

THE CULTIVATION OF RAT COLON TUMOR WITH PLACENTAL MULTIPOTENT STEM CELLS

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The aim of the research was to assess the impact of rat placental multipotent stem cells on the levels of proliferation and apoptosis in organotypic coculture with slices of colon tumors. Placental multipotent stem cells were isolated by explant method, colon cancer was modeled by administering 20 mg/kg dimethylhydrazine weekly for 20 weeks, tumors of the colon were cut into 500 μm slices. There were no changes in proliferative activity of tumor tissue, or in the level of apoptosis. Absence of influence of placental multipotent stem cells on colon tumor growth is a unique feature of these cells, and extends the application of placental multipotent stem cells in therapy.

Key words: placental multipotent stem cells, colon cancer, organotypic cocultivation.

Colon cancer remains the second abundant oncological disease in Europe [1, 2]. The most acute problem is treatment of late-diagnosed patients. Thus, if colon cancer is diagnosed at an early stage, five-year survival rate is 87–92%, but at late stages — only 11–69% [3]. Therefore, a search for biopreparations capable of influencing the growth of mid- to late-stage tumors, is an urgent problem.

A tumor is a “consequence” of several various genetic disruptions in cells, and so by its nature is heterogeneous. Currently we know such genes, whose mutations are linked to colon cancer: KRAS, APC, DCC/MADH2/MADH4 and TP53 [4]. Regardless of the fact that genetically, cell lines of colon cancer are close to spontaneous human tumors [5], current models of cocultivation do not take into account the whole complex of interactions between different cell types that take place in a heterogeneous tumor. To evaluate the complex of all processes occurring between malignitized cells, intact epitheliocytes, tissue macrophages, lymphocytes, fibroblasts we used organotypic

cultures of rat colon tumors at late stages. A novel approach to treating colorectal cancer is employing mesenchymal stem cells (MSC) [6]. Multipotent stem cells, isolated from mature placenta (MSCP), have properties similar to the properties of MSC of bone marrow [7, 8], but placenta as stem cell source has a number of advantages: obtaining it does not require invasive procedures, the amount of donor material is fully sufficient, no ethical problems. Besides that, experiments on C57/BL6 mice showed that human placenta tissue extract can be used to prevent the growth of melanoma tumors [9]. A heat shock gp96 protein isolated from placenta is also being studied for inoculation against melanoma and breast cancer [10]. Use of placental extract fraction lighter than 3 kDa was patented as an anti-tumor agent and tested on cell lines of hepatoma Hep3B and HepG2, lung cancer H460 and melanoma A2058 [11]. Thus, our goal was to evaluate the influence of MSCP on the level of proliferation and apoptosis in rat colon tumor tissue under direct-contact and non-contact cocultivation.

Materials and Methods

MSCP isolation and cultivation

All animal experiments were performed in accordance with the international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European Convention, Strasbourg, 1986) and article 26 of the Law of Ukraine "On protection of animals from cruelty" (No 3447-IV, 21.02.2006), in addition to following all norms of bioethics and biosafety. The protocol was approved by The Bioethics Committee of The Education and Scientific Centre "Institute of Biology" of Taras Shevchenko National University of Kyiv (Protocol Number 8, 03.04.14).

Pregnant rats were scarified at the 21st day of pregnancy by asphyxiation using carbon dioxide. Placentas were cut out and washed in a sterile Hank's balanced salt solution with 2.5 mkg/ml amphotericin B, 50 mkg/ml streptomycin and 100 units/ml penicillin (Sigma, USA). From that point the tissue was treated in sterile environment. The amniotic and decidual layers were excised. The villous tissue was washed in Hank's balanced salt solution and cut with sterile surgical scissors into fragments of about 1–5 mm³. The fragmented tissue was put into T75 flasks (Sarstedt, USA) with adhesive covers. Over it was poured 25 ml of the whole medium of such composition: DMEM (DMEM–Dulbecco's Modified Eagle Medium) with high glucose content (Sigma, USA) with the addition of 10% fetal bovine serum (FBS) (HyClone, USA), 20 mkl/ml 50 X RPMI-1640 solution of amino acids (Sigma, USA). Primary cultures were cultivated in a CO₂-incubator in humid air with 5% CO₂ at +37 °C. Culture medium was changed twice a week. If the monolayer reached 90% confluence, the cells were treated with 0.05% trypsin and 0.02% EDTA (Biochrom, USA) until they were all dislodged, but not longer than 5 min. The resulting cell suspension was centrifuged at 300 g for 5 min and resuspended-dissolved in the whole medium (5·10³ cells/cm²).

The obtained cultures successfully differentiated in two mesodermal directions and had the CD90+CD44+CD29+/lowCD45-immune phenotype, which supports the multipotency of placental cultures [12].

Modeling colon cancer in rats

To model colorectal cancerogenesis we used two-months-old male albino Wistar rats ($n = 180$) weighing 180–200 g, obtained from the Institute pharmacology and toxicology of NAMS of Ukraine. 1,2-Dimethylhydrazine (DMH) (Sigma, USA) was diluted in physiological solution of NaCl and adjusted to pH 6.5 using 2 M NaOH directly before using. To induce the tumor's development, the rats were injected subcutaneously, once a week for twenty weeks, with 20 mg/kg body mass of DMH in 0.1 ml of physiological solution [13].

Coculture of MSCP with the colon cancer tissue

One day before cocultivation MSCP of the 3rd passage were sown into 7 wells of a 12-well plate at 25·10³ cells/well, and a flask of T75 MSCP of the 2nd passage was left for direct-contact cocultivation. After 24 hr, the rats at the 20th week of colorectal cancer modeling were terminated by asphyxiation using carbon dioxide. Every tumor was cut in halves, one part was fixed in 10% buffered formalin, and the other one was put into 15 ml of sterile culture medium DMEM with high glucose content (Sigma, USA) with 10% FBS (HyClone, USA), cut into slices of 500 mkm thickness using automated chopper (McIllwain, England) in aseptic environment. Slices from every tumor were carefully washed in sterile DMEM medium and 3 slices were placed at three different inserts (Millipore, Germany) with pore diameter 1 mkm. Slices from every tumor were used simultaneously for direct-contact, non-contact culture and for control. Inserts with slices were put into wells of every one of the three 12-well plates: for non-contact coculture the inserts were placed into wells with already sown MSCP; for direct-contact cultivation, to an insert with slices we added 25·10³ MSCP cells; for control, the insert with slices was placed into a well with whole medium. We used 7 different tumors in total. Coculture was kept for 72 hr, changing the whole medium in the wells every 24 hr to provide sufficient nutrition for slices and cells. After this the slices were carefully washed with Hank's solution and fixed for an hour in 10% buffered formalin.

Immunocytochemical analysis of expression of PCNA and caspase 3 in slices

The fixed slices were carried into wells of a 96-wells plate, washed in 1X Dako

Wash salt solution (Dako, Denmark). Non-specific binding was blocked for an hour at room temperature using 0.1 M phosphate buffer containing 1% of normal goat serum (Sigma, USA), 0.3% Triton X-100 and 0.5% bovine serum albumine (Sigma, USA). The blocking solution was poured off and a mixture of primary antibodies was added: Mouse Monoclonal Anti-PCNA (Proliferating Cell Nuclear Antigen) antibody, clone PC10 (ThermoScientific, MS-106-P1, USA) diluted 1:100 and Rabbit Polyclonal Caspase-3 Antibody (Novus Biologicals, NB100-92149, USA) diluted 1:500 in 0.1 M phosphate buffer with 0.3% Triton X-100, incubated at +4 °C for 24 hours. After incubation the preparations were washed from primary antibodies thrice for 10 min in 0.1 M phosphate buffer. Then the samples were incubated for 2 hr at room temperature with secondary antibodies Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 647 conjugate, #A-31571 (Life technologies, USA) diluted 1:1 000 and Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 555 conjugate, #A-31572, diluted 1:1 000 (Life Technologies, USA) in 0.1 M phosphate buffer. After incubation, preparations were washed from secondary antibodies thrice for 10 min in 0.1 M phosphate buffer and the nuclei were stained with Hoechst 33342 (Life Technologies, USA) diluted 1:5 000. Slices were carefully carried to glass slides, the histological preparations were made using Mowiol 4-88 medium (Sigma, USA). Slices were studied using confocal Zeiss LSM 510 Meta microscope (Zeiss, Germany) with Zeiss LSM Image Browser software.

Statistical analysis

In every slice we randomly chose three fields, for which made 5 optical sections of different depth using the confocal microscope, and so evaluated 15 fields per slice. The experiment was carried out in three the experiment was carried out three times. Using Zeiss LSM Image Browser and ImageJ we counted the nuclei with caspase 3, PCNA and the total number of nuclei. After that, we determined the percent of caspase 3 or PCNA signals (proliferating cell nuclear antigen) among optical sections of every field and counted the mean value for slices of a tumor. The fractions of proliferating and apoptotic cells were compared using Student's paired *t*-test in Statistica 8.0. The figures show mean data \pm SD.

Results and Discussion

The histological analysis showed that colon carcinomas at different stages of invasion into the intestine wall that we chose for cocultivation were medium- and low-differentiated (Table, Fig. 1). Determination of the differentiation level has high prognostic value and depends on the structure of the epithelium of colonocytes. In highly differentiated tumors there are all cell types of normal colon epithelium (colonocytes, goblet cells), smooth nuclei layer, smooth outlines of nucleus. Highly differentiated tumors have less ability to metastase and are less aggressive by growth rates. In medium-differentiated tumors the usual structure of colonic crypts is broken, the nuclei and epithelium are stacked into several layers

Histological analysis of colon tumors used for organotypic coculture

Tumor number	Invasion stage	Level of differentiation
1	Submucosa	Medium
2	Submucosa	Medium
3	Serosa	Low
4	Serosa	Low
5	Serosa	Medium
6	Serosa	Low
7	Submucosa	Medium

(Fig. 1, *a, b*). In low-differentiated tumors there are no structures characteristic of healthy colon epithelium, and the typical crypt structure cannot be seen (Fig. 1, *c, d*), cells lose their ability to differentiate into colonocytes, weakly hold onto the basal membrane, uncontrollably proliferate and easily metastase.

Important parameters used to evaluate tumor growth are proliferation and apoptosis rates of malignized cells. To take into account both processes in tumor slices under cocultivation with MSCP we counted PCNA+ and caspase 3+ cells. PCNA expression grows twice in the nucleus during late G1 phase, directly before the start of DNA synthesis, peaks in S-phase and decreases during G2 and M. Its level correlates with the rate of cell proliferation and DNA synthesis [14, 15]. Caspases are a family of cellular proteases mediating cell death and are main effectors of apoptosis. Since caspase 3 is activated by caspase 8

and caspase 9 and is a point of convergence of different signaling pathways, it is a good marker of the apoptosis process [16].

Our non-contact coculture of slices of rat colon tumors with rat MSCP showed the absence of the cells' impact on apoptosis levels in tumor tissue (Fig. 2, *a*), and in only one case (tumor 7) the number of proliferating cells grew significantly under the action of MSCP (Fig. 2, *c*). However, the paired t-test for all tumors we found no statistically significant changes. This result is unexpected, since the literature contains contradictory reports of the impact of MSC on tumor growth under the effect of soluble factors secreted by MSC. For example, we showed that MSC secrete neuregulin 1 that activates the cascade PI3K/AKT/BAD after activating receptors HER2/HER3 in colon tumor cells and so stimulates the invasive growth of the latter [17]. We also identified the soluble factor PAI-1, secreted by MSC,

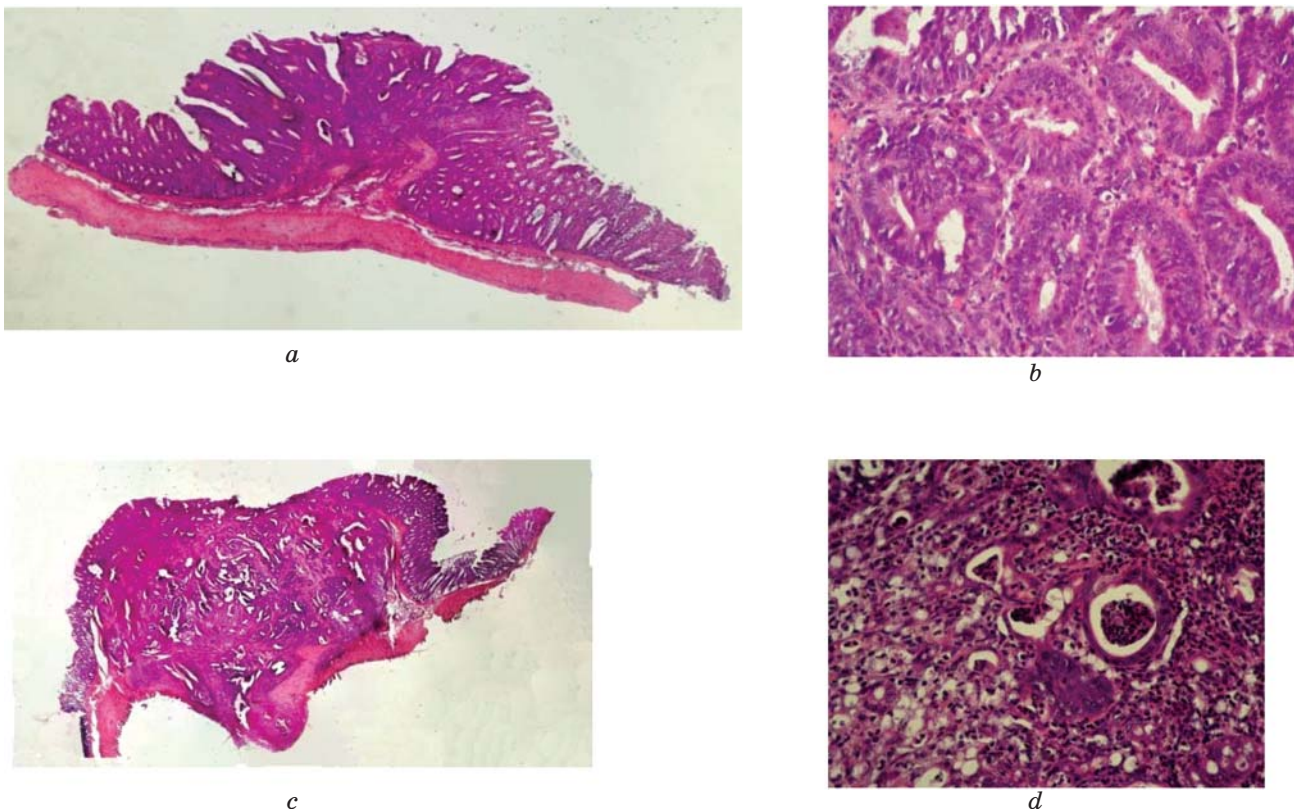


Fig. 1. Histological analysis of colon tumors:

- a* — histological section of tumor 1, hematoxylin eosin staining, $\times 20$;
b — structure of crypts of a medium-differentiated tumor. Several layers of nuclei, several layers of epithelium, hematoxylin eosin staining, $\times 400$;
c — histological section of tumor 4, hematoxylin eosin staining, $\times 20$;
d — epithelium of a low-differentiated tumor, unstructured crypts, hematoxylin eosin staining, $\times 400$

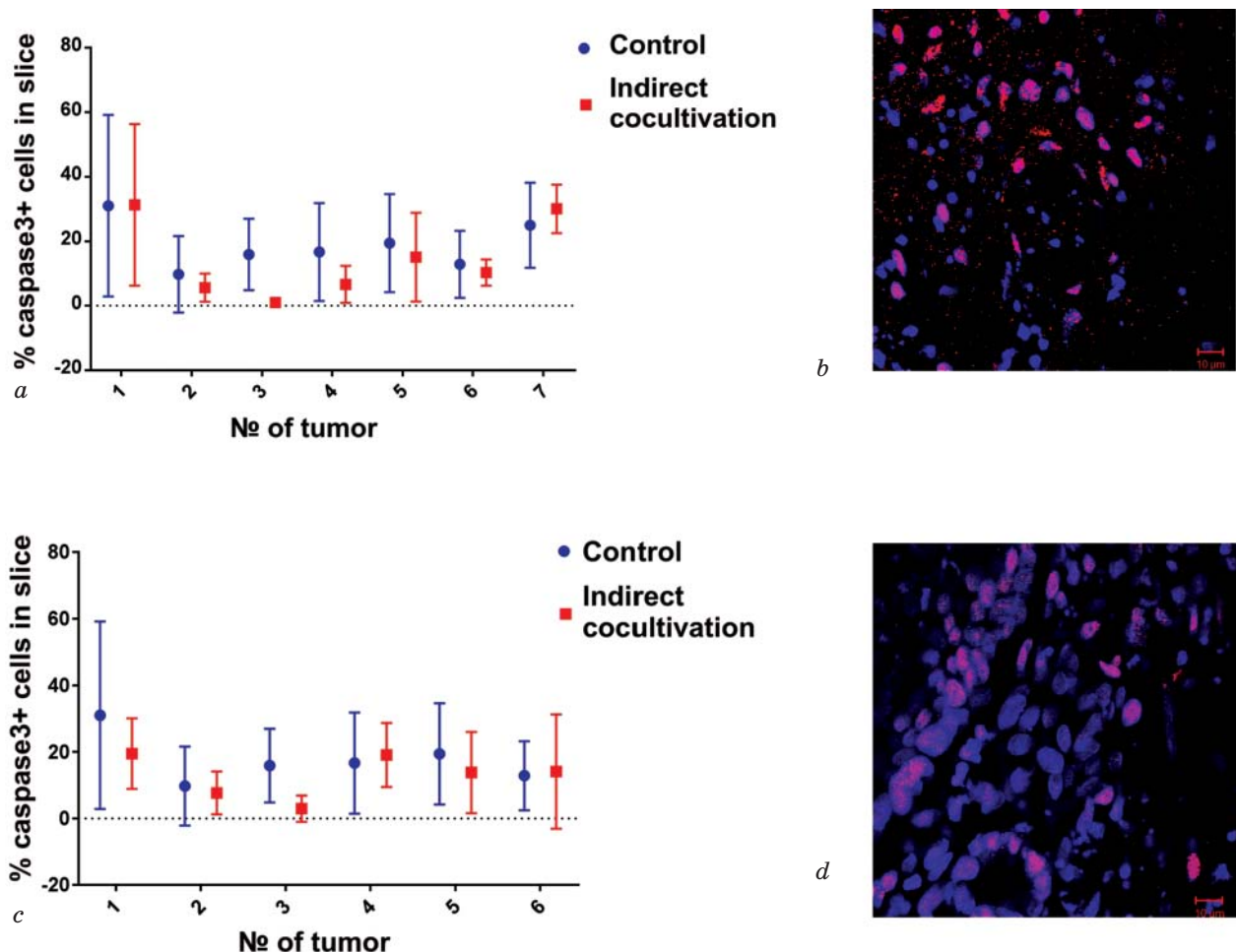


Fig. 2. Parameters of proliferative activity in slices of colon tumors under non-contact cocultivation:
a — mean percent of caspase 3+ cells in three slices of every tumor;
b — immunocytochemical staining of a slice using antibodies to caspase 3 (red signal), blue signal — nuclei (representative analysis of a slice of tumor 7);
c — mean percent of PCNA+ cells in three slices of every tumor;
d — immunocytochemical staining of a slice using antibodies to PCNA (pink signal), blue signal — nuclei (representative analysis of a tumor 7 slice)

which promotes colon cancer cell migration under non-contact coculture with cell lines of colorectal cancer HT29 and HCT-116 [18]. However, we established that MSC, isolated from cord matrix under cocultivation with cell lines of esophagus WHCO1 and breast MDA MB 231 cancer, activate proteins p53 and p21 in cancer cells, initiate apoptosis processes and so inhibit cancer growth [19].

We showed that under systemic introduction MSC migrate directly to tumor tissue, so one should expect contact effect of stem cells on tumor growth. Using Kaposi's sarcoma model we showed that MSC inhibit growth of malignized cells in a dose-

dependent manner after contact interaction of stem and malignized cells [20]. This phenomenon is based on the inhibition of proteinkinase Akt in tumor cells. The organotypic coculture did not lead to observable changes in both the proliferative activity of tumor cells and apoptosis (Fig. 3).

The lack of impact of MSCP on proliferation and apoptosis in colon tumor tissue is a peculiarity of stem cells of placental origin. Although the placental tissue and its extracts have anti-cancer properties, isolated MSCP gain certain properties, similar to MSC from bone marrow, namely the ability to accelerate regenerational processes in the

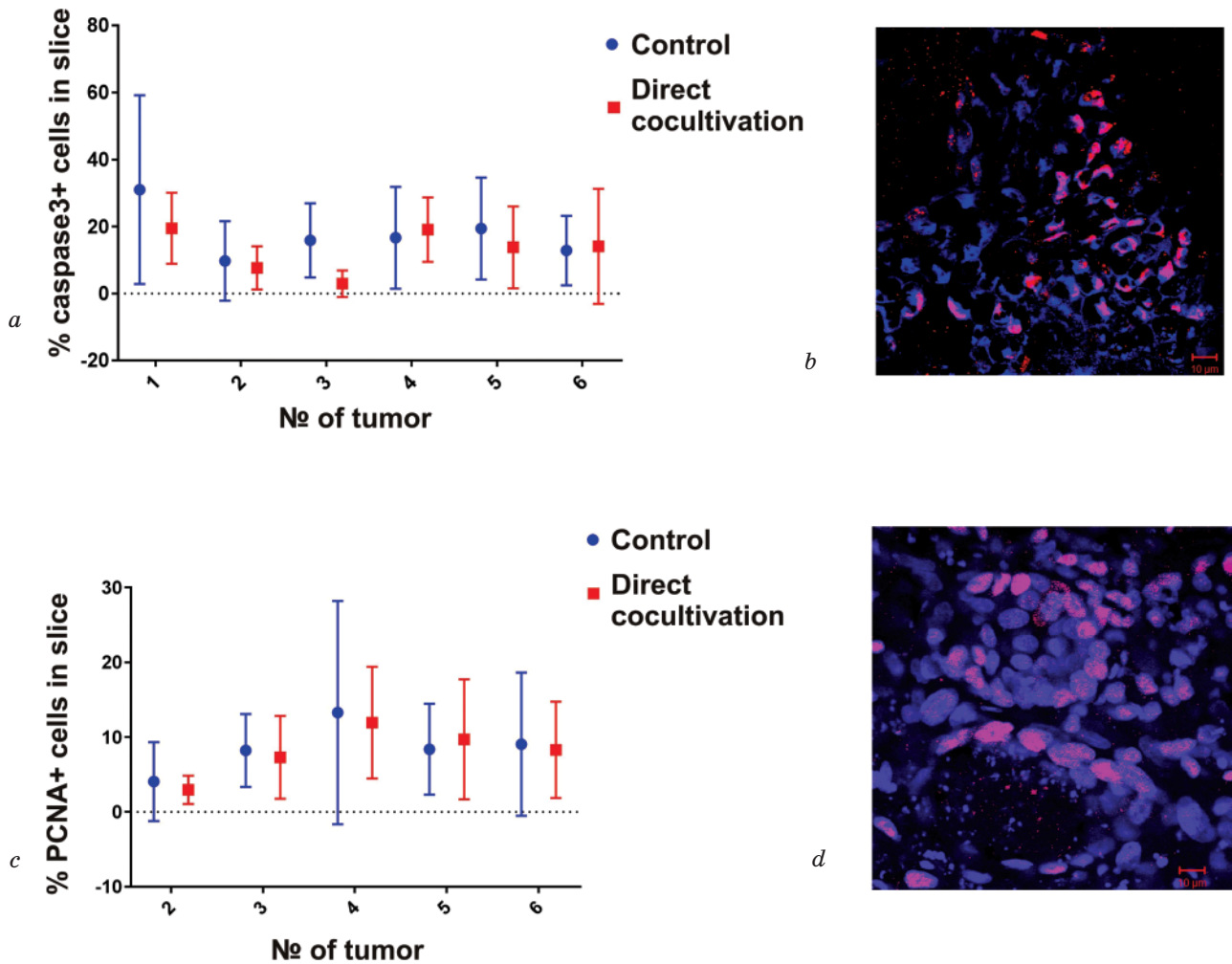


Fig. 3. Parameters of proliferative activity in colon tumor slices under contact cocultivation:

- a* — mean percent of caspase 3+ cells in three slices of every tumor;
- b* — immunocytochemical staining of the slice using antibodies to caspase 3 (red signal), blue signal — nuclei (representative analysis of a tumor 7 slice);
- c* — mean percent of PCNA+ cells in three slices of every tumor;
- d* — immunocytochemical staining of the slice with antibodies to PCNA (pink signal), blue signal — nuclei (representative analysis of a tumor 3 slice)

tissues where they migrate, yet lose anti-cancer abilities.

The model of organotypic coculture is one of novel biotechnological, cell-biological approaches to determination of tissue-specific and morphogenetical changes during cocultivation of not only different separate cell populations, but even different cells integrating into the tissue without disrupting its structure. Rats' MSCP under either direct-contact or non-contact cocultivation with slices of colon tumors do not affect proliferative activity and apoptosis levels in tumors.

The absence of impact of multipotent stem cells of placenta on the growth of colon tumors is a unique peculiarity of these cells that opens new ways to use multipotent placental stem cells in therapy.

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КУЛЬТИВУВАННЯ ТКАНИНИ ПУХЛИНИ ТОВСТОГО КИШКОВИКА З ПЛАЦЕНТАРНИМИ МУЛЬТИПОТЕНТНИМИ СТОВБУРОВИМИ КЛІТИНАМИ ЩУРІВ

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Метою роботи було оцінювання впливу мультипотентних стовбурових клітин плаценти на рівень проліферації і апоптозу за органотипового співкультивування зі зрізами клітин пухлин товстого кишковика щурів. Мультипотентні стовбурові клітин плаценти виділяли методом експлантів, рак товстого кишковика моделювали введенням 20 мг/кг маси тварин диметилгідразину щотижня впродовж 20 тижнів, пухлини товстого кишковика нарізали на зрізи завтовшки 500 мкм. У результаті поставленого співкультивування змін у проліферативній активності зрізи клітин пухлин та рівні апоптозу не спостерігали. Відсутність впливу мультипотентних стовбурових клітин плаценти на ріст пухлини товстого кишковика є унікальною особливістю цих клітин і розширює можливість застосування мультипотентних стовбурових клітин плаценти в терапії.

Ключові слова: мультипотентні стовбурові клітини плаценти, рак товстого кишковика, органотипове співкультивування.

КУЛЬТИВИРОВАНИЕ ОПУХОЛЕЙ ТОЛСТОГО КИШЕЧНИКА С ПЛАЦЕНТАРНЫМИ МУЛЬТИПОТЕНТНЫМИ СТВОЛОВЫМИ КЛЕТКАМИ КРЫС

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Целью работы была оценка влияния мультипотентных стволовых клеток плаценты на уровень пролиферации и апоптоза при органотипическом сокультивировании со срезами опухолей толстого кишечника крыс. Мультипотентные стволовые клетки плаценты выделяли методом экплантов, рак толстого кишечника моделировали путем введения 20 мг/кг массы диметилгидразина еженедельно в течение 20 недель, опухоли толстого кишечника нарезали на слайсы толщиной 500 мкм. В результате поставленного сокультивирования изменений в пролиферативной активности слайсов опухолей и уровне апоптоза не наблюдали. Отсутствие влияния мультипотентных стволовых клеток плаценты на рост опухоли толстого кишечника является уникальной особенностью этих клеток и расширяет возможности применения мультипотентных стволовых клеток плаценты в терапии.

Ключевые слова: мультипотентные стволовые клетки плаценты, рак толстого кишечника, органотипическое сокультивирование.