ISJ 13: 94-101, 2016 ISSN 1824-307X

RESEARCH REPORT

Dynamics of hemocyte subsets from ascidian *Halocynthia aurantium* in response to tissue damage: a comparative analysis of flow cytometry vs confocal microscopy data

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Accepted March 21, 2016

Abstract

The current study was aimed at investigating the dynamics of circulatory ascidian hemocyte subsets from *Halocynthia aurantium* in response to tunic damage. By using flow cytometry and confocal microscopy, it was demonstrated that the relative amount of hemoblasts and hyaline amebocytes was increased 24 h after cutting ascidian tunic and subjacent muscle layer. By applying a broad panel of fluorescently labeled monoclonal antibodies against human adhesion molecules expressed by lymphoid and stem cells both assays allowed to detect two cross-reactive epitopes (CD54 and CD90) on the surface of ascidian hemocytes. Upon that, the expression of CD54-like epitope was found to be downregulated on ascidian cells after tissue damage, whereas binding to CD90-like epitope was upregulated in all examined cell subsets.

Key Words: ascidian; hemocytes; flow cytometry; confocal microscopy; tunic damage

Introduction

Dynamics and renewal of cell subsets in multicellular organisms are among the basic issues of current biology. Most explicitly, the importance of such scientific area is related to the discovery, the investigation and the potential clinical use of human and animal circulatory stem cells. In the meantime, a question regarding tissue-specific stem cells and dynamics of cell subsets in multicellular organisms was most clearly raised in fundamental studies done by Zavarzin (Zavarzin, 1953). There is an important evolutionary aspect in examining dynamics of cell subsets and mechanisms underlying functioning of stem cell systems. Whereas stem cells of various potency in invertebrates are mainly amassed as tissue-specific regeneration nests, nodes and other structures, chordates and particularly vertebrates possess tissue-specific oligopotent stem cells as well as new system of circulatory pluri- and multipotent stem cells displaying high plasticity and regenerative potential. This led to changes in tissue reparation process resulting from overall increase in level of organization of all chordates, particularly vertebrates, with more pronounced tissue specialization, but, mainly, from the appearance of close circulatory system. Directed trafficking of circulatory stem cells is accompanied by increasing role of body integrative systems, enhanced nervous regulation, establishing network of soluble cytokines and growth factors, which are specifically demonstrated in models of tissue reparation caused by various insults.

According to this viewpoint, investigation of dynamic changes in ascidian circulatory hemocyte subsets may provide an important evidence on the initial stages of developing system of mobile stem cells. In the larva stage, ascidians have close circulatory system, however it seems possible that the transition to a sedentary lifestyle of these benthic filter-feeders, coupled with loss of close circulatory system, operational principles of stem cell system are not altered (Polevshchikov *et al*., 2005). This is one of the reasons why, over the years, ascidians have been extensively used for examining specialization of circulatory cells (Sukhachev *et al*., 2013) as well as their involvement in defense and reparative reactions (Smith, 1970).

Our study was aimed at analyzing dynamic changes in circulatory hemocyte subsets during response to tunic damage of the solitary ascidian *Halocynthia aurantium* from the Sea of Japan, which were assessed by flow cytometry and confocal microscopy.

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Table 1 Changes in the percentage of subsets of circulatory hemocytes isolated from ascidian *H. aurantium* in response to tunic damage assessed by flow cytometry

Hemocyte subset	Control	24 h after
Hemoblasts and hyaline amebocytes	12.04±0.66	14.98±0.93*
Macrophage-like cells	13.60±0.59	10.90±2.35
Morula cells and granulocytes	66.96±1.86	64.15±3.35
Total identified hemocytes	92.60	90.03

hereinafter, significant differences at 24 h after tunic damage vs. control group are marked by asterisks (: $p < 0.05$). Data were analyzed by using Student's t-test and are presented as mean ± s.e.m., n ≥ 24 at each time point.

Materials and Methods

Animals

The solitary ascidian *Halocynthia aurantium* (Stolidobranchia: Pyuridae) was used in experiments. Animals were harvested during July-August 2013 - 2015 at the biological station "Vostok", Marine Biology Institute, Far East Branch of the Russian Academy of Science. Prior to the study, animals were kept for 4 days in aquarium with running seawater and forced aeration. Totally, more than 120 animals were used during the study.

Model of ascidian tunic damage

Tissue injury consisted of a standardized cutting (length: 1 cm, depth: 0.5 cm) on tunic and subjacent tissues including the wall of the circulatory. After the 1st tunic damage 9 ml of flowing from the wound hemolymph was immediately collected from each specimen (0 h, control) into test tubes with 30 mM EDTA (Sigma-Aldrich, USA) prepared in filtered seawater for preventing hemocyte clotting. To examine dynamics in circulatory cell subsets, 24 h later each ascidian repeatedly received another cutting followed by hemolymph collection and the same volume of hemolymph was collected. Cell suspension was centrifuged at 100*g* for 10 min 4 ºC followed by washing in Dulbecco's Phosphate Buffered Saline (PanEco, Russia), without calcium chloride and magnesium chloride, pH 6.0, containing 34 g/l NaCl that matches its concentration in water from Sea of Japan. Final cell concentration after washing was 10^7 cell per 1 ml of modified Dulbecco's solution.

Flow cytometry

For performing flow cytometry assay, 500 μl of cell suspension (5×10^6 cells) were transferred into Eppendorf tube and added with equal volume of cooled (4 °C) 8 %-formalin (Sigma-Aldrich, USA) for storage at 4 °C until use. Cell samples were analyzed by flow cytometry on FACSCalibur™ (Becton Dickinson, USA) equipped with CellQuest Pro software, according to the earlier described method (Sukhachev *et al*., 2015). Cross-reactivity of surface antigenic epitopes of hemocytes from *H. aurantium* and adhesion molecules expressed by human peripheral blood leukocytes was evaluated using to the above-described method by using a panel of monoclonal mouse, allophycocyanin-conjugated, anti-human antibodies (mAbs) against CD166 (ALCAM, Activated Leukocyte Cell Adhesion Molecule), CD117 (c-kit), CD29 (integrin β1-chain), CD34 (adhesion molecule typical of mammalian hematopoietic stem cells), CD15 (3-fucosyl-N-acetylglucosamine), CD54 (ICAM-1, Intercellular Adhesion Molecule), CD62L (L-selectin), CD62P (P-selectin), CD90 (evolutionarily conserved Thy-1 antigen, typical of nervous system and differentiating T cells), CD94 (C-type lectin found within NKG2 heterodimers of mammalian NK cells). Allophycocyanin-conjugated MOPC-21 immunoglobulin (all mAbs from BD Biosciences, USA) was used as an isotype-match control antibody for determining the extent of non-specific binding. Ascidian cells were incubated with antibodies at concentrations recommended by the manufacturer. For the assay, 100 μl of cell suspension from each animal, harvested at different time points, were incubated with the primary antibody, in microtubes, for 3 h in the dark at RT (20 - 25 °C) followed by adding 500 μl of BD FACS Fixative Solution (BD Biosciences, USA) according to the manufacturer's instructions. In order to properly exclude all events, which did not match morphometric parameters of ascidian hemocytes (by size and granularity) from analysis, gating strategy was applied based on particle distribution according to forward and side scattering. Fluorescence data were summarized per 10,000 cells gated according to their size and granularity. The expression of antigenic epitopes cross-reactive with anti-human Abs raised against CD15, CD29, CD34, CD54, CD62L, CD62P, CD90, CD94, CD117, and CD166 was presented as a percentage of cells stained with specific Abs as revealed by fluorescence of higher intensity than the upper threshold of isotype-match control antibody staining.

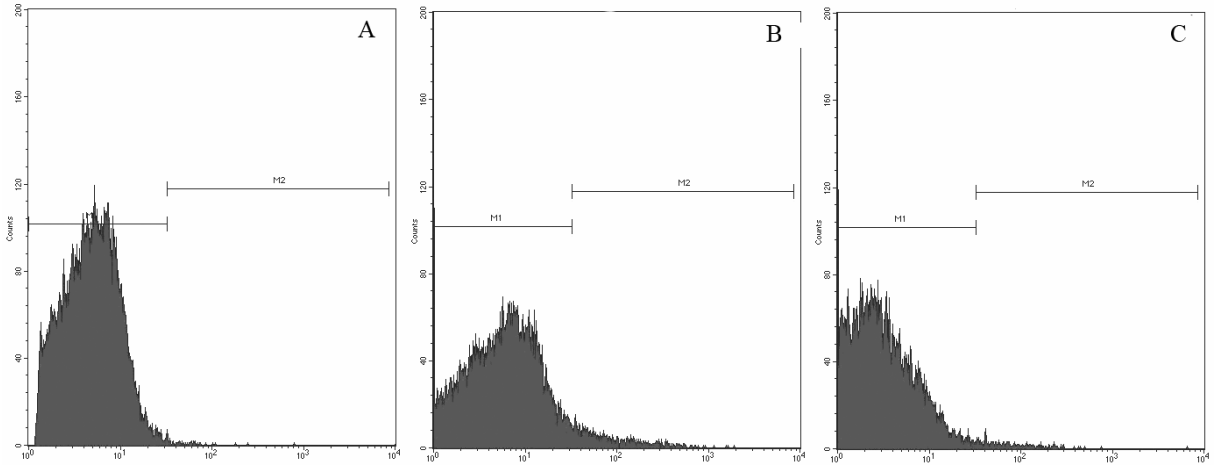

Fig. 1 Binding of anti-human CD54 mAbs with ascidian *H. aurantium* hemocytes in response to tissue damage assessed by flow cytometry. Staining of the cells with: A - antibody of isotype control, B - 0 h, C - 24 h after tunic damage. M1-gate - staining with isotype-match control antibody, M2-gate - CD54-positive cells. X-axis: FL4 channel (logarithmic scale); Y-axis: number of events (cells).

Confocal microscopy

Confocal microscopy was used to detect surface adhesion molecules using the panel of Abs mentioned above. Briefly, after washing, cell suspension was placed on the Culture Slides BD Falcon™ (Becton Dickinson, USA) followed by sedimenting the cells for 2 h in the dark at RT. Hemocytes were then incubated with 50 μl of diluted antibodies and incubated for 2 h in the dark. After that, specimens were washed with phosphate-buffered saline followed by fixing with 4 %-formalin solution. Immediately before analysis, specimens were additionally stained with DAPI (Sigma, USA) and mounted with Mowiol. Specimens were analyzed by using confocal microscope Zeiss LSM 510 Meta (Germany) and LSM 510 (Release Version 4.2) software. Additionally, image processing was performed using Image Browser software. Fluorochrome-conjugated Abs were excited by two lasers with wavelength 364 and 633 nm, and signals were detected using 435 - 485 nm and 650 - 704 nm fluorescence filters for DAPI and allophycocyanin, respectively. Spectrum channel PMT1 and PMT2, for detecting DAPI and allophycocyanin, respectively, were coded by blue and red color. Overall, >150 images including phase-contrast imaging were taken at magnification 40x as well as 63x for oil-immersion microscopy.

Statistical analysis

Statistical analysis was performed according to previous recommendations. Data were presented as mean ± SEM. The statistical analysis was made using Statistic 7.0 software (StatSoft, USA). Results are presented as the mean and SEM. The significance of differences was assessed using Student's t-test. Significance level was set at $p < 0,05$.

Results and Discussion

Previously, by applying standard morphology methods (Sukhachev *et al.,* 2013) and flow cytometry (Sukhachev *et al*., 2015) 5 main subsets of circulatory hemocytes, in ascidian *H. aurantium* were described: hemoblasts, granulocytes, hyaline amoebocytes, macrophage-like and morula cells.

During the current study, we assessed dynamics of the major hemocyte subsets in response to tissue injury 24 h after tunic damage in the same species (Table 1). It was demonstrated, that more than 90 % of circulatory hemocytes were available for phenotyping and included into analysis, which is a good yield given technical limitations of

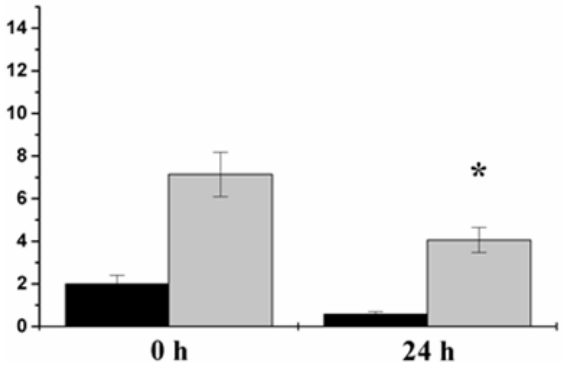

Fig. 2 Analysis of CD54-like marker expression in total population of circulatory hemocytes assessed by flow cytometry. X-axis: time after tunic damage; Y-axis: percentage of positive cells. Bars: black - the cells stained with antibody of isotype control, gray - the cells stained with mAbs against cell marker. Data expressed as mean ± s.e.m., n ≥ 12.

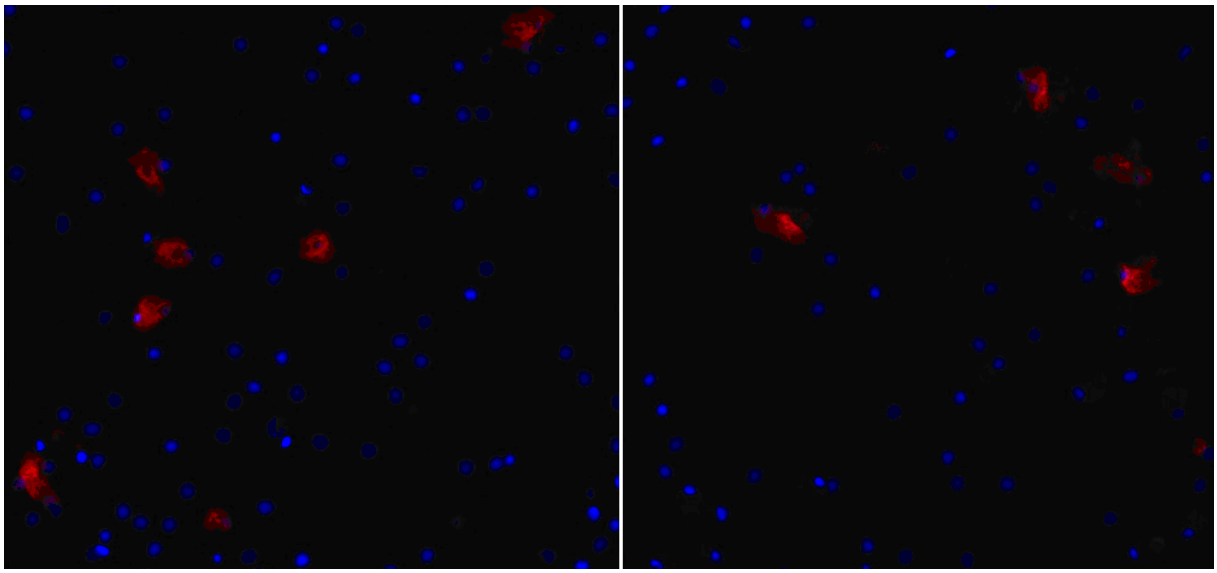

Fig. 3 Binding of anti-human CD54 Abs to circulating hemocytes from the ascidian *H. aurantium*, assessed by confocal microscopy and stained with APC-conjugated Abs (650 - 704 nm, red color) and DAPI (435 - 485nm, blue color). Left: 0 h; right: 24 h after tunic damage.

flow cytometry. Moreover, it was found that percentage of hemoblasts and morphologically similar hyaline amebocytes was significantly increased in the circulation 24 h after tunic damage. Conversely, the amount of circulating macrophage-like and morula cells as well as granulocytes was only slightly, not significantly, decreased, probably due to their role in thrombogenesis.

The capacity of antibodies to cross-react with homologous or analogous epitopes on hemocytes was examined in both intact and operated ascidians. In the last case, tissue repair was triggered tissue reparation. No binding to any hemocyte subset from *H. aurantium* was detected by flow cytometry or confocal microscopy was observed with the five mAbs (CD15, CD62L, CD62P, CD94, CD166) used during the experiments.

In the case of two Abs (CD54 and CD90), binding to ascidian hemocytes was observed in both assays. However, the dynamics of expression of both the surface epitopes detected by these Abs was different: the expression of CD54-like epitope was decreased after tissue damage, whereas, for CD90-like epitope, labelling was upregulated on all examined hemocyte subsets after tissue damage.

CD54 (ICAM-1) belongs to immunoglobulin superfamily and is involved in the interaction between antigen-presenting cells and naïve T cells. Molecules from immunoglobulin superfamily were unequivocally confirmed to be expressed in ascidians (Dehal *et al*., 2002; Cima *et al*., 2004). In addition, it was firmly established that ascidians lack TcR and BcR (Dehal *et al*., 2002). Therefore, in case of using monoclonal antibody against human ICAM-1 apparently it might be correct to interpret such data as detection of cross-reactive epitope on some hemocyte surface molecule belonging to immunoglobulin superfamily or containing, along with lectin, one or more immunoglobulin domains. Moreover, a search for CD54-like molecules in ascidians has never been done before with anti-human CD54 Abs (Figs 1 - 3).

Table 2 Dynamics of CD54- and CD90-positive hemocytes isolated from ascidian *H. aurantium* in response to tunic damage assessed by flow cytometry

Hemocyte subset	CD54		CD90	
	Control	24 h after	Control	24 h after
Hemoblasts and hyaline amebocytes	1.76±0.52	5.19±0.88*	19.10±4.85	42.56±3.77*
Macrophage-like cells	2.79±0.71	8.79±1.81*	29.18±5.23	64.00±3.66*
Morula cells and granulocytes	8.24±1.46	3.52±0.62*	34.03±3.21	51.96±3.30*

All comments as in Table 1.

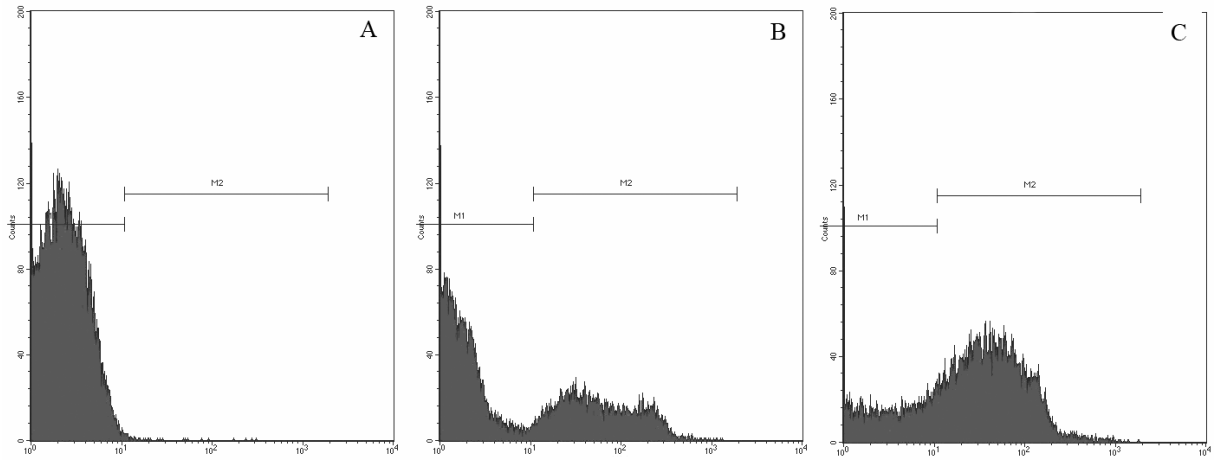

Fig. 4 Binding of anti-human CD90 to hemocytes from the ascidian *H. aurantium* in response to tunic damage assessed by flow cytometry. Figure captions as in Figure 1

Expression level for molecule bearing epitope cross-reactive with human ICAM-1 was found to be at low level in intact animals (M2-gate, Fig. 1B), which was downregulated by ~2-fold after tunic damage (Fig. 2): intact ascidians contained 7.14 ± 1.04 %, 24 h later after tissue damage its expression declined down to 4.06 % ± 0.59 % out of total hemocyte population. By using confocal microscopy it was shown that intact ascidians contained 9.67 % ± 2.19 % ICAM-1-like-positive cells, whereas 24 h later after tissue damage its percentage decreased to 7.33 ± 3.84 %. It should be noted that a huge amount of granulocytes and morula cells was lost after placing them on the slides, whereas this preparation technique did not affect amount of phagocytes and hemoblasts by allowing to detect them at full amount. Therefore, the quantitative data obtained by flow cytometry and confocal microscopy due to the features of these assays must be compared with caution.

In addition, flow cytometry allowed assessing dynamic changes in binding of anti-human CD54 Abs to the earlier described phenotypic subsets of hemocytes from *H. aurantium*. In particular, it was found that subset-specific dynamics in expression of CD54-like marker differed from that one described in total hemocyte population (Table 2). Morula cells were found to represent the majority of CD54-positive cells (8.24 ± 1.46 %), whereas hemoblasts and hyaline amebocytes expressed this marker at the lowest level (0.23 ± 0.06 %). Importantly, 24 h after the onset of tissue damage, only 3.52 ± 0.62 % of morula cells were positive to anti-CD54 antibody ($p < 0.05$), whereas in undifferentiated hemoblasts the percentage of positive cells increased from 1.76 ± 0.52 % up to 5.19 ± 0.88 % ($p < 0.01$), and in macrophage-like cells - from 2.79 ± 0,71 % up to 8.79 ± 1.81 % ($p < 0.01$). By taking into consideration, that morula cells is the most abundant cell subset among circulatory hemocytes in *H.aurantium* (~66 % out of total cells, according to previously published data (Sukhachev *et al*., 2015), it may be concluded that the decreased binding of anti-human Abs to CD54 in total hemocyte population may be of importance, as this was exactly the hemocyte subset containing the highest amount of positive cells, therefore, being responsible for overall drop in expression level, despite the fact that its expression was shown to be upregulated in other hemocyte subsets. It seems essential that such CD54-like molecule most likely represents an analogue of adhesion molecule involved in inter-cellular interactions.

In human, anti-CD90 Abs bind to Thy-1 homologue, which is typical of thymocytes and determines T-cell lineage differentiation. Therefore, a search for a molecule with cross-reactive epitope on ascidian hemocytes by anti-CD90 Abs is of interest in terms of evolutionary histology and comparative immunology. Anti-human CD90 Ab recognized a great number of hemocytes (Figs 4 - 6)

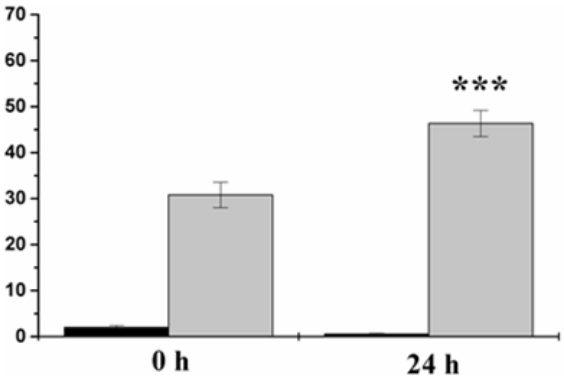

Fig. 5 Analysis of CD90-like marker expression in total population of circulatory hemocytes assessed by flow cytometry. Figure captions as in Figure 2.

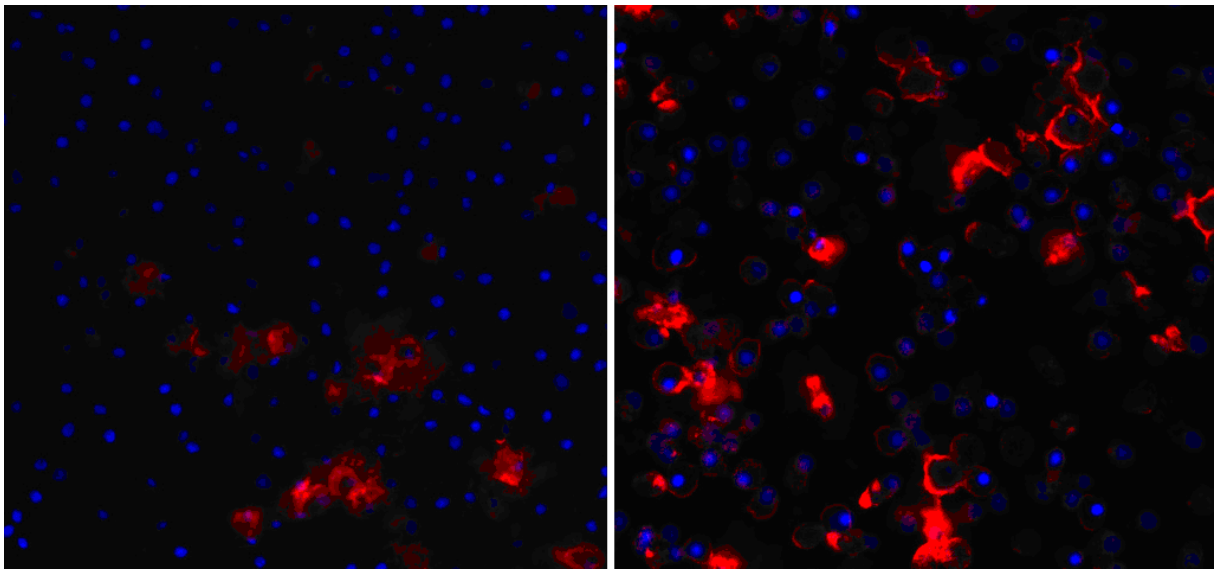

Fig. 6 Binding of anti-human CD90 Abs to circulating hemocytes from the ascidian *H. aurantium* assessed by confocal microscopy. Figure captions as in Figure 3.

which, in intact animals, amounted to 30.79 ± 2.78 %, it rose up to 46.33 ± 2.85 % ($p < 0.001$) of total circulatory hemocytes (Figs 4, 5). Similar tendency was observed on specimens for confocal microscopy, wherein percentage of CD90-like-positive cells increased from 46.66 ± 19.06 % up to 70.00 ± 2.45 % (Fig. 6).

Antibody against human CD90 was shown to bind to the all examined ascidian cell subsets. Nonetheless, the amount of morula cells bearing CD90 cross-reactive epitope was 34.03 ± 3.21 %, *i.e.,* 1.78- and 1.17-fold higher than of hemoblasts, hyaline amebocytes and macrophage-like cells compared to total hemocytes (Table 2). Moreover, 24 h after tunic damage the percentage of CD90+-like cells was upregulated in all examined cell subsets. Analyzing the percentage of $CD90^{+}$-like cells in various hemocyte subsets, it was found that it increased by 2.1-, 2.2- and 1.8-fold in hemoblasts, macrophage-like and morula cells, respectively. This suggests both the direct participation of CD90 homologue/analogue and the cells expressing it in tissue repair as well as the presence, in ascidians, of evolutionary T cell precursors, which are involved in reparative responses.

Finally, three other mAbs (CD29, CD34, CD117) used in our study provided an incomplete match between the data obtained by both the assays. Whereas flow cytometry detected a very low binding for such Abs, confocal microscopy allowed to observe positive staining only in some of the cells. However, in this case positive signal was detected not only along the contour of the cells, as shown for anti-CD90 Abs (Fig. 6), but on the entire area of the cells as exemplified for anti-CD29 Abs (Fig. 7). Probably, the fluorescent staining detected by confocal microscopy, reflects the higher sensitivity of this approach as well as previously described autofluorescence emitted by granules of morula cells (Sukhachev *et al.,* 2015). In addition, one should not exclude the possibility of interaction between antibodies and epitopes located inside the cell cytosol, which, however, should be further investigated in detail.

At least five major cell subsets were detected in the circulation of the ascidian *H. aurantium*. Among them, hemoblasts, hyaline amebocytes, macrophage-like and morula cells (Sukhachev *et al*., 2013). In terms of morphology, hemoblasts from *H. aurantium* virtually did not differ from hemoblasts of other ascidian species. In all tunicates, hemoblasts are characterized by a high nucleus-cytoplasm ratio, rarely exceeding 5 μm in diameter (Hirose *et al*., 2003). In addition, it is worth mentioning that morphologically ascidian hemoblasts are similar to mammalian lymphocytes so that many investigators call them as lymphocyte-like cells (Fuke, 2001). In response to tunic damage, an increased amount of hemoblasts as well as hyaline amebocytes, which might be histogenetically related to them (Ballarin and Cima, 2005), was observed in circulation.

Recently, flow cytometry was widely applied in various scientific areas (Kudryavtsev *et al*., 2012). Annually, a rising number of studies dedicated to investigating circulatory cells of invertebrates and lower vertebrates is published. During our study, we applied flow cytometry to examine hemocytes from the ascidian *H. aurantium*. We found that flow cytometry data on examined cell subsets are in good agreement with the results obtained by light microscopy and data published elsewhere (Hirose *et al*., 2003; Sukhachev *et al*., 2013, 2015).

However, whether hemoblasts are able to differentiate only into circulatory cells or they represent stem cells with a broader range of potential lineage-specific transitions still remains unclear. At present, an open question is whether

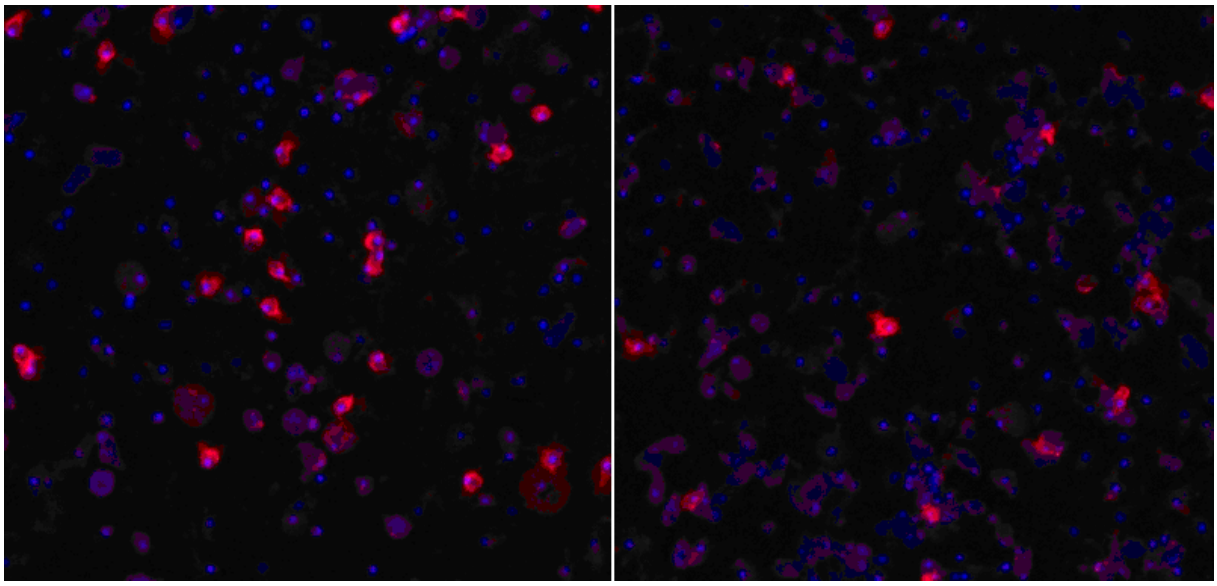

Fig. 7 Binding of anti-human CD29 Abs to circulating hemocytes from the ascidian *H. aurantium* assessed by confocal microscopy. Figure captions as in Figure 3.

ascidians contain circulatory pluripotent stem cells as well as oligopotent stem cells in various tissues. However, it is generally accepted that hemoblasts form different morphotypes of circulatory cells and that the latter (*e.g.*, morula cells) may take part in formation of distinct tissue structures, *e.g.*, tunic layers (Smith, 1970; Smith and Peddie, 1992; Ballarin and Cima, 2005). Amount of morula cells as the most abundant subset of circulatory hemocytes decreases after tissue injury, because they are responsible for "wound sealing" (Smith and Peddie, 1992).

Altogether, it clearly justified the reason for performing quite a broad examination of changes in adhesion molecule repertoire on hemocytes in response to tissue damage. It is evident that response to tunic damage coupled with extensive loss of circulatory cells was paralleled with significant downregulation of these markers on morula cells and granulocytes. On the conversely, the only exclusion from this rule was the upregulated expression of the marker bearing epitope that cross-reacted with anti-human CD90 Abs. Quite possibly, upregulated expression on all examined hemocyte subsets of a molecule cross-reacting with anti-CD90 Abs exactly reflects an enhanced intercellular cooperation in response to tissue damage. By taking into consideration that morula cells and granulocytes are the most differentiated subsets of hemocyte population (Chaga, 1998) as well as the fact that they are involved in the recovery of ascidian tunic in response to tissue damage these cell types were consumed *in vivo* much faster compared to the other ones. Upon that, flow cytometry data showed that percentage of these cell subsets was insignificantly decreased (Table 1).

Thus, by using flow cytometry and confocal microscopy, it was suggested that the relative level of hemoblasts and hyaline amebocytes was increased in response to tunic damage in ascidian *H. aurantium*. This process was accompanied by altered expression repertoire of membrane receptors, wherein some of them bear antigenic epitopes cross-reactive with antibodies against adhesion molecules of human lymphoid (CD54, CD90) and stem cells (CD90). However, in order to unequivocally conclude that ascidians possess an established system of circulatory stem cells, it is necessary to assess proliferative activity of stem cells both in circulation and at site of tissue damage.

Acknowledgements

This study was supported by Program №1326 of the Ministry of Education and Science, Russian Federation, and Grant of The Russian Fund of Basic Researches (№15-04-05093).

References

Ballarin L, Cima F. Cytochemical properties of *Botryllus schlosseri* haemocytes: indications for morpho-functional characterization. Eur. J. Histochem. 49: 255-264, 2005.

Chaga OYu. Blood cells from ascidian *Styela (Goniocarpa) rustica*. I. Histology analysis. Tsitologiya 40: 31-44, 1998 (paper in Russian).

Cima F. Sabbadin A, Ballarin L. Cellular aspects of allorecognition in the compound ascidian *Botryllus schlosseri*. Dev. Comp. Immunol. 28: 881-889, 2004.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A *et al.* The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298: 2157-2167, 2002.

Fuke M. Cell types involved in allogeneic contact reactions of the solitary ascidian, *Halocynthia roretzi*. Zool. Sci. 18: 195-205, 2001.

Hirose E, Shirae M, Saito Y. Ultrastructures and classification of circulating hemocytes in 9 Botryllid ascidians (Chordata: Ascidiacea). Zool. Science 20: 647-656, 2003.

Kudryavtsev IV, Khaydukov SV, Zurochka AV, Chereshev VA. Flow cytometry in experimental biology. Ekaterinburg*:* RIO UrO RAN, p.192, 2012 (paper in Russian).

Polevshchikov AV, Kudryavtsev IV, Dyachkov IS, Kharazova AD. Theory of tissue parallelism in comparative immunology. Vestnik. Sankt-Peterburgskogo universiteta 3: 68-74, 2005 (paper in Russian).

Smith MJ. The blood cells and tunic of the ascidian *Halocynthia aurantium (Pallas)*. I. Hematology, tunic morphology, and partition of cells between blood and tunic. Biol. Bull*.* 138: 354-378, 1970.

Smith VJ, Peddie CM. Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis (L).* Biol. Bull. 183: 211-219, 1992.

Sukhachev AN, Dyachkov IS, Romanyuk DS, Kumeiko VV, Sinitsina VF, Korolkova ED, *et al.* Morphology analysis of hemocytes from ascidian *Halocynthia aurantium*. Tsitologiya 55: 901-906, 2013 (paper in Russian).

Sukhachev AN, Dyachkov IS, Kudryavtsev IV, Kumeiko VV, Tsibulsky AV, Polevshchikov AV*.* Experience of using flow cytometry for analysis of circulatory hemocyte subsets from ascidian *Halocynthia aurantium* (Pallas, 1787). J. Evol. Biochem. Physiol. 51: 246-253, 2015.

Zavarzin AA. Essays on evolutionary histology of blood and connective tissue. Moscow - Leningrad: USSR Academic Publishing 4: 720, 1953 (paper in Russian).