THE LEVEL OF PLATELET AGGREGATION INDUCED BY AN ARACHIDONIC ACID AND METASTASIS IN MICE WITH CARCINOMA 3LL

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УРОВЕНЬ АГРЕГАЦИИ ТРОМБОЦИТОВ, ИНДУЦИРОВАННОЙ АРАХИДОНОВОЙ КИСЛОТОЙ, И ПОКАЗАТЕЛИ МЕТАСТАЗИРОВАНИЯ У МЫШЕЙ С КАРЦИНОМОЙ 3LL

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The research of the metastatic level in mice with pulmonary carcinoma (3LL) upon administration of inhibitors of the haemostasis after resection of primary tumor is carried out. In the groups of animals different preparations were introduced: 1) heparin (during 6 days after surgery); 2) calcium nadroparin (CN) (before operation and during 6 days after it); 3) CN as in group 2 + ticlopidine during 7-th–21-th days. For monitoring of the levels of tumor dissemination the volumes of pulmonary metastasis (VPM) and level of the VEGF were determined. For evaluation of platelet activity level, the level of platelet aggregation, induced by arachidonic acid (PAIAA) was used. It has been shown that the application of antihaemostatic preparations contributes to the decrease in the level of tumor dissemination especially if CN in combination with ticlopidine are applied. Also, the slowing of metastasis was shown to correlate with inhibition of platelet aggregating function upon introduction of antiaggregants.

***Key Words*: platelet aggregation, arachidonic acid, vascular endothelial growth factor, pulmonary metastasis, heparin, calcium nadroparin, ticlopidine.**

Проведено исследование уровня метастазирования при действии ингибиторов системы гемостаза у мышей с карциномой легких 3LL после удаления первичной опухоли. Для снижения активности гемостатических реакций животным из разных групп вводили: 1) гепарин в течение 6 сут после операции; 2) надропарин кальция до операции и в течение 6 сут после нее; 3) надропарин кальция по вышеприведенной схеме и дополнительно — с 7-х по 21-е сут — тиклопидин. Для мониторинга уровня диссеминации опухолевого процесса определяли объем метастазов в легких (ОМЛ) и количество VEGF. Для исследования тромбоцитарной активности использовали метод определения агрегации тромбоцитов, индуцированной арахидоновой кислотой (АТИАК). Установлено, что применение антигемостатических препаратов способствует снижению уровня диссеминации опухолевого процесса, наблюдаемого в наибольшей степени при введении надропарина кальция в комплексе с тиклопидином. Также выявлено, что снижение метастазирования коррелирует с угнетением агрегационной функции тромбоцитов при действии антиагрегантов.

***Ключевые слова*: метастазирование, ангиогенный фактор, агрегация тромбоцитов, арахидоновая кислота, гепарин, надропарин кальция, тиклопидин.**

The tumor cells that circulate in blood vessels secrete proaggregants and procoagulants. Interacting between themselves and activated platelets, fibrin filaments, lymphocytes and polymorphonuclear leucocytes [1, 2], tumor cells form thromboongenic emboli that adhese to vessel intima. Those events promote tumor cell implantation and strongly influence the frequency of metastasis. Also, angiogenesis in primary and secondary tumor occurs with active participation of fibrin serving as a matrix for endothelial cells [3]. In the tumors and in blood plasma of the patients with cancer of lungs, ovaries, breast and bones, the increased level of thromboxan A_2 and decreased content of prostacyclin I_2 linked to increased aggregating capacity of platelets was reported [4–7].

Experimental studies have shown that the introduction of inhibitors of platelet aggregation decreases tumor cell adhesion to subendothelial matrix *in vivo* [4, 5]. The majority of antiaggregants (nonsteroid anti–inflam–

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Abbreviations used: CN — calcium nadroparin; 3LL — Lewis lung carcinoma; LMWH — low molecular weight heparin; PAIAA — platelet aggregation, induced by arachidonic acid; Tp — ticlopidine; VEGF — vascular endothelial growth factor; VPM — volume of pulmonary metastasis; UFH — unfractionated heparin.

matory agents) blocks cyclooxigenase way of platelet activation. However, significant decrease of metabolic products of arachidonic acid may result in destabilization of haemostasis and immunity and cause some side effects [1, 6]. The search for harmless ways of inhibition of platelet function resulted in the production of antiaggregant of new generation — ticlopidine (Tp). Tp acts as competitory inhibitor of receptors of platelet plasmalemma that became unable to contact with ADP and fibrinogen [8–10]. Among known antiaggregants, Tp has the lowest number of side effects.

For decrease the level of fibrinogenesis in cancer it is supposed reasonable to apply anticoagulants of direct action [1, 11–13]. Heparins influence coagulant and platelet haemostasis in different ways. Low molecular weight heparin (LMWH) decreases the level of blood coagulation nearly as the unfractionated heparin (UFH) does, but has lower level of interaction with platelets and doesn't cause heparin–induced thrombocytopenia.

The present study was aimed on *in vivo* evaluation of efficacy of antiaggregant therapy for slowing tumor progression.

MATERIALS AND METHODS

In the study, C57Bl/6 mice weighting 20–25 g, bred in the vivarium of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine) were used. As experimental model, Lewis lung carcimoma (3LL) was chosen. Tumor transplantation was performed by injection of 0.01–002 ml of tumor cell suspension ($2 \cdot 10^5$ cells) in pedical area. At 18–th day after transplantation when the primary tumor reached the volume 0.8–1.0 cm^3, the foot with tumor was resected under ether narcosis. Animals were housed in 5 groups: 1) intact animals whose indexes were used for estimation of normal PAIAA values (control 1); 2) animals that received surgery only (control 2); 3) animals after surgery that received UFG subcutaneously (10 MO daily) during 6 days; 4) animals received subcutaneous injection of 3 MO of calcium nadroparine in 0.01 ml of physiologic solution of NaCl (CN — Sanofi–Synthelabo, France) 2 h before operation and the same injection daily during 6 days after surgery; 5) animal received the same treatment as those from group 4 + intramuscular Tp (Sanofi–Synthelabo, France) injections from day 7 till day 21 after surgery (Tp, 0.8 mg/0.3 ml physiologic saline solution daily). In special terms (before operation, on days 3, 7, 14 and 21 after surgery) animals were killed using ether narcosis and the blood samples were collected.

The level of metastasis was evaluated by the calculation of the volume of each lung metastasis by formula:

$V=4/3\ \pi R^3$,

where V — the volume of metastasis, R — its radius.

The content of VEGF was determined by immunoenzyme method with the use of polyclonal anti–VEGF–antibodies according to [14].

For study of platelet aggregation the blood samples were stabilized by addition of 3.8% three–substituted sodium citrate. For determination of PAIAA the method described in [15] was applied. The photometric registration of platelet aggregation in citrate–containing plasma was carried out on aggregometer "Thromlite" (Russia) and with the use of arachidonic acid ("Sigma", USA).

The calculation of indexes was reformed with the use of methods for variation statistics [16, 17].

RESULTS AND DISCUSSION

The alterations of the volume of pulmonary metastasis (VPM) in the studied groups of animals are presented in Table 1. On the day 18 after tumor transplantation (before operation) the lung metastasis were yet not detecteble visually. On the day 3 after surgery metastasis became visible. The differences in VPM values between the groups are presented on Table 2 and yield up to 23.6% between groups 2 and 3, 21.0% — between groups 2 and 4, 16.4% — between groups 2 and 5. The decrease of metastatic level in groups 3–5 in comparison with the control group may be caused by the administration of anticoagulants. On the day 7, 14 and 21 after surgery the gradual increase in metastasis was registered; the differences in VPM between the control and experimental groups 3–5, respectively, were 19.5%, 41.0%, 51.8% for at the day 7; 16.0%, 31.0%, 57.14% at the day 14; 24.8%, 39.8%, 57.5% at the day 21. So, the most pronounced decrease in metastasis was achieved in the group of animals treated with LMWH and Tp.

The results of determination of VEGF level in the sera of experimental animals are presented in Table 3. Before the surgery the level of VEGF was nearly equal in all groups and decreased by a factor 7–8 at the 3–rd day after surgery. The differences between the indexes of the control and experimental groups (3, 4, 5 respectively) were: on the 3–rd day after surgery — 27.5 %, 10.4%, 11.0%; at the 7^{th} day — 21.3%, 21.8%, 21.4%; at the 14–th day — 16.4%, 23.2%, 36.5%; at the 21–th day — 18.1%, 48.5%, 61.5% (Table 4). The data presented pointed to the significant decrease of VEGF synthesis by tumor cells in the groups of animals treated with CN and CN +Tp.

The alteration of PAIAA indexes are presented in Table 5. Before surgery, in animals from groups 2–5 the moderate increase in PAIAA level in comparison with group 1 was recorded, at the day 3 after surgery — its decrease, at the 7^{th} day — its elevation. Those data may be possibly explained by the renewal of haemostasis, tumor progression and, in the group 3, — by platelet activation by UFH. Later (at 14^{th}–21–th day after surgery) the PAIAA level remained unaltered in the group 3, lightly decreased in the group 4, and significantly decreased in the group 5. The differences in PAIAA indexes between control and experimental groups were nonsignificant before operation ($p > 0.05$) as well as at the 3^{rd} day after surgery. At the day 7 after surgery PAIAA values in the group 3 were lightly higher than those in group 2 (by 10.1%, $p > 0{,}05$), at the 14th day — lower ($p > 005$); at the 21–th day the differences in PAIAA values between groups 3 and 2 were 28.62%, ($p > 0.001$) (Table 6). The differences in PAIAA values between groups 4 and 2 became significant only at 21–th day after surgery and were 29.1% ($p < 0.001$).

The differences in PAIAA values between groups 5 and 2 were significant starting from the day 14–th after surgery (by 47.2%, $p < 0.001$) and increased at day 21–th up to 69.0% (see Table 6).

PAIAA alterations may be due to the decrease in tumor progression and also by the lack of additional activation of platelets by LMWH. The decrease of PAIAA levels in the group 3 may be caused by Tp application. It is known that Tp blocks platelet activation by "weak" inductors and significantly decreases conformational alterations of platelet plasmalemma; as a consequence, the activation of phospholipase A2 is suppressed and cyclooxigenase cycle is strongly influenced. The application of arachidonic acid as inducer of platelet aggregation allows to suppose that the blocking of aggregation on the stage of the binding of inducer to the membrane receptors strongly influences functional activity of the platelets. The decrease of platelet activity and retention between tumor cells inhibit the formation of thromboncogenic emboli.

The high correlation indexes between VPM and VEGF levels (Table 7) and VPM and PAIAA levels (Table 8) in the control and experimental groups of animals were revealed. Those results showed the dependence between aggregation function of platelets and metastasis volume in the progression and dissemination of 3LL carcinoma upon applied treatment schedule. The similar correlation was observed between VEGF and PAIAA indexes and was linear in groups 3, 4, and 2. The Tp administration resulted in significant decrease of the functional activity of platelets and moderate but stable increase of VPM and VEGF levels; so, the inversed correlation between platelet activity and indexes of tumor progression was found.

So, all applied schemes were found to be effective and decrease the level of tumor progression. The most

Table 1. Pulmonary metastasis volumes in experimental groups of animals, mm^3

Group	Terms of observation								
	Before surgery	*p*	3rd day	*P*	7th day	*P*	14th day	*p*	21th day
3 (n = 15)	0	< 0.001	14.9 ± 0.2	< 0.001	46.8 ± 0.4	< 0.001	70.6 ± 0.2	< 0.001	95.1 ± 0.3
4 (n = 15)	0	< 0.001	15.3 ± 0.1	< 0.001	34.3 ± 0.1	< 0.001	58.0 ± 0.2	< 0.001	72.2 ± 0.1
5 (n = 15)	0	< 0.001	16.3.00 ± 0.16	< 0.001	28.00 ± 0.23	< 0.001	36.0 ± 0.2	< 0.001	51.0 ± 0.1
2 (n = 10)	0	< 0.001	19.5 ± 0.1	< 0.001	58.1 ± 0.2	< 0.001	84.0 ± 0.3	< 0.001	120.0 ± 0.2

Table 2. The dynamics of the differences between VPM indexes in the experimental groups of animals

Differences between the groups	Terms of observation				
	Before surgery	3rd day after surgery	7th day after surgery	14th day after surgery	21th day after surgery
2 and 3	0	4.6	11.3	13.4	24.9
2 and 4	0	4.1	23.8	26	47.8
2 and 5	0	3.2	30.1	48	69

Table 3. Alterations of VEGF level in the blood sera of experimental animals, ng/ml of the serum

Group	Terms of observation								
	Before surgery	*p*	3rd day	*p*	7th day	*P*	14th day	*P*	21th day
3 (n = 10)	167.01 ± 0.004	< 0.001	21.03 ± 0.01	< 0.001	29.04 ± 0.02	< 0.001	35.98 ± 0.01	< 0.001	81.11 ± 0.11
4 (n = 10)	164.03 ± 0.015	< 0.001	26.01 ± 0.01	< 0.001	28.87 ± 0.02	< 0.001	33.05 ± 0.1	< 0.001	51.02 ± 0.23
5 (n = 15)	162.30 ± 1.6	< 0.001	25.82 ± 0.01	< 0.001	29.00 ± 0.02	< 0.001	27.30 ± 0.1	< 0.001	38.10 ± 0.21
2 (n = 10)	171.00 ± 0.014	< 0.001	29.02 ± 0.01	< 0.001	35.91 ± 0.02	< 0.001	43.02 ± 0.01	< 0.001	99.00 ± 0.01

Table 4. The dynamics of the differences between VEGF levels in the experimental groups of animals

Differences between the groups	Terms of observation				
	Before surgery	3rd day after surgery	7th day after surgery	14th day after surgery	21th day after surgery
2 and 3	3.99	7.99	7.87	8.03	17.89
2 and 4	6.97	3.01	8.04	9.97	47.98
2 and 5	8.7	3.2	7.91	10	60.90

Table 5. Alterations of platelet aggregation induced by arachidonic acid in the groups of experimental animals, absolute units (1 a.u. = 1% of aggregated platelets), (for group 1, normal value is 34.2 — 35.4%)

Group	Terms of observation								
	Before surgery	*p*	3rd day	*p*	7th day	*P*	14th day	*P*	21th day
3	35.1 ± 0.98	< 0.001	28.4 ± 1.35	< 0.001	39.4 ± 1.72	> 0.05	40.5 ± 1.57	> 0.05	42.4 ± 0.86
4 (n = 10)	36.7 ± 1.35	< 0.001	26.4 ± 0.74	< 0.001	32.7 ± 0.92	< 0.05	38.1 ± 2.11	> 0.05	42.1 ± 1.96
5 (n = 15)	34.9 ± 1.07	< 0.02	27.3 ± 2.53	> 0.05	32.4 ± 1.84	< 0.001	22.5 ± 1.23	> 0.05	18.4 ± 1.62
2 (n = 10)	35.2 ± 1.42	< 0.002	29.2 ± 1.42	< 0.002	35.8 ± 1.85	< 0.001	42.6 ± 1.26	< 0.001	59.4 ± 1.06

Table 6. The dynamics of the differences between PAIAA indeces in the experimental groups of animals

Differences between the groups:	Terms of observation				
	Before surgery	3rd day after surgery	7th day after surgery	14th day after surgery	21th day after surgery
2 and 3	0.1	0.8	–3.6	2.1	17
2 and 4	–1.5	2.8	3.1	4.5	17.3
2 and 5	0.3	1.9	3.4	20.1	41

Table 7. Correlation indexes between VPM and VEGF level in experimental groups of animals

Group	Correlation indeces, r
3	0.89
4	0.88
5	0.99
2	0.88

Table 8. Correlation indexes between PAIAA and VPM values in experimental animals

Group	VPM	VEGF
3	0.91	0.66
4	1.00	0.87
5	–0.76	–0.82
2	0.97	0.97

pronounced antimetastasis effect was revealed by combined application of CN + Tp.

In conclusion, after the resection of the primary tumor, the inhibition of the primary and secondary haemostasis is accompanied by the decrease of tumor dissemination. The proaggregant activity of unfractionated heparin is decreasing the antimetastasis efficacy of its application. The minimal activating influence of LMWH on platelets along with its anticoagulant action promotes its antitumor action. The sequential application of CN and Tp has the best results in inhibition of 3LL carcinoma cells dissemination. Possibly, the decrease of metastasis level may depend on nondirect action of anticoagulants toward tumor angiogenesis.

REFERENCES

1. **Baluda VP, Baluda MV, Tlepshukov IK, Tsyb AF.** Cancer and thrombosis. Baluda VP, ed. Moskow—Obninsk 2001. 153 p (In Russian).

2. **Gasic GJ.** Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metastasis Rev 1984; **3**: 99–114.

3. **Olander JV, Bremer ME, Marasa JC, Feder J.** Fibrin enhanced endothelial cell organization. J Cell Physiol 1985; **125**: 1–9.

4. **Schwalke MA, Tzanakakis GN, Vezeridis MP.** Effects of prostacyclin on hepatic metastases from human pancreatic cancer in the nude mouse. J Surg Res 1990; **49**: 164–7.

5. **Honn KV, Tang DG, Chen YQ.** Platelets and cancer metastasis: more than epiphenomenon. Semin Thromb Hemost 1992; **18**: 392–415.

6. **Barkagan ZS, Momot AP.** Diagnosis and controlled therapy of disturbancies in haemostasis. Moskow: NewaMed, 2001. 296 p (In Russian).

7. **Heinonen PK, Metsa-Ketela T.** Prostaglandin and thromboxane production in ovarian cancer tissue. Gynecol Obstet Invest 1984; **18**: 225–9.

8. **Schor K.** Antiplatelet drugs. A comparative review. Drugs 1995; **50**: 7–28.

9. **Nobble S, Goa KL.** Ticlopidine. A review of its pharmacology, clinical efficacy and tolerability in the prevention of cerebral ischaemia and stroke. Drugs 1996; **8**: 214–32.

10. **Herbert JM, Frehel D, Vallee E, Kieffer G, Gouy Y.** Clopidogrel, a novel antiplatelet and antithrombotic agent. Cardiovasc Drug Rev 1993; **2**: 180–98.

11. **Letai A, Kuter DJ.** Cancer, coagulation and anticoagulation. Oncologist 1999; **4**: 443–9.

12. **Barkagan ZS.** Pathogenesis and therapy of disturbancies of haemostasis in cancer patients. Ther Arkh 1997; **7**: 65–7 (In Russian).

13. **Gulyaev DV.** Thrombosis in nonsurgical patients with malignancies: prophylaxis and therapy. Oncology 2001; **3**; 113–8 (In Russian).

14. **Alistratov AV, Lisniyak IA, Yatsenko SM, Vinnitsky VB.** The dependence of the level of VEGF from characteristics of 3LL carcinoma development in C57BL6 mice. Exp Oncol 2002; **24**: 64–8 (In Russian).

15. **Ivanov EP.** Diagnosis of the disturbancies of haemostasis. Minsk: Byelorus, 1983. 222 p (In Russian).

16. **Baily N. Statistics in biology.** Moscow: Izd Inostr Lit, 1962. 260 p (In Russian).

17. **Lapach SN, Chubenko AV, Babich PN.** Methods of statistics in medical and biological studies with the use of Excel. Kyiv: Morion, 2000. 320 p (In Russian).