Innovative Approaches to Inactivation of Cancer Stem Cells in Experimental Ehrlich Carcinoma Model

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Received Date: 01 December, 2016; Accepted Date: 31 January, 2017; Published Date: 8 February, 2017

Abstract

One of the tasks of current oncology is identification of Cancer Stem Cells (CSCs) and search of therapeutic means capable of their specific inhibition. The paper presents the data on phenotype characteristics of Ehrlich Carcinoma (EC) cells as convenient and easy to follow model of tumor growth. The evidence of CSCs as a part of EC and significance of CD44+ and CD44− subpopulations in maintaining the growth of this type of tumor were demonstrated. A high (10-fold) tumorigenic activity of the EC CD44+ cells if compared to CD44− cells was proven. In this pair of comparison the CD44+ cells had a higher potential of generating in peritoneal cavity (PC) of CD44high, CD44+CD24−, CD44+CD24+ cell subpopulations, highlighting the presence of CSCs in a pool of CD44+ cells.

In this study the ability of synthesized hybrid nanocomplexes, comprising the nanoparticles of rare earth orthovanadates GdYVO4: Eu3+ and cholesterol, to inhibit the tumor growth and to increase the survival of the animals with tumors was established. A special contribution in tumor-inhibiting effect is made by each of its components. Treatment of EC cells with two component hybrid complex resulted in maximum reduction in the concentration of the most tumorigenic CD44high cells with simultaneous rise in the number of CD117+ cells, that decreased an intensity of tumor growth by 74.70±4.38% if compared with the control.

Keywords: Cancer Stem Cells; Ehrlich Carcinoma Cells; Nanocomplexes

Introduction

The problem of malignant growth remains one of the most urgent in medicine. In recent decades, there has been some progress in developing new treatments for cancer. This is due to the revision of the classical concept of cancer, and the discovery of Cancer Stem Cells (CSCs), which are capable of unlimited self-renewal and can be identified by a number of phenotypic markers. Most of these cells are resistant to radio- and chemotherapies, causing relapse of the malignant growth and metastasis. There are mastered the new methods of anticancer therapy, namely allowing selectively inactivate tumor cells with minimal damage to normal tissue [1].

The CSCs were for the first time identified and described in 1997 by M. Dick team [2]. The authors investigated an acute myeloid leukemia wherein the subpopulation being 0.01-1% of the total population of cells could cause leukemia when transplanted to the immunodeficient NOD/SCID (Nonобese Diabetic-Severe Combined Immunodeficiency) mice. These tumor-inducing cells were phenotypically characterized as the CD34+CD38-. In 2003, M. Al-Hajj and M.S. Wicha succeeded to identify the CSCs in a solid form of Human Breast Cancer (BC) [3]. It has been found that the unseparated population of primary breast cancer exhibited a tumorigenic potential in 100% of cases (10/10) when administered to NOD/SCID mice at a concentration of 5x10⁴ cells/mouse. Reducing the concentration of cells administered down to 1x10³ cells/mouse diminished their tumorigenic activity 4 times (3/12) [3]. CD24+CD44+ fraction when administered at various...
doses (2x10^4 down to 100 cells/mouse) did not allow the tumor growth. Herewith CD44^+ CD24^low subpopulation possessed significantly higher tumorigenic activity, demonstrating the formation of tumors in 100% of cases when administered 103 cells / mouse. The most pronounced ability to form tumors was inherent to the subpopulation of CD44^+ CD24^low ESA^+ phenotype. Administering to mice just 200 of these cells resulted in the formation of solid tumors in 100% (4/4) 5 months later their injection [3]. These studies were continued by Ponti D. et al., who showed the ability of certain populations of breast cancer biopsy samples to form the mammospheres in vitro in serum-free culture [4]. Most of the cells of the obtained mammospheres were of CD44^+ CD24^low phenotype as well as an increased tumorigenic potential in vivo when administered to SCID (Severe Combined Immunodeficiency) mice. The capability of forming tumor in this subpopulation was 1,000 times higher versus that for traditionally transplanted line of breast carcinoma MCF7 [4]. However, the authors have shown that only 20% of CD44^+ CD24^low cells had the capacity for self-renewal. This may be due to heterogeneity of this subpopulation, namely, the presence of additional markers (ESA, ALDH), determining the function of cells and also can be related to the CD44 expression rate. The papers published during the last two years show that the CSCs with a high expression of the marker (CD44^high) have the highest tumorigenic activity [5,6]. In orthotopic implantation of 5×10^5 CD44^high-RAS-transformed and CD44^low_ cells to NOD/SCID mice, it has been found that a subpopulation CD44^low_ possessed low tumorigenicity (tumor was formed in 30% of cases), while the CD44^high_ cells were capable of forming tumors in 100% of cases [6].

Summarizing the published data the differentiation row of subpopulations of breast cancer cells can be represented as follows:

CD44^high_ → CD44^+ CD24^− → CD44^+ CD24^+ → CD44^+ CD24^+

A number of cells bearing other markers, particularly Sca-1+ claims the CSCs stage. The data on a reduction of tumor growth in the Sca-1 knock-out mice indicate in favor of the hypothesis of tumor-initiating role of Sca-1+ cells at an early stage of tumorogenesis [7]. Recently much more attention of the researchers has been attracted not only by CSCs, but also the cells making their accessory-regulatory microenvironment. The CD117^+ cells, which are traditionally detected in a pool of blood stem cells deserve a particular attention among them [8]. The total population of human breast carcinoma cells comprises the so-called carcinoma-associated fibroblasts of stroma with CD117^+ phenotype. They support tumor growth, promoting its angiogenesis [9, 10]. An assumption of the presence in Ehrlich Carcinoma (EC) population of stem cell, study of tumorigenic potential of CD44^+ fraction and role of CD117^+ cells in maintaining the tumor development requires an extra evidence.

Most of the experiments to passage CSCs in vivo were performed in SCID or NOD/SCID mice. These mice do not respond with an immune reaction to xenotransplantation of human cells. The search for adequate and relevant experimental models to study and assess the antitumor activity of various therapeutic agents is in progress. One of them is the in vivo transplanted tumor cell line of EC, which was obtained from a spontaneous breast cancer of mice [11]. However, there are virtually no publications as for subpopulation composition of EC cells and their phenotypic characteristics, presence of CSCs and their importance in maintaining the growth of this type of tumor. Taking into account histogenetic similarity of EC and BC it can be assumed that in initiation and development of the simulated tumor the same genes controlling proliferation of cancer cells can be involved as well as similar biochemical pathways leading to the expression of tumor marker proteins. However, the supposition on the presence of CSCs in the EC population and study of their tumorigenic potential needs additional evidence, which was one of the objectives of the present study.

No less urgent problem of current oncology is to find the drugs, not only specifically recognizing, but also inactivating the CSCs. The very this concept of understanding of the problem was the basis formed in the direction of the moment “theranostics” (therapy+diagnosis). Within the frames of “theranostics” there are developed the technological approaches of using the medicines and tools of simultaneous diagnosis and therapy of cancer. The successful solution of this problem contributes to the rapid development of nanobiotechnology, namely the use of nanoparticles (NPs) of rare-earth metals (such as vanadium and its compounds). So, it has been shown that a vanadium dichloride can significantly inhibit cell proliferation as a result of accumulation in nuclear heterochromatin with subsequent induction of mitotic aberrations transient suppression of mitoses, leading to accumulation of cells in late S and G2 phases [12]. Promising for the treatment of malignant tumors can be the use of hybrid nanocomplexes based on rare-earth based NPs of orthovanadates GdYVO_4: Eu^{3+} and cholesterol, developed at the Institute for Scintillation Materials of the National Academy of Sciences of Ukraine [13].

The purpose of their creation was to enhance a therapeutic effect of anticancer agents due to the presence in the composition of nanocomplexes having an affinity for the target cell membranes. One is cholesterol which is actively “withdrawn” from the bloodstream by proliferating cancer cells to build the biomembranes. This is facilitated by the presence on the surface of a large number of tumor cells SR - B1 (scavenger receptor, class B type 1) and caveolin-1 (Cav-1) receptors, which can bind with the free bloodstream cholesterol [14].

Thus, the aim of this work was to identify the subpopulation
composition of EC cells, including those with the signs of CSCs, as well as their tumorigenic activity after pretreatment with hybrid nanocomplexes.

**Materials and Methods**

The experiments were performed in 8-month-old female Balb/C mice. All the procedures for animal maintenance and euthanasia were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine (Rec. №4 of 06.10.2015) and conformed to the European Convention on the Use of Experimental Animals (Strasbourg, 1986), approved by the First National Congress of Ukraine in Bioethics (Kiev, 2004).

**Culturing of EC cells in vivo** Ehrlich Carcinoma (EC) cells were passaged in the Peritoneal Cavity (PC) of Balb/C mice. The cryopreserved in ascitic fluid EC cells were used as a primary culture [15].

After thawing the EC cells were three times transplanted in vivo, to the mitigation of an influence of the factors of freeze-thawing and gaining by them of morphological and functional features of native cells [16]. “Stabilized” thereby EC cells were intraperitoneally injected in a dose 3x10^6 cells/mouse in 0.3 ml saline and cultured for 7 days in vivo. After 7 days, the experimental animals were removed from the experiment under light ether anesthesia. Ascitic fluid from PC was taken by a syringe through a needle of 2.69 mm inner diameter and placed into 10 ml measuring tube. Absolute cell number was determined by the volume of accumulated ascitic fluid therein with recording the number of EC cells counted in the Goryaev chamber. An increase in total number up to 35.00x10^7 EC cells in PC of mice to day 7 was a criterion of carcinoma development [16]. In future, the very these cells served as the object of study.

**Phenotypic assessment of EC subpopulations** was performed with a flow cytometer «FACS Calibur» («Becton Dickinson», USA) using monoclonal antibodies (US «BD Biosciences») to CD44 (FITC) (number 553133, clone IM7), CD117 (FITC) (№ 553352, clone 2B8) and Sca-1 (FITC) (№ 553333, clone E13-161.7) and CD24 (PE) (№ 553262, clone M1/69). As a control, the samples with adding nonimmune FITC- and PE-labeled monoclonal antibodies of the same isotopes («BD Biosciences»), (№ 553988, clone A95-1 and № 553989, clone A95-1), as antibodies to the tested marker were used. An immunophenotypic double staining was performed using CD44 (FITC) and CD24 (PE) monoclonal antibodies. The cells with an average fluorescence of CD44 marker higher 10^3 (according to a logarithmic scale) were referred to CD44^high subpopulations. Recording and analysis of the results were performed with “WinMDi 2.9” software (Joseph Trotter, La Jolla, USA).

**Separation of CD44^+ Fraction of EC Cells Using Immunomagnetic Sorting**

Keeping in mind that the CSCs with a high expression level of the CD44 marker (CD44^high), comprised by heterogeneous population of CD44^+ cells, possess the highest tumorigenic activity, it was isolated from a total EC population with a magnetic sorter (BDTM Imagnet). To isolate CD44^+ fraction there were used primary unlabelled monoclonal antibodies for marker CD44 (BD, 558739) and secondary Mouse IgG1 Magnetic Particles-DM (BD, 557983) according to the manufacturer’s protocol. Separation purity for CD44^+ cells from total EC population was 90%.

To determine the tumorigenic ability of cells of total population and isolated CD44^- and CD44^- fractions of EC there was used the described above method of culturing in vivo. The experimental setup is shown in Figure.
In the first set of experiments we evaluated the tumorigenic ability of total population and isolated CD44+ and CD44- EC fractions when administering them to animals in a standard dose used for EC initiation (3x10^6 cells in 0.3 ml saline).

Animals were divided into the following groups (n = 10):
- Group 1.1-administering of total population of EC cells (3x10^6 cells / animal)
- Group 2.1-administering of CD44+ fraction of EC cells (3x10^6 cells / animal)
- Group 3.1-administering of CD44- fraction EC cells (3x10^6 cells / animal)

In 7 days after the inoculation in each of the experimental groups an absolute amount of cells in PC of animals was counted, phenotypic characteristics of cells were assessed (as described above) and CD44^{high}/CD117^{+} ratio of EC cells, determined as the ratio of CD44^{high} percentage to CD117^{+} cells [17]. Proliferative potential of cells of total EC population and isolated CD44+ and CD44- fractions was estimated basing on the data as follows: Multiplicity Factor (MF) of cell population surplus during culturing time: $M = N/N_0$; and time doubling (TD):

$$TD = (\log_2)^t \frac{1}{\log_2 \left( \frac{N}{N_0} \right)}$$

where $t$ is the time of cell culturing (hrs), $N$ is the number of cells at t time; $N_0$ is initial cell number [18].

In the second set of experiments there was estimated a minimum dose of the administered cells of total population and isolated CD44+ and CD44- EC fractions, inducing tumor growth. Total cell suspension and isolated CD44+ and CD44- EC fractions were intraperitoneally administered to mice at the doses of 3x10^6, 3x10^5, 3x10^4 and 3x10^3 cells per mouse in 0.3 ml saline and cultured for 7 days in the PC.

Animals used in this set of experiments have been divided into the following groups (n = 10):
- Group 1.1-administering of total population of EC cells (3x10^6 cells / animal)
- Group 1.2-administering of total population of EC cells (3x10^5 cells / animal)
- Group 1.3-administering of total population of EC cells (3x10^4 cells / animal)
- Group 1.4-administering of total population of EC cells (3x10^3 cells / animal)
- Group 2.1-administering of CD44+ fraction of EC cells (3x10^6 cells / animal)
- Group 2.2-administering of CD44+ fraction of EC cells (3x10^5 cells / animal)
- Group 2.3-administering of CD44+ fraction of EC cells (3x10^4 cells / animal)
- Group 2.4-administering of CD44+ fraction of EC cells (3x10^3 cells / animal)
Group 3.1-administering of CD44+ fraction EC cells (3x10⁶ cells / animal)  
Group 3.2-administering of CD44+ fraction EC cells (3x10⁵ cells / animal)  
Group 3.3-administering of CD44+ fraction EC cells (3x10⁴ cells / animal)  
Group 3.4-administering of CD44+ fraction EC cells (3x10³ cells / animal)  

In every experimental group an absolute number of cells in PC and that of animals with ascites development were determined 7 days later the EC inoculation.

**Synthesis of Nanocomplexes**

Hybrid nanocomplexes containing spherical nanoparticles (NPs) (of 2-3 nm diameter) at a concentration of 1.30 g/l and sheep cholesterol in concentration of 0.55 g/l («Acros organics», Belgium) were synthesized at the Institute for Scintillation Materials of the National Academy of Sciences of Ukraine (Kharkiv) according to the methods [13]. NPs based on orthovanadates of rare earth elements GdYVO₄: Eu³⁺ of spherical form in a concentration of 1.30 g/l were prepared as described by [19]. Aqueous colloidal solutions based on orthovanadates have been purified of impurities by dialysis using the membranes “Cellu Sep H1” 3.5 KDa.

In hybrid nano complex the negatively charged NPs are localized along the periphery of cholesterol particles due to van der Waals and hydrophobic interactions, play the role of stabilizer due to electrostatic interactions and provide nanocomplexes of not more than 100 nm. In addition, the NPs exhibit antioxidant properties and are not subjected to oxidation. This fact contributes to the rise in resistance of aqueous dispersion of cholesterol in relation to the reactive oxygen species. Schematic structure of hybrid nanocomplex is shown in Figure 2.

![Figure 2: Hybrid nanocomplex](a) schematic representation  
(b) transmission electron microscopy photomicrography of hybrid nanocomplexes, procured from cholesterol aqueous solution placed on carbon network.

To record an accumulation of hybrid nanocomplexes in cells during the studies *in vitro*, hydrophobic fluorescent dye 1,1’-dioc-tadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) could be additionally introduced into of cholesterol aqueous dispersion, allowing in local luminescent spectroscopy to evaluate the dynamics of integration of the complex into a cell membrane by the ratio of the monomer – «J-aggregate” luminescence bands [20]. Our earlier studies have shown that hybrid nanocomplex is able to be integrated not more than into 10% of cells of total EC population and virtually to all the cells of isolated CD44+ fraction having the highest carcinogenic potential. This allows the use of hybrid nanocomplex in this modification (NPs + cholesterol + DiI) as a method of identifying local accumulation of nanocomplexes in cancer cells [21,22].

**Pre-Treatment of EC Cells with Nanomaterials**

Total suspension of EC cells with hybrid nanocomplexes or NPs was incubated in a solution of 5% glucose (“Infusion” CJSC, Kyiv) at room temperature for 3 hrs. Such an incubation time was previously found as an optimal for binding nanocomplexes to cells [21].

The following variants of EC cells pre-treatment with nanomaterials were tested:

Variant 1-to 900 µl of EC cells (1x10⁷) 100 µl of spherical NPs (1.3 g/l) were added  
Variant 2-to 900 µl of EC cells (1x10⁷) 100 µl of hybrid complex (spherical NPs (1.3 g/l) + cholesterol (0.55 g/l)) were added  
Control was the cells of EC total population, which were incubated in a solution of 5% glucose with no treatment with nano composites. The number of animals in each experimental group...
was not less than 20.

After incubation, the EC cells of all the tested groups were washed three times with saline (1:1) by centrifugation (10 minutes at 300 g).

Intensity of EC development after pretreatment with nanomaterials was evaluated by intraperitoneal injection in a dose 3x10⁶ cells in 0.3 ml saline. In 7 days after EC cell inoculation all the studied groups were determined:

- an absolute number of cells in PC
- inhibition rate (Ri) of EC growth according to the formula: 
  \[ Ri = \frac{(V(c) - V(e))}{V(c)} \times 100\% \], where \( V(c) \) – absolute number of EC cells in PC of the control group, \( V(e) \) – absolute number of EC cells in PC of the experimental group (variants 1-2);
- growth rate (Rg) of EC was calculated using the formula: 
  \[ Rg = \frac{(c)}{\text{Ri}} \], where \( Rg \) – growth rate of tumors of experimental group of animals; \( Rg(c) \) - growth rate of tumor of the control group, \( Ri \) - inhibition rate of EC growth in experimental group of animals;
- \( CD44^{\text{high}}/CD117^{\text{+}} \) ratio (ratio of \( CD44^{\text{high}} \) percentage to \( CD117^{\text{+}} \) cells)
- Animal survival was assessed to day 20 after intraperitoneal injection of untreated and treated with nanocomposites EC cells.
- Statistical processing was performed using non-parametric U-Mann-Whitney test in Statistica 6.0.
- Software Differences were considered statistically significant at P <0.05.

Results

The obtained results indicate the presence of a heterogeneous population of EC cells with signs of CSCs of various differentiation levels and those could be attributed to accessory-regulatory elements of microenvironment. The concentrations of cells with these characteristics in total EC pool (group 1.1) are shown in Table 1 and are completely consistent with the previous findings on EC subpopulation composition [23].

The most informative in terms of phenotypic identification of CSCs is CD44 molecule expression, which either itself or in combination with other surface markers are used to isolate this cell population from various tumors, including EC. The most important role in implementing a tumorigenesis is played by an expression rate of the molecule. Indeed, in contrast to leukocytes for adhesion of those normally a low expression rate of CD44 receptor is required, triggering and self-maintenance in CSCs are implemented its much greater density on a cell surface [24]. It was previously found that a minor subpopulation of CD44^{\text{high}} cells has a high proliferative potential and plays a critical role in EC developing [15].

It is known that CD44- glycoprotein is a hyaluronic acid (HA) receptor, a main component of extracellular matrix. The emerging set of HA-CD44 activates many receptor tyrosine kinases, resulting in activation of PI3K/Akt/ mTOR way [25,26], which plays the role of a single universal signal transmission mechanism to the translation apparatus and is responsible for the integration of proliferative stimuli.

Among two known CD44 isoforms in normal hematopoietic cells its standard isoform (CD44s) are predominantly expressed [27]. In most malignant tissues there were detected both CD44s and variable isoforms of CD44+ molecule (CD44+), resulting from alternative splicing of exons 6-15. Namely alternative splicing leads to a lengthening of CD44 extracellular domain, promoting its greater interaction with HA and tumor metastasis [28].

According to the classical concepts, tumor differentiation is accompanied by a reduced intensity of CD44 expression with its gradual disappearance and appearance of the cells expressing CD24 [3]. These changes of phenotypic markers are believed to cause the tumor expansion by enhancing the “cell-to-cell” and “cell-to-extracellular matrix” interactions.

With the development of breast cancer in its classical manifestation the interaction of tumor cells with P-selectin via CD24 is one of the most important ways of their adhesion and ensures metastasis by promoting tumor dissemination in the sites with more favorable conditions for its development [29]. The research performed by Shipitsin M et al. has shown that CD44+ and CD24+ cells in breast cancer development are cell populations with different genetic profiles [30]. CD24+ cells have been noted to be more differentiated, while more progenitor-like functions are inherent to CD44+ cells. The authors suggest that CD24+ cells can be derived from CD44+ cells [30]. Fillmore C. and Kuperwasser C. supposed that CD24+ population was mainly characterized by less differentiated basal type of breast cancer, and CD44+ cells caused the development of luminal form of breast cancer, being more differentiated type of tumor [31].

Our hypothesis about the dependence of EC on functional activity of a subpopulation of CD44+ cells was tested when evaluating the intensity of tumor growth induced by CD44+ and CD44 fractions and EC total population. Table 1 demonstrates that the highest tumor-inducing activity was inherent to the cells of CD44+ fraction. Actually, after administering 3x10⁶ CD44+ cells (group 2.1), an absolute number of cells in PC was 23 times higher than after that of total population of EC cells (group 1.1), and 105 times more than when CD44 fraction was introduced (Group 3.1).
Here with there were found the changes of not only quantitative but also qualitative compositions of a developing tumor. The fraction of CD44+ formed the ascites with a predominant content of CD44+ cells, i.e. CD44high, CD44’CD24- and CD44+CD24+ cells. Besides, the concentration of CD44high cells was 2 times higher if compared with group 1.1 and 16 times higher in group 3.1. The fraction of CD44+, in contrast, formed a tumor which contained more mature cells, namely, those with CD44+CD24+ phenotype. The very this redistribution of subpopulation composition of cells in group 3.1 apparently determined the minimum absolute content of cells in the PC.

Sca-1 is known as a marker of stem cells [32], including breast cancer progenitors [33]. In murine models of breast cancer it has been shown that the expression rate of the marker by cells is determined by an animal genetic profile. It has been established [34] that in transgenic mice with a spontaneous development of breast tumors the expression of Sca-1 exceeded 3 times that of healthy animals due to the activation of an oncogene Wnt, while when activating MMTV-neu oncogene the presence of Sca-1 was inherent only for 6-10% of the cells [34]. The data presented in Table 1 indicate that in EC development almost all the cells are the carriers of Sca-1+ marker.

Analyzing the patterns of tumor development, the classic hypothesis of “seed and soil” looks very actual [35], which postulated that an appropriate microenvironment (soil) is required for optimal growth of tumor cells (CSCs). Most often the carcinoma-associated fibroblasts (CAFs) act as a tumor stroma in breast cancer and pancreatic cancer [36]. It has been shown that the CAFs, derived from invasive forms of human breast carcinomas, activated much stronger the growth of human breast cancer cell line MCF-7-Ras when administered to immunodeficient mice if compared with normal fibroblasts [9]. This function is implemented by the microenvironment cells due to the secretion by them of cytokines, chemokines and growth factors [10,37]. Although so far the phenotypic identification of these cells has remained a subject of debate, most often used for this purpose the surface markers of primitive hematopoietic and endothelial cells, including c-kit (CD117), CD133, VE-cadherin, VEGFR-2 and endoglin are used [38].

The established by us dependence of tumor growth rate on the presence/absence of CD117+ cells and their concentration relationship with CD44high cells makes sense. Previously, after analysis of the significance of the content ratios for different subpopulations of EC cells when maintaining tumor growth, we proposed to use the CD44high/CD117+ ratio as a prognostic criterion of tumor development [17]. As Table 1 shows, when initiating the EC by introducing the total cell population (group 1.1.) in the PC there were formed 34.80 ± 1.27x106 cells at the CD44high/CD117+ ratio, which was equal to 0.02 relative units.

Tumorigenic potential of CD44+ fraction was 4 times lower (group 3.1) that was manifested by a reduced CD44high/CD117+ ratio to the same extent (4 times) if compared with group 1.1. This change in CD44high/CD117+ index was mainly due to a decrease in CD44high concentration (in 8.5 times) on the background of the reduced content of CD117+ cells (in 2 times) as well.

### Table 1: Absolute number of cells in peritoneal cavity and their phenotypic parameters to day 7 after administration of total population and isolated CD44+ EC fractions.

<table>
<thead>
<tr>
<th>Index</th>
<th>Group 1.1. administration of total population of EC cells (3x10⁶ cells/animal)</th>
<th>Group 2.1. administration of CD44+ EC cell fraction (3x10⁴ cells/animal)</th>
<th>Group 3.1. administration of CD44- EC cell fraction (3x10⁷ cells/animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44high</td>
<td>0.17±0.03</td>
<td>0.32±0.05*</td>
<td>0.02±0.002*,**</td>
</tr>
<tr>
<td>CD44’CD24-</td>
<td>3.78±0.51</td>
<td>5.65±0.41*</td>
<td>3.81±0.28**</td>
</tr>
<tr>
<td>CD44+CD24-</td>
<td>2.89±0.22</td>
<td>4.21±0.50*</td>
<td>2.98±0.18**</td>
</tr>
<tr>
<td>CD44 CD24-</td>
<td>5.33±0.64</td>
<td>2.00±0.15*</td>
<td>8.50±0.59*,**</td>
</tr>
<tr>
<td>Sca-1+</td>
<td>90.32±5.33</td>
<td>89.05±4.35</td>
<td>81.30±6.41</td>
</tr>
<tr>
<td>CD117+</td>
<td>7.81±0.83</td>
<td>-</td>
<td>3.56±0.06*</td>
</tr>
<tr>
<td>CD44high/CD117+ ratio (arb.units)</td>
<td>0.02</td>
<td>-</td>
<td>0.005</td>
</tr>
<tr>
<td>Absolute number of cells in the peritoneal cavity (x10⁶)</td>
<td>35.80±2.27</td>
<td>826.5±6.53*</td>
<td>7.82±0.94*,**</td>
</tr>
<tr>
<td>MF (arb.units)</td>
<td>116.67±9.60</td>
<td>2755±9.34*</td>
<td>26.07±1.84*,**</td>
</tr>
<tr>
<td>TD (hrs)</td>
<td>24.47±2.75</td>
<td>14.70±1.35*</td>
<td>35.71±2.53*,**</td>
</tr>
</tbody>
</table>

Note: the indices are determined to day 7 after administration of total population and isolated CD44+ EC fractions; EC – Ehrlich carcinoma; MF – multiplicity factor; TD – time doubling; (*) differences are statistically significant if compared with group 1.1; (**) differences are statistically significant if compared with group 2.1 (P<0.05).
When evaluating the intensity of ascites growth, generated by CD44+ fraction, a significant increase in absolute number of cells in PC (almost 24-fold if compared to group 1.1) was noted. As well an important is the excess in two times of CD44high concentration and lack of CD117+ cells. In initial material of CD44+ fraction (prior to culturing) according to flow cytometric analysis of the data the content of CD44high cells was 15 times higher than in total population of EC cells (data not presented).

To summarize the mentioned above, we may argue that a crucial role in the initiation of EC is played by CSCs with a high expression rate of CD44 marker (CD44high), while one of the most important functions of CD117+ subpopulation is a regulation (“restricting”) of tumorigenic activity of CD44high cells. The absence of CD117+ cells (group 2.1) seems to multiply the proliferative and differentiation potential of the entire pool of CD44+ cells, causing a significant rise in total number of cells in the PC.

Analysis of proliferative potential of a total pool of EC cells and CD44+ fraction favors this interpretation. It is shown that the multiplication factor (MF) in the overall population cultured in the PC in group 2.1 during 7 days increased almost in 24 times if compared with group 1.1. This was accompanied by a decrease in cell doubling time from 24.47 ± 2.75 hrs in group 1.1 to 14.70 ± 1.35 in group 2.1 that may characterize a population of ascites cells grown from CD44+ fraction, as more actively proliferating one (Table 1).

To prove the special role of CD44+ cells in initiation and maintenance of tumor when administered EC even in minimal doses, it was of interest to comparatively assess the tumorigenic capacity of isolated CD44+ and CD44+ fractions when administered at various concentrations. It has been found that after the introduction of 3x10⁶ cells of total EC population, tumor growth was observed in 100% of animals (10/10) (Table 2). Reducing 10 times the dose of cells administered (3x10⁵) resulted in a proportional decrease in absolute number of cells in the PC, tumor developed only in 50% of animals (Table 2). Reducing the administered dose of total EC population of cells down to 3x10⁴ did not lead to tumor formation in the PC.

Initiations of EC by introducing of CD44+ cells at concentrations of 3x10⁶ and 3x10⁵ cells per animal resulted in almost 100% tumor development for both cases. Herewith tumorigenic potential of CD44+ fraction exceeded that of total population of EC cells administered in the same doses (in 23 and 21 times, respectively). Moreover, introduction of 3x10⁴ cells of CD44+ fraction caused a tumor formation in 33% of animals, while total population of EC cells used in the same dose, did not cause the formation of ascites. With the introduction of 3x10³ cells of CD44+ fraction no animals with the developed EC have been identified.

Fraction of CD44+ cells just in a dose of 3x10⁵ was capable of forming tumors in 50% of animals, the number of cells in the PC in this case was 4.5 times less than when introducing the total population and in 105.9 times less than when inducing by CD44+ fraction. Thus, the results of this part of research suggest that CSCs are mainly present in the pool of cells with CD44+ phenotype. This emphasizes the importance of this subpopulation of cells in initiation and development of EC.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Absolute number of EC cells in the peritoneal cavity (x10⁶) when administrating under various concentrations</th>
<th>Number of animals with EC development to day 7 after administration of various concentrations of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3x10⁶</td>
<td>3x10⁵</td>
</tr>
<tr>
<td>admin. of total pop. of EC cells</td>
<td>10/10</td>
<td>5/10</td>
</tr>
<tr>
<td>admin. of CD44+ EC cell fraction</td>
<td>826.51±6.53*</td>
<td>65.00±1.27*</td>
</tr>
<tr>
<td>admin. of CD44+ EC cell fraction</td>
<td>7.82±0.94*</td>
<td>-</td>
</tr>
</tbody>
</table>

**Note:** number of animals with EC development was calculated by considering the number of animals with evident EC degree to day 7 (absolute number of cells in the peritoneal cavity was not less than 35.00x10⁶), correlated to the number of animals in each group (n = 10); differences are statistically significant as compared with administration of total population of EC cells (*); (P<0.05).

**Table 2:** Tumorigenic activity of total population, isolated CD44+ and CD44+ EC fractions.

As noted above, identification and inactivation of CSCs is a major theoretical and practical issue of oncology. On this basis, the next task of our study was to investigate the impact of hybrid nanocomplexes designed at the Institute for Scintillation Materials of National Academy of Sciences of Ukraine on the tumorigenic activity of EC cells.

As Table 3 demonstrates an incubation of EC cells with only NPs as a component of hybrid nanocomplexes (Variant 1) decreased the concentration of CD44high virtually twice if compared to the control and 5 times the content CD44+CD24+ cells in ascites formed in vivo. The number in it more differentiated CD44+, CD44+CD24+ cells remained practically unchanged.
if compared to the control. In this group there was established a reduction of CD117+ cells (35%) at a slightly changed content of Sca-1+ subpopulation. Based on the data, the inhibition rate of EC growth (59.41 ± 3.45%) in Variant 1 was accompanied by a two-fold decrease in the concentrations of CD44high cells in comparison with the control that was also reflected in the reduction of CD44high / CD117+ ratio (Table 3).

<table>
<thead>
<tr>
<th>Index</th>
<th>Pre-treatment variants</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of cells with corresponding phenotype, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD44high</td>
<td>0.09±0.01*</td>
<td>0.02±0.001*, **</td>
</tr>
<tr>
<td>CD44CD24+</td>
<td>0.75±0.05*</td>
<td>0.40±0.02 *, **</td>
</tr>
<tr>
<td>CD44CD24−</td>
<td>2.75±0.28</td>
<td>9.77±0.62 *, **</td>
</tr>
<tr>
<td>CD44CD24</td>
<td>6.18±0.32</td>
<td>7.85±0.44 *</td>
</tr>
<tr>
<td>Sca-1+CD117</td>
<td>81.82±5.28</td>
<td>83.77±5.73</td>
</tr>
<tr>
<td>CD44high/CD117+ ratio (arb.units)</td>
<td>5.04±0.32</td>
<td>10.47±0.83 *, **</td>
</tr>
<tr>
<td>Absolute number of cells in the peritoneal cavity (x10⁷)</td>
<td>14.53±0.72 *</td>
<td>9.06±0.25 *, **</td>
</tr>
<tr>
<td>Inhibition rate of EC growth (Ri) (%)</td>
<td>59.41±3.45</td>
<td>74.70±4.38 **</td>
</tr>
</tbody>
</table>

Note: number of animals with EC development was calculated by considering the number of animals with evident EC degree to day 7 (absolute number of cells in the peritoneal cavity was not less than 35.00x10⁷), correlated to the number of animals in each group (n = 10); differences are statistically significant as compared with administration of: the Control (*); Variant 1 (**)(P<0.05).

Table 3: Change in composition and functional activity of in vivo cultured EC cells after pretreatment with hybrid nanocomplexes.

Pretreatment of EC cells with hybrid nanocomplexes (Variant 2), reduced almost 10 times the concentration of CD44high and CD44CD24− cells in the developed ascites if compared to the control (Table 3). It should be noted that the concentration of more differentiated CD44CD24+ and CD44CD24− cells after this treatment increased slightly if compared to the control. The redistribution pattern of EC subpopulation composition in this option was accompanied with a pronounced enhancement of tumor growth inhibition of compared to Variant 1 (74.70 ± 4.38 and 59.41 ± 3.45%, respectively, p<0.05), that underlined the importance of cholesterol as a targeted compound of antitumor therapy. It is noteworthy that in this option of pretreatment there was found a maximum reduction of CD44high / CD117+ ratio (10 times as compared with Variant 1, that again confirmed a specific role of ration of these cell subpopulations in the EC growth.

The main component in manifesting an antitumor effect of the synthesized hybrid nanocomplexes are obviously, spherical NPs. Introduction of cholesterol having affinity to tumor cell membranes into composition of hybrid nanocomplexes enhanced an inhibitory activity of NPs. Similar data were obtained by Betker J.L. et al. after analysis of the structure and functioning principles of the membranes of tumor cells [39]. The authors concluded that the incorporation of cholesterol into membranes of tumor cells could be a prerequisite for a targeted delivery of liposomes with therapeutic agents directly into a cell. In this case, many cells of total EC pool could serve as such a target (both direct and indirect), but first and foremost could be those with CD44high phenotype, as the most potent CSCs forming an entire subsequent row of advanced in differentiation tumorigenic cells.
A significant decrease in the actual concentrations in a growing pool of EC after pretreatment with hybrid nanocomplexes clearly coincided with a reduced tumor growth rate. As well a crucial role in determining the nature of EC growth is also played by the ratio of CD44$^{high}$ and CD117$^+$ cell subpopulations. For all the types of EC pretreatment the reduction of CD44$^{high}$/CD117$^+$ ratio was accompanied by a decrease in tumor growth rate and increased survival of animals to day 20 of EC development (Figure 3).

Conclusions

1. On the base of the findings of phenotypic assessment and functional potential studies the Ehrlich carcinoma is a heterogeneous population of tumor cells of varying differentiation extent referred to high and less potent tumor-inducing precursors, as well as the cells composing their microenvironment.

2. A high (10-fold) tumorigenic activity of the EC CD44$^+$ cells if compared to CD44- cells was proven. In this pair of comparison the CD44$^+$ cells had a higher potential of generating in PC of CD44$^{high}$, CD44$^+$CD24-, CD44$^+$CD24$^+$ cell subpopulations, highlighting the presence of CSCs in a pool of CD44$^+$ cells.

3. There was found an ability of the synthesized nanocomplexes based on rare earth orthovanadates and cholesterol to inhibit the growth of CD44$^+$ cell pool (CD44$^{high}$, CD44$^+$CD24-, CD44$^+$CD24$^+$), that was accompanied by a reduced intensity of EC growth (by 75%) and increased survival of the animal with tumors (in 3.5 times) in comparison with the control.

4. It has been shown that the reduction in tumor growth rate after pretreatment with hybrid nanocomplexes was accompanied with a change in the composition of EC subpopulation, that was reflected in a decrease in the CD44$^{high}$/CD117$^+$ ratio. This ratio can be offered as one of diagnostic and prognostic tests of the severity and extent of oncology inactivation.

References


