Reduction of Interstitial Cells of Cajal (ICC) Associated With Neuronal Nitric Oxide Synthase (n-NOS) in Patients With Achalasia

Ines Gockel, M.D., Ph.D.,1 Juergen R.E. Bohl, M.D.,2 Volker F. Eckardt, M.D. Ph.D.,3 and Theodor Junginger, M.D., Ph.D.1

1Department of General and Abdominal Surgery, Johannes Gutenberg-University, Mainz, Germany; 2Institute of Neuropathology, Johannes Gutenberg-University, Mainz, Germany; and 3Department of Gastroenterology, German Diagnostic Clinic, Wiesbaden, Germany

BACKGROUND: The etiology of achalasia is still unknown. The current theories of chronic inflammation leading to autoimmune response with destruction and loss of the inhibitory myenteric ganglion cells enlighten its pathogenesis in a limited way only. Interstitial cells of Cajal (ICC) have been shown to be involved in nitrergic neurotransmission of the lower esophageal sphincter (LES).

AIM: To investigate the significance of ICC and neuronal nitric oxide synthase (n-NOS) in esophageal wall tissue of patients undergoing surgery for achalasia.

METHODS: In 53 patients with a median age of 45 (6–78) yr undergoing surgery for achalasia, the immunoreactivity of ICC (CD117/c-kit) and n-NOS was assessed. In 42 patients, biopsies were taken from the LES high-pressure zone during Heller myotomy, whereas in 11 patients with end-stage achalasia and a decompensated megaesophagus, the complete esophagus was resected. A semiquantitative analysis was carried out and ICC and n-NOS impairments were classified into four grades. Staining intensity was correlated with preoperative clinical, radiologic, and manometric findings and with long-term postoperative Eckardt score.

RESULTS: Grade III/IV ICC reduction (severe reduction to complete loss) was seen in 59.5% of all biopsy specimens of the LES high-pressure zone. Patients with grade III/IV ICC reduction had a significantly longer duration of achalasia symptoms (3 [0–43] yr) than patients with minor to marked (grade I/II) impairment (1 [0–16] yr, \( P = 0.028 \)). A majority (72.5%) of tissue samples revealed severe reduction to complete loss of n-NOS immunoreactivity. The preoperative Eckardt score was statistically significantly different between patients with grade I/II and those with grade III/IV n-NOS reductions (\( P = 0.031 \)). CD117 (c-kit) positivity was statistically significantly correlated with n-NOS staining intensity (correlation coefficient \( r = 0.781, P < 0.0001 \)).

CONCLUSION: The present results suggest that in the pathogenesis of achalasia, especially in the development of the LES high-pressure zone, depletion of ICC networks and potential changes in the electrical activity of smooth muscle cells may play a crucial role. The reduction in CD117-positive ICC in a few patients also seemed to be of relevance, even if the cells of Auerbach’s plexus were unscathed. The associated reduced NOS release might underlie the profound ICC impairment and could possibly be responsible for the lack of LES relaxation, because of missing inhibitory neurotransmission. It is unclear, however, whether the ICC loss is primarily caused by the accelerated attrition of mature cells or their impaired regeneration.

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INTRODUCTION

Achalasia is a primary esophageal motility disorder of unknown etiology, characterized by aperistalsis of the esophageal body and a hypertensive, nonrelaxing lower esophageal sphincter (LES) due to the lack of inhibitory neurotransmission. The current theories on the underlying pathophysiologic mechanisms in achalasia seem multidimensional. A cascade of inflammatory events, leading to myenteric plexus alteration, following an initial insult to the esophagus, possibly a viral infection or an unknown environmental factor, has been considered (1). The inflammation then leads to an autoimmune response in a susceptible population, possibly genetically predisposed. These autoimmune mechanisms...


and neural fibrosis accompany the continuing progress of achalasia. The number of ganglion cells and the degree of inflammation or fibrosis are believed to change with the stage of achalasia (2). Injury can extend to vagal terminal endings within the esophagus and neuronal bodies at the dorsal motor nucleus of the vagal nerve in the brainstem (3).

A study of the muscular ultrastructure in achalasia revealed no alterations in smooth muscle fibers other than hyperplasia, and primary myopathy was virtually excluded (4). However, our own previous histopathological examinations of the esophageal wall high-pressure zone of the LES in patients undergoing Heller myotomy for achalasia showed that the primary lesion in achalasia could also be a severe visceral myopathy, playing an important role in smooth muscle morphologic alteration and dysfunction (5).

Abnormal esophageal motor function in achalasia is due to an imbalance between the excitatory and inhibitory innervations of the esophagus. Nitric oxide (NO)- and nitric oxide synthase (NOS)-deficient functions have been implicated in the etiology of achalasia.

Interstitial cells of Cajal (ICC) are proposed to modulate the neural inhibitory stimulus in different regions of the gastrointestinal tract (6). Forming neuronal networks spread in submuscular, intramuscular, and intermuscular layers, they generate pacemaker potentials that determine the spontaneous electrical and mechanical activities of smooth muscle cells (7). Impairment in NOS-containing nerves in the esophagus has been confirmed in achalasia (8, 9). Although the c-kit (CD117)-positive ICC seem to form a close physical and functional association with nitricergic nerves, no clear correlation between the two parameters has been demonstrated so far.

The purpose of our study was to investigate the different degrees of reduction in ICC and neuronal NOS (n-NOS) in esophageal wall specimens from patients with achalasia as well as their correlation with clinical, radiologic, and manometric parameters and with long-term clinical course.

PATIENTS AND METHODS

Patients

Tissue samples from 53 patients undergoing surgery for achalasia were evaluated in this study. The study duration for biopsies taken from the LES while performing a Heller myotomy (N = 42) was from February 2003 to September 2006. Esophageal resectates from patients with decompensated megaesophagus (N = 11) were investigated between December 1990 and July 2005. The preoperative diagnosis was confirmed by clinical evaluation according to the Eckardt score. A symptom score ranging from 0 to 3 was determined depending on whether dysphagia, regurgitation, and chest pain occurred occasionally, daily, or several times during the day. In addition, a symptom score of 0 to 3 was assigned to the degree of weight loss. Thus, a completely asymptomatic patient would have a symptom score of 0, whereas a severely affected patient could have a symptom score up to 12 (10).

At diagnosis, each patient underwent esophageal manometry, using a low-compliance capillary perfusion method and a barium esophagogram with assessment of the maximum diameter of the esophageal body and the narrowest diameter of the esophagogastric junction. Upper gastrointestinal endoscopy was carried out in all patients preoperatively to rule out secondary achalasia. Follow-up after surgery was performed by structured interviews according to the Eckardt score in all patients at regularly defined intervals.

Tissue Samples

In 42 patients undergoing open Heller myotomy, smooth muscle biopsies with 20 × 15-mm longitudinal segments of the myenteric plexus were taken from the LES high-pressure zone for histopathological examination. The complete esophageal body was available from 11 additional patients with resection for advanced and decompensated achalasia with megaesophagus.

Esophageal tissue specimens from six controls (autopsy material) taken from the high-pressure zone of the LES were investigated for comparison with the achalasia biopsies.

The tissue specimens were fixed in formalin and embedded in paraffin. In selected cases, tissue specimens were embedded in epoxy resin for semithin sections.

Six different biopsies were taken from each achalasia patient (cryostat sections), and at least 15 sections with different types of staining were done in each subject. The number of low- and high-power fields per slide was dependent on the length of the biopsy. In small biopsies, there was a maximum of six high-power fields and three low-power fields per slide, and in large biopsies, a maximum of 12 high-power fields and 6 low-power fields was available. The cellular distribution of ICC and n-NOS was considered for Auerbach’s plexus only.

Only one neuropathologist (J.R.E.B.) was involved in the interpretation of the histopathological and immunohistochemical findings, and the investigator was not blinded in his readings of ICC and n-NOS as to the patients’ characteristics. The slides were read individually as each patient entered the study, and control slides were used throughout the study to maintain uniformity in reading the biopsy specimens. The histologic sections of the muscle were oriented in a similar plane when read.

Immunoreactivity and Quantification of ICC and n-NOS

The c-kit (CD117) and n-NOS immunohistochemical staining was performed on consecutive sections.

Fresh biopsies from the esophageal muscle were immediately divided into three different portions. The first was used for frozen sections and for enzyme-histochemical methods (−30°C), the second was fixed in a buffered solution of formaldehyde (4%) and was then embedded in paraffin for histopathological and immunohistochemical investigations, and the third portion of the biopsy was fixed in a buffered solution of glutaraldehyde (3%) and then embedded in epoxy resin.
for semithin sections or for ultrastructural studies. Semithin sections were stained with para-phenylendiamin (PPD) or methylene blue. The following antibodies against CD117 and n-NOS were used: rabbit anti-CD117 (c-kit), Zytomed catalog number 503-1444, and rabbit anti-n-NOS, Zytomed catalogue number 214-0424 (Zytomed Systems GmbH, Berlin, Germany).

The immunohistological reactions were detected with either the alkaline phosphatase-anti-alkaline phosphatase (APAAP) or the peroxydase-anti-peroxydase (PAP) method. The antibody used against CD117 recognizes a protein of 145 kDa, which is identified as CD1217/p145kit. It is known to be relatively specific to human and canine Cajal cells in tissue samples of the gastrointestinal tract, but sometimes some inflammatory cells are also stained. By considering the shape and location of the positive cells, it is possible in most cases to identify ICC among other positively stained cells. The sensitivity of the reaction depends on the quality of the tissue sample, that is, on the amount and intensity of the artificial damage of the muscle tissue and also on the degree of dilution of the antibody.

The antibody used against n-NOS recognizes a 155-kDa protein (a 16-residue synthetic peptide [residues 724–739] with a cysteine [C] residue added and the peptide coupled to keyhole limpet hemocyanin [KLH]) corresponding to the apparent molecular mass of neuronal nitric oxide synthase I (n-NOS). It is relatively specific to human n-NOS and also reacts with n-NOS of rat, mouse, cat, and monkey. Nerve cell bodies as well as small nerve fibers usually give a distinct positive reaction in all layers of the esophageal muscle. The sensitivity depends on the quality of the tissue sample and is comparable with that of the neurofilament antibodies.

A semiquantitative analysis was carried out and the impairments in ICC and n-NOS were classified into four grades (corresponding staining intensity and density of positive reactions of ICC and n-NOS in parentheses): grade I (3): numerous positive cells, equally distributed in most investigated areas, i.e., normal conditions or minor reduction; grade II (2): patchy distribution of positive cells, easily found in high-power fields, i.e., marked reduction; grade III (1): only rare and single positive structures, i.e., severe reduction; grade IV (0): no reaction at all, i.e., complete loss of ICC and respective n-NOS.

**Statistical Analysis**

The SSPS 12.0 software package was used for statistical data analyses (SSPS, Inc., Chicago, IL). Clinical data from patients with achalasia were prospectively collected and retrospectively analyzed. Data are expressed as median with range (minimum to maximum), or as percentage (%).

Associations between patients’ demographic, clinical, manometric, and radiologic data and long-term postoperative course and the semiquantitatively assessed immunoreactivities of ICC and n-NOS of esophageal wall tissue samples were assessed. The grading system of ICC and n-NOS reductions was subdivided into two groups: grade I/II (minor to marked reduction) versus grade III/IV (severe reduction to complete loss). The Fisher’s exact test or the χ² test was used for univariate comparisons between the two groups and the Mann-Whitney U-test served as the nonparametric analytic method. Spearman correlation was applied for linear regression analysis. A P value of <0.05 was considered statistically significant for all procedures.

**RESULTS**

**Patient Characteristics**

Tissue specimens were obtained from 53 patients undergoing surgery for achalasia. There were 27 female and 26 male patients. The median age at the time of surgery was 45 (6–78) yr, whereas the median duration of achalasia symptoms was 3 (0.25–63) yr. Twenty-four of 53 patients had undergone pneumatic dilation therapy prior to surgery (1–15 dilations), and in 16 patients, a previous surgical procedure had been performed and the tissue sample was taken at the second surgery.

Patients undergoing Heller myotomy, in whom a biopsy specimen (BS) of the LES high-pressure zone could be obtained (N = 42), were younger (42 [6–78] yr) than those with esophageal resection (ER) for end-stage achalasia (N = 11) (63 [42–78] yr). The durations of achalasia were also different in the two groups: median 2.25 (0.25–43) yr (BS) versus 25 (4–63) yr (ER) (Table 1).

All controls had died of extraesophageal diseases and their age ranged from 23 to 82 yr (4 men, 2 women).

**Clinical, Radiologic, and Manometric Data**

The preoperative Eckardt score was 6 (2–12) in patients with biopsies and 6.5 (1–9) in patients in whom the complete esophagus was resected. The maximum diameter of the esophageal body and the narrowest diameter of the esophagogastric junction were larger in patients undergoing esophagectomy, whereas the resting pressure of the LES was higher in patients with Heller myotomy and consecutive biopsy (Table 2).
Table 2. Clinical, Radiologic, and Manometric Parameters Prior to Surgery

<table>
<thead>
<tr>
<th></th>
<th>Biopsy Specimens (N = 42)</th>
<th>Esophageal Resectates (N = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eckardt score</td>
<td>6 (2–12)</td>
<td>6.5 (1–9)</td>
</tr>
<tr>
<td>Max. diameter of the</td>
<td>45 (22–70)</td>
<td>55 (35–85)</td>
</tr>
<tr>
<td>esophageal body (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Min. diameter of the</td>
<td>2 (1–10)</td>
<td>8 (3–15)</td>
</tr>
<tr>
<td>esophagogastric junction (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting pressure of the lower esophageal sphincter (mmHg)</td>
<td>31.3 (16.4–115)</td>
<td>12 (10.2–22.5)</td>
</tr>
</tbody>
</table>

**Follow-Up**

Follow-up of patients with achalasia undergoing surgery was 100%. All patients were followed with structured interviews at regularly defined intervals: 6 months after surgery and thereafter every 2 yr, or if patients had new symptoms. The median follow-up in the group with biopsy specimens was 13.5 (2–68) months with a postoperative Eckardt score of 1 (0–4), whereas the group with complete resections of the esophagus was followed over a median period of 41 (7–188) months and its Eckardt score was 1 (0–9).

Patients were followed to their last interview or death. At the time of closing the study in May 2007, one patient in the BS group and three patients in the ER group had died.

**Immunoreactivity of ICC**

Biopsies of the LES HIGH-PRESSURE ZONE. Semi-quantitative grading of Cajal cell reduction in 42 patients with biopsies of the high-pressure zone revealed grade I in 7 (16.7%), grade II in 10 (23.8%), grade III (Fig. 1A and B) in 14 (33.3%), and grade IV in 11 (26.2%) patients. Subdividing the whole group into patients with minor to marked reduction versus severe reduction to complete loss of ICC, the relation was 17:25 (40.5:59.5%). Patients with grade III/IV ICC reduction displayed a significantly longer duration of achalasia symptoms (3 [0–43] yr) than patients with less severe impairment (grade I/II) (1 [0–16] yr, P = 0.028). The parameters: age at surgery, preoperative Eckardt score, preoperative maximum diameter of the esophageal body, minimum diameter of the esophagogastric junction, LES resting pressure, and postoperative Eckardt score showed no statistically significant differences between grade I/II and grade III/IV ICC reductions (Table 3). Using Spearman correlation for linear regression, the correlation coefficient between the reduction in ICC and LES resting pressure was \( r = 0.093 \) (P = 0.594).

**Esophageal Resectates.** In 11 cases with end-stage achalasia, the total resected esophagus was investigated systematically and several tissue specimens were taken from all regions of the enlarged esophagus. An obvious loss of CD117-positive cells was seen in both layers of the esophagus and this reduction seemed to be focally distributed.

Within the same level of esophageal wall region, totally depleted CD117-positive cells coexisted in close proximity to areas with a nearly normal number of cells. This irregular pattern of Cajal cell distribution seemed more pronounced in the caudal portions of the esophagus, where the texture of the smooth muscle layer also seemed focally distributed.
Table 3. Clinical, Radiologic, and Manometric Parameters in Patients With Minor to Marked Reduction (Grade I/II) Versus Severe Reduction to Complete Loss (Grade III/IV) of ICC in Patients With Biopsies of the LES High-Pressure Zone

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade I/II (N = 17)</th>
<th>Grade III/IV (N = 25)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at surgery (yr)</td>
<td>43 (6–78)</td>
<td>42 (11–74)</td>
<td>0.780</td>
</tr>
<tr>
<td>Duration of achalasia symptoms (yr)</td>
<td>1 (0–16)</td>
<td>3 (0–43)</td>
<td>0.028*</td>
</tr>
<tr>
<td>Preop. Eckardt score</td>
<td>7 (2–10)</td>
<td>6 (3–12)</td>
<td>0.106</td>
</tr>
<tr>
<td>Preop. max. diameter of esophageal body (mm)</td>
<td>40 (22–50)</td>
<td>45 (25–70)</td>
<td>0.779</td>
</tr>
<tr>
<td>Preop. min. diameter of esophagogastric junction (mm)</td>
<td>2 (1–8)</td>
<td>2 (1–10)</td>
<td>0.591</td>
</tr>
<tr>
<td>Preop. LES resting pressure (mmHg)</td>
<td>39.3 (16–58)</td>
<td>29.1 (11–115)</td>
<td>0.695</td>
</tr>
<tr>
<td>Postop. Eckardt score</td>
<td>1 (0–4)</td>
<td>1 (0–3)</td>
<td>0.500</td>
</tr>
</tbody>
</table>

*Statistically significant.

Table 4. Clinical, Radiologic, and Manometric Parameters in Patients With Minor to Marked Reduction (Grade I/II) Versus Severe Reduction to Complete Loss (Grade III/IV) of n-NOS in Patients With Biopsies of the LES High-Pressure Zone

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Grade I/II (N = 11)</th>
<th>Grade III/IV (N = 29)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at surgery (yr)</td>
<td>42 (6–74)</td>
<td>38 (12–78)</td>
<td>0.857</td>
</tr>
<tr>
<td>Duration of achalasia symptoms (yr)</td>
<td>1 (1–6)</td>
<td>2.5 (0–43)</td>
<td>0.091</td>
</tr>
<tr>
<td>Preop. Eckardt score</td>
<td>8 (4–10)</td>
<td>6 (3–12)</td>
<td>0.031*</td>
</tr>
<tr>
<td>Preop. max. diameter of esophageal body (mm)</td>
<td>38 (22–50)</td>
<td>45 (23–70)</td>
<td>0.689</td>
</tr>
<tr>
<td>Preop. min. diameter of esophagogastric junction (mm)</td>
<td>2 (1–6)</td>
<td>2 (1–10)</td>
<td>0.911</td>
</tr>
<tr>
<td>Preop. LES resting pressure (mmHg)</td>
<td>39.3 (16–58)</td>
<td>30.5 (11–115)</td>
<td>0.748</td>
</tr>
<tr>
<td>Postop. Eckardt score</td>
<td>1 (0–4)</td>
<td>1 (0–3)</td>
<td>0.084</td>
</tr>
</tbody>
</table>

*Statistically significant.

Correlation Between ICC and n-NOS Reductions
CD117 (c-kit) positivity was statistically significantly correlated with n-NOS staining intensity (r = 0.781, P < 0.0001) (Fig. 3). Using Spearman linear regression analysis, there was no further significant correlation between the immunoreactivity of ICC and the respective n-NOS reduction and clinical (age at surgery, duration of achalasia symptoms, and Eckardt score), radiologic (maximum diameter of the esophageal

Immunoreactivity of n-NOS
Only biopsies of the LES high-pressure zone were stained with antibodies against n-NOS. Due to the inhomogeneous reactions in the complete resectates and the presence of four carcinomas in this group, they were not considered for n-NOS reactivity interpretation. Semiquantitative grading of n-NOS reduction in 40 patients with biopsies of the high-pressure zone revealed grade I in 2 (5%), grade II (Fig. 2) in 9 (22.5%), grade III in 14 (35.0%), and grade IV in 15 (37.5%) patients. Subdividing the group into patients with minor to marked reduction versus severe reduction to complete loss, the relation was 11:29 (27.5:72.5%).

The preoperative Eckardt score was statistically significantly different between patients with grade I/II and those with grade III/IV reductions in n-NOS (P = 0.031). Thus, there was no significant difference for the variables: age at surgery, duration of achalasia symptoms, and preoperative radiologic and manometric parameters, as well as for the postoperative Eckardt score (Table 4). Using Spearman correlation for linear regression, the correlation coefficient between the reduction in n-NOS and LES resting pressure was r = 0.105 (P = 0.550).
body and minimum diameter of esophagogastric junction), or manometric (LES resting pressure) parameters ($P > 0.05$).

**DISCUSSION**

The present results suggest that in the pathogenesis of achalasia, especially in the development of the LES high-pressure zone, depletion of ICC networks and potential changes in the electrical activity of smooth muscle cells may play a crucial role. It is unclear, however, whether the ICC loss is primarily caused by the accelerated attrition of mature cells or their impaired regeneration.

A remarkable finding of this study was the significant correlation between CD117 positivity and n-NOS staining intensity in tissue specimens of the LES high-pressure zone in patients undergoing Heller myotomy for achalasia. The associated reduced NOS release might underlie the profound ICC impairment and could possibly be responsible for the lack of LES relaxation, because of the missing inhibitory neurotransmission.

Thus, the entire role of ICC and the enteric nervous system in the integrative control of gastrointestinal function, especially of spontaneous rhythmic activity, is still unknown. In the esophagus, ICC are present scattered among the smooth muscle cells in both the longitudinal and circular muscle layers (11, 12). Here, they are frequently located between nerve endings and smooth muscle cells, establishing synapse-like contacts and gap junctions (11). At present, it appears that the role of ICC is to depolarize the smooth muscle syncytium to increase the opening probability of voltage-dependent ion channels expressed by the smooth muscle cells. Depolarization of the smooth muscle cells activates $Ca^{2+}$ entry mechanisms (13). Slow wave propagation occurs through the networks of electrically coupled ICC (14). The electric impedance of the smooth muscle syncytium tends to prohibit regenerative propagation of action potentials, and the cell-by-cell smooth muscle response to the conducting slow wave depolarization is, therefore, likely to be a relatively localized response.

The LES displays prominent NO-dependent hyperpolarization of the resting membrane potential and relaxation in response to activation of enteric inhibitory neurons, thereby suggesting significant regulation by nitricergic mechanisms (15). The presence and location of NOS have been studied by immunohistochemistry in surgical specimens obtained from

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**Figure 3.** Correlation between ICC and n-NOS immunoreactivity in 40 biopsies of the LES high-pressure zone in patients with achalasia (correlation coefficient $r = 0.781$, $P < 0.0001$). Semiquantitative assessment of ICC and n-NOS immunoreactivity: 0 = grade IV reduction/complete loss; 1 = grade III reduction; 2 = grade II reduction; 3 = grade I reduction.
the gastroesophageal junction in patients with achalasia and of nonachalasic surgical controls, demonstrating, biochemically and immunologically, the absence of NOS in achalasia, while, in contrast, specimens from control subjects clearly showed activity of the NO-generating enzyme (8, 9). In addition, administration of NO donors relaxed the LES in both controls and achalasia patients, suggesting that smooth muscle cell transductional responses were intact (16, 17).

Recent evidence suggests that ICC are involved in NO-mediated inhibitory neuromuscular transmission (18). Functionally, intramuscular ICC may be the effectors that transduce NO signals into hyperpolarizing responses. The (dys)functional interaction between ICC and nitrergic nerve terminals is not yet understood. The close physical relationships of ICC to neurotransmitters and neuropeptides, especially n-NOS, vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY) nerve fibers, have been investigated in animal models (19, 20) and in humans in detail (21).

However, data on the association between ICC and nitrergic nerves have been conflicting so far. Ward et al. suggested that the lack of ICC could potentially lead to a hypertensive LES, unable to relax by impaired NO-dependent hyperpolarization of the smooth muscle cells (6). According to Sivarao et al., in contrast to our data, no clear correlation between injury to or loss of intramuscular ICC and loss of nitrergic nerves at both the light and electron microscopy levels was found (22). A possible explanation for the controversial findings of the ICC/n-NOS association in the literature might be that the examinations have been performed at different levels of chronological sequence in the microstructural pathologic changes. As proposed by Zarate et al., injury and loss of intramuscular ICC in achalasia take many more years than loss of nitrergic nerves (23). Different degrees of ICC injury, as observed on electron microscopy, were scattered lamella bodies, autophagosomes, scarce cytoplasm, and enlarged mitochondria. Loss of n-NOS immunoreactivity in the Zarate et al. study on 11 patients with achalasia was completed within 3 yr of acquiring achalasia, and thereafter, progressive ultrastructural injury to the remaining nerve endings was evident (23).

Interestingly, the reduction in CD117-positive ICC in a few patients in our study also seemed to be of relevance, even if the cells of Auerbach’s plexus were unscathed. Although the data of Zarate et al. suggest no linear correlation between ICC loss and duration of disease, albeit in a small number of patients only, in our study, patients with severe reduction to complete loss of ICC had a significantly longer history of achalasia symptoms than those with minor to marked depletion.

As a drawback of this study, there were no properly matched controls to achalasia patients, but only samples from autopsies without any esophageal pathology available, making data on the possible decrease in ICC or n-NOS difficult to interpret. Also, an age-related progressive depletion of ICC and n-NOS, in analogy to diminution of esophageal ganglia cells as described by Eckardt and LeCompte (24), is considerable but could not be proved due to the lack of age-matched controls. Controls of patients undergoing surgery for esophageal disease other than achalasia would also be problematic with respect to possible morphological or immunological changes (e.g., gastroesophageal reflux or esophageal carcinoma). With any kind of previous intervention (e.g., preoperative pneumatic dilation or previous surgery), inflammation, bleeding, and even scarring within the distal esophagus and LES are considerable, which could lead to destruction of ICC and n-NOS. Thus, there was no statistically significant correlation between the number or kind of preoperative intervention and the ICC and n-NOS reductions.

Using a method with three-dimensional sections would possibly have been more informative, but was limited by the length and number of biopsies available to us. This would also have necessitated a different technique for immunohistochemical reactions, using consecutive sections of 250 μm, and with this thickness, all other examinations would not have been possible.

The present study does not allow us to draw any conclusions on the functional changes in ICC with consecutive dysfunctional nerve interaction potentially preceding the numerical reduction. In this context, despite a normal or only slight reduction in the number and/or distribution of ICC using c-kit (CD117) immunohistochemistry, ultrastructural damage might have been present and might have remained undetected at the light microscopy level. Furthermore, our data provide no information about the damage to gap junctions and other special connections between smooth muscle cells and ICC within the muscle bundles. As severe myopathic changes, in particular the frequent finding of visceral myopathy (possibly as a consequence of neuronal degeneration and denervation), were found, particularly at advanced stages of achalasia, in our patients, an association with the pathologic course of the disease in the context of ICC reduction could be considerable.

An open question remains regarding the possible process of compensating mechanisms acquired by the remaining ICC subset after significant loss. It may be hypothesized that a reduced subpopulation of surviving CD117-positive cells might be able to regenerate the electrical networks and interference with smooth muscle cells and neuronal structures.

Discovering new ways to manipulate the development of ICC depletion or to stimulate regeneration may provide a dramatic new therapy for patients suffering from achalasia.

ACKNOWLEDGMENT

Parts of this study were presented at the Digestive Disease Week (DDW) (AGA) in May 2006 in Los Angeles, CA, and at the International Society for Diseases of the Esophagus (ISDE) in February 2006 in Adelaide, Australia, where it was awarded “Prize for Best Abstract.”
STUDY HIGHLIGHTS

What Is Current Knowledge

- Current theories of the underlying pathophysiologic mechanisms in achalasia are a cascade of inflammatory events, leading to myenteric plexus alteration, following an initial insult to the esophagus, possibly a viral infection or an unknown environmental factor.
- Inflammation then leads to an autoimmune response in a susceptible population, possibly genetically predisposed.
- These autoimmune mechanisms and neuronal fibrosis accompany the continuing progress of achalasia.

What Is New Here

- The present results suggest that in the pathogenesis of achalasia, especially in the development of the high-pressure zone, depletion of interstitial cells of Cajal (ICC) networks and potential changes in the electrical activity of smooth muscle cells may play a crucial role.
- The associated reduced neuronal nitric oxide synthase (n-NOS) release might underlie the profound ICC impairment and could possibly be responsible for the lack of relaxation of the lower esophageal sphincter, on account of the missing inhibitory neurotransmission.

REFERENCES


CONFLICT OF INTEREST

Guarantor of the article: Ines Gockel, M.D., Ph.D.
Specific author contributions: Ines Gockel and Juergen R.E. Bohl initiated the study and Volker F. Eckardt and Theodor Junginger supervised the study. Ines Gockel, Juergen R.E. Bohl, Volker F. Eckardt, and Theodor Junginger performed the study design. Volker F. Eckardt diagnosed all patients included in the study and performed the postoperative
follow-up at regularly defined intervals. Theodor Junginger operated on all patients. Juergen R.E. Bohl performed the neuropathological examinations of all patients. Ines Gockel collected the data, performed the statistical analysis, and wrote the first draft of the manuscript. All authors approved the final version of the manuscript.

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**Potential competing interests:** None.