THE STUDY OF THE INTERACTION OF MESENCHYMAL STEM CELLS AND THE TUMOR USING THE METHODS OF FLUORESCENT BIOIMAGING

UDK 575.1/.2:611-018.82:612.419
Received 13.07.2012

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The aim of the investigation is to study the pathogenesis of tumor development when interacting with mesenchymal stem cells transfected by a gene of red fluorescent protein using the method of vital bioimaging.

Materials and Methods. There were used adipose-derived adult stem (ADAS) cells taken from human adipose tissue. ADAS were transfected by a gene of red fluorescent protein Turbo FP635 (Close Joint Stock Company "Eurogene", Russia) by the method of lentiviral transfection. Tumors were implanted to nude mice by subcutaneous injection of Hela Kyoto tumor cells (cervical cancer). ADAS labeled by fluorescent protein were injected to animals at different stages of carcinogenesis (0 and 8 days after tumor culture injection) by various ways: locally — in the tumor node forming region, and systemically — intravenously, in the tail vein. There were formed the following groups of animals: 1st group — an early stage of carcinogenesis (immediately after the injection) and systemic injection of ADAS; 2nd group — an early stage of carcinogenesis and local injection of ADAS; 3rd group — the stage of developed tumor (8 days) and systemic injection of ADAS. The control group consisted of the animals with induced tumor without stem cells injection.

Results. ADAS isolated and characterized with the help of immunocytochemical analysis had the phenotype of mesenchymal stem cells (there were expressed CD105, CD49d, STRO-1) and were differentiated *in vitro* in adipogenic, osteogenic, and chondrogenic approaches in induction media cultivation. The efficiency of transfection of ADAS by red fluorescent protein Turbo FP635 was 75%. The stem cells under study — ADAS labeled by red fluorescent protein Turbo FP635 in systemic injection were shown to be able to migrate in spleen, and in systemic and local injection — in bone marrow, lungs, and recipient's tumor tissues. The methods of fluorescent bioimaging and laser scanning microscopy can be used to study the interaction between the tumor and mesenchymal stem cells. They effectively complement each other in gaining general knowledge of the distribution of migratory fluorescent cells.

Key words: mesenchymal stem cells; adipose-derived adult stem (ADAS) cells; red fluorescent protein Turbo FP635; tumor Hela Kyoto; vital bioimaging; laser scanning microscopy; transfection; carcinogenesis.

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Currently, the study of stem cells (SC) role in tumorgenesis follows two main directions: fundamental science investigates cell flexibility and gene mechanisms underlying cancer diseases, and applied science — the capabilities of applying SC of different origin for cell-based therapy in the management and prevention of oncological diseases.

The studies of different experimental models "Tumor-stem cell" have stated SC to be able to transform spontaneously into malignant cells [1], and maintain the development of existing tumors [2, 3]. In animal studies (*in vivo*) [4] stem cells have been shown to contribute to tumor growth and be fully engaged in: tumor neoangiogenesis; niche creation for maintaining tumor cells growth and activity; modulation of organism's immune response; metastases formation [4].

On the other hand, these studies *in vitro* [5] give the evidence that SC are also able to generate a negative impact on tumor cell growth in culture, that is: to reduce tumor cell proliferation level; take part in tumor cells apoptosis; inhibit invasion of tumor cells.

Thus, the role of SC of different origin (hemopoietic, stromal, tissue-specific, mesenchymal) in oncogenesis still remains a subject for researches. New information is relevant both for understanding the mechanisms of neoplastic growth maintenance and the search of new tumor therapy approaches.

Mesenchymal SC are precursors of mesenchymal origin tissue cells, and have unique immune and pro-angiogenic properties. Except bone marrow, the cells with mesenchymal cell characteristics are found in many organs and tissues of adult body: skeletal muscles, vascular walls, dental pulp, fat tissue, etc. Adipose-derived adult stem (ADAS) cells do not differ in morphology, immune phenotype, and developmental potential from those of bone marrow, though they have some undisputed advantages:

accessibility and non-traumatic way of receiving (the sources are lipoaspirates of patient's adipose tissue);

ease of administration and maintenance in culture;

a wide range of possible differentiations.

To study SC participation in oncogenesis and observe their distribution in recipient's organism, fluorescence methods of biomedical imaging are used. Owing to bright fluorescent agents working in the sphere of tissue "spectral window", the development of conjugation and fluorophore targeting technologies, as well as compact sources and high sensitivity optical receivers, the methods of fluorescence bioimaging have helped to breakhrough in the study of many biological processes [6–8]. The most of fluorescent bioimaging modalities are based on exogenous administration of fluorophore (or its precursors) into body (cell), and the detection of labeled objects fluorescence in natural environment. Fluorescent (GFP-like) proteins of different groups, the genes of which are embedded in experimental cells (of cancerous lines, mesenchymal SC) are of major interest as markers (genetic marks), as they enable to trace the location of marker-positive cells and their products, as well as their changes [9].

Among fluorescent bioimaging techniques, *in vivo* observational methods emerge to prominence, as they open possibilities to observe a biological object as an entire one, as well as study the functions and procession on one animal for a long time taking into account its individual peculiarities. The easiest realization of *in vivo* fluorescent imaging is surface fluorescent imaging that enables to assess promptly (1–2 s) the size of fluorescent area that is close to the surface of a biological object under study. The application of high sensitivity receivers also permits to detect deep fluorescence, the image of deep fluorescent sources being significantly blurred due to light scattering by biotissues.

The aim of the investigation. Within the scope of this work the following tasks have been set:

1) to isolate, study and transfect adipose tissue stem cells by red fluorescent protein;

2) to study the pathogenesis of tumor development when interacting with stem cells using the method of vital surface fluorescent imaging;

3) to study the migration of marker-positive stem cells in potential niches in the body.

Materials and Methods.

Cell culture. There were used ADAS isolated from human lipoaspirates or adipose tissue taken in abdominal plasty. ADAS were isolated according to P.A. Zuk [10] method with modifications. To reveal the potential of these cells to differentiate in adipogenic, chondrogenic, and osteogenic directions, ADAS were cultured in appropriate induction media [10, 11]. Immunocytochemical analysis was performed to determine mesenchymal SC markers [12]. ADAS were transfected by a gene of red fluorescent protein Turbo FP635 (Close Joint Stock Company "Eurogene", Russia) by the method of lentiviral transfection. This protein has maximum absorption at wave length of 585 nm, and emission peak — at wave length of 635 nm (Fig. 1).

Tumor models. *Nude* female mice aged 6 months, weighting 20 g were used. 5 million Hela Kyoto tumor cells (cervical cancer) were injected subcutaneously in left thigh area, suspended in 100 μl PBS (phosphate-buffered saline).

Experiment design. ADAS labeled by fluorescent protein were injected to animals at different stages of carcinogenesis: 0 and 8 days after tumor culture injection. The cells in amounts *of* 1.5 million were injected to the animals by various means*:* locally — in the tumor node forming region, and systemically — intravenously, in the tail vein. There were formed the following groups of animals:

1st group — an early stage of carcinogenesis (0 days) and systemic ADAS injection;

2nd group — an early stage of carcinogenesis and local ADAS injection;

3rd group — the stage of developed tumor (8 days) and systemic ADAS injection;

control group — with induced tumor, without SC injection.

The injection of labeled cells at early stages of carcinogenesis was based on the assumption that ADAS can be the source of neoplastic vascular net formation, be included into tumor stromal structures. Local SC injection was used to simulate the interaction between tissue specific

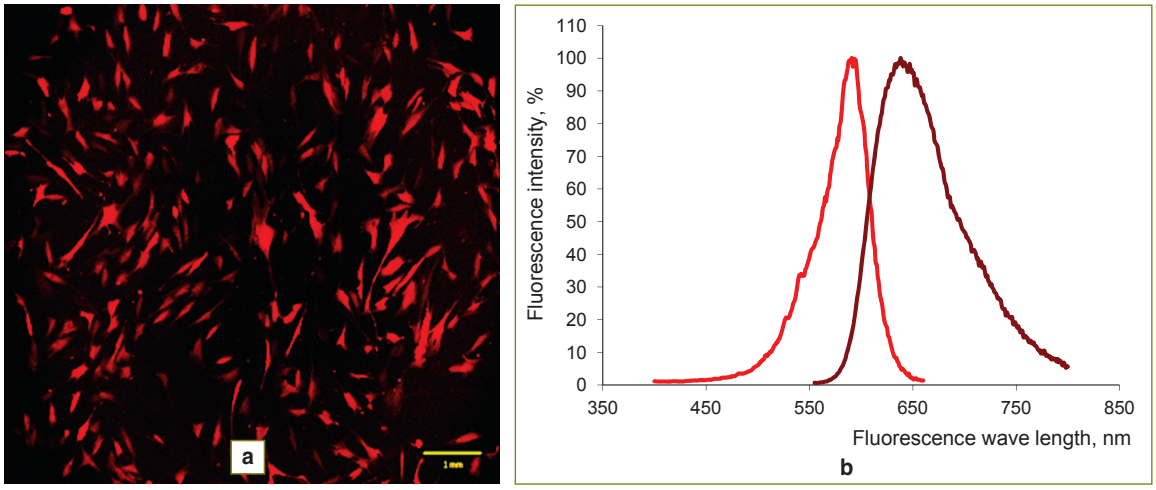

Fig. 1. ADAS transfected by a gene of protein Turbo FP635: *a* — fluorescent image (fluorescent microscope Olympus X71, Japan/Germany; fluorescence excitation — 545–580 nm, registration — 610–650 nm), x10; *b* — spectrum of excitation and emission of protein Turbo FP635 (www.evrogen.ru)

ADAS and tumor tissues in the tumor node forming region, and systemic — to simulate the interaction between tumor and ADAS migrating from other niches (e.g. bone marrow). In developed tumor stage (mature circulatory system and stroma) ADAS were injected to control the effect of mesenchymal SC on further tumor growth, invasion, and metastases formation. In all three groups of animals we supposed to state possible additional niches of mesenchymal SC proliferation (bone marrow, spleen). The attention was also given to the organs of possible ADAS redistribution in a recipient's body (lungs), and the organs of waste products elimination (kidneys, liver). Each group consisted of three animals. Tumor growth was measured every 2–3 days with a vernier caliper.

Tumor volume analysis. The change of tumor volume was calculated by Schrek formula modified by I.S. Amosov et al. [13]:

$$V=0{,}5\times[(a+b)/12]^3,$$

V — tumor volume, mm^3; *a, b* — bidirectional tumor sizes, mm.

Monitoring of labeled ADAS migration by surface fluorescent imaging method. For vital monitoring of fluorescence-labeled cells in the recipient's body, there was used surface fluorescent imaging unit developed in Institute of Applied Physics of Russian Academy of Sciences (Nizhny Novgorod, Russia). In the course of measurements an animal was placed in a "black" chamber and exposed to a wide light beam in a narrow spectrum range (to excite Turbo FP635 protein fluorescence there was used radiation at wave length of 585 nm, with band of 20 nm). Fluorescence excited on animal surface was recorded by refrigerated CCD camera (ORCA II BT-512G, Hamamatsu Photonics K.K., Japan). Interference filter (Chroma Technology Co., USA) with pass band of 628–672 nm was mounted on CCD-camera lens to separate fluorescence and probe radiation. Exposure time of CCD-camera was 2 s. Before measurements the animals were anesthesized with intraperitoneal injection of Zoletil (Vibrac, France), the concentration being 20 mg/ml, and fixed horizontally on the base. Protein fluorescence was examined immediately after the injection, and then every day within 14 days.

Monitoring of labeled ADAS by confocal laser scanning microscopy (LSM). The animals were euthanized by intravenous injection of Zoletil, in concentration of 40 mg/ml, to analyze the organ distribution of labeled cells in a recipient's body *ex vivo*. Then there was carried out the acquisition of organ samples, 3–4 mm in size, and bone marrow smears for further study on laser scanning microscopic unit (LSM 510 META 23, Carl Zeiss, Germany) on the basis of mounted inverted microscope (Axiovert 200M) equipped by spectral module to detect epifluorescence spectra in visible range with resolution of 10 nm. Confocal images were captured using oil-emission lens, with magnification of 63. Fluorescent images were registered in single-photon excitation by argon laser, wave length being 543 nm, power — 12 microW on a sample.

Results.

ADAS characteristic. These cells were isolated and characterized by means of immunocytochemical analysis. ADAS were found to have the phenotype of mesenchymal SC (express CD105, CD49d, STRO-1) and be able to differentiate *in vitro* in adipogenic, chondrogenic, and osteogenic directions when being cultured in induction media. For transfection there were used ADAS at early culture passages. The efficiency of ADAS transfection by a gene of red fluorescent protein Turbo FP635 was 75% (See Fig. 1, *a*).

Tumor growth dynamics. According to the measurement data of tumor linear dimensions and fluorescent imaging, in the course of the study no differences were revealed in tumor growth dynamics in experimental models (both with and without stem cells injection). Exogenous injection of ADAS appears to have no significant effect on tumor growth.

Monitoring of labeled ADAS migration by surface fluorescent imaging in vivo. In trial experiments there was

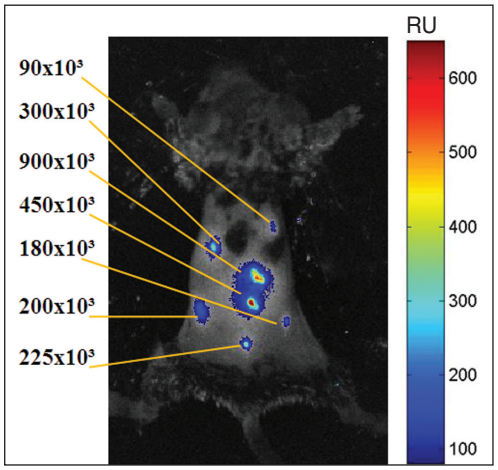

Fig. 2. The determination of sensitivity of the surface fluorescence bioimaging unit *in vivo* to visualize ADAS labeled by protein Turbo FP635. The observations of different amounts of labeled stem cells, site of injections are indicated

studied fluorescence of subcutaneously injected fluorescent cells in different concentrations to determine the sensitivity of the unit to detect labeled SC. Minimum amount of fluorescent ADAS detected by the unit was 90 thousand cells (Fig. 2).

90 images were captured in the observation process of animals with tumors of all four groups using surface fluorescent imaging unit.

Control animals in the process of tumor growth monitoring demonstrated slight tissue fluorescence due to the presence of endogenous porphyrinic structures [14, 15], excitation peak of which being in the range of 400–500 nm, and emission spectrum having two peaks — 635 and 690 nm. Background signal on the obtained images related to a nonideal interference filter results in artifacts due to irregular scattering of radiation on animal skin (pigmentation and skin defects).

In group 1 (early stage of carcinogenesis and systemic ADAS injection) the fluorescence of labeled ADAS was found in all animals in spleen area on day 9 and persisted up to 14 days (Fig. 3). In tumor cells of these animals we failed to reveal fluorescence consistent with labeled ADAS accumulation.

In the 2nd group of animals (early stage of carcinogenesis and local ADAS injection) there was registered fluorescence of the injection site of labeled ADAS that eventually attenuated, and by the third day was not seen at all. Fluorescence consistent with the accumulation of labeled ADAS in other animal body tissues and organs were not revealed. Observation time was 14 days (Fig. 4).

In the 3rd group of animals (developed tumor and systemic ADAS injection) ADAS fluorescence was found on the 5th day in spleen area. There was increasing fluorescence in

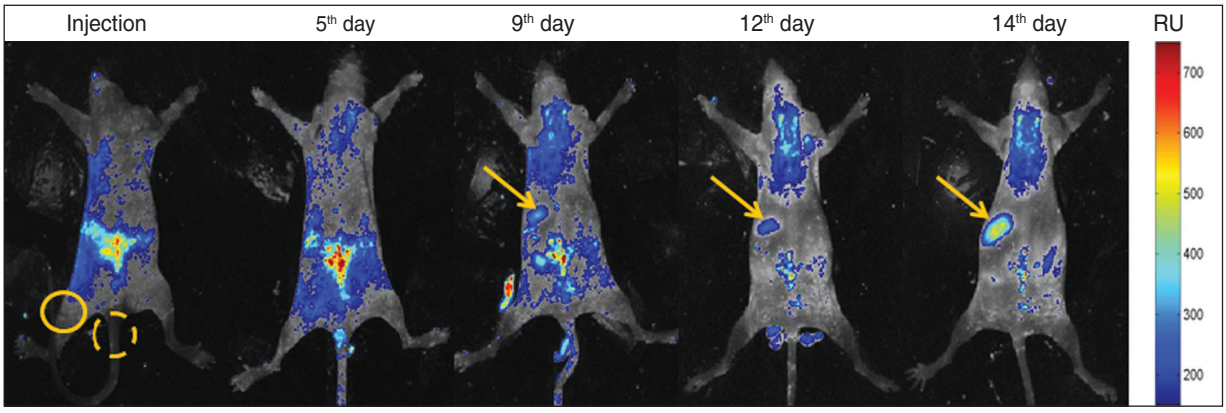

Fig. 3. Monitoring of fluorescence-labeled ADAS by the method of fluorescence bioimaging in animals of group 1 (early stage of carcinogenesis, systemic ADAS injection). A full line indicates the site of tumor cell injection, a dot line — the injection site of fluorescence-labeled ADAS, arrows — the localization of fluorescence-labeled ADAS

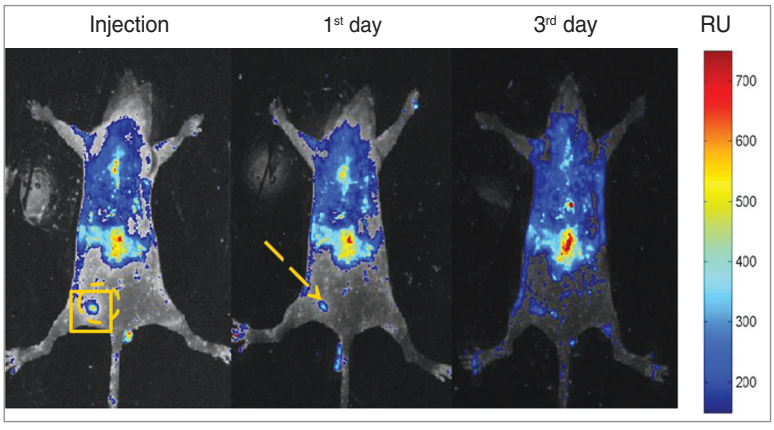

Fig. 4. Monitoring of fluorescence-labeled ADAS by the method of fluorescent bioimaging in animals of group 2 (early stage of carcinogenesis, local ADAS injection). A square indicates the injection site of tumor cells, a dot line — the injection site of fluorescence-labeled ADAS, a dotted arrow — fluorescence of ADAS injection site

the following days (up to the 10th day), similar to the first group. No fluorescence of labeled ADAS in tumor node and niches was found.

Monitoring of labeled ADAS by the method of confocal laser scanning microscopy. LSM method was used to verify the results obtained by surface fluorescent imaging technique. To obtain control spectrum of protein Turbo FP635 using LSM equipment there were analyzed spectral characteristics of ADAS cultures expressing this protein for further comparison with tissue spectra of the organs under study. In contrast to the spectrum of purified plasmid protein Turbo FP635 (See Fig. 1, *б*) the obtained spectrum of the cells transfected by gene vector of this protein had two evident peaks — at 613 and 635 nm (Fig. 5). The appearance of an additional peak could be related to either protein mutation in ADAS culture, or the autofluorescence effect of cells.

In the control group there was assessed the autofluorescence intensity of organs and tumor tissues when excited by laser radiation at wave length of 543 nm. Autofluorescence in skin, muscles, heart, brain, lungs, intestine and tumor tissue was found to be extremely low, while in the tissues of spleen, kidneys and liver it was bright (Fig. 6), spectral characteristics of tissues being different from those of fluorescence-labeled ADAS and having no evident peaks — at 613 nm and 635 nm.

In experimental groups of animals with injected labeled ADAS, there was intense autofluorescence of splenic, renal and hepatic tissues when excited by laser radiation, with wave length being 543 nm that exceeded the fluorescence of Turbo FP635. However, in these experimental animals the areas of fluorescence consistent with the spectrum of cells with protein Turbo FP635 were revealed in the tissues with low level of fluorescence: tumors, bone marrow, and the lungs (Fig. 7, *a*) that gave the evidence of the accumulation (proliferation) of labeled cells in these niches.

Fluorescence of labeled ADAS was found in tumor tissues of the 2nd group animals (Fig. 7, *b*). The animals of other groups had no fluorescence in tumor tissue consistent with the accumulation of labeled ADAS.

Bright cell accumulations with spectral characteristics of labeled ADAS were revealed in bone marrow of the animals of group 2. However, in bone marrow of the animals of the 1st and 3rd groups just dull autofluorescence was observed.

In lung tissues of the animals of group 3 there were found accumulations of cells with fluorescence consistent with the target spectrum, in other cases only dull autofluorescence was observed.

Discussion. To study the interaction between ADAS and the tumor, as well as the monitoring of ADAS migration in a recipient's body, two techniques were used: surface fluorescence imaging and confocal laser scanning microscopy.

The method of vital bioimaging in animals with local ADAS injection was used to observe the redistribution of labeled ADAS from the injection site. The fluorescence of labeled ADAS was registered in spleen area in animals-tumor carriers with systemic ADAS injection at early stages of carcinogenesis and with developed tumor. In the course of time fluorescence was increasing indicating the migration and/or growth of labeled ADAS in this organ in the detectable amounts (over 90 thousand cells). The findings have corroborated the available information that spleen is the niche for ADAS in systemic injection [16, 17].

The failure to find other organs of SC accumulation in their redistribution in a recipient's body in first three groups using the method of surface fluorescence imaging is due to low fluorescence intensity of labeled ADAS compared to autofluorescence of animal tissue that is caused by the following:

1) small amount of labeled ADAS in corresponding niches;

2) deep localization of niches (e.g. bone marrow, lungs). However, in sufficient amount of fluorescence-labeled cells and their shallow localization, surface fluorescent imaging can be used in similar studies.

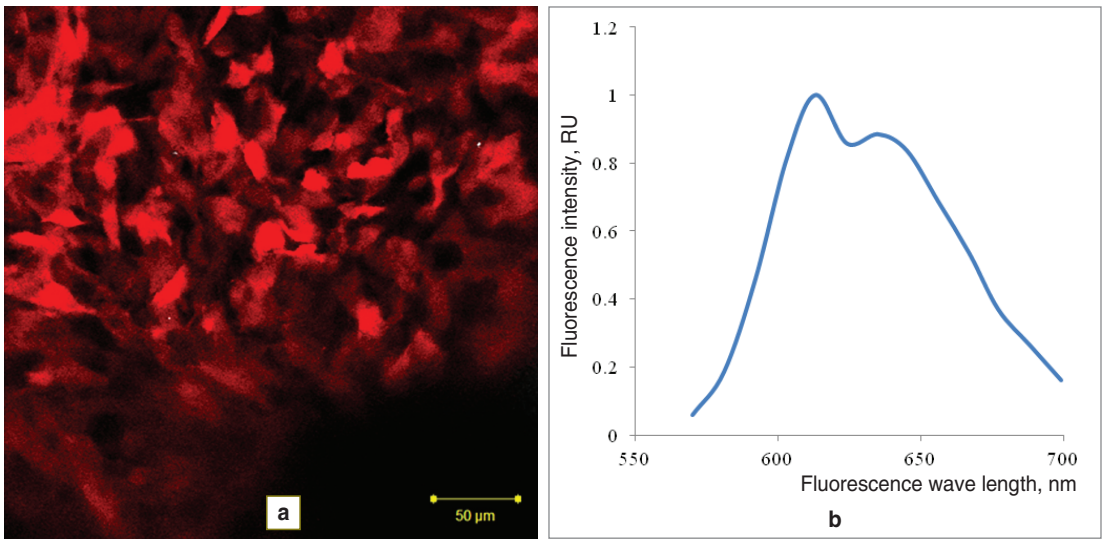

Fig. 5. The results of confocal laser scanning microscopy of ADAS labeled by protein Turbo FP635: *a* — Fluorescent image of cells (fluorescence excitation — 543 nm, registration 650–710 nm), *b* — an appropriate fluorescence spectrum in the range of 597–694 nm, x40

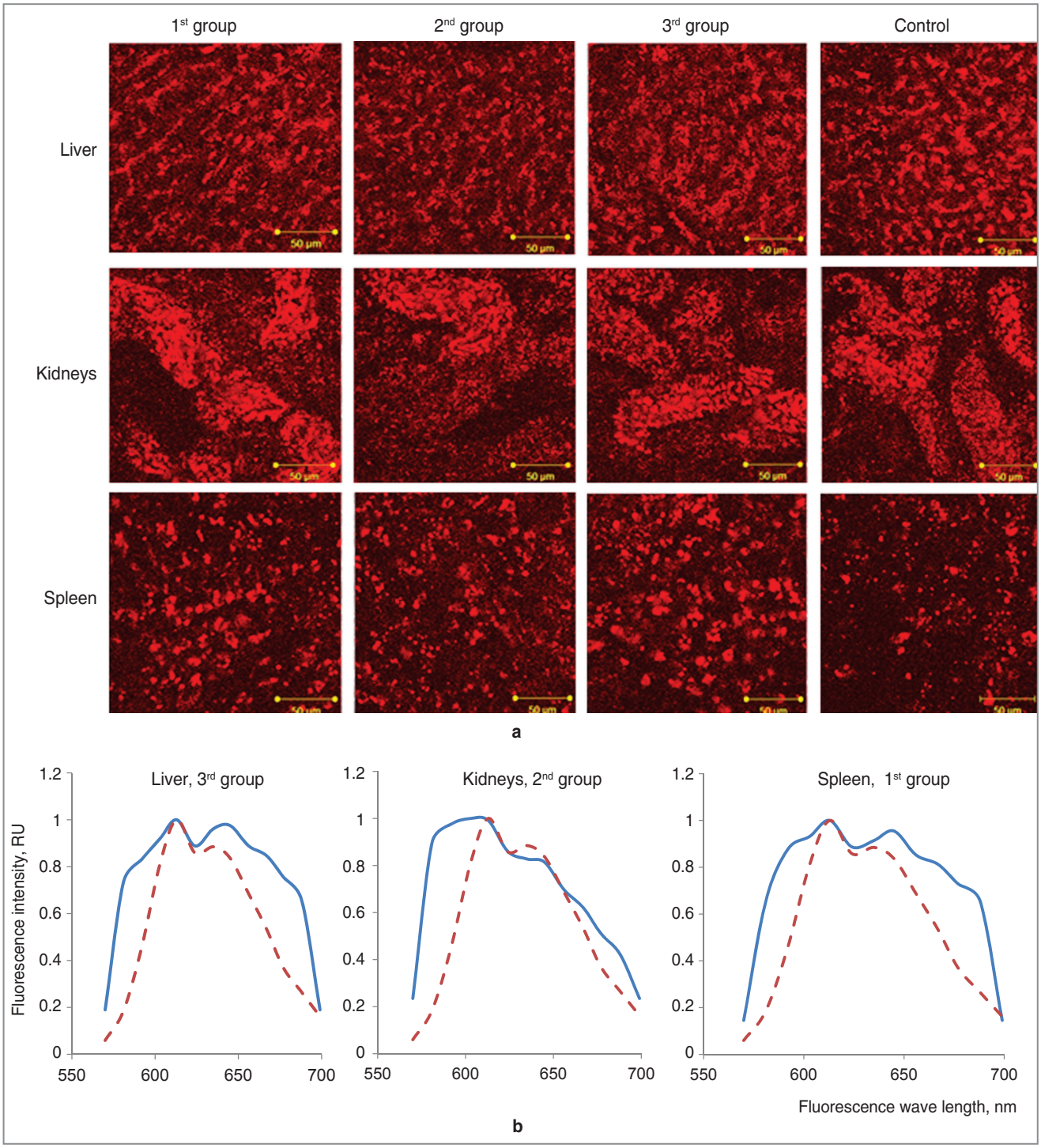

Fig. 6. The results of confocal laser scanning microscopy of organ tissues *ex vivo*: *a* — fluorescence images (fluorescence excitation — 543 nm, registration — 650–710 nm); *b* — fluorescence spectra of animal tissues within the range of 597–694 nm in different groups; a full line indicates the fluorescence spectra of animal tissues, a dot line — spectrum of ADAS labeled by protein Turbo FP635

LSM revealed fluorescence-labeled ADAS in tumor tissues of animals with local injection of cells that can give the evidence of the modeling of interaction between SC and the cells of surrounding tumor tissues (including the participation in forming tumor blood vessels). Labeled ADAS were not found in tumor sites in animals with systemic SC injection.

Despite the fact that fluorescence of splenic tissues was registered by the method of vital surface fluorescent imaging in the groups of animals with systemic implantation of mesenchymal SC, LSM technique failed to differentiate fluorescence of Turbo FP635. It could be related to intense autofluorescence due to a large amount of endogenous porphyrinic structures being the components

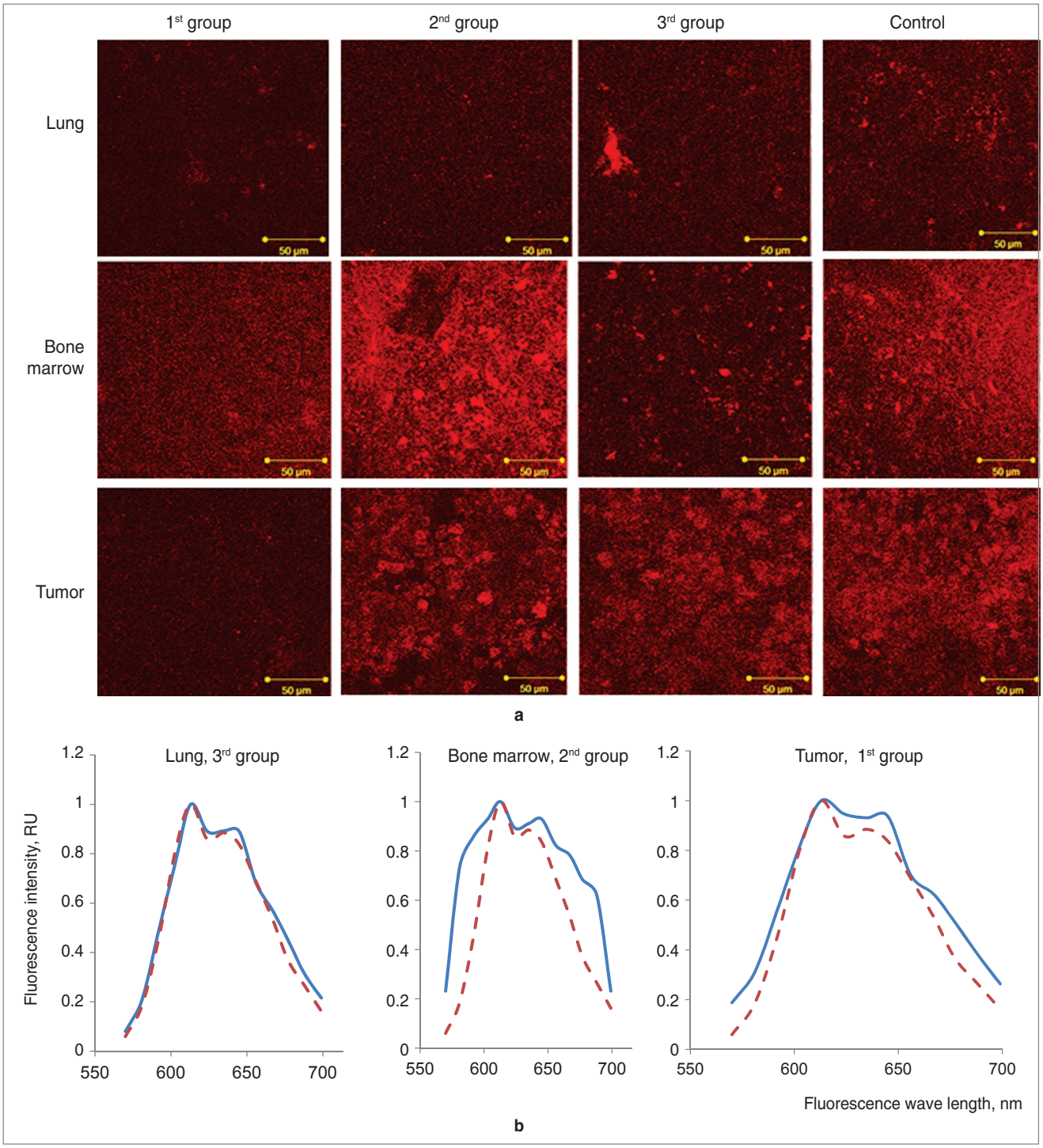

Fig. 7. The results of confocal laser scanning microscopy of organ tissues *ex vivo*: *a* — fluorescence images (fluorescence excitation — 543 nm, registration — 650–710 nm); *b* — fluorescence spectra of animal tissues within the range of 597–694 nm in different groups; a full line indicates the fluorescence spectra of animal tissues, a dot line — spectrum of ADAS labeled by protein Turbo FP635

of hemoglobin, myoglobin, catalase, peroxidase, and numerous cytochromes. Moreover, wave length of probe radiation in LSM equipment (543 nm) is not optimal for excitation of Turbo FP635 fluorescence. Furthermore, the excitation of labeled ADAS fluorescence in the unit for surface fluorescent imaging was performed at wave length of 585 nm, in consequence of which we managed to reveal the fluorescence of ADAS labeled by Turbo FP635 against the background of autofluorescence of the organ.

The presence of labeled ADAS in bone marrow of animals with local ADAS implantation at early stage of carcinogenesis appeared to be unexpected, while in bone marrow of animals of other groups these cells were not found.

One more localization of labeled ADAS — the lungs — was revealed in animals with systemic injection of ADAS in developed tumor. It is known that in similar experimental models, the lungs of animals serve as premigration place where SC injected systemically accumulate by the 3–4 day after injection, and by the 7 day they redistribute in a recipient's body. In this case, there must have been found a part of ADAS and their descendants that did not left lung tissues by the 14th day of the experiment.

Conclusion. The methods of fluorescent bioimaging and laser scanning microscopy can be used to study the interaction of the tumor and mesenchymal SC. They effectively complement each other in gaining general knowledge of the distribution of migratory fluorescent cells. The cells under study — adipose tissue stem cells — labeled by a gene of red fluorescent protein Turbo FP635 in systemic injection are able to migrate in spleen, and in systemic and local injection — in bone marrow, lungs, and tumor tissues of the recipient.

The work is carried out with the support of Russian Foundation for Basic Research No.11-02-01199 "Fluorescent bioimaging of "Tumor-stem cell" system" and Grant of Russian Federation government for support of scientific researches carried out under the supervision of leading scientists (Contract No.11.G34.31.0017 dated November 24, 2010), "Fluorescent proteins: new approaches to the study of the mechanisms of physiological and pathological processes in living systems".

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