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Studies of the Role of Phagocytes in Superficial Wound Healing in the Holothurian *Eupentacta fraudatrix*

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Abstract—The abundance and functional activity of two types of phagocytes of the sea cucumber *Eupentacta fraudatrix* have been studied at individual stages of wound healing. It is shown that the dynamics of the number of individual types of phagocytes and their viability do not correspond to those for coelomocytes. In this case, injury causes a shift in the proportion and opposite changes in the functional activity of different types of phagocytes, which also had opposite directions at the early and later stages of wound healing. The administration of proteins isolated from the coelomic fluid of regenerating holothurians to wounded individuals at different stages caused multidirectional shifts in the ratio of the two types of phagocytes and their activity compared to the effect of the wound itself. It has been concluded that the two types of phagocytes can play different roles in wound healing.

Keywords: sea cucumbers (holothurians), phagocytes, nitric oxide, coelomic fluid proteins, wound healing

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INTRODUCTION

Wound healing is an important problem in biology and medicine, and the frequent development of prolonged non-healing wounds, which can lead to disability, is also becoming a social problem (Lindholm and Searle, 2016). Wounds, including superficial ones, affects different tissues and cell types (Yanez et al., 2017); therefore, understanding the general mechanisms of regulating their healing seems especially important for therapy. Inflammation is a fundamental type of tissue response to injury and a necessary stage in wound healing. It has recently been shown that the key role in the regulation of inflammation and tissue repair in vertebrates is played by macrophages (Guo et al., 2016). In tissues, they are activated ("polarized") by different exogenous and endogenous stimuli via two main pathways with the formation of two cell subpopulations called M1 and M2 macrophages, which are characterized by different markers. The main markers for M1 macrophages are high levels of nitric oxide (NO) and the expression of inducible NO synthase (iNOS), and the main marker for M2 macrophages is high arginase activity (Dolmatova and Dolmatov, 2021). M1 and M2 macrophages have different functions; at the early inflammatory stage of healing, the preferential polarization of tissue macrophages is observed along the M1 pathway; along the M2 pathway, it is recorded at the recovery stage (Ferrante and Leibovich, 2012). Activation of M1 macrophages depends on cytokines secreted by Th1 lymphocytes and activation of M2 macrophages depends on Th2 cytokines (Dolmatova and Dolmatov, 2021). In turn, cytokines and chemokines secreted by wounded macrophages are important for wound healing and their amount changes over time (Ishida et al., 2012). At the same time, the polarization of macrophages is influenced by changes in the surrounding tissues during wound healing (Sipka et al., 2022). The mechanism for maintaining the balance of pro- and anti-inflammatory activity of macrophages, necessary for normal physiological wound healing, remains poorly understood. Therefore, model systems for studying the interaction of macrophages with tissues are in demand. Immune cells of echinoderms have been of particular interest among these model systems. The phylum Echinodermata includes several classes of marine aquatic organisms evolutionarily close to vertebrates, that, along with the availability of cells freely circulating in the coelomic cavity of animals, makes them a convenient object of study (Pinsino et al., 2007). It was shown that holothurians (Echinodermata, Holothuroidea) had a high capacity for regenerating the lost body parts and effective wound healing (Dolmatov et al., 2021). In particular, an extract (SCE) with significant wound healing properties was

obtained from the tissues of Far Eastern species of sea cucumbers (Dolmatova and Ulanova, 2014).

Sea cucumber phagocytes are also involved in wound healing (Ramírez-Gómez et al., 2010). At the same time, the presence of individual functionally different types of phagocytes has been established in echinoderms (Prompoon et al., 2015; Dolmatova et al., 2019). Thus, two types of phagocytes (P1 and P2) with different morphofunctional characteristics have been identified in the sea cucumber *Eupentacta fraudatrix* Djakonov and Baranova, 1958 (Dolmatova and Smolina, 2022). In particular, high level of NO is a marker of P1, but not P2 phagocytes (Dolmatova et al., 2019). However, the role of individual types of phagocytes in wound healing and mechanisms of regulation of their activity are unclear.

It is known that the composition of protein fractions in the blood significantly changes under inflammatory conditions accompanying wound healing (Ermolaev and Nikulina, 2010). Similar changes in the protein composition of the coelomic fluid were found after injury in a representative of one of the echinoderm classes, the starfish *Asterias rubens*. The detected synchronicity of changes in the proteome and coelomocyte concentration suggests the presence of a functional relationship between them (Shabelnikov et al., 2019).

The purpose of this study was to determine the dynamics of the number of coelomocytes and P1 and P2 phagocytes during the healing of a superficial wound in the sea cucumber *E. fraudatrix* and identify proteins expressed during wound healing and establish their influence on NO production by P1 and P2 phagocytes.

MATERIALS AND METHODS

Holothurians. *E. fraudatrix* were collected by divers in spring from the Vostok Bay (Peter the Great Bay, Sea of Japan). Before the experiment, the animals were adapted in aquariums with running sea water at seasonal temperature for no less than two weeks.

Sea cucumber extract. SCE was obtained from the tissues of *E. fraudatrix* according to the previously described method (Dolmatova et al., 2007). The dry extract was diluted in phosphate-buffered saline with the addition of 36 g/L NaCl (PBSN) at pH 7.6.

Experimental design. At the first stage of the experiment, a superficial 5-mm long incision was made in sea cucumbers with a body length of 5.01± 0.43 cm using a sterile scalpel. Immediately after this incision, SCE (0.3 μg/g) or PBSN was injected into the coelomic cavity of the experimental animals ($n = 8$) through the intact body wall using a syringe. Control animals were injected with PBSN and a separate group was injected with SCE. After 1 day or 7 days, coelomic fluid was collected from the sea cucumbers and the concentration and viability of the coelomocytes were determined. For further isolation of phagocytes and study of protein fractions, samples from two individuals were combined. In the coelomic fluid samples obtained 1 day after the beginning of the experiment, after cell sedimentation by centrifugation, the distribution of proteins by molecular mass was studied and the protein components, the content of which significantly changed during the wounding and wound healing under SCE administration, were collected. At the second stage of the experiment, we used sea cucumbers with a body length of 5.36 ± 0.46 cm. The selected proteins were injected into the coelomic fluid of sea cucumbers ($n = 3$), which also underwent a superficial incision. Control animals were injected with PBSN. The coelomic fluid of individual animals was obtained 1 or 7 days after the beginning of the experiment. Phagocytes were isolated from the resulting samples and the quantitative ratio of P1 and P2 phagocytes (P1/P2) and NO level in the cells were determined.

Isolation of phagocytes. P1 and P2 phagocytes were isolated from the coelomic fluid by centrifugation in a ficoll–verografin density gradient, as previously described (Dolmatova et al., 2019), and resuspended in modified medium 199 (Dolmatova et al., 2003).

Concentration and viability of cells. The concentration of cells was determined in a Goryaev chamber, and their viability was tested using the trypan blue dye exclusion test (Phillips, 1973).

Determination of the molecular mass distribution of proteins and peptides. The molecular mass was determined by gel permeation chromatography (GPC) on an Agilent Technologies 1260 Infinity chromatograph (United States). Column: TSK gel G 3000PWXL (7.8 mm × 30 cm) (TOSOH Corporation, Tokyo, Japan). Flow rate 0.3 mL/min, temperature 25°C. Mobile phase 0.1 N NaCl, 20 mM Tris-HCl buffer, pH 7.8. Detection was performed at 280 nm. The molecular mass was determined using a calibration graph constructed based on standard protein samples (Sigma-Aldrich Co., United States): bovine serum albumin (66.3 kDa), egg albumin (44.3 kDa), myoglobin (18.0 kDa), cytochrome C (12.4 kDa), aprotinin (6.5 kDa), and bacitracin (1.4 kDa). All samples were filtered through a PVDF 0.2 syringe filter (Whatman). The sample volume introduced for separation was 30–50 μL. Fractions were collected in a continuous automatic mode at a volume of 100 μL/fraction. For further study, fractions containing individual proteins were combined in accordance with the chromatographic profile. All measurements were performed in triplicate and the retention time calculation data are presented as mean with standard deviation for components with molecular mass above 6 kDa and as a range for components with molecular mass below 6 kDa. The molecular mass is given as mean calculated from mean retention time. Protein concentrations in fractions were determined by Bradford method.

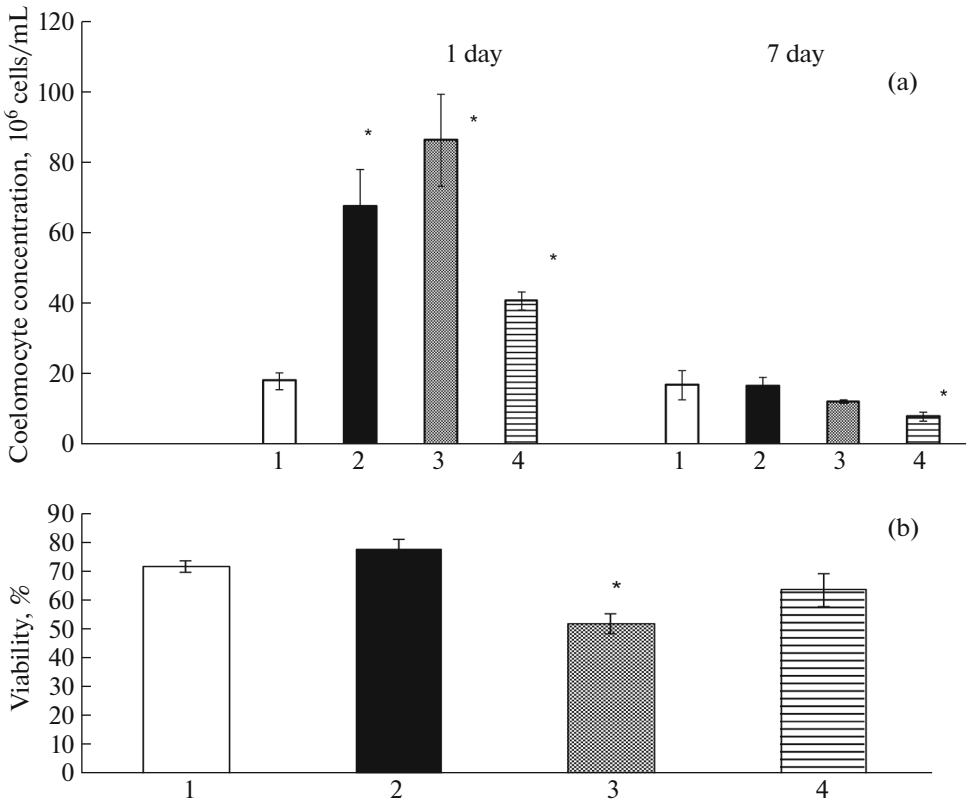

Fig. 1. Concentration of *E. fraudatrix* coelomocytes (a) 1 and 7 days after wounding and their viability (b) after 7 days. The abscissa axis shows the number of the experimental group: (1) control (PBSN), (2) SCE, (3) wounding + PBSN, (4) wounding + SCE. $N = 8$. * $P < 0.05$ compared to control.

Measurement of NO levels. NO levels were determined using the Griess reagent according to the previously described method (Torika et al., 2016).

Statistical analysis. Data were analysed statistically using the GraphPad InStat software, v. 3.01 (GraphPad Software, Inc.). The data for experimental groups are given as mean value ± standard error of the mean. The significance of differences was determined using Student's *t*-test. The difference between the samples was considered significant at $P < 0.05$.

RESULTS

Effect of SCE on Concentration and Viability of Coelomocytes during Injury

Superficial wounding, as well as SCE injection, induced a significant increase in the concentration of coelomocytes after 24 h (Fig. 1a); however, when SCE was administered to wounded animals, the concentration tended to decrease to the control values, although it still remained significantly elevated. Seven days after the beginning of the experiment, the number of circulating phagocytes returned to the control values except that in the group of wounded animals receiving SCE, where the concentration of coelomocytes even decreased compared to the control.

The viability of coelomocytes did not differ from the control level one day after wounding or SCE administration (data not shown). At the same time, the viability of coelomocytes decreased only in the group of wounded sea cucumbers after 7 days (Fig. 1b).

Effect of SCE on the Quantity and Viability of Phagocytes

Injection with PBSN induced a significant increase in the number of P1 and P2 phagocytes 1 day after the beginning of the experiment compared to the intact animals (Fig. 2a). SCE did not induce significant changes at this time compared to the effect of PBSN. After 7 days, in the groups administered with SCE or PBSN, the quantity of both types of phagocytes decreased significantly less than that in the intact animals. Although the quantity of both types of cells decreased after SCE administration, the content of P1 cells was just over a tenth of that of P2, while after PBSN administration it decreased to only 1.3 times. Therefore, SCE induced a shift in the ratio of P1 and P2 phagocytes (P1/P2) in favor of the latter.

The viability of P1 phagocytes significantly decreased 1 day after both wounding and SCE injec-

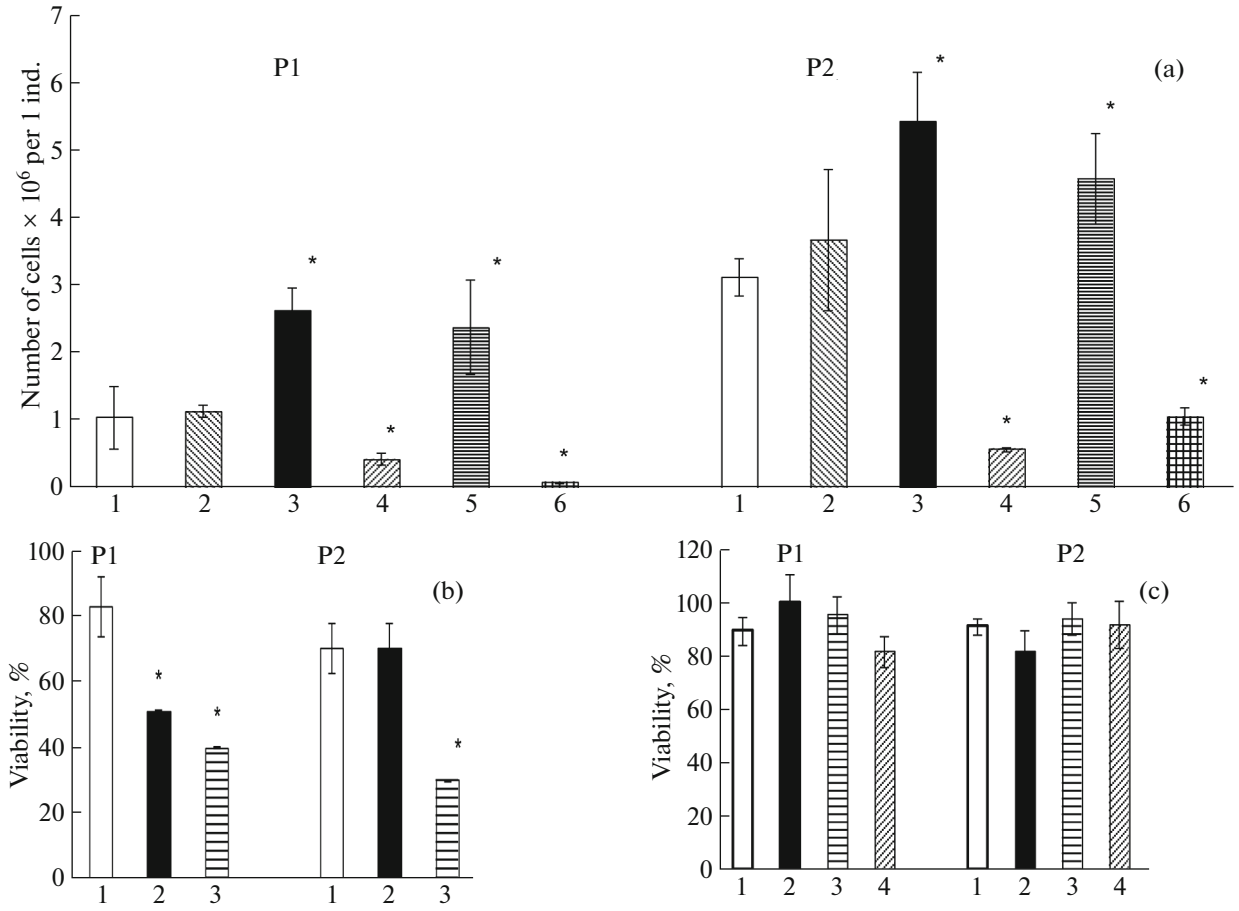

Fig. 2. Effects of SCE injection on the content of P1 and P2 phagocytes in the coelomic fluid of *E. fraudatrix* (a) and SCE injection and wounding on the viability of phagocytes after 1 day (b) and 7 days (c). The abscissa axis shows the number of the experimental group. (a): (1) intact individuals, (2) PBSN injection, 0 min, (3) PBSN injection, 1 day, (4) PBSN injection, 7 days, (5) SCE, 1 day, (6) SCE, 7 days; (b): (1) PBSN injection, 1 day, (2) SCE, 1 day, (3) wounding + PBSN, 1 day; (c): (1) PBSN injection, 7 days, (2) SCE, 7 days, (3) wounding + PBSN, 7 days, (4) wounding + SCE, 7 days. $N = 4$. * $P < 0.05$ compared to control.

tion compared to the control. However, the viability of P2 phagocytes decreased only after wounding (Fig. 2b). On the contrary, the viability of both types of phagocytes did not change 7 days after either wounding or SCE injection.

Molecular Mass Distribution of Coelomic Fluid Proteins

The molecular composition of the coelomic fluid proteins of the sea cucumber was analyzed using gel permeation chromatography in the isocratic elution mode (Fig. 3). The molecular mass distribution of proteins in the coelomic fluid samples is given in Table 1. As can be seen from the table, the coelomic fluid contains predominantly water-soluble proteins with a molecular mass of up to 40 kDa. Proteins with a molecular mass of more than 100 kDa constituted a minor group, reaching only 0.6–0.7% of the total protein content in intact animals and after introduction of SCE. However, their content increased with injury, maximally (up to 3.7%) with wounding (1 day).

All the samples contain two major proteins with a molecular mass of 9.2 and 7.6 kDa, the relative total content of which is approximately the same and varies from 68.6% in intact animals to 89.5% for samples obtained from animals administered with SCE. The samples taken from intact animals contain components with a molecular mass of 16.8 and 13.7 kDa, the relative content of which is 15.6 and 9.1%, respectively. The ratio of contents of proteins with a molecular mass of 9.2 and 7.6 kDa in intact animals is 1.5 and drops to 0.5 immediately after the control injection, rising again to 1 after 1 day. For the group of animals injected with SCE, the ratio of contents of proteins with molecular masses 9.2 and 7.6 kDa is 3 : 1.

In the sample taken from the control animals immediately after the administration of PBSN, a triple chromatographic peak of proteins with close retention times and molecular masses of 9.2, 9.1, and 8.8 kDa is noteworthy. Moreover, whereas the ratio of proteins

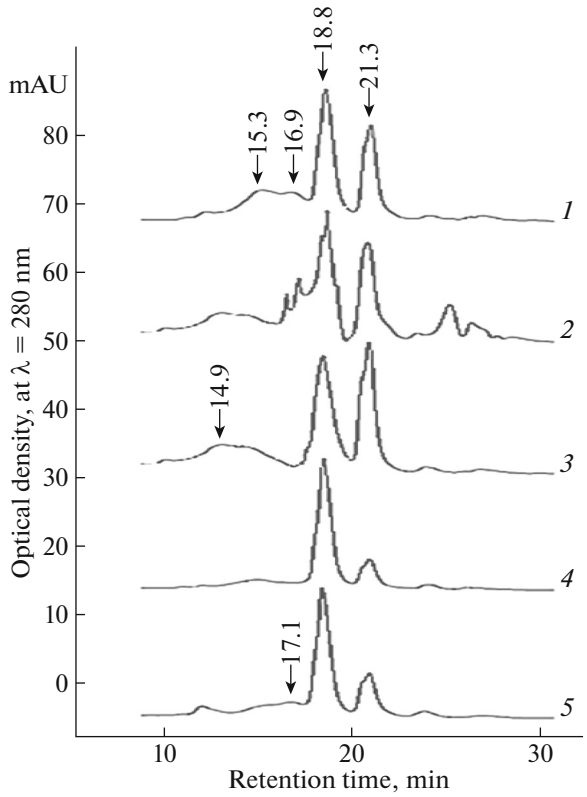

Fig. 3. Examples of chromatograms of coelomic fluid samples. (*1*) Intact animals, (*2*) PBSN injection, 0 min, (*3*) PBSN injection, 1 day, (*4*) SCE, 1 day, (*5*) wounding, 1 day.

with a molecular mass of 9.2 and 9.1 is 1 : 1, the proportion of the third minor component with a molecular mass of 8.8 kDa is 4.1%, or 20% relative to the proteins with a molecular mass of 9.2 and 9.1 kDa. In the other samples, we found only individual components with a molecular mass of 9.2 kDa.

The total content of minor components with a molecular mass of less than 6 kDa is constant in all samples and varies from 3.5 to 5.2%. The only exception is the sample of the control group on the 1st day; the proportion of low-molecular minor components reaches 16.4% in this sample.

It should be noted that there are significant differences in the molecular composition of the main protein components of the samples studied. Thus, the protein with a molecular mass of 39.1 is present only in a sample taken from the control group of animals (PBSN injection) after 1 day; the relative content of this component is 10.3%. The sample from animals that received SCE injection contains a protein with a similar retention time and a molecular mass of 33.9 kDa; its content is 5%, which is half the content of the protein with a molecular mass of 39.1 in the sample from the control animals.

Another feature is the presence of proteins with a molecular mass of 10.0 and 10.9 kDa in the samples immediately after the administration of the buffer and 1 day after wounding, with a relative content of 5.6 and 13.5%, respectively. These components are absent in other samples.

The sample from intact sea cucumbers contains components with a molecular mass of 16.8 and 13.7 kDa, the relative content of which is 15.6 and 9.1%, respectively. The control sample obtained immediately after the injection of PBSN contained a protein with a molecular mass of 17.8 kDa; however, its content was only 1.4%. The protein with a molecular mass of 16.0 kDa is discovered 1 day after PBSN injection and it disappears in all other samples, similarly to the protein with a molecular mass of 13.7 kDa.

At a further stage of the study, it was of interest to identify the effect on phagocytes of protein components with molecular masses of 39.1 (hereinafter, protein 39) and 33.9 (hereinafter, protein 34) kDa, which exclusively appear in the samples one day after PBSN or SCE injection, respectively.

Effect of Coelomic Fluid Proteins on the Ratio of P1 and P2 Phagocytes

Taking into account that the effect of SCE was characterized by a shift in the ratio of P1 and P2 phagocytes, the effects of the proteins were further estimated using the percentage ratio of the amount of P1 and P2 phagocytes (P1/P2, %). One day after wounding (Fig. 4a), P1/P2 decreased by 40% compared to the control. Both studied proteins significantly influenced changes in the ratio of P1/P2 phagocytes in the healing dynamics. At the same time, protein 39 at concentrations of 119 and 650 ng/g, but not 26 ng/g, stimulates an increase in the number of P1 phagocytes in the coelomic fluid in wounded animals on the first day, while protein 34 (30 and 110 ng/g) has almost no effect on the phagocyte ratios compared to those in wounded animals injected with PBSN. After 7 days (Fig. 4b), when the content of P1 phagocytes, on the contrary, increases by 2.5 times in wounded animals compared to the control, protein 39 reduced the phagocyte ratio in a direct concentration-dependent manner in favor of the predominance of P2 phagocytes, even below the control value (starting from a concentration of 119 ng/g). Protein 34 at a concentration of 110 ng/g, but not 30 ng/g, also reduced the phagocyte ratio.

Effect of Coelomic Fluid Proteins on NO Content in Phagocytes

The NO level in the control was 1.9 times higher in P1 phagocytes than in P2 cells after 1 day (Fig. 5a). After wounding, this index remained almost at the control level in P2 phagocytes, while it sharply decreased in P1 phagocytes, almost by 20 times. A

Table 1. Molecular mass distribution of proteins and peptides in the coelomic fluid of *E. fraudatrix* according to the GPC results

Experimental group	Retention time, min	Molecular mass (MM), kDa	Amount of protein fraction with the given MM, % of the total content
Intact	<12.0	>100	0.6
	15.3 ± 0.6	16.8	15.6
	16.9 ± 0.8	13.7	9.1
	18.8 ± 0.8	9.2	41.3
	21.3 ± 0.1	7.6	27.3
	24.4–47.1	<6	3.1
PBSN injection 0 min	16.7 ± 0.5	17.8	1.4
	17.4 ± 0.9	10.0	5.6
	9.3 ± 0.2	9.2	18.3
	9.5 ± 0.5	9.1	18.8
	9.7 ± 0.2	8.8	4.1
	10.5 ± 0.5	7.7	36.1
	11.9–14.4	<6	16.4
PBSN injection 1 day	<12.0	>100	1.8
	14.93 ± 0.4	39.1	10.3
	15.3 ± 0.4	16.0	12.3
	18.7 ± 0.5	9.2	37.3
	21.2 ± 0.6	7.6	34.7
	24.3–34.4	<6	3.5
SCE 1 day	<12.0	>100	0.7
	15.2 ± 0.4	33.9	5.0
	18.7 ± 0.3	9.2	72.9
	21.3 ± 0.4	7.6	16.6
	24.3–27.0	<6	4.0
Wound 1 day	<12.0	>100	3.7
	17.1 ± 0.4	10.9	13.5
	18.8 ± 0.4	9.2	56.8
	21.3 ± 0.7	7.6	20.9
	24.2–34.7	<6	5.1

trend towards an increase in NO content in P1 phagocytes was recorded for proteins 39 and 34 at concentrations of 119 and 30 ng/g, respectively. On the contrary, the proteins almost completely suppressed its generation in P2 phagocytes.

After 7 days (Fig. 5b), the NO level was three times higher in the P1 phagocytes than in the P2 phagocytes in the control animals. Wounding led the NO level in the P1 phagocytes to halve, while the NO level in P2 doubled compared to the control. Protein 39 contributed to an even more significant decrease in the marker level in the P1 phagocytes in a direct concentration dependence and reduced the NO level in the P2 phagocytes in an inverse concentration-dependent manner compared to wounding alone. Protein 34 in a small concentration significantly reduced the NO level in the P1 phagocytes compared to the group of wounded animals; however, at a higher concentration, it almost completely suppressed the increase in the NO level in P2 phagocytes.

DISCUSSION

Vertebrate macrophages are regulators of regeneration processes. Different roles were established for their two main types (M1 and M2) at individual stages. M1 macrophages are involved in the first inflammatory stage of regeneration, during which they protect against infectious pathogens and purify the tissues from the remains of destroyed cells (Kuninaka et al.,

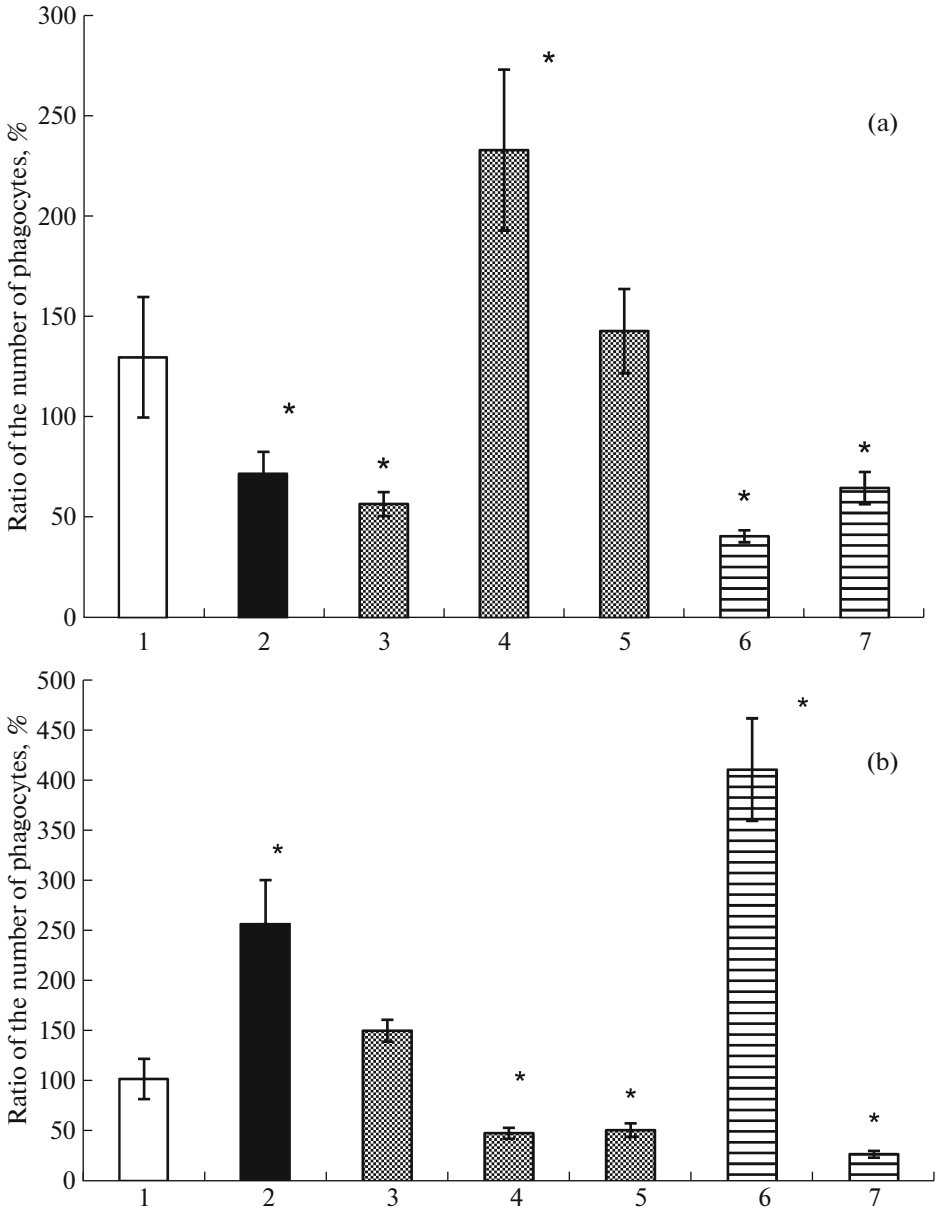

Fig. 4. Effect of wounding and proteins isolated from the coelomic fluid of wounded animals on the ratio of P1 and P2 phagocytes (P1/P2) 1 day (a) and 7 days (b) after wounding. The abscissa axis shows the number of the experimental group: (1) PBSN, (2) wounding + PBSN, (3) wounding + protein 39, 26 ng/g, (4) wounding + protein 39, 119 ng/g, (5) wounding + protein 39, 650 ng/g, (6) wounding + protein 34, 30 ng/g, (7) wounding + protein 34, 110 ng/g. $N = 3$. * $P < 0.05$ compared to control.

2022). M2 macrophages are involved in tissue repair and remodeling at the recovery stage by stimulating angiogenesis, proliferation, migration, and differentiation of fibroblasts (Hesketh et al., 2017). At the same time, most modern vertebrates have lost the ability to regenerate body parts, which is characteristic of many evolutionarily lower animal taxa (Dolmatov, 2020).

Sea cucumbers (Holothuroidea, Echinodermata) are among the most regenerative animals (Dolmatov et al., 2021). A number of researchers have found that superficial wounds are healed quite rapidly in these animals and the edges of a wound are closed after 3–6 days (Menton and Eisen, 1973; San Miguel-Ruiz and Garcia-Arraras, 2007) or even 2 days (Szulgit and Shadwick, 1998) in different species**,** although the healing process may not yet be complete (Menton and Eisen, 1973). We previously showed that the edges of the superficial cut wound in *E. fraudatrix* were closed by the 7th day in most individuals (Dolmatova and Ulanova, 2014).

Echinoderm coelomocytes are a heterogeneous cell population, in which amoebocytes (or phagocytes) and morula-like cells are most numerous (Isaeva and Korenbaum, 1989). Phagocytes and

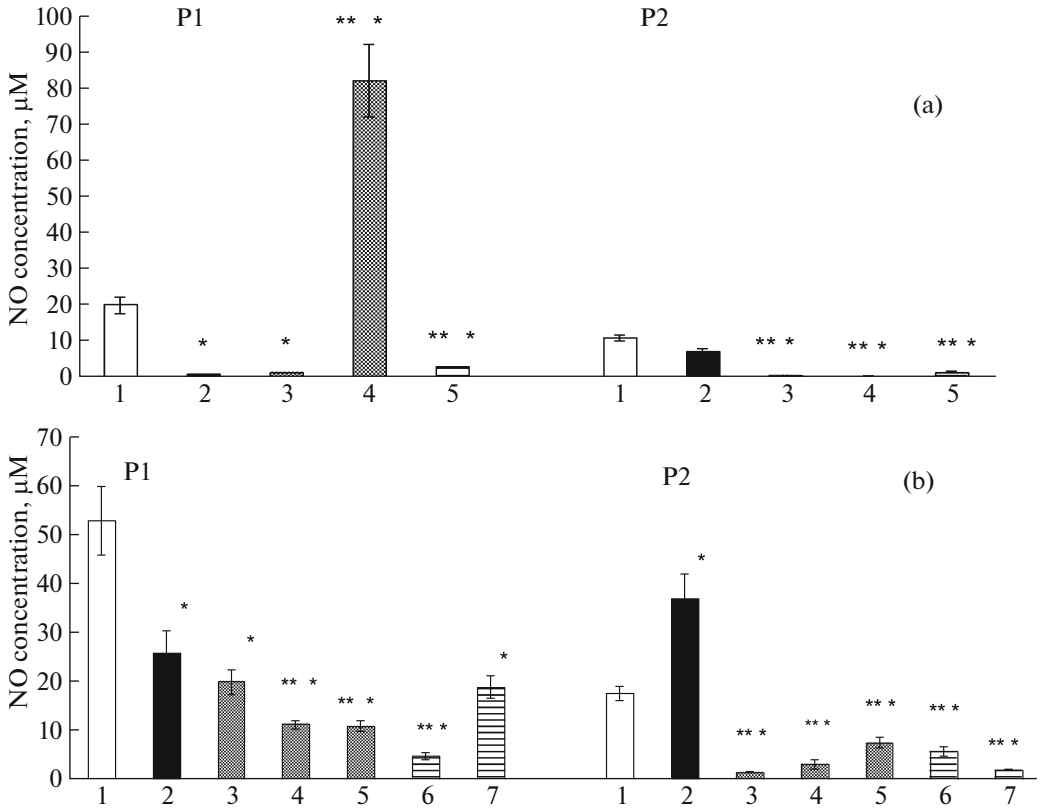

Fig. 5. NO concentration in P1 and P2 phagocytes 1 day (a) and 7 days (b) after wounding and injection of protein components of coelomic fluid. The abscissa axis shows the number of the experimental group. (a): (1) PBSN injection, (2) injury + PBSN, (3) wounding + protein 39, 119 ng/g, (4) wounding + protein 39, 650 ng/g, (5) wounding + protein 34, 30 ng/g; (b): (1) PBSN, (2) wounding + PBSN, (3) wounding + protein 39, 26 ng/g, (4) wounding + protein 39, 119 ng/g, (5) wounding + protein 39, 650 ng/g, (6) wounding + protein 34, 30 ng/g, (7) wounding + protein 34, 110 ng/g. $N = 3$. * $P < 0.05$ compared to control, ** $P < 0.05$ compared to injury.

spherulocytes and/or morular cells (in different species) are involved in wound regeneration; different studies differently assess the importance of phagocytes compared to morular cells (Menton and Eisen, 1973; Canicattí and Farina-Lipari, 1990; San Miguel-Ruiz and Garcia-Arraras, 2007). The role of phagocytes was studied mainly in terms of their adhesive properties and ability to directly participate in thrombus formation (Canicattí and Farina-Lipari, 1990). At the same time, echinoderm phagocytes are also heterogeneous and their different fractions are identified in many species when separated by gradient centrifugation (Dolmatova and Smolina, 2022).

In recent years, there has been evidence that arginase expression in macrophages in response to various stimuli can be manifested without the involvement of Th2 cytokines IL-4 and IL-13, which indicates that the associated regulation of wound healing can be performed by innate immune cells (Dzik, 2014). In addition, enzymes involved in arginine metabolism (oxide synthase and arginase) were described and a negative correlation was established between NO levels and arginase activity in the coelomocytes of the sea cucumber *Apostichopus japonicus* (Yina et al., 2016). It was later shown that, similarly to M1 and M2 macrophages, the phagocytes of the sea cucumber *E. fraudatrix* (P1 and P2), separated by gradient centrifugation, had markers such as NO and arginase activity, respectively (Dolmatova et al., 2019) and had morphofunctional similarities with the two types of macrophages (Dolmatova and Smolina, 2022).

In this paper, it was shown at the first stage of the study that superficial injury (both injection and incision) induced a significant increase in the concentration of coelomocytes after 1 day. The increase in the number of circulating cells in the coelomic fluid is a response of echinoderms to different stimuli, including tissue damage (Pinsinoet al., 2007; Vazzana et al., 2015; Petukhova et al., 2019); the number of spherulocytes (morular cells) generally increased at the injury site in *Holothuria glaberrima* (San Miguel-Ruiz and Garcia-Arraras, 2007) and an increase in the proportion of spherular cells in the total number of coelomocytes was recorded in the sea urchin *Paracentrotus lividus* after natural injury (Matranga et al., 2000). However, it should be noted that an increase in the number of circulating coelomocytes is usually observed after an initial decrease in the very first hours after injury

(Vazzana et al., 2015). We previously showed that wounding in *E. fraudatrix* also induced a significant decrease in the number of coelomocytes after 1 h (Dolmatova and Ulanova, 2014). The decrease in the concentration of circulating cells is determined by their recruitment to the injury site (Vazzana et al., 2015). Seven days after the beginning of the experiment, the number of circulating coelomocytes returned to the control values. SCE, which previously showed a wound-healing effect (Dolmatova and Ulanova, 2014), induced a lower increase in the concentration of coelomocytes in wounded sea cucumber than the wound itself after 1 day, and the concentration of coelomocytes under its effect was even lower than the control level after 7 days. It can be assumed that the wound-healing effect of SCE is associated with the increase in the recruitment of coelomocytes to the tissues. At the same time, SCE prevented a decrease in the viability of coelomocytes in wounded individuals without affecting the viability of coelomocytes in unwounded specimens, which supports the assumption of absence of SCE cytotoxicity and presence of SCE anti-inflammatory properties.

Injection of PSBN into the control animals also induced an increase in the number of P1 and P2 phagocytes after 1 day compared to the intact animals; however, it decreased below the control level after 7 days, which apparently reflects the depletion of the phagocyte pool. Comparison with changes in the coelomocyte concentration indicates that the recruitment of individual types of coelomocytes is selective; in particular, phagocytes of the two types are recruited into tissues more intensively than the total pool of coelomocytes. SCE injection did not influence the number of phagocytes of both types compared to the control (PBSN) after 1 day; similarly to PBSN injection, it reduced the number of both cell types after 7 days compared to the intact animals and, at the same time, induced a shift in the ratio of P1 and P2 phagocytes towards the latter compared to that in the control. This effect of SCE indicates that P1 and P2 phagocytes, like M1 and M2 macrophages, play different roles in wound healing and P2 phagocytes are important at the stage of tissue recovery.

Although the viability of coelomocytes generally did not change in wounded specimens after 1 day, the viability of P1 and P2 phagocytes significantly decreased. SCE injected into intact animals reduced the viability of P1 phagocytes only, which suggests the predominant activity of P1 phagocytes at the early stage of healing after SCE injection.

After 7 days, the viability of both types of phagocytes, as well as that of coelomocytes, did not change, which may indicate that circulating phagocytes reduce their activity by this time.

The coelomic fluid of echinoderms contains soluble substances secreted by different body tissues and coelomocytes (Canicattí and Farina-Lipari, 1990; Shabelnikov et al., 2019). The content of these substances changes under different physiological and pathological conditions. The proteome of the coelomic fluid changes in echinoderms during injury. A significant increase in the number of proteins was shown in the starfish *A. rubens* during the first hours after injury; however, it decreased even below the normal level by the 3rd day (Shabelnikov et al., 2019). *Holothuria tubulosa* showed a significant activation of hydrolases in the coelomic fluid after wounding (Mauro et al., 2021), as well as a significant increase in the expression of heat shock proteins 90 (HSP90) (Vazzana et al., 2015), reaching the maxim level in the first hours after injury, with a return to the control level after 3 days.

Analysis of the molecular composition of proteins in the coelomic fluid of the sea cucumber revealed significant changes in the composition and quantity (relative content) of many proteins in the molecular mass range up to 40 kDa (which form the main group in terms of the proportion of total protein) one day after tissue damage (injection or incision). Minor components with a molecular mass below 6 kDa retained a constant number in all samples; they increased only in the samples of the control group on the 1st day. Minor components with a molecular mass of more than 100 kDa increased their presence after injury, especially after wounding

Of note is the appearance of a protein with a molecular mass of 10 kDa in individuals immediately after PBSN injection and 1 day after incision; however, this was not observed in intact or control animals on the 1st day or those which received SCE. This protein is probably expressed in response to acute stress, which is also supported by the increase in its relative content in wounded animals compared to those injected with PBSN. Although protein identification was not covered by this study, it can be assumed with some probability that this is a protein of the evolutionarily conservative family of heat shock proteins, which includes several proteins that regulate a response to different stress stimuli and are expressed immediately after injury (Vazzana et al., 2015). The smallest protein with respect to the molecular mass is HSP10 (10 kDa). This protein is involved in immunomodulation and cell proliferation and differentiation and inhibits the immune response to stress (Jia et al., 2011). HSP10 expression was not observed during injury to *H. tubulosa* (Vazzana et al., 2015); however, this work used a limited set of antibodies to heat shock proteins, which did not include antibodies to HSP10. On the other hand, this study did not establish proteins with a molecular mass of 90, which are recorded for HSP90; this may be due to species differences or differences in the nature of the damage. Undoubtedly, only the subsequent identification of 10-kDa protein will make it possible to determine what protein family it belongs to; however, it can be considered as an acute stress protein.

On the contrary, intact animals contained proteins that were absent or present in smaller quantities in other groups (16.8 and 13.7 kDa).

Two proteins (34 and 39 kDa) appeared in the coelomic fluid of the sea cucumber only after the administration of a wound-healing agent or PBSN (1st day). These proteins were used at the next stage of the experiment to study the response of P1 and P2 phagocytes in wounded animals to the effect of presumably anti-inflammatory substances.

Numerous studies indicate that the effect of a certain exposure depends on the ratio of M1 and M2 macrophages, rather than on their absolute number (Dan et al., 2020; Kuninaka et al., 2023). During wound healing, the two types of macrophages play different roles, and the imbalance in their polarization causes impaired regeneration (Kuninaka et al., 2023). When determining the effects of proteins isolated from regenerating animals on the two types of phagocytes, we also studied the ratio of the number of phagocytes, P1/P2.

The P1/P2 ratio significantly decreases in the coelomic fluid within 1 day after wounding, while this trend changes for its significant increase after 7 days, which indicates the preferential recruitment of P1 phagocytes into the tissue on the first day and P2 phagocytes on the seventh day. Taking into account the greater oxidative activity of P1 phagocytes compared to that of P2 phagocytes, previously identified under the influence of bacterial toxin (Dolmatova et al., 2019), it can be assumed that the P1 phagocytes recruited into tissues are involved in the inflammatory response at the first stage of healing. In turn, the preferential recruitment of P2 phagocytes at the next stage of healing (after 7 days) may be associated with their anti-inflammatory role by analogy with M2 macrophages. Studies on humans (Kuninaka et al., 2023) showed that M1 macrophages quantitatively exceeded M2 macrophages at the injury site starting from the first day after a skin wound was inflicted, and the M1/M2 ratio decreased starting from the 9th day. The regeneration stage in humans takes no less than 14 days (Deng et al., 2022). The comparatively early increase in P1/P2 in the present study, presumably determined by the recruitment of the larger number of P2 phagocytes into the tissues, may be explained by more rapid wound healing in sea cucumbers. On the whole, the opposite changes in the P1/P2 ratio in the coelomic fluid at different times after wounding indicate different roles of P1 and P2 phagocytes at different healing stages.

Both proteins studied significantly influenced changes in the P1/P2 phagocyte ratio during the healing dynamics. Protein 39 at concentrations of 119 and 650 ng/g in wounded animals stimulated the preferential recruitment of P2 phagocytes into the tissues on the first day compared to the control or prevented an increase in the recruitment of P1 phagocytes, which indicates a putative anti-inflammatory effect of this protein. On the contrary, it decreased the P1/P2 ratio compared to the control and variant with wounding after 7 days, starting from a concentration of 119 ng/g, while it returned the M1/M2 ratio to the control value at a concentration of 26 ng/g. Apparently, protein 39 can accelerate the recruitment of P2 macrophages in a concentration-dependent manner at the first stage of healing and promote the replenishmentof P2 phagocytes in the coelomic fluid at the second stage, thereby exerting an anti-inflammatory effect. However, these assumptions require further verification.

Unlike protein 39, protein 34 in the concentration range of 30–110 ng/g stimulated the recruitment of P1 phagocytes after 1 day; at a concentration of 30 ng/g, it was even more pronounced than the recruitment after wounding. After 7 days, only the higher concentration contributed to the accumulation of P2 phagocytes. Since the inflammation is a necessary stage for successful tissue regeneration (Kuninaka et al., 2023), it can be assumed that protein 34, which, in lower concentration, apparently contributed to increased recruitment of P1 phagocytes in the early period after injury and, conversely, P2 phagocytes at a later stage, may be a mediator of the wound healing effect of SCE.

It should be noted that both proteins, the appearance of which was recorded under different effects, in the range of similar concentrations (26–119 ng/g) had a largely opposite effect on the P1/P2 ratio of injured animals, which differed from that due to the wounding itself. The ability of proteins isolated from the coelomic fluid of injured animals to stimulate thrombus formation (which is associated with the activity of coelomocytes) was also previously shown in experiments with the starfish *A. rubens* (Holm et al., 2010). At the same time, the opposite change in the P1/P2 ratio after exposure to proteins at different healing stages supports the assumption of their different roles in this process.

NO is a mediator of acute and chronic inflammation (Ghazanfari et al., 2009) and is produced almost exclusively in M1 cells in vertebrate macrophages (Dolmatova and Dolmatov, 2021). Studies conducted on the starfish *Asterias forbesi* (Beck et al., 2001) showed that invertebrate coelomocytes could produce NO, which is an evolutionarily conserved mediator of antibacterial defense. The involvement of NO in antibacterial defense was also confirmed in experiments with *A. japonicus* (Guanghui et al., 2023), which established mechanisms of apoptosis regulated by it in coelomocytes. It was previously shown that NO was also a marker of P1 phagocytes of the sea cucumber *E. fraudatrix*, in which its level is significantly higher than that in P2 phagocytes. One of the mechanisms of immunosuppressive action of the thermostable toxin of the bacterium *Yersinia pseudotuberculosis* was the suppression of NO production in P1 phagocytes, but not P2 phagocytes (Dolmatova et al., 2019).

In the present study, a decrease in the NO level was shown in P1 phagocytes of wounded animals one day after the injury compared to the control, which indicates a decrease in the activity of P1 phagocytes and their transformation into the P2 phenotype. Therefore, injury causes a decrease in both the number of P1 phagocytes in the coelomic fluid and their functional activity. At the same time, the NO level in P2 phagocytes of wounded animals did not differ from the control level. Numerous researchers have noted that humans exhibit similar significant immunosuppression one day after injury in response to proinflammatory cytokines expressed in the first hours after the injury, which is primarily manifested by a shift in the ratio of Th1/Th2 activity towards the predominance of Th2 cells (Menger and Vollmar, 2004; Savchenko et al., 2011), which corresponds to the shift of the macrophage phenotype towards the M2 type. No such lymphocytes have been found in echinoderms and protein regulators of phagocyte activity have been little studied; however, the established shifts in the ratio of P1 and P2 phagocytes confirm the above statements that the immune regulation of wound healing in echinoderms can be implemented at the level of innate immunity (Dzik, 2014). The pattern of changes in the NO level in P1 phagocytes under the effect of proteins 39 and 34 also corresponded to changes in the P1/P2 index, while it had the opposite direction with this index in P2 phagocytes. A trend towards a decrease in wound-induced immunosuppression was recorded under the influence of both proteins.

After 7 days, the NO level remains low in the P1 phagocytes of sea cucumbers compared to the control; however, it tends to increase compared to the 1st day. The pattern of change in the NO level in P1 phagocytes during this period is opposite to changes in the ratio of P1/P2 phagocytes. In addition, the NO level in P2 phagocytes is even significantly higher than the control one during this period. The mechanisms of such transformation are unclear in this case; however, they may presumably reflect an increase in the functional activity of P2 phagocytes.

The changes in NO level in P1 phagocytes after the administration of proteins 39 and 34 were also opposite to the changes in P1/P2 after 7 days, similar to changes after wounding alone. In P2 phagocytes, the changes in NO level were opposite to changes in P1/P2; however, this effect differed from the effect of wounding alone. The proteins contributed to an even greater transformation of P1 phagocytes towards the P2 phenotype and promoted a decrease in the functional activity of P2 phagocytes themselves, which indicates their anti-inflammatory action.

In addition, the opposite direction of NO changes in P1 and P2 phagocytes after wounding and the opposite concentration dependence of protein effects on NO levels in P1 and P2 phagocytes also indicate different functional activities and probably different roles of these cells during injury, similarly to M1 and M2 macrophages.

CONCLUSIONS

Our study shows that the superficial wounding of the body in the coelomic fluid of the sea cucumber involves significant changes both in the total number of coelomocytes and in the number of individual types of phagocytes (P1 and P2), which apparently reflects their recruitment into the tissues. The number of phagocytes of both types, as well as the total number of coelomocytes, increased 1 day after wounding. However, the direction of change in the number of phagocytes did not coincide with that of the total number of coelomocytes after 7 days, which indicates a specific role of phagocytes in wound healing. It is shown for the first time that the quantitative ratio of circulating P1/P2 phagocytes at the early (1 day) and later (7 days) stages of wound healing changed in the opposite way. Taking this into account, as well as the previously established higher oxidative activity of P1 phagocytes compared to P2 phagocytes, P1 cells, which apparently prevail in tissues at the early stage of healing, are involved in inflammatory reactions. P2 cells, which dominate at a later healing stage, may presumably perform anti-inflammatory and regenerative functions; this assumption is also supported by the shift in the P1/P2 ratio towards P2 upon injection of the wound-healing agent (sea cucumber extract) into intact sea cucumbers.

Changes also cover the functional activity of circulating P1 and P2 phagocytes, which was estimated by the NO level. The observed decrease in the functional activity of P1 phagocytes with increase in that of P2 cells after 1 day and the preservation of this activity after 7 days indicate immunosuppression, which presumably develops under the influence of regulators appearing in response to inflammation. The study of the protein components of the coelomic fluid revealed a number of differences in the proteome of intact and wounded sea cucumbers. In particular, a 10 kDa protein was detected; it is expressed immediately after injury and is apparently an inflammation acute-phase protein. At the same time, some proteins expressed in intact specimens are not detected in wounded individuals. When wound-healing SCE was injected into the intact animals, they produced a protein that was not detected in other experimental groups. Injection of this protein and another protein component, determined in the control animals only after post-stress regeneration, into wounded sea cucumbers was accompanied by a decrease in immunosuppression after 1 day (increase in NO in P1 phagocytes and decrease in P2 phagocytes) and by a higher level of NO in P1 phagocytes with respect to P2 phagocytes after 7 days. Proteins also stimulated an increase in P1/P2 in the coelomic fluid in the opposite concentration-dependent manner after 1 day, thereby, apparently,

accelerating the preferential recruitment of P2 phagocytes to the injury site. Thus, these proteins contributed to a decrease in immunosuppression at the early inflammatory stage of wound healing and stimulated the recruitment of P2 phagocytes, which is apparently important for later healing stages. This indicates the ability of coelomic fluid proteins, produced under conditions of active regeneration or under the influence of regeneration activators, to regulate the activity of P1 and P2 phagocytes, exerting the opposite effect on the latter.

On the whole, the resulting data support the idea of different functional activity of P1 and P2 phagocytes in sea cucumbers and assume their different roles in wound healing. It is necessary to carry out further studies, including the determination of phagocyte activity at the injury site, which will make it possible to expand our understanding of the features of immune regulation of healing in sea cucumbers and assess evolutionary changes in its mechanisms. Further study of low-molecular proteins of the coelomic fluid, which are involved in wound healing in sea cucumbers, also seems promising for developing therapeutic agents.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and vertebrate animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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