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MICROENVIRONMENT OF INTESTINAL ANASTOMOSES WITH COMPARATIVE ASSESSMENT OF IMMUNE STATUS IN PATIENTS WITH SMALL INTESTINE ANASTOMOSES

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The purpose of the study was to assess local immune status as a marker for the risk of intestinal anastomosis failure. The research was based on the study of local immune mechanisms of intestinal anastomoses failure in the comparative aspect. The material of the study was the anastomosis tissue, areas of the intestine at a distance of 1-3 cm from the anastomosis, as well as the patients' blood samples taken before surgery, on the first and the fifth days after the formation of the anastomosis in subgroups of the study. The occurrence of intestinal anastomoses failure is mainly determined by the interaction of immunocompetent cells and products of their activity - cytokines. Immunological disorders in the intestine tissues in the anastomosis area cause the failure pathogenesis. The microenvironment of intestinal anastomoses by immunological parameters is a field that promotes further progression of inflammation or serves as a buffer zone that protects tissue from pathological changes. The study revealed the formation of secondary immune deficiency in patients with resection of intestinal segments; recovery of immune status indices in patients with uncomplicated postoperative period occurs at an earlier date and to the fuller extent.

Key words: intestinal anastomoses, failure, immunology.

The work is a fragment of the initiative research project "To develop technologies for the prediction, prevention and surgical treatment of anastomotic failure in patients with small bowel surgery" at the stage of planning.

An intestinal anastomosis is a surgical procedure performed to establish a connection between two previously distant parts of the intestine. This procedure restores the continuity of the intestine after the removal of a pathological condition that affects the intestine [3].

The history of the intestinal suture study goes back over 200 years and it is no secret that great progress has been made in this field of abdominal surgery. Numerous clinical and experimental studies provide various information on the efficacy of an intestinal suture's particular type. It is known that the main pathophysiological aspect in patients with intestinal anastomoses (IA) failure are volemic and hemodynamic disorders, which are caused by reduced arterial inflow and impaired venous outflow due to compression of intramural vessels, sequestration of fluid into the lumen of the intestine and abdominal cavity [3, 4].

Endogenous intoxication and metabolic disorders in IA failure are of complex and multicomponent nature [2, 6]. The main source of endotoxemia in patients with IA failure is the intestine [5]. Impairment of the intestinal wall's barrier function leads to endotoxemia, which is progressive in the absence of adequate treatment [4, 5, 9].

Chronic inflammation to some extent may serve as a background for the intestinal anastomoses failure against the background of proliferation in the intestinal mucosa. The starting point for the proliferation are the epithelial cells of the intestinal mucosa [1]. The immune system of the gastrointestinal tract (GIT) is represented by lymphoid tissue associated with the intestine (GALT-gut associated lymphoid tissue), located in three parts - diffusely distributed by its own plate under the intestinal epithelium, in the epithelium and in organized lymph follicles, such as Peyer's patches. It is in close contact with a huge flow of microbial and allergenic material coming from the intestinal lumen, and serves as the first barrier in its path [9].

The following factors are crucial in the further development of pathological changes: gradual depletion of the liver detoxification potential; translocation of intestinal microflora and its acquisition of pathogenic properties; growth in the total mass of toxic products in internal environments; development of systemic microcirculatory disorders in organs and tissues, disorders of cellular metabolism; development and progression of peritonitis, the second source of intoxication [6, 9].

In our opinion (and this is confirmed by various studies [1, 7, 8, 9]), it is important to study the role of immunocompetent cells and cytokines in the formation of the intestinal anastomoses failure.

The purpose of the study was to assess the local immune status as a marker for the risk of intestinal anastomoses failure.

Materials and methods. The work was based on the study of local immune mechanisms of intestinal anastomoses failure in the comparative aspect. The material of the study was the anastomosis tissue, as well as areas of the intestine at a distance of 1-3 cm from the anastomosis. Clinical material (paraffin blocks)

sampled from 52 patients who were selected by random sampling, who were performed resections of small bowel segments with the formation of intestinal anastomoses, as well as blood samples taken before surgery, on the first and the fifth days after the formation of the anastomosis, in SI "V.T. Zaitsev Institute of General and Urgent Surgery of NAMSU" in the period from 2001 to 2018. The mean age of patients was 54.7 ± 5.9 years. Of all the operated there were - 29 (55.9%) women and 23 (44.1%) men.

The patients were divided into two subgroups: subgroup I consisted of patients with uncomplicated postoperative period (23 patients), subgroup II included 29 patients with complicated postoperative period (who were diagnosed with intestinal anastomoses failure).

Immunological typing of tissue lymphocytes (CD3 +, CD3 + CD4 +, CD10 + CD8 +, CD16 + CD56 +, CD19 +, T-lymphocytes with receptors) and cytokines (TNF-, IL-1, IL-2, IL-6, IL-8, IL-10), as well as studies of markers (antibodies): Vimentin, Ki - 67, were evaluated using monoclonal antibodies by immunohistological staining with Ventana Medical Systems, Inc. automatic system using Ventana kits (Ventana, Tucson, AZ) in compliance with the manufacturer's instructions.

Serial paraffin sections were prepared and applied to adhesive-coat glass. The study was performed according to the method of C.R. Taylor, R. Cote. Pre-treatment of the sections was performed by the method of restoration of antigenic tissue determinants (S.H Shi, 1991 and M. Sowa; Y. Kato, I. Nakanishi et al., 1992). Incubation with primary antibodies lasted 30 min at room temperature. The sections were thoroughly washed in the buffer, dried, and then treated with the En Vision system (Daco, Denmark). Monoclonal antibodies were to desmin (Daco, RTU dilution), to vimentin (Daco, RTU dilution), to Ki - 67 (Daco, RTU dilution). The study was performed at different types and methods of anastomoses formation.

The study of the total population of T-lymphocytes (CD3⁺), subpopulations of T-lymphocytes - T-helpers (CD4⁺), T-suppressors (CD8⁺) and β -lymphocytes (CD19⁺ and CD20⁺) in blood serum was performed using monoclonal antibodies CD3⁺, CD4⁺, CD8⁺, CD19⁺ and CD20⁺ by the immunofluorescence method with STAT-FAX303 enzyme-immunoassay analyzer, USA. The content of immunoglobulins A, M, G (IgA, IgM, IgG), total immunoglobulin E (IgE) in the blood serum was studied using enzyme-immunoassay systems produced by Tov. NVL "Granum-Ukraine", and the content of allergen-specific IgE was studied using enzyme-immunoassay systems produced by NVT "Microgen" (Russia). Studies of circulating immune complexes were performed in blood serum by the method of Gashkova et al. (1977), as well as tumor necrosis factor alpha (TNF- α) in the blood serum was detected using enzyme-immunoassay systems manufactured by VAT "Protein Contour" (Russia) and "Diacclone" (France).

Statistical analysis was performed using the modern software package STATISTICA 7.0 (StatSoft Inc., USA) and MedCalc (version 9.3.5.0).

Results of the study and their discussion. Assessment of local immune status in the intestinal anastomoses failure revealed that the content of T-helper-induction lymphocytes (CD3⁺, CD4⁺) in the tissue of the anastomosis was reliably ($p \leq 0.05$) higher by 44.7%. Respectively, the CD4⁺ / CD8⁺ index was by 1.7 times and by 55.0% higher than that of relatively healthy tissue ($p < 0.05$), but the number of CD8⁺ cells and B lymphocytes was reduced by 21 % and 23.5 % respectively compared to relatively healthy tissue and by 42.2% (table 1).

In the intestinal tissue, which was sampled at the distance of 1-3 cm from the anastomosis, on the contrary, there was a higher content of CD19⁺ cells by 37.2%, but also a lower number of cytotoxic cells (CD8⁺) (18.0%) compared to the indices along the line of resection ($p < 0.05$) (table. 1). According to other criteria, no reliable differences were found. Studies of local immune status in different parts of the intestine did not reveal significant differences.

Table 1

Immunohistochemical parameters of lymphocyte subpopulation in intestinal tissues of patients with intestinal anastomoses in subgroups

Subpopulation of lymphocytes	Patients of group I			Patients of group II		
	line of resection	1-3 cm from anastomosis	7-10 cm from anastomosis	line of resection	1-3 cm from anastomosis	7-10 cm from anastomosis
CD3 ⁺	76.2 \pm 3.1	57.5 \pm 2.8*	71.6 \pm 2.6*	65.3 \pm 1.6*	58.7 \pm 3.0	51.4 \pm 2.2*•
CD3 ⁺ /CD4 ⁺	40.7 \pm 2.4*•	24.5 \pm 3.1*•	31.1 \pm 2.7*	34.8 \pm 1.5*	24.2 \pm 1.4*	23.3 \pm 1.2*•
CD3 ⁺ /CD8 ⁺	34.8 \pm 2.0	31.3 \pm 2.6	35.3 \pm 2.1*	29.0 \pm 1.3 *	28.5 \pm 1.7*	35.6 \pm 2.5*•
CD16 ⁺ /CD56 ⁺	9.2 \pm 2.1	20.5 \pm 2.2*	12.4 \pm 1.6	12.1 \pm 1.6	11.1 \pm 1.5	10.1 \pm 1.2*
CD19 ⁺	12.2 \pm 2.4	20.1 \pm 1.5*•	16.3 \pm 2.4*	17.3 \pm 2.1 *	31.8 \pm 2.0 *	24.0 \pm 1.8*
CD4 ⁺ /CD8 ⁺	1.9 \pm 0.33	0.95 \pm 0.21*	1.15 \pm 0.36	1.6 \pm 0.25*	1.14 \pm 0.26	0.97 \pm 0.13*•
CD10 ⁺	21.2 \pm 1.8	16.5 \pm 1.45*	24.1 \pm 2.0	11.5 \pm 1.3	9.5 \pm 0.88*	12.4 \pm 1.1

Note - statistically significant compared to the resection line (7-10 cm - conditionally healthy tissue): * - by Student's test, $p < 0.05$; • - by the Z-criterion, $p < 0.05$

A study was performed on the CD10 immunohistochemistry relevance for diagnosis in this clinical situation, isolating the intestinal mucosa. Uniformly positive CD10-immunostaining was detected in the normal intestinal mucosa, but variable expression loss was revealed under the conditions of intestinal anastomosis failure. In particular, CD10 staining was lost in 80% of the mucosa in intestinal anastomoses failure, usually in the presence of active inflammation. There was no expression of CD10 in normal intestinal mucosa 7-10 cm from the anastomosis. Therefore, although CD10 immunostaining identifies normal intestinal mucosa with 100% specificity, negative staining does not definitively exclude anastomotic failure.

In general, in patients with intestinal anastomoses failure, the process was characterized by a high content of tumor-infiltrating T-helper-inducer lymphocytes ($CD3^+$, $CD4^+$, $CD3^+ / CD4^+$, $CD3^+ / CD8^+$) and low levels of cytotoxic ($CD3^+$, $CD8^+$ and $CD19^+$). However, the resection line may have maintained an immune barrier due to the increased content of B-lymphocytes ($CD19^+$) compared to the intact tissue.

Cytokines are known to be the major products of immunocompetent cells. We have studied the level of tissue cytokines (IL-2, TNF- α , IL-6, IL-8, IL-10, IL-1) in the intestinal anastomosis and in the surrounding tissues (table 2).

Table 2

Immunohistochemical parameters of interleukins in intestinal tissues of patients with intestinal anastomoses in subgroups

Subpopulation of interleukines	Subgroup I (n=23)			Subgroup II (n=29)		
	line of resection	1-3 cm from anastomosis	7-10 cm from anastomosis	line of resection	1-3 cm from anastomosis	7-10 cm from anastomosis
IL-1	43.3 \pm 4.1*•	49.9 \pm 5.16*	58.5 \pm 6.4*•	270.3 \pm 25.5*•	80.29 \pm 9.95*•	100.12 \pm 13.1*
IL-2	35.7 \pm 5.1*	32.6 \pm 3.7*•	60.3 \pm 6.6*•	18.1 \pm 3.31*	7.65 \pm 1.42*	5.55 \pm 0.74*•
IL-6	21.9 \pm 4.7*	25.5 \pm 5.1	39.11 \pm 8.4*	190.9 \pm 23.1*•	16.8 \pm 1.22*•	55.21 \pm 4.6*•
IL-8	181.1 \pm 16.7*	166.2 \pm 17.7*	169.4 \pm 13.3*	520.1 \pm 19.0*•	242.5 \pm 20.4*	130.2 \pm 15.7*•
IL-10	26.6 \pm 2.5*	15.5 \pm 1.8*	17.4 \pm 1.9*	8.56 \pm 1.94	6.22 \pm 1.55*	8.24 \pm 1.75
TNF- α	7.08 \pm 1.13	5.9 \pm 1.05	5.33 \pm 0.91*	17.32 \pm 1.12	11.73 \pm 1.51*	12.07 \pm 0.65*

Note. Statistically significant for the resection line (7-10 cm - conditionally healthy tissue): * - by Student's test, $p < 0.05$; • - by the Z-criterion, $p < 0.05$

Generally, all the studied cytokines in patients with intestinal anastomoses failure had a high level, except for IL-10. Thus, the content of IL-2, IL-6, IL-8, IL-1 in tumor tissue was by 3 times higher than compared to the resection area. In the area of the resection line, the level of IL-8 was statistically reliably ($p < 0.05$) by 2 times lower compared to the area of 1-3 cm from the anastomosis (table 2).

Analysis of the distant intestinal mucosa obtained from patients with intestinal anastomoses failure, revealed the presence of the crypts in the basal parts, proliferation of Ki67 positive cells, and single cells that expressed Vim and were located among the epithelium of the crypts (fig. 1).

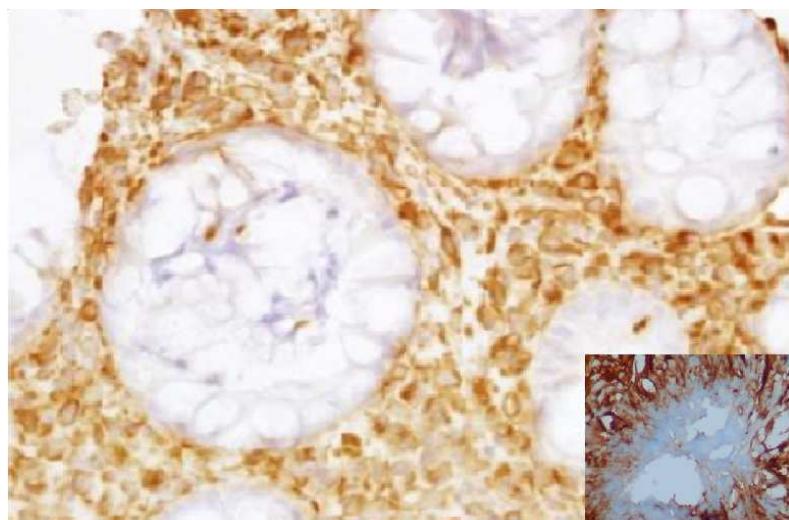


Fig. 1. Immunohistochemical expression of Vimentin in the intestinal mucosa of patients with intestinal anastomoses failure.

As a result the immunohistochemical examination of the mucous membrane revealed differences in the main and the control groups. Thus, the proliferation index for Ki67 in group II with intestinal anastomoses failure was 88.42%, in group I (control) - 0% ($p = 0.0001$) Vimentin.

When studying the initial parameters of immune status in patients of the studied subgroups, before surgery and on the first day after surgery ($p > 0.05$), reliably significant differences were revealed. It was found that in patients with complicated postoperative period (subgroup II) immunogram values were reliably ($p \leq 0.05$) lower. In the uncomplicated course of the postoperative period (subgroup I), this index is almost close to normal values ($63.47 \pm 0.68\%$), while in subgroup II by the end of treatment there was only a tendency to increase the content of $CD3^+$ in the blood ($55.53 \pm 0.65\%$, 65%).

When determining the level of CD20⁺ in the patients of both subgroups it was revealed almost twofold increase in this index compared to normal values, but in patients with uncomplicated postoperative period there was a more pronounced tendency to normalize the level of CD20⁺ in patients of subgroup I (22.6±0.29%) than in patients of subgroup II (25.67±0.37%, p < 0.05).

In addition, there was a reliable (p < 0.05) increase in the level of NK cells, which may be associated with an impairment of the intestinal mucosa barrier function and with the penetration of the intestinal flora antigens into the submucosal layer. The results of the lymphocyte subpopulations study are presented in table 3.

Table 3

**Dynamics of lymphocyte subpopulations and phagocytic activity
of neutrophils in peripheral blood samples of patients in subgroups under study**

Indices	Norm, %	Subgroup I (n=23)		Subgroup II (n=29)	
		Day 1,%	Day 5,%	Day 1,%	Day 5,%
CD3 ⁺ (T-lymphocytes)	66.2±0.5	47.12±0.91*	63.47±0.68	50.93±0.83*	55.53±0.65**
CD4 ⁺ (T-helpers)	43.9±0.8	29.12±0.61*	40.51±0.46	30.77±0.6*	33.3±0.66**
CD8 ⁺ (T-cytotoxic)	27.0±0.9	15.21±0.35*	22.11±0.5	16.2±0.3*	18.45±0.41
CD16 ⁺ (NK-cells)	13.5±0.7	19.12±0.45*	12.56±0.34	18.97±0.41*	14.73±0.32
CD20 ⁺ (B- lymphocytes)	14.0±0.2	27.4±0.27*	22.6±0.29	27.67±0.25*	25.67±0.37**
CD4 ⁺ /CD8 ⁺	1.9±0.02	1.92±0.18	1.86±0.15	1.87±0.16	1.7±0.15
Neutrophil phagocytic activity (%)	66.32±2.11	46.68±1.14*	64.2±1.65	45.23±0.89*	53.03±1.03**
Phagocytic number	5.5±0.4	2.77±0.05*	4.72±0.11	2.80±0.06*	3.85±0.1**

Note: * p < 0.05 compared to normal values, ** p < 0.05 compared to subgroup I.

In the examined patients a significant decrease in neutrophil phagocytic activity and in phagocytic number was revealed. Faster and more complete recovery of phagocytic activity (64.2±1.65%) and phagocytic number (4.72±0.11%) indices occurred in subgroup I compared to subgroup II (53.03±1.03% and 3.85±0.1%, respectively), which created the preconditions for the restoration of phagocytosis and its completion, and, consequently, to reduce the risk of complications in the postoperative period.

In patients of subgroup I there was an increase in the level of Ig A by 2 times (5.11±0.07 g / l) at a normal value of 2.5±0.08 g / l, this is due to the fact that immunoglobulins of the IgA class are "the first line of the body's defense" on the mucous membranes of the gastrointestinal tract. In the study of the level of the IgM and IgG classes immunoglobulins, no reliably (p < 0.05) significant deviations from normal values were established (table 4).

Table 4

**The level of Ig A, Ig M, Ig G immunoglobulins in peripheral blood samples
of patients in subgroups under study**

Indices	Norm, g/l	Subgroup I (n=23)		Subgroup II (n=29)	
		Before treatment, g/l	Day 5, g/l	Before treatment, g/l	Day 5, g/l
Ig A	2.5±0.08	5.11±0.07*	3.75±0.06	4.85±0.07*	4.27±0.05**
Ig M	1.51±0.05	1.18±0.05	1.30±0.04	1.22±0.05	1.25±0.04
Ig G	14.7±0.42	16.3±0.2	14.84±0.10	15.55±0.07	15.17±0.09

Note: * p < 0.05 compared to normal values, ** p < 0.05 compared to subgroup I.

It should be noted that the state of cellular and humoral immunity in patients with small intestine surgery suffers markedly, and is characterized, to a greater extent, as general immune depression in patients with anastomotic failure, which largely correlates with studies on complicated surgical pathology [7]. Data on the T-lymphocytes reduction against the background of increased duration of the pathological process and the severity of inflammation, which we observed in patients of subgroup II, significantly correlates with literature data, and necessitates further study of the impact of immunocompetent cells on the processes of small intestinal anastomoses healing [1]. Patients examined in the study showed a decrease in phagocytic activity of neutrophils, as well as in phagocytic number, in addition, the recovery of phagocytic activity and phagocytic number was slower in subgroup II, which may be associated with activation of the immune response against infectious agents, which may be factors in gastrointestinal pathologies [9].

Conclusion

Development of intestinal anastomoses failure is largely determined by the interaction of immunocompetent cells and products of their activity - cytokines. Local immunological disorders in

intestinal tissues along the line of resection cause pathogenetic initiation of anastomosis failure. The microenvironment of intestinal anastomoses according to its immunological parameters is a field that promotes further progression of inflammation or serves as a buffer zone that protects tissue from pathological changes.

Studies have shown that in patients who underwent resection of intestinal segments, secondary immune deficiency is formed, which is determined by abnormalities in the system of cellular and humoral immunity. Earlier and complete recovery of immune status occurs in patients with uncomplicated postoperative period.

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Реферати

МІКРООТОЧЕННЯ КИШКОВИХ АНАСТОМОЗІВ З ПОРІВНЯЛЬНОЮ ОЦІНКОЮ ІМУННОГО СТАТУСУ У ПАЦІЄНТІВ З АНАСТОМОЗАМИ ТОНКОЇ КИШКИ

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Метою дослідження є оцінка локального імунного статусу як маркера ризику неспроможності кишкового анастомозу. Дослідження базувалося на основі вивчення в порівняльному аспекті локальних імунних механізмів неспроможності кишкових анастомозів. Матеріалом дослідження служили тканина анастомозу, ділянки кишки на відстані 1-3 см від анастомозу а також зразки крові пацієнтів, забрані в операції, на першу і п'яту добу після формування анастомозу в підгрупах дослідження. Виникнення неспроможності кишкових анастомозів в основному визначається взаємодією імунокомпетентних клітин і продуктів їх активності - цитокинів. Імунологічні порушення в тканинах кишки в зоні анастомозу обумовлюють патогенез неспроможності. Мікрооточення кишкових анастомозів за імунологічними параметрами є полем, сприяє подальшому прогресуванню запалення або служить буферною зоною, захищає тканину від патологічних змін. Дослідження виявило формування вторинної імунної недостатності у пацієнтів з резекцією сегментів кишечника; відновлення показників імунного статусу у пацієнтів з неускладненим післяопераційним періодом відбувається в більш ранні терміни і в більш повному обсязі.

Ключові слова: Кишкові анастомози, неспроможність, імунологія.

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МІКРООКРУЖЕННЯ КИШЕЧНИХ АНАСТОМОЗОВ СО СРАВНИТЕЛЬНОЙ ОЦЕНКОЙ ИМУННОГО СТАТУСА У ПАЦИЕНТОВ С АНАСТОМОЗАМИ ТОНКОЙ КИШКИ

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Целью исследования является оценка локального иммунного статуса как маркера риска несостоятельности кишечного анастомоза. Исследование базировалось на основе изучения в сравнительном аспекте локальных иммунных механизмов несостоятельности кишечных анастомозов. Материалом исследования служили ткань анастомоза, участка кишки на расстоянии 1-3 см от анастомоза а также образцы крови пациентов, забранные в операции, на первые и пятые сутки после формирования анастомоза в подгруппах исследования. Возникновение несостоятельности кишечных анастомозов в основном определяется взаимодействием иммунокомпетентных клеток и продуктов их активности - цитокинов. Иммунологические нарушения в тканях кишки в зоне анастомоза обуславливают патогенез несостоятельности. Микроокружение кишечных анастомозов по иммунологическим параметрам является полем, способствует дальнейшему прогрессированию воспаления или служит буферной зоной, защищает ткань от патологических изменений. Исследование выявило формирования вторичной иммунной недостаточности у пациентов с резекцией сегментов кишечника; восстановление показателей иммунного статуса у пациентов с неосложненным послеоперационным периодом происходит в более ранние сроки и в более полном объеме.

Ключевые слова: Кишечные анастомозы, несостоятельность, иммунология.

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