IMPACT OF TUMOR ON THE CELL CYCLE AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

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Today, there is a theory that proliferative potential of hematopoietic stem cells is depleted, and the balance of committed precursor cells shifts towards suppressors during the development of cancer. However, differentiation of hematopoietic stem cells can vary depending on the tumor type, localization, and microenvironment specifics. The study aimed to assess the impact of tumors of various origins on the $CD34^+$ hematopoietic stem cells (n = 10). Assessment of the cell cycle and cell differentiation via both direct contact with the tumor and exchanging humoral factors only in transwells was conducted by flow cytometry. In the co-culture with K562, the number of hematopoietic stem cells being in their synthesis phase was 2.1%, while in the control it was 11.2% (p = 0.01); in the co-culture with SK-mel37, the number of hematopoietic stem cells being in the G_2–M cell cycle phase was reduced to 0.3% (p < 0.05). 1301 and K562 directed the hematopoietic stem cell differentiation towards granulocyte-macrophage precursor cells (p < 0.05), while 1301 and SK-mel37 directed it towards common multipotent progenitor cells. It is interesting that the number of pluripotent hematopoietic stem cells significantly increased (2-fold) compared to control after incubation with K562 in transwells (24.17% and 10.19%, respectively). Thus, properties of hematopoietic stem cells can vary depending on both tumor type and the way of interacting with these cells.

Keywords: hematopoietic stem cell, cell differentiation, committed precursor cells, proliferation, flow cytometry, T-cell leukemia, chronic myelogenous leukemia, melanoma

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Research Institute of Fundamental and Clinical Immunology (protocol No. 145 dated 19 April 2024). All the conditionally healthy donors submitted the informed consent to participation in the study.

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ВЛИЯНИЕ ОПУХОЛИ НА КЛЕТОЧНЫЙ ЦИКЛ И ДИФФЕРЕНЦИРОВКУ ГЕМОПОЭТИЧЕСКИХ СТВОЛОВЫХ КЛЕТОК

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На сегодняшний день существует теория о том, что пролиферативный потенциал гемопоэтических стволовых клеток истощается, а баланс коммитированных предшественников смещается в сторону супрессоров в ходе развития онкологии, однако дифференцировка гемопоэтических стволовых клеток может варьироваться в зависимости от типа, локализации и специфики микроокружения опухоли. Целью исследования было оценить влияние опухолей различного генеза на $CD34^+$-гемопоэтические стволовые клетки (n = 10). С помощью метода проточной цитометрии проводили анализ клеточного цикла и дифференцировки клеток как через прямой контакт с опухолью, так и через обмен только гуморальными факторами в трансвеллах. В ко-культуре с K562 количество ГСК, находящихся в синтетической фазе, составило 2,1%, в контроле — 11,2% (p = 0,01); в ко-культуре с SK-mel37 количество клеток, находящихся в фазе G_2–M клеточного цикла, снижалось до 0,3% (p < 0,05). 1301 и K562 направляли дифференцировку ГСК в сторону гранулоцитарно-макрофагальных предшественников (p <0,05), а 1301 и SK-mel37 в сторону общих мультипотентных предшественников. Интересно, что после инкубации с K562 в трансвеллах статистически значимо увеличивалось количество плюрипотентных гемопоэтических стволовых клеток в два раза по сравнению с контролем (24,17% и 10,19% соответственно). Таким образом, свойства гемопоэтических клеток могут меняться как от вида опухоли, так и от способа взаимодействия с ними.

Ключевые слова: гемопоэтическая стволовая клетка, дифференцировка клеток, коммитированные предшественники, пролиферация, проточная цитометрия, Т-клеточный лейкоз, хроническая миелогенная лейкемия, меланома

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It is well known that hematopoietic stem cells (HSCs) that form the pool of blood cells after maturation and differentiation throughout the life are an important component of the bone marrow niche. The niche represents specific microenvironment with humoral factors and specific cell contacts for HSCs, ensuring the strictly regulated processes of self-maintenance or self-renewal and cell differentiation. HSCs have a unique cell cycle with asymmetric division for maintenance and renewal of the pool of pluripotent HSCs and, at the same time, for generation of the essential common or more differentiated progenitor cells. The HSC activity depends directly on the cell cycle, on its duration or time before entering the cell cycle, as well as on the cell division rate. It is important to note that under homeostatic conditions the majority of HSCs should be through the G_0 phase of the cell cycle or the resting phase (up to 95%) to prevent premature depletion of cells, while self-renewal and differentiation of cells occur in phase G2 [1]. Since under conditions of the niche HSCs are usually in the resting state mediated by the intra- and extracellular mechanisms, including some proliferation inhibitors, such as CXCL4 and TGFβ, secreted by megakaryocytes [2], alterations of cell-to-cell contact or concentration, as well as the emergence of new humoral factors can bring HSCs out of the resting state, induce their proliferation and differentiation, similar to HSC proliferation associated with blood loss, irradiation or effects of pro-inflammatory cytokines [3]. In turn, this will lead to the decrease in HSC function, aberrant cell cycle regulation and even malignancy [4, 5]. It is interesting that cell division is not an essential phase preceding differentiation into common myeloid, megakaryocyte-erythroid and pre-megakaryocytic progenitors [6]. Along with these progenitors, common multipotent and lymphoid progenitors are distinguished in the hematopoietic process. Cells can be distinguished by differentiation markers acquired by HSC during specialization under exposure to various stimuli, including the combination of colony-stimulating factors. Cytokines also represent conventional stimuli, but these can have both negative and positive effects on differentiation. Thus, GM-CSF, G-CSF, M-CSF, EPO, TPO, SCF/KL, FL, TNF, LIF, IL12, IL11, IL6, IL5, IL4, IL3, IL1, SDF-1, FGF-4 can induce myelopoiesis, while TNF, IL4, TGFβ , IFN, MIP-1, IL10, IL13 can suppress myelopoiesis; IL2, IL4, IL7 are essential for activation of lymphopoiesis, and TGFβ , IL4 are essential for suppression of lymphopoiesis [7]. A broad range of factors potentially capable of directing HSC differentiation into these or other common or more specialized progenitors is synthesized in the tumor microenvironment due to the presence of a large number of tumor-associated cells. Thus, under certain conditions, it is possible to expand the pool of HSCs, HSC progenitors and, therefore, alter their functional activity, which can result in the disease process development in the body or worsening of the course of the existing disease process. For example, tumor process is associated with formation of specific microenvironment, in which, according to the latest data, an important role is played by hematopoietic stem cells. It is well known that the tumor can recruit cells during carcinogenesis, and HSCs are no exception. Furthermore, all the cells derived from HSCs in the solid tumor microenvironment are involved in tumor invasion, growth, progression, and chemoresistance [8]. HSCs or the so-called tumor-associated HSCs often represent tumor-initiating cells capable of causing differentiation of other cells into fibroblasts, macrophages and endothelial cells, which support tumor growth and recurrence via production and secretion of growth factors and extracellular matrix components, in addition to triggering angiogenesis [9]. Thus, along with other tumor-associated cells, hematopoietic stem cells contribute actively to the tumor process maintenance and progression. However, aspects of the interplay between hematopoietic stem cells and tumor cells are poorly understood. That is why it is necessary to assess the influence of both cell-to-cell contact and humoral factors on the key components of the HSC vital activity, such as proliferative capacity, cell cycle, and differentiation, which constituted the aim of our study.

METHODS

The $CD34^+$-separated hematopoietic stem cells (HSCs) of the donors (n = 10, average age 38.1 ± 3.4 years) were obtained at the Immunopathology Clinic of the Research Institute of Fundamental and Clinical Immunology. The study also involved the use of human T-cell leukemia 1301 cell line, human chronic myelogenous leukemia K562 cell line (European Collection of Authenticated Cell Cultures, Sigma Aldrich, Merck KGaA, Germany), and human melanima Sk-mel-37 cell line kindly provided by the Laboratory of Cellular Technologies of the Research Institute of Fundamental and Clinical Immunology (Novosibirsk, Russia).

HSC culture

The cryopreserved CD34-positive HSC samples were thawed and washed in accordance with the standard guidelines for frozen precursor cells [10, 11]. HSCs were enumerated in the 0.01% (10 mg/mL) methylene blue solution (Biolot, Russia) to determine cell viability using the Goryaev chamber. The cells obtained were cultured in different quantities (from 100,000 to 1,000,000 cells/mL) with appropriate RPMI-1640 medium (PanEco, Russia) or Stemline II (STEM) medium for hematopoietic stem cell reproduction (Sigma Aldrich Co. LLC, USA) used as positive control, supplemented with 50 mg/mL gentamicin (Dalfarma, Russia), 25 mg/mL tienam (Merck Sharp & Dohme Corp., Kenilworth, New Jersey, USA) within different time depending on the series of experiments at 37 °C, 5% CO_2 in the humidified atmosphere.

Tumor cell line culture

The suspension tumor cell lines K562 and 1301 and the adherent cell line SK-mel37 were cultured under standard conditions using the complete culture medium RPMI-1640 + 2 mM glutamine + 10% fetal bovine serum (HyClone, USA). The cultures were maintained within 100,000–1,000,000 cells/mL at 5% CO_2 and 37 °C. The conditioned culture media were selected taking into account the series of the experiment in the tumor cell line exponential growth phase and frozen for future use.

HSC viability and proliferation

HSCs (1×10^5 cells/250 mL) were incubated with the conditioned media from the 1301, K562, Sk-mel 37 tumor cell lines in different dilutions (100%, 50%, 10%) added the complete culture medium RPMI-60 with the 10% human albumin (Octapharma Pharmazeutika Produktionsgesellschaft, m.b.H., Austria) to the specified volume in the 96-well flat bottom plate (TPP, Switzerland) in triplets for 3, 5, and 7 days. The STEM specialized culture medium was used as positive control. DMSO was used as negative control. HSCs were cultured under standard conditions: at 37 °C, 5% CO_2, 90% relative humidity.

Furthermore, HSCs (1×10^5 cells/250 mL) were incubated with the tumor cell lines in specific plates to completely avoid cell-to-cell contact, i.e. in the 12-well transwells with the pore

size of 0.4 μm and the insert diameter of 6.5 mm (Corning Incorporated, Costar, Arizona, USA) for three days under the same conditions.

The HSC viability and proliferative activity were assessed using the WST-1 reagent (Takara Bio Inc., Kusatsu, Japan). The samples were analyzed by colorimetry using the Tecan Infinite F50 microplate reader (Austria) at the wavelength of 450 nm (standard 650 nm).

HSC cell cycle

HSCs were previously stained with the CFSE dye (Invitrogen, Eugene, Oregon, USA) in accordance with the manufacturer's protocol to ensure these would differ from tumor cells when co-cultured in the plate.

HSCs in a ratio of 1 : 1 — 1 × 10^5 cells/mL and 10 : 1 — 1 × 10^6 cells/mL to tumor cells were incubated in the 24-well plate as the co-culture to assess the influence of direct contact with tumor cells on the stem cells and in the 12-well transwells to assess the impact of humoral factors only for three days under the same conditions; the intact cells in the RPMI and STEM media were used as controls. Then the cells were transferred to flow cytometry tubes and fixed in the ice cold 70% alcohol on ice for 2 h, triple washed, then added 1 mg/mL of the staining solution based on EtBr (Serva Electrophoresis GmbH, Heidelberg, Germany), 5 μg/mL of RNAase A (Microgen, Russia), 10% fetal bovine serum and PBS + EDTA, and stained for 30 min at 37 °C. The ready-made samples were analyzed using the LongCyte 14-color flow cytofluorometer (Challenbio, China) with the ModelFlower software.

HSC differentiation

HSCs in a ratio of 10 : 1 – 1 × 10^6 cells/mL to tumor cells (since the larger number of cells is necessary for assessment of committed precursor cells) were incubated in the 24-well plate as the co-culture and in the 12-well transwells under the same conditions. Then the cells were transferred to flow cytometry tubes and stained using monoclonal antibodies against CD10 PE (BioLegend, USA), CD34 APC (BioLegend, USA), CD38 PE-Cy7 (ElabScience, China), CD45RA PerCP (ElabScience, China), CD90 APC-Cy7 (Cloud-Clone Corp., USA), Lin- (cocktail CD3/14/16/19/20/56) FITC (BioLegend, USA). The test samples were analyzed using the LongCyte 14-color flow cytofluorometer (Challenbio, China) with the ModelFlower software. When assessing fluorescence for each monoclonal antibody, the fluorescent minus one (FMO) control was used. Precursor cells were typed based on the surface markers as follows: pluripotent hematopoietic stem cells (pHSCs) $Lin^-CD34^+CD38^-CD45RA^-CD90^+$; common multipotent progenitors (cMPPs) Lin^- $CD34^+CD38^-CD45RA^-CD90^-$; common lymphocyte progenitors (CLPs) $Lin^-CD34^+CD38^+CD45RA^+CD90^-$; myeloid and megakaryocyte-erythroid progenitors (MEPs) $Lin^-CD34^+CD38^+CD45RA^-CD10^-$; granulocyte-monocyte progenitors (GMPs) $Lin^-CD34^+CD38^+CD45RA^+CD10^-$; B cell and NK cell precursors (B-NKP) $Lin^-CD34^+CD38^+CD45RA^+CD10^+$. Furthermore, the relative number of progenitors was assessed before plating to ensure differentiation control ("before").

Statistical data processing was performed using the GraphPad Prism 9.0.0 software. The Friedman test was used for estimation of intergroup differences, and $p < 0.05$ was considered significant. The data were presented as median (25th percentile; 75th percentile) ± interquartile range.

RESULTS

In the first phase we assessed viability and proliferative activity of hematopoietic stem cells at three time points with different share of humoral factors from tumor cells line of various origin. Thus, we showed that HSC viability at the level of the control was maintained on day 3 in the conditioned media from the tumor cell lines of varying concentration. On day 5, not only viability was preserved, but also proliferation began in all dilutions of the conditioned media; almost the same proliferation level was maintained on day 7 (Fig. 1). Furthermore, on days 3 and 5, HSC viability was higher in the 50% and 100% conditioned medium from SK-mel37 (Fig.1A, B), however, HSC viability decreased rapidly on day 7 (Fig. 1C). Perhaps, this was due to the fact that HSC proliferation was more intense in the conditioned medium from SK-mel37, and the cells began to die by day 7 (presumably due to the lack of nutrients).

The importance of humoral influence on the HSC properties is also confirmed by the results obtained when culturing HSCs with tumor cell lines under conditions precluding the cell-to-cell contact; significant differences showed that proliferative activity of HSCs was higher in transwells, than in the control, with the almost equal number of cells (Fig. 2B). The fact that SK-mel 37 shows higher proliferative capacity in transwells with HSCs compared to the equivalent quantity of the 1301 cell line also attracts attention (Fig. 2A).

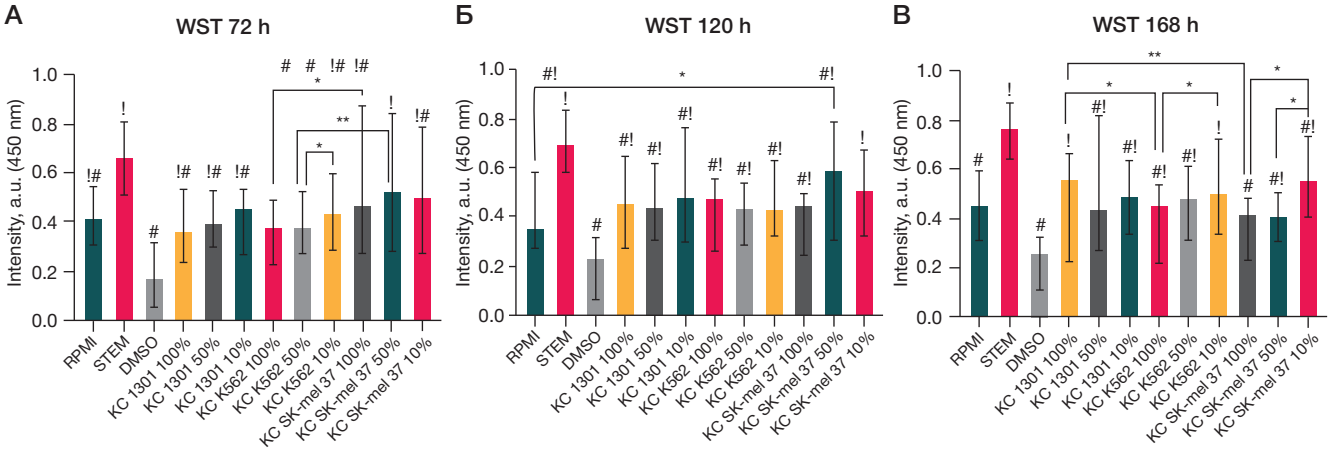


Fig. 1. Assessment of viability and proliferative activity of the hematopoietic stem cells co-cultured (c-c) with the conditioned media from the tumor cells lines 1301, K562, and SK-mel (10%, 50%, and 100% dilution) for 72 h **(A)**, 120 h **(B)**, and 168 h **(C)** (WST). Friedman test, significant differences $p < 0.05$, data are provided as median and interquartile range; # — significant differences from the control STEM; ! — significant differences from the control DMSO; * — significant differences, $p < 0.05$; ** — significant differences, $p < 0.005$

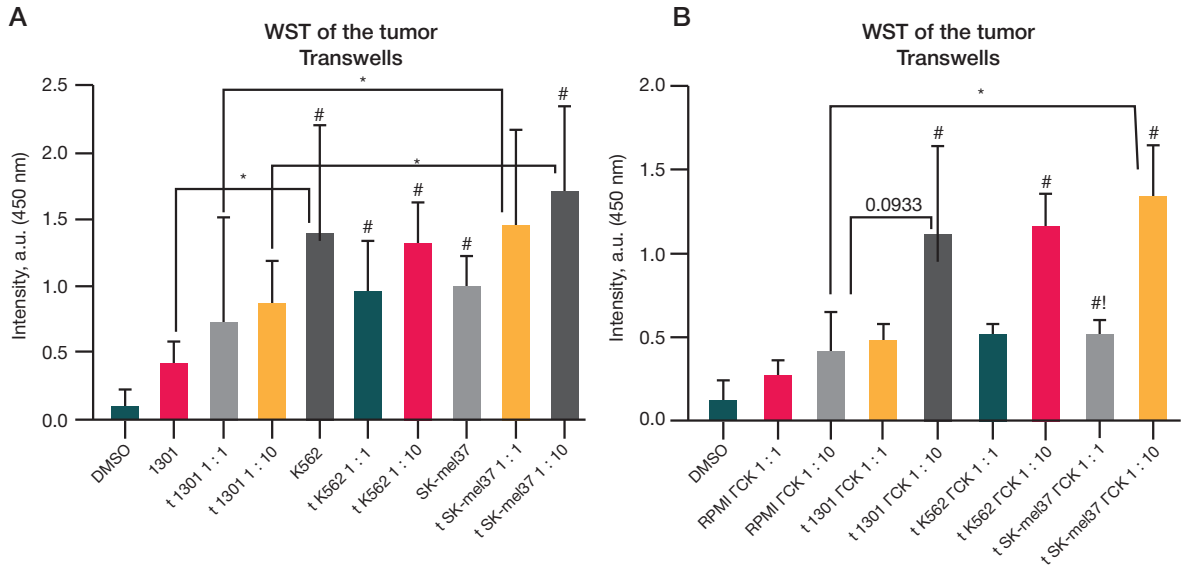


Fig. 2. Assessment of viability and proliferative activity of the tumor cell lines 1301, K562, and SK-mel37 **(A)** and hematopoietic stem cells **(B)** co-cultured in transwells (t) for 72 h. Friedman test, significant differences $p < 0.05$, data are provided as median and interquartile range; # — significant differences from the control DMSO; ! — trend compared to the SK-mel 37 HSC 1:10, $p = 0.05$; * — significant differences, $p < 0.05$

Then we assessed the influence of co-culturing on the HSC cell cycle phases. Thus, the number of cell being through the G2–M phase was higher in the co-culture with SK-mel37 compared to other tumor cell lines. The larger number of cells being through the S phase of the cell cycle was reported in cases of cell-to-cell contact with 1301. We also showed a rapid decrease in the number of HSCs cultured in transwells with SK-mel37. This was due to cell death, since 89.4% of cells were through the Sub-G1 phase (Table). It is interesting that HSC viability and proliferative activity also decreased in the conditioned media from SK-mel, but by day 7.

In the final phase, when assessing differentiation of hematopoietic stem cells, we found that the ratio of progenitors was different. Thus, after three days the number of HSCs and common multipotent progenitors increased (Fig. 3A), and the number of the latter increased much more (Fig. 3C), while the number of common lymphoid, megakaryocyte, erythroid, and myeloid progenitors decreased (Fig. 3B). It is interesting that the number of HSCs and MPP was significantly lower (20.3%) in transwells with K562 compared to other tumors in transwells (Fig. 3A), while the value of pluripotent hematopoietic cells with the $Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ phenotype was higher compared to both other tumors under the same conditions and control samples (Fig. 4). It should be noted that the larger relative number of granulocyte-monocyte progenitors was observed in the co-culture with 1301 and K562 compared to the cultures in transwells with the same tumors and the control medium (Fig. 4). The number of common progenitors of platelets, red blood cells, and cells of myeloid type decreased when cultured with the SK-mel-37 melanoma cells under conditions of both direct cell-to-cell contact and exchange of humoral factors. However, no significant differences between transwells and the co-culture in the plate were reported (Fig. 4).

Thus, the relative number of pluripotent HSCs increased under exposure to the K562 tumor humoral factors; the number

Table. Relative number of hematopoietic stem cells in various phases of the cell cycle during co-cultivation with the tumor cell lines 1301, K562, and SK-mel37 in the co-culture (c-c) and transwells (t) for 72 h.

	G2/M	S	G0–G1	Sub-G1
RPMI 1/1	3.1 (0.2–6.7)	8.6 (3.6–25.7)	77.4 (63.9–86.9)	3.5 (0.9–4.7)
RPMI 1/10	0.5 (0.4–1.9)	11.2 (5.1–13.9)	82.6 (74.1–87.0)	1.3 (1.1–4.3)
c-c 1301 1/1	15.7 (8.3–21.9)	3.9 (1.1–11.2)	76.8 (65.6–81.0)	5.1 (0.2–7.5)
c-c 1301 1/10	11.5 (3.0–20.9)	13.0 (5.9–31.3)#	79.6 (63.6–95.4)	2.2 (0.5–3.5)
t 1301 1/1	3.0 (2.5–9.2)!	3.8 (2.2–24.1)	77.5 (69.1–88.9)	4.4 (0.8–6.8)
t 1301 1/10	3.2 (1.3–5.8)!	7.2 (1.9–11.3)	86.9 (84.5–93.3)	1.3 (0.5–2.3)!!
c-c K562 1/1	10.1 (0.4–27.2)	5.8 (1.8–32.1)	72.9 (59.1–83.5)	7.9 (3.7–12.6)
c-c K562 1/10	4.4 (1.3–7.3)	2.1 (1.4–3.8)**	83.9 (78.7–91.9)**	4.6 (1.9–6.6)
t K562 1/1	1.2 (0.4–4.4)	6.6 (3.5–22.6)	81.9 (72.9–88.7)	3.2 (0.9–5.6)
t K562 1/10	1.9 (1.2–16.6)	6.5 (3.6–7.6)	84.5 (72.7–89.6)	2.9 (1.1–3.6)
c-c SK-mel37 1/1	0.3 (0.1–0.6)*	7.2 (4.3–19.4)	71.9 (66.6–93.6)	6.8 (1.7–14.9)
c-c SK-mel37 1/10	1.0 (0.2–2.8)	12.5 (4.9–16.8)	86.1 (53.4–87.9)	3.8 (0.5–5.8)
t SK-mel37 1/1	1.6 (0.6–14.8)	6.4 (3.1–28.5)	78.9 (76.6–89.1)	1.2 (0.6–4.4)
t SK-mel37 1/10	0.5 (0.3–6.0)	4.7 (2.0–12.0)	1.6 (0–6.61)$	89.4 (84.9–91.8)***

Note: Friedman test, significant differences $p < 0.05$; * — significant differences compared to the c-c K562 1/1, c-c 1301 1/1; ** — significant differences compared to the c-c 1301 1/10, c-c SK-mel37 1/10, RPMI 1/10; *** — significant differences compared to the t SK-mel37 1/1, c-c SK-mel37 1/10, t K562 1/10, t 1301 1/10, RPMI 1/10; ! — significant differences from the control RPMI in equivalent concentrations; !! — significant differences compared to the t 1301 1/1; # — trend, $p = 0.05$ compared to c-c 1301 1/1, t 1301 1/10; $ — significant differences compared to the t SK-mel37 1/1, c-c SK-mel37 1/10, t K562 1/10, t 1301 1/10, RPMI 1/10

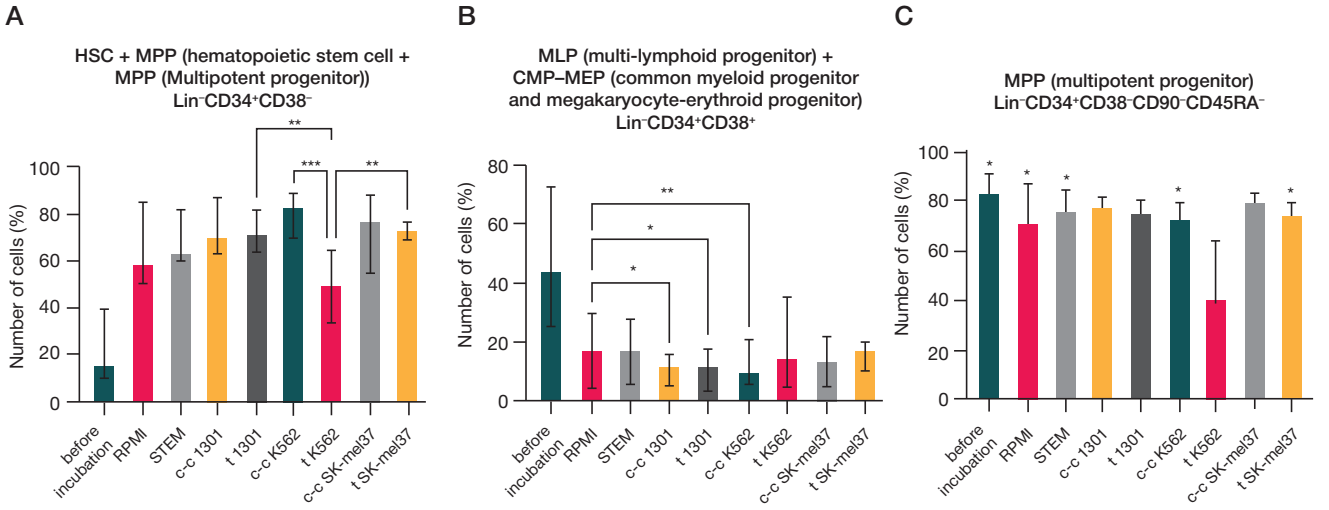


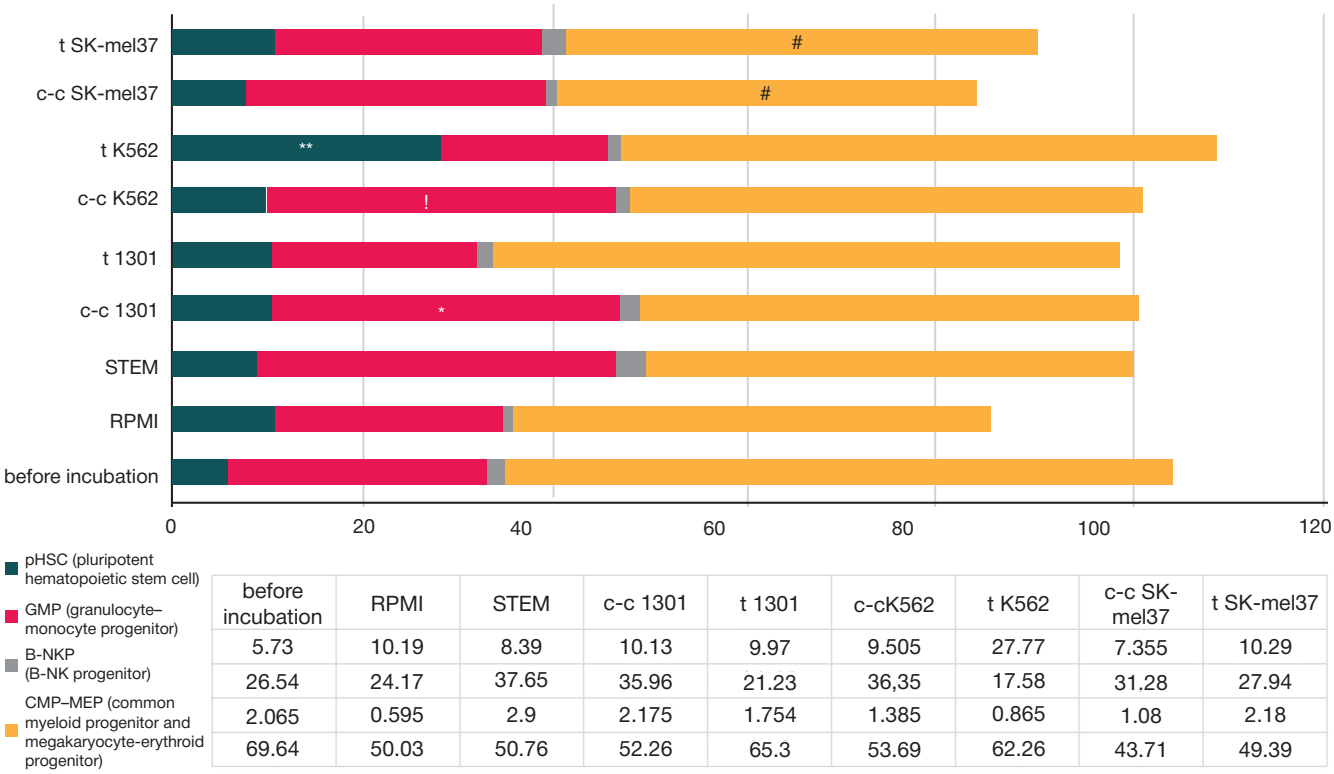
Fig. 3. Relative number of the common progenitors of hematopoietic stem cells co-cultured with the tumor cell lines 1301, K562, and SK-mel37 in the co-culture (c-c) and transwells (t) for 72 h. Friedman test, significant differences $p < 0.05$, data are provided as median and interquartile range; * — significant differences compared to t K562, $p < 0.05$

of granulocyte-monocyte progenitors increased in cases of cell-to-cell contact between HSCs and K562 or 1301.

DISCUSSION

Tumor microenvironment is a complex dynamic structure that represents the carcinogenesis regulator. The issue of studying tumor microenvironment in experimental models still remains relevant. Two major components of tumor microenvironment can be distinguished: synthesis and exchange of humoral factors, as well as formation of cross-links between cells under conditions of cellular neighborhood. Cells can change their properties and functions when influenced by the tumor. There is information about specific cells having their own functions in the tumor microenvironment, these are the so-called tumor-associated macrophages, fibroblasts, dendritic cells, etc. [12–14] involved in carcinogenesis. Today, these cells can be considered as both informative marker and therapeutic target. Since we have discussed the fact that hematopoietic stem cells are found in the tumor microenvironment, it is important to assess the impact of the tumor on the HSC properties.

According to our data, HSC differentiation in the culture occurred on day 3, when the cells were activated and proliferation started by day 3, and differentiation started by day 7. It is noteworthy that HSCs can enter early differentiation on day 3 under exposure to various factors [15]. In general, the



before incubation	RPMI	STEM	c-c 1301	t 1301	c-cK562	t K562	c-c SK-mel37	t SK-mel37
5.73	10.19	8.39	10.13	9.97	9.505	27.77	7.355	10.29
26.54	24.17	37.65	35.96	21.23	36,35	17.58	31.28	27.94
2.065	0.595	2.9	2.175	1.754	1.385	0.865	1.08	2.18
69.64	50.03	50.76	52.26	65.3	53.69	62.26	43.71	49.39

Fig. 4. Relative number of hematopoietic stem cell progenitors co-cultured with the tumor cell lines 1301, K562, and SK-mel37 in the co-culture (c-c) and transwells (t) for 72 h. Friedman test, significant differences $p < 0.05$, data are provided as median and interquartile range; * — significant differences from the control RPMI, t K562; ** — significant differences compared to all test groups, $p < 0.005$; ! — trend compared to the t K562, $p = 0.06$; # — trend compared to the control before incubation and RPMI, $p = 0.05$

HSC counts and differentiation depend on the fact, whether HSC interact with tumor cells directly or their interaction is mediated by humoral factors. The common HSC differentiation pattern was reported for 1301 and K562, most likely because both tumor types are clones of hematopoietic cells in origin. It should be also noted that the shift towards granulocyte-monocyte cells is associated with high risk of metastasis [16], which is generally typical for leukemia. However, HSCs cultured with a solid tumor, melanoma, showed a slightly different cell ratio with the decreased number of common progenitors of myelopoiesis and increased number of multipotent progenitors (p = 0.05). It is interesting that, according to the literature, in solid tumors HSC differentiation is directed not only towards myeloid cells, specifically myeloid suppressors [17], but also towards less differentiated cells with preserved multipotency [18], which is consistent with our data.

CONCLUSIONS

Proliferative activity, cell division and differentiation change depending on the tumor type. The K562 and 1301 leukemia tumor cell lines affect viability and differentiation of hematopoietic stem cells in the same way, while melanoma SK-mel-37, the solid tumor, has a different effect on the same processes, which teaches us about both isolated and common patterns of the tumor influence on the vital activity of the hematopoietic stem cell. For better understanding of the impact of tumors on the HSC properties it is necessary to use a more comprehensive approach involving assessment of the cell secretome, recruiting markers, and association with the tumor process, and to use more complex and relevant methods to co-culture cells taking into account the complex, dynamic, and heterogeneous structure of tumor microenvironment.

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