Decreased Vascular Endothelial Growth Factor Expression Is Associated With Cell Apoptosis in Low-Dose Aspirin-Induced Gastric Mucosal Injury

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Abstract: *Background:* The use of low-dose aspirin (LDA) has emerged as an important cause of gastrointestinal ulcers. The aim of this study was to investigate the association between LDA-induced gastric mucosal injury and the expression of vascular endothelial growth factor (VEGF) and cell apoptosis in elderly Chinese patients. *Methods:* A total of 136 patients aged 60 to 80 years with LDA-induced (100 mg/d for at least 1 month) gastric mucosal injury and 48 age-matched healthy subjects were enrolled in this study. The patients were divided into a low-severity group and a high-severity group based on their modified Lanza scale scores. Biopsy specimens of gastric mucosa from all participants were subjected to immunohistochemical staining for VEGF expression and terminal deoxynucleotidyl transferase dUTP nick end labeling staining for cell apoptosis. Staining indices and apoptotic indices were applied to assess VEGF expression level and the extent of cell apoptosis. *Results:* VEGF expression decreased significantly in the 2 patient groups, whereas the extent of cell apoptosis significantly increased compared with the control group. Furthermore, Spearman's correlation coefficients suggest that VEGF expression levels and the extent of cell apoptosis in gastric mucosae shared a significant correlation with the severity of LDA-induced gastric mucosal injury. Receiver operating characteristics analysis further confirmed these results. *Conclusions:* Our findings provide important clues as to the underlying molecular mechanism behind gastric mucosal injury resulting from exposure to LDA in elderly adults, and also suggest that interventions specifically targeting the pathways associated with angiogenesis and apoptosis may help facilitate the healing process.

Key Indexing Terms: Aspirin; Gastric mucosal injury; Cell apoptosis; Vascular endothelial growth factor. **[Am J Med Sci 2015;349(2):110–116.]**

In the main guidelines set forth by the societies of Europe and the United States, taking low-dose aspirin (LDA) daily (<330 mg/d) is recommended for long-term antithrombotic prophylaxis in all patients with cardiovascular and/or cerebrovascular diseases, or with atherosclerotic risk factors.[1,2] However, it is well known that aspirin causes gastroduodenal mucosal injury and that the use of LDA has emerged as an important cause of gastrointestinal (GI) ulcers.[3] According to recent studies, daily doses of 75 to 300 mg aspirin can result in a 2- to 4-fold increase in the risk of GI ulcers.[4] Additionally, although LDA-induced mucosal injury is often asymptomatic, this injury is more likely to induce severe complications to develop in elderly adults, such as bleeding or perforation, which can lead to increased morbidity and mortality rates due to comorbidities.[5] Therefore, it is necessary that we understand the molecular pathogenesis associated with LDA-induced mucosal injury to explore novel therapeutic strategies for this disease, especially in elderly subjects.

Damage to the gastric mucosa integrity is a key pathogenic feature of LDA-induced mucosal injury.[6] Generally, the integrity of the gastric mucosa is maintained by pre-epithelial, epithelial and postepithelial barriers that allow the mucosa to be resistant to various damage-causing factors.[7,8] The postepithelial barrier is formed by endothelial cells lining the mucosal blood microvessels (capillaries, arterioles and collecting venules), which allow for the exchange of oxygen, CO_2 and nutrients as well as the removal of toxic metabolites.[8,9] Additionally, this barrier is involved in the generation, concentration and transportation of various vasoactive substances, hormones and growth factors, which are essential in maintaining the structure and function of the GI mucosa.[10–12]

Aspirin, as a nonsteroidal anti-inflammatory drug (NSAID), causes gastric mucosal damage primarily by inhibiting cyclooxygenase-1 (COX-1), which consequently reduces the mucosal generation of protective prostaglandins such as prostaglandin E2 (PGE2) and prostacyclin (PGI2).[3] In addition, previous *in vitro* and *in vivo* studies have shown that aspirin use could result in mucosal injury by inhibiting physiological angiogenesis as well as by inducing epithelial cell apoptosis.[13–16] However, there is still a lack of direct clinical evidence that supports aspirin having these 2 mechanisms of action. Furthermore, it remains unclear whether its association with mucosal injury severity is related to the reduction of angiogenesis and the increase in cell apoptosis. Therefore, in this study, we performed immunohistochemistry (IHC) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to examine the expression of the angiogenesis marker vascular endothelial growth factor (VEGF) and the levels of cell apoptosis in elderly patients with LDA-induced gastric mucosal injury of various severities. Based on the findings of our IHC and TUNEL examinations, we explored the association between the severity of injury, VEGF expression and cell apoptosis.

PATIENTS AND METHODS

Patient Population

From December 2008 to January 2011, a total of 476 patients with coronary artery disease who had been treated with LDA in the Heart Center of the First Affiliated Hospital of Tsinghua University were consecutively selected for this study. LDA was defined as 100 mg/d of aspirin, which was recommended by Guidelines and Consensus on Prevention and Treatment of Cardiovascular Diseases in China 2007 for Chinese population.[17]

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To ensure the quality of our study, strict inclusion and exclusion criteria were followed. Inclusion criteria were (1) age between 60 and 80 years, (2) taking daily LDA treatment for at least 1 month before enrollment, (3) suffering from gastric mucosal injury that was confirmed by endoscopy at the time of enrollment and (4) expressing willingness to participate in the study. Exclusion criteria were (1) taking other NSAIDs, antacids, misoprostol or antisecretory drugs during LDA treatment, (2) undergoing major gastric surgery within 1 month before enrollment, (3) having severe hepatic or renal insufficiency and (4) having a history of peptic ulcer disease or other hemorrhagic esophagitis/gastritis before starting LDA treatment. Additionally, 48 consecutive age-matched healthy subjects were included as controls during the study period.

All eligible patients and healthy subjects were fully informed of the study. Written informed consent was obtained from all participants. At the time of enrollment, all participants were tested for serum *Helicobacter pylori* antibody using an enzyme-linked immunosorbent assay kit (Biohit, Helsinki, Finland). Their smoking and alcohol habits were also recorded. The study protocol was approved by the Ethical Committee of the authors' institute.

Endoscopy and Collection of Gastric Mucosal Samples

Endoscopy was performed at the time of enrollment to determine patients' eligibility. The patients with gastric mucosal injury on endoscopy were eligible for enrollment. For eligible patients, the severity of LDA-induced gastric mucosal injury was also assessed. Briefly, during endoscopy, more than 40 pictures were taken by an experienced technician who was blinded to the subjects' clinical information. Endoscopic images were also recorded on video. Then, based on the modified Lanza scale (MLS) system as presented in Table 1,[18,19] the grade of LDA-induced gastric mucosal injury was evaluated independently by 2 other experienced endoscopists who were also blinded to the subjects' clinical information. If there was any disagreement regarding the score assigned, a third endoscopist was consulted and a decision was made by consensus. Patients were divided into 2 groups based on their MLS scores: a low-severity group (MLS scores = 1 and 2) and a high-severity group (MLS scores = 3 and 4).

Three biopsy specimens were taken from 3 sites of the gastric mucosa free of macroscopic mucosal injury in both eligible patients and healthy controls: 1 from the minor curvature of the gastric antrum, 1 from the incisura angularis and 1 from the minor curvature of the lower part of the gastric corpus. The collected biopsies were then used for IHC staining and TUNEL staining.

TABLE 1. Modified Lanza scoring system

Score	Description[a]
0	Normal mucosa, no visible lesions
+1	1 mucosal hemorrhage or erosion
+2	2–10 mucosal hemorrhages or erosions
+3	11–25 mucosal hemorrhages or erosions
+4	>25 mucosal hemorrhages or erosions, or an peptic ulcer

[a] Mucosal hemorrhage is defined as a small reddened hemorrhagic area with an intact mucosa. Erosion is defined as a mucosal defect less than 5 mm in diameter but having no depth. Peptic ulcer is defined as a mucosal defect greater than 5 mm in diameter with a well-defined crater.

IHC Staining of VEGF and TUNEL Staining

IHC staining was performed to examine VEGF expression levels in all mucosa specimens taken from the 3 sampling sites. Briefly, the specimens were fixed with 10% neutral buffered formalin and then embedded in paraffin. The paraffin sections (4–5 μm thick) were prepared for IHC staining with a polyclonal rabbit antibody against VEGF (Fuzhou Maxim Biotech Inc, Fuzhou, China) and for TUNEL staining with a commercially available kit (Fuzhou Maxim Biotech Inc). The immunoreactivity of VEGF was detected using a biotinylated secondary antibody and a streptavidin-peroxidase kit (Fuzhou Maxim Biotech Inc). Hematoxylin and eosin (H&E) staining was also performed on each paraffin section according to a standard protocol.

Evaluation of IHC Staining and TUNEL Staining Results

Two experienced pathologists who were blind to participants' clinical information reviewed all of the immunostained and TUNEL-stained sections. Image analysis was performed using Image Pro Plus software (version 6.0; Media Cybernetics Inc, Silver Spring, MD). Any discrepancy in results between the 2 pathologists resulted in a re-evaluation to obtain a consensus result.

Two to 5 sections per sampling site were randomly selected for analysis of VEGF expression. For each section, the degree of IHC staining was assessed using a semiquantitative and subjective scoring system that took into consideration the proportion of cells expressing VEGF as well as the intensity of staining.[20,21] The percentage of VEGF-positive cells was scored on a scale of 0 (<4%), 1 (5%–24%), 2 (25%–49%), 3 (50%–74%) and 4 (75%–100%). Staining intensity was graded on a scale of 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The 2 scores were multiplied to determine a staining index (SI, values from 0 to 12). In regards to TUNEL staining, 2 to 3 sections per sampling site were randomly selected for analysis. TUNEL signal-positive cells were counted in each section by viewing samples of approximately 500 cells taken from 10 different fields. The percentage of TUNEL signal-positive cells among the total number of cells counted was calculated to obtain an apoptotic index (AI). The median value of the SI or AI of all selected sections was used for subsequent statistical analysis.

Statistical Analysis

All statistical analysis was performed using SPSS 15.0 software (SPSS Inc, Chicago, IL). The normality of continuous variables was tested using the Kolmogorov-Smirnov test. Data with normal distribution are presented as mean ± SD. For discrete variables, data are presented as counts and percentages. In regards to the demographic data of the study groups, the statistical significance for continuous and discrete variables was assessed using analysis of variance and the χ^2 test, respectively. As for the IHC staining and TUNEL staining results, the statistical significance between the groups was assessed using the nonparametric Kruskal-Wallis test for multiple comparisons. Spearman's rank order correlation analysis was used to examine the correlation between MLS scores, SI and AI in patient with LDA-induced gastric mucosal injury. Receiver operating characteristic (ROC) analysis was applied to validate the results obtained from the Kruskal-Wallis test. A P value <0.05 was considered sufficient for statistical significance.