Perioperative changes in peripheral regulatory B cells of patients with esophageal cancer

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Abstract. Current treatments for esophageal cancer (EC) rely on tumor eradication by surgery or chemoradiotherapy. However, such treatments do not account for the assessment and adjustment of the immune status of the patients. Regulatory B cells (Bregs) have been confirmed as a negative regulatory subtype in B-cell populations. However, to the best of our knowledge, there have been no direct studies on Bregs in patients with EC. The present study enrolled sixty patients with EC and sixty healthy donors to detect the presence of Bregs in peripheral blood and to determine their clinical significance. The percentage of peripheral Bregs was measured using flow cytometry with fluorescence-labeled antibodies against cluster of differentiation (CD) 5, CD19, interleukin (IL)-10, forkhead box protein 3 (Foxp3) and transforming growth factor-β1 (TGF-β1) prior to and following radical surgery. The level of circulating Bregs in patients with EC was observed to be significantly higher than that in the healthy donors. However, this level was observed to decrease following surgery. The percentage of circulating TGF-\beta-producing Bregs and Foxp3-expressing Bregs in patients with EC also decreased following surgery. By contrast, the percentage of peripheral

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Abbreviations: EC, esophageal cancer; Bregs, regulatory B cells; Foxp3, forkhead/winged-helix protein; TGF-β, transforming growth factor-β; IL-10, interleukin-10; B10, IL-10-producing regulatory B cell; Br3, TGF-β-producing regulatory B cells

Key words: regulatory B cells, esophageal cancer, interleukin-10, transforming growth factor- β , forkhead/winged-helix protein

IL-10-producing Bregs (B10s) significantly increased in patients with advanced EC following surgery. These findings suggest that Bregs have a negative immunoregulatory role in the development and progression of EC. Furthermore, postoperative combination therapies against Bregs, particularly B10s, may improve the outcome of patients with EC following resection.

Introduction

Esophageal cancer (EC), the eighth leading cause of cancer-related mortality globally, has a poor prognosis among digestive tract malignancies and an annual diagnosis of approximately half a million individuals worldwide (1,2). The incidence of EC has markedly increased over the past three decades (3). A total of 482,000 new EC cases are diagnosed annually worldwide, resulting in 407,000 mortalities (2). Surgery and radiotherapy are limited to treating local tumors, whereas chemotherapy is limited by toxicity due to its low tumor specificity (1). Considerable evidence suggests that the immune system recognizes and destroys tumor cells, particularly EC cells (4-7).

B cells perform several immunological functions and have been identified to be positive regulators of immune responses and central contributors to the pathogenesis of immune-related diseases as a result of their capacity to produce antigen-specific antibodies (8). However, over the past 30 years, evidence has supported a negative regulatory function for B cells (8,9). Advances in B-cell biology have demonstrated that regulatory B cells (Bregs) release numerous cytokines, with certain Bregs involved in the production of negative regulatory cytokines, including interleukin (IL)-10 (B10s) and transforming growth factor- β (TGF- β) (Br3s), and others expressing the transcription factor forkhead box protein 3 (Foxp3) (10). Previous studies have demonstrated that Bregs have a significant role in the development and resolution of numerous chronic diseases, including experimental autoimmune encephalomyelitis, inflammatory bowel disease and contact hypersensitivity (11-13). The regulatory mechanisms associated with Bregs in the immune system include protection from lethal inflammation, modulation of the development of autoimmune diseases (13-15) and inhibition of anti-tumor responses in various tumor models (16-19). However, few studies have assessed the role of Bregs in EC development.

While evidence has indicated the importance of Bregs in tumor development, there has, to the best of our knowledge, been no research into the functions of Bregs in cancer, particularly in patients with EC (20). The present study investigated the perioperative changes in the Bregs in patients with EC and the association between these cells and clinical phenotypes.

Material and methods

Patient selection. A total of 60 patients with EC were recruited into this case-control study, including 36 males and 24 females with an age range of 50-70 years and a mean age of 64 years. Patients with EC were recruited from the Department of Cardiothoracic Surgery, Lianyungang Hospital Affiliated to Bengbu Medical College (Lianyungang, China). All cases of EC had been histologically confirmed prior to the study. None of the patients with EC had received any invasive treatment, such as radiotherapy or neoadjuvant chemotherapy, prior to tumor resection. Patients with EC were analyzed using the seventh edition of the American Joint Committee on Cancer tumor, node and metastasis staging system in EC for the assessment of disease severity (21). Age- and gender-matched healthy controls were recruited from the Medical Examination Centre of Lianyungang Hospital Affiliated to Bengbu Medical College between July 2011 and July 2012. None of the subjects had a history of autoimmune diseases or tumors.

This study was approved by the Ethical Committee of Lianyungang Hospital Affiliated to Bengbu Medical College (no. 2011-108) and was conducted in compliance with the Declaration of Helsinki. All participants were informed about the investigative nature of the study and signed an informed consent document prior to enrollment in the study.

Sample collection and preparation. Peripheral blood samples were obtained from venous blood immediately subsequent to admission but prior to treatment intervention, in addition to at one and seven days after tumor resection. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood samples using density gradient separation. Whole blood samples were overlaid onto Ficoll[®] separation media (GE Healthcare, Waukesha, WI, USA) following 1:1 dilution with Hank's Balanced Salt Solution (Gibco-BRL, Carlsbad, CA, USA). PBMCs were then centrifuged at 1,500 x g for 30 min, collected at the plasma interface and washed three times following centrifugation at 1,500 x g for 10 min.

The isolated PBMCs were resuspended in a complete RPMI-1640 GlutaMaxTM medium (Gibco-Invitrogen, Breda, The Netherlands) at $5x10^6$ cells/ml in two FalconTM tubes. GolgiPlugTM (BD Biosciences, San Jose, CA, USA) was then added to each tube and cells were incubated for 5 h in 5% CO₂ at 37°C. Following incubation, PBMCs were stained for 15 min in the dark using phycoerythrin-labeled anti-cluster of differentiation (CD) 19 and fluorescein isothiocyanate-labeled anti-CD5 (BD Biosciences) monoclonal antibodies. PBMCs were then permeabilized/fixed using the FIX&PERM[®] kit (ADG, Kaumberg, Austria). Cells were successively stained

for 15 min in the dark using allophycocyanin-labeled anti-IL-10 and -TGF- β 1 monoclonal antibodies and peridinin chlorophyll-cyanine5.5-labeled anti-FOXP3 monoclonal antibodies (BD Biosciences), prior to washing once with phosphate-buffered saline (PBS). Cells were resuspended in 500 μ l PBS prior to flow cytometric analysis.

Flow cytometry. Flow cytometric analysis was conducted using a BD FACSCalibur flow cytometer (BD Biosciences). For the analysis of Bregs, the acquisition and analysis gates were restricted to the live lymphocyte population. For myeloid-derived suppressor cell analysis, all live cells were included. CD5⁺CD19⁺ cells were calculated as the percentage of live CD19⁺ lymphocytes. Bregs were identified as CD5⁺CD19⁺ cells and the production of IL-10, FOXP3 and TGF β 1 was calculated as the percentage of cytokines.

Statistical analysis. All values are presented as the mean \pm standard error of the mean. Statistical analyses were performed using SPSS 11.0 statistical software (SPSS Inc., Chicago, IL, USA). The percentage of Bregs among the groups was analyzed using one-way analysis of variance, followed by an unpaired student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Perioperative changes in peripheral Bregs. As shown in Fig. 1, the percentage of peripheral Bregs in patients with EC prior to surgery (ECb) was observed to be significantly higher than that in the group of healthy controls $(3.40\pm0.60 \text{ vs.} 1.76\pm0.23\%; P<0.05)$. No significant difference was observed in the percentage of Bregs between patients with EC at one day after surgery (ECa1) and the ECb group $(2.99\pm0.39 \text{ vs.} 3.40\pm0.60\%; P>0.05;$ Fig. 1). However, a significant reduction in the percentage of Bregs was observed in patients with EC at seven days after tumor resection (ECa7) compared with the ECb $(1.92\pm0.39 \text{ vs.} 3.40\pm0.60\%; P<0.05;$ Fig. 1) and ECa1 $(1.92\pm0.39 \text{ vs.} 2.99\pm0.39\%; P<0.05;$ Fig. 1) groups.

Perioperative changes in peripheral B10s. As shown in Fig. 2, the percentage of B10s in the ECb group was observed to be significantly higher than that in the healthy controls $(2.52\pm0.41 \text{ vs. } 1.56\pm0.28\%; \text{ P}<0.05)$. Furthermore, the percentage of B10s in the patients with EC significantly increased over time following tumor resection $(3.59\pm0.29\% \text{ in ECa1 vs. } 8.36\pm0.51\% \text{ in ECa7; P}<0.05)$.

Perioperative changes in peripheral Br3s. The percentage of Br3s in the ECb group was not observed to be significantly different from that of the healthy controls (6.13 ± 0.57 vs. $5.91\pm0.23\%$; P>0.05; Fig. 3). However, the percentage of Br3s was observed to decrease in the ECa1 group compared with the ECb group (4.54 ± 0.41 vs. $6.13\pm0.57\%$) prior to increasing in the ECa7 group ($5.61\pm0.42\%$). The percentage of Br3s in the ECa7 group remained below that in the ECb group, although the difference was not significant (P>0.05) (Fig. 3).

Perioperative changes in peripheral Foxp3-expressing Bregs. As shown in Fig. 4, the percentage of Foxp3-expressing Bregs



Figure 1. Percentage of peripheral Bregs (CD5⁺CD19⁺ cells) in the study groups. The percentage of peripheral Bregs was determined in healthy controls (Healthy) and patients with EC prior to surgery (ECb), at one day (ECa1) and at seven days after surgery (ECa7). (A) Flow cytometric analysis of peripheral Bregs with a representative percentage of Breg cells indicated for each group. (B) Percentage of Bregs in the total lymphocyte population in peripheral blood. Data are presented as the mean \pm standard error of the mean. *P<0.05 vs. the Healthy group; *P<0.05 vs. the ECb group and &P<0.05 vs. the ECa1 group. Bregs, regulatory B cells; EC, esophageal cancer; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin.



Figure 2. Percentage of peripheral B10s in the study groups. The percentage of peripheral B10s was determined in healthy controls (Healthy) and in patients with EC prior to surgery (ECb), at one day (ECa1) and at seven days after surgery (ECa7). (A) Flow cytometric analysis of peripheral B10s with a representative percentage of B10 cells indicated for each group. (B) Percentage of B10s in the total Breg population in peripheral blood. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. the Healthy group; #P<0.05 vs. the ECb group and *P<0.05 vs. the ECa1 group. Bregs, regulatory B cells (CD5+CD19+ cells); B10, IL-10-producing Breg; EC, esophageal cancer; IL, interleukin; APC, allophycocyanin.

was observed to be significantly higher in the ECb group than that in the healthy controls $(8.35\pm1.04 \text{ vs}. 2.91\pm0.23\%; P<0.05)$. However, the percentage of Foxp3-expressing Bregs was shown to significantly decrease over time following tumor resection $(8.35\pm1.04 \text{ in ECb vs}. 4.84\pm0.16 \text{ in ECa1}$ and $4.02\pm0.66\%$ in ECa7; P<0.05). The frequency of Foxp3-expressing Bregs was observed to be lower in the ECa7 group compared with that in the ECa1 group; however, this difference was not significant $(4.84\pm0.16 \text{ vs}. 4.02\pm0.66\%; P>0.05)$ (Fig. 4).

Discussion

As a functionally unique subgroup of B lymphocytes, Bregs promote tumor progression in various malignancies, including sarcomas, melanomas, breast carcinomas and hepatocellular carcinomas (5,22,23). However, current studies on Bregs have been predominantly conducted using animal models. Thus, the disease-specific patterns of peripheral Bregs in patients with EC are yet to be elucidated. The present study investigated the functions of CD5⁺CD19⁺ B-cell-derived IL-10, TGF- β and FOXP3 in patients with EC.

IL-10 is a 178-amino acid protein secreted by various cells, primarily Type 2 helper T-cell (Th2) clones (24). IL-10 has multiple functions in immune modulation and exhibits anti-inflammatory and suppressive effects on hematopoietic cells (17). A number of studies have identified IL-10-producing CD19⁺ B cells, which are known as B10s (8,10,12). Lee *et al* (25,26) described human IL-10-producing Bregs that



Figure 3. Percentage of peripheral Br3s in the study groups. The percentage of peripheral Br3s was determined in healthy controls (Healthy) and in patients with EC prior to surgery (ECb), at one day (ECa1) and at seven days after surgery (ECa7). (A) Flow cytometric analysis of peripheral Br3 with a representative percentage of Br3 cells indicated for each group. (B) Percentage of Br3 in the total Breg population in peripheral blood. Data are presented as the mean \pm standard error of the mean. *P>0.05 vs. the Healthy group; 4 P>0.05 vs. the ECb group and 8 P<0.05 vs. the ECa1 group. Bregs, regulatory B cells (CD5⁺CD19⁺ cells); Br3, TGF- β -producing regulatory B cell; EC, esophageal cancer; TGF- β ; transforming growth factor- β ; APC, allophycocyanin.



Figure 4. Percentage of peripheral Foxp3-expressing Bregs in the study groups. The percentage of peripheral Foxp3-expressing Bregs were determined in healthy controls (Healthy) and in patients with EC prior to surgery (ECb), at one day (ECa1) and at seven days after surgery (ECa7). (A) Flow cytometric analysis of peripheral Foxp3-expressing Bregs with a representative percentage of Foxp3-expressing cells indicated for each group. (B) Percentage of Foxp3-expressing Bregs in the total Breg population in peripheral blood. Data are presented as the mean \pm standard error of the mean. *P<0.05 vs. the Healthy group; #P<0.05 vs. the ECb group. Bregs, regulatory B cells (CD5+CD19+ cells); EC, esophageal cancer; Foxp3, forkhead box protein 3; PerCP-Cy5.5, peridinin chlorophyll-cyanine5.5.

expressed a CD5⁺CD19⁺ phenotype in PBMCs. The present study demonstrated that the percentage of B10s was higher in patients with EC than that in healthy controls and was markedly increased following surgery, with the in the ECa7 group exceeding that in the ECa1 group. These findings are consistent with the significant perioperative changes observed in peripheral Bregs in patients with hepatocellular carcinoma reported by Chen *et al* (23).

TGF- β belongs to a superfamily of cytokines that regulates cell proliferation and differentiation, developmental patterning and morphogenesis, and disease pathogenesis (24). TGF- β 1 plays critical roles in tumor development through its influence on the apoptotic pathways (27-29). Miller *et al* (30) demonstrated that TGF- β 1 is crucial in the progression of EC due to the fact that it induces the activation of extracellular signal-regulated protein kinases 1 and 2. In a study by Tian *et al* (31), an additional Breg subgroup, which produced TGF- β 1 following *in vitro* stimulation with lipopolysaccharides, was identified. Furthermore, Lee *et al* (32) recently reported the presence of Br3s in human peripheral blood. The present study revealed that the percentage of peripheral Br3s was higher in patients with EC than that in healthy controls. In addition, the percentage of Br3s was observed to markedly increase following surgery in patients with EC. Therefore, the systemic inflammatory state induced by EC cells may promote peripheral Br3 production.

Foxp3 is a transcription factor that controls the development of regulatory T cells and is reported to be expressed in mouse CD4⁺ T cells (33). CD4⁺Foxp3⁺ T cells are known as Tregs and are involved in the negative regulation of immune responses (33). A study by Huang and Fu (7) reported that CD4+Foxp3+ T cells are present in EC tissues at levels 10-fold higher than those in non-EC tissues. Furthermore, Wang et al (34) demonstrated that FOXP3 is overexpressed in EC cells, but not found in normal esophageal mucosal cells. A study has also indicated that CD5⁺CD19⁺Foxp3⁺ Bregs are present among human PBMCs (35). The results of the present study demonstrate that the percentage of peripheral CD5⁺CD19⁺Foxp3⁺ Bregs is higher in patients with EC than that in healthy controls. Furthermore, the percentage of CD5⁺CD19⁺Foxp3⁺ Bregs was observed to significantly decrease in patients with EC following surgery. However, the percentage of these cells in the peripheral blood of the ECa7 group was not observed to differ significantly from that of the ECa1 group. These results suggest that FOXP3 overexpression may be significantly correlated with tumor staging and lymph node metastasis.

To the best of our knowledge, the present study is the first to demonstrate the perioperative changes in circulating Bregs in patients with EC prior to and following radical surgery. Further studies are required to determine whether the increases in circulating Bregs following surgery are a consequence of the tumor removal or of the extensive esophageal surgery. The mediators of Br1, Br3 and Bregs should be profiled and validated in order to elucidate the mechanisms associated with their interactions. Network biomarkers that show protein-protein interactions within these regulatory cells should be investigated based on protein annotations, interactions and signaling pathways (36,37).

In conclusion, the current study demonstrated that the percentage of peripheral Br3s and Foxp3-expressing Bregs decreased following surgery in patients with EC, whereas the percentage of circulating B10s was significantly increased in patients with advanced EC following surgery. Furthermore, Foxp3 and TGF- β may be involved in regulating the number and function of Bregs in patients with advanced EC who undergo surgery. The findings of the present study suggest that, in patients with EC, the level of IL-10 reduced to the levels of healthy controls via medical intervention, therefore this may confer an enhanced prognosis following surgery.

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