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ORIGINAL ARTICLE

Units of fibrinolytic system in mice with urokinase gene knockout in presence of growing B16/F10 melanoma

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ABSTRACT

Purpose of the study. Was to reveal the effect of urokinase gene knockout in male and female mice with transplanted B16/F10 melanoma on the functions of the fibrinolytic system units.

Materials and methods. Male and female mice were used: main group with genetically modified mice C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); control group with C57Bl/6 (uPA+/+) mice. B16/F10 melanoma was transplanted by the standard methods to the animals, and levels of plasminogen (PG), plasmin (PAP), urokinase receptor uPAR, content (AG) and activity (act) of uPA, t-PA and PAI-I were measured with ELISA (Cussabio, China) in 10 % tumor homogenates and peritumoral area after 3 weeks of tumor growth.

Results. The activity and levels of urokinase in intact uPA-/- animals were significantly (by 100–860 times) inhibited, compared to uPA+/+, but uPAR levels were unchanged in females and were 1.9 times lower in males. PAP levels in uPA-/- mice were 2.1–4.2 times higher than in uPA+/+ animals. The growth of B16/F10 melanoma in uPA-/- mice was slower and metastasizing was suppressed, but their survival was not improved. The dynamics of changes in components of the fibrinolytic system in presence of melanoma growth differed in uPA-/- mice, compared to uPA+/+ animals: PAP levels in tumor samples decreased by over 2 times, uPA levels and activity were not increased, PAI was practically unchanged, but activity of t-PA elevated by 3.8–8.2 times, as well as in uPA+/+ mice.

Conclusion. Despite the suppression of the growth and metastasis of the primary tumor nodes in uPA-/- mice, their average survival was not improved, which indicates that the mechanisms of tumor are complex and there are alternative biological pathways supporting melanoma to survive in conditions of the urokinase gene knockout.

Keywords: urokinase gene knockout, mice, melanoma B16/F10, fibrinolytic system

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Compliance with ethical standards: the work with animals was carried out in compliance with the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Directive 86/609/EEC) and the Helsinki Declaration, as well as in compliance with the International Guiding Principles for Biomedical Research Involving Animals, and Order No. 267 of the Ministry of Health of the Russian Federation dated 06/19/2003 "On approval of the rules for laboratory practice". The Bioethics Commission of the National Medical Research Center of Oncology dated 12/24/2019, approved the research protocol (Protocol of the Ethical Committee No. 15/75) on working with Balb/c Nude mice

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ОРИГИНАЛЬНАЯ СТАТЬЯ

Звенья фибринолитической системы у мышей с нокаутом по гену урокиназы на фоне роста меланомы B16/F10

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РЕЗЮМЕ

Цель исследования. Изучение влияния нокаута по гену урокиназы у мышей обоего пола с перевитой меланомой B16/F10 на функционирование звеньев фибринолитической системы.

Материалы и методы. Были использованы мыши обоего пола: основная группа генмодифицированная линия C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); группа контроля – линия C57Bl/6 (uPA+/+). Животным по стандартной методике перевивали меланому B16/F10 и через 3 недели роста в 10 % гомогенатах опухоли и ее перифокальной зоне ИФА методом определяли уровень: плазминогена (ПГ), плазмина (PAP), рецептора урокиназы uPAR, содержание (АГ) и активность (акт) uPA, t-PA и PAI-I (Cussabio, Китай).

Результаты. У интактных животных uPA-/- в коже оказалась существенно подавлена, по сравнению с uPA+/+ активность и содержание урокиназы (в 100–860 раз), однако у самок не изменился уровень uPAR, тогда как у самцов снизился в 1,9 раза. Уровень плазмина у uPA-/- мышей был выше в 2,1–4,2 раза, по сравнению с uPA+/+ животными. Рост меланомы B16/F10 у uPA-/- мышей был замедлен, тормозилось метастазирование, однако не увеличивалась продолжительность жизни. Динамика изменений компонентов фибринолитической системы при росте меланомы у uPA-/- мышей отличалась от uPA+/+: в образцах опухоли снижался уровень PAP более чем в 2 раза, не повышался уровень и активность uPA, практически не реагировала PAI, однако, как и у uPA+/+ возрастала активность t-PA в 3,8–8,2 раза.

Заключение. Несмотря на подавление роста первичного узла опухоли и процессов метастазирования у мышей uPA-/-, средняя продолжительность жизни не увеличивалась, что свидетельствует о сложных механизмах опухолевой болезни и наличии альтернативных биологических путей, позволяющих меланоме прогрессировать в условиях нокаута гена урокиназы.

Ключевые слова: нокаут по гену урокиназы, мыши, меланома B16/F10, фибринолитическая система

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Соблюдение этических стандартов: работа с животными проводилась в соответствии с правилами «Европейской конвенции о защите животных, используемых в экспериментах» (Директива 86/609/EEC) и Хельсинкской декларации, а также в соответствии с «Международными рекомендациями по проведению медико-биологических исследований с использованием животных» и приказом Минздрава России от 19.06.2003 г. № 267 «Об утверждении правил лабораторной практики». Комиссией по биоэтике ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации от 24.12.2019 г., был одобрен протокол исследования (протокол этического комитета № 15/75) по работе с мышами линии Balb/c Nude

Финансирование: финансирование данной работы не проводилось

Конфликт интересов: все авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи

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INTRODUCTION

The fibrinolytic system is considered one of the leading mechanisms of carcinogenesis, due to the destruction of cell membranes, proliferation, migration and invasion of cells [1].

Urokinase-type (uPA) and tissue-type (t-PA) plasminogen activators are serine proteases that convert plasminogen into plasmin after binding to the uPA receptor (uPAR) [2]. uPA is found on the surface of tumor cells, and its overexpression at the final stage of transformation of malignant cells contributes to the processes of metastasis [3]. The activation of the fibrinolytic system and the formation of plasmin stimulates metalloproteinases, vascular growth factors, this in turn consequently destroys the physical barrier to the migration of tumor cells and stimulates tumor growth [4].

Several researchers believe that understanding the molecular mechanisms of the biological action of the plasmin/plasminogen system and inhibition of angiogenesis by blocking serine proteases may allow improving therapeutic strategies for regulating the growth of malignant tumors and disorders associated with neovascularization [5, 6].

It has been previously shown that changes in the links of the fibrinolytic system of the skin occur in the growth dynamics of B16/F10 transfused melanoma in C57Bl/6 mice with wild type genes, characterized by increased activity of all components of the plasminogen activation system, subsequently leading to an increased content of plasmin in it. The comorbid disease, i. e. chronic neurogenic pain, has a modifying effect on the studied indicators [7–9].

Experimental models of tumors make it possible to find out the causes, study the pathogenesis of the tumor process, develop methods for its prevention and treatment, while the use of various animal lines, including those with genetically determined characteristics, is justified [10]. Models of genetically engineered mice have been successfully used for decades in modeling the tumor process [11]. There are certain types of transgenic mice used in studies of the malignant process in which oncogenes can be constitutively or conditionally expressed. In such animal models, tumor suppressor genes can be suppressed using traditional methods such as retroviral infection, microinjection of DNA constructs and the so-called "gene-directed" transgenic approach. To date, transgenic models have become traditional and are successfully used in carcinogenesis studies [12].

For us, the mice with uPA gene knockout were of the greatest interest, obtained using a molecular genetic method during which changes are made to the nucleotide sequence of the uPA gene, as a result of which urokinase is not bound by the urokinase-type plasminogen activator receptor (uPAR). These mutant animals can be used in the study of inflammation, oncogenesis, and fibrinolysis mechanisms in tumors and surrounding tissues.

The aim of the study was to study the effect of knockout by the urokinase gene in mice of both sexes with B16/F10 transplanted melanoma on the functioning of the fibrinolytic system links.

MATERIALS AND METHODS

The study used genetically modified female and male mice of the C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-) line with an initial weight of females – 24–26 g, 31–33g for males. The rodents were btained from the nursery of laboratory animals "Pushchino" Branch of the Institute of Bioorganic Chemistry named after Academicians M. M. Shemyakin and Yu. A. Ovchinnikov (Pushchino, Moscow region). Animals with urokinase knockout gene (uPA-/-) can be used in studies of chronic tissue inflammation, mechanisms of fibrinolysis, oncogenesis and vascular growth in the tumor and surrounding tissue. Mice of both sexes of the C57BL/6 (uPA+/+) line with an initial weight of 21–23 g obtained from the Andreevka Scientific Center for Biomedical Technologies (FMBA) (Moscow Region) were used as controls. The animals were kept under natural lighting conditions with free access to water and food. The study was conducted in accordance with the "International Recommendations for conducting biomedical research using animals" and the Order of the Ministry of Health of the Russian Federation No. 267 dated 06/19/2003 "On approval of the rules of laboratory practice".

The study was performed on 64 male and 64 female mice. The animals were divided into groups of 10 individuals each: intact females and males of the C57BL/6 line (uPA+/+); intact females and males of the C57BL/6 line -Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-); control group females and males of the C57BL/6 line (uPA+/+) 3 weeks after trans-

plantation of melanoma B16/F10; the main group of females and males of the C57BL/6-Plautm1.1Bug – ThisPlauGFDhu/GFDhu (uPA-/-) line 3 weeks after transplantation of melanoma B16/F10. The study period – 3 weeks after the transplantation of melanoma B16/F10 was chosen because it was the stage of mass death for male mice and the beginning of death of females, in addition, after 3 weeks, maximum differences in average tumor volumes in animals of uPA-/- and uPA+/+ lines were noted. Groups of uPA-/- (12 individuals of each sex) and uPA+/+ (25 individuals of each sex) animals were separately identified for the study of average life expectancy.

This work used a cell line of mouse melanoma B16/F10 metastasizing to the lungs, obtained from the N. N. Blokhin Russian Research Center of the Russian Academy of Medical Sciences (Moscow). Melanoma B16/F10 was transferred by subcutaneous injection of 0.5 ml of tumor tissue suspension in saline solution (1:10) into the right hind leg of a mouse according to the standard procedure [13]. With standard grafting, the tumor appears in 100 % of cases, grows quite quickly and on the 12th-16th day of growth metastasizes mainly hematogenously to the lungs (60–90 %), less often to the liver and spleen [14]. For the experiment, the second passage of melanoma B16/F10 transplantation in C57BL mice was used/6.

Tumor growth was assessed by daily measuring its diameters in three mutually perpendicular areas, followed by calculating the volume of the tumor as the product of its three measurements.

Intact animals, as well as mice of the control and main groups, were decapitated 3 weeks after the transplantation of melanoma, and the following were isolated in the cold: tumor, perifocal zone, skin. The samples were mechanically homogenized, 10 % homogenates were obtained from the tissues, prepared on a 0.1M potassium phosphate buffer pH 7.4 containing 0.1 % Twin-20 and 1 % BSA In tissue homogenates, the level of plasminogen (PG), plasmin (PAP), urokinase receptor uPAR, content (AG) and activity were determined using enzyme immunoassay methods (act) uPA, t-PA and PAI-I (Cussabio, China).

Statistical processing of the obtained results was carried out using the Statistica 10.0 program. For all quantitative data, the group arithmetic mean (M) and standard error (m) were calculated. All the results obtained were checked for compliance with the law of normal distribution (Shapiro-Wilk criterion (for small samples)). When the sample corresponded to the normal distribution, parametric statistics were used (Student's criterion), and when there was a discrepancy, nonparametric statistics were used (Wilcoxon-Mann-Whitney criteria). The differences were considered statistically significant at $p < 0.05$.

STUDY RESULTS

It was found that the process of carcinogenesis in genetically modified female mice (uPA-/-) compared with the control (uPA+/+) had features consisting in a reduction in the preclinical period of melanoma development and a decrease in the average volume of tumor nodes at all stages of observation (from 1 to 4 weeks). Single lung metastases were diagnosed in the females of the experimental group, whereas metastatic lung and liver damage was observed in the control group. In males, tumors were characterized by a fairly active, "spasmodic" growth, and their average volume at 4 weeks after transplantation did not differ from those in mice with a normal genome. In uPA-/-males, no visible metastases to internal organs were detected at all stages of the growth of B16/F10 transfused melanoma, but hemorrhages to the lungs were detected. The average life expectancy in uPA-/- and uPA+/+ mice had no significant differences and was 34.67±0.67 versus 30.25 ± 1.67 in females, and 23.33 ± 3.18 versus 22.1 ± 0.82 in males, respectively [15].

Based on the previously obtained results of differences in the growth of malignant tumors in animals with a knockout of the urokinase gene and a wild type of gene, it was interesting to find out what differences in the content and activity of the main links of the fibrinolytic system of the skin are characteristic of animals with a knockout of the urokinase gene (Tables 1, 2).

Compared with animals of the control group, only traces of uPA were recorded in the skin of intact uPA-/- mice of both sexes: a decrease in uPA levels and activity was noted by 100–860 times (Tables 1, 2). In intact uPA-/- females, the high content of PPB attracts attention, exceeding 4.2 times the same indicator in female uPA+/+ mice with a 1.3-fold (p 0.05) reduced PG content.

In intact uPA-/-males, concentrations of both PAP and PG were increased 2.1-fold and 1.8-fold in the skin, compared with those in the skin of intact uPA+/+ mice (Table 2).

In conditions of uPA deficiency with a high content of PAP, an increase in the level of the second activator of PG – tPA was expected, However, its content and activity were reduced only in uPA-/- males by 4.3 times and 1.7 times, respectively. In uPA-/- females, a decrease was revealed, relative to the data in uPA+/+ mice, only tPA activity by 2.5 times, despite an increase in its content by 1.7 times.

The amount of the uPAR receptor in uPA-/- female mice was at the same level as in uPA+/+ mice, whereas in males it was reduced by 1.9 times. Significant differences with the norm were also observed for PAI-1: in females, uPA-/- PAI-I activity and its content were 15.0 and 3.0 times lower than normal, respectively, in males by 4.9 times and 9.8 times, respectively.

Since the average tumor size in female uPA-/- mice was smaller after 3 weeks of the experiment than in uPA+/+ females [15], a comparative analysis of the links of the fibrinolytic system in samples of melanoma of animals with knockout and mice with wild genome type was further performed.

Table 1. The content and activity of fibrinolytic system components in the skin, tumor and perifocal zone of female uPA -/- mice with melanoma B16/F10 3 weeks after transplantation *(M ± m)*

Indicators	Intact mice skin (normal)	Skin	Tumor volume cm³	Perifocal zone
uPA -/- female mice (*n* = 10)				
uPA-act (u/g t)	0.010 ± 0.001[3]	0.009 ± 0.0009[2,3]	0.025 ± 0.002[3]	0.01 ± 0.001[3]
uPA-AG (ng/g t)	0.220 ± 0.02[3]	0.24 ± 0.018[2,3]	0.14 ± 0.011[1,3]	0.73 ± 0.06[1,2,3]
uPAR (pg/g t)	58.20 ± 4.3	56.2 ± 4.7	66.2 ± 5.3[3]	59.5 ± 5.3[3]
PAP (ng/g t)	45.0 ± 3.4[3]	18.75 ± 1.6[1]	19.7 ± 1.4[1,3]	24.4 ± 2.2[1,3]
PG (ng/g t)	7.70 ± 0.6[3]	10 ± 0.97[1,3]	8 ± 0.77[3]	20 ± 1.7[1,2,3]
tPA-act (u/g t)	0.240 ± 0.02[3]	0.16 ± 0.014[1,2,3]	1.95 ± 0.16[1]	0.155 ± 0.014[1,2,3]
tPA-AG (ng/g t)	0.670 ± 0.05[3]	0.34 ± 0.028[1,2,3]	0.69 ± 0.05[3]	1.55 ± 0.13[1,2,3]
PAI-I-act (u/g t)	1.60 ± 0.1[3]	1.85 ± 0.15[2,3]	3.05 ± 0.29[1,3]	5.25 ± 4.4[1,2,3]
PAI-I-AG (ng/g t)	3.30 ± 0.3[3]	2.2 ± 0.17[1.3]	2.7 ± 0.24[3]	2.8 ± 0.22[3]
uPA +/+ female mice (*n* = 10)				
uPA-act (u/g t)	1.6 ± 0.12	2.5 ± 0.2[1]	2.8 ± 0.19[1]	2.6 ± 0.17[1]
uPA-AG (ng/g t)	31.7 ± 2.1	187.5 ± 13[1,2]	335.5 ± 23[1]	186.5 ± 12.5[1,2]
uPAR (pg/g t)	56.06 ± 4.5	65.36 ± 5.6[2]	141.8 ± 11.7[1]	112.0 ± 9.6[1]
PAP (ng/g t)	10.7 ± 0.7	19.9 ± 1.1[1.2]	36.4 ± 1.5[1]	18.9 ± 1.2[1,3]
PG (ng/g t)	10.25 ± 0.9	13.5 ± 1.1[1]	16.7 ± 1.4[1.]	11.03 ± 0.9
tPA-act (u/g t)	0.6 ± 0.04	0.7 ± 0.03[2]	2.2 ± 0.19[1]	0.7 ± 0.06[3]
tPA-AG (ng/g t)	0.4 ± 0.02	2.0 ± 0.15[1,2]	12.3 ± 0.9[1]	2.5 ± 0.13[1,3]
PAI-I-act (u/g t)	24.0 ± 0.16	24.0 ± 1.4[2]	71.1 ± 4.2[1]	81.0 ± 5.8[1]
PAI-I-AG (ng/g t)	9.9 ± 0.4	24.5 ± 1.8[1,2]	79.5 ± 6.3[1]	71.6 ± 5.2[1]

Note: [1] – the differences are statistically significant relative to the norm in animals; [2] – compared with a tumor; [3] – compared with similar samples in uPA+/+ animals ($p < 0.05$)

In the tumor samples of uPA-/- females, compared with the tumor samples of uPA+/+ females, the indicators of the determined factors were significantly lower: the activity and content of uPA by 112 times and by 2396 times, the level of uPAR by 2.1 times, PP and PG by 1.8 times and 2.1 times, the content of tPA by 17.8 times, the activity and content of PAI-1 by 23.3 times and 29 times, respectively. Only the activity of tPA did not have significant differences in tumor samples depending on the urokinase gene.

That said, 3 weeks after the transplantation of melanoma B16/F10 in uPA+/+ females in tumor samples, compared with the corresponding intact skin, an increase in all studied parameters of the fibrinolytic system was noted, whereas in uPA-/- females in melanoma samples such stimulation was not detected, except for an increase in activity, but not the content tPA.

There were also differences in the studied parameters in the perifocal zone and the skin unaffected by tumor growth. Thus, in the perifocal zone of uPA -/- females after 3 weeks of melanoma growth, uPA activity and level were lower than in the perifocal zone of uPA+/+ mice by 260 and 255 times, respectively, and the concentration of uPAR was also 1.9 times lower. The level of tPA, as well as its activity, were

Table 2. Content and activity of fibrinolytic system components in the skin, tumor and perifocal zone in male uPA -/- mice with melanoma B16/F10 3 weeks after transplantation *(M ± m)*

Indicators	Intact mice skin (normal)	Skin	Tumor volume cm^3	Perifocal zone
uPA -/- male mice				
uPA-act (u/g t)	0.010 ± 0.001[3]	0.009 ± 0.0007[2,3]	0.013 ± 0.0011[1,3]	0.015 ± 0.001[1,3]
uPA-AG (ng/g t)	0.250 ± 0.02[3]	0.25 ± 0.018[2,3]	0.10 ± 0.009[1,3]	0.39 ± 0.03[1,3]
uPAR (pg/g t)	56.90 ± 4.3[3]	67.4 ± 5.9	90.8 ± 7.6[1]	67.55 ± 5.5[2]
PAP (ng/g t)	30.0 ± 2.5[3]	18.13 ± 1.4[1,3]	14.4 ± 0.9[1,3]	18.13 ± 1.7[1]
PG (ng/g t)	12.50 ± 0.9[3]	9.1 ± 0.77[1,3]	10 ± 0.07[3]	12.2 ± 0.8
tPA-act (u/g t)	0.320 ± 0.02[3]	0.17 ± 0.015[1,2,3]	1.22 ± 0.78[1.3]	0.17 ± 0.014[1,2,3]
tPA-AG (ng/g t)	0.70 ± 0.05[3]	0.66 ± 0.06[2,3]	0.46 ± 0.04[1.3]	0.88 ± 0.071[2,3]
PAI-I-act (u/g t)	2.60 ± 0.2[3]	1.77 ± 0.13[1,2,3]	2.87 ± 0.21[3]	2.37 ± 0.18[3]
PAI-I-AG (ng/g t)	4.10 ± 0.3[3]	2.43 ± 0.21[1,2,3]	3.9 ± 0.33[3]	4.8 ± 0.43[3]
uPA +/+ male mice				
uPA-act (u/g t)	1.561±0.10	1.65 ± 0.143	2.7 ± 0.21	1.9 ± 0.17
uPA-AG (ng/g t)	215.3 ± 16.8	181.6 ± 17.1[3]	300.4 ± 24	210.3 ± 19
uPAR (pg/g t)	110.3 ± 6.5	65.13 ± 5.7[1]	73.48 ± 5.3[1]	85.96 ± 7.2[1]
PAP (ng/g t)	14.52 ± 0.9	30.7 ± 2.9	48.8 ± 4.1	19.8 ± 1.7
PG (ng/g t)	6.851 ± 0.5	15 ± 1.2	21.6 ± 1.9	13 ± 1.1
tPA-act (u/g t)	0.551 ± 0.04	0.86 ± 0.07	2.4 ± 0.19	0.8 ± 0.06
tPA-AG (ng/g t)	2.981 ± 0.2	4.8 ± 0.38[1]	11.6 ± 0.9	5.5 ± 0.42
PAI-I-act (u/g t)	12.61 ± 1.02	52.5 ± 4.7	41.3 ± 3.8	59.4 ± 5.6
PAI-I-AG (ng/g t)	40.0 ± 3.5	28 ± 2.5	39 ± 3.4	54.8 ± 4.5

Note: [1] – the differences are statistically significant relative to the norm in animals; [2] – compared with a tumor; [3] – compared with similar samples in uPA+/+ animals ($p < 0.05$)

1.6 times and 4.5 times lower than in animals without knockout, respectively. The activity of PAI-I and its content were reduced by 15.4 times and 25.6 times. Despite this, the level of PAP and PG in the perifocal zone of uPA-/- females turned out to be 1.3 times and 1.8 times higher than in uPA+/+ females, respectively.

That said, in the perifocal zone in female uPA+/+ mice, after 3 weeks of melanoma growth, almost all links of the fibrinolytic system (with the exception of PG and tPA activity) exceeded the indicators in the skin of intact animals, whereas in uPA-/- mice in the perifocal zone, compared with the skin of intact animals, only an increase in the content was detected PG, uPA and tPA, without increasing their activity, as well as increased activity of PAI-I.

In the skin of uPA-/- females unaffected by tumor growth, almost all indicators of the fibrinolytic system were reduced, except for the absence of differences in PAP and uPAR, compared with skin samples in uPA+/+ females. Thus, in skin samples with tumor growth in females, uPA-/- activity and uPA content were lower by 277.8 times and 781.3 times; tPA activity and content by 4.4 times and 5.9 times; PAI-I activity and content by 13 times and 11.4 times, respectively. After 3 weeks of tumor growth, the dynamics of changes in the studied parameters in unaffected skin in uPA+/+ females, compared with intact mice, generally corresponded to the orientation in the tumor and perifocal zone – activation of the fibrinolytic system was observed, whereas in uPA-/- females, on the contrary, either no changes or a decrease in the level of RAR, activity and the content of tPA and the content of PAI-I, compared with intact mice of the same line.

At the stage 3 weeks after transplantation, the volumes of primary tumors in uPA-/- males were smaller than in animals with wild type genes [15].

In the tumor samples of uPA-/- males, compared with similar samples in uPA+/+ males, the level of plasmin was reduced by 3.4 times and plasminogen by 2.2 times (Table 2). The activity and content of uPA in males with urokinase gene knockout were 208 times and 3004 times lower than in wild-type animals, respectively, and the content and activity of tPA were 25.2 times and 2 times lower, respectively. In addition, a decrease by 14.4 times and 10 times in the activity and concentration of PAI-I was revealed as well.

Only the uPAR content did not differ depending on the state of the urokinase gene. So if in males uPA+/+ in tumor samples, compared with intact skin (normal), almost all the studied parameters of the fibrinolytic system have increased, with the exception of the receptor level, in melanoma in males uPA-/- on the other hand only an increase in tPA and uPAR activity was noted.

In the perifocal zone of uPA-/- males, compared with the perifocal zone of uPA+/+ males, the activity and level of uPA were 127 times and 538 times lower, and tPA was 25 times and 11.4 times, and PAI-I was 25 times and 8.8 times, respectively. At the same time, the level of uPAR, plasmin and PG in the perifocal zone did not carry any significant differences depending on the state of the urokinase gene. It turned out that with tumor growth in uPA+/+ males in the perifocal zone, the content of PAP, PG, as well as the activity and content of tPA and PAI-I increased compared with the skin of the corresponding intact animals, whereas in males uPA-/- either did not change compared with intact skin, or decreased.

In the samples of unaffected skin in uPA-/- males with melanoma B16/F10, compared with the indicators in unaffected skin in uPA+/+ males, the concentrations of PAP and PG were on average 1.7 times lower, the activity and content of uPA 183 times and 726 times, the activity and content of tPA 5.1 times and by 7.3 times, the activity and concentration of PAI-I by 29.7 times and 11.5 times. Only the uPAR level had no significant differences. At the same time, it should be noted that in uPA+/+ males and in mice with urokinase gene knockout, only the absence of changes in the activity and content of iRA, as well as a decrease in PAI-I levels, turned out to be the same in unaffected skin with melanoma growth, as well as a decrease in the level of PAI-I, compared with the indicators of healthy skin of the corresponding intact animals. The rest of the studied parameters changed in different directions – in uPA-/- males either decreased (PAP, PG, tPA activity) or did not change (PAI-I activity, tPA content, uPAR), whereas activation was detected in uPA+/+ (with the exception of uPAR).

DISCUSSION

Currently, it is known that urokinase (uPA) is secreted in many malignant cells, including pancreatic, breast, and colorectal cancers, and its expression often correlates with the prognosis of the

disease [4, 16]. The biological role of this protease is to bind to the uPAR receptor to stimulate the proteolytic cascade and convert inactive proteases such as plasmin and matrix metallopeptidase 9 (MMP-9) into active forms, thereby endowing tumor cells with the ability to destroy the components of the extracellular matrix, activate the growth and metastasis of tumor cells [17–19]. Therefore, the role of uPA in migration, invasion and metastasis of tumor cells is undeniable [18].

Previously, we received confirmation of the effect of urokinase gene knockout on the tumor process, namely, significant suppression of tumor volume growth and metastasis in animals of both sexes [15]. We found that in intact uPA-deficient mice of the C57BL/6-PlautmI.IBug-ThisPlau6FDhu/GFDhu line, almost the entire cascade of PG regulators was suppressed in the skin (with the exception of the urokinase receptor uPAR and tPA content only in females). We expected to detect an increase in the activity of a number of enzymes, but in intact uPA-/- mice, an increased content of plasmin alone was recorded. With uPA deficiency in C57BL/6-PlautmI.IBug-ThisPlau6FDhu/GFDhu mice, plasmin activity could have found other targets in our experiment. We believe that an increase in the content of PAP in knockout mice is a kind of compensation, contributing to a sharp decrease in urokinase, cleavage of its receptor.

Despite the significant suppression of the fibrinolytic system in mice, uPA-/- transfused melanoma grew, and although it had significantly smaller volumes (especially in females) and rarely metastasized (males had no visible metastases), the life expectancy of animals of the two lines did not have significant differences. In addition, the level of plasmin in the skin of intact uPA-/- mice exceeded the values in animals of the C57BL/6 line. These points prove the presence of alternative biological pathways that melanoma "uses" for its survival in conditions of knockout of the urokinase gene.

One of the alternative pathways in urokinase gene knockout conditions may be uPAR, known as CD-87, which is highly expressed in various tumor cells, and various signals regulated by uPAR play an important role in neoplasm proliferation and metastasis, tumor-related glycolysis, as well as tumor microenvironment and angiogenesis [20]. There is evidence that it is uPAR that regulates the migration of melanoma cells by assembling them in complex regulatory units with transmembrane receptors [21].

Our study showed that on the background of significant suppression of urokinase, the level of the uPAR receptor in intact skin in females did not change, and in males. Although an almost twofold decrease in its concentration was detected. However, its content in the tumor and surrounding tissues on the background of the growth of melanoma B16/F10 has no significant differences from those in animals with wild the type of genome. It is known that uPAR competes with uPA for participation in many non-proteolytic biological processes, such as migration, adhesion, cell proliferation and angiogenesis [22]. Thus, uPA-/- uPA functions could be performed by uPAR in mice. In our study, the uPA-/- urokinase receptor level in the studied samples did not change in relation to the parameters in intact animals, whereas in males the tumor samples increased, which was accompanied by large volumes of melanoma in males, compared with females.

Series of studies confirm that a decrease in uPAR expression on the cell surface mitigates the development of characteristic cancer signs caused by PIK-3CA and KRas mutations in colorectal cancer [23], and by interacting with uPA and IGF1R, uPAR is able to enhance the malignant potential of triple negative breast cancer [24]. Clinical observations are confirmed by experimental studies in which knockout of the uPAR gene in mice leads to G2/M arrest, thereby suppressing cell proliferation [25]. There are studies on the possibility of using uPA inhibitors to slow tumor growth and metastasis [26].

It is believed that overexpression of uPAR in human melanoma cells controls the invasive and glycolytic phenotype. uPAR-mediated pathways have already been established, including the integrin-dependent association of uPAR with at least four IL-TKR systems: *EGFR, IGFR, PDGFR* and *MET* [27]. The results of our studies showed a significant increase in the level of uPAR in tumor samples and its perifocal zone in uPA+/+ mice, whereas in uPA-/- females such a pattern was not observed and only in uPA-/- males the concentration of the urokinase receptor increased in tumor samples. The complexity of various molecular pathways allows malignant cells to continue to proliferate and migrate even in conditions of urokinase deficiency, using uPA-independent pathways of proteolytic

activation of angiogenesis factors. This was confirmed by our previous studies of angiogenesis factors in animals with urokinase gene knockout, demonstrating an increased content of VEGF-A and especially VEGF-C in unaffected skin in female uPA-/- mice [28].

At the same time, it should be considered that knockout by the urokinase gene is a kind of artificially induced genetic comorbid disease, as a result of which the fibrinolytic system is suppressed not only in the skin, but also in other organs and systems. The involvement of the fibrinolytic system in various physiological processes, wound healing, as well as in the preservation of brain neurons after various ischemic injuries indicates a possible insufficiency of these processes in uPA-/- animals.

CONCLUSIONS

The fact that in uPA-/- mice, despite the extremely small volumes of the primary tumor and rare metastasis, tumor disease caused the death of animals at the same time as in uPA+/+ animals, indicates a significant effect of tumor disease on all regulatory systems of the body, regardless of the size of the neoplasm. Our study confirmed the claim that the use of drugs that inhibit the urokinase pathway may be promising in the treatment of the disease by slowing the growth of neoplasm volume and its metastasis, but is not a panacea, since the effect of a malignant tumor on the body is much more complex, therefore further studies of the pathogenesis of malignant growth are required.

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