Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Fundamental aspects of arm repair phase in two echinoderm models

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ARTICLE INFO

Keywords:
Starfishes
Brittle stars
Emergency reaction
Wound healing
Collagen
Immune/inflammatory response

ABSTRACT

Regeneration is a post-embryonic developmental process that ensures complete morphological and functional restoration of lost body parts. The repair phase is a key step for the effectiveness of the subsequent regenerative process: in vertebrates, efficient re-epithelialisation, rapid inflammatory/immune response and post-injury tissue remodelling are fundamental aspects for the success of this phase, their impairment leading to an inhibition or total prevention of regeneration. Among deuterostomes, echinoderms display a unique combination of striking regenerative abilities and diversity of useful experimental models, although still largely unexplored.

Therefore, the brittle star *Amphiura filiformis* and the starfish *Echinaster sepositus* were here used to comparatively investigate the main repair phase events after injury as well as the presence and expression of immune system and extracellular matrix (*i.e.* collagen) molecules using both microscopy and molecular tools.

Our results showed that emergency reaction and re-epithelialisation are similar in both echinoderm models, being faster and more effective than in mammals. Moreover, in comparison to the latter, both echinoderms showed delayed and less abundant collagen deposition at the wound site (absence of fibrosis). The gene expression patterns of molecules related to the immune response, such as *Ese-fib-like* (starfishes) and *Afi-ficolin* (brittle stars), were described for the first time during echinoderm regeneration providing promising starting points to investigate the immune system role in these regeneration models.

Overall, the similarities in repair events and timing within the echinoderms and the differences with what has been reported in mammals suggest that effective repair processes in echinoderms play an important role for their subsequent ability to regenerate. Targeted molecular and functional analyses will shed light on the evolution of these abilities in the deuterostomian lineage.

1. Introduction

All animals face and heal wounds regardless of their phylogenetic position and the life stage of individuals, though the final result of the restoration process can be remarkably different. The first post-traumatic events and the specific regulation and cross talk of the numerous cytotypes and molecules involved are fundamental to address the final outcome: tissue repair *versus* tissue regeneration and functional recovery (White et al., 2009). In vertebrates, the main steps of wound repair are re-epithelialisation, inflammatory/immune response, formation of the granulation tissue, and extracellular matrix (ECM) deposition and remodelling (Xue and Jackson, 2015). The impairment of these events, such as the absence/reduction of re-epithelialisation, the misregulation of the inflammatory/immune response and the occurrence of fibrosis, can be correlated with limited regenerative ability.

Wound healing *via* a complete and functional epithelial layer is a

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http://dx.doi.org/10.1016/j.ydbio.2017.09.035
Received 29 April 2017; Received in revised form 5 June 2017; Accepted 28 September 2017

critical step to ensure effective repair (Pastar et al., 2014): for example, in mammals impaired epidermal restoration leads to chronic non-healing wounds, causing severe medical problems, such as ulcers and absence of tissue regeneration (Sivamani et al., 2007).

Functional repair is achieved also thanks to a highly tuned inflammatory and immune response. The immune system is fundamental during haemostasis and throughout the whole inflammation phase (Park and Barbul, 2004; MacLeod and Mansbridge, 2015). In mammals, several molecules, such as fibrinogen, lectins, ficolins, cytokines (*i.e.* TNF-α and TGF-β) and interleukins (*i.e.* IL-1, IL-2, IL-6, IL-8), are key players during the inflammation process and their misregulation as well as local and systemic factors may affect proper wound healing (Guo and DiPietro, 2010) and subsequent tissue restoration.

The constant and finely regulated remodelling of the ECM components (mainly collagen) is a further key event needed for effective wound healing (Xue and Jackson, 2015). Exaggerated inflammatory response during the first phase of repair can lead to fibro-proliferative disorders (Tredget et al., 1997; Singer and Clark, 1999) which in turn result in excessive deposition of collagen and other ECM molecules (fibrosis) (Ben Amar and Bianca, 2016) and occasionally also in pathological hypertrophic scar or keloid formation. Over-deposition of collagen and its reduced remodelling are known to impair proper healing and regeneration of the damaged tissues (Bock and Mrowietz, 2002; Rahban and Garner, 2003; Diegelmann and Evans, 2004).

It is noteworthy that vertebrates are able to heal minor injuries but most of them possess restricted ability to completely restore lost body parts (Sánchez Alvarado, 2000). Some fishes (Akimenko et al., 2003), amphibian urodeles (Brockes and Kumar, 2002) and reptiles (Bateman and Fleming, 2009) can repair and regenerate after severe or debilitating wounds but the most striking regenerative abilities are still and by far found among the invertebrate clades. Cnidarians (Bosch, 2007), planarians (Saló et al., 2009), annelids (Bely, 2006), and echinoderms (Candia Carnevali, 2006) are the most representative examples. Echinoderms (Arnone et al., 2015) in particular show the maximum extent of regenerative potential among deuterostomes: indeed, they can regenerate body appendages, such as arms (Candia Carnevali, 2006), internal organs (Mozzi et al., 2006; Mashanov and García-Arrarás, 2011), and even whole animals from an isolated body fragment (Ducati et al., 2004). Moreover, representatives of all the five extant classes display regenerative capabilities (Hyman, 1955) with clear examples also found in fossils (Oji, 2001), suggesting that these are ancient and widespread features of the phylum. Therefore, echinoderms are promising models to study this phenomenon and, thus, they provide us with a valid comparative perspective with non-regenerating models, humans included.

Arm regeneration is one of the most extensively studied processes in echinoderms (for a review see Candia Carnevali and Bonasoro, 2001; Biressi et al., 2010; Ben Khadra et al., 2017). Regardless of the species, different critical events take place during the first hours/days post-amputation, including wound closure, re-epithelialisation and a rapid inflammatory response. As for mammals (Stroncek and Reichert, 2008), tissue remodelling at the wound site is also observed. During sea cucumber gut regeneration tissue remodelling is one of the last phenomena occurring in the repair phase and this was suggested to be directly related to their high efficiency of regeneration (Quiñones et al., 2002; Cabrera-Serrano and García-Arrarás, 2004). Furthermore, immune-related molecules have been described in sea urchins and sea cucumbers (Pancer et al., 1999; Rast et al., 2006; Ramírez-Gómez et al., 2008, 2009, 2010; Ramírez-Gómez and García-Arrarás, 2010; Smith et al., 2010) and their presence/role needs to be comparatively investigated in the repair processes of other echinoderms. This should lead to a deeper understanding of the process and to shed light on evolutionary divergences/similarities within the phylum and with non-regenerating models.

Among the different echinoderms, starfishes (Asteroidea) and brittle stars (Ophiuroidea) are becoming valid experimental models to study arm regenerative process (Ben Khadra et al., 2017; Biressi et al., 2010; Czarkwiani et al., 2013, 2016). Nevertheless, in both classes, the cellular/tissue and molecular aspects of the repair phase have never been simultaneously and comparatively investigated and with a multidisciplinary approach.

Therefore, this research aims to describe and compare the phenomena occurring during the repair phase after traumatic arm amputation using both the brittle star *Amphiura filiformis* (Ophiuroidea) and the starfish *Echinaster sepositus* (Asteroidea). Classical histological and ultrastructural methods are employed for the description of the main repair events from a cell/tissue perspective, whereas molecular techniques are used to investigate the involvement of inflammatory/immune responses and the ECM (mainly collagen). Overall, a detailed knowledge on how echinoderms heal severe wounds, and actually regenerate, will possibly shed light on similarities and/or differences with other animals able to regenerate whole lost body parts and, also, with those unable to do it, humans included.

2. Materials and methods

2.1. Animal collection, maintenance and regeneration tests

Adult (disc diameter ~ 0.5 cm) specimens of *Amphiura filiformis* were collected at the Sven Lovén Centre for Marine Sciences in Kristineberg (Sweden). Adult (diameter ~ 12 cm) specimens of *Echinaster sepositus* were collected by SCUBA divers at depth of 5–8 m in the Marine Protected Areas of Portofino (Ligurian Sea, Italy) and of Isola di Bergeggi (Ligurian Sea, Italy). All experimental animals were left to acclimatise for about one-two weeks and maintained in aerated aquaria of artificial sea water (ASW) (Instant Ocean®) at 14°C and 34‰ salinity (brittle stars) or 18°C and 37‰ salinity (starfishes). Chemical-physical ASW parameters were constantly checked. Animals were fed twice a week with Microvore Microdiet (Brightwell Aquatics; brittle stars) or small pieces of cuttlefish (starfishes). Traumatic arm amputation was performed using a scalpel: for brittle stars a maximum of two arms per animal were amputated at 1 cm from the disc, whereas for starfishes the distal third of one arm was removed. Brittle stars were previously anaesthetised in 3.5% $MgCl_2$ ($6H_2O$) solution (pH 8.3) in a 1:1 mix of filtered ASW and milliQ water. Animals were then left to regenerate in the aquaria for pre-determined periods, namely 24 and 72 hours (h) and 1 week (w) post-amputation (p.a.) for *E. sepositus* and 8, 16, 24, 48, 72 h and 5 days (d) p.a. (corresponding to stage 2 of Czarkwiani et al., 2016) for *A. filiformis*. Brittle star samples at 8 d (stage 4) and 2–3 w p.a. (> 50% DI; Dupont and Thorndyke, 2006; from now on called > 50%) were collected and processed as well in order to confirm/complete some *in situ* hybridisation results (see below and Supplementary Materials). Regenerating arms were collected including part of the stump and differently processed according to the subsequent analyses.

2.2. Microscopy analyses

2.2.1. Light (LM) and transmission electron microscopy (TEM)

For Epon resin embedding regenerating samples were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (pH about 7.4) with 1.2% (brittle stars) or 1.4% (starfishes) NaCl and washed overnight at 4°C in 0.1 M cacodylate buffer. They were then processed as described by Ben Khadra et al. (2015a) with only slight modifications in decalcification step that was performed after osmium tetroxide post-fixation at 4°C for at least 2–3 days using a 1:1 solution (v/v) of 2% L-ascorbic acid and 0.3 M NaCl in distilled water. Semi-thin sections (1 μm) were obtained using a Reichert-Jung Ultracut E ultramicrotome with glass knives, stained with crystal violet and basic fuchsin and then observed under a Jenaval light microscope provided with a DeltaPix Invenio 3S 3M CMOS camera and DeltaPix Viewer LE Software or a Zeiss AxioImager M1 microscope equipped with a Zeiss AxioCamHRc camera.