GERD is associated with shortened telomeres in the squamous epithelium of the distal esophagus

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Souza RF, Lunsford T, Ramirez RD, Zhang X, Lee EL, Shen Y, Owen C, Shay JW, Morales C, Spechler SJ. GERD is associated with shortened telomeres in the squamous epithelium of the distal esophagus. Am J Physiol Gastrointest Liver Physiol 293: G19–G24, 2007. First published March 29, 2007; doi:10.1152/ajpgi.00055.2007.—Telomeres are repetitive DNA sequences located at the ends of chromosomes. Telomeres are shortened by repeated cell divisions and by oxidative DNA damage, and cells with critically shortened telomeres cannot divide. We hypothesized that chronic gastroesophageal reflux disease (GERD)-induced injury of the esophageal squamous epithelium results in progressive telomeric shortening that eventually might interfere with mucosal healing. To address our hypothesis, we compared telomere length and telomerase activity in biopsy specimens of esophageal squamous epithelium from GERD patients and control patients. Endoscopic biopsies were taken from the esophageal squamous epithelium of 38 patients with GERD [10 long-segment Barrett’s esophagus (LSBE), 15 short-segment (SSBE), 13 GERD without Barrett’s esophagus] and 16 control patients without GERD. Telomere length was assessed using the terminal restriction fragment assay, and telomerase activity was studied by the PCR-based telomeric repeat amplification protocol assay. Patients with GERD had significantly shorter telomeres in the distal esophagus than controls [8.3 ± 0.5 vs. 10.9 ± 1.5 (SE) Kbp, P = 0.043]. Among the patients with GERD, telomere length in the distal esophagus did not differ significantly in those with and without Barrett’s esophagus (LSBE 7.9 ± 0.8, SSBE 8.6 ± 0.9, GERD without BE 8.7 ± 1.0 Kbp). No significant differences in telomerase activity in the distal esophagus were noted between patients with GERD and controls (4.0 ± 0.39 vs. 5.2 ± 0.53 RIUs). Telomeres in the squamous epithelium of the distal esophagus of patients who have GERD, with and without Barrett’s esophagus, are significantly shorter than those of patients without GERD despite similar levels of telomerase activity.

gastroesophageal reflux disease; Barrett’s esophagus; telomerase

AT THE 3’ ENDS of all eukaryotic chromosomes are long regions of noncoding, repetitive DNA sequences called telomeres, which are needed to maintain normal chromosomal stability and function (3, 4, 6). During DNA replication, the cell’s replication machinery cannot copy completely the 3’ ends of chromosomes and, consequently, 50 to 200 bp of telomeric DNA are lost during each round of cell division (31). Oxidative damage can also shorten telomeres, which appear to be preferential sites for oxidative DNA injuries (11, 13, 22, 32). When telomeric shortening due to repeated cell divisions and oxidative damage reaches a certain critical level, it triggers exit from the cell cycle and entry into a permanent, growth-arrested state called senescence (1).

Telomeric shortening is judged to be the major intrinsic mechanism that ultimately limits the proliferative capacity of normal cells. Telomeric shortening from cell divisions and oxidative DNA damage can be halted if cells can express sufficient levels of telomerase, a cellular ribonucleoprotein enzyme that catalyzes the synthesis of new telomeric DNA (5). For some proliferating cells, telomerase expression prevents the critical telomeric shortening that otherwise would result in senescence.

A number of studies have shown that gastroesophageal reflux disease (GERD) increases proliferation in esophageal squamous cells (15, 34). Severe GERD is also associated with the generation of reactive oxygen species (ROS) that cause oxidative DNA damage in the esophageal squamous epithelium (23, 30, 33). The GERD-induced increase in cellular proliferation and oxidative DNA damage would be expected to result in shortened telomeres if the squamous cells are not able to produce sufficient levels of telomerase to counterbalance their loss of telomeric DNA.

We hypothesized that repeated cycles of GERD-induced injury and regeneration of esophageal squamous cells cause a progressive shortening of telomeres. It is possible that the GERD-induced loss of telomeric DNA might eventually reach a critical level that triggers senescence, thereby interfering with esophageal healing and promoting epithelial repair through alternative mechanisms like metaplasia. The aim of our study was to compare telomere length and telomerase activity in biopsy specimens of esophageal squamous epithelium from GERD patients and control patients.

MATERIALS AND METHODS

Study patients, endoscopic examination, and biopsy protocol. Patients scheduled for elective endoscopy at the Dallas VA Medical Center were invited to participate in the study. The study was approved by the IRB committee at the Dallas VA Medical Center and written informed consent was obtained from all patients. Patients were considered to have GERD if they had heartburn (off antireflux medications) at least once per month and/or endoscopic evidence of

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reflux esophagitis or Barrett’s esophagus during the study endoscopic examination or during a previous endoscopic examination. Control patients had no heartburn and no endoscopic signs of reflux esophagitis or Barrett’s esophagus.

During the endoscopic examination, reflux esophagitis was scored using the Los Angeles grading system (17). The gastroesophageal junction (GEJ) was identified as the most proximal extent of the gastric folds with the stomach partially inflated. Barrett’s esophagus was suspected if columnar epithelium was seen to extend proximal to the GEJ, and confirmed if biopsy specimens of the esophageal columnar epithelium showed specialized intestinal metaplasia. Patients with Barrett’s esophagus were further categorized as long-segment (LSBE) if specialized intestinal metaplasia extended ≥3 cm above the GEJ, and as short-segment (SSBE) if specialized intestinal metaplasia extended <3 cm above the GEJ. Biopsy specimens were taken for study purposes in all patients using jumbo biopsy forceps (Olympus FB-50K-1) as follows: 1) two specimens at the squamo-columnar junction (Z-line), 2) five specimens from the distal esophagus at 1 cm above the Z-line, 3) five specimens from the proximal esophagus at 20 cm from the incisor teeth, and 4) five specimens from the gastric fundus at 5 cm below the GEJ. The two specimens from the Z-line were used for histological evaluation only. For the other sets of five biopsy specimens, two were sent for histological evaluation and the other three were used for analyses of telomerase activity and telomere length.

Histological scoring of reflux esophagitis. Hematoxylin and eosin-stained slides of esophageal squamous epithelium were graded for inflammation by consensus of two pathologists (Y.S. and E.L.L.), who were blinded to clinical information [based on the number of eosinophils, lymphocytes, or neutrophils per high-power field (HPF)] as none (0 cells per HPF), mild (1 cell per HPF), moderate (2–5 cells per HPF), or severe (>5 cells per HPF).

Telomerase activity and telomere length analysis. Telomerase activity was studied using the PCR-based TRAP-eee Telomerase Detection kit (Intergen, Burlington, MA) as previously described (12). Briefly, 1 μg of protein isolated from frozen biopsy specimens was first incubated with a telomerase substrate Cy5-conjugated primer; an internal control. The telomerase-generated products and ITAS were amplified by PCR, and resolved on 10% nondenaturing acrylamide gels. The gel was denatured, dried, and hybridized with a 32P-labeled telomeric (CCCTAA)4 probe, complementary to the telomeric sequence. The gel was exposed on a PhosphorImager and mean telomere length was calculated using the program TELORUN provided by C. Harley, R. Allsopp, and H. Vaziri, as previously described (24). Statistical analysis. Statistical comparisons of telomerase activities and telomere lengths between biopsy specimens from GERD patients and controls were performed using an unpaired Student’s t-test using the Instat for Windows statistical software package (GraphPad Software). Statistical analyses of telomerase activity and telomere length of biopsy specimens and of patient characteristics between GERD and control patients with and without intestinal metaplasia were performed using an ANOVA using the Instat for Windows statistical software package (GraphPad Software). Statistical comparisons of telomerase activity between the gastric fundus and the distal and proximal esophagus within patient groups were performed using a paired ANOVA using the Instat for Windows statistical software package (GraphPad Software). P values of ≤0.05 were considered significant for all analyses.

RESULTS

Study population. The study population comprised 38 patients with GERD (including 15 patients with LSBE, 10 patients with SSBE, and 13 patients who had GERD without Barrett’s esophagus) and 16 control patients. Clinical characteristics of the patients are shown in Table 1.

Telomere length in the distal squamous-lined esophagus is shorter in GERD patients than in controls. In the distal squamous-lined esophagus (1 cm proximal to the Z-line), mean telomere length was significantly shorter in the 38 patients with GERD than in the 16 control patients [8.3 ± 0.5 (SE) vs. 10.9 ± 1.5 Kbp, P = 0.043; Fig. 1]. No significant differences in mean telomere length were noted between GERD patients and controls for the proximal esophagus (GERD 9.4 ± 0.7, control 11.0 ± 1.5 Kbp) or for the gastric fundus (GERD 8.8 ± 0.4, controls 11.0 ± 1.5 Kbp).

We performed subgroup analyses to determine whether there is an association between Barrett’s esophagus and the length of telomeres in the esophageal squamous epithelium. We found no significant differences in mean telomere lengths among

Table 1. Clinical characteristics of study patients

<table>
<thead>
<tr>
<th></th>
<th>SSBE (n = 10)</th>
<th>LSBE (n = 15)</th>
<th>GERD, no BE (n = 13)</th>
<th>Controls (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, yr (range)</td>
<td>55.7 (42–76)</td>
<td>60.9 (52–74)</td>
<td>66 (52–80)</td>
<td>61.1 (41–78)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1 (10%)</td>
<td>1 (6.7%)</td>
<td>2 (15%)</td>
<td>1 (6.2%)</td>
</tr>
<tr>
<td>Male</td>
<td>9 (90%)</td>
<td>14 (93.3%)</td>
<td>11 (85%)</td>
<td>15 (93.8%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>9 (90%)</td>
<td>15 (100%)*</td>
<td>10 (77%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>African-American</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (23%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (10%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3 (19%)</td>
</tr>
<tr>
<td>Acid-suppressive</td>
<td>10 (100%)</td>
<td>14 (93%)</td>
<td>12 (92%)</td>
<td>6 (37.5%)*</td>
</tr>
<tr>
<td>Endoscopic esophagitis</td>
<td>1 (10%)</td>
<td>3 (20%)</td>
<td>4 (31%)</td>
<td>0</td>
</tr>
<tr>
<td>LA grade</td>
<td>(1 A)</td>
<td>(1 C; 2B)</td>
<td>(1A; 2B; 1C)</td>
<td></td>
</tr>
<tr>
<td>Specialized intestinal metaplasia in biopsies</td>
<td>at the Z-line</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSBE</td>
<td>10 (100%)</td>
<td>15 (100%)</td>
<td>0 (0%)</td>
<td>8 (50%)</td>
</tr>
</tbody>
</table>

SSBE, short-segment Barrett’s esophagus; LSBE, long-segment Barrett’s esophagus; BE, Barrett’s esophagus; GERD, gastroesophageal reflux disease. *P < 0.05.
Patients with GERD and control patients have similar levels of telomerase activity in the squamous-lined esophagus. Although patients with GERD had significantly shorter telomeres in the distal esophagus than control patients, we found no significant differences in telomerase activities between GERD patients and controls for the distal squamous esophagus [GERD 4.0 ± 0.39 (SE) relative intensity units (RIUs), control 5.2 ± 0.53 RIUs] (Fig. 3), for the proximal esophagus, or for the gastric fundus (data not shown). We also found no significant differences in telomerase activities at any site between control patients with and without IM at the GEJ (data not shown).

Telomerase activities are lower in the gastric fundus than in the esophagus in both patient groups. Telomerase activities in the gastric fundus (GERD 0.38 ± 0.15, control 0.64 ± 0.24 RIUs) were significantly lower than those in the distal esophagus (GERD 4.0 ± 0.39, control 5.2 ± 0.53 RIUs) and in the proximal esophagus (GERD 5.3 ± 0.31, control 5.6 ± 0.55 RIUs) for both patient groups ($P < 0.05$; Fig. 4). There were no significant differences in telomerase activities in the esophagus and stomach between the two groups.

patients who had GERD with and without Barrett’s esophagus for the distal squamous-lined esophagus (SSBE 8.6 ± 0.9, LSBE 7.9 ± 0.8 SE, GERD without Barrett’s esophagus 8.7 ± 1.0 Kbp; Fig. 2), for the proximal esophagus (SSBE 9.2 ± 1.5, LSBE 9.6 ± 1.2, GERD without Barrett’s esophagus 9.3 ± 1.1 Kbp), or for the gastric fundus (SSBE 9.1 ± 1.2, LSBE 8.9 ± 0.68, GERD without Barrett’s esophagus 8.4 ± 0.62 Kbp).

Although our control patients had no symptoms of GERD and no endoscopic signs of esophagitis or Barrett’s esophagus, biopsy specimens at the Z-line revealed intestinal metaplasia in 8 of the 16 patients. This condition is called intestinal metaplasia at the GEJ (IM at the GEJ), and its etiology is disputed (28). Whereas some authorities contend that this condition may be a manifestation of GERD, we performed subgroup analyses to determine whether there is an association between IM at the GEJ and the length of telomeres in the esophageal squamous epithelium. We found no significant differences in mean telomere lengths between control patients with and without IM at the GEJ for the distal esophagus (control 11.6 ± 2.1, control with IM 10.2 ± 2.2 Kbp), for the proximal esophagus (control 11.5 ± 2.5, control with IM 10.6 ± 2.0 Kbp), or for the gastric fundus (control 11.2 ± 2.4, control with IM 10.8 ± 1.8 Kbp).
Endoscopic and histological esophagitis scores did not differ significantly between the two groups. We considered the possibility that telomere length and/or telomerase activity could vary with the severity of acute reflux esophagitis. At the time of endoscopy, however, the large majority of our GERD patients had been treated with antisecretory medications to control their reflux esophagitis (see Table 1). Consequently, we found no significant difference in the histological esophagitis scores between our GERD and control patients (GERD 1.7 ± 0.17, control 1.8 ± 0.28, P = 0.8).

**DISCUSSION**

Esophagitis due to GERD is most severe in the distal esophagus and infrequently involves the proximal esophagus. We found that telomeres in squamous cells from the distal esophagus of patients with GERD are significantly shorter than those of patients without GERD. In contrast, we found no significant differences in telomere lengths in the proximal esophagus of patients with and without GERD. These findings support our hypothesis that repeated bouts of GERD-induced injury result in a loss of telomeric DNA in the distal squamous-lined esophagus.

One mechanism whereby GERD may cause a loss of telomeric DNA in the distal esophagus is by increasing cellular proliferation, presumably as a result of peptic injury and inflammation. Telomeres are shortened by repeated cell divisions, and numerous studies have shown that GERD increases the proliferation rate of esophageal squamous cells. In a rat model of chronic GERD induced by esophagoduodenostomy, for example, the esophageal squamous epithelium exhibits an expanded basal proliferative zone with increased staining for the proliferation marker Ki-67 (34). In patients with severe reflux esophagitis, furthermore, biopsy specimens of esophageal squamous epithelium exhibit an expanded basal proliferative zone with increased uptake of tritiated thymidine (15).

Another mechanism whereby GERD may shorten telomeres is by generating ROS that cause oxidative damage to telomeric DNA. Elevated levels of ROS have been demonstrated in the esophageal squamous epithelium of patients with reflux esophagitis (33). In patients with severe GERD, furthermore, oxidative DNA damage has been demonstrated in the esophageal squamous epithelium by comet assay (which detects DNA strand breaks) and by the incorporation of the Fapy-DNA glycosylase enzyme (which detects 8-OHdG) (23). In a transgenic rat, which is engineered so that DNA mutations can be detected in vivo, the surgical induction of GERD has been shown to increase the frequency of DNA mutations in the esophageal squamous mucosa (30). Some studies also suggest that telomeres may be preferential sites for oxidative DNA injury (11, 13, 22, 32).

Telomerase is a ribonucleoprotein enzyme that catalyzes the synthesis of telomeric DNA by using an RNA component (hTR-human RNA telomerase) as a template and a telomerase catalytic (hTERT-human telomerase reverse transcriptase) subunit protein to add repetitive DNA sequences to the 3' ends of chromosomes (7, 19). In some proliferating cells, telomerase expression may compensate for the progressive telomere loss that eventually would lead to replicative senescence. Although our GERD patients exhibited shortened telomeres in the distal esophagus, we found no significant differences in esophageal telomerase activities between patients with and without GERD. This suggests that the decreased telomere length in GERD patients did not result from decreased telomerase activity. Nevertheless, it is conceivable that the “normal” levels of telomerase in GERD patients may not be sufficient to prevent...
the progressive loss of telomeric DNA. In other words, an upregulation of telomerase activity might be required to prevent telomere shortening in patients with chronic GERD.

We did find that telomerase activities in the gastric fundus were significantly lower than those in the proximal and distal esophagus in both GERD and control patients. Telomerase activity is found in epithelial cells that maintain their proliferative capacities, whereas telomerase activity usually is absent in connective tissues (2, 10). Therefore, when determining telomerase activity in mucosal biopsy specimens, the presence of submucosal connective tissues that are devoid of the enzyme can result in an underestimation of epithelial telomerase activity. Whereas we have noted that endoscopic biopsy specimens of the stomach may contain more submucosal tissue than those of the esophagus, some of the difference in telomerase activity levels between the two organs may be spurious (i.e., the result of greater contamination of the gastric specimens with telomerase-poor submucosal tissues). Nevertheless, our findings are consistent with those of Bachor et al. (2) who also observed lower telomerase activities in endoscopic biopsy specimens taken from the stomach than in those obtained from the squamous-lined esophagus. Furthermore, the magnitude of the difference between gastric and esophageal telomerase activities appears to be too large to be explained by submucosal contamination alone. The explanation for the differences in telomerase activities between these two gastrointestinal organs is not known.

We considered the possibility that the telomere shortening that we observed in our GERD patients might have been the result of advanced age. Telomere lengths are known to decrease with age, but the ages of our patients and control patients did not differ significantly (9, 14). We also considered the possibility that acute reflux esophagitis might cause confounding effects on telomere length and telomerase levels. At the time of endoscopy, however, the large majority of our GERD patients had been treated with antisecretory medications to control their reflux esophagitis. Thus we found no differences in the degree of histological grade of esophagitis between the GERD patients and controls.

In most patients with reflux esophagitis, the peptic esophageal injury heals through the regeneration of more squamous cells. In some, however, healing occurs through a metaplastic process in which an abnormal, intestinal-type epithelium replaces the injured squamous one. This condition is called Barrett’s esophagus, and the metaplastic cells of Barrett’s esophagus are predisposed to develop adenocarcinoma (27). It is not known why only a minority of patients with GERD develop Barrett’s esophagus. We found significantly shortened telomeres in the distal esophagus of patients with GERD. We speculate that esophageal squamous cells with critically shortened telomeres may become senescent and unable to regenerate in response to further peptic injury. This situation might favor mucosal healing through alternative mechanisms such as metaplasia.

Several studies found that telomeres in Barrett’s metaplasia are shorter than those in the stomach (8, 18, 21). Despite the finding of short telomeres, biopsy specimens of metaplastic Barrett’s epithelium exhibit higher levels of mRNA expression for the telomerase reverse transcriptase catalytic subunit (TERT) than normal squamous epithelium (16). Using in situ hybridization, Morales et al. (20) found expression of telomerase RNA in benign Barrett’s epithelium in up to 70% of cases. They also found that increased telomerase expression correlated with the degree of dysplasia, with strong expression demonstrated in 100% of cases with high grade dysplasia and adenocarcinoma in Barrett’s esophagus. Furthermore, inhibition of telomerase in Barrett’s cancer cells in vitro leads to telomeric shortening, senescence, and apoptosis, suggesting that telomerase expression is essential for the survival of Barrett’s cancer cells (25, 26).

To the best of our knowledge, ours is the first study to evaluate systematically the telomere length and telomerase activity in the squamous esophagus of patients with GERD, with and without Barrett’s esophagus. A subgroup analysis of our GERD patients revealed no significant differences in squamous cell telomere length among those with long- and short-segment Barrett’s esophagus and those who had GERD without Barrett’s esophagus. Thus it is not clear that telomere shortening contributes to the pathogenesis of Barrett’s esophagus. Nevertheless, further studies on this issue are clearly warranted.

REFERENCES

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