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REGULAR ARTICLE

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Myogenesis during holothurian intestinal regeneration

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Abstract Echinoderms are well known as being able to regenerate body parts and thus provide excellent models for studying regenerative processes in adult organisms. We are interested in intestinal regeneration in the sea cucumber, *Holothuria glaberrima*, and focus here on the regeneration of intestinal muscle components. We have used immunohistochemical techniques to describe the formation of the intestinal muscle layers. Myoblasts are first observed within the regenerating structure, adjacent to the coelomic epithelia. Within a few days, these cells acquire muscle markers and form a single cell layer that underlies the epithelia. Animals injected with BrdU at various regeneration stages have been subsequently analyzed for the presence of muscle differentiation markers. BrdU-labeled muscle nuclei are observed in myocytes of 3-week regenerates, showing that these cells originate from proliferating precursors. The peak in muscle precursor proliferation appears to occur during the second week of regeneration. Therefore, new muscle cells in the regenerating intestine originate from precursors that have undergone cell division. Our results suggest that the precursor cells arise from the coelomic epithelia. We also provide a comparative view of muscle regeneration in an echinoderm, a topic of interest in view of the many recent studies of muscle regeneration in vertebrate species.

Keywords Regeneration · Digestive tract · Organogenesis · Muscle · *Holothuria glaberrima* (Echinodermata)

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Introduction

The regeneration of a complete digestive system is one of the most dramatic regenerative processes that can be observed in deuterostome animals. This process occurs in echinoderms of the class Holothuroidea, or sea cucumbers, following natural or induced evisceration (Hyman 1955). Digestive tract regeneration implies a series of coordinated events that can be used to study the process of organogenesis in adult organisms. Our laboratory has been studying the process of intestinal regeneration in the sea cucumber *Holothuria glaberrima* for the past few years (García-Arrarás et al. 1998, 1999; García-Arrarás and Greenberg 2001; Quiñones et al. 2002). Data from our group have shown that intestinal regeneration begins from a thickening of the free edges of the mesenteries and involves processes of cell migration from the esophageal and cloacal remnants and possibly from the mesentery itself. Regeneration of the intestine is essentially completed in a 3– to 4-week period with the formation of an organ that has the same tissue layers and cell composition as the original, albeit smaller in size.

Previous studies have focused on the cellular events that form the mucosal (luminal epithelium) and submucosal (internal connective tissue) layers and the enteric nervous system (García-Arrarás et al. 1998, 1999). We have shown that the muscle layer, like other intestinal tissues, is present from early in the regenerating structure and is organized, in the 4-week regenerate, in a pattern that closely resembles the non-eviscerated organs (García-Arrarás et al. 1998). Information on the precursors of the muscle cells, the origin of these precursors, and the mechanisms of new muscle formation is lacking. Moreover, as with other regenerating processes, it remains unclear whether muscle cells originate from dividing precursors (possibly epigenetic) or from non-dividing cells that differentiate into muscle cells.

We have now used several muscle-specific markers to dissect the temporal and spatial events by which muscle cells appear within the regenerating intestine and are incorporated into a functional organ. Our results show that

intestinal muscle regeneration includes proliferation and differentiation events. The results provide not only a clearer view of visceral muscle regeneration but also helpful insights into the process of echinoderm regenerative organogenesis.

Muscle regeneration is not limited to echinoderms. Indeed, there has recently been a surge in studies of muscle regeneration in vertebrate species (Carlson 2003; Corbel et al. 2003; Gojo and Umezawa 2003 Charge and Rudnicki 2004). Muscle regeneration has been shown to occur in both skeletal and cardiac muscle of amphibians and mammals, and increasing interest has focused on the role of multipotential stem cells involved in this process. This current interest in regenerative processes makes comparative studies necessary in order to be able to determine the differences and similarities among the regenerative processes and the animal species in which it occurs (Tsonis 2000; Sanchez-Alvarado 2000; Brockes and Kumar 2002).

Materials and methods

Animals

Holothuria glaberrima specimens were obtained from the north and west coasts of Puerto Rico and kept in indoor aquaria in the laboratory. Evisceration was induced by injecting 3–5 ml 0.35 M KCl into the coelomic cavity. Animals were dissected at various days post-evisceration (dPE). Further details of animal upkeep and dissection were as given previously (García-Arrarás et al. 1998, 1999).

Immunohistochemistry

The immunohistochemical techniques used were as described previously (García-Arrarás et al. 1998, 1999). Briefly, tissues were fixed in either picric acid/formaldehyde (Zamboni) or 4% paraformaldehyde. After the tissue had been rinsed and embedded in optimal cutting temperature (OCT, Tissue Tek) mounting media, they were sectioned (10–20 μm) in a cryostat. Sections were treated with primary antibodies overnight at room temperature followed by the appropriate secondary antibody for 1 h at room temperature. In cases in which double-labeling was performed, the two primary antibodies were added together, and the two secondary antibodies were added together (see García-Arrarás 1993). In order to label nuclei, some sections were immersed in a bath of Hoechst (1 μM) for 5 min, once the primary antibodies had been rinsed away. Two more washes in 0.1 M phosphate-buffered saline (PBS) followed, and the secondary antibody was then added.

Double-labeling experiments were also performed with rhodamine-labeled phalloidin (Rh-phalloidin, Sigma) and a muscle-specific monoclonal antibody, HgM1 (see García-Arrarás et al. 1998). HgM1 hybridoma supernatant was added to the sections and left overnight. On the following day, a solution of equal amounts of Rh-phalloidin (1/100) and the secondary antibody, viz., goat anti-mouse fluorescein isothiocyanate (GAM-FITC, Biosource, Calif.; 1/50), was added to the sections. The subsequent steps of the procedure were as described above. As controls, single-labeling experiments were run at the same time. Rh-phalloidin (1/200) was applied to the tissue sections for 1 h, and the sections were then rinsed and mounted as usual.

Triple-labeling was performed by combining two primary antibodies: a polyclonal anti-actin (1/100, Sigma) and a monoclonal anti-bromodeoxyuridine (BrdU; 1/2-1/5; Amersham) followed by Hoechst treatment. The secondary antibodies used for these studies were GAM-FITC (1/50) and goat anti-rabbit cyanine 3 (GAR-Cy3; Biosource, Calif.; 1/1,000).

Sections were observed either on a Leitz Laborlux fluorescence microscope with N2, I2/3, and D filters or on a Nikon Eclipse E600 fluorescence microscope with FITC, R/DII, and DAPI filters. Measurements were made with the help of an optical micrometer.

Cell division studies

Experiments to determine if and when muscle cell precursors were dividing were performed by using the thymidine analog BrdU. Animals were injected daily with 100 μl of a 1 mg/ml BrdU solution (approximately 0.01 mg/g wet weight) for three consecutive days. Injection regimes included: 1–3 dPE, 3–5 dPE, 6–8 dPE, 9–11 dPE, 12–14 dPE, and 15–17 dPE. Animals were sacrificed at 16 or 19 dPE. The regenerating intestines were fixed and treated as described above for immunohistochemistry. Antibodies against BrdU were used to detect the cells that had incorporated BrdU. Immunocytochemistry was performed as described above but including an additional bath of 2N HCl prior to the addition of the BrdU antibody in order to improve the accessibility of the antibody to the BrdU epitope, followed by two additional PBS washes.

Western blot

Homogenates were made from the large intestine of *H. glaberrima* specimens by using Triton X-100 and Laemmli sample buffer (2.5% SDS). Some samples were treated with B-mercaptoethanol (20 μl/ml) and urea (0.3 g/ml). From each sample, the equivalent volume to 40 μg of the soluble fraction was run on 13% gels by SDS-polyacrylamide gel electrophoresis. Biotinylated broad-range standards with avidin-horseradish peroxidase were used for molecular weight determination (BioRad). Gels were equilibrated in Towbin transfer buffer with 15% methanol and transferred to polyvinylidene fluoride membranes (Amersham Pharmacia) in an electrophoresis transfer cell at 50 V for 2.5 h (Quiñones et al. 2002). After

transfer, membranes were blocked with 5% non-fat milk and rinsed with PBS with 0.1% Tween 20 (pH 7.4). Membranes were incubated with the antibody (HgM1) at a 1/50 dilution of the clone supernatant. The secondary antibody (sheep anti-mouse Ig coupled to horseradish peroxidase; Amersham Pharmacia) was used at a 1:5,000 dilution. Enhanced chemoluminescence (ECL) was performed following the manufacturer's instructions (Amersham Pharmacia).

Results

Antigen characterization

The present experiments were possible, in large part, because of the availability of monoclonal antibody HgM1. This antibody recognizes an as yet uncharacterized epitope found in holothurian muscle (Fig. 1A). Labeling is muscle-specific and includes the labeling of other muscles in the animal, such as the longitudinal body wall muscles (not shown). The labeled tissues are essentially the same that are labeled with actin and phalloidin (see Figs. 1, 4, 6). Labeling of the non-eviscerated holothurian enteric muscle was essentially similar to that described previously (García-Arrarás et al. 1998). In the digestive tract of *H. glaberrima*, the antibody labels the two muscle intestinal layers, viz., the longitudinal layer adjacent to the coelomic epithelium and the circular layer adjacent to the internal connective tissue or submucosa. The labeling of these two layers is continuous with the labeling of the mesenteric muscle, which also follows a longitudinal orientation.

Several correlated pieces of evidence provide information on the nature of the epitope recognized by HgM1. First, from the label distribution, the epitope seems to reside outside the nucleus, since in all cases, the nuclear area is unlabeled. Second, although HgM1 co-localizes in cells with phalloidin labeling, the spatial distribution of the two labels differ (Fig. 1B, C). Phalloidin labeling, as expected, is restricted to the contractile apparatus of the muscle cells, whereas the labeling of HgM1 is peripheral to the phalloidin labeling and seems to lie in the plasma membrane. Indeed, in cross sections of enteric muscle, little or no labeling is observed in the central area of the myotubes, in which the contractile apparatus is present.

Western blots of normal un-eviscerated intestine show several immunoreactive bands labeled with HgM1 antisera (Fig. 1A, inset). The principal labeling consists of a band or possibly a duplet of around 8 kDa. This band presents the strongest labeling of the immune reaction and has consistently been observed in all Western blots. However, when the fluorescent detection reaction is prolonged, other weak immunoreactive bands of higher molecular weights (around 53, 40, 31, and 24 kDa) have been found.

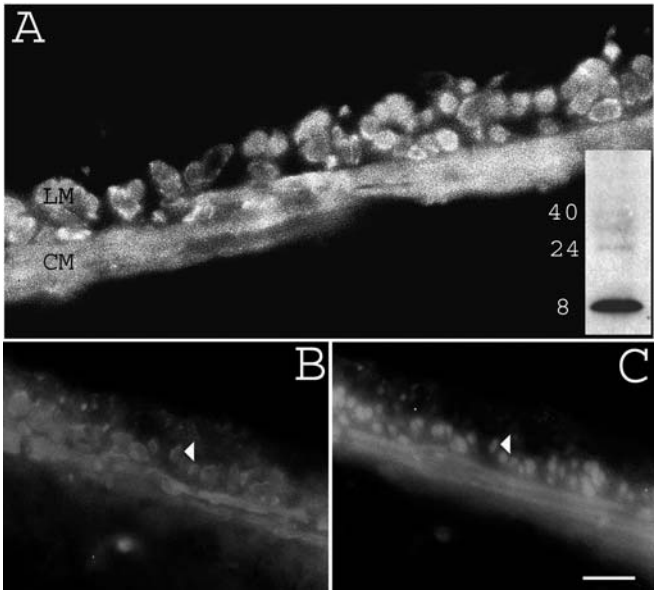

Fig. 1 Monoclonal antibody HgM1 labels the enteric muscle layers of *Holothuria glaberrima*. **A** Transverse section of the holothurian intestine showing the labeling of the longitudinal and circular muscle layers. Double-labeling of the intestine with **B** HgM1 and **C** phalloidin show that the two markers are differentially localized (*arrowhead* one muscle fiber showing that phalloidin labels the central contractile apparatus, whereas HgM1 appears to label a membrane component). *Inset*: Western blot revealing the major protein band immunoreactive to HgM1 at 8 kDa and weakly immunoreactive bands of higher molecular weights. *Bar* 10 μm (**A**), 20 μm (**B, C**)

Appearance of muscle precursors and formation of muscle layers

The epitope labeled by HgM1 is expressed by muscle precursors prior to the appearance of other muscle markers, thus facilitating the study of muscle origin and formation. In the first 3 dPE, the regenerating structure, a blastema-like structure at the free end of the mesentery that forms soon after wound healing, does not contain immunoreactive elements. Indeed, muscle cells within the mesentery, adjacent to this intestinal primordium, begin to dissapear, and little, if any, immunoreactivity to HgM1 can be observed. HgM1 labeling is first observed within the regenerating structure at 4 dPE (Fig. 2A, F, K). At this stage, labeling consists of a few isolated rounded cells, or myoblasts, localized close to or within the coelomic epithelium. The cells show immunoreactivity throughout their cell bodies, excluding the nuclei, and short extensions are usually observed that contact the coelomic border and/or the area in which the basal lamina is found. In all cases, the cells appear to be moving inwardly, away from the coelomic epithelium. Over the next few days, the cells increase in number and move farther from the coelomic epithelium forming a cellular layer underneath the coelomic epithelium. The movement of these muscle precursors from the coelomic epithelium to form the muscle layer has been quantified by measuring the distance of the HgM1-labeled cells from the apical end of the coelomic

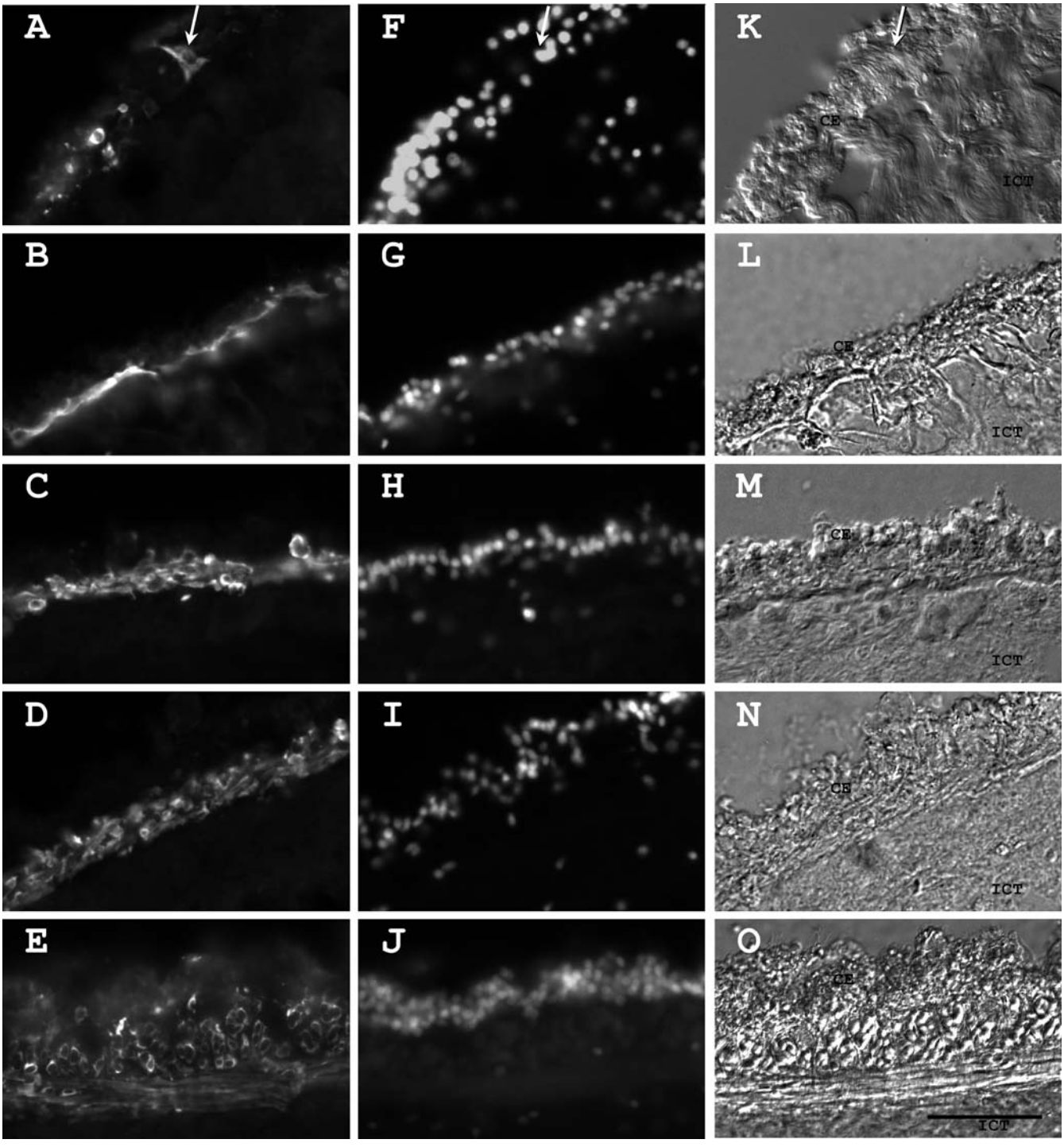

Fig. 2 Regeneration of the enteric muscle layers (*ICT* internal connective tissue, *CE* coelomic epithelium). Muscle regeneration observed by using HgM1 labeling (**A–E**). The same tissue sections are shown with Hoechst nuclei (**F–J**) and in phase contrast (**K–O**) to provide a better view of the regeneration process at the hisotological level. **A, F, K** Muscle precursors can be identified first with HgM1 (**A**) at 4–5 days post-evisceration (dPE) as isolated cells adjacent to, or within, the coelomic epithelium. One of the labeled cells (*arrow*) shows a cellular extension that reachs the apical edge of the coelomic epithelium. **B, G, I** At 7 dPE, the HgM1-labeled cells have elongated and form a single cell layer underneath the coelomic epithelium. **C, H, M** This layer continues to grow in size but remains somewhat disorganized at 14 dPE. **D, I, N** Organization of the muscle into the circular and longitudinal layers occurs between the third and fourth week PE. **E, J, O** Muscle organization in the unevisceratedlarge intestine. *Bar* 50 μm

epithelium. This distance triples between 4 and 28 dPE (Fig. 3).

By the end of the first week of regeneration (7–8 dPE), the cellular morphology of the labeled cells begins to change, as they elongate from rounded to a spindle-shape

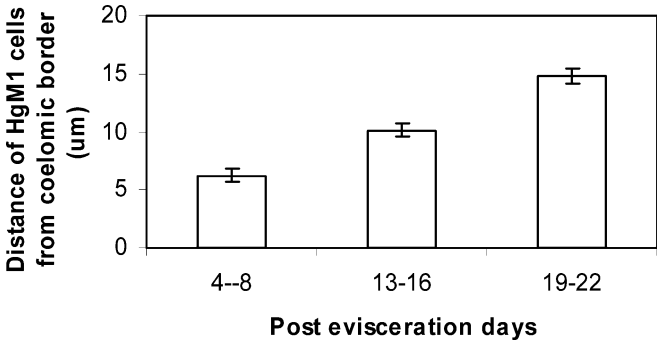

Fig. 3 Localization of HgM1-labeled cells relative to the coelomic border. The distance between the labeled cells and the coelomic border was measured. Muscle precursors expressing the HgM1 label were found close to the coelomic border during early regenerative stages and moved apart from the coelomic border as regeneration proceeded, thus forming the circular and longitudinal muscle layers. Each point represents the mean percentage ± SE from at least three animals

(Fig. 2B, G, L). This elongation occurs under the coelomic epithelium in a circular direction and, as a consequence, the distance between each cell decreases from 64 μm at 4 dPE to 5 μm at 13 dPE. The outcome of the elongation of the cells is the formation of a continuous single-cell-thick muscle layer. These cells now have the characteristic shape of myocytes. The formation of this initial muscle layer is completed during the second regeneration week. Prior to myoblast elongation, the width of the layer containing the muscle precursors is about 6.6 μm. The width of the layer increases with the formation of the continuous muscle layer to around 8.7 μm. For the next few weeks, the muscle layer continues to develop, initially in a disorganized fashion in which new myocytes continue to appear in the muscle layer that is forming under the coelomic epithelia. During the next 2 weeks (15–28 dPE), the width of the muscle layer increases to about 10–11 μm (Fig. 2C, H, M). At 21 dPE, the muscle layer has increased in size but has no clear organization. Nevertheless, from this stage on, the muscle layer begins to organize itself and, by 28 dPE, the two muscle layers (longitudinal and circular layers) can be clearly distinguished. Thus, by the end of the fourth week of regeneration, the two muscle layers can be identified (Fig. 2D, I, N). However, during our period of study, the width of the muscle layer of a regenerating animal never reached that of a non-eviscerated animal; the intestine diameter of a regenerating animal is significantly smaller than that of the non-eviscerated animal (Fig. 2E, J, O).

Differentiation of muscle precursors

The process of cell differentiation that gives rise to the muscle cells in the regenerated intestine can be followed by using two other markers. (1) Hoechst nuclear staining has revealed a distinct change in muscle cell nuclear morphology during the differentiation process. (2) Phalloidin, a toxin that binds to actin myofilaments, provides a distinct marker for a muscle-specific characteristic, viz., the contractile machinery that includes organized actin myofilaments. The contractile machinery is acquired by the muscle precursors during their differentiation. Both Hoechst and phalloidin can identify possible stages in the formation of a muscle cell from an undifferentiated precursor (or myoblast) and serve to visualize differentiation as a step by step process in which mature muscle properties are acquired and some of the undifferentiated cellular precursor properties are lost, thereby forming the characteristic myocyte.

Double-labeling with Hoechst treatment and HgM1 has shown the changes in nuclear morphology that occur during muscle cell differentiation. In tissues at 5 dPE, the nuclei of the HgM1-labeled cells are small and rounded and cannot be distinguished from the nuclei of other cells within the intestine primordia. However, later in intestinal regeneration, as myoblasts elongate to form the muscle layers, their nuclei acquire an elongated shape, and the intensity of the Hoechst-induced fluorescence is reduced when compared with other cells in the coelomic epithelium. This nuclear morphogenesis can first be detected during the first week of regeneration (Fig. 4A, B). The nuclear changes observed with Hoechst, together with the HgM1 labeling, have been used to quantify the number of myocytes within the regenerating structure. Our results show an increase in the number of cells that are positive to HgM1 and that have muscle-type Hoechst nuclei labeling within the regenerating intestine (Fig. 5). Thus, the regeneration of the muscle layer not only occurs by early changes in muscle precursor morphology but also depends on an increase in the number of myocytes during the first 3 weeks of regeneration.

The elongation of muscle precursors to form the muscle layer also coincides with the appearance of phalloidin labeling. In the non-eviscerated animal, phalloidin labeling is found in the contractile machinery of the two enteric muscle layers. In the early stages of regeneration, no phalloidin labeling can be seen. The first signs of phalloidin labeling are observed around 7 dPE, several days after the time at which the labeling with the HgM1 begins. However, the staining is sparse and limited to small fiber bundles that run circularly beneath the coelomic layer. Well-defined bundles can be observed by 14 dPE (Fig. 4C, D), coinciding with the stage at which the continuous muscle layer is formed, as determined by HgM1 labeling.

Muscle regeneration involves cell division

To determine whether the increase in muscle cells observed during intestinal regeneration was attributable to muscle precursor differentiation exclusively or whether cell proliferation had occurred, BrdU studies were performed. Our results demonstrated unequivocally that the muscle cell layer was formed by cells that had undergone division (Fig. 6A–C). This could be seen after the BrdU labelling of muscle nuclei. Two different

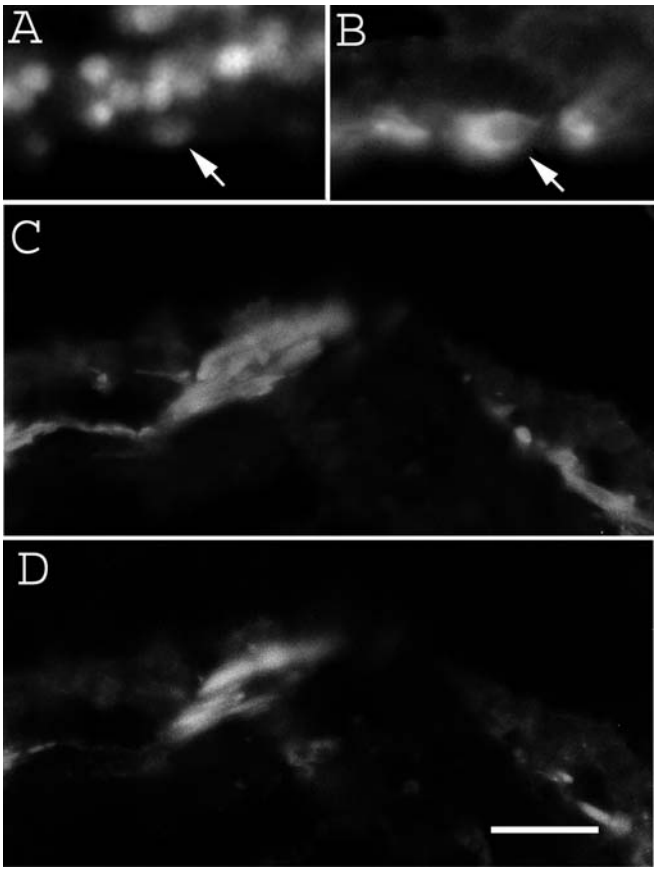

Fig. 4 Double-labeling immunocytochemistry demonstrating the appearance of muscle markers during intestinal regeneration. **A, B** Elongated nucleus (*arrows*) with less intense Hoechst fluorescence (**A**) is observed in differentiating muscle cell also labeled by HgM1 (**B**). **C, D** Double-labeling also demonstrates the appearance of phalloidin staining (**D**) in HgM1-labeled cells (**C**). *Bar* 21 μm (**A, B**), 26 μm (**C, D**)

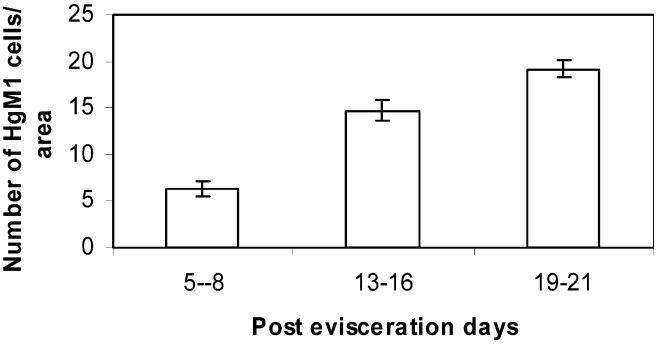

Fig. 5 Number of HgM1 cells per microscope field of view. The number of HgM1-labeled cells in the microscope field of view (an area of about 10 mm^2) was determined by using double-labeling with HgM1 and Hoechst nuclei stain. The number of muscle cells per area was found to increase between the first regeneration week when compared with the number of cells in the third regeneration week. Each point represents the mean percentage ± SE of at least three animals

methods of identifying muscle nuclei were used. First, BrdU labelling was observed in myocytes recognized by their elongated Hoechst-labeled nuclei. Second, double-labeling with antibodies against actin and BrdU revealed myocytes within the muscle layer (also with elongated nuclei) that expressed both the BrdU and actin markers.

The experimental strategy used also served to determine the temporal pattern of muscle cell division, since groups of animals were injected at several days post-evisceration and sacrificed at the same date during the third week of regeneration. Thus, cells that had incorporated the BrdU label were those that had been dividing during the days on which BrdU injection was performed. The data showed that some division of the muscle precursor occurred early in regeneration, as animals that were injected at 3–5 dPE showed labeled muscle nuclei at 19 dPE (Fig. 6D). Nevertheless, muscle precursor division was an ongoing process, since all injection groups showed labeled muscle nuclei when sacrificed during the third week of regeneration. Muscle precursor division appeared to peak during the 12–14 dPE period, since the group of animals injected on those days had more muscle nuclei labeled with BrdU.

Discussion

Enteric muscle layers are formed in a series of stages

The regeneration of the intestinal muscle layers, similar to other developmental processes, appears to occur in a series of stages. The first detectable event in intestinal muscle regeneration is the appearance of the HgM1-labeled cells within the blastema-like structure. These cells can be defined as myoblasts or the precursors of the enteric muscle. Other than HgM1 labeling, they express no marker that might serve to distinguish them from other cell types. The next stage in enteric muscle development is the differentiation of these muscle precursors into myocytes. This occurs when myoblasts acquire the morphological and biochemical properties of muscle, e.g., contractile mechanisms (as determined by phalloidin labeling), elongated cellular morphology, and characteristic nuclear morphology. The next stage is the formation of a continuous single-cell muscle layer underlying the coelomic epithelium of the regenerated blastema. This layer then increases in size, possibly by the incorporation of new muscle cells in a somewhat disorganized fashion. Finally, the muscle cells are organized into the circular and longitudinal muscle layers found in the non-eviscerated intestine. As in vertebrate embryogenesis, the formation of the circular muscle layer appears to precede the formation of the longitudinal layer (Kordylewski 1983). This organization of the intestinal muscle corresponds to the formation of a regenerated organ that, although smaller in size, has the same tissue layer proportions as the normal non-eviscerated organ. An additional event that we have not studied in our organisms is the continuous growth of the intestinal muscle as the regenerated intestine acquires the size of the original organ.

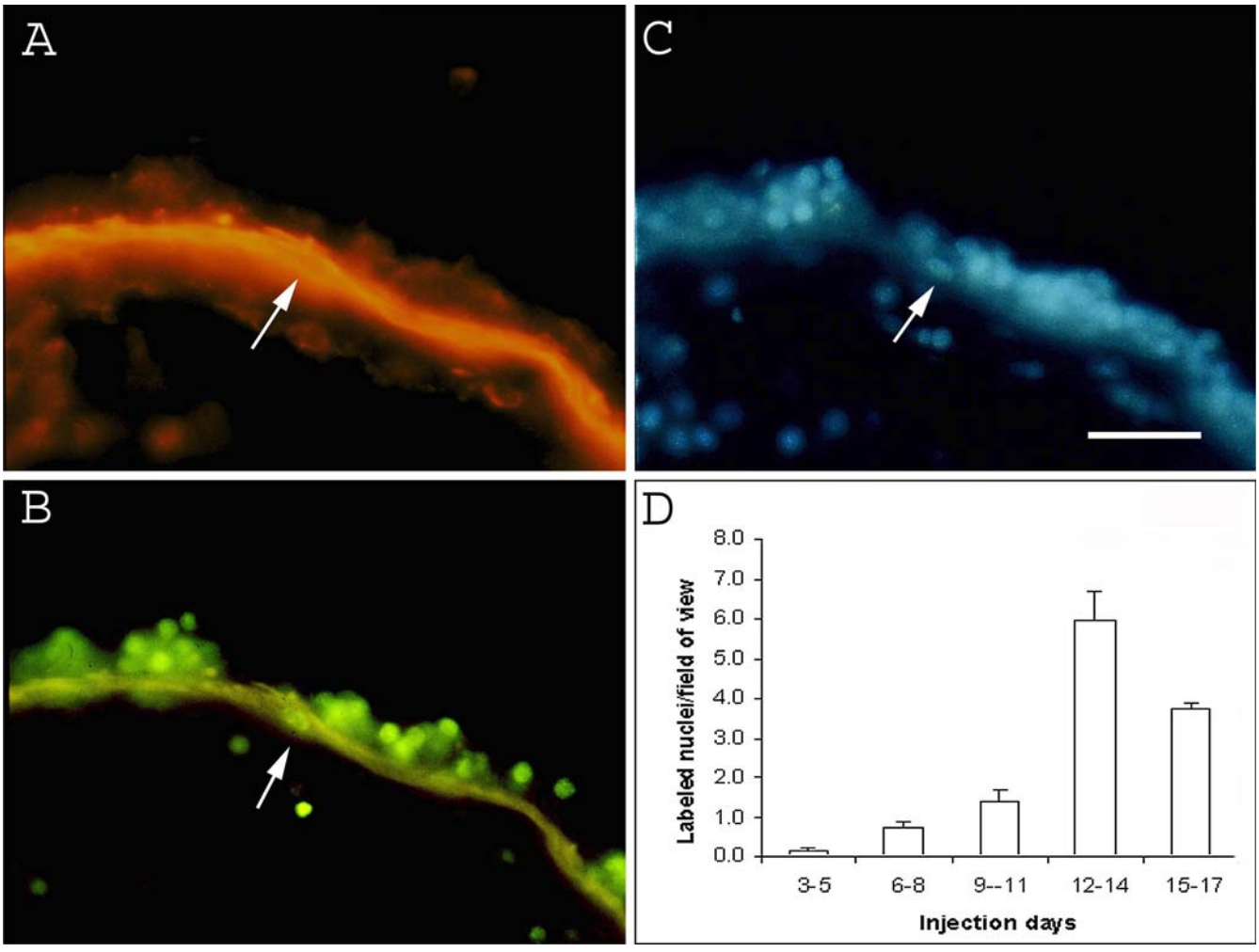

Fig. 6 Cellular proliferation of muscle precursors determined by BrdU labeling. Animals were injected with BrdU at various days post-evisceration and allowed to regenerate for 3 weeks. Triple labeling of regenerating *H. glaberrima* intestine for **A** actin, **B** BrdU, and **C** cell nuclei (Hoechst labeling). **A** Actin labeling identified the muscle cells within the intestinal muscle layers. **B** The BrdU labeling revealed the dividing cells that had incorporated the thymidine analogue in a three-week-old regenerating intestine. **C** The total number of cells present in the section could be observed with Hoechst nuclear labeling. *Arrows* in **A–C** indicate a double-labeled nucleus in a muscle cell as determined by actin labeling and by the elongated morphology of the muscle nucleus. *Bar* 24 μm (**A–C**). **D** Timing of muscle cell division during the first 3 weeks of intestinal regeneration. The number of muscle cells labeled with BrdU per area in the regenerated animals was found to peak during the second week of regeneration. Each point represents the mean percentage + SE of at least three animals

Muscle cell origins

Where do myoblasts originate? There are several possible sources of the new muscle cells. These could include specific muscle precursors or multipotential stem cells within other tissues (particularly within the mesentery), coelomocytes within the coelomic fluid, and cells of the coelomic epithelia. Several investigators have indeed suggested the possibility that, during the regeneration of other echinoderm tissues, the regenerating cells (not necessarily muscle cells) originate from cells of the coelomic fluid (Candia-Carnevali et al. 1997). In addition, results from both protostomes and deuterostomes have suggested the presence of multipotent stem-like cells in the circulating fluids. Amebocytes in crayfish hemolymph have been proposed as the origin of crustacean muscle cells (Uhrik et al. 1989). More recently, the possibility that blood-borne cells can give rise to muscle tissue in mammals has been suggested (Ground et al. 2002; LaBarge and Blau 2002), although this possibility remains highly controversial.

Although mainly based on correlated evidence, our studies support the idea that the new muscle cells within the regenerating intestine originate from the coelomic epithelia. The finding that the first myoblasts can be recognized within the coelomic epithelia, sometimes still with some extensions into the apical border, offers some ideas regarding the process of the coelomic epithelial production of muscle cells. As the cells differentiate, the newly formed myoblasts distance themselves from the overlying epithelium and form the initial single cell layer.

Echinoderm coelomic epithelial cells have been proposed to be multipotent precursors for several cell types, including muscle, nerve, and coelomocytes (Mladenov et

al. 1989; Candia-Carnevali et al. 1993, 1995; Dolmatov et al. 1996; VandenSpiegel et al. 2000). Moreover, studies from other regenerating echinoderm species further support the coelomic epithelium as the origin of the new muscle cells. The role of the coelomic epithelium in longitudinal muscle regeneration has been elegantly shown by Dolmatov and colleagues in several holothurian species (Dolmatov et al. 1996; Dolmatov and Ginanova 2001). Using a coelomic epithelium-specific antibody or labeled thymidine together with microscopic analysis, they have shown that, when the longitudinal muscle is damaged, cells of the overlying coelomic epithelia differentiate into myocytes. Similarly, electron-microscopic studies of crinoid regenerating arms have also pinpointed the coelomic epithelium as the source of new myocytes (Candia-Carnevali et al. 1998; Candia-Carnevali and Bonasoro 2001). Finally, in regenerating Cuvierian tubules of the sea cucumber *H. forskali*, muscle cells have been found to regenerate from the coelomic epithelium compartment (VandenSpiegel et al. 2000).

The possibility that coelomic epithelia give origin to muscle cells is attractive from an evolutionary point of view. Rieger and Lombardi (1987) propose that myocytes might have evolved through the invagination or immigration from myoepithelia. In their evolutionary model, the muscle cells, although underlying the coelomic epithelia, are still attached to a common basal lamina. This would thus present a case in which the regenerative process of coelomic epithelial cells giving rise to the myoblasts and eventually the myocytes recapitulates the evolutionary process. Moreover, according to some authors, the coelomic epithelial cells together with the muscle and nerve elements constitute a single tissue layer named the mesothelium (Smiley 1994). All the cells within this mesothelium, including the adluminal cells, neurons, and myoepithelial cells, share a common origin and rest on the same basal lamina.

Cellular proliferation during muscle regeneration

Cell proliferation is known to occur during regenerative processes of some echinoderms. In crinoid arm regeneration, cell proliferation is active within the coelomic epithelia (Candia-Carnevali et al. 1995). In starfish regenerating arms, active proliferation is also observed in epithelial structures of the water and perihemal canals, and these epithelia provide cells that migrate to the regenerating structure (Mladenov et al. 1989; Moss et al. 1998). Furthermore, our own studies and those of others have shown cell proliferation in the coelomic and luminal epithelia of sea cucumbers (Leibson 1992; García-Arrarás et al. 1998). However, in none of the echinoderm studies described above is there any direct evidence to show that muscle cells do indeed originate from dividing precursors.

In contrast, our BrdU labeling results clearly show that, whatever the origin of the muscle precursors, most muscle cells divide before the third week of regeneration. Proliferation of muscle precursors begins early in the regenerative process, possibly prior to the acquisition of most of the mature muscle properties, and peaks during the third week when the muscle layers are increasing in size and being organized into two distinct layers. Indeed, if we take the number of muscle cells in the third week regenerate as being close to 20 per microscope field of view (Fig. 5), we can estimate that around 13 of these cells undergo cell division prior to differentiation (the sum of the number of cells in the BrdU experimental stages, Fig. 6D). Thus, more than half of the muscle cells in the 3-week regenerated intestine have arisen from dividing precursors. This number is probably an underestimate since not all dividing cells are necessarily labeled during BrdU injection periods.

Muscle regeneration model

Visceral muscle formation during regenerative events has been seen in other systems. In the aquapharyngeal complex of *Eupentacta fraudatrix*, visceral muscle has been found to regenerate from myoepithelial cells in the coelomic epithelium; these cells undergo a process of de-differentiation and subsequent differentiation into muscle cells (Dolmatov 1992). Similarly, in the regeneration of Cuvier organs (intestine-associated organs that serve a defensive role) in *H. forskali*, new muscle cells are also formed from precursors in the coelomic epithelia (VandenSpiegel et al. 2000)

Our ongoing hypothesis is that enteric muscle cells originate from dividing coelomic epithelial cells (a model is presented in Fig. 7). The process by which this occurs is an epimorphic mechanism that can be described as follows. Once the rupture site of the mesentery heals and a new coelomic epithelium has been formed to cover the exposed area, the cells within the coelomic epithelium are able to divide to produce additional coelomic epithelial cells needed as the mesentery thickens and the regenerating primordia grows. Some of the progeny of these coelomic epithelial cells de-differentiate to produce the myoblasts. These myoblasts migrate under the coelomic epithelia, eventually differentiating into myocytes and giving rise to the muscle layers. Continued production of muscle precursors is needed as the regenerating structure grows in size and as the various muscular layers are formed. Thus, these new muscle cells are continuously produced by either further division from the coelomic epithelium or by dividing myoblasts within the forming muscle layers. The formation of the muscle cells is similar to our proposed model for the formation of the enteric nervous system (García-Arrarás et al. 1998). However, in contrast to the more localized area, confined to the region of the mesenterial thickening as has been proposed for enteric neuron production, new muscle cell formation appears to occur along the whole epithelia of the regenerating intestine. Nonetheless, the possibility that the coelomic epithelium can give rise to both neurons and muscle cells provides further support to the model of

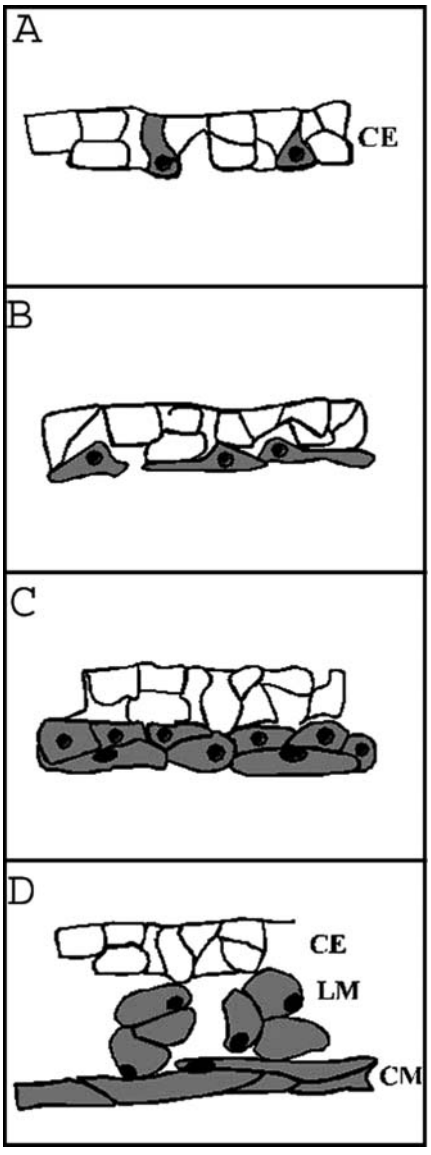

Fig. 7 Proposed model for the regeneration of the enteric muscle in *H. glaberrima*. Representation of a cross-section of the regenerating intestine at approximately weekly intervals. **A** Our results suggest that muscle cells in the regenerating intestine originate from dividing precursors in the coelomic epithelia (*CE*). **B** These cells migrate basally and elongate to form, initially, a single-cell muscle layer. **C** Subsequent incorporation of cells to this cell layer occurs without any apparent organization. **D** Eventually, cells within the muscle layer are organized into the circular (*CM*) and longitudinal (*LM*) components found in the non-eviscerated intestine

muscle evolution proposed by Rieger and Lombardi (1987).

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