THE NEW ARMENIAN MEDICAL JOURNAL
Vol.9 (2015), No 2, p.60-67

MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL FEATURES OF THYMUS IN THE PRESENCE OF COLON CANCER

Struchko G.Y.*, Merkulova L.M., Moskvichev E.V., Kostrova O.Y., Mikhailova M.N., Drandrova E.G.

Department of Normal and Topographic Anatomy with Operative Surgery,
Chuvash State University after I.N. Ulianov, Cheboksary, Russia

Received 10/22/2014; accepted for printing 02/28/2015

Abstract

Morphological features of the thymus of rats were studied using of entirehistochemical, luminescent-histochemical and immunohistochemical methods over 5 months after the use of a carcinogen 1,2-dimethylhydrazine.

It was found that the development of colon cancer was accompanied by the development of accidental involution of the thymus. Meanwhile, the area of thymic lobules was reduced in size along with a simultaneous reduction of both cortical and medullary substance and a growth of adipose tissue around the gland.

Introduction of carcinogen leads to an increase in the number of thymus cells expressing CD68, protein S-100 and synaptophysin, active T-lymphocytes and cells of thymopoietic microenvironment, and to a change in the ratio between the cortical and medullary $CD3^+$ thymocytes with a predominance of the latter. Increase in the number of granule cells and luminescent levels of serotonin, histamine and catecholamines was reported therein.

Thus, we suppose that an evolving tumor is capable of providing a short-term stimulatory effect on the production of T-helper cells five months later, which is combined with its toxic effect on the bone marrow and impaired supply of thymopoiesis precursors to the thymus.

Keywords: *thymus, carcinogenesis, colon, accidental involution.*

Introduction

The problem of the growth of malignant tumors is worldwide. About 10 million cases of malignant tumors are diagnosed each year. Meanwhile, about 8 million people die of cancer. The steady growth of cancer causes the need to reform the organization of cancer care. This will solve the old problem in a new, more qualitative level and to ensure that standards of newly-emerged social medical work in oncology.

According to statistics, malignancies are a major cause of adult mortality, ranking third in its morbidity pattern after diseases of the cardiovascular system, injury and poisoning [*Kulikova OM et al., 2012*]. Only in 2011 in Russia there were found more than 522,000 new cases of malignancies. The total prevalence of tumors was more than 2,000 per 100,000 in the population. The absolute number of those with the disease was 15% more in 2011 than in 2001. The share of malignant neoplasms of the digestive organs in women was 23.9%, in men – 31.7%, including colon – 7 and 5.8%, respectively. The absolute number of first-time diagnoses of malignant tumors of the colon in 2011 exceeded 33,000 for the first time, yielding in number only to tumors of the skin, breast, stomach, and respiratory organs [*Chissov VI et al., 2013*].

Despite the oncologists' best efforts to reduce the incidence of colon tumors, the problems of their early diagnosis and timely treatment are highly topical and relevant [*Egorenkov VV, 2011*].

According to the statistical reports, in Chuvash Republic the indicator of primary malignant tumors in this period amounted to 253.5 per 100,000 people, which is comparable to the nationwide indicator. In the morbidity pattern of malignant neo-

Address for Correspondence:
Moskovski prospect 45, Cheboksary 428015, Russia,
Medical faculty of I.N. Ulianov Chuvash State University
Tel.: (+7) 8352-45-26-97; (+7) 908-309-8737
E-mail: glebstr@mail.ru

plasms colon and rectum tumors in men rank 5th (7.3%), in women - 4th (6.4%).

Epidemiological and experimental studies conducted so far have concluded that the development of cancer is associated with lifestyle factors [*Zabezhenski MA, 2011*] and the human environment [*Vlasov AD, 2009*]. Among lifestyle factors significantly increasing the risk of cancer in recent years, greater attention is given to improper nutrition, physical inactivity, emotional stress, and bad habits [*Egorenkov VV, 2011; Subramanian S et al., 2011*].

It is known that an important role in the implementation of a carcinogenic effect also belongs to the immune system [*Molchanov OE, 2002; Zabezhinski MA, 2011*]. The collected data led to the development of independent scientific direction of modern oncology – immunology of malignant growth, which was formed due to intensive work of local and foreign researchers.

Immunological status of the patients depends on the type of tumor, stage of the process, and individual features [*Grinevich YA, Kamenez LY, 1986; Glyshkov AN, 1991*].

At this point, the nervous and endocrine systems are known to work closely with the immune system. A huge role in organizing the interaction between these systems belongs to thymus. Morphological features of thymus involution in the presence of malignant tumors have long been described, however, analysis of published data shows that in the presence of tumors thymus involution and associated impaired replenishment of peripheral T lymphocytes underlie the development of T-cell immunodeficiency [*Krilov AV, 2008; Kiseleva EP, 2010; Moskvichev EV et al., 2012*].

Mechanisms of thymic involution in tumor growth are still not fully elucidated [*Kostrova OY et al., 2012*]. Hormonal and cytokine hypothesis are known and some researches suppose that, thymus involution can be induced by tumor breakdown products, components of extracellular matrix, metabolic factors, and also the vascular endothelial growth factor, which is produced directly by tumor cells [*Ohm JE et al., 2003*]. According to Kiselev E.P. (2002), vascular endothelial growth factor possibly enhances thymocytes apoptosis.

In connection with the above, study of the thymus structures after carcinogen injection is very important. Comprehensive study of accidental involution of the thymus in terms of tumor development provides a new, more holistic understanding of the pathogenesis of malignant growth that will further allow developing more effective methods of prediction, monitoring and treatment of tumors.

The objective of the study is to analyze of morphological and immunohistochemical features of the thymus 5 months after carcinogen injection.

Materials and Methods

The trial included 70 white non-pedigree male rats weighing 180-220 grams. The animals were kept in a vivarium, caring for them was carried out in accordance with the "Rules of the work using experimental animals." The animals were kept in a vivarium and taken care of in accordance with the "Guidelines on Experimental Animal Care". The animals were divided into 2 groups: group I (control), comprised of 20intact rats and group II (test), comprised of 50 rats, intraperitoneally injected with the carcinogen (1,2-dimethylhydrazine) at 20 *mg/kg* 1 time a week during four weeks, in accordance with the model of R.F. Jacoby et al (1991). Withdrawal from the experiment was conducted by decapitation 5 months after the completion of carcinogen administration. The thymus was the object of the study.

During autopsy of the test group animals, their abdominal cavity was subjected to operative exploration the colon. Post mortem examination took into account the incidence of tumors, their morphological features, and localization. Animals that did not exhibit tumor formation in the study were not considered. During pathomorphological research were taken into account the incidence of tumors, their morphological features, localization. Animals that did not have tumor formation were exempted from the experiment.

The following methods were used in the study:

Immunohistochemistry [*Kumar GL, Rudbeck L, 2011*]:

a) **using monoclonal antibodies** (MCAB) (Santa Cruze Company, USA and NovoCastra, UK)

- against pan cytokeratin for non-selective identification of the epithelial cells of the thymus lobes;
- against CD3 to identify mature thymocytes;
- against CD30 for identification of activated T-lymphocytes of the cortex;
- against CD68 to identify macrophages thymic lobules;

- against IgM and IgG for identification of B lymphocytes and plasma cells in the microenvironment structures;
- against cell proliferation marker Ki-67 for identification of cells in the mitotic, G1, S and G2 phases of the cell cycle;

b) **using polyclonal antibodies** (PCAB) (Santa Cruze Company, USA and NovoCastra, UK) :

- against S-100 protein for the identification of cells of neuroectodermal histogenesis and dendritic cells;
- against synaptophysin to identify cells of neuroendocrine origin;
- against the protein p53 to identify apoptotic cells in modified structures thymic lobules;
- against bcl-2 protein to determine the orientation of apoptotic responses in structures thymic lobes;

Hematoxylin-eosin staining was used to investigate the morphology of the thymus.

Computer morphometry. Digital images of micro preparations were based on Leica DM4000B microscope (Leica Microsystems, Germany) using color camera Leica DFC 425 and Leica Application Suite 3.6.0 licensed software. Linear morphometric measurements were made with the same software;

Falk-Hillarp luminescent-histochemical method was used to detect serotonin and catecholamines in the structures of the thymus [*Falk B et al., 1962*];

Luminescent-histochemical method of Cross, Evan, and Rost was used to detect histamine in the structures of the thymus [*Cross SA et al., 1971*];

Cytospectrophotometric method to measure the levels of serotonin, histamine and catecholamine in the structures of thymus. Measurements were performed with FMEL-1A tubus, which was placed on a fluorescent microscope LUMAM-4 (Lomo PLC, Russia). Content of biogenic amines in the structures of the thymus was assessed in units of fluorescence (arbitrary units on a scale recording device) [*Kalmikov VL, 1982*].

The evaluation of statistical significance of the data was performed by Student t-test.

Results and Discussion

We found that 5 months after the introduction of the carcinogen into the colon of rats in the presence of precancerous changes in the form of crypt cell proliferation and cell dysplasia of varying degrees, one-two tumors with the morphology of highly differentiated adenocarcinoma are formed (Fig. 1).

The morphology of the rat thymus against tumor development differs significantly from that of intact animals. If normal thymus lobules have a polygonal

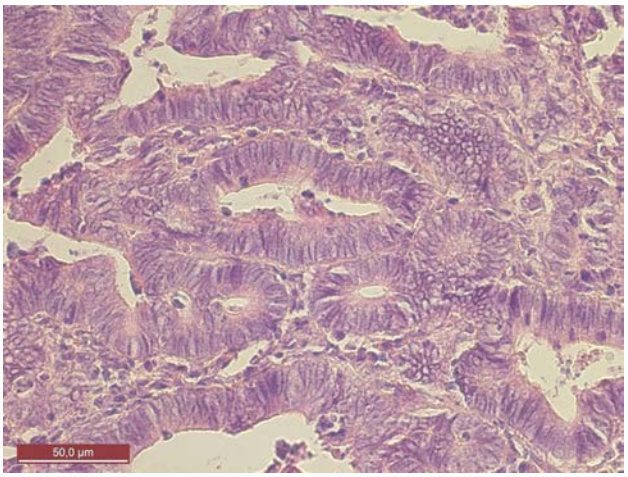

Figure 1. *Large intestine. Five months after the administration of the carcinogen. Highly differentiated adenocarcinoma of proximal segment. Hematoxylin and eosin. 400x*

shape with a highly visible boundary between the cortex and medulla, the introduction of carcinogen is accompanied by strong involutional changes, while there is a decrease in the size and formation of "multiblade" lobules of the thymus (Fig. 2a). The boundary between the slices changes at random and a "merging" of lobules with the total cortex is formed, while in the center of the organ are located large lobules with observable boundary between the cortex and the medulla, and smaller ones with poorly observable boundary on the periphery.

Fluorescent microscopy shows medulla as spindle-shaped or with an improper amoeboid shape, which is virtually free of luminescent cells. The cortical substance contains multiple bright luminescent granule cells (LGC) different in size, which are unevenly distributed from the corticomedullary zone to the periphery (Fig. 2b). LGC corticomedullary zone has irregular polygonal shape and contain large granules with a whitish-yellow or yellow-orange luminescence. The diameter of these cells was 13.4±0.9 μm (Fig. 2c). On the periphery of the cortex in the subcapsular zone smaller cells with yellowish-green glow are scattered randomly. The subcapsular cell diameter is 5.9±0.4 μm.

Cytospectrophotometric method detected elevation of serotonin, catecholamines and histamine

Table 1.

The luminescence intensity of bioamines (U) in the structures of the thymus in intact rats and 5 months after the carcinogen administration

Structures of the thymus		Control group			Test group		
		serotonin	histamine	catechol-amines	serotonin	histamine	catechol-amines
Luminescent granule cells	corticomedullary zone	235.6±10.4	280.2 ± 21	105.2 ± 5.0	595.2±25.9*	555.7±21.3*	160.1±3.8*
	subcapsular zone	127.4 ± 4.3	144.1± 7.3	50.7± 0.4	321.7±22.5*	279.6±2.8*	108.5±4.8*
Thymocytes	cortical	102.7 ± 2.7	125.7 ± 2.9	36.9 ± 1.4	159.1±2.7*	231.1±5.6*	71.4±4.5*
	medulla	60.05 ±1.1	97.6 ± 1.0	24.2 ± 0.8	104.3±3.2*	163.8±7.3*	32.2±0.5*
Mast cells		99.8 ± 2.5	223.3 ± 7.9	35.2 ± 0.5	222.4±1.6*	264.6±7.7	76.7±3.8*

Note: * $p<0.001$

in all cell structures of the thymic lobules and in the lymphocytes of their microenvironment. Especially distinct increase of histamine and serotonin content is observed in LGC corticomedullary and subcapsular zones by 2-2.5 times, which leads to an increase in their level in the thymic parenchyma by 1.7-2 times (Table 1).

In determining the morphometric parameters of the lobule a significant 29% decrease in its area is observed, while the relative thymus weight is reduced by 22%. It should be noted that unlike age-related involution, when the lobule area reduction is caused mainly by cortical atrophy, more substantial reduction both in the area of the medullary substance (37%) and cortical thickness (16%) is observed in carcinogenesis. In addition, the immunohistochemical examination revealed a significant 1.65-fold increase in the expression of $CD3^+$ thymocytes in the cortex and 14% increase of $CD3^+$ thymocytes in the medulla (Fig. 2d).

The quantity and cytoarchitectonics of the thymus epithelial cells changes significantly. Immunohistochemical reaction with pan cytokeratin reveals a more than 2 times increase in the number of epithelial cells in the cortex, and 1.86-fold increase in the medullary substance (Table 2). Tails of cortical epithelial cells anastomose with each other and form a dense network in the cells of which are located lymphocytes. Medullary epithelial cells, in contrast, form compact clusters of a large number of cells, which are located around blood vessels in the central parts of the medullary areas, but do not come into contact with cortical epithelial cells (Fig. 2e.

Immunohistochemical examination of the thymus 5 months after the carcinogen found significant increase in activated $CD30^+$ lymphocytes of the cortex, $CD68^+$ macrophages (Fig. 2f), $S\text{-}100^+$ cells of neuroectodermal histogenesis and dendritic cells as well as $synaptophysin^+$ cells of the neuroendocrine origin (Table 2).

Immunohistochemical examination of the medulla of peripheral lobules exposed to atrophy shows a significant difference of the cellular composition compared with intact rats. The bulk of the cells represented mature plasma cells, which give an intense reaction with MCAB against IgM and IgG, whose number amounts to 44% and 55% re-

Table 2.

Number of epithelial cells, expression of CD68, S-100, synaptophysin and CD30 in thymus structures of control and test groups

Indices	Control group	Test group
Cortical epithelian cells (%)	15.71±1.6	32.4±3.4**
Medullar epithelian cells (%)	16.92±1.7	31.5±2.8**
$CD68^+$ cellules (units in p/s)	48.36±3.82	69.8±3.21*
$S\text{-}100^+$ cellules (units in p/s)	34.29±5.3	66.7±4.6**
$Synaptophysin^+$ cellules (units in p/s)	22.47±3.45	50.3±6.2**
$CD30^+$ cellules (units in p/s)	20.01±3.18	34.1±4.9**

Note: * $p<0.01$, ** $p<0.001$

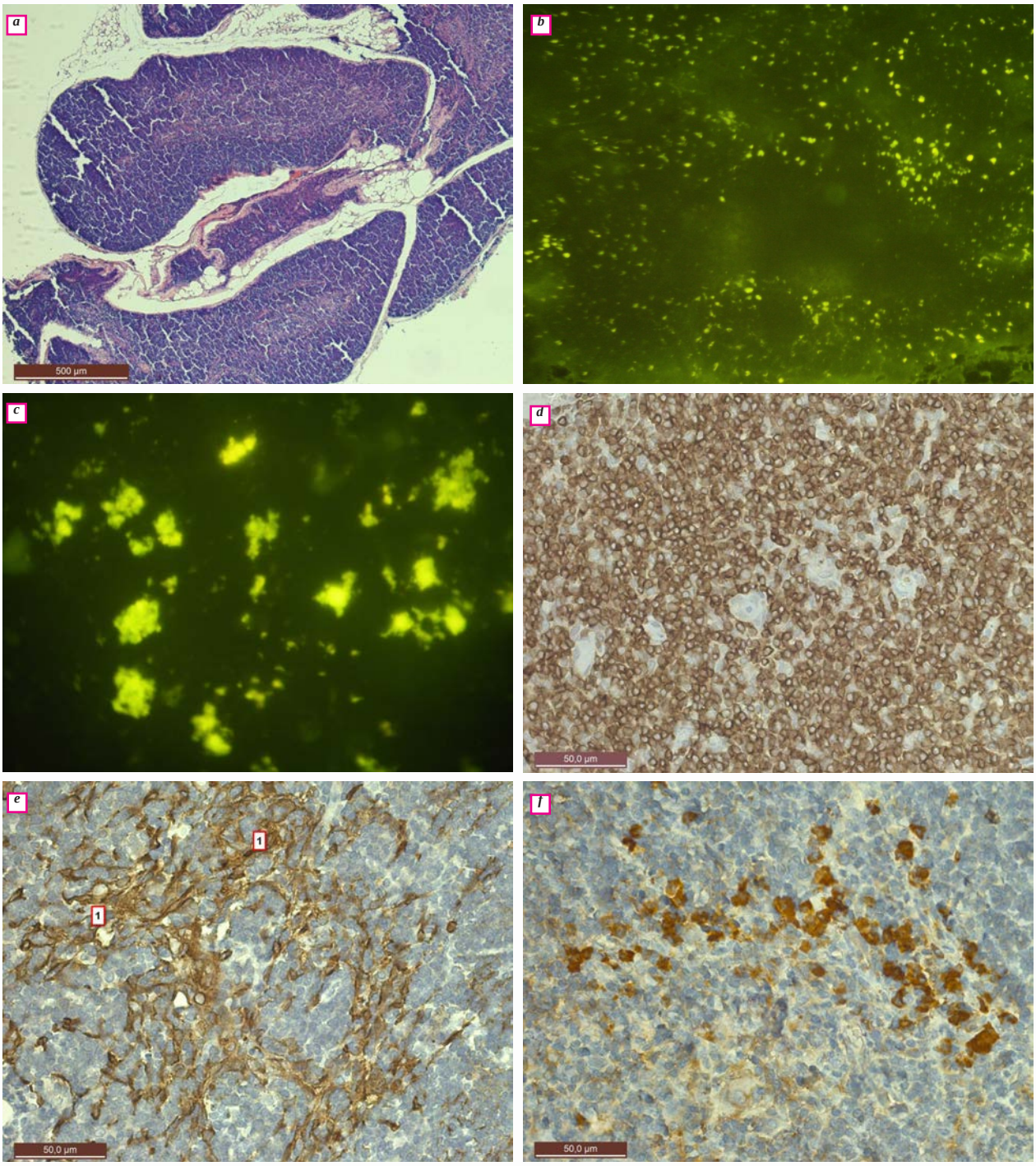

Figure 2. *Thymus. Five months after the administration of the carcinogen.*
a. *A lobule with manifestations of accidental involution (in the center). Hematoxylin and eosin, 200x*
b. *Slice of irregular shape with randomly arranged granular cells. Falk Method. 100x*
c. *Accumulation of large luminescent granule cells of the corticomedullary zone. Falk Method. 600x*
d. *High expression of CD3 in the cortical and medullary thymocytes. Immunohistochemical reaction to CD3. 400x*
e. *A dense network of cortical epithelial cells (**1**). Immunohistochemical reaction to pan cytokeratin. 400x*
f. *Increase in the number of macrophages in the corticomedullary zone of the lobule. Immunohistochemical reaction with CD 68. 400x*

spectively in the field of view. Besides the medulla lobules located on the periphery, a significant number of cells positive for IgM and IgG are present in the interlobular septs of the thymus' central part. In preserved lobules the number of cells positive for IgM and IgG was significantly higher than in intact rats by almost 5 times. When determining apoptosis regulatory proteins in the structures of the animals' thymus lobules exposed to carcinogen, 5 months later a more than 8-fold increase in bcl-2 expression was established, and a more than 25% decrease in p53 protein in both cortical thymocytes and medullar substance. The use of MCAB against cell proliferation marker Ki-67 allowed establishing increase of its expression in the cortex and medulla thymocytes by 24 and 41% respectively.

Thus, as shown in our experiments that administration of a carcinogen leads to an increase in cell number in the thymus, bcl-2 expression, S-100, and synaptophysin. As is known, anti-apoptotic bcl-2 protein is localized in immune memory cells, and its increase serves as a marker of high intensity of cell division in immune disorders [*Reed JC et al., 1987*]. P53 is also a regulator of apoptosis and oversees the state of DNA. Expression of this protein in the cell may indicate that the high intensity of apoptosis, while in malignancy transformation it indicates the presence of gene mutation. During the activation of bcl-2 in the cytoplasm it enters in a complex with p53 protein and inactivates it, thereby blocking apoptosis [*Israels LG, Israels ED, 1999*]. Furthermore, changes in the expression of apoptosis regulatory proteins in conjunction with the increased number of dendritic cells and APUD-series indicates the reaction to development of thymus tumors still at the stage of preneoplastic changes, which have been previously demonstrated [*Struchko GY et al., 2011*].

Increased expression of cell proliferation marker Ki-67, which coincides with an increase in the number of active T-lymphocytes and cells of thymopoetick microenvironment may indicate increase in thymopoiesis. We suppose that these changes are caused by the immune system as a response to the tumor growth and overproduction of T-helper type 2 required for the formation of tumor immune tolerance [*Nishimura T et al., 2000; Barishnikov AY, 2003*].

Development of colon cancer occurs due to the change in the correlation between $CD3^+$ thymocytes and cerebral cortex with predominance of the latter. Reduction in the number of mature medullary $CD3^+$ thymocytes may explain their increased output to the periphery due to tumor growth and increase in cell number due to environment changes of the thymus involution. Increased number of cortical $CD3^+$ cells may point to an enhanced thymocytes differentiation, and to reduction in the number of immature $CD3^-$ forms due to insufficient influx from the bone marrow.

The increase in LGC and the level of biogenic amines in them may be the consequence of the increase in the number of dendritic cells that secrete IL-1, IL-6 and TNF-α [*Leplina OY, 2011*]. It is known that IL-1 and IL-2 can reach the hypothalamus, and, along with catecholamines, stimulate the synthesis of corticotropin-releasing factor. Glucocorticoids, in turn, reduce the degree of pathological process as suppressors by inhibiting production of this factor by hypothalamus [*Semenkov VF et al., 2011*].

Great value in the ability of the thymus to produce lymphocytes belongs to the epithelial network of the cortex. It is known that epithelial cells produce thymic hormones, including thymulin and thymopoietin, which are necessary for the TCR receptors rearrangements in the thymocytes differentiation process. [*Sempowski GD et al., 2002*]. We found that involutive changes after exposure to carcinogen do not lead to disruption of cortical epithelial cells of the network; however, they are accompanied by a significant increase in their number. There is an opinion that keeping the network of epithelial cells intact is a prerequisite for proper thymopoiesis in the process of involution [*Flores KG et al., 1999*].

Thus, we believe that 5 months later a progressing tumor is able to provide a short-term stimulatory effect on the production of T-helper cells, which is combined with its toxic effects on the bone marrow and impaired delivery of thymopoiesis precursors to the thymus. On the one hand, progressing tumor inhibits delivery of thymopoiesis precursors to the gland, and on the other it enhances the proliferation and differentiation of thymocytes into mature forms.

REFERENCES

1. *Barishnikov AY*. [Interaction of the tumor and the immune system] [Published in Russian]. Practical Oncology. 2003; 4(3): 127-130.
2. *Chissov VI, Starinski VV, Petrova GV*. [Malignancies in Russia in 2011 (morbidity and mortality)] [Published in Russian]. Moscow: P. Herzen MORI, Russian Ministry of Health. 2013. 289 p.
3. *Cross SA, Ewen SW, Rost FW*. A study of methods available for cyto-chemical localization of histamine by fluorescence induced with o-phtaldehyde or acetaldehyde. Histochem J. 1971; 3(6): 471-476.
4. *Egorenkov VV*. [Prevention of gastric cancer and colon] [Published in Russian]. Practical oncology. 2011; 12(2): 70-75.
5. *Falk B, Hillarp N, Thieme G, Torp A*. Fluorescence of catecholamines and related compounds condensed with formaldehyde. J Histochem. Cytochem. 1962; 10: 348-354.
6. *Flores KG, Li J, Sempowski GD., et al*. Analysis of the human thymic perivascular space during aging. J Clin Invest. 1999; 104 (8): 1031-1039.
7. *Kumar GL, Rudbeck L*. Immunohistochemistry: Guide. M.: 2011. 224 p.
8. *Glushkov AN*. [Autonomous behavior of tumor immunology] [Published in Russian]. Immunology. 1991; 5: 11-14.
9. *Grinevich YA, Kamenez LY*. [Basics of clinical tumor immunology] [Published in Russian]. Kiev: Health. 1986. 160 p.
10. *Israels LG, Israels ED*. Apoptosis. The Oncologist. 1999; 4(4): 332-339.
11. *Jacoby RF, Lior X, Teng BB., et al*. Mutations in the K-ras oncogene induced by 1,2-dimethylhydrazine in preneoplastic and neoplastic rat colonic mucosa. J Clin Invest 1991; 87(2): 624-630
12. *Kalmikov VL*. [Modern methods for the quantitative determination of catecholamines and serotonin] [Published in Russian]. Lab part. 1982; 7: 31-36.
13. *Kiseleva EP*. [The role of neuronal and angiogenic factors in the mechanisms of thymic involution in tumor growth] [Published in Russian]. Med Ac Jour. 2010; 10(4): 201-209.
14. *Kiseleva EP*. [Mechanisms of thymus involution and activation of mononuclear phagocytes during the growth of experimental tumors] [Published in Russian]. dissertation abstract of doctor of medical science. Saint-Petersburg. 2002. 38 p.
15. *Kostrova OY, Mihailova MN, Struchko GY, Merkulova LM, Bessonova KV., et al*. [Accidental involution of the thymus of rat on the background of adenocarcinoma of the colon induced by 1,2-dimethylhydrazine amid splenectomy] [Published in Russian]. Vestnik of The Chuvash State University. 2012; 3: 416-423.
16. *Krilov AV*. [Gene expression of vascular endothelial growth factor and thrombospondin - 1 in thymus cells and mouse peritoneal macrophages in tumor growth] [Published in Russian]. dissertation abstract of candidate of biological sciences. Saint-Petersburg. 2008. 21 p.
17. *Kulikova OM. Lyuboshenko TM, Fomenko AA*. [Prediction of cancer incidence in the regions of the Russian Federation] [Published in Russian]. Electronic scientific journal: Modern Problems of Science and Education. 2012; 3.
18. *Leplina OY*. [Characterization of interferon-alpha-induced dendritic cells and their therapeutic potential in the treatment of cancer and infectious diseases] [Published in Russian]. dissertation abstract of doctor of medical science. Novosibirsk. 2011. 39 p.
19. *Molchanov OE*. [Cytokine therapy of malignant tumors with interleukin-2] [Published in Russian]. Benefit for doctors. Saint-Petersburg, Ed.: «Clear light». 2002. 38 p.
20. *Moskvichev EV, Merkulova LM, Struchko GY*. Immunohistochemical characterization of the cellular proliferation and apoptosis in the thymus in experimental colon tumors. Immunology. 2012; 33(6): 303-305.

21. *Nishimura T, Nakui M, Sato M, Iwakabe K, Kitamura H., et al*. The critical role of Th1-dominant immunity in tumor immunology. Cancer Chemother Pharmacol. 2000; 46: 52-61.

22. *Ohm JE*. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. Blood. 2003; 101(12): 4878-4886.

23. *Reed JC, Tsujimoto Y, Alpers JD., et al*. Regulation of bcl-2 proto-oncogene expression during normal human lymphocyte proliferation. Science. 1987; 236: 1295-1299.

24. *Semenkov VF, Karandashov VI, Mihailova TA*. [Stress and human aging] [Published in Russian]. Vestnik of the Russian Academy of Natural Sciences. 2011; 4: 72-78.

25. *Sempowski GD, Rhein ME, Scearce RM, Haynes BF*. Leukemia inhibitory factor is a mediator of Escherichia coli lipopolysaccharide-induced acute thymic atrophy. Eur J Immunol. 2002; 32: 3066-3070.

26. *Struchko GY, Merkulova LM, Moskvichev EV.* [Morphological and immunohistochemical features of gastrointestinal tumors on a background of immune deficiency] [Published in Russian]. Vestnik of The Chuvash State University. 2011; 3: 450-455.

27. *Subramanian S, Velizheeva P, Samulenko AN*. [Prevention of lung cancer] [Published in Russian]. Practical oncology. 2011; 12(2): 90-96.

28. *Vlasov AD*. [Anthropogenic carcinogenesis as a major cause of cancer morbidity] [Published in Russian]. Proceedings of the University of Kazan. Series of natural sciences. 2009; 154(3): 247-254.

29. *Zabezinskii MA*. [Principles of primary prevention of cancer] [Published in Russian]. Practical oncology. 2011; 12(2): 57-61.