Regulatory T cells and monocytes crosstalk in patients with gastric cancer

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INTRODUCTION: *Primary function of regulatory T(Treg) cells is to control and regulate the immune responses. In many patients with tumor tissues, increased Treg cell numbers have been reported. In this study, we aimed to measure the cellular content of blood samples in patients with gastric cancer (GC) and define their role in tumor progression.*
METHODS: *We prospectively evaluated 34 gastric cancer cases and 20 healthy control samples. The blood was collected from both the gastric coronary and peripheral veins of the patients and only from the peripheral vein of the control group. Cellular content and lymphocyte subset including, regulatory T cells, were determined by flow cytometric analysis.*
RESULTS: *The GC patients revealed similar percentages of T cells, B cells, neutrophils, and eosinophils in the venous samples from periphery vein and gastric coronary. The percentage of monocytes from the tumor-draining gastric coronary vein was significantly lower than monocytes from the peripheral vein in gastric cancer patients (p=0.03). T-regulatory cells had a higher percentage in samples obtained from gastric cancer patients compared with the control group.*
CONCLUSION: *Our findings confirmed that patients with gastric cancer have a significantly higher percentage of regulatory T cells than the control group, suggesting that they may contribute to the tumor progress. Regulatory T cells and monocytes interact in patients with GC, which can be used as a parameter in the clinical follow-up of patients with GC.*

KEY WORDS: Gastric cancer, Treg cell, Flow cytometry, FoxP3

Introduction

Gastric cancer (GC) is one of the deadliest cancers worldwide. While GC is the leading cause of cancer-related deaths in many Asian countries, deaths are less common in many developed countries [1-2]. It is known that in all cancers, tumor tissues are infiltrated by immune system cellular structures, including natural killer cells, macrophages, T lymphocytes, and many others [3]. The prognosis of tumor types is affected by tumor-associated immune cells [4]. Examination of pathological specimens and peripheral blood evaluations in GC revealed that with the progression of nodal involvement, natural killer (NK) and dendritic cell infiltration decrease, which may indicate that t-regulatory cells might have an essential mission in the progression of gastric cancer [5-8].

In cancer patients, it was found that T-regulatory cells reduced the effective functional activity against the tumor. This situation was seen as dysfunction in T and Dendritic cells (DCs) in the cancer-bearing host. [9]. Numerous T-regulatory cells have been shown in cases with different types of cancer, such as GC and esophageal cancer [9,10,11]. These findings triggered to suppose that cancer-bearing hosts of patients with advanced cancer have raised T reg cells, which may reduce the body's resistance to cancer and decrease the effectiveness of T cells formed against tumor-specific cells. The task of T-reg cells against autoimmune diseases is to directly or indirectly suppress effective T cells that have reacted with their self-antigens [12, 13]. Most antigens produced by neoplastic cells are self-antigens. T reg cells suppress the immune response against the tumor by blocking the antigen presentation of DCs, thus contributing to the tumor escape mechanism [13,14].

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Regulatory T cells suppress immune cells with multiple mechanisms, including soluble cytokines IL-10 and TGF-beta that they produce [11]. The higher proliferation rate and the lower apoptosis rate of Treg cells at the tumor sites may promote the accumulation of Treg cells in GC tissue [11,13]. Additionally, the enhanced recruitment by adhesion molecule and the decreased emigration due to CCR7 down-regulation on the Treg surface may increase this accumulation [11,13]. The cellular composition of venous blood from the tumor microenvironment of breast and lung cancers has been described by others [9,15]. However, in GC patients, whether lymphocytes' cellular composition and immunologic properties in the tumor-draining veins are similar to those of peripheral blood has not been explored.

In this study, we compared the cellular composition of blood samples from the gastric coronary vein to the peripheral vein by flow cytometric characterization in 34 gastric cancer patients at the beginning of surgery. This study may contribute to the identification of critical biological features in immune cells and tumor cells.

Materials and Methods

Patients and Controls

We have analyzed blood samples from 34 gastric cancer patients and 20 control samples in this study. The age ranges between 41-85 years inpatients and 40-65 years in the control group. The majority of GC patients were male (26/8). Pathological identification was determined for whole the cases, and all cases were diagnosed as high-grade tumors with adenocarcinoma. Whole cases were preoperatively untreated and scheduled to carry out GC surgery. Patients did not take chemotherapy, radiotherapy, immunotherapy, or any therapy regimen that affected the cellular composition.

Blood Samples

A blood sample from controls via an antecubital vein and two samples from GC patients via a gastric coronary vein and antecubital vein were collected with the anti-coagulated tube. The blood sample from gastric cancer patients was obtained when the shortest possible time with the least possible dissection events in the surgery. The percentages and characteristics of cellular subsets, including Treg cells, were measured from these samples by flow cytometry.

The Immune Phenotype of Cells With Flow Cytometry

The erythrocytes in the pellet fraction from 3 ml blood taken from the patients and healthy control group were lysed with a lysis solution (both from gastric coronary vein and peripheral vein). Afterward, leukocytes were separated from this solution by washing twice with cell washing. (Becton Dickinson (BD) (Biosciences, San Diego, CA).

Autofluorescence of leukocytes was evaluated with 5.0 µg/mL monoclonal isotype antibodies. Cell markers were analyzed from this solution taken into four tubes. 10 µL incubation was applied for each antibody: CD3-FITC, CD16+56-PE, CD19-APC, CD45-PerCP; CD3-FITC, alfa-beta-PE, gamma-delta-APC, CD45-PerCP; FoxP3-PE CD4-FITC, CD8-APC, CD25-PC7; CD14-FITC, CD45-PerCP. These antibodies were used for staining and the data acquisition by flow cytometer, FacsCanto II (BD Biosciences, San Diego, CA).

Immunophenotypic characterization was determined according to the antibody staining; T cells were characterized by the expression of alfa-beta+, CD3+ cells, whereas NK cells were characterized as CD56+, CD3- cells. However, helper T cells were characterized by the expression of both CD3+ and CD4+ and a lack of CD8- expression, and Treg cells by expression of both CD3, FoxP3, CD4, CD25 or CD3, CD8, CD25. B cells were characterized as CD19+, CD3- cells, and monocytes as CD14+ cells.

Statistical Analysis

Data were described as mean ±, standard deviation. Statistical significance was evaluated using the Wilcoxon test in both samples from patients. Statistical analysis was carried out by SSPS statistical software, and statistical significance was evaluated as $p < 0.05$.

Results

Overall Patient Characteristic

Fifty-four blood samples were enrolled in this study, including from 34 gastric cancer patients and from 20 control samples. The majority of the GC patients were male (26/8), and ages ranged from 44 to 81 years, with a mean of 62.15 ± 10.5 years. The blood samples were collected from the gastric coronary vein, an antecubital vein in patients, and an antecubital vein in the control group were analyzed by flow cytometry on the same day.

The Peripheral and Coronary Vein Cellular Contents in Gastric Cancer Patients

We have compared the cellular characteristic of samples from the gastric coronary vein with that of peripheral blood of the same patient, and the flow cytometry data shows that the gastric cancer cases revealed similar per-

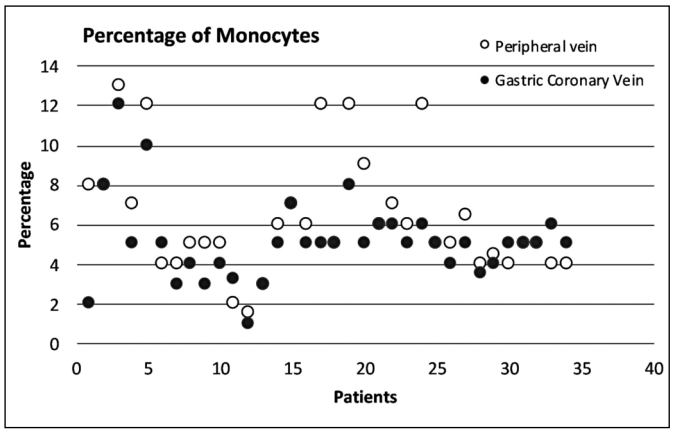

Fig. 1: The percenteges of Monocytes at blood samples from gastric coronary vein and peripheral vein in 34 patients.

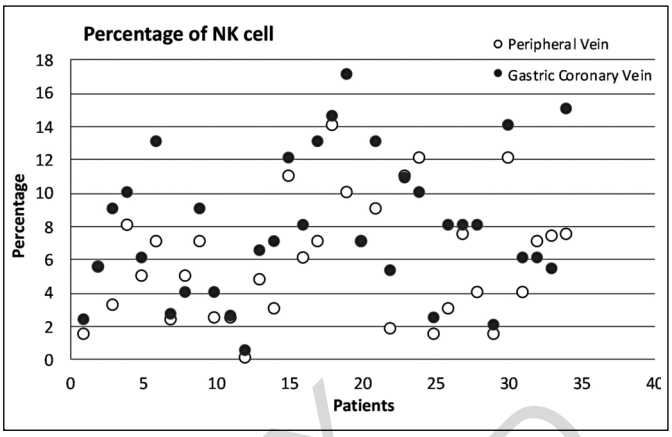

Fig. 2: The percenteges of NK cells at blood samples from gastric coronary vein and peripheral vein in 34 patients.

centages of T cells, B cells, neutrophils, and eosinophil in both samples including the regulatory T cells (Table I). Nevertheless, the results revealed that a remarkable increase in the percentage of NK cells and a remarkable decrease in the percentage of monocytes were observed in the blood samples from a tumor-draining gastric coronary vein in cases (p=0.04 and p=0.03, respectively) (Table I), (Figs. 1, 2). Our results show no remarkable difference in the regulatory T cell ratios in coronary and peripheral venous samples from GC cases.

The Comparison of Peripheral Veins Cellular Contents Between Patients and Controls

Although the peripheral blood samples from GC patients showed that T cells, B cells, and NK cells were slightly higher than the control group, these distinctions were not statistically remarkable (p_T=0.2, p_B=0.2, and p_{NK}=0.3, respectively).

Moreover, to these cells, monocytes were slightly higher in patients with gastric cancer, but this distinction was not statistically remarkable (p=0.07).

The percentage of T helper cells CD4+, cytotoxic T cells CD8+, and T regulatory cells FoxP3+CD25+ were also measured by flow cytometry. We found no statistically remarkable difference between the both CD4+ and CD8+ T cell percentages in both groups except the regulatory T cells.

Regulatory T cells were remarkably higher in samples of GC cases compared to control group, regulator helper T-cell CD4+CD25+ (p=0.01), regulator cytotoxic T-cell CD8+CD25 (p= 0.03) (Table II).

Table I - *Cellular percentage samples collected from the tumor draining coronary vein in gastric cancer patients and peripheral blood in both patients and controls.*

	Patient		Control
	Coronary vein (%)	Peripheral vein (%)	Peripheral vein (%)
Monocytes	5.0 ± 0.5*	6.2 ± 0.5	4.7 ± 1.7
Neutrophils	66.7 ± 2.4	67.0 ± 2.4	59.0 ± 8.0
Eosinophils	2.2 ± 0.5	3.0 ± 0.5	2.3 ± 1.4
T cells (CD3+)	15.2 ± 1.3	15.2 ± 1.3	17.9 ± 1.5
B cells (CD19+)	3.0 ± 0.7	2.7 ± 0.7	10.1 ± 1.4
NK cells (CD56+)	7.9 ± 0.7*	5.9 ± 0.7	6.0 ± 2.2

*p<0.05 and p-value representing Wilcoxon test in both samples from patients.

Table I - *The percentage of regulator T cells in peripheral vein samples from both gastric cancer patients and controls.*

Regulator T Cells subgroups	Patient (%)	Control (%)
Regulator T Helper (CD4+ 25+)(Percentage in all T helper)	21.4 ± 1.2 *	16.4 ± 1.6
Regulator T Cytotoxic (CD8+ 25+)(Percentage in all T cytotoxic)	3.3 ± 0.5 *	1.0 ± 0.7

*p<0.05

Discussion

In this study, we first compared the cellular components of blood samples from the tumor-draining venous system and the periphery in patients with gastric tumors. Secondly, we have compared the cellular content and percentage of peripheral blood samples from GC patients and the control group. The cellular contents and percentages of both samples from the GC patients showed that the ratios of T cells, B cells, neutrophils, and eosinophils are almost similar. The ratios of T cells, B cells, and others were also similar in both groups (Table I).

In many tumor types, it has been noticed that regulatory T cells are increased at the tumor site and contributed to tumor-induced immune suppression, cancers including melanoma, breast, ovarian, head and neck, liver, stomach, and pancreas [16-18]. We have also analyzed all lymphocytes and the subset of T cells in our patients and controls. The percentage of CD4+ T helper cells, CD8+ cytotoxic T cells, and FoxP3+CD25+ regulatory T cells were identified by flow cytometry. The percentage of the Treg, both helper and cytotoxic, was markedly higher in the peripheral blood samples from gastric cancer patients than in the control group (CD4-Treg p=0.01 and CD8-Treg p=0.03, respectively) (Table II). The latest data demonstrated that raised numerous Treg cells either in the peripheral circulation or tumor microenvironment of GC patients upon pathological examination of specimens [4].

Treg cells mainly suppress T cells and B cells, and NK cells, so the lymphocytes proliferation pathway is affected directly, and dendritic cells and macrophages are indirectly affected. So, tumor antigen-presenting cells might not work correctly when the Treg percentage increases [19,20]. Treg cells and their cytokines (IL-10, TGF-B) differentiate DCs to dysfunctional (tolerogenic and immunosuppressive) or apoptotic cells in the tumor microenvironment [19]. Our results showed that although monocytes percentage higher in GC patients than in the control group, a remarkable decrease in the percentage of monocytes was observed in the blood samples from tumor-draining gastric coronary vein compared to peripheral samples of the same patients (p=0.03) (Fig. 1).

The main reason for this event might be the penetration of monocytes from the capillary of the gastric coronary vein to the tumor tissue and differentiates to the new DCs to compensate for the inactivation of dendritic cells by Treg. So low monocytes percentage in gastric coronary vein and increased Treg cell in these patients might have a reciprocal interaction and could be helpful for clinical follow-up. Future research related to the percentage of monocytes and replacement of the tumor inactivated dendritic could help understand the crosstalk between monocytes, Treg cells, and dendritic cells.

Additionally, a remarkable increase in the percentage of NK cells was detected in the blood samples from a tumor-draining gastric coronary vein in cases when compared to peripheral samples of the same cases, and this might be a sign of NK cell activity in gastric tumor tissue (p=0.04) (Fig. 2). However, additional studies with a test like CD158, CD159, and CD137 for NK cell receptors, might be needed in the cases to evaluate the interplay between tumor inactivated DCs and NK cells in both tumor-draining vein and peripheral vein [21].

Although the Treg cells were significantly higher in the GC patients than in the control group, we could not detect a difference between the patient and control Treg cells regarding the expression levels of CD25, T cell activation marker. So, different kinds of specific antibodies for Treg cells might give different characteristics of Treg in tumor microenvironments. Further, the stemness factors Sox2 and Oct3/4 were correlated with induced pluripotent stem cells [22], recommending a correlation between these stemness factors and cancer stem cells. Sox2-positive expression or Oct3/4-negative expression might be correlated with invasion of GC. Sox2 and Oct3/4 might be independent prognostic factors for cases with GC [23].

Results have shown that immune-phenotypic distinctions in the cellular composition of blood samples from tumor-draining veins and peripheral blood. Both samples give similar results for Treg cells percentage, and a peripheral blood sample could be helpful for Treg evaluation in GC patients. The results also showed that patients have a significantly higher percentage of regulatory T cells than controls suggesting that Treg may contribute to the tumor pathogenesis. This information improves our understanding of properties of gastric tumor and might be helpful for the diagnostic study related with Treg heterogeneity in patients with GC and need future investigation focused on Treg cell and monocytes crosstalk.

Main Point

Immunophenotyping of blood samples taken from both coronary and antecubital veins of the cancer patients [33] and only from the antecubital vein of the control patients [17], who were planned for control patiens was performed by flow cytometry method. The diagnosis of the patients was made by clinical examination, upper endoscopy, USG, and histopathological evaluation of the tissue sample. In our study, we reached the following results:

I. We describe a method that has not been used before in gastric cancer patients to obtain blood from the gastric tumor microenvironment via the vein system.

II. We compared cellular immunity in gastric cancer, close to and far from the tumor. We analyzed the blood samples taken from two different regions of the same patient by flow cytometry method. We found that the percentages of NK CD56+ cells were statistically sig-

nificantly different in the coronary and antecubital veins of the cancer patients. The statistical excess was in favor of Gastric coronary veins of the cancer patients.
III. We also found that the percentage ratios of Monocyte and CD64+ cells were statistically significantly different in the coronary and antecubital veins of the cancer patients. We showed that the statistical excess of monocytes and CD64+ cells, in contrast to NK cells, favored antecubital veins of the cancer patients.
IV. We found a statistically significant relationship between NK, pathological grade, and stage.
V. When the percentages of CD4/CD25 and CD8/CD25 (Treg) cells were compared between the antecubital vein of the cancer patients and the antecubital vein of the control patients, we showed that there was a statistically significant difference in both. We found that the statistical excess is in favor of the antecubital vein of cancer patients.
VI. There was no statistically significant correlation between the percentage of CD4/CD25+ cells with T Helper (CD3+ CD4+ CD8-) regulator and stage from T reg cells. We showed a statistically significant correlation between the percentage of CD8/CD25+ cells with T cytotoxic regulator (CD3+ CD8+ CD4-) and stage.

Riassunto

La funzione primaria delle cellule T regolatorie (Treg) è controllare e regolare le risposte immunitarie. In molti pazienti affetti da tumore è stato riscontrato un aumento del numero di cellule Treg. In questo studio, abbiamo misurato il contenuto cellulare di campioni di sangue in pazienti con cancro gastrico (GC) e definire il loro ruolo nella progressione del tumore.
Si tratta di uno studio prospettico su 34 casi di cancro gastrico e 20 campioni di controllo sani. Il sangue è stato raccolto sia dalle vene coronariche gastriche che periferiche dei pazienti e solo dalla vena periferica del gruppo di controllo. Il contenuto cellulare e il sottogruppo di linfociti, inclusi i linfociti T regolatori, sono stati determinati mediante analisi citofluorimetrica.
Risultati: I pazienti con GC hanno rivelato percentuali simili di cellule T, cellule B, neutrofili ed eosinofili nei campioni venosi della vena periferica e della coronaria gastrica. La percentuale di monociti dalla vena coronarica gastrica drenante il tumore era significativamente inferiore rispetto ai monociti dalla vena periferica nei pazienti con cancro gastrico (p=0,03). Le cellule T-regolatorie avevano una percentuale più alta nei campioni ottenuti da pazienti con cancro gastrico rispetto al gruppo di controllo.
Conclusione: I nostri risultati hanno confermato che i pazienti con cancro gastrico hanno una percentuale significativamente più alta di cellule T regolatorie rispetto al gruppo di controllo, suggerendo che possono contribuire al progresso del tumore. Le cellule T regolatorie ei monociti interagiscono nei pazienti con GC, che possono essere utilizzati come parametro nel follow-up clinico dei pazienti con GC.

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