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CYTOBIOLOGY

Comparative Morphological Analysis of in Vitro Cultured Hemocytes from Two Species of Bivalve Mollusks[1]

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Abstract—A comparative analysis of the morphology of hemocyte spreading on cover glasses from two bivalve species, the Japanese scallop *Mizuhopecten yessoensis* and Pacific mussel *Mytilus trossulus*, was carried out. Parameters for the quantitative analysis of cell morphology were suggested. The interspecific comparison of single spreading hemocytes and cell communities from two and three adjoining cells revealed significant differences in a number of studied parameters, which testified to the taxa specificity of the given cell features and the fundamental possibility of the numerical description and differentiation of complicated forms of cell spreading in vitro.

Key words: Japanese scallop *Mizuhopecten yessoensis*, Pacific mussel *Mytilus trossulus*, hemocytes.

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The morphology of cells is defined by a set of factors, including the behavior of the cells, as well as their genetic, functional [9, 11], biochemical, and physiological [4, 14, 21, 27] features. Cell morphology can vary in response to the effects of outer agents, including hormones and cytokines [20, 25]. This depends on the features of the extracellular matrix [15, 22, 28], the mechanical properties of the substrate [3], cytoskeletal structures [6, 10, 12, 16, 19, 23, 24], and on the set of cell adhesion molecules [5]. Thus, the modeling of morphogenesis at the cellular level is relevant for understanding the features of cell behavior and physiology. Such modeling needs, in turn, a quantitative description of cell morphology.

In Zavarzin's work [2] based on light microscopy, the morphology of hemocytes of invertebrates was described in detail, and the classification of hemocytes by phylogenetic, physiological, and morphological criteria was suggested. A.A. Zavarzin hypothesized that cells of the connective tissue and blood of invertebrates are rather poorly differentiated and are presented by several basic types, which are common to all invertebrates. The individual specialized types of cells that occur in various invertebrate species or the morphofunctional distinctions of cells are variations of the initial general types.

Hine [13] systematized the data on the cell structure and typology of the hemolymph cells of bivalves. He distinguished granular hemocytes as a uniform group and subdivided agranular hemocytes into three types, blastoid cells with basophilic cytoplasm, macrophagoid cells with basophilic cytoplasm, and hyalinocytes with colorless cytoplasm. Granulocytes most likely had agranular predecessors. To identify distinctly agranular and granular cell is impossible, as, unlike leukocytes of vertebrates, they are insufficiently well differentiated and distinguishable. Moreover, functional heterogeneity is possible within each morphological type.

The conventional morphological criteria for the differentiation of types of molluscan hemocytes are cell size, nucleus/cytoplasm ratio, and the size of granules contained in the cytoplasm. Compared with granulocytes, the hyaline cells, as a rule, are of smaller size and have a larger nucleus, i.e., they are characterized by a high nucleocytoplasm ratio, and their cytoplasm is more electron dense, because of a significant number of small (50 nm in diameter) inclusions [18].

Hemocytes of the Pacific mussel *Mytilus edulis* are subdivided into three classical groups, viz., hyalinocytes and two types of granulocytes [7]. The large, eosinophilic granulocytes of *M. edulis*, compared with hyalinocytes, have a more pronounced ability for phagocytosis, development of active forms of oxygen, and manifestation of cytotoxic activity [8].

According to the literature, the cellular structure of hemolymph of the Japanese scallop *Mizuhopecten yessoensis* is heterogeneous and consists of cell forms that are morphologically transitive between different types of cells. Large agranulocytes compose the base of the cell population and granulocytes are about 4% of the total number of amoebocytes [1]. Data that demon-

[1] The article was translated by the authors.

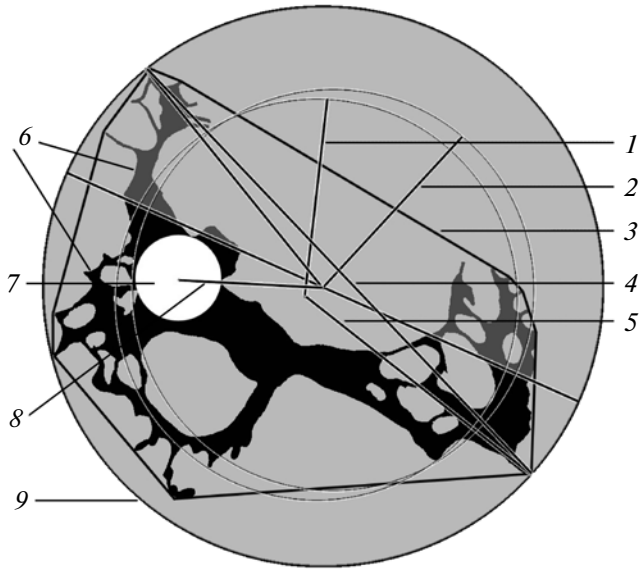

Fig. 1. Morphological parameters of hemocytes. *1*, average radius from the center of mass to the convex hull (AR); *2*, average radius from the center of bounding circle to the convex hull (ARCR); *3*, convex hull; *4*, straight line connecting the most remote points of the convex hull (MRCH); *5*, the maximum radius from the center of mass of the convex hull to boundary of convex hull (MR); *6*, cell parts in different halves of the cell circumscribing circle (CA); *7*, circle inscribed in a cell; *8*, the distance from the center of the bounding circle to the center of the inscribed circle (DCC); *9*, cell-circumscribing circle.

strate the absence of granulocytes in *M. yessoensis* [26] have been published.

Our work is the first attempt to describe and compare the hemocyte spreading for two species of marine bivalve in identical conditions in vitro on the basis of a quantitative analysis of their morphology.

MATERIAL AND METHODS

The hemocytes of two bivalve species, the Japanese scallop *Mizuhopecten yessoensis* (the Pectinidae family) and Pacific mussel *Mytilus trossulus* (the Mytilidae family) were chosen for comparative description.

The animals were sampled in the middle of September in the Vostok Bay of Peter the Great Bay, Sea of Japan. After they were caught, the animals were opened and a sample of hemolymph was taken with a syringe from the pericardium of the Japanese scallop and the Pacific mussel, which immediately was dropped on a cover glass. The glasses were kept for 1 h at room temperature, then attached to glass cells, where they were fixed with 4% formaldehyde in sea water. The cells were stained with hematoxylin–eosin up to saturation, dehydrated, and embedded in balm for study under the light microscope. The contours of the spread cells were outlined, using an RA-6 drawing device with an Ergaval and a Carl Zeiss Jena light microscope.

These 2D-cell images were scanned and digitized (Fig. 1). The morphological parameters of the cells were assessed using the FracLac 2.5 plug in for ImageJ image analysis software and Adobe Photoshop CS2 image editor. The statistical analysis was carried with the Statistica 6.0 software package.

The greater part of the parameters were used for quantitative analysis of single cells and cell communities; the last (5th) group of parameters was applied only for single cells:

(1) attributes based on measurement of the convex hull (CH), viz., a polyline outlining of the 2D image of the cell without acute angles:

—the area confined by the convex hull (ACH);

—the maximum remoteness of the points of the convex hull (MRCH) is a line connecting remote points, which cannot pass through the center of mass of the convex hull;

—the maximum radius from the center of mass of the convex hull to the boundary of the convex hull (MR);

—the average radius from the center of mass of the convex hull to the convex hull (AR); the AR and MR differ significantly if the cell has a long outgrowth;

—the maximum radius from the center of the cell-boundary circle to the border of the convex hull (MRCR);

—the center of mass of the convex hull and the center of the boundary circle may not coincide, which distinguishes this parameter from the maximum radius of the center of mass of the convex hull to the boundary of the convex hull, although both parameters may correlate;

—the average radius drawn from the center of the cell is the boundary circle to the boundary of the convex hull (ARCR);

—the ratio of the greatest and least axes of the convex hull (RGLA);

—the ratio of the maximum and minimum radii drawn from the center of mass to the boundary of the convex hull (RRCMB);

—the ratio of the maximum and minimum radii drawn from the center of cell;

—the boundary circle to the boundary of the convex hull (RRCAC);

—the ratio of the area limited by the cell; and

—the boundary circle to the area limited by the convex hull (Scir/Scon).

(2) An attribute based on measurement of the circumscribing circle (the circle of the minimum radius that can circumscribe the cell); this characterizes the space occupied by the cell, taking the symmetry of rotation into account: the diameter of the circumscribing circle (DCC).

(3) The cell area (Sc) is a parameter that characterizes the size of the cell and its degree of flat spreading.

(4) The density is equal to the ratio of the cell area to the area of the background in the convex hull.

(5) The parameters used only for single cells include:

—the ratio of the areas of the cell parts in different halves of the cell boundary circle (CA);

—the degree of asymmetry of the cell (the diameter of the boundary circle divides the cell in such a manner that the two parts resulting from the division are different in their area from each other by as much as possible);

—the ratio of the total cell area to the area of the inscribed circle (Sc/Sinc)

—the circle inscribed in the cell not adjoining the extracellular space reflects the area of the continuous surface of the spreading cell. Closed perforations of a cell layer in the inscribed circle are admissible, while their total area does not exceed the area filled with the cell;

—the distance from the center of cell boundary circle to the center of the inscribed circle (DCC) characterizes the remoteness of the area with the greatest continuous cell covering from the geometrical center of the cell. As the concentration of the main mass of the cell substance often falls on the region with the maximum area of continuous surface of the spreading cell, this parameter can partly describe the asymmetry of the allocation of the main mass of the cell substance relative to the geometrical center of cell;

—the maximum extent of the cell boundary (MECB) is defined by the size of cell, but to a greater degree by the irregularity of its boundaries and number of processes. During the assessment, both the outer boundaries and the ruptures of the spreading cell that were not connected to the extracellular space surrounded by the plasma membrane and closed cavities formed by jointed pseudopodia were measured;

—roundness (Rot) is the roundness of the convex hull. $Rot = 4\pi S/P^2$ (S is the area limited by the CH; P is the CH perimeter, for a circle this value is equal to one.

RESULTS AND DISCUSSION

The external morphology of single spreading cells that are not contiguous to other cells and of communities of two and three contiguous cells was analyzed (Fig. 2). The spreading in vitro hemocytes of the studied species of invertebrates were visually distinguishable, i.e., they were species specific morphologically. The formal quantitative description of spreading cells was complicated by the great variety of forms of these biological objects, which were difficult to formalize, and "wrong" from the point of view of Euclidean geometry.

Morphometric parameters of hemocytes of *Mizuhopecten yessoensis* and *Mytilus trossulus* ± standard error of the mean

Parameters	Hemocytes	
	M. yessoensis	*M. trossulus*
ARCR	304.5 ± 7.61	135.2 ± 3.45
AR	301.9 ± 7.63	133.4 ± 3.37
RGLA	1.6 ± 0.05	1.4 ± 0.04
RRCAC	1.7 ± 0.08	1.6 ± 0.06
RRCMB	1.8 ± 0.05	1.7 ± 0.06
CA	3.0 ± 0.35	1.7 ± 0.08
DCC	78.5 ± 6.26	30.2 ± 2.38
Density	0.4 ± 0.02	0.7 ± 0.01
ACC	216525.3 ± 11450.93	47291.7 ± 2228.83
Rotundity	0.8 ± 0.01	0.9 ± 0.01
MRCR	338.4 ± 8.69	151.2 ± 4.06
MR	375.1 ± 9.92	164.0 ± 4.77
MRCH	672.3 ± 17.43	300.1 ± 8.11
DCC	676.8 ± 17.39	302.3 ± 8.11
Scir/Scon	1.8 ± 0.05	1.6 ± 0.06
Sc/Sinc	2.8 ± 0.13	2.2 ± 0.06
SC	87841.4 ± 3785.51	33545.6 ± 1703.21
MECB	4373.333 ± 251.2263	1201.057 ± 52.1318

Single hemocytes of the Pacific mussel had higher values than the cells of Japanese scallops for almost all linear parameters, except image density and roundness (see the table). This showed the greater size of the hemocytes of the Pacific mussel spread on glass compared with the hemocytes of the Japanese scallop, both for single cells and for communities of two and three cells.

The higher values of the Sc/Sinc parameter that were obtained for the hemocytes of the Pacific mussel show greater partition, perforation, or stretching of the cells; the area of the continuous space of the cell surface (the area of the inscribed circle) was significantly less than the total cell area. This coordinated with the visual assessment of the hemocytes of both studied molluscan species; the cells of the Pacific mussel had a greater number of perforations and long thin processes.

The higher value of cell density in the Japanese scallop also showed the denser filling of space by the cell and the compactness of its form. This was caused by the lower level of partitioning and the considerable roundness of the cell and the existence of a smaller number of processes and perforations; this coordinated with the visual assessment of the cell form of the hemocytes.

Cells of the considered species did not differ significantly in their RGLA, RRCMB, RRCAC, and

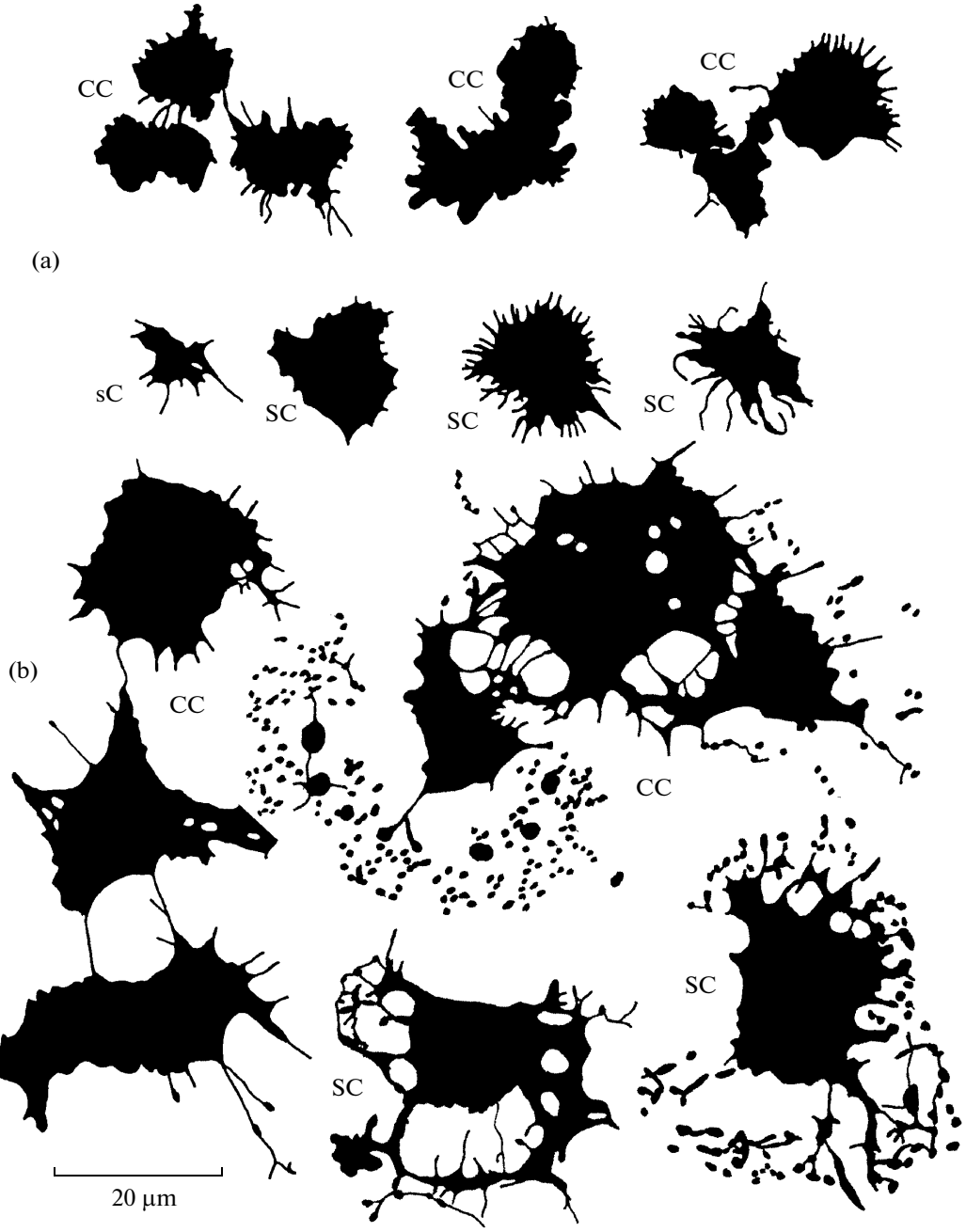

Fig. 2. Single cells (SC) and communities of two and three contacting hemocytes (CC) in the studied bivalve species in vitro. (a) *Mizuhopecten yessoensis*, (b) *Mytilus trossulus*.

Scir/Scon parameters (table). The first three attributes are connected to the form of the convex hull. The lack of differences in the fourth attribute shows the high correlation between the value of the circle circumscribing the area and the convex hull in both species (the value of the correlation was 0.8065, with $p = 0.000$ for cells of the Japanese scallop and 0.8828, with $p = 0.00$ for cells of the Pacific mussel).

It is worth noting that for a great number of linear parameters the confidence intervals were wider for the parameters of the hemocytes of the Pacific mussel for most of the linear parameters (MRCH, ACC, MR, AR, DCC, MRCR, ARCR, CA, Sc/Sinc, DCC, and Sc); this showed the greater dispersion of morphological, including dimensional, attributes of the hemocytes in this species.

The roundness of the convex hull was a little greater in the more compact single hemocytes of the Japanese scallop. The roundness value for cell ensembles that contain a small number of cells should greatly depend

on the relative arrangement of the cells. Visually, there were no differences in the arrangement of neighboring cells recorded in various molluscan species. Various possible spatial combinations were observed: cells were arranged in rows, triangles, merged in a way that resembled a uniform cell, or contacted only a few processes; filopodial forms were found next to lamellipodial forms and significantly spread forms with less spread forms. No significant differences in roundness between two and three-cell ensembles of molluscan hemocytes were revealed.

Compared with single cells, two and three-cell conglomerates of hemocytes of Pacific mussels and Japanese scallops differed in their significantly higher values of the dimensional area characteristics, MRCH, ACC, MR, AR, DCC, MRCR, and ARCR. Three-cell conglomerates were distinguished by significantly lower, values of density due to an increase in intercellular spaces compared with single cells.

There were no significant differences between single cells and cell communities of the same species for the RGLA, RRCMB, and RRCAC parameters, which characterize cell form. Comparison of single cells and three-cell groups of the Japanese scallop revealed that the roundness parameter significantly decreased; the convex hull of the single cells of this mollusk was closer to the roundish form than that in the conglomerates of three cells, which were characterized by greater variability in their relative positioning. The numerical value of the roundness in single cells of the Japanese scallop was also higher than that in the cells of the Pacific mussel; this coordinated with the visually lower level of variability and greater roundness of the form of the hemocytes of the Japanese scallop compared with the Pacific mussel. The variation of this parameter in one to three cells of the Pacific mussel was great, which does not allow its wide use in the roundness estimation of hemocytes.

The obtained results displayed significant differences in the spreading of the hemocytes of *M. trossulus* and that of the hemocytes of *M. yessoensis* in culture according to a number of morphological parameters. The revealed differences are obviously related to species-specific features of the organization of the cytoskeleton, with the concentration and distribution of cell adhesion molecules, with the features of cell behavior, and with other factors that affect the form of the spreading cell; therefore, the approach used in this work may be effective for the characteristics of the morphological condition of hemocytes and is promising for the widest spectrum of studies on the morphogenesis of hemocytes, both, in vivo and in vitro, in particular, for studies of the rearrangement of the cytoskeleton depending on culture conditions.

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