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Secretory phospholipase A_2 is required to produce histologic changes associated with gastroduodenal reflux in a murine model

Ashok Babu, MD, Xianzhong Meng, MD, PhD, Anirban M. Banerjee, PhD, Fabia Gamboni-Robertson, PhD, Joseph C. Cleveland, MD, Sagar Damle, MD, David A. Fullerton, MD, and Michael J. Weyant, MD

Objective: The earliest response of esophageal mucosa to gastric reflux is the development of oxidative damage and inflammation. These processes contribute to the development of metaplasia known as Barrett's esophagus, as well as the progression to malignancy. Secretory phospholipase A_2 is a mediator of inflammation with levels that are increased in Barrett's metaplasia and carcinoma when compared with levels in normal samples. Our goal is to determine the role of secretory phospholipase A_2 in the development of reflux-associated changes in the esophageal mucosa.

Methods: Secretory phospholipase A_2–deficient mice (C57BL/6, n = 5) and mice known to express high levels of secretory phospholipase A_2 (BALB/c, n = 5) underwent side-to-side surgical anastomosis of the first portion of the duodenum and gastroesophageal junction, allowing exposure of esophageal mucosa to duodenal and gastric contents duodeno-gastroesophageal anastomosis. Control animals (n = 5) of each strain underwent laparotomy with esophagotomy and repair. Tissue was frozen in embedding medium. Hematoxylin and eosin staining and Ki67 and secretory phospholipase A_2 immunohistochemistry were used to evaluate esophageal tissue and its response to duodeno-gastroesophageal anastomosis.

Results: Immunofluorescent staining confirmed the absence of secretory phospholipase A_2 in C57BL/6 mice and its presence in BALB/c mice. Hematoxylin and eosin staining demonstrated significant thickening of the esophageal mucosa in response to gastroesophageal reflux in the presence of secretory phospholipase A_2. Mice known to express high levels of secretory phospholipase A_2 also demonstrated increased numbers of proliferating cells. Secretory phospholipase A_2–deficient mice were immune to the early changes induced by mixed reflux.

Conclusions: The presence of secretory phospholipase A_2 appears necessary for early histologic changes produced by exposure of the esophagus to gastroduodenal contents. This enzyme is identified as a promising target for evaluation of mechanisms of carcinogenesis and chemoprevention of esophageal carcinoma.

The incidence of esophageal adenocarcinoma of the gastroesophageal junction is rapidly increasing, and it is currently the cancer with the fastest-increasing incidence in the United States. Since 1970, its incidence in some populations has increased by more than 800%.[1] Many possible risk factors, including obesity and tobacco use, have been identified, but the most common risk factor is the increased exposure of the esophagus to refluxed gastric contents.[1] One case–control study estimated that patients with long-standing severe gastroesophageal reflux disease (GERD) were 43 times more likely to have adenocarcinoma of the esophagus.[2]

The actual mechanism by which GERD initiates the development of adenocarcinoma is not proved but is thought to be the result of the development of the precursor lesion Barrett's esophagus.[3] The development of Barrett's esophagus because of GERD has been attributed to multiple mechanisms, including alteration in gene expression levels caused by repeated injury during exposure of refluxed material and

From the Department of Surgery, Division of Cardiothoracic Surgery, University of Colorado at Denver and Health Sciences Center, Denver, Colo.

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Address for reprints: Michael J. Weyant, MD, Department of Surgery, Division of Cardiothoracic Surgery, University of Colorado at Denver and Health Sciences Center, 4200 East Ninth Ave, C310, Denver, CO 80262 (E-mail: Michael.Weyant@UCHSC.edu).

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Abbreviations and Acronyms

ANOVA	= analysis of variance
DGEA	= duodeno-gastroesophageal anastomosis
EGF	= epidermal growth factor
GERD	= gastroesophageal reflux disease
PBS	= phosphate-buffered saline
$sPLA_2$	= secretory phospholipase A_2

migration of gastric cardia tissue more proximally or by conferring a competitive advantage to a mutant clone, allowing a population of cells to predominate in the mucosa.[3] The study of the events that lead to the development of this preneoplastic lesion can provide valuable insight into potential treatments that might prevent the development of esophageal adenocarcinoma.

The earliest response of esophageal mucosa to the presence of gastric reflux is the development of oxidative damage and inflammation.[4] Inflammation is manifested in several ways, including alterations in cytokine production, infiltration of inflammatory cells, and upregulation of inflammatory mediators. Histologic evidence of exposure of esophageal mucosa to reflux includes basal cell hyperplasia, acanthosis (or thickening of the squamous epithelium), and eosinophilic infiltration of the mucosa.[5]

Given the widely established link between the mucosal injury produced by GERD and the development of both Barrett's esophagus and carcinoma, the study of mediators of this inflammatory response has been intense. Molecules, such as cyclooxygenase-2,[6] nuclear factor κB,[7] and tumor necrosis factor α,[8] have been studied extensively regarding their role in esophageal mucosal inflammation. Recently, the group of phospholipase A_2 enzymes[9] has been implicated as a mediator of intestinal inflammation and identified as playing a possible role in tumor development. This group of enzymes is responsible for liberating arachidonic acid from phospholipids for eicosanoid production (Figure 2). A subtype of this group, group IIa secretory phospholipase A_2 ($sPLA_2$), is thought to play a significant role in the pathogenesis of inflammatory bowel disease.[10] Levels of $sPLA_2$ have also been shown to be increased in samples of human Barrett's esophagus, as well as adenocarcinomas, compared with levels in normal mucosa, indicating a potential role of $sPLA_2$ in the development of both of these pathologic lesions.[11]

A report by Kennedy and colleagues[12] first demonstrated that several species of inbred mice have a disruption in the $sPLA_2$ gene.[12] The occurrence of a naturally occurring murine knockout model has led to investigation into the possible role that $sPLA_2$ plays in cellular function. Insights gained from these studies have demonstrated that $sPLA_2$ might play a role in apoptosis in gastric mucosal cells[13] and contractile function of cardiac muscle,[14] as well as influencing antigen-presenting cell–mediated intestinal tumorigenesis.[15] We report our observations regarding the influence of the presence of $sPLA_2$ on the early response of esophageal mucosa to gastroduodenal reflux in a murine model.

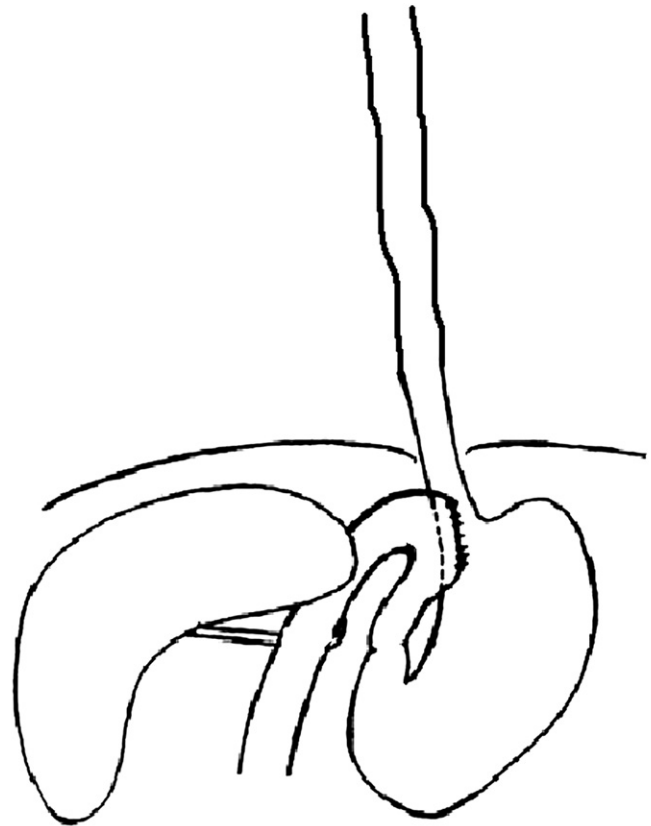

Figure 1. Schematic representation of the surgical model of creating mixed gastroduodenal (DGEA) reflux.

Materials and Methods

Generation of Gastroduodenal Reflux in a Murine Model

Eight-week-old BALB/c (n = 12) and C57BL/6 (n = 10) mice (Jackson Labs, Bar Harbor, Me) aged 8 to 12 weeks and weighing 18 to 22 g were fed regular chow (Harlan Teklad #2018, Madison, Wis) and water ad libitum. Animals were allowed to acclimatize for 10 days before surgical intervention. Animals were fasted but allowed access to water for 24 hours before the experimental procedure. Mice were anesthetized by means of the intraperitoneal injection of ketamine (80 mg/kg; Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (12 mg/kg; VEDCO, St Joseph, Mo). Body temperature was monitored rectally and maintained at 36.5°C by using a heating lamp. Under sterile conditions and with the aid of an operating microscope (Leica MZ95, Wetzlar, Germany), a side-to-side anastomosis was performed between the first portion of the duodenum and the gastroesophageal junction by using 10-0 nylon sutures (duodeno-gastroesophageal anastomosis; n = 7 BALB/c mice; n = 5 C57BL/6 mice; Figure 1).[16] Animals were then recovered under a heating

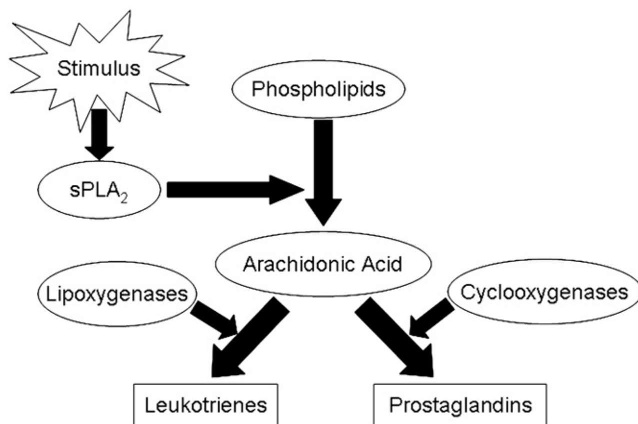

Figure 2. Diagram demonstrating the role of secretory phospholipase A_2 ($sPLA_2$) in the arachidonic acid pathway.

lamp. Control animals underwent similar anesthesia and laparotomy with incision and closure of the esophagus superior to the gastroesophageal junction without anastomosis (n = 5 in each strain). Animals then were fed ad libitum and weighed weekly to monitor weight gain. The Animal Care and Use Committee at the University of Colorado at Denver Health Sciences Center approved the protocol to perform the necessary survival operation and tissue harvesting for this project (protocol no. 77205206[05]1E).

Tissue Harvesting

The mice were sacrificed 28 days after surgical induction of gastroduodenal reflux by using inhaled carbon dioxide. The entire esophagus and stomach were then removed and flushed with OCT medium (OCT Tissue-Tek, Torrance, Calif). Care was taken to identify and use only tissue above the anastomosis for study. Three segments of tissue cut in 5-mm lengths originating just above the anastomosis were then cut, embedded in OCT medium, and frozen in a way that would allow axial sectioning of the esophageal lumen. Serial 5-μm sections were then mounted onto glass slides for histologic analysis. This study presents data obtained only from the blocks that were closest to the anastomosis, thus comparing the same segment from all animals.

Morphologic and Immunohistochemical Analysis of Esophageal Tissue

Hematoxylin and eosin staining was performed to evaluate mucosal morphology. Four digital images taken around the circumference of each specimen were acquired, and 3 measurements of mucosal thickness were made at equal intervals within each digital image by a blinded observer. Data were compared by means of analysis of variance (ANOVA) with the post-hoc Tukey test.

Detection of $sPLA_2$ Protein in Esophageal Tissue by Using Immunofluorescence

Esophageal tissue frozen in OCT medium was cut and placed on slides. Slides were then fixed in acetone/methanol (1:1), blocked in 5% donkey serum containing 1% albumin in phosphate-buffered saline (PBS), and then incubated with a polyclonal antibody to human $sPLA_2$ (goat anti-human antibody; Santa Cruz Biotechnology, Santa Cruz, Calif) in a dilution of 1:50 in 1% albumin/PBS for 1 hour at room temperature. After washing in PBS, the slides were then incubated with a Cy3-conjugated Donkey anti-goat antibody (Jackson Immunoresearch, West Grove, Pa) in a 1:150 dilution for 45 minutes at room temperature, washed, and placed under coverslips over antiquenching medium. Visualization of staining for $sPLA_2$ was performed on a Zeiss confocal microscope (Thornwood, NY).

Identification of Proliferating Cells by Using Ki67 Immunohistochemistry

Endogenous peroxidase activity was blocked by incubating slides for 10 minutes in 0.3% H_2O_2 in methanol. Slides were then fixed in acetone/methanol (30:70) for 5 minutes. After washing in PBS, slides were fixed with 4% paraformaldehyde for 10 minutes. Antigen retrieval was performed with a citrate buffer bath for 20 minutes. Slides were then bathed in distilled water and washed in PBS. Slides were then incubated in 5% blocking serum with 0.3% Triton in PBS (sheep or rabbit) for 30 minutes. Samples were then incubated in rabbit polyclonal antibody to Ki67 (NovusBio, Littleton, Colo) 1:25 in 0.3% Triton in PBS for 12 hours at 4°C. After washing in PBS 3 times, the slides were incubated with a biotinylated sheep anti-rabbit secondary antibody (Serotec, Raleigh, NC) 1:250 with 0.3% Triton in PBS for 1 hour at room temperature. After washing in PBS and incubating in horseradish peroxidase complex for 30 minutes, slides were developed with 3,3′-diaminobenzidine. Four digital images of each of 3 esophageal segments for each animal were acquired by using a 40× objective. In each image the total number of cells in the esophageal mucosa with nuclei that stained positive for Ki67 was counted by a blinded observer.

Statistical Methods

Data were compared by means of ANOVA with the post-hoc Tukey test.

Results

Outcome of Surgical Procedure and Health of Mice

Overall, 90% of the animals survived the procedures and study period. Two of the 7 BALB/c mice died perioperatively after the study (duodeno-gastroesophageal anastomosis) procedure, and no animals died either after the sham procedure or during the period of 24 hours after surgical intervention and the 28-day end point of the experiment. The 2 animals that died might have died of excessive anesthesia, hemorrhage, or intestinal ischemia, but a definitive cause was not recognized. The mean body weights in all of the animal groups were not significantly different at the end of the study period (C57BL/6 mice, $P = .87$; BALB/c (DGEA) mice, $P = .39$; Table 1). The BALB/c duodeno-gastroesophageal anastomosis mice initially have slower weight gain but recover to normal at least 2 weeks before the end of the study period.

The animals appeared grossly normal at the termination of the experiment. On visual inspection of the esophageal tissue after harvest, there grossly did not appear to be any differences among the study groups. Tumor tissue was not grossly identified in any of the specimens. The anastomotic areas in all DGEA animals were identified to be patent, as demonstrated by passing a probe through the anastomosis in each animal.

TABLE 1. There were no significant differences in weight (group average ± standard error of the mean) between animals that underwent sham operations and those undergoing reflux operations

	Week 0	Week 1	Week 2	Week 3	Week 4
BALB/c DGEA	22.9 ± 0.69	22.1 ± 0.98	22.6 ± 0.93	23.9 ± 1.00	26.3 ± 0.80
BALB/c CTRL	23 ± 0.46	23.5 ± 0.69	24.24 ± 0.67	25.5 ± 0.68	26.1 ± 0.67
C57BL/6 DGEA	20.8 ± 0.79	22.6 ± 0.44	23.36 ± 0.25	24.9 ± 0.24	25.5 ± 0.41
C57BL/6 CTRL	20.8 ± 0.33	22.8 ± 0.15	22.8 ± 0.32	24.5 ± 0.43	25.3 ± 0.43

P values for week 4 are shown in the text. *DGEA*, duodeno-gastro esophageal anastomosis; *CTRL*, control.

Microscopic Analysis of Esophageal Tissue

We evaluated by means of immunofluorescence the presence of the $sPLA_2$ protein in esophageal tissue in the study animals. We clearly identified the presence of $sPLA_2$ in the esophageal mucosa of BALB/c mice, but the C57BL/6 mice had no identifiable $sPLA_2$ in esophageal mucosa when compared with the negative control animals (Figure 3).

Microscopically, DGEA induced observable changes in the esophageal mucosa of BALB/c, $sPLA_2^{+/+}$ mice ($P <$ .0001, ANOVA). These changes included a significant thickening of the mucosal layer similar to that described in human subjects (BALB/c DGEA mice vs BALB/c sham mice: $P \leq$ .001).[17] These changes were most clearly evident in the 5 mm of esophagus immediately superior to the anastomotic area (which is the data presented in this study). The changes appeared to dissipate in a graded fashion, moving more proximally in the esophagus (data not shown). Epithelial thickness was not significantly different in the $sPLA_2^{-/-}$ C57BL/6 mice with surgically induced reflux (C57BL/6 DGEA mice vs C57BL/6 sham mice: $P = .47$, Tukey post-hoc test), with no significant microscopic changes seen by means of hematoxylin and eosin staining in any portion of the esophagus compared with that seen in control animals, thus indicating the potential role that this enzyme might play in these mucosal changes (Figure 4). Evaluating the presence of proliferating cells through the use of Ki67 staining demonstrated that the BALB/c mice had a significant increase in the number of proliferating cells when compared with the control animals ($P < .0001$ [ANOVA] and $P <$ .001 [Tukey post-hoc test] for BALB/c DGEA mice vs BALB/c sham mice). These proliferating cells appeared to be most prominent in the basal layers, which is a consistent pathologic finding in human gastroesophageal reflux.[17] Again, there was no significant difference between the treatment and control groups in the $sPLA_2$ null mice ($P = .22$, Tukey post-hoc test, for C57BL/6 DGEA mice vs C57BL/6 sham mice; Figure 5).

Discussion

Histologic changes that are produced in response to gastroduodenal reflux include thickening (acanthosis) of the mucosa,

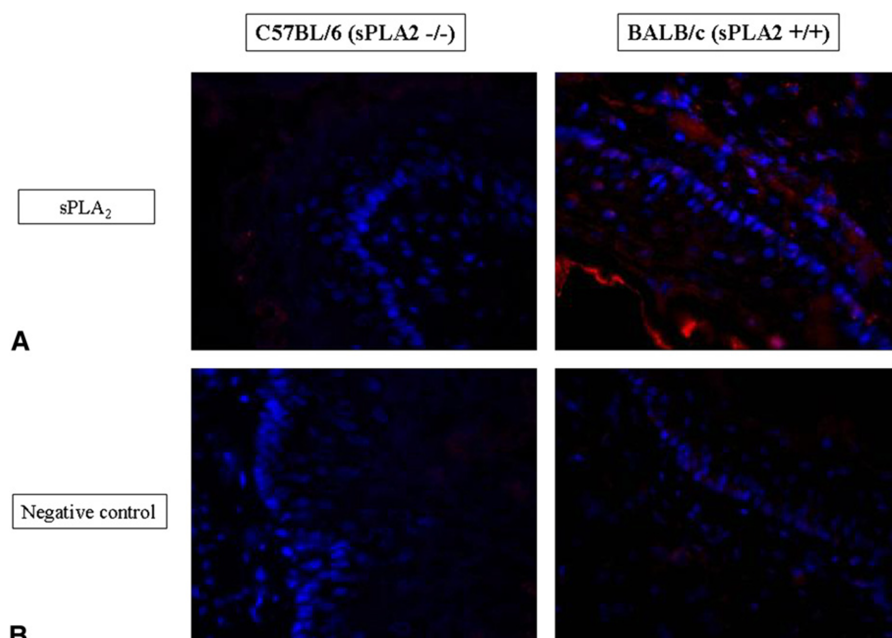

Figure 3. Immunofluorescence analysis of DGEA esophageal tissue in study animals demonstrating no identifiable protein in C57BL/6 mice and identifiable secretory phospholipase A_2 ($sPLA_2$) protein (red staining) in BALB/c mice (A) compared with that seen in negative control animals (B).

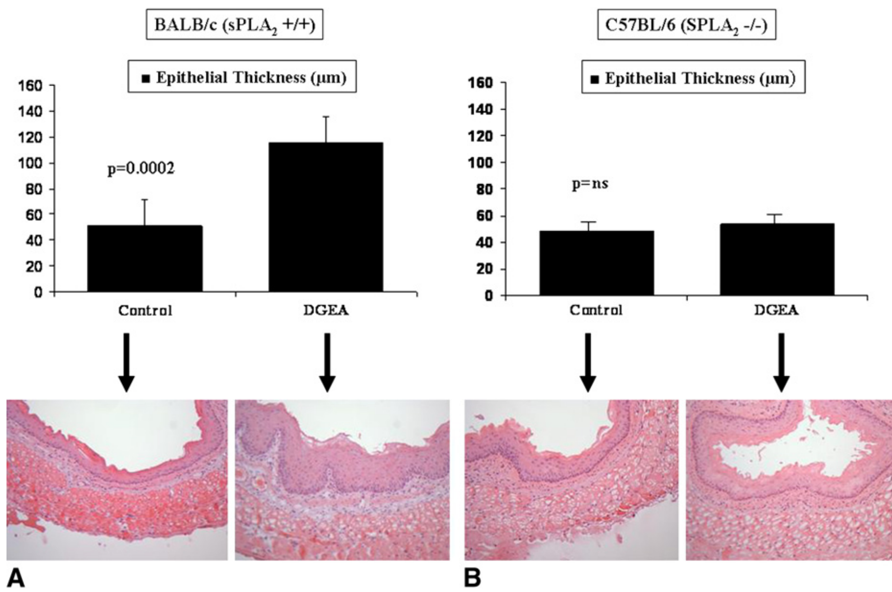

Figure 4. Hematoxylin and eosin analysis of mucosal thickness at 20× magnification. Significant increase in mucosal thickness in response to DGEA in BALB/c secretory phospholipase A_2 ($sPLA_2$) +/+ mice (n = 5; A) compared with C57BL/6 $sPLA_2^{-/-}$ mice (n = 5; B) is shown.

hyperproliferation, and eosinophilic infiltration.[5,17] Here we have shown in a murine model that histologic changes in response to gastroduodenal reflux are similar to those seen in human and other animal models. The model we have adapted was originally described to produce esophageal adenocarcinoma and changes similar to Barrett's esophagus in rats over a 40-week period.[16] We have previously demonstrated that the technical aspects of the same model can be successfully applied in mice (Babu and Weyant and associates, manuscript in preparation), and our observations show that with the introduction of the DGEA, there is no significant alteration in such nutritional parameters as food intake and body weight during the study period. The time period of 4 weeks was chosen to allow the dissolution of the effects of surgical intervention and healing on the esophageal mucosa, yet provide an opportunity to observe the early effects of reflux.

In this study we have demonstrated that histologic changes caused by gastroduodenal reflux might be influenced by the genetic background of the host animal. We take advantage of a naturally occurring disruption of the $sPLA_2$ gene that occurs in C57BL/6 mice and use it to study the effects of the presence of this enzyme during stimulation of esophageal mucosa with DGEA by comparing them with BALB/c

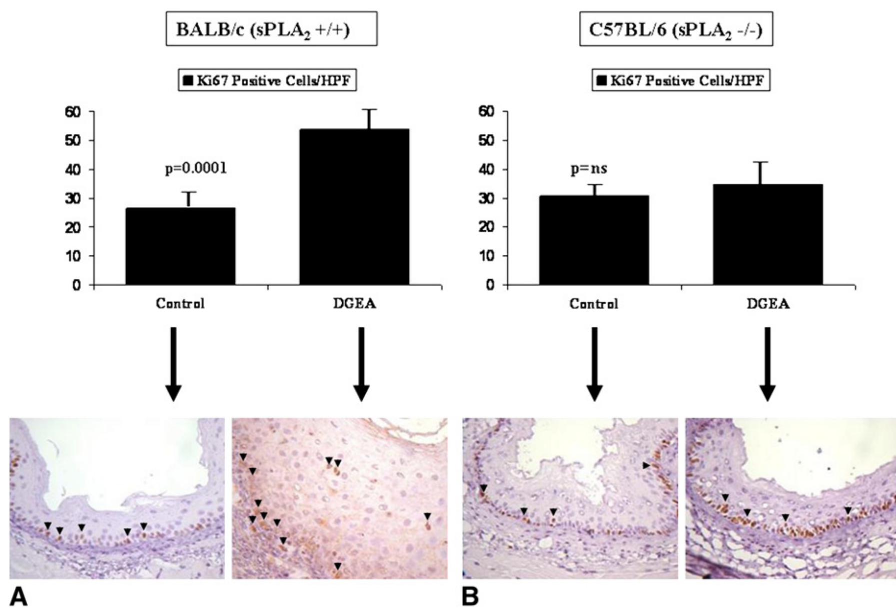

Figure 5. Ki67 immunohistochemistry demonstrating a significant increase in proliferating cells in response to DGEA in BALB/c secretory phospholipase A_2 ($sPLA_2$) +/+ mice (n = 5; A) compared with C57BL/6 $sPLA_2^{-/-}$ mice (n = 5; B). *Black arrowheads* point to some of the positive nuclei as an example. *HPF*, High-powered field.

mice known to express high levels of the enzyme. The convenience of a naturally occurring disruption of the $sPLA_2$ gene has led to several observational studies comparing these 2 strains of mice, demonstrating functional differences in such areas as cardiac contractile function and susceptibility to the development of intestinal tumorigenesis.[14,15]

The most well-described role of the group of $sPLA_2$ enzymes is to catalyze the hydrolysis of membrane phospholipids, leading to the generation of arachidonic acid, thus providing the substrate for inflammatory mediators, such as prostaglandins and leukotrienes.[18] The induction and overexpression of $sPLA_2$ enzymes are implicated in a variety of pathologic processes, such as septic shock and inflammatory bowel disease.[18] It is not surprising that there is significant interaction with other known pathogenic enzymes, such as cyclooxygenase-1 and -2.

Our observations suggest that $sPLA_2$ might play a role in regulating esophageal mucosal growth and the hyperplasia produced by GERD. The role of $sPLA_2$ as a potential growth-regulating protein is demonstrated in other in vivo and in vitro models. Grass and colleagues[19] reported that in the same C57BL/6 mouse with human group IIa $sPLA_2$ reintroduced into the genome, there was epidermal hyperplasia at baseline, indicating an effect of the reconstituted enzyme on cell proliferation, apoptosis, or both. Other in vitro studies have demonstrated that arachidonic acid metabolites are required for epidermal growth factor (EGF)–mediated cell proliferation.[20,21] This is important considering that EGF/EGF receptor signaling is an important pathway regulating cell proliferation in the esophageal mucosa.[22] In macrophages $sPLA_2$ has been demonstrated to mediate phosphorylation of Akt, which leads to a downstream growth regulatory effect in these cells.[23] In murine small intestinal tissue an $sPLA_2$–like molecule has been shown to bind to EGF and influence cell proliferation.[24] Given the reported role of the EGF receptor in all types of esophageal carcinoma, this represents an important relationship to be studied in this model.[25] In human subjects the observation that levels of this enzyme are increased in both Barrett's mucosa and esophageal adenocarcinoma indicates a possible role of $sPLA_2$ in growth and metaplastic transformation of these cells.[11] Characterization of the presence of $sPLA_2$ activity, as well as downstream mediators, in these animal strains exposed to reflux will be an important direction of future study to make a mechanistic link to epithelial growth regulation.

We demonstrate that mice with an intact $sPLA_2$ gene appear to have an enhanced development of histologic changes associated with gastroduodenal reflux as early as 4 weeks after the surgical induction of reflux. Importantly, we demonstrate the ability to identify the $sPLA_2$ protein in esophageal tissue in BALB/c mice, as well as showing its absence in the C57BL/6 mice by means of immunofluorescence. Recent studies using other methods of detection have not demonstrated high levels of this protein in BALB/c esophageal tissue. The ability to clearly identify the presence of the enzyme in our study animals might indicate the finding that the enzyme is upregulated in response to surgical intervention. Further studies to clarify this finding are underway.[26] The relevance of histologic changes seen in this model with respect to Barrett's esophagus, as well as esophageal carcinoma, remain unknown. However, in similar rodent models the progression from epithelial hyperplasia to Barrett's metaplasia has been demonstrated, suggesting that these are indeed clinically significant changes.[27]

Given the available data demonstrating the presence of this genetic anomaly, it would be unwise to assume that this might be the only contributing factor to the epithelial changes seen here. Nonetheless, these findings allude to the involvement of $sPLA_2$ in the manifestation of histologic changes early in the response to DGEA. This murine model also demonstrates significant utility in the study of hyperplastic changes related to reflux. Our findings highlight $sPLA_2$ as a potential agent to be studied in the treatment of GERD, as well as chemoprevention of esophageal cancer.

References

1. DeMeester SR. Adenocarcinoma of the esophagus and cardia: a review of the disease and its treatment. *Ann Surg Oncol*. 2006;13:12-30.
2. Lagergren J, Bergstrom R, Lindgren A, Nyren O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med*. 1999;340:825-31.
3. Maley CC. Multistage carcinogenesis in Barrett's esophagus. *Cancer Lett*. 2007;245:22-32.
4. Lee JS, Oh TY, Ahn BO, Cho H, Kim WB, Kim YB, et al. Involvement of oxidative stress in experimentally induced reflux esophagitis and Barrett's esophagus: clue for the chemoprevention of esophageal carcinoma by antioxidants. *Mutat Res*. 2001;480-481:189-200.
5. Takubo K, Honma N, Aryal G, Sawabe M, Arai T, Tanaka Y, et al. Is there a set of histologic changes that are invariably reflux associated? *Arch Pathol Lab Med*. 2005;129:159-63.
6. Altorki N. COX-2: a target for prevention and treatment of esophageal cancer. *J Surg Res*. 2004;117:114-20.
7. O'Riordan JM, Abdel-latif MM, Ravi N, McNamara D, Byrne PJ, McDonald GS, et al. Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am J Gastroenterol*. 2005;100:1257-64.
8. Eksteen JA, Scott PA, Perry I, Jankowski JA. Inflammation promotes Barrett's metaplasia and cancer: a unique role for TNFalpha. *Eur J Cancer Prev*. 2001;10:163-6.
9. Diaz BL, Arm JP. Phospholipase A(2). *Prostaglandins Leukot Essent Fatty Acids*. 2003;69:87-97.
10. Haapamaki MM, Gronroos JM, Nurmi H, Irjala K, Alanen KA, Nevalainen TJ. Phospholipase A2 in serum and colonic mucosa in ulcerative colitis. *Scand J Clin Lab Invest*. 1999;59:279-87.
11. Lagorce-Pages C, Paraf F, Wendum D, Martin A, Flejou JF. Expression of inflammatory secretory phospholipase A2 and cytosolic phospholipase A2 in premalignant and malignant Barrett's oesophagus. *Virchows Arch*. 2004;444:426-35.
12. Kennedy BP, Payette P, Mudgett J, Vadas P, Pruzanski W, Kwan M, et al. A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains. *J Biol Chem*. 1995;270:22378-85.
13. Wang TC, Goldenring JR, Dangler C, Ito S, Mueller A, Jeon WK, et al. Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with Helicobacter felis infection. *Gastroenterology*. 1998;114:675-89.

14. Stull LB, Hiranandani N, Kelley MA, Leppo MK, Marban E, Janssen PM. Murine strain differences in contractile function are temperature- and frequency-dependent. *Pflugers Arch*. 2006;452:140-5.
15. Markova M, Koratkar RA, Silverman KA, Sollars VE, MacPhee-Pellini M, Walters R, et al. Diversity in secreted PLA2-IIA activity among inbred mouse strains that are resistant or susceptible to Apc Min/+ tumorigenesis. *Oncogene*. 2005;24:6450-8.
16. Chen X, Yang G, Ding WY, Bondoc F, Curtis SK, Yang CS. An esophagogastroduodenal anastomosis model for esophageal adenocarcinogenesis in rats and enhancement by iron overload. *Carcinogenesis*. 1999;20:1801-8.
17. Dent J. Microscopic esophageal mucosal injury in nonerosive reflux disease. *Clin Gastroenterol Hepatol*. 2007;5:4-16.
18. Touqui L, Alaoui-El-Azher M. Mammalian secreted phospholipases A2 and their pathophysiological significance in inflammatory diseases. *Curr Mol Med*. 2001;1:739-54.
19. Grass DS, Felkner RH, Chiang MY, Wallace RE, Nevalainen TJ, Bennett CF, et al. Expression of human group II PLA2 in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate. *J Clin Invest*. 1996;97:2233-41.
20. Handler JA, Danilowicz RM, Eling TE. Mitogenic signaling by epidermal growth factor (EGF), but not platelet-derived growth factor, requires arachidonic acid metabolism in BALB/c 3T3 cells. *J Biol Chem*. 1990;265:3669-73.
21. Sellmayer A, Uedelhoven WM, Weber PC, Bonventre JV. Endogenous non-cyclooxygenase metabolites of arachidonic acid modulate growth and mRNA levels of immediate-early response genes in rat mesangial cells. *J Biol Chem*. 1991;266:3800-7.
22. Katayama M, Shoji M, Satomi S. Differential growth properties of normal and malignant esophageal epithelial cells: a possible cross talk between transforming growth factor-beta1 and epidermal growth factor signaling. *Tohoku J Exp Med*. 2005;206:61-71.
23. Park DW, Kim JR, Kim SY, Sonn JK, Bang OS, Kang SS, et al. Akt as a mediator of secretory phospholipase A2 receptor-involved inducible nitric oxide synthase expression. *J Immunol*. 2003;170:2093-9.
24. Mulherkar R, Rao R, Rao L, Patki V, Chauhan VS, Deo MG. Enhancing factor protein from mouse small intestines belongs to the phospholipase A2 family. *FEBS Lett*. 1993;317:263-6.
25. Wilkinson NW, Black JD, Roukhadze E, Driscoll D, Smiley S, Hoshi H, et al. Epidermal growth factor receptor expression correlates with histologic grade in resected esophageal adenocarcinoma. *J Gastrointest Surg*. 2004;8:448-53.
26. Eerola LI, Surrel F, Nevalainen TJ, Gelb MH, Lambeau G, Laine VJ. Analysis of expression of secreted phospholipases A2 in mouse tissues at protein and mRNA levels. *Biochim Biophys Acta*. 2006;1761:745-56.
27. Ireland AP, Peters JH, Smyrk TC, DeMeester TR, Clark GWB, Mirvish SS, et al. Gastric juice protects against the development of esophageal adenocarcinoma in the rat. *Ann Surg*. 1996;223:358-62.

Discussion

Dr Steven DeMeester *(Los Angeles, Calif)*. I would like to congratulate Dr Weyant and his colleagues both for an excellent presentation and a very clearly written manuscript.

In this study the authors evaluated the role of sPLA$_2$ in a mouse model of surgically induced gastroduodenoesophageal reflux. By using an inbred mouse strain deficient in the gene for phospholipase A$_2$, they were able to ascertain the role of this enzyme in early esophageal mucosal changes associated with reflux. Compared with normal mice, the knockout mice showed significantly less increase in epithelial thickness and in Ki67 activity, with levels in the knockout mice equivalent to those in control animals. They conclude that phospholipase A$_2$ is involved in the early squamous mucosal changes associated with gastroduodenoesophageal reflux.

The authors are to be congratulated for several things. First, they successfully developed this mouse model of surgically induced reflux, which has been a hurdle for many investigators in the past. Second, they demonstrate that the phospholipase A$_2$ protein can be found in esophageal tissue in the mouse. Third, they designed an elegant first experiment by using a mouse knockout model for this gene to demonstrate the role of phospholipase A$_2$ in early squamous esophageal mucosal injury associated with reflux. I have 3 questions for you.

First, changes in esophageal mucosal thickness or the old (Ishmael Bagay) criteria for reflux are known to be quite nonspecific. Recent work by Ray Orlando and colleagues using transmission electron microscopy to look at dilated intracellular channels suggests that this might represent a very sensitive and specific way to assess early mucosal changes associated with reflux. Have you considered using transmission electron microscopy to evaluate in the mouse the presence or absence of these dilated intracellular channels?

Dr Weyant. I am aware of the technique, but at our institution, it is difficult to achieve the use of that specific technique, although it is something that we are certainly interested in looking at in the future.

Dr Demeester. Second, the really significant (ie, premalignant) changes that occur with reflux involve the transformation of the squamous esophageal mucosa to columnar cardiac mucosa and then subsequently to intestinal metaplasia. Do you anticipate that with a longer duration of reflux in this mouse model that these histologic changes will occur? In other words, can you replicate in the mouse model what has been shown to occur in the rat model of gastroduodenoesophageal reflux?

Dr Weyant. I believe we can. I am certainly very optimistic that we can. Even after 4 weeks in some of these mice, we are actually able to see changes that appear to be the early morphologic changes consistent with Barrett's esophagus. I did not have the time to show the slides here, but our next phase of these studies is going to be to take these mice and reflux them for several weeks, up to 40 weeks, and harvest the mice at several different time periods so that we have the whole spectrum of carcinogenesis of gastroesophageal junction adenocarcinoma; that will then allow us to study all of these molecular markers at these different time points in this model, as well as using the similar model in other genetic backgrounds, such as a P53 knockout mouse and others that are available, where we can apply this model now that we are proficient in it.

Dr Demeester. Lastly, although there is no doubt that a link between inflammation and cancer exists, the nature of that relationship is very complex. Rebecca Fitzgerald, in addition to work from our own laboratory, has shown that there is an inflammatory gradient within Barrett's esophagus with maximal expression of inflammatory genes proximally in a Barrett's esophagus segment, yet many of the cancers occur distally in areas with much lower levels of inflammatory gene expression. In light of this, can you give us your thoughts on the role of phospholipase A$_2$ and what its role might be in carcinogenesis within Barrett's esophagus? Do you think it is an early mediator of progression in Barrett's esophagus or merely a marker for injury?

Congratulations again and thank you for the opportunity to discuss this paper.

Dr Weyant. Thank you very much for your comments, Dr DeMeester.

Part of our next phase of these studies also goes along with what our next main hypothesis is regarding the function of sPLA$_2$ in that

our initial observations showed of these quick and dirty findings of hyperplasia and thickening of the mucosa, but what that really points to for me is that $sPLA_2$ is somehow a growth regulator in esophageal mucosa. Some of the articles I have listed on one of the previous slides are some of the less well-known literature regarding $sPLA_2$, and not one of them has sort of hit on the main point that it interacts with EGF receptor and its ligand, which we know is already a growth factor in esophageal mucosa, as well as other growth factors. Again, these are reported in other tissues using different models, but I think our next step is to apply those hypotheses to this model to see really how $sPLA_2$ acts as a growth factor in esophageal mucosa, and I think we will.

Doctor. I have a quick follow-up question for you, which speaks to my inherent suspicion of knockout mice. Can you comment more on these collateral secretory phenotypic changes associated with the black-6 mouse? In other words, I appreciate that your inhibitor studies validate that phospholipase A_2 is at least a necessary cofactor in this preparation, but are there any other changes that result from this particular genetic variation as relates to acid or bile salt excretion?

Dr Weyant. As far as other experience goes, no. That is a continual concern of ours, and data that I did not present here that we have also recently accumulated in the laboratory—there is actually a mouse available with a black-6 background that has the $sPLA_2$ gene reintroduced into it. When we do this same model here comparing a black-6 wild-type mouse and a black-6 mouse that has the $sPLA_2$ gene reintroduced, we get the same findings of the hyperplasia and thickening of mucosa. Between the inhibitor studies and what we call the ''knock-in'' studies, that is as confident as I can be that we are actually seeing a real change here. The reason we went to these lengths of using the inhibitor study and the knock-in mouse was because of those concerns that we shared also.

Doctor. I agree. Excellent job. Elegant work, and everybody on the project should be commended on technically pulling this off. That is a real feat of technical expertise. Congratulations.