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Foxp3+ lymphocyte count in Barrett's esophagus tissue is higher than in inflamed esophageal tissue

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Abstract: Introduction: The number of Foxp3+ lymphocytes is increased in patients with esophageal carcinoma. Little is known about Foxp3+ cells count in Barret's esophagus, which is a precancerous state for esophageal cancer.
Aim: To count the number of Foxp3+ lymphocytes in tissue samples from patients with Barrett's and compare it with samples from individuals with esophagitis and esophageal cancer.
Materials and methods: 43 patients were enrolled to the study: 14 with esophageal carcinoma, 15 with Barrett's esophagus and 14 with non-metaplastic esophagitis. Every patient undergone gastroscopy during which a tissue sample was taken. Foxp3+ lymphocytes and CD4+ lymphocytes were detected by using immunohistochemistry.
Results: Mean density of Foxp3+ cells in patients with esophagitis was 7.37/10HPF (range: 1–9), 18.5/10HPF (range: 5–29) and 26.8/10HPF (range: 16–40) in patients with dysplastic and non-dysplastic BE, respectively and 47.92/10HPF in individuals with esophageal a carcinoma. These intergroup differences turned out to be statistically significant ($p = 0.000$; Fig. 3). Patients, either with dysplasia or without, presented with significantly higher Foxp3+ cell counts than the subjects with esophagitis ($p = 0.0003$ and $p = 0.0006$, respectively). Also the number of Foxp3+ lymphocytes in esophageal adenocarcinoma specimens turned out to be significantly higher than in esophagitis ($p = 0.0001$), non-dysplastic and dysplastic BE tissue ($p = 0.016$ and $p = 0.00047$, respectively).
Conclusions: Barrett's metaplasia, either with dysplasia or without, is associated with an evident increase in the number of Foxp3 lymphocytes within the esophagogastric junction mucosa. Restoration of lymphocyte balance in esophageal tissue might prevent malignant transformation of Barrett's metaplasia.

Key words: Regulatory T-cells, Barrett's esophagus, Immunohistochemistry, Esophageal cancer.

Introduction

The pathophysiology of Barrett's esophagus (BE) is poorly understood. This condition is defined as a reparative metaplastic remodeling of the esophagogastric junction mucosa in response to chronic inflammation associated with reflux disease. BE is an established risk factor for esophageal adenocarcinoma [1, 2]. During the course of BE, normal stratified squamous epithelium of the esophagus is replaced by a cylindrical epithelium characteristic for lower gastrointestinal tract. This is considered to be a factor predisposing to esophageal adenocarcinoma, a condition with particularly poor prognosis.

Only few previous studies dealt with the immunological aspects of BE pathogenesis. This condition is postulated to be primarily associated with the mechanisms of humoral immunity. Nevertheless, lymphocytic infiltration of the metaplastic esophageal epithelium has been observed as well. Some of the infiltrating lymphocytes are the so-called T-regulatory cells (Tregs) with TCD4+CD25+Foxp3+ phenotype. This lymphocyte subpopulation is currently a subject of extensive research owing its involvement in carcinogenesis. However, the available evidence is inconclusive. While Treg count in inflamed mucosa of patients with gastroesophageal reflux disease was shown to be reduced [3], concentration of Foxp3+ lymphocytes in esophageal cancer tissue seems to be higher than in normal esophagus, and is even considered a prognostic factor [4]. Tregs promote carcinogenesis due to induction of local immunosuppression. However, we still do not know when exactly these cells start to accumulate within the altered tissue, and if they induce local immunosuppression already at early stages of carcinogenesis, thus enabling survival of cancer cells that would otherwise be recognized and destroyed by human immune system. Finally, it is unclear If Tregs play also a role in BE pathogenesis.

Objective

The aim of the study was to compare the density of Foxp3+ lymphocytes in biopsy specimens from Barrett's glandular metaplasia, esophageal adenocarcinoma and inflamed non-metaplastic esophageal mucosa, in order to analyze the evolution of immune response at consecutive stages of the esophagogastric junction pathology.

Methods and material

The study included subjects with clinical or endoscopic indications for the esophagogastric junction biopsy (suspected inflammation or BE) and patients with esophageal carcinoma, all examined at the John Paul II Hospital in Krakow.

Individuals with a history of previous anticancer treatment, confirmed immunodeficiency or diabetes mellitus, as well as pregnant women, were excluded from the study. Written informed consent was sought from all the study subjects. The

protocol of the study was approved by the Local Bioethics Committee (decision no. KBET/288/B/2014).

Tregs were detected immunohistochemically (IHC) in paraffin-embedded tissue specimens. Selected paraffin blocks were cut into 4 μm-thick sections and mounted on glass slides. The slides were triple deparaffinized in three changes of xylene for 10 min, and dehydrated in ethanol gradient (70%, 86% and 96%, 5 min each). The activity of endogenous peroxidase was blocked by 10-min incubation with 3% H_2O_2. Subsequently, the slides were placed in EDTA buffer (pH 8.0, 0.01M) and heated for 30 min at a water bath (98°C) to unmask the epitopes. Then, monoclonal mouse anti-human FOXP3 antibody (Abcam, 236A/E7, 1:100) was applied, and the slides were incubated at room temperature for 30 min. After washing off the residual unbound antibody with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7,6; DAKO Corp.), antigen-antibody complexes were visualized with Ultra Vision LP Value Detection System (Lab Vision Corp.) with 3,3-diaminobenzidine tetrahydrochloride (DAB, DAKO Corp.) as a chromogen. Finally, cellular nuclei were counterstained with Mayer's hematoxylin for 1 min, the slides were cover slipped and sealed with Cytoseal XYL (Thermo SCIENTIFIC). The slides were examined under an Olympus CX41 microscope; the number of IHC-positively stained cells was determined in ten high-power fields (HPF, x 400) in the areas of greatest density. The density of Tregs was calculated as the mean absolute number of IHC-positively stained cells showing nuclear reaction to the anti-FOXP3+ antibody.

Statistical analysis was conducted with Statistica 12 package. The significance of intergroup differences in Treg count was verified with Mann-Whitney U-test and Kruskal-Wallis ANOVA.

Material

The study included 14 patients with esophageal carcinoma and 15 with BE; the latter group was divided into two subsets: with esophageal dysplasia (n = 9) and without (n = 6). Control group was comprised of 14 patients with non-metaplastic esophagitis. Women constituted 42% of the study subjects. Median age of the participants was 61.42 years (range: 32–83 years; Table 1).

Table 1. Patient characteristics.

Type of lesion	Number of patients	Mean age (range)	Mena density of Foxp3+ T cells (range)
Esophagitis	14	61.2 (40–83)	3.50 (1–9)
Non-dysplastic Barrett's esophagus	9	61.7 (52–74)	26.75 (16–40)
Dysplastic Barrett's esophagus	6	55.0 (32–80)	17.40 (5–29)
Esophageal cancer	14	65.0 (51–78)	47.90 (36–68)

Results

All examined specimens contained Foxp3+ lymphocytes. The number of Tregs was not influenced by patient sex. Mean density of Foxp3+ cells was 7.37/10HPF (range: 1–9; Fig. 1) in subjects with esophagitis, 18.5/10HPF (range: 5–29) and 26.8/10HPF (range: 16–40) in patients with dysplastic and non-dysplastic BE, respectively (Fig. 2), and 47.92/10HPF in individuals with esophageal a carcinoma. These inter-group differences turned out to be statistically significant ($p = 0.000$; Fig. 3). Post-hoc analysis demonstrated that BE patients, either with dysplasia or without, presented with significantly higher Foxp3+ cell counts than the subjects with esophagitis ($p = 0.0003$ and $p = 0.0006$, respectively). Also the number of Foxp3+ lymphocytes in esophageal adenocarcinoma specimens turned out to be significantly higher than in esophagitis ($p = 0.0001$), non-dysplastic and dysplastic BE tissue ($p = 0.016$ and $p = 0.00047$, respectively). No statistically significant differences in Foxp3+ cell counts were found between the subsets of patients with dysplastic and non-dysplastic BE ($p = 0.12$).

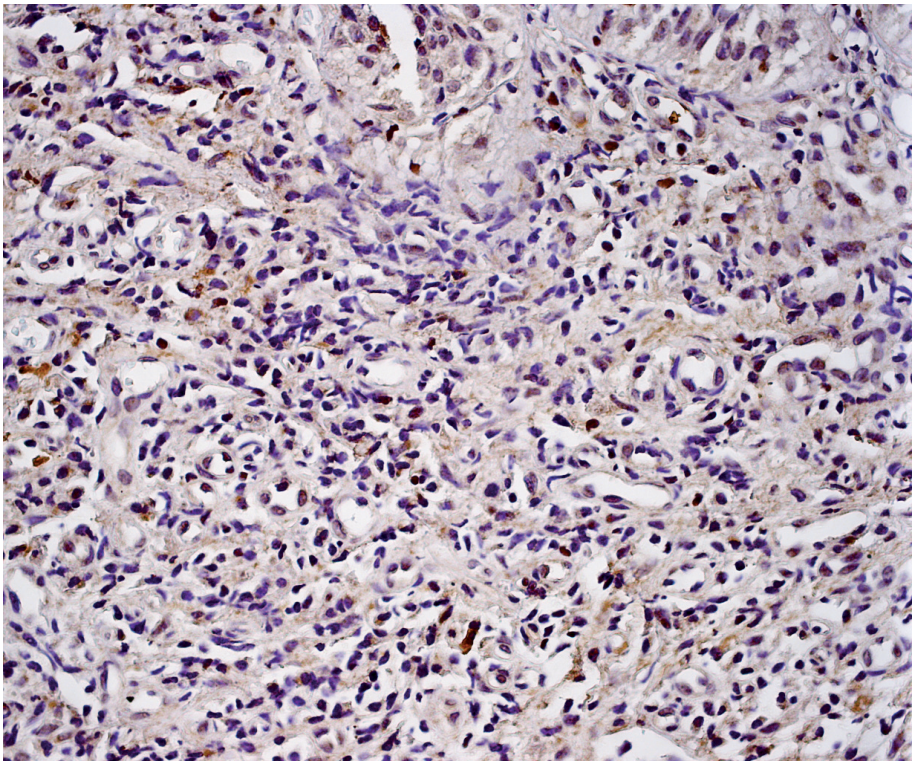

Fig. 1. Foxp3+ T cells in inflamed esophageal mucosa.

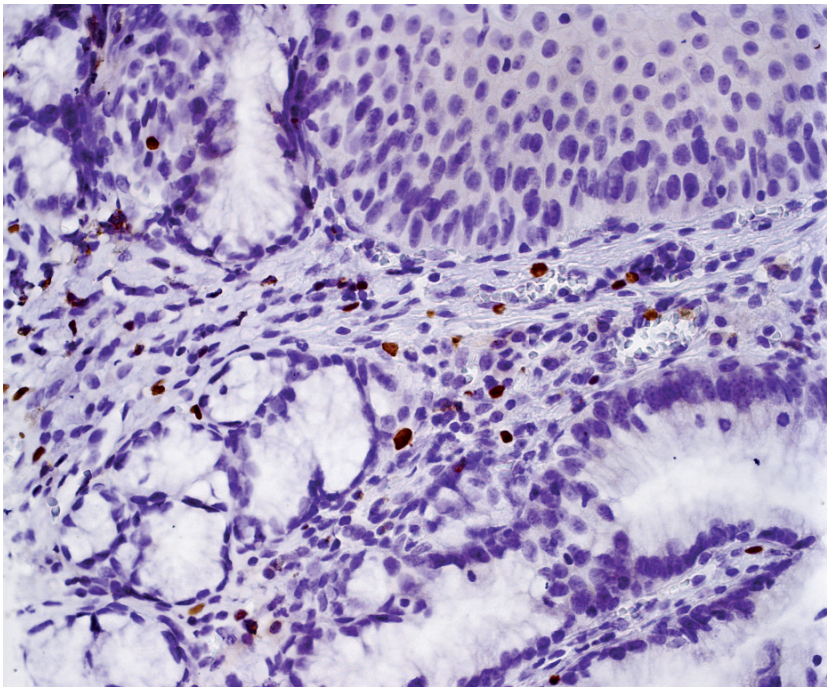

Fig. 2. Foxp3+ T cells in a biopsy specimen of dysplastic Barrett's esophagus.

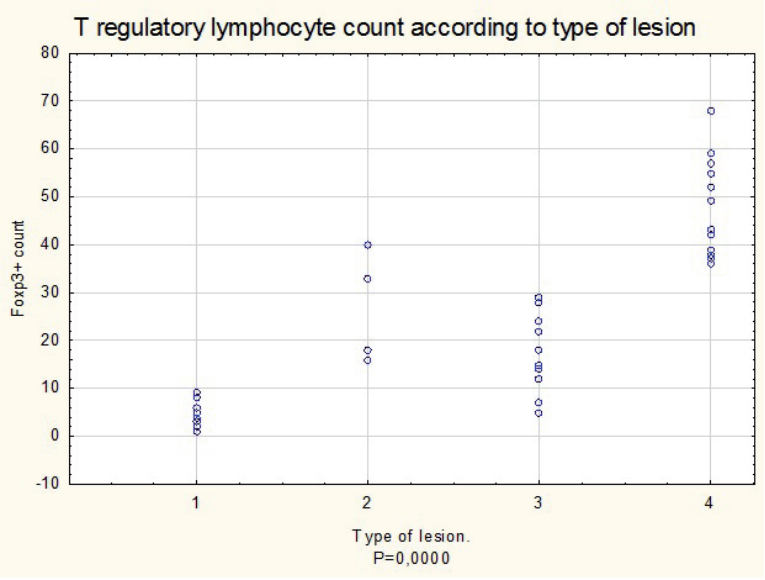

Fig. 3. Density of Foxp3+ T cells, stratified according to the type of lesion (1 — esophagitis, 2 — non-dysplastic Barrett's esophagus, 3 — dysplastic Barrett's esophagus, 4 — esophageal cancer).

Discussion

Glandular metaplasia of esophageal mucosa is postulated to be a potent risk factor for malignant transformation [5]. Previous studies demonstrated that lymphocytic infiltration is present already at early stages of both esophagitis and Barrett's metaplasia [6]. Contrary to the inflammatory lesions, whereby the key role is played by Th1 cells, a shift toward Th2-mediated immune response is observed during the course of progression to BE, which results in the synthesis of pro-inflammatory cytokines [7].

Also dendritic cells are implicated in the etiopathogenesis of BE. The accumulation of these cells has been observed in biopsy specimens from the esophagogastric junction of patients with established Barrett's metaplasia [8]. Moreover, dendritic stem cells co-cultured with esophageal adenocarcinoma and BE cell lines with various degree of dysplasia were shown to stimulate the differentiation of Foxp3+ Tregs from "naive" CD4+ T cells [9].

To the best of our knowledge, the present study is the first to demonstrate that the number of Tregs in esophageal mucosa increases substantially already at the stage of Barrett's metaplasia. This implies that BE may represent an early stage of local immunosuppression and some its underlying processes may resemble those taking place within the invasive cancer tissue.

Previous studies demonstrated with the level of Tregs in peripheral blood of patients with esophageal cancer is higher than healthy persons [10]. Moreover, the distribution of T cells is known to change with the progression of inflammatory lesions to Barrett's metaplasia and then to esophageal cancer. Specifically, an increase in the amount of 'naïve' CD7+ cells and a concomitant decrease in the number of CD4+ and NK cells are observed within the esophageal tissue [6].

Wang *et al.* demonstrated that the density of Foxp3+ lymphocytes infiltrating the tumor correlates positively with the level of anti-inflammatory interleukin 10, thus contributing to immunosuppression. The same study showed that patients with greater density of Tregs are more prone to develop synchronous malignancies [11].

Recently, Li *et al.* revealed that the so-called tumor-derived microvesicles may induce formation of B-regulatory cells that can suppress proliferation of cytotoxic CD8 T lymphocytes [12]. A transmission electron microscopic study demonstrated the presence of similar microvesicles in normal esophagus, and showed that their number increases during the course of metaplasia-dysplasia-adenocarcinoma sequence [13].

Functional suppression of Tregs is a subject of ongoing research as a potential method to improve the treatment outcomes in patients with esophageal dysplasia. Reginato *et al.* analyzed the effects of photodynamic therapy (PDT), a method

for ablation of esophageal dysplasia, on the activity of Tregs. While an increase in peripheral neutrophil count and serum level of interleukin-6 has been observed at 7 days post-PDT, the number of Tregs remained unchanged which likely corresponded to their functional suppression [14].

The effect of BE treatment on lymphocyte balance is still unclear. The results of previous studies point to argon plasma coagulation (APC) of metaplastic epithelium as a promising therapeutic option in BE [15]. Since the underlying mechanisms of BE and esophageal adenocarcinoma likely overlap, future research should center around the restoration of lymphocyte balance during the course of APC and other ablative therapies, such as HALO and PDT. Importantly, APC was already shown to reduce the overexpression of cyclooxygenase-2 (COX2) within the esophagogastric junction, another factor involved in BE etiopathogenesis [16]. In view of this promising preliminary evidence, a study of APC-induced changes in the number of Tregs in biopsy specimens from patients with BE is currently conducted at our clinic.

Conclusions

Barrett's metaplasia, either with dysplasia or without, is associated with an evident increase in the number of Foxp3 lymphocytes within the esophagogastric junction mucosa. Restoration of lymphocyte balance in esophageal tissue might prevent malignant transformation of Barrett's metaplasia.

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Mateusz Rubinkiewicz was responsible for study design and manuscript preparation.
Marcin Migaczewski was responsible for manuscript preparation and statistical analysis.
Jerzy Hankus was responsible for immunohistochemistry.
Piotr Major was responsible for recruiting the patients to the study.
Michał Pędziwiatr as responsible for recruiting the patients to the study.
Piotr Budzyński was responsible for data base management.
Krzysztof Okoń was responsible for critical review of the manuscript.
Andrzej Budzyński was responsible for critical review of the manuscript.

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Conflict of interest

None declared.

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