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Research Report

Wound repair in the marine worm *Sipunculus nudus* (Sipunculidae)

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Abstract

The cells and molecules involved in the wound healing of *Sipunculus nudus* were studied. An incision, 5 mm in length, was cut longitudinally at a site opposite the anus and 10 mm from the introvert. The histological study performed at different times showed an involvement of both Type I and Type II granulocytes in the process of healing. The former were capable of extracellular digestion and they were immunoreactive to anti-IL-4, -IL-10 and -epidermal growth factor (EGF) antibodies (Abs); the latter were involved in the synthesis of connective tissue from 24 h after the incision, thereby causing the initial closing of the wound. After 70 h, a continuous layer of Type II granulocytes was found on the sides of the wound where the future muscle tissue would be formed; many of these granulocytes had been partially degranulated. It was not possible to establish any existing relationship between the functions of Type I granulocytes and their reactivity to anti-IL-4, -IL-10 and -EGF Abs.

Kew words: marine worm; *Sipunculus nudus*; wound repair; cytokines

Introduction

The process of wound healing has been the subject of intensive research, mainly in vertebrates (Redd ***et al***., 2004; Harvey, 2005; Whitney, 2005). With regard to invertebrates, Kindred (1924) observed that the removal of a fragment of the body wall in echinoderms led to the healing of the wound, by the proliferation and infiltration of cells from the surrounding connective tissue. In *Asterias* the aggregation of coelomocyte and of amoebocytes from adjacent tissue was seen to contribute to the wound healing (Anderson, 1962, 1965). In the sea cucumber *Stichopus tremulus* numerous morula cells, found in proximity of the incision, were supposed to play a significant role in healing wounds, and to be homologous with the vertebrate mastocytes (Rollefsen, 1965). In contrast, in *Stichopus badionotus,* after superficial cutaneous incisions Cowden (1968) observed a rapid repair with complete fibrogenesis without the intervention of morula cells.

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The migration of epidermal and pigment cells from the periphery of the wound margin in *Thyone briareus* caused re-epithelialization in the absence of evident mitotic activity, an morula cells seemed to be involved in this migration although their precise role is unknown (Menton and Eisen, 1974). Fibroblast-like cells, pigment cells and numerous coelomocytes were the first cells to arrive at wound sites in the *Holothuria polii* and the newly formed collagen fibres were synthesized by Type II spherula cells. Cutaneous lesions performed after antigenic stimulation healed more slowly than controls since Type II spherula cells were also actively involved in the immune reaction by forming brown bodies in cooperation with the amoebocytes (D'Ancona, unpublished). Consequently, in this case, a lower number of Type II spherula cells are available for the synthesis of connective components (Canicattì and D'Ancona, 1989).

Recently, Franchini and Ottaviani (2000) have studied the effects of platelet-derived growth factor (PDGF-AB) and transforming growth factor (TGF-β)1 in the repair mechanism of wounds in the mollusc *Limax maximus.* The main repair stages include an initial infiltration phase in which the hemocytes migrate and stratify at wound margins, actively phagocitize cell debris and damaged tissue and were immunoreactive to anti-IL-1α, -IL-8 and -tumor necrosis factor (TNF)-α antibodies (Abs). This was followed by the formation of granulation tissue, the synthesis and depositing of

extracellular matrix components, such as fibronectin, collagen fibres and reticular fibres. Finally, re-epithelialization of the wound occurred. The exogenous administration of PDGF-AB and TGF-β1 stimulated the tissue healing process though a general acceleration of the activities involved.

In the coelomic fluid of *S. nudus* two types of granulocytes (Type I and Type II), are distinguishable on the basis of their morphology and chemical composition. Type I granulocytes do not have a phagocytic capability, even though they contain lytic enzymes; Type II granulocytes show phagocytic activity. Furthermore, haemerythrocytes, signet-ring cells, urna cells complexes, empty vesicles, vesicle fragments, laminar structures, aggregates of stem cells and brown bodies were also found (D'Ancona *et al*., 2004). In the present paper the mechanism of wound repair in *Sipunculus nudus* was studied. Furthermore, it was also examined if cells reactive to anti-IL-4, -IL-10 and -EGF Abs were present and involved in the wound healing of the marine worm.

Materials and Methods

Twenty adult specimens of *Sipunculus nudus* were incised with a scalpel in the frontal part of the body, producing a wound in a site opposite to the anus and 10 mm from the introvert. The incision, 5 mm in length, was performed longitudinally and it involved the integument and the circular muscle structure below. The animals were sacrificed and fixed in Bouin mixture at different times after the incision (3, 15, 18, 22, 24, 71 and 96 h) and unwounded specimens were used as controls. Fragments including the wound and 2 mm of surrounding healthy tissue were included in paraffin and 7 μm thick sections were stained with Gomori triple staining (the connective tissue resulted green and the Type I granulocytes red) and Alcian blue-PAS (Type II granulocytes stained green and pink) (Ganter and Jòlles, 1969; Mazzi, 1977).

The immunocytochemical reactions were performed by incubating sections for 1 h at room temperature (RT) in mouse primary monoclonal Abs (Euroclon, Celbio, Italy), raised against IL-4, IL-10 and EGF, diluted 1:100 in phosphate-buffered saline (PBS) (1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4 and 0.065 M Na_2HPO_4), rapidly washed in PBS, incubated for 30 min at RT in biotinylated goat anti-mouse immunoglobulins (Kit DAKO cytomation, Denmark), washed in PBS, incubated in streptavidin peroxidase conjugate for 30 min at RT. After washing, sections were stained for 15 min in the chromogen aminoethylcarbamate. Sections incubated with normal non-immune rabbit serum were used as controls.

Results

In physiological conditions the body walls of *S. nudus* consist of a cubic epithelium, secreting an outer cuticle, a derma, a layer of circular muscle, a layer of longitudinal muscle and the peritoneum. The derma contains fine fibres, connective cells and coelomatic, longitudinal canals, which communicate with each other and with the general coelom (Hyman, 1959).

As far as the wounded specimens are concerned, 3 h after the incision, the wound did not show any signs of healing, the layers of muscle and connective tissue were still damaged and the cuticle was missing. On the outer part of the wound, coelomocytes were present, most of which were acidophilic Type I granulocytes (Fig. 1). Furthermore, Type II granulocytes and a few haemerythrocytes were observed. Three h after the incision, an intense immunoreaction with anti-EGF Ab was observed in Type I granulocytes, including those which were present in the coelomatic cavity (Fig. 2). Type II granulocytes and haemerythrocytes were negative. No changes in the response were observed up to 15 h after the incision, but for an increase in exocytised acidophilic material**.**

Eighteen h after incision, new muscle fibres were not observed but fine connective fibres were evident. Both partially degranulated Type I granulocytes and Type II granulocytes were present, in addition to transparent spherical cells on the outer part and the sides of the wound.

Twenty-two h after incision, the wound had been externally closed up by fine collagen fibres and internally by numerous coelomocytes (haemerythrocytes, Type I granulocytes and various Type II granulocytes). At this time, EGF-like material was found in all Type I granulocytes, while other coelomocytes were negative (Fig. 3).

Twenty-four h after incision, the wound had closed: on the internal part of the wound, acidophilic Type I granulocytes were present and the circular, muscle tissue was interrupted towards the central part of the wound. In its place there was newly synthesized connective tissue with numerous Type I and Type II granulocytes nearby. Type I granulocytes were highly reactive to the anti-IL-4 Ab, while Type II granulocytes, were less numerous and negative (Figs 4, 5).

In incisions of the entire animal wall, both the derma and muscle tissue were interrupted and the circular muscle fibres displayed rounded extremities, which were covered by connective tissue (Fig. 6). The wound had been closed by various thin, connective lamina distributed over 2-3 layers at the extremities. Each lamina contained Type II granulocytes and a few transparent cells (Fig. 7). Some Type II granulocytes start to loose their basophilic core, while others devoid of the basophilic granules, were amoeboid in shape with a flattened nucleus and were involved in collagen fibre production (Fig. 8). Twenty-four h after the incision, large spaces containing degranulating Type I granulocytes were highlighted near the tissue on the internal part of the wound, and these degranulated cells flattened and formed a sort of barrier that trapped haemerythrocytes and Type II granulocytes (Fig. 9). Type I granulocytes located towards the coelomatic cavity were almost degranulated with a weak reaction to anti-IL-10 Ab. Type II granulocytes did not react with this Ab, while degranulated material resulted highly immunopositive (Fig. 10). Ninety-six h after the incision, the walls of *S. nudus* had been reconstituted but the connective tissue was not always well compacted and the muscle fibres did not show their typical circular arrangement (Fig. 11). In the lateral parts of the wound, the muscle fibres were interrupted and a great number of "spongy" Type II granulocytes,

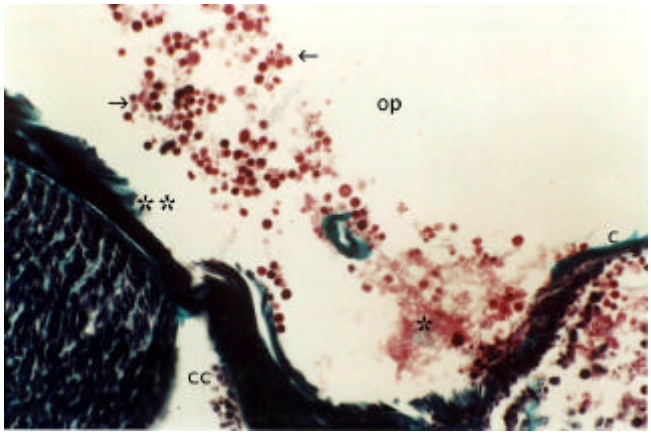

Fig. 1 Histological section 3 h after incision, stained with Gomori triple staining. Note, on the outer part of the wound (op), coelomocytes (←) with acidophilic material and degranulated acidophilic material (*). The connective tissue and muscle tissue (**) are still damaged and the cuticle (c) is also missing. Coelomatic cavity (cc).

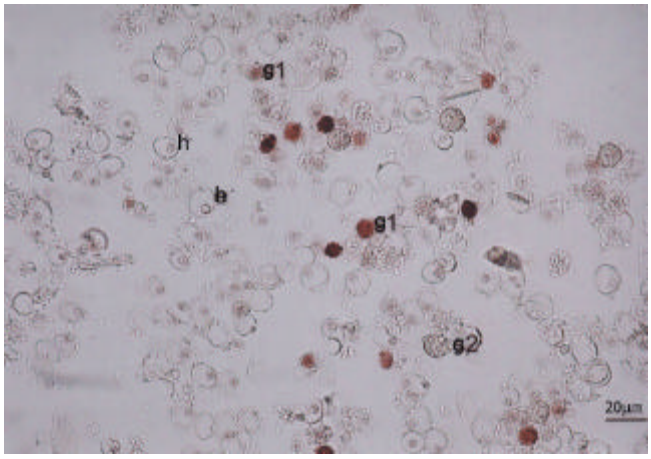

Fig. 2 Immunocytochemical reaction with anti-EGF Ab 3h after incision. Positive Type I granulocytes (g1); negative Type II granulocytes (g2) and haemerithrocytes (h) in the coelomatic cavity.

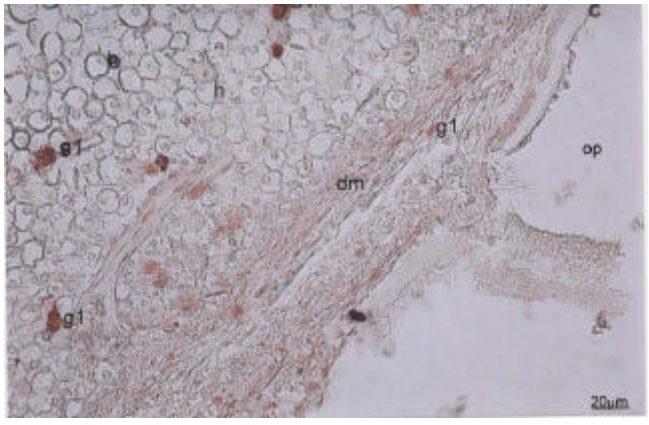

Fig. 3 Immunocytochemical reaction with anti-EGF Ab 22 h after incision. Positive Type I granulocytes (g1) and degranulated material (dm); negative haemerithrocytes (h). Outer part of the wound (op), Cuticle (c).

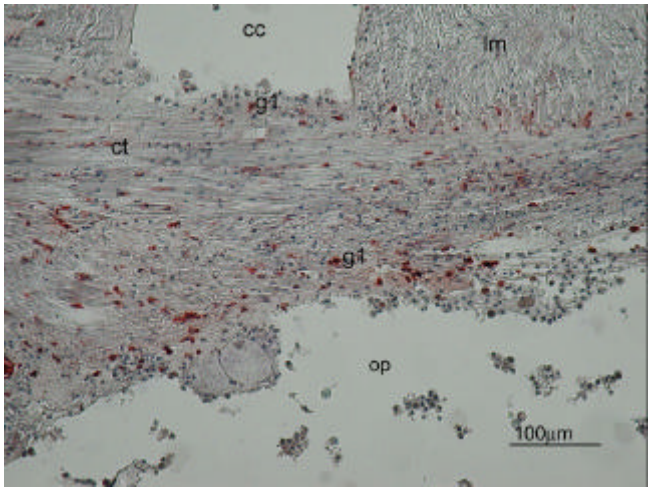

Fig. 4 Immunocytochemical reaction with anti-IL-4 Ab 24 h after incision. Positive Type I granulocytes (g1) are present in the connetive tissue (ct). Coelomatic cavity (cc); longitudinal muscle (lm), outer part of the wound (op).

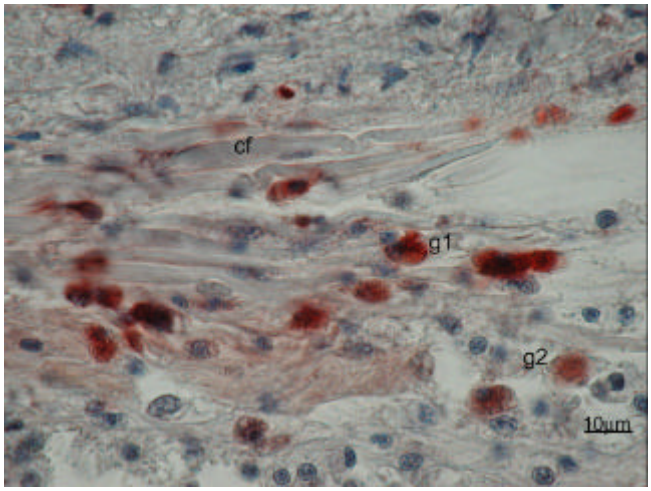

Fig. 5 Detail of Fig. 4. Type I granulocytes (g1) positive and Type II granulocytes (g2) negative to anti-IL-4 Ab. Collagen fibres (cf).

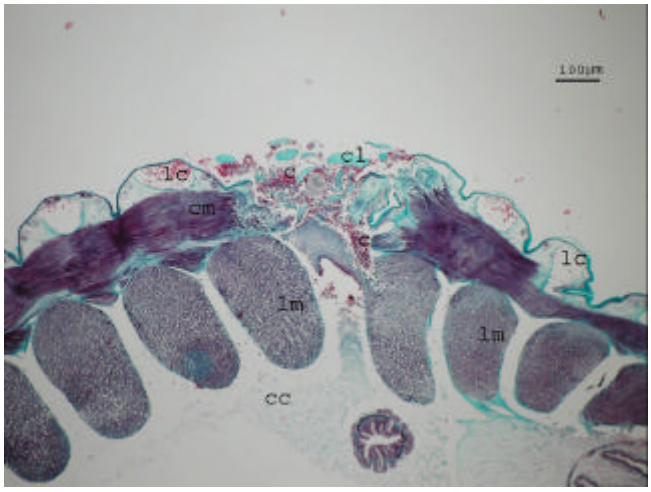

Fig. 6 Histological section 24 h after wound, stained with Gomori triple staining. The incision concerned the entire thickness of the animal wall. The wound is closed by connective lamina (cl). Longitudinal canal (lc); coelomocytes and acidophilic material (c); circular muscle fibres (cm); coelomatic cavity (cc); longitudinal muscle (lm).

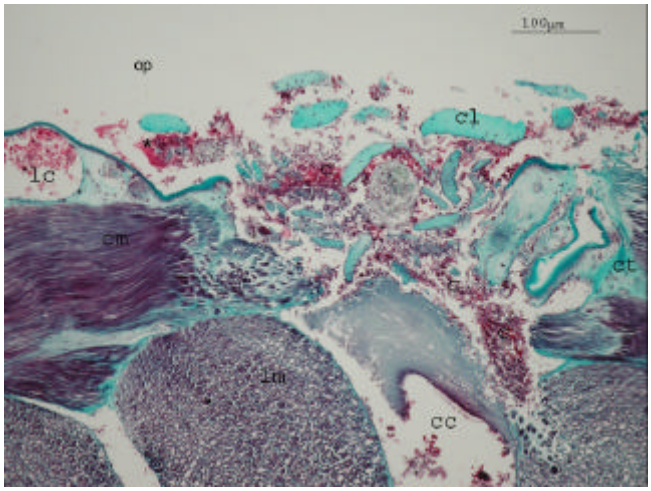

Fig. 7 Detail of Fig. 6. Note longitudinal canal (lc); degranulated acidophilic material (*); coelomocytes (c); connective lamina distributed over 2-3 layers (cl); circular muscle fibres with rounded extremities (cm); coelomatic cavity (cc); longitudinal muscle (lm); outer part of the wound (op).

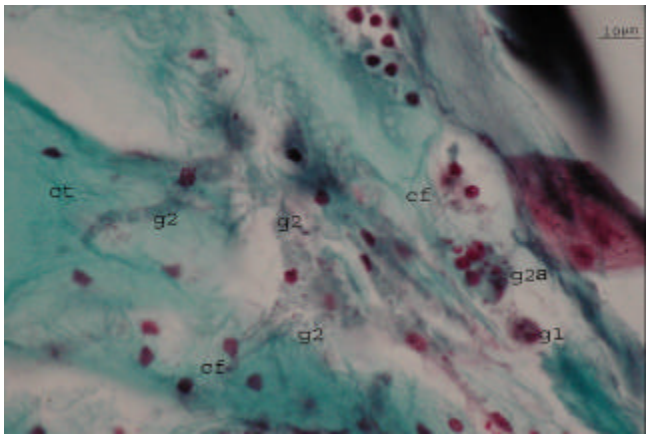

Fig. 8 Detail of Fig. 6. External surface of the wound. Note type I granulocyte (g1); connective tissue (ct) where Type II granulocytes (g2a) displayed their basophilic core and Type II granulocytes shaped like a triangle (g2) are producing thin bundles of collagen fibrils (cf).

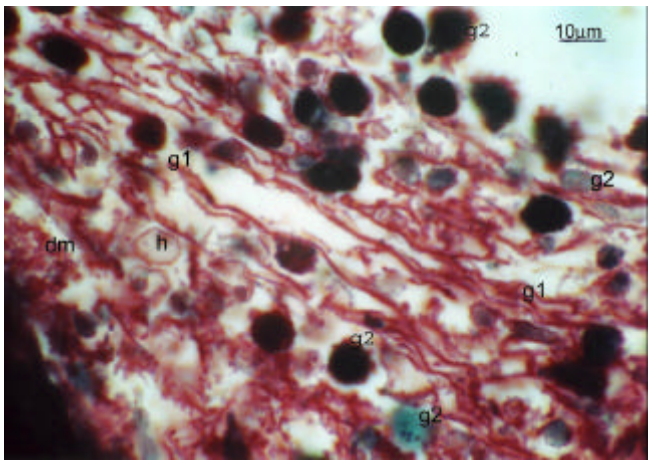

Fig. 9 Histological section stained with Gomori triple staining 24 h after a deep incision. Note a barrier made up of flattened Type I granulocytes (g1), which have trapped haemerythrocytes (h), type II granulocytes (g2) and degranulated material (dm).

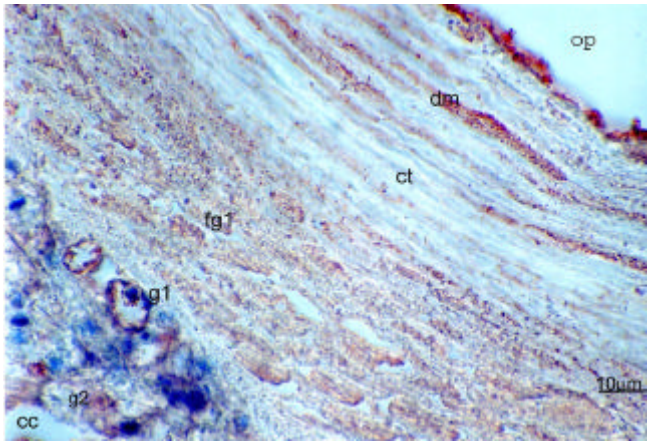

Fig. 10 Immunocytochemical reaction of section with anti-IL-10 Ab 24h after incision. Partially degranulated Type I granulocytes (g1) were moderately reactive whilst the degranulated material (dm) was highly reactive. Negative Type II granulocyte (g2). Note Type I flattened granulocytes (fg1); connective tissue (ct); outer part of the wound (op); coelomatic cavity (cc).

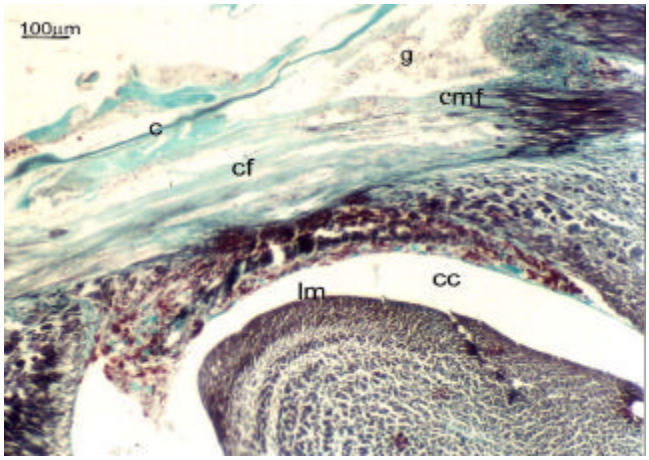

Fig. 11 Histological section 96 h after incision stained with Gomori triple staining. The wound is partially healed. Note cuticle (c); granulocytes; poorly compacted collagen fibrils (cf); circular muscle fibres (cmf) being formed; coelomic cavity (cc); longitudinal muscle (lm).

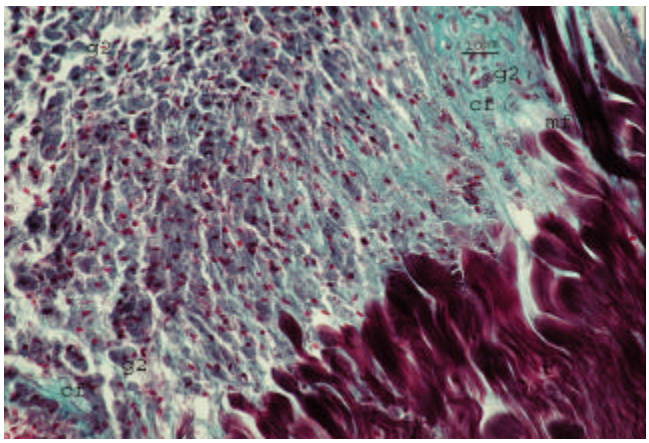

Fig. 12 Detail of Fig. 11 in the lateral parts of wound. Note the interrupted muscle fibres (mf) and a great number of spongy type II granulocytes (g2), separated by connective fibres (cf).

separated by connective material, formed a base onto which the muscle fibres were formed (Fig. 12). Elongated Type I granulocytes, with varying amounts of acidophilic granules, were found between the forming muscle fibres.

Discussion

The histological study performed 3-96 h after the incision in *S. nudus* revealed that Type I and Type II granulocytes from the coelomatic cavity are responsible of the wound repair. Type I granulocytes are the first cells to be activated and they degranulated thereby causing the transfer of their material to the surrounding area and, in particular, on Type II granulocytes. The contribution of the coelomocytes was not always the same. In most cases, the coelomocytes migrated from the coelomic cavity to the lesion zone, due to the probable presence of various chemotactic substances produced *in situ* (Abercrombie, 1972; Postletwaite, 1976). Type I granulocytes contain lysosomal enzymes (Matozzo *et al.*, 2001; D'Ancona *et al.*, 2004) that could act in the digestion of cell debris and damaged tissue. The urna cell complexes present in the coelomatic liquid of *S. nudus* (D'Ancona *et al.*, 2004) and endowed with phagocytic activity (Bang and Bang, 1962) did not participate in wound repair process. Unlike the hemocytes found in the wound repair of *L. maximus* (Franchini and Ottaviani, 2000), Type I granulocytes do not take part directly by means of phagocytosis but they secrete enzymes into the extracellular environment.

Type I granulocytes also have an important hemostatic function. Indeed, they move from the coelomatic cavity towards the wound in great amounts in order to create an aggregate that can prevent the passage of coelomatic material towards the outer and of pathogens towards the inner side of the body. These cells flattened after degranulation, stuck along the lower margins of the wound and form a lamina which entrap other cells, especially haemerythrocytes. Many of these, in turn, flocked around the wound with the aim of supplying oxygen, which is necessary for metabolic activity (Steins *et al.*, 2001).

Rollefsen (1965) hypothesized that morula cells, present in the hydrovascular system and dermal connective tissue of *S. tremulus,* can be involved in the production of the intercellular substance of connective tissue, as they are both metachromatic and PAS positive. Morula cells may form fibres (Endean, 1966) which are responsible for connective tissue growth and repair (Smith, 1981). In other echinoderms polysaccharides and proteins are present in the granular inclusions of spherula cells (Endean, 1958; Hetzel, 1963; Johnson, 1969; D'Ancona and Canicattì, 1990). Type II granulocytes in *S. nudus* showed the same cytological characteristics of the morula cells of *S. tremulus* and *H. poli* Type I spherula cells (D'Ancona and Canicattì, 1990). They also have the same histochemical affinity of connective tissue and it can, therefore, be hypothesized that Type II granulocytes may be the main cell producers of connective material.

The granulation tissue, typical of wound healing in vertebrates, was also found in *L. maximus* (Franchini and Ottaviani, 2000). In this mollusc the main cell type involved in the different phases of repair process is the hemocyte. Indeed, this cell was immunoreactive to cytokines, was able to phagocytize cell debris and damaged tissue and showed fibroblast-like activity, as found by Sminia *et al.* (1973) in *L. stagnalis*.

In *S. nudus* numerous Type I granulocytes are found everywhere and they continuously exocytise acidophilic material between other cells and muscle fibres. Moreover these cells contain hydrolytic enzymes, able to remove cell debris and damaged tissue, and were also immunoreactive to EGF-, IL4- and IL10 Abs. In marine worm it can be supposed, as in vertebrates**,** that in the first hours after incision, an inflammatory response occurs, involving cellular proliferation of granulocytes (D'Ancona *et al*., 2004).

In mammals and some invertebrates, growth factors such as PDGF and TGF-β, play an important role in healing wounds as they are chemotactic and proliferative factors (Seppa *et al.*, 1982; Deuel *et al.*, 1982; Senior *et al.*, 1983; Postlethwaite *et al*., 1987; Wahl *et al.*, 1987; Clark *et al*., 1997; Ottaviani *et al.*, 1997; Franchini and Ottaviani, 2000). The presence of EGF-, IL-4- and IL-10-like material in *S. nudus* does not exclude the possibility that other cytokines or other factors, found in other invertebrates, could also be present in Type I granulocytes, and that they could be directly involved in the process of wound healing. However, it is so far impossible to attribute a specific function to EGF-, IL-4- and IL-10-like material in the process of the healing of wounds in *S. nudus*.

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