Zhang et al., 1998). Either acid or bile acids up-regulate the expression of COX-2 in Barrett's esophagus biopsy explant ex vivo culture systems (Shirvani et al., 2000). Ottignon et al. reported that patients with both erosive and nonerosive esophagitis exhibit increased levels of PGE2, $PGF_{2\alpha}$, and PGD2 compared to healthy controls and that PGE2 levels appear to parallel the degree of tissue damage, being significantly higher in those with erosive esophagitis compared to those with nonerosive disease in esophageal biopsy tissues (Ottignon and Deschamps, 1988). Another institute reported that patients with esophagitis have also been shown to release increased amounts of PGE2 into the lumen and the degree of increase in PGE2 has been shown to correlate with the degree of endoscopic esophagitis (Marcinkiewicz et al., 1995; Sarosiek et al., 1995). However, there was no

investigation regarding COX-2 expression and PGE2 production in normal human esophageal epithelium in contact with gastroduodenal components such as bile acids, acid and pancreatic juice. The aim of this study is to clarify what kind of components, including in the gastroduodenal contents affect COX-2 expression and production of PGE2 in the normal esophageal epithelium. Therefore we studied COX-2 expression and PGE2 production in normal cultured human esophageal epithelial cells using bile acid (chenodeoxycholic acid), trypsin, acidic condition and different combination of these three factors. We also studied the effect of the selective COX-2 inhibitor JTE-522 (Wakitani et al., 1998).

Materials and methods

Antibodies and reagents

Monoclonal antibody raised against human COX-1 and COX-2 was obtained from Cayman Chemical Laboratories (Ann Arbor, MI. USA). Monoclonal antibody raised against human cPLA2 was purchased from Transduction Laboratories (Lexington, KY, USA). Monoclonal antibody raised against humanβ-actin was purchased from PIERCE (Rockford, IL, USA). Secondary antibody (anti-mouse immunoglobulin G alkaline phosphatase conjugate) was obtained from PIERCE (Rockford, IL, USA). COX-1 and COX-2 standard for immunoblot were obtained from Cayman Chemical Laboratories (Ann Arbor, MI, USA) and Oxford Biochemical Research, Inc (Oxford, MI, USA). Keratinocyte serum-free media supplemented with bovine pituitary extract and epithelial growth factor were from Gibco BRL, Life technologies, Inc (Rockville, MD, USA). Hydrochloric acid (0.5 mol/L) was from Wako Pure Chemical Industries (Osaka, Japan). Trypsin was from Gibco BRL, Life technologies, Inc (Rockville, MD, USA). Chenodeoxycholic acid and arachidonic acid were from Sigma (St Louis, MO, USA). JTE-522, a COX-2 selective inhibitor was provided by Japan Tobbaco, Inc (Tokyo, Japan). Chenodeoxycholic acid was dissolved in ethanol to 200 mM as stock solution. Trypsin was dissolved in the keratinocyte serum-free media to 1000 U/ml as stock solution. JTE-522 was dissolved in DMSO (dimethyl sulfoxide) to 100 μmol/L as stock solution.

Cell culture

Human normal esophageal epithelial cells were cultured from normal esophageal epithelium, which were obtained from the resected esophagus of a patient in our department suffering from esophageal carcinoma. Tissues were confirmed to contain no macroscopic tumor tissue or histologically detectable metaplastic cells or cancer cells. The tissues were incubated in 1 ml Dispase at 37°C for 30 min. The detached epithelium was minced into small pieces and incubated in phosphate buffer saline containing 0.1% trypsin 0.02% EDTA at 37°C for 15 min. The dissociated cells were collected and seeded into 5 cm collagen IV –coated culture dishes. Cells were grown in keratinocyte serum-free media (pH 7.4) supplemented with bovine pituitary extract and epithelial growth factor in a 5% CO_2/water-saturated incubator at 37°C. In these conditions, cells were able to passage more than 30 times without senescence. Written informed consent was obtained from the patient for surgery and use of his resected samples.

Immunoblot analysis

Cells (5 × 10^5/ dish) were plated in 10 cm dishes and grown to 80% confluence. The medium was replaced with keratinocyte serum free media containing various gastroduodenal contents such as bile acid (chenodeoxycholic acid, 50 ~ 200 μmol/L) for 12 hours. Trypsin (1 ~ 10 units/ml) was added for the final hour (hour 11 to hour 12) by adding the appropriate volume of stock solution. Concentrations of chenodeoxycholic acid and trypsin were determined as previously reported [22–28]. For acidic culture conditions, the media was acidified with 0.5 mol/L HCl (~ 2% vol/vol, pH 7.0 ~ 5.5) and cells were cultured in the media for 12 hours. Cells were not able to survive in media below pH 5.5. And cells were also cultured with combinations of these three factors such as chenodeoxycholic acid plus trypsin, chenodeoxycholic acid plus acid, trypsin plus acid, and chenodeoxycholic acid plus trypsin plus acid simultaneously at the same concentration and time course of the single- factor trials. For COX-2 inhibitor experiments, cells were exposed to JTE-522, a selective inhibitor of COX-2, for the entire 12 hours. For example, for chenodeoxycholic acid plus trypsin plus acidic culture condition trials, media was changed to acidified media by HCl with 200 μmol/L chenodeoxycholic acid at the beginning, and trypsin was added at the one hour pulse into the well appropriate volume of stock solution from the 11th hour after to 12th hour. Therefore, the total time course of all studies was 12 hours (Fig. 1). Vehicles of components absent from each study were added at suitable times and final concentrations. We also compared time course for induction of COX-2 by chenodeoxycholic acid (200 μmol/L) form 0 to 36 hours. Lysates for the detection of COX-2 were prepared by treating cells with lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 5 mmol/L EDTA, 0.5% NP-40, 2 mM Na_3VO_4, 0.1% SDS supplemented with the following protease inhibitors: soy bean trypsin inhibitor (5 μg/ml), leupeptin (1 μg/ml), aprotinin (2 μg/ml), pepstatin (1 μg/ml) and phenylmethylsulfonylfluoride (100 μg/ml). Lysates were sonicated for 20 seconds on ice and centrifuged at 14,000 rpm for 20 minutes to remove the cell debris. The total protein of the supernatant was measured using a BCA protein assay kit (PIERCE, Rockford, IL) with bovine serum albumin as a standard. Beta-mercaptoethanol was added (final concentration was 0.24 mol/L) to the

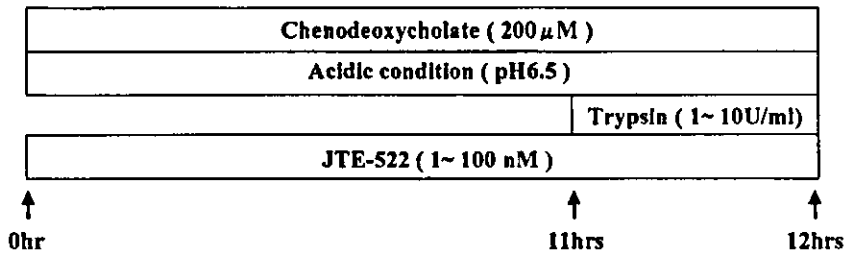

Fig. 1. Time course of each study. Normal human esophageal epithelial cells were cultured with chenodeoxycholic acid (CD) and acidic culture condition for 12 hours. Trypsin was added for the final hour (from hour 11 to hour 12) by adding the appropriate volume of stock solution. Cells were also cultured with combinations of these three factors such as CD plus trypsin, CD plus acid, trypsin plus acid, and CD plus trypsin plus acid simultaneously at the same concentration and time course as the single- factor trials. For COX-2 inhibitor experiments, cells were exposed to JTE-522, a selective inhibitor of COX-2, for the entire 12 hours. Vehicles of components absent from each study were added at suitable times and final concentrations. Total time course of all studies was 12 hours (except the study for time course of COX-2 expression cultured with CD, Fig. 2B).

supernatant, boiled for 5 minutes and analyzed. A 30 μg sample of protein was loaded per lane and SDS-poly acrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (1970). Proteins were electrophonically transferred to PVDF membranes. The PVDF membranes were then incubated with mouse monoclonal anti COX-1, COX-2, cPLA2 and β-actin antibody respectively. Blots were probed with the corresponding secondary antibody to IgG conjugated to alkaline phosphatase using the BCIP/NBT alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instruction. Quantitative image analysis of the protein on the membrane was performed on a Macintosh computer using the public domain National Institute of Health Image program version 1.62 (developed at the US National Institute of Health).

ProstaglandinE2 production and cell survival

Cells (1×10^5 cells/well) were plated in 6-well dishes and grown to 80% confluence, and the medium was replaced in the same manner as for immunoblot analysis (Fig. 1). At the end of the treatment period, the culture media was collected to determine the amount of PGE2 secreted spontaneously by these cells. Fresh medium containing 30 μmol/L arachidonic acid was then added for 30 minutes when we intended to examine the effect of the substrate. After the arachidonic acid treatment, the medium was collected for detection of PGE2. The levels of PGE2 released by the cells were measured by an enzyme immunoassay (Cayman Chemical Co, Ann Arbor, MI, USA) according to manufacturer's instruction. Number of cells in each dish was counted by Coulter Counter and haemocytometer. Rates of PGE2 production were normalized to the number of the cells surviving at the end of the treatment period. Cell survival was assessed by number of cells and trypan blue dye exclusion test.

Amount of the Arachidonate in the cells

Cells were cultured in the same manner as for immunoblot analysis. At the end of the treatment period, the cells were scraped and sonicated for 20 seconds in phosphate buffered saline on ice and centrifuged at 14,000 rpm for 20 minutes. The supernatants were used for quantification of arachidonic acid by gaschromatography. Total fatty acid was extracted by Folch solution from the supernatant and evaporated to dryness under nitrogen gas in a dry block bath at 37°C. After the dryness, the fatty acids were transesterified by adding K-methoxide and BF_3/methanol. The samples were then evaluated by gaschromatography. Fatty acid analyses were performed by using a Shimazu model GC14A gaschoromatograph equipped with a Shimazu model CR-4A data processor.

cPLA2 activity

Cells (5×10^5/ dish) were plated in 10 cm dishes and grown to 80% confluence, the medium was replaced in the same manner as for immunoblot analysis (Fig. 1). At the end of the treatment period, the cells were scraped and sonicated for 20 seconds in a buffer (50 mM Hepes, pH 7.4, containing 1 mM EDTA) on ice and centrifuged at 14,000 rpm for 20 minutes at 4°C to remove the cell debris. The supernatant was collected to determine the cPLA2 activity in the cells. cPLA2 activity was measured by cPLA2 assay kit (Cayman Chemical Co, Ann Arbor, MI) according to manufacturer's instruction.

Statistical analysis

Statistical evaluations were performed using the Turkey-Kremer test with a level of significance of $P < 0.05$. Graphically, data were shown schematically as vertical bars plotting the means + SEM. Stat view J-5 (Abacus Concepts, Inc, Berkeley, CA) was used for statistical analysis.

Results

Immunoblot analysis of COX-1 and COX-2

Fig. 2A shows the effect of chenodeoxycholic acid on the expression of COX-2 protein. Chenodeoxycholic acid up-regulated COX-2 protein expression in a dose dependent manner. We also compared time course for induction of COX-2 by chenodeoxycholic acid (Fig. 2B). COX-2 protein expression was enhanced after 6 hours of treatment and maximal induction continued from 12 hours to 36 hours after initial treatment with chenodeoxycholic acid. Fig. 2C shows the effect of trypsin on the expression of the COX-2 protein. The COX-2 protein was slightly induced in the presence of trypsin. Acidic conditions also induced lower-grade COX-2 protein expression than bile acid stimulated condition (Fig. 2D). Furthermore, we compared the expression of COX-1 and COX-2 protein stimulated by combinations of these three factors and JTE-522. Fig. 3 shows that neither the

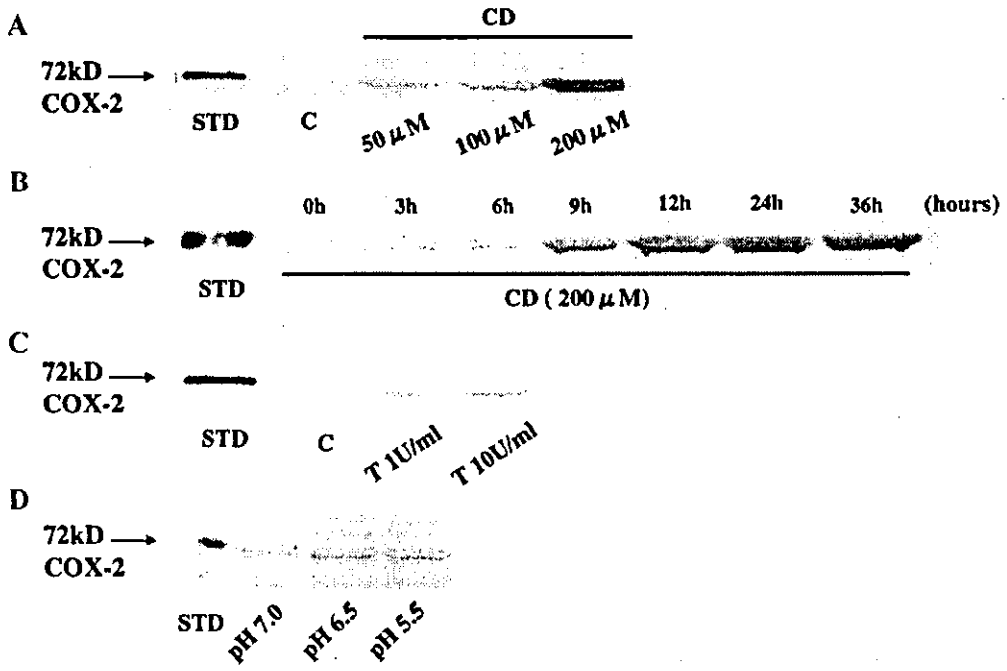

Fig. 2. Effect of respective chenodeoxycholic acid (CD), trypsin (T) and acidic condition on COX-2 expression, assessed by immunoblotting, in normal human esophageal epithelial cells. (A) Cultured with 50, 100 and 200 μmol/L CD for 12 hours. (B) Time course of changes in COX-2 expression cultured with chenodeoxycholic acid. (C) Cultured with 1 to 10 units/ml trypsin for a one-hour pulse. (D) Cultured in acidic condition (pH 7.0 to 5.5) for 12 hours.

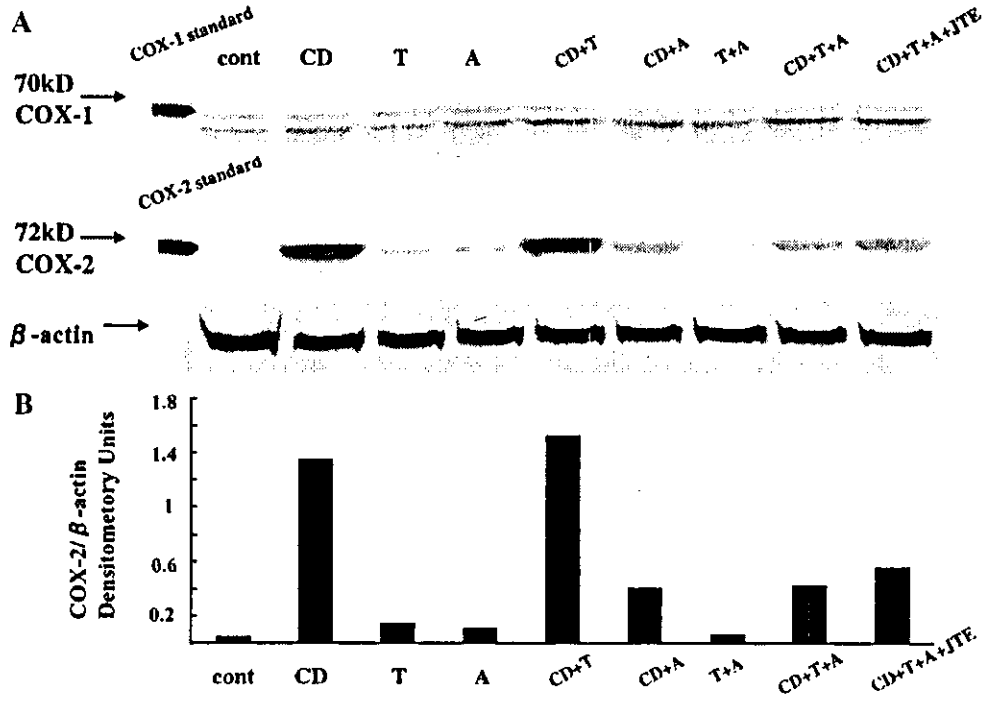

Fig. 3. (A) Immunoblots for COX-1 and COX-2 expression in normal human esophageal epithelial cells cultured with three factors of gastroduodenal contents. The interaction of chenodeoxycholic acid (200 μmol/L) for 12 hours, a 1-hour trypsin pulse (10 units/ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours, and JTE-522 for 12 hours. cont, control vehicle; CD, chenodeoxycholic acid; T, trypsin; A, acidic condition (pH 6.5); JTE, JTE-522 (100 nmol/L). Immunoblots for β-actin was also performed as the loading control. (B) Densitometory of COX-2 protein, standardized to β-actin, for the conditions listed above in A.

combination of bile acid and acid nor all three components had a synergistic effect on COX-2 expression larger than bile acid itself. However, the expression of COX-2 via bile acid was reduced by treatment with acid or acid and trypsin. JTE-522 did not affect COX-2 expression in the presence all three components. Trypsin and acidic condition did not have a synergistic effect on COX-2 expression. Low-level COX-1 expression was observed in all conditions and COX-2 expression did not depend on the COX-1 expression.

Production of prostaglandinE2

Fig. 4 shows the effect of the various conditions on the production of PGE2. Compared with the control, culture with chenodeoxycholic acid, trypsin, acidic condition did not reveal a significant increase in PGE2 production individually. But, culture with chenodeoxycholic acid plus trypsin revealed a 2.5-fold quantitative increase in PGE2 production ($p < 0.01$). Such an increase was even more pronounced in cultures of chenodeoxycholic acid with acidic condition and trypsin plus acidic condition, which revealed a 3.2-fold increase in PGE2 production ($p < 0.01$). Cultures with chenodeoxycholic acid plus trypsin plus acidic condition showed a 6.3-fold enhanced production compared with the control ($p < 0.01$) and JTE-522 completely suppressed PGE2 production in a dose dependent manner. After the treatment, control wells, chenodeoxycholic acid culture wells and chenodeoxycholic acid plus trypsin plus acidic condition wells were subsequently

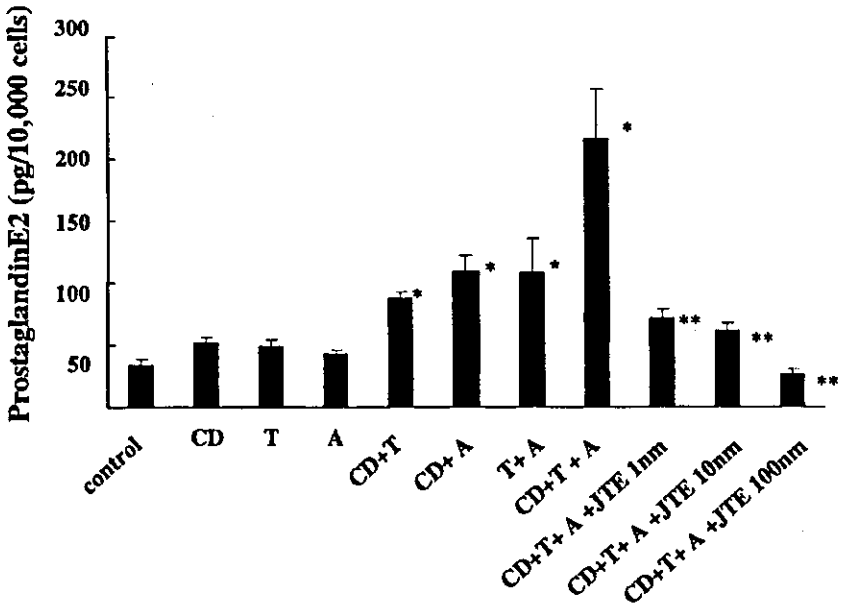

Fig. 4. ProstaglandinE2 secreted in culture media produced spontaneously by normal human esophageal epithelial cells cultured with three factors of gastroduodenal contents. The interaction of chenodeoxycholic acid (200 μmol/L) for 12 hours, a 1-hour trypsin pulse (10 units/ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours, and JTE-522 (1 to 100 nmol/L) for 12 hours. CD, chenodeoxycholic acid; T, trypsin; A; acidic condition (pH 6.5); JTE, JTE-522. $^{*}P < 0.05$ compared with control, $^{**}P < 0.01$ compared with CD plus trypsin plus acid, n = 3.

incubated with fresh medium containing 30 μmol/L arachidonic acid for 30 minutes. After this additional treatment, production of PGE2 was significantly increased in the media culture with chenodeoxycholic acid, although the control and cultured with the three components exhibited only a slight increase in PGE2 production ($p < 0.01$). And the amount of PGE2 was also significantly suppressed by JTE-522 ($p < 0.01$, Fig. 5).

Cell survival

After medium was collected from each well as samples for PGE2 measurement, the surviving cells were counted as described in Materials and Methods. Fig. 6 shows the survival rate of each trial compared to the control ($p < 0.01$). Except for the acidic condition, culture with each factor significantly decreased cell survival. The combination of chenodeoxycholic acid and other factors had a synergistic effect on cytotoxicity and contact with all three factors was most harmful in the conditions. The combinations of JTE-522 and all three components did not affect cell survival compared with all three components without JTE-522.

Arachidonate release in cells

In order to verify the cause of up-regulation of PGE2 production, we measured the amount of arachidonate as a substrate for COX. Fig. 7 shows the amount of arachidonate released in cells. Arachidonate release in the cell was significantly increased in cultures of trypsin plus acidic condition and of chenodeoxycholic acid plus trypsin plus acidic condition compared with control. There was a

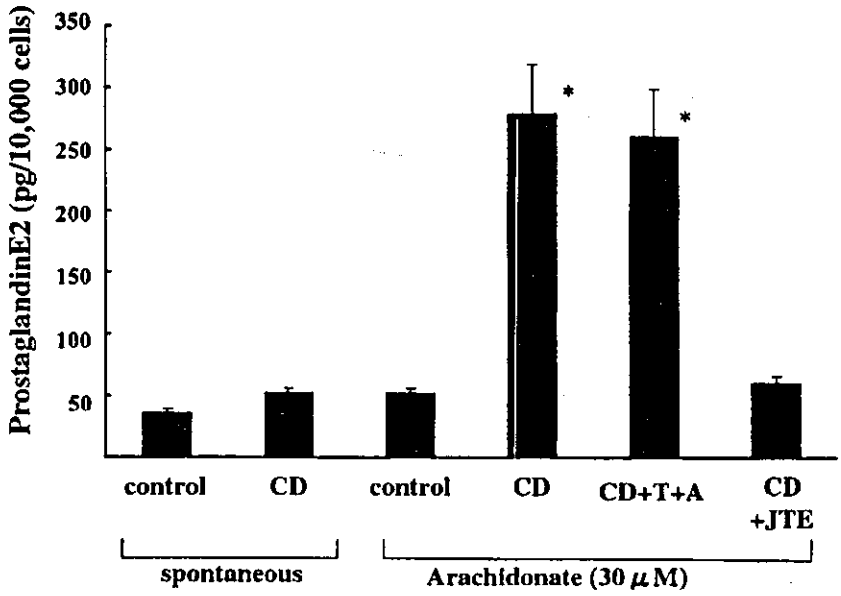

Fig. 5. ProstaglandinE2 secreted in culture media produced by normal human esophageal epithelial cells spontaneously and under excess arachidonic acid (30 μmol/L) condition after 12 hours stimulation of control vehicle, chenodeoxycholic acid (200 μmol/L), a 1-hour trypsin pulse (10 units/ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours and JTE-522 (100 nmol/L) (see Materials and methods). CD, chenodeoxycholic acid; T, trypsin; A; acidic condition (pH 6.5); JTE, JTE-522 (100 nmol/L). *$P < 0.05$ compared with spontaneous PGE2 production in control and CD or control and CD plus JTE with arachidonic acid. n = 3.

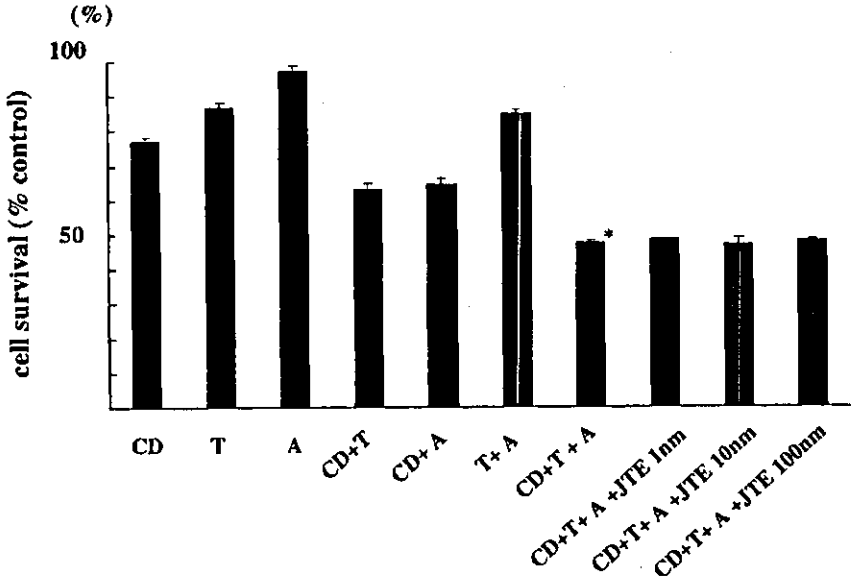

Fig. 6. Effect of gastroduodenal components on cell survival compared with mean value of control. Cultured with chenodeoxycholic acid (200 μmol/L) for 12 hours, a 1-hour trypsin pulse (10 units/ ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours and JTE-522 (1-100 nmol/L) for 12 hours. CD, chenodeoxycholic acid; T, trypsin; A, acidic condition; JTE, JTE-522 (100 nmol/L). $P < 0.05$ compared with other single or combination trials, n = 3.

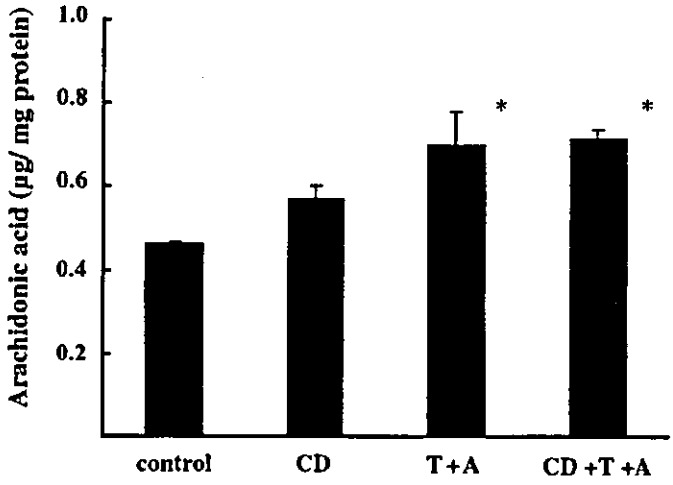

Fig. 7. Arachidonic acid release in the cells cultured with three factors of gastroduodenal contents. The interaction of chenodeoxycholic acid (200 μmol/L) for 12 hours, a 1-hour trypsin pulse (10 units/ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours. *$P < 0.05$ compared with control, n = 3.

tendency for the amount of arachidonate to increase in the cultures with these three components compared with chenodeoxycholate alone.

cPLA2 expression and activity

Since cytosolic phospholipaseA2 is considered as one of the main enzyme to release arachidonate from cytomembrane in most cell types, we evaluated the expression and activity of cPLA2. The expression of cPLA2 protein by immunoblot analysis did not reveal any alteration in the conditions (data not shown). cPLA2 tended to be activated in the cells cultured with the three components compared with the control or bile acid alone (Fig. 8).

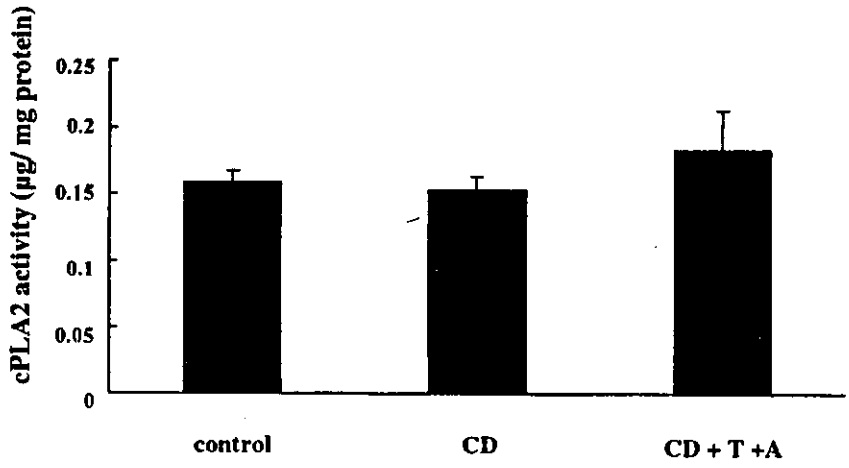

Fig. 8. cPLA2 activity in the normal esophageal epithelial cells cultured with three factors of gastroduodenal contents. The interaction of chenodeoxycholic acid (200 μmol/L) for 12 hours, a 1-hour trypsin pulse (10 units/ml, from 11 hours after the beginning of treatment to 12 hours), acidic condition (pH 6.5) for 12 hours. n = 5.

Discussion

Our data clearly showed that culture with bile acid plus trypsin plus acidic condition was produced the largest amount of PGE2 in human normal esophageal epithelial culture cells. A series of studies showed that various grades of esophagitis and Barrett's esophagus are related to gastroesophageal reflux (DeMeester et al., 1980; Goldberg et al., 1969; Stein et al., 1992). However, reflux of duodenal contents alone or with gastric juice may also cause esophageal mucosal damage and Barrett's esophagus (Champion et al., 1994; Gotley et al., 1992). Kauer et al. reported that 58% of patients with gastroesophageal reflux disease were found to have increased esophageal exposure to gastric and duodenal juice. The degree of mucosal damage increased when duodenal juice was refluxed into the esophagus, in that patients with Barrett's metaplasia had a significantly higher prevalence of abnormal esophageal bilirubin exposure than did those with erosive esophagitis or with no injury. Patients with Barrett's metaplasia also had greater esophageal bilirubin exposure compared with patients without Barrett's changes, with or without esophagitis (Kauer et al., 1995). They also reported that the correlation of pH and bilirubin showed that 87% of esophageal bilirubin exposure occurred when the pH of the esophagus was between 4 and 7. It is also reported that unconjugated bile acid and trypsin caused the most damage at its optimal pH activity range of pH 5 to 8 (Harmonn et al., 1981; Kivilaakso et al., 1980). In addition, pH in the stomach of a patients with duodenogastroesophageal reflux treated with proton pump inhibitors is typically in five to six range, and trypsin and bile acid also present. In our experiment the acidic condition culture was pH 6.5. Therefore, remarkably similar circumstances that we used in the culture systems may occur in patients with reflux disease. Our study indicated that normal human esophageal epithelial cells expressed COX-2 under the stimulation of bile acid. COX-2 expression was also induced by trypsin and acidic conditions, but the levels were lower than the levels of COX-2 expression in bile acid cultures. On the other hand, spontaneous production of PGE2 was not particularly increased by bile acid, although the COX-2 expression was significantly up-regulated. Interestingly, combination of bile acid and other components up-regulated the spontaneous PGE2 production without additional arachidonic acid. Our results explain, in part, the cause of PGE2 up-regulation in the patients with esophageal diseases resulting from reflux of gastroduodenal contents. We speculated that COX-2 expression is up-regulated by bile acid in advance, and after up-regulation, trypsin and gastric acid (buffered or regulated by duodenal juice, saliva and proton pump inhibitors to some degree) or both of these components up-regulate the PGE2 production in esophageal epithelial cells during reflux (Fig. 9). We are not able to explain the discrepancy between COX-2 expression and PGE2 production in this study. However, treatment with chenodeoxycholic acid significantly increased the synthesis of PGE2 in the presence of excess arachidonic acid, whereas the control and cultures with the three components did not exhibit such a drastic change in PGE2 production. Therefore, the culture system that we used may have contained arachidonic acid insufficient for PGE2 production by catalytic reaction of over-expressed COX-2. Spontaneous over-production of PGE2 caused by contact with these three components was dramatically inhibited by JTE-522; therefore the PGE2 was produced by catalytic reaction of COX-2. So our results partly suggest that trypsin or acid may supply substrates of the arachidonic acid cascade and this supply of substrates resulted in high production of PGE2 while COX-2 induction was low. In fact, the amount of arachidonic acid in the cells was significantly increased when the cells was cultured with these three component compared with control. The up-

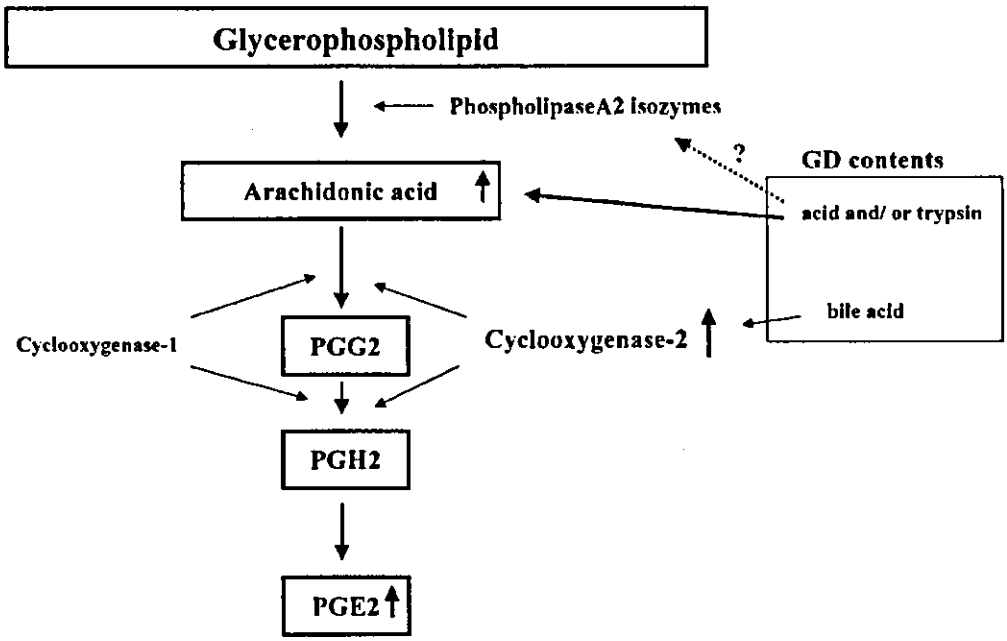

Fig. 9. Effect of gastroesophagoduodenal contents to arachidonic acid cascade in esophageal epithelium. This diagram illustrates the effect of gastroesophagoduodenal content to PGE2 production via arachidonic acid cascade in esophageal epithelial cells. For details, see text.

regulation of arachidonic acid seems slightly lower than we expected. This may have been due to the fact that the released arachidonic acid may already have been converted, in part, to PGH2 by the catalytic reaction of COX-2 which is induced by bile acid. We evaluated the expression and activity of cPLA2, one of the main enzymes that supplies the arachidonic acid to COX. The expression of cPLA2 did not reveal any difference in the conditions. The cPLA2 tended to be activated in the cells cultured with the three components, compared with the control or bile acid alone, but there was no significant difference in these conditions. There is a possibility that other PLA2 isozymes as well as cPLA2 may affect the reaction. For example, trypsin activates pancreatic type phospholipaseA2 (typeIB sPLA2), which was found as a digestive enzyme at first but also confirmed in other organs, and sPLA2 IB stimulates PGE2 synthesis via a specific PLA2-I receptor (Kishino et al., 1994; Tohkin et al., 1993). Further examinations are needed in this issue.

In conclusion, COX-2 expression and prostaglandinE2 production were significantly up-regulated in normal human esophageal cells by bile acid in combination with trypsin and acid. This information will contribute to the consideration of causes and treatments of the esophageal diseases resulting from the duodenogastroesophagal reflux.

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