Effect of aging on gastric mucosal defense mechanisms: ROS, apoptosis, angiogenesis, and sensory neurons

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Kang JM, Kim N, Kim J, Oh E, Lee B, Lee BH, Shin CM, Park JH, Lee MK, Nam RH, Lee HE, Lee HS, Kim JS, Jung HC, Song IS. Effect of aging on gastric mucosal defense mechanisms: ROS, apoptosis, angiogenesis, and sensory neurons. Am J Physiol Gastrointest Liver Physiol 299: G1147–G1153, 2010. First published August 19, 2010; doi:10.1152/ajpgi.00218.2010.—Aging changes in the stomach lead to a decreased capacity for tissue repair in response to gastric acid. The aim of this study was to determine the mechanism associated with the increased susceptibility to injury of aging mucosa including reactive oxygen species (5), apoptosis, angiogenesis, and sensory neuron activity. Fischer 344 rats at four different ages (6, 31, 74 wk, and 2 yr of age) were studied. The connective tissue indicators [salt-soluble collagen and sulfated glycosaminoglycan (sGAG)], lipid hydroperoxide (LPO), myeloperoxidase (MPO), and hexosamine were assessed. We also evaluated the expression of early growth response-1 (Egr-1), phosphatase and tension homologue deleted on chromosome 10 (PTEN), caspase-9 (index of apoptosis), VEGF (index of angiogenesis), calcitonin gene-related peptide (CGRP, index of sensory neurons), and neuronal nitric oxide synthase (nNOS). The histological connective tissue area in the lower part of rat gastric mucosa increased with aging, with increase of salt-soluble collagen and sGAG. LPO and MPO in old rats were significantly greater than in the young rats, whereas hexosamine was significantly reduced. These results indicate that the lower part of rat gastric mucosa was found to be replaced by connective tissue with accumulation of oxidative products with aging. In addition, impairment of apoptosis, angiogenesis, and sensory neuron activity via theactivation of Egr-1 and PTEN might increase the susceptibility of gastric mucosa to injury during aging.

Egr-1; PTEN; caspase-3; glycosaminoglycan; aging gastropathy; CGRP; lipid hydroperoxide; reactive oxygen species

WITH AGE, THE STOMACH LINING’s capacity to resist damage decreases, which in turn may increase the risk of peptic ulcer disease, especially in people who use aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (26). Several studies have reported an increased susceptibility of the gastric mucosa to various injuries in old animals (3, 12, 25, 48). The increased susceptibility of rat gastric mucosa has been explained by alteration of the gastric defense mechanisms in several aspects. Animal and human studies have indicated that aging gastric mucosa has impaired mucosal defenses with decreased mucus and bicarbonate secretion (8, 11, 25, 48). In addition, healthy older people have a reduced mucosal prostaglandin concentration compared with younger individuals (8). The cyclooxygenase enzyme is involved in this pathway, which is significantly reduced with age (50). In old rats, a reduction in basal gastric blood flow has been demonstrated. In addition, the increase in gastric blood flow in response to injury has been shown to be attenuated with advancing age (24). Moreover, studies have shown that aging gastric mucosa has reduced nitric oxide synthase (NOS) activity and impaired sensory nerve response to luminal acid (34). In addition, reactive oxygen species (ROS) (5) have been shown to play a role in tissue damage and aging (27). However, the majority of previous studies were performed on tissue from other sites such as brain, liver, lung, and heart, not from the gastrointestinal tract (31, 55).

The phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a 403-amino acid peptide with a high degree of homology to protein tyrosine phosphatase and the cytoskeletal protein tensin. PTEN functions a tumor suppressor. In addition, PTEN regulates several fundamental cellular processes such as cell adhesion, growth, migration, and apoptosis (43). The early growth response gene-1 (Egr-1) is an 80- to 82-kDa transcription factor that is rapidly and transiently activated by a number of extracellular stimuli, including growth factors, cytokines, and injury-related stimuli (46). Egr-1 can directly regulate PTEN, triggering the initial step in this apoptotic pathway (49). Although a recent study demonstrated that PTEN could induce apoptosis in aged stomach (44), most studies of PTEN have focused on its role in tumor cell biology.

Our previous study demonstrated that calcitonin gene-related peptide (CGRP) and VEGF expression were important factors for gastric protection and healing of ulcers in young rats 8–10 wk of age (21, 22). Aging has been associated with the absence of periarteriolar axon containing CGRP from the rat stomach (13). In addition, impaired angiogenesis in ischemic tissues of old animals was found to be associated with reduced expression of VEGF (37, 38); these prior experiments were performed on endothelial cell lines and musculoskeletal tissue, but not on gastrointestinal tract tissue including the stomach. Furthermore, there has been no comprehensive study regarding the link between the morphology and molecular changes.
among young, middle aged, old, and very old rats. Therefore, the goal of this study was to determine the influence of aging on the gastric mucosal defense mechanisms in terms of ROS, angiogenesis, and sensory neuron activity as well as hypoxia, apoptosis, and anatomical changes, by using rats at four different ages.

**MATERIALS AND METHODS**

**Animals.** Male Fischer 344 rats, *Helicobacter pylori* free and virus free (Orient, Seoul, Korea), 6, 31, and 74 wk and 2 yr of age were used for the experiments. The animals were housed in a cage maintained at 23°C, with 12:12-h light-dark cycles under specific pathogen-free conditions. The rats were starved but allowed water for 12 h prior to the experiments. All experimental procedures described here were approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital.

**Mucosal histology.** The rats were euthanized, and gastric specimens were obtained and fixed in 10% buffered formalin for histology. The specimens were embedded in paraffin and routinely processed and stained with hematoxylin and eosin (H&E). The gastric mucosal histology was evaluated on coded gastric specimens stained with H&E by two independent gastrointestinal pathologists (H. E. Lee and H. S. Lee). The area of the connective tissue was quantified in the lower one-third of the mucosa and the total area of each specimen (n = 10) by using the MetaMorph 7.0 video image analysis system (Molecular Devices, Downingtown, PA). The connective tissue area was expressed as a % of the total area.

**Measurement of salt-soluble collagen, sulfated GAG, lipid hydroperoxide, MPO, and hexosamine.** For the molecular biology studies, the excised stomach was extensively washed with ice-cold saline, and the gastric mucosa was scraped with slide glass and rapidly frozen into liquid nitrogen. It was kept at −70°C until used. Salt-soluble collagen was measured according to the manufacturer’s instructions (Biocolor, Newtownabbey, Northern Ireland). Briefly, 100 mg tissue was homogenized with 0.05 M Tris-HCl (pH: 7.5) containing 1 M sodium chloride and centrifuged at 13,000 g for 30 min. The Sircol dye reagent was added to each sample and standardized and mixed for 30 min. The collagen-dye complex was precipitated by centrifugation at 12,000 g for 10 min. Bound dye was released and dissolved via addition of alkali reagent and the absorbance was measured at 555 nm.

Sulfated GAG (sGAG) content was assayed by using the Blyscan kit (Biocolor). The Blyscan dye reagent was added to each sample and standardized and mixed for 30 min. The insoluble pellet of sGAGs was precipitated by centrifugation at 12,000 g for 10 min. Bound dye was released with the dissociation reagent, and the absorbance was measured at 650 nm.

The lipid hydroperoxide (LPO) content was measured in tissue homogenates by use of a LPO kit (Calbiochem, San Diego, CA). LPOs were extracted by homogenizing isolated gastric mucosa in 500 μl of ice-cold 30 mM HEPES buffer, pH 7.4, 1 mM DTT, and 0.1 mM EDTA. The samples were deproteinized by adding 500 μl of metaphosphoric acid (20 mg/ml in methanol) to each sample and the precipitates were removed by centrifugation (5,000 g for 5 min). The nonpolar LPOs were extracted from each supernatant by adding 2 ml of chloroform per sample, followed by centrifugation for 5 min at 2,000 g. An aliquot of 200 μl was removed from each organic layer and spectrophotometrically assayed for LPOs by using a LPO kit (Calbiochem). LPOs are highly unstable and readily promote the conversion of Fe"⁻ to Fe"⁺ and were detected by using thiocyanate ions as the chromogen.

An assay of gastric mucosal MPO concentration was measured. Three hundred milligrams of scraped mucosa was homogenized for 30 s with a Polytron homogenizer in 1.0 ml of ice-cold 0.5% hexadecyl-trimethylammonium bromide in 50 mM of phosphate buffer (pH 6.0). Hexadecyltrimethylammonium bromide was used to negate the pseudoperoxidase activity of hemoglobin and to solubilize the membrane-bound MPO. The homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged for 20 min at 18,000 g. The supernatant was isolated and evaluated for the determination of the enzyme concentration, by utilizing an ELISA kit (Immundiagnostik, Bensheim, Germany).

A stock solution of glucosamine hydrochloride (Fluka, Buchs, Switzerland) was prepared fresh daily and stored at 5°C. Acetylcarnitine (0.3 ml; Sigma) was added to 10 ml of a carbonate buffer of pH 10 (8 g of sodium bicarbonate and 2.1 g of sodium bicarbonate in 100 ml), which solution was prepared immediately before use. The Ehrlich's reagent was prepared immediately before use by dissolving 0.4 g of p-dimethylenobenzaldehyde (Sigma) in 1.5 ml of concentrated HCl and 13.5 ml of isomyl alcohol. Gastric mucosal mucin extracted with Triton X-100 was hydrolyzed with 1 ml of 3 N HCl. Finally, hexosamine obtained from hydrolyzed mucin was assayed by the method described by Neuhaus and Letzring (35).

**Real-time PCR for Egr-1, PTEN, caspase-9, VEGF, CGRP, and neuronal nitric oxide synthase.** Egr-1 is an 80- to 82-KDa transcription factor is rapidly and transiently induced by a number of extracellular stimuli, including growth factors, cytokines, and injury-related stimuli (46). Egr-1 can directly regulate PTEN, triggering the initial step in this apoptotic pathway (49). PTEN is a 403-amino acid peptide and known to regulate several fundamental cellular process such as cell adhesion, growth, migration, and apoptosis. Thus we measured both of Egr-1 and PTEN by real-time PCR.

Briefly, RNA was extracted from the gastric mucosa using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA samples were diluted to a final concentration of 0.5 mg/ml in RNase-free water and stored at −80°C until use. Synthesis of the cDNA was performed with 1 mg of total RNA with Moloney murine leukemia virus (MMLV) reverse transcription reagents (Invitrogen, Carlsbad, CA). The 20-μl reverse transcription reaction consisted of 4 μl of first-strand buffer, 500 mM deoxynucleoside triphosphate mixture, 2.5 mM oligo (8)₁₂₋₁₈ primer, 0.4 U/ml ribonuclease inhibitor, and 1.25 U/ml MMLV reverse transcriptase (Invitrogen). The thermal cycling parameters for the reverse transcription were 5 min at 65°C, 50 min at 37°C, and 15 min at 70°C. Real-time PCR amplification and determination were performed by using the ABI PRISM 7000 sequence detection system, TaqMan universal PCR master mix, commercially available predesigned, gene-specific primers, and FAM-labeled probe sets for quantitative gene expression (TaqMan Gene Expression Assays, rodent VEGF, mouse β-actin; Applied Biosystems, Foster City, CA). All of the probes used in these experiments spanned an exon-intron boundary. The Egr-1, PTEN, VEGF, CGRP, neuronal nitric oxide synthase (nNOS) and β-actin mRNA were quantified by parallel estimation. The thermal cycler conditions were 2-min hold at 50°C and 10-min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Western blotting for PTEN and caspase-9.** The gastric mucosa was homogenized with lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM DTT (1 mM leupeptin (10 μg/ml), aprotinin (10 μg/ml), PMSF (1 mM), and Triton X-100 (0.1%). Briefly, the proteins (each sample, 30 μg) were separated by SDS-PAGE (7.5% wt/wt gel) and transferred to nitrocellulose membranes. All procedures were done in Tris buffer (40 mM, pH 7.5) containing 0.3 M of NaCl and 0.3% Tween 20. The membranes were then blocked with dried milk (6% wt/vol) and subsequently incubated with PTEN (monoclonal, 1:500 Santa Cruz Biotechnology, Santa Cruz, CA) and caspase-9 (rabbit polyclonal, 1:500; Santa Cruz Biotechnology) at 4°C overnight. The blots were incubated with secondary antibody (goat polyclonal antibody, 1:1,000; Santa Cruz Biotechnology) and an imaging analyzer was used to measure the band densities.

**Statistical analysis.** All statistical calculations were performed using SPSS software (version 12.0; SPSS, Chicago, IL). The results were compared by the Mann-Whitney U-test and the Wilcoxon’s
Influence of aging on connective tissue area, sGAG, salt-soluble collagen, LPO, MPO, and hexosamine. Histological finding showed that there was no definite mucosal atrophy of the gastric glands in the upper mucosa, but the basal one third of the mucosa was replaced with hyaline-like connective tissue as age increased (Fig. 1, A–D). Quantification of the connective tissue area demonstrated that the basal one third of the mucosa had a marked (>3-fold) increase in connective tissue as the rats increased in age (Fig. 2A). In terms of inflammation there was no increase of inflammatory cell infiltration depending on age. In addition, there was no Helicobacter-like organism in any of rat mucosa. In terms of atherosclerosis,
A mild thickening of submucosal vessel was found in one 74-wk-old and one 2-yr-old rat. The sGAG composition of the gastric mucosa of the 2-yr-old rats was significantly increased compared with the rats 74 wk of age (Fig. 2B). The salt-soluble collagen composition of the gastric mucosa of the rats at 2 yr of age was also significantly increased compared with the 6-, 31-, and 74-wk-old rats (Fig. 2C). The LPO of the gastric mucosa in the 74-wk and 2-yr-old rats was significantly increased compared with the 6-wk-old rats (Fig. 2D). The mucosal levels of MPO in the 2-yr-old rat were significantly increased compared with the rats 6 and 31 wk old (Fig. 2E). In contrast, the mucosal levels of hexosamine at 74-wk-old and 2-yr-old rat were significantly decreased compared with the 31-wk-old rat (Fig. 2F).

Influence of aging on Egr-1 and PTEN by real-time PCR and Western blotting. The real-time PCR of the hypoxia-inducible transcription factor Egr-1 mRNA in the 2-yr-old rats showed significant increase compared with the 74-wk-old rats (Fig. 3A). PTEN mRNA of the 2-yr-old rats also significantly increased compared with the 74-wk-old rats by real-time PCR (Fig. 3B). Similarly, PTEN protein expression of the 2-yr-old rats was significantly increased compared with the 6-, 31-, and 74-wk-old rats (Fig. 4A) by Western blotting.

Influence of aging on VEGF, CGRP, and nNOS by real-time PCR. The expression of VEGF in the 74-wk-old and 2-yr-old rats was significantly decreased compared with the 31-wk-old rats by real-time PCR (Fig. 3C). The expression of CGRP in the 31-wk-old rats was significantly increased compared with the 6-wk-old rats by real-time PCR (Fig. 3D). However, the expression of CGRP in the 74-wk-old and the 2-yr-old rats was significantly decreased compared with the 31-wk-old rats (Fig. 3D). Similar to CGRP, the expression of nNOS in the 74-wk and the 2-yr-old rats was also significantly decreased compared with the 31-wk-old rats (Fig. 3E).

Influence of aging on caspase-9 by Western blotting. The expression of apoptotic protein caspase-9 of the 2-yr-old
rats was significantly increased compared with the 6-, 31-, and 74-wk-old rats by Western blot analysis (Fig. 4B).

DISCUSSION

The results of previous studies have demonstrated that aging is associated with marked changes in the structural and functional properties of tissues of the digestive tract including the stomach (10, 20). For example, aging has been shown to cause glandular atrophy that is replaced by connective tissue (14, 30). In the present study, the connective tissue areas markedly increased in the 2-yr-old rats up to more than three times those of the rats that were 6 wk of age. In addition, these findings were supported by the increase in sGAG and salt-soluble collagen, which are major components of connective tissue in old rats.

Aging is known to closely associate with increased proinflammation conditions (7). ROS, free radicals, and oxidative products such as LPOs have been shown to participate in tissue injury and chronic inflammation during the onset and progression of degenerative diseases in humans (4). Lipid peroxidation mediated by free radicals is thought to be an important cause of membrane destruction, cell damage, and atherosclerosis (41). MPO is a key enzyme of neutrophil to produce potent oxidants (52). Specifically, water immersion restraint stress in rats causes a significant increase in the gastric mucosal LPO (40). In addition, one study has reported an age-related increase of LPO and MPO levels in the mouse heart and kidneys (42, 47).

However, little is known about the effects of aging on LPO and MPO levels in the gastric mucosa. In the present study, LPO and MPO of the gastric mucosa of 2-yr-old rats were significantly increased compared with the rats 6-wk old. The molecular sources of ROS that contribute to the aging process are still a matter of debate. Recently the role of the NOX family of superoxide-generating NADPH oxidase in ROS generation with aging process has been suggested. Actually, increased gastric mRNA expression of the NOX2 was reported in the elderly (39). In the present study there was an increase of MPO in the aged rats, which suggests that ROS generation during aging could be related with MPO. However, there was no age-related difference observed in inflammatory cell infiltration in the present study, which is different from \textit{H. pylori}-induced glandular atrophy. Thus the elevated MPO activity during age might be caused by increased activity of MPO in the recruited cells rather than increased recruitment of activated inflammatory cells. In terms of atherosclerosis, although there was a thickening of submucosal vessel in one 74-wk-old and one 2-yr-old rat, there were no significant changes among different age groups. Further study is needed to investigate the mechanism of ROS generation in the relation with aging.

Egr-1 is an 80- to 82-kDa transcription factor that is rapidly and transiently induced by a number of extracellular stimuli, including growth factors, cytokines, and injury-related stimuli (46). Egr-1 is functionally implicated in many critical biological processes, including inflammation, cell proliferation, cell differentiation, apoptosis, vascular wound response, and cancer progression (33, 46). Upregulation of Egr-1 in gastric epithelial cells by \textit{H. pylori} has previously been described (2, 23). One study showed an increased expression of Egr-1 in old rats (44), between the ages of 3 and 24 mo. In the present study, the levels of Egr-1 were stable until 74 wk, and then it rapidly increased in the rats 2 yr of age, suggesting that this phenomenon occurs late in life. Taken together, an increase of Egr-1 might be associated with susceptibility to gastric injury and carcinogenesis, common events associated with aging.

PTEN is a dual-specificity phosphatase that has activity with both phosphorylated peptides and phospholipids. PTEN indirectly inhibits the activation of Akt, which is important for several processes, including cell proliferation, migration, survival, and angiogenesis (6, 32, 53). Inhibition of Akt following tissue injury by activated PTEN would, therefore, be expected to impair most of the above processes essential for the healing of an injury. Recently, the impaired mucosal defenses of old rats has been explained by an overexpression of PTEN, which inhibited cell survival and induced apoptosis (programmed cell death) by a reduction of antiapoptosis factors such as the expression of survivin (44). In the gastric epithelium, apoptosis plays an essential role in maintaining tissue integrity. Normally, the rate of cell loss by apoptosis is matched by the rate of cell proliferation. The results of previous studies have shown that apoptosis in mucosal cells of the small intestine was increased in old rats (15). Furthermore, Tarnawski et al. (44) reported an increase of caspase-3 and caspase-9 and a reduction in survivin in the old mucosa, which might result in gastric atrophy. In the present study, the expression of protein as well as the mRNA of PTEN was significantly increased in the 2-yr-old rats, supporting the findings of previous reports. In addition, the expression of caspase-9 was also significantly increased in the 2-yr-old rats. These findings suggest that overexpression of caspase-9 via overexpression of PTEN in the gastric mucosa of old rats might be one possible mechanism associated with an imbalance between proapoptotic and antiapoptotic factors, resulting in gastric atrophy. Microarray for many other apoptosis-related genes would support or enhance the present study results. However, one study reported that the number of colon mucosal cells undergoing apoptosis was lower in the older animals (54). Therefore, further study including microarray for many other apoptosis-related genes is needed to determine whether the effect of aging on apoptosis is similar or different in the stomach compared with the colon tissue.

Aging has been associated with lower capabilities for cellular repair in the gastric mucosa (9, 28, 36). This age-related deficiency in mucosal repair is secondary to reduced expression of various growth factors such as EGF and TGF-\(\alpha\) in the stomach of old rats (29, 36). Impaired angiogenesis in ischemic tissues of old animals was found to be associated with expression of VEGF (38). However, angiogenesis in old gastric mucosa has not been examined previously. In the present study, VEGF expression was significantly decreased in the 74-wk-old and 2-yr-old rats compared with the 31-wk-old rats by real-time PCR. Several studies have reported that overexpression of PTEN inhibited cell survival and angiogenesis in the endothelial cells (18, 45). Taken together, reduced expression of VEGF could be related to overexpression of PTEN in the gastric mucosa of old rats and might be another mechanism associated with impaired angiogenesis and delayed healing. However, Abdel-Latif et al. (1) showed that exposure of gastric cells to acidic conditions induced the expression of the Egr-1 transcription factor with downstream induction of VEGF. Thus further study is needed to elucidate the effects of Egr-1 on angiogenesis and aging in the gastric mucosa.
Recently, it has been shown that sensory neurons regulate a variety of cytoprotective functions mainly through CGRP (16). If the mucosal barrier is disturbed or disrupted in the presence of luminal acid, the surge of acid intruding into the lamina propria stimulates spinal afferents, which cause prompt hyperemia in the gastroduodenal mucosa via local CGRP release and NO formation (16, 17). Miyake et al. (34) demonstrated that dysfunction of primary afferent neurons in old animals could lead to an increase of mucosal susceptibility to acid injury. Moreover, it was suggested that the impaired mucosal defenses and reduced repair in old animals (24–30 mo old) might be due to a decreased density of CGRP-staining nerve fibers around the submucosal blood vessels, resulting in reduced activity of sensory neurons (13). However, little is known regarding the effects of aging on tissue expression of gastric CGRP. In the present study, the expression of CGRP in the 74-wk and 2-yr-old rats was significantly decreased compared with the 31-wk-old rats. These findings suggest that decrease of CGRP in old gastric mucosa might be another mechanism responsible for susceptibility to gastric injury in the old rats.

NO plays a critical role in modulating several components of mucosal defense and is catalyzed by three kinds of NOS: neuronal, endothelial, and inducible (nNOS, eNOS, and iNOS). Of them, the source of the NO that strengthens the mucus barrier appears to be nNOS (51). In addition, CGRP has been reported to stimulate mucin synthesis through an NO-dependent mode of action in the rat gastric corpus (19). Experimental and human studies indicate that aging has impaired gastric mucosal defense, e.g., decreased mucus and bicarbonate secretion (8, 26), and reduced NOS activity (13). In the present study, nNOS RNA expression and gastric mucosal hexosamine (an index of gastric mucus synthesis) significantly decreased with age, which are consistent findings with previous reports.

Taken together, the results of the present study demonstrated an age-dependent increase in lipid peroxidation and decrease in the VEGF and CGRP expression in the gastric mucosa in four different aged groups (6, 31, 74 wk and 2 yr), which differs from previous study that used a young vs. an old group (44). The expression of Egr-1 and PTEN were significantly increased at 2 yr of age, with onset at 74 wk of age. These findings suggest that the impairment associated with aging likely depends depending on the factors studied; that is, some may occur in a stepwise fashion whereas others occur in a gradual pattern. However, further study is necessary to elucidate the link between the Egr-1/PTEN pathway and VEGF or CGRP.

In conclusion, the results of this study suggest that during the aging process the gastric mucosa is replaced by connective tissue combined with an increase in gastric mucosal collagen and lipid peroxidation by oxidative injury. Impairment of apoptosis, angiogenesis, and sensory neuron activity during aging appears to be mediated by the activation of Egr-1 and PTEN. This impairment of mucosal defensive mechanisms during aging may predispose old animals to gastric mucosal injury. This provides an explanation for the frequent occurrence of NSAID-associated ulcers and ulcer complications in patients of advanced age.

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DISCLOSURES
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