Apurinic/apyrimidinic endonuclease-1 is associated with angiogenesis and VEGF production via upregulation of COX-2 expression in esophageal cancer tissues

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Submitted 4 March 2013; accepted in final form 10 November 2013

Apurinic/apyrimidinic endonuclease-1 (APE-1) is a key enzyme responsible for DNA base excision repair and is also a multifunctional protein such as a redox effector for several transcriptional factors. Our study was designed to investigate APE-1 expression and to study its interaction with cyclooxygenase (COX)-2 expression and vascular endothelial growth factor (VEGF) production in esophageal cancer tissues. The expression of APE-1, COX-2, monocyte chemoattractant protein (MCP)-1, CC-chemokine receptor (CCR)2, and VEGF were evaluated by immunohistochemistry in 65 human esophageal squamous cell carcinoma (ESCC) tissues. Real-time PCR and Western blotting were performed to detect mRNA and protein expression of APE-1 and p-signal transducer and activator of transcription 3 (pSTAT3) expression in MCP-1-stimulated ESCC cell lines (KYSE 220 and EC-GI-10). siRNA for APE-1 was used to determine the role of APE-1 in the regulation of COX-2 expression, VEGF production, and antiapoptotic effect against cisplatin. In human ESCC tissues, nuclear localization of APE-1 was observed in 92.3% (60/65) of all tissues. There was a significant relationship (P = 0.029, R = 0.49) between nuclear APE-1 and cytoplasmic COX-2 expression levels in the esophageal cancer tissues. In KYSE 220 and EC-GI-10 cells, MCP-1 stimulation significantly increased mRNA and protein expression of APE-1. Treatment with siRNA for APE-1 significantly inhibited p-STAT3 expression levels in MCP-1-stimulated cells. Furthermore, treatment of siRNA for APE-1 significantly reduced COX-2 expression and VEGF production in MCP-1-stimulated esophageal cancer cell lines. Treatment with APE-1 siRNA significantly increased apoptotic levels in cisplatin-incubated KYSE 220 and EC-GI-10 cells. We concluded that APE-1 is overexpressed and associated with COX-2 expression and VEGF production in esophageal cancer tissues.

Apurinic/apyrimidinic endonuclease-1; esophageal cancer; cyclooxygenase-2; vascular endothelial growth factor; monocyte chemoattractant protein-1
Given the role that APE-1 may play in the development of esophageal cancer, we examined the coexpression of APE-1 and COX-2 expression as one predictive factor in esophageal cancer tissues and determined whether APE-1 was associated with VEGF production in the development of esophageal cancer tissues through induction of COX-2 protein.

MATERIALS AND METHODS

Patients. A total of 65 paraffin-embedded esophageal squamous cell carcinoma (ESCC) tissues were obtained from archived patients with esophageal cancer who had undergone esophagectomies and/or received chemoradiotherapy in Nippon Medical School Hospital. The protocol of this study was approved by the Ethics Committee of Nippon Medical School, and written informed consent was obtained from all patients. Among these patients, there were 47 males and 18 females, ranging in age from 50–86 yr. Sixty-five tissues were categorized according to UICC (International Union Against Cancer) TNM classification as follows: 3 stage 0, 10 stage I, 10 stage IIA, 1 stage IIB, 19 stage III, 5 stage IVA, 17 stage IVB.

Cell culture and treatments. ESCC cell lines, KYSE 220, and EC-GI-10 (purchased from National Institute of Biomedical Innovation, Osaka, Japan) were maintained in RPMI 1640 or Ham F12 (Nikken Bio Medical Laboratory, Nagoya, Japan) supplemented with 10% fetal bovine serum (Nippon Bio-Supply Center, Tokyo, Japan) at 37°C in 5% CO2. Group 1 without any treatments served as the control, group 2 was stimulated by human recombinant MCP-1 (0.1 μmol/l) without pretreatment of siRNA for APE-1, whereas group 3 was stimulated by MCP-1 (0.1 μmol/l) after 24-h pretreatment with siRNA for APE-1. Cisplatin (0, 1, 3, and 6 μl/ml) was then added to the KYSE220 cells with or without pretreatment of siRNA for APE-1.

Immunohistochemical analysis of APE-1, COX-2, MCP-1, CCR2, and VEGF. Expression of APE-1, COX-2, MCP-1, CCR2, and VEGF in the esophageal cancer tissues was evaluated by immunostaining. Briefly, 4-μm sections were deparaffinized, antigens were retrieved by microwaving for 5 min in 5% urea solution, and endogenous peroxidase activity was blocked with 3% H2O2 in methanol. The samples were incubated overnight at 4°C with rabbit anti-APE-1 antibody (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-COX-2 antibody (diluted 1: 150; Cayman Chemical, Ann Arbor, MI), rabbit anti-MCP-1 antibody (diluted 1:100; Abcam, Cambridge, MA), anti-CCR2 antibody (diluted 1:20; Abcam), and anti-VEGF antibody (diluted 1:200; Santa Cruz Biotechnology). After being washed, the secondary antibody was detected by LSAB 2 kit (DAKO) with diaminobenzidine as chromogen. For the negative

Fig. 1. Localization of apurinic/apyrimidinic endonuclease-1 (APE-1), cyclooxygenase (COX)-2, monocyte chemoattractant protein (MCP)-1, CC-chemokine receptor 2(CCR2), and VEGF in esophageal cancer tissues. APE-1- (A), COX-2- (C), MCP-1- (D), CCR2- (E), and VEGF-positive cells (F) in esophageal cancer tissues were identified by immunohistochemistry. B: APE-1-positive cells were not detected in normal esophageal mucosa. (Magnification ×125 for A, E, F, ×150 for B, C, and D). G: specialized columnar epithelial cells could be seen in Barrett’s epithelium (hematoxylin and eosin staining; original magnification, ×80). H: APE-1 was observed at low levels in specialized columnar epithelial cells in Barrett’s epithelium (original magnification, ×80). Arrows show APE-1 immunoreactivity in a faint nuclear pattern. I: staining was not seen in esophageal cancer tissues incubated with control IgG antibody (original magnification, ×200).
control, primary antibodies were replaced with isotype-matched immunoglobulin.

Double-labeling immunofluorescence methods and confocal laser scanning microscopy were used to evaluate the coexpression of immunoreactivity for the pair of mouse anti-human COX-2 (diluted 1:40; Cayman Chemical) and rabbit anti-human APE-1 (diluted 1:20; Santa Cruz Biotechnology). Sections were incubated overnight at 4°C with a mixture of the two primary antibodies and then with FITC or Texas red-conjugated secondary antibodies [horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and goat anti-rabbit IgG (Vector Laboratories), for COX-2 and APE-1, respectively] followed by nuclear counterstaining with 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO).

The immunostaining scoring system as follows was established by experienced pathologists. For APE-1, immunoreactivity was graded from 0 to 3 (0:0%, 1:0–33%; 2:33–66%; 3:67–100%) based on the proportion of positive nuclei to total cell number in representative areas of the cancer tissue (9). For COX-2, immunoreactivity was determined by the intensity of staining and the percentage of areas with positive staining. The intensity of staining was graded from 0 to 3, and the area of positivity was estimated as a percentage of total area of the tumor. These two variables constituted the actual score: 0, 1, 2, 3, as described previously (22). Six fields were selected to determine the average score of APE-1 and COX-2. For MCP-1, sections with more than 30% positive cells were considered positive, whereas the sections with 30% or less positive cells were considered negative, as described previously (24). Since Kitadai et al. (17) have reported that MCP-1 was significantly correlated with VEGF immunoreactivity in esophageal cancer tissues (positive staining for VEGF was defined as the presence of VEGF immunoreactivity >30% of esophageal cancer cells), we have also determined the definition of MCP-1 positivity based on previous studies (24).

Western blotting analysis of APE-1 and p-STAT3 expression levels in treated KYSE 220 and EC-GI-10 cells. Stimulated [H2O2: (200 μmol/l); MCP-1 (0.03, 0.1 and 0.3 μmol/l)] KYSE 220 and EC-GI-10 cells were lysed in a buffer solution containing 0.1% NP-40, 10 mM NaCl, 5 mM MgCl2, 10 mM Na2HPO4, 65 mM Na-orthovanadate, and protease inhibitor cocktail. APE-1 protein, partially purified, was determined by the intensity of staining and the percentage of areas with positive staining. The intensity of staining was graded from 0 to 3, and the area of positivity was estimated as a percentage of total area of the tumor. These two variables constituted the actual score: 0, 1, 2, 3, as described previously (22). Six fields were selected to determine the average score of APE-1 and COX-2. For MCP-1, sections with more than 30% positive cells were considered positive, whereas the sections with 30% or less positive cells were considered negative, as described previously (24). Since Kitadai et al. (17) have reported that MCP-1 was significantly correlated with VEGF immunoreactivity in esophageal cancer tissues (positive staining for VEGF was defined as the presence of VEGF immunoreactivity >30% of esophageal cancer cells), we have also determined the definition of MCP-1 positivity based on previous studies (24).

Real-time PCR of APE-1 expression levels in treated KYSE 220 and EC-GI-10 cells. Real-time quantitative PCR was performed to detect mRNA expression levels of APE-1 from MCP-1-stimulated KYSE 220 and EC-GI-10 cells. Briefly, RNA extracted from these cells was reversetranscribed, and subsequently cDNA was amplified in 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) with primers, dual-labeled fluorogenic probes, and a TaqMan PCR Reagent kit (Applied Biosystems). The primers and probes for CA) with primers, dual-labeled fluorogenic probes, and a TaqMan in 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) with primers, dual-labeled fluorogenic probes, and a TaqMan

RESULTS

Localization of APE-1, MCP-1, CCR2, COX-2, and VEGF in the esophageal cancer tissues. In human ESCC tissues, nuclear localization APE-1 was observed in 92.3% (60/65) of all tissues (Fig. 1A). In contrast, in normal esophageal tissues, APE-1 expression could not be observed (Fig. 1B). COX-2, APE-1 IS ASSOCIATED WITH ANGIOGENESIS AND VEGF PRODUCTION

Fig. 2. COX-2 and APE-1 protein expression levels in MCP-1-positive esophageal cancer tissues. Expression levels of APE-1 and COX-2 in MCP-1-positive esophageal cancer tissues (n = 41) were significantly higher compared with MCP-1-negative tissues (n = 24) (∗P < 0.001; **P < 0.001, respectively). Data are expressed as means ± SE of each score in 65 esophageal cancer tissues.
MCP-1, and CCR-2 were primarily expressed in the cytoplasmic compartment in the esophageal cancer cells (Fig. 1, C–E). VEGF was also expressed in the cytoplasm in esophageal cancer cells (Fig. 1F). Specialized columnar epithelial cells could be seen in Barrett’s epithelium (Fig. 1G). APE-1 was expressed at low levels in specialized columnar epithelial cells in Barrett’s epithelium (Fig. 1H). In addition, staining could not be seen in esophageal cancer tissues using control IgG antibody (Fig. 1I).

**COX-2 and APE-1 protein expression levels in MCP-positive esophageal cancer tissues.** Because MCP-1 has been reported to play important roles in tumorigenesis in esophageal cancer tissues, we determined whether MCP-1 was associated with APE-1 and COX-2 expression in esophageal cancer tissues. In immunohistochemical analysis, both APE-1 and COX-2 expression scores in MCP-1-positive esophageal cancer tissues \( (n = 41) \) were significantly \( (P < 0.01, P < 0.01, \) respectively) greater compared with MCP-1-negative esophageal cancer tissues \( (n = 24) \) (Fig. 2).

**Correlation between COX-2 and APE-1 expression in esophageal cancer tissues.** Because APE-1 and COX-2 were strongly expressed in the esophageal cancer tissues, in this study, we determined the relationship between COX-2 and APE-1 expression levels in esophageal cancer tissues. FITC-labeled (green) cells in the esophageal cancer tissues in Fig. 3A show COX-2 immunoreactivity (Fig. 3A). Texas red-conjugated cells in Fig. 3B show APE-1 immunoreactivity for the same section (Fig. 3B). Double-positive-stained cells (yellow cells) for the same section (Fig. 3C). However, we have addressed that nuclear APE-1 and cytoplasmic COX-2 expressions were mainly coexpressed in the esophageal cancer tissues (Fig. 3C). In addition, we observed a significant relationship \( (P = 0.029, R = 0.49) \) between COX-2 and APE-1 expression levels in 65 esophageal cancer tissues (Fig. 3E). Moreover, we have also determined that there was a significant relationship \( (P = 0.021, R = 0.41) \) between APE-1 and CD34-positive cells in esophageal cancer tissues (Fig. 3F).

**APE-1 expression levels in MCP-1-stimulated esophageal cancer cell lines.** To clarify whether MCP-1 stimulation induced APE-1 expression in esophageal cancer tissues, we examined APE-1 expression levels induced by \( \text{H}_2\text{O}_2 \) and MCP-1 stimulation in esophageal cancer cell line, KYSE 220 and EC-GI-10 cells, by Western blotting analysis. \( \text{H}_2\text{O}_2 \) (200

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**Fig. 3.** The relationship between APE-1 and COX-2 expression in esophageal cancer tissues. A: COX-2-positive cells (green cells) in the esophageal cancer tissues (original magnification, \( \times 150 \)). B: APE-1-positive cells (red cells) in the esophageal cancer tissues (original magnification, \( \times 150 \)). C: nuclear APE-1 and cytoplasmic COX-2 expressions were mainly coexpressed in the same section. Double-positive cells (yellow cells) reveal COX-2-and APE-1-positive cells in the same section (original magnification, \( \times 150 \)). D: 4’,6-diamidino-2-phenylindole staining (blue cells) (original magnification, \( \times 150 \)). E: there was a significant relationship \( (P = 0.029) \) between APE-1 and COX-2 expressions in the 65 patients with esophageal cancer. F: there was a significant relationship \( (P = 0.021, R = 0.41) \) between APE-1 and CD34-positive cells in the esophageal cancer tissues.
APE-1 expression was also observed in 200 μmol/l H2O2-stimulated KYSE 220 and EC-GI-10 cells. APE-1 protein levels were low in unstimulated KYSE 220 and EC-GI-10 cells (Fig. 4).

**DISCUSSION**

In this study, we aimed to investigate the localization of APE-1 expression in esophageal cancer tissues and to determine whether APE-1 was associated with development of esophageal cancer through elevation of COX-2 expression and VEGF production. Our major findings are as follows: 1) APE-1 was overexpressed and found primarily in esophageal cancer cells with nuclear localization; 2) a significant relationship between APE-1 and COX-2 expression scores in esophageal cancer tissues was demonstrated; 3) MCP-1 stimulation significantly increased APE-1 protein level in KYSE 220 and EC-GI-10 cells; and 4) reduction of APE-1 by specific siRNA significantly reduced COX-2 mRNA expression levels and VEGF production in MCP-1-stimulated KYSE 220 cells.
To our knowledge, this is the first report of APE-1 overexpression and a significant relationship between COX-2 and APE-1 in esophageal cancer tissues. Previous studies have shown that overexpression of APE-1 was associated with resistance to chemoradiotherapy in HeLa cells (12), glioma cell (27), medulloblastoma, and primitive neuroectodermal tumors (3), as well as nonsmall cell lung cancer (33). Overexpression of APE-1 is found in many kinds of malignancies of digestive tract-associated organs, including cytoplasmic expression of APE-1 in colorectal cancer (16), hepatocellular carcinoma (6), and nuclear expression in pancreatic cancer (15). Our results demonstrate that APE-1 was not detected in normal esophageal tissues, but it was expressed in 73.3% of cells in esophageal cancer tissues with nuclear localization of APE-1 observed in 92.3% (60/65) of these tissues. The nuclear localization of APE-1 may be associated with chemoradiotherapy resistance and a poor survival (18).

Previous studies have reported that MCP-1 is expressed in esophageal cancer tissues and plays a pivotal role in tumorigenesis of esophageal cancer (24). Because we found that APE-1 expression levels in MCP-1-positive esophageal cancer were significantly increased compared with those with MCP-1-negative esophageal cancer, we investigated whether APE-1 expression levels were affected by MCP-1 stimulation using Western blotting analysis. We showed that APE-1 expression in KYSE220 and EC-GI-10 cells was increased by MCP-1 stimulation. Our results suggest that MCP-1 may play an important role in APE-1 induction in esophageal cancer tissues. Further studies are needed to clarify the relationship between MCP-1 and APE-1 expression in esophageal cancer tissues.

In immunohistochemical analysis, there was a significant relationship between COX-2 and APE-1 in esophageal cancer tissues. We have previously reported that a selective COX-2 inhibitor, celecoxib, inhibited APE-1 expression via reduction of IkBα phosphorylation (8). We have recently reported that MG-132, an NF-κB inhibitor, abrogated the p65 and APE-1 expression levels in MCP-1-stimulated esophageal cancer cell lines (28). In addition, APE-1 has been reported to stimulate the DNA binding activity of NF-κB. Therefore, reduction of APE-1 in stimulated esophageal cancer cell lines could lead to inhibition of stimulated COX-2 expression through downregulation of NF-κB activation. However, in this study, basal COX-2 expression levels in unstimulated esophageal cancer cell lines such as KYSE 220 and EC-GI-10 cells were not affected by siRNA treatment for APE-1 through reduction of DNA binding activity of NF-κB. Constitutive basal COX-2 expression levels in unstimulated esophageal cancer cell lines might be partly induced in NF-κB-independent pathway as previously reported (13). Previous studies have reported that APE-1 interacts with HIF-1α, STAT3, and activates hypoxygen-induced expression of VEGF (10, 35). In this study, we have also reported that inhibition of APE-1 expression was associated with reduction of p-STAT3 expression levels in stimulated esophageal cancer cell lines. Zou et al. (37) have also reported...
that the APE-1-specific inhibitor, E-3330m inhibited tumorigenesis via reduction of growth of endothelial cells and VEGF production (37), and, in another report, inhibition of APE-1 was associated with reduction of angiogenesis in vitro (14). In addition, Hall et al. (11) have also reported that APE-1 is also cytoprotective against endothelial apoptosis induced by hypoxia and by tumor necrosis factor-α, through the transcriptional upregulation of NF-κB-dependent survival signals, as well as NF-κB-independent mechanism. Previous studies and our study suggest that inhibition of APE-1 were partly linked to the reduction of VEGF production through reduction of activation of STAT3. In addition, although, in our data, there was not colocalization of APE-1 and CD34 in esophageal cancer tissues, we have also demonstrated that APE-1 expression levels were significantly associated with CD34 expression levels in esophageal cancer tissues. Collectively, these results suggest that APE-1 may be modulated to prevent the development of esophageal cancer through reduction of angiogenesis.

APE-1 overexpression has been reported to be associated with resistance to various anticancer drugs. Several studies have shown that targeted reduction of APE-1 protein by specific antisense oligo or siRNA sensitized to methylmethane sulfonate, H2O2, bleomycin, and gemcitabine (3, 19, 25, 30). Whether such enhanced sensitivity is solely due to the loss of the DNA repair activity of APE-1 or also due to the loss of its transcriptional regulatory function or both is still unknown. Our data showed that, in APE-siRNA-treated KYSE220 and EC-GI-10 cells, the antiapoptotic function of APE-1 against cisplatin was abolished. Hall et al. (11) have reported that depletion of the redox-sensitive domain of APE-1 reduced the antiapoptotic function of APE-1 in both hypoxia and tumor necrosis factor-α-induced endothelial cell death (11). Furthermore, Bhattacharyya et al. (2) reported that H. pylori-mediated acetylation of APE-1 suppressed Bax expression and prevented p53-mediated apoptosis. Although there were relatively few (n = 23) patients treated with chemoradiotherapy, we found a negative trend (P = 0.09, R = 0.512) between effective ratio against chemoradiotherapy and APE-1 expression levels in esophageal cancer tissues. Further studies will be needed to clarify whether APE-1 expression is associated with chemoresistance in esophageal cancer tissues. Taken together with these prior reports, our results suggest that APE-1 affects development of esophageal cancer through upregulation of VEGF production and reduction of apoptosis.

In conclusion, our data demonstrate that a significant relationship exists between APE-1 and COX-2 expression in esophageal cancer tissues. Moreover, reduction of APE-1 by specific siRNA significantly reduced COX-2 mRNA expression levels and VEGF production in MCP-1-stimulated KYSE 220 and EC-GI-10 cells. We believe that a greater understanding of the role of COX-2 in the regulation of APE-1 expression could provide potential future therapeutic targets in esophageal cancer.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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