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RESEARCH ARTICLE

RGD-Containing Peptides Inhibit Intestinal Regeneration in the Sea Cucumber *Holothuria glaberrima*

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The sea cucumber *Holothuria glaberrima* is an echinoderm capable of regenerating its viscera. Previous studies from our group have shown a striking remodeling of the extracellular matrix (ECM) during intestinal regeneration. To study the role of the ECM during regeneration, we have focused on the RGD sequences present in many ECM molecules. Regenerating animals were treated with an RGDS (Arg-Gly-Asp-Ser) peptide that competes with the interaction between RGD sequence and cellular integrins. Saline and RGES (Arg-Gly-Glu-Ser) peptide injections were done as controls. The size of the regenerating intestine was determined, and the regenerating structures were analyzed by immunohistochemistry for the presence of collagen and fibronectin, as well as for muscle and other cells. The results show a delay in intestinal regeneration in animals injected with the RGDS peptide, suggesting that the ECM–integrin interaction plays an important function in the regenerative process. *Developmental Dynamics 231:171–178, 2004.*

Key words: echinoderm; regeneration; extracellular matrix; digestive tract; MMP

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INTRODUCTION

Echinoderms are well known for their capacity to regenerate their tissues and organs. While this regenerative capacity is found in all echinoderm classes, it is particularly striking in members of the class Holothuroidea, known to be able to regenerate most of their internal organs following a process known as evisceration (Hyman, 1955). We have been using the sea cucumber *Holothuria glaberrima* as an animal model to study the cellular and molecular events involved in intestinal regeneration (García-Arrarás et al., 1998, 1999; García-Arrarás and Greenberg, 2001; Quinoñes et al., 2002). In this organism, the intestinal system is the first organ to regenerate. It originates from a thickening at the edges of the torn mesentery and in a 3- to 4-week period forms what appears to be a functional intestine. One of our main interests has been to study the role of the extracellular matrix (ECM) in the regeneration of the holothurian intestine.

The ECM is composed of different types of macromolecules such as fibronectin, laminin, collagens, and proteoglycans, among others (Alberts et al., 2002). ECM molecules are known to participate in many processes, including cell migration, differentiation, and organ formation. The interactions between cells and ECM molecules are mediated by receptors in the cellular membrane, the best studied of which is the integrin family. Integrins are surface glycoproteins that recognize an arginine-glycine-aspartate (RGD) sequence found in some ECM molecules (Ruoslahti and Pierschbacher, 1987). Actually, RGD has been established as the minimum necessary sequence for the cell surface receptors to recognize some ECM molecules such as fibronectin, vitronectin and collagen type 1 (Ruoslahti and Pierschbacher, 1987). This interaction can be inhibited by peptides containing the RGD sequence. In fact, multiple studies us-

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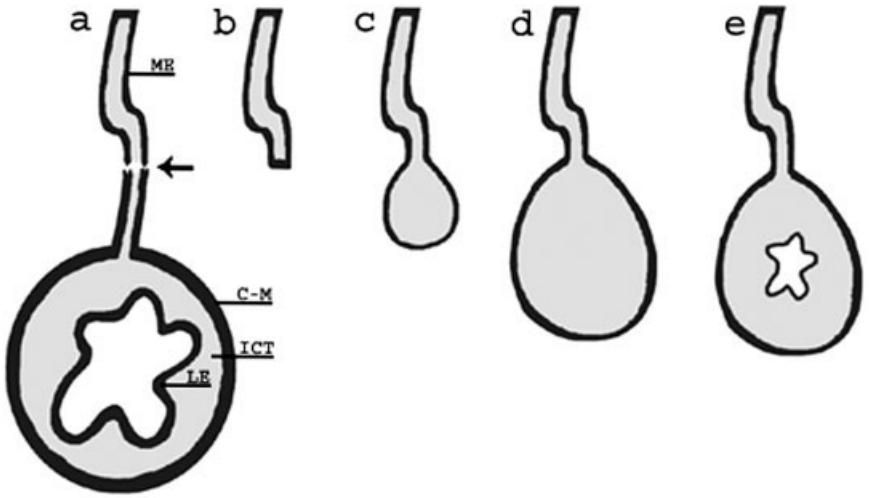

Fig. 1. Drawings of the intestine of the sea cucumber *Holothuria glaberrima* in cross-sections depicting the events that occur during evisceration and regeneration. **a:** At the histological level, the holothurian intestine is composed of a coelomic epithelium layer overlying the muscle layers (longitudinal and circular) (these layers are shown together as C-M), an inner connective tissue layer (ICT), and a luminal epithelium layer (LE). During evisceration, the intestine detaches (at arrow) from the mesentery (ME) and is expelled through the cloaca. **b:** Within 24 hr after evisceration, a wound-healing process takes place where the coelomic epithelia covers the tissue layers exposed at the rupture plane. **c:** Regeneration continues during the first week with a thickening of the mesenterial edges forming a blastema-like structure. **d:** This mesenterial thickening continues to increase in size during the second week of regeneration. **e:** Formation of the luminal cavity and epithelial layer occur during the second week by cells that migrate from the esophagus and cloaca into the blastema-like structure. Subsequent events not shown here include organization of the muscle layer into longitudinal and circular layers and general growth of the organ.

ing RGD-containing peptides have shown that inhibition of the ECM–integrin interactions can cause striking alterations of cellular processes (Pierschbacher and Ruoslahti, 1984a,b; Yamada and Kennedy, 1984; Gehlsen et al., 1988; Perris et al., 1989). More recently, studies have suggested a mechanistic link between matrix metalloproteinase activity and the production or exposure of RGD sequences (Davis et al., 2000; Holliday et al., 2003).

Our research group previously had demonstrated changes in the ECM molecules of the intestine and mesentery of *H. glaberrima* during the process of regeneration (Quinoñes et al., 2002). We have also obtained evidence for the involvement of metalloproteinases in the ECM remodeling that accompanies intestinal regeneration. However, no direct proof of cellular–ECM interactions has been presented. In this study, we investigate the possible role of ECM–integrin interactions in the intestinal regeneration of *H. glaberrima* by means of the treatment of regenerating animals with synthetic peptides with and without the RGD sequence. We use microscopy and immunohistochemical analysis to observe changes in the regenerated tissues after 7 days. Our results show that intestinal regeneration is inhibited in animals treated with synthetic RGD-containing peptides. Thus, the results suggest that ECM–integrin interactions indeed play an important role in the regeneration of the digestive tract of *H. glaberrima* and possibly in other regenerative processes of echinoderms.

RESULTS

H. glaberrima specimens that had been eviscerated and left to regenerate for a week form a mesenterial thickening at the edge of the torn mesentery, which has been described previously (Fig. 1). This thickening, which resembles a regeneration blastema, is the intestinal primordium that eventually becomes the regenerated intestine. Therefore, the size of this structure serves as an indicator of the ongoing regenerative process.

In preliminary experiments, regenerating animals were injected with peptides containing the RGD sequence, GRGDTP (Gly-Arg-Gly-Asp-Thr-Pro), RGDS (Arg-Gly-Asp-Ser), and RGD (Arg-Gly-Asp) ranging in concentrations between 0.8 and 10 mg/ml. Peptides without the RGD sequence, p-EHP (Glu-His-Pro) and RGSE (Arg-Gly-Glu-Ser), were used as controls. Although all animal groups injected with peptides containing RGD sequences showed a smaller regenerating structure than controls, these differences were not statistically significant because of the inherent variability in the regeneration process among the different animals and the small sample size-(data not shown). Nonetheless, these experiments provided some encouraging results and served to test our selection of dosage and injection protocol.

The role of RGD-mediated interactions in the regeneration process was tested by comparing the effects of two tetrapeptides at the same dose (160 μg in 200 μl), one with the RGD sequence (RGDS) and a very similar peptide that lacked the RGD sequence (RGES). That these tetrapeptides differed in only one of the amino acids provided an exquisite control. Phosphate-buffered saline (PBS) injections served as an additional control. The results from these experiments showed that RGDS-injected animals had a significantly smaller regenerating structure (approximately 50% smaller) when compared with either RGES- or PBS-injected controls (Fig. 2).

The thickening of the mesenteric edges to form a blastema-like structure is only one of the events that occur during intestinal regeneration. Among the other events that have been described to occur are the degradation of the collagen within the ECM (Quinoñes et al., 2002), the formation of the muscle layer (García-Arrarás et al., 1998; Murray and García-Arrarás, manuscript submitted for publication), and the accumulation of a spherule-containing cell population (García-Arrarás et

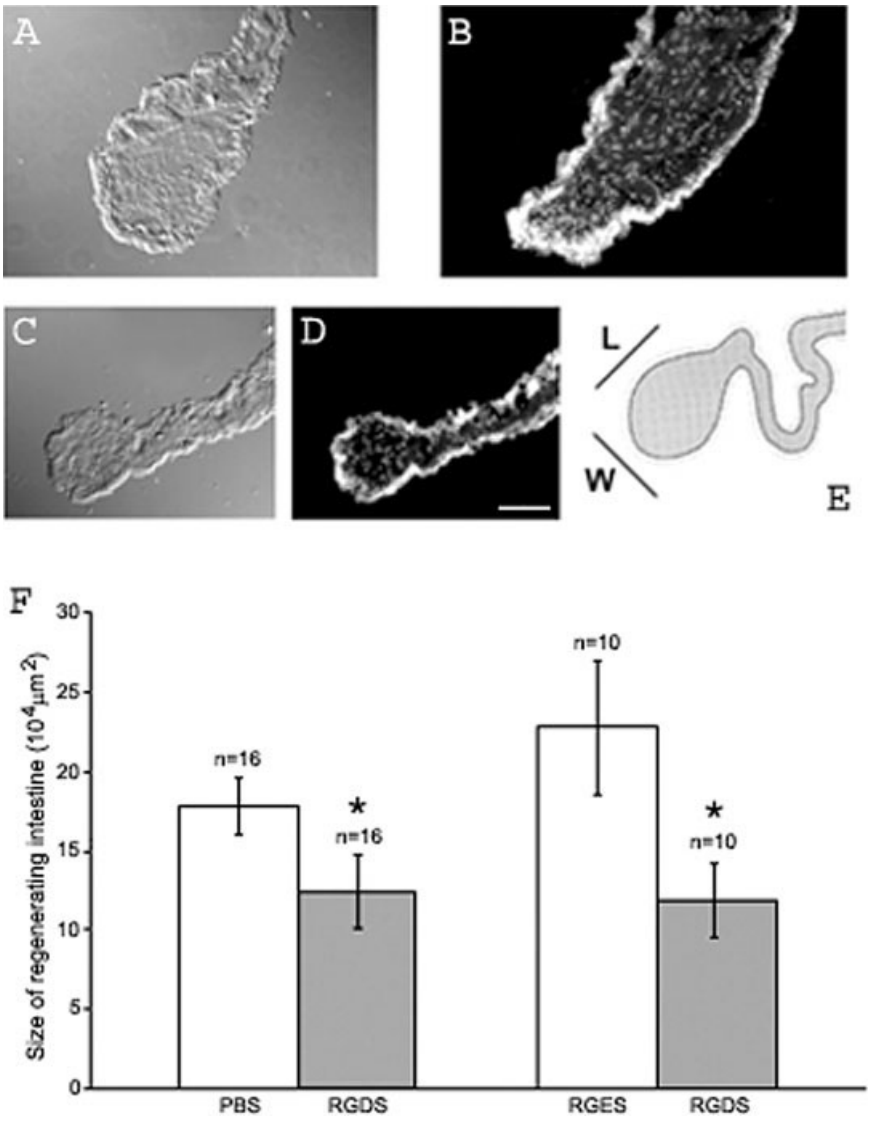

Fig. 2. Injections of RGDS decrease the size of the regenerating intestinal primordia. **A–D:** Sections of regenerating intestine from day 7 PBS-injected (A, phase contrast) and RGES-injected (B, Hoescht) control animals show the large size of the regenerates when compared with RGDS-injected animals (C, phase contrast) and (D, Hoescht). **E:** The area of the regenerating intestine was determined by measuring the width and length of the structure. **F:** Measurements of the regenerate relative area show a significant decrease in the size of the structure in RGDS when compared with either PBS- or RGES-injected animals. Values are expressed as the mean ± SE of the area. n, number of animals. Scale bar = 100 μm. Asterisks indicate difference from control (PBS or RGES), $P < 0.05$.

al., 1998; Schenk et al., unpublished observations). Therefore, to determine what other changes occurred due to the RGD-interference, we used immunohistochemistry to analyze the degree of collagen degradation, muscle formation, and the presence of the spherule-containing cell population within the regenerating structure and mesentery.

There were no observable differences in collagen expression among the experimental and control groups (Fig. 3A,B). This finding was made evident by observation of collagen with immunohistochemistry and confirmed after a subjective analyses giving a numerical value to the degree of collagen immunoreactivity (2.9 ± 0.1, 2.8 ± 0.05, and 2.9 ± 0.1 for RGDS, RGES, and PBS, respectively (mean ± SE)). In all specimens, collagen expression showed spatial patterns and immunoreactivity similar to what has been reported previously (Quinoñes, 2002); collagen was present in the mesentery and was largely absent from the mesenteric edge thickening. In fact, in the regenerating intestine, collagen has been shown to be degraded during the first 2 weeks of regeneration, with a subsequent loss of collagen immunoreactivity. In all our animals, whether injected with RGDS, RGES, or PBS, collagen immunoreactivity was stronger than in noninjected animals, suggesting a delay in the degradation of collagen possibly due to the injection protocol but not due to the action of the peptides.

In contrast, the formation of the muscle layer was altered in RGDS-injected animals. The muscle layer in the 1-week regenerate is formed by a single muscle layer beneath the coelomic epithelium (García-Arrarás et al., 1998). In animals injected with RGDS, this cell layer was not fully formed or appeared thinner than in controls (Fig. 3C,D). Sections were classified according to the relative muscle layer formed (see Experimental Procedures section). The values obtained for these experiments (PBS, 2.2 ± 0.16, RGES, 2.1 ± 0.11, and RGDS, 1.4 ± 0.15) showed a difference between the RGDS-injected animals and RGES- and PBS-injected controls.

Finally, we have shown that there are changes in the cell populations within the intestine during the regeneration process (García-Arrarás et al., 1998). Recent studies in our laboratory have identified a monoclonal antibody that recognizes a spherule-containing cell type present in the mesentery and in the mesenteric edge thickening. We used the monoclonal antibody to determine the number of cells in RGDS-injected animals and in control animals by counting the number of cells per each 1.4-mm section of mesentery between the regenerating intestine and the body wall, as measured with an optical micrometer. RGDS-injected animals showed a significant decrease in the number of cells in the mesentery when compared with the RGES-injected controls (Fig. 4). The RGES-injected animals showed similar number of cells as PBS-injected animals. Approximately 20 spherule-containing cells per length of mesentery were found in both PBS- and

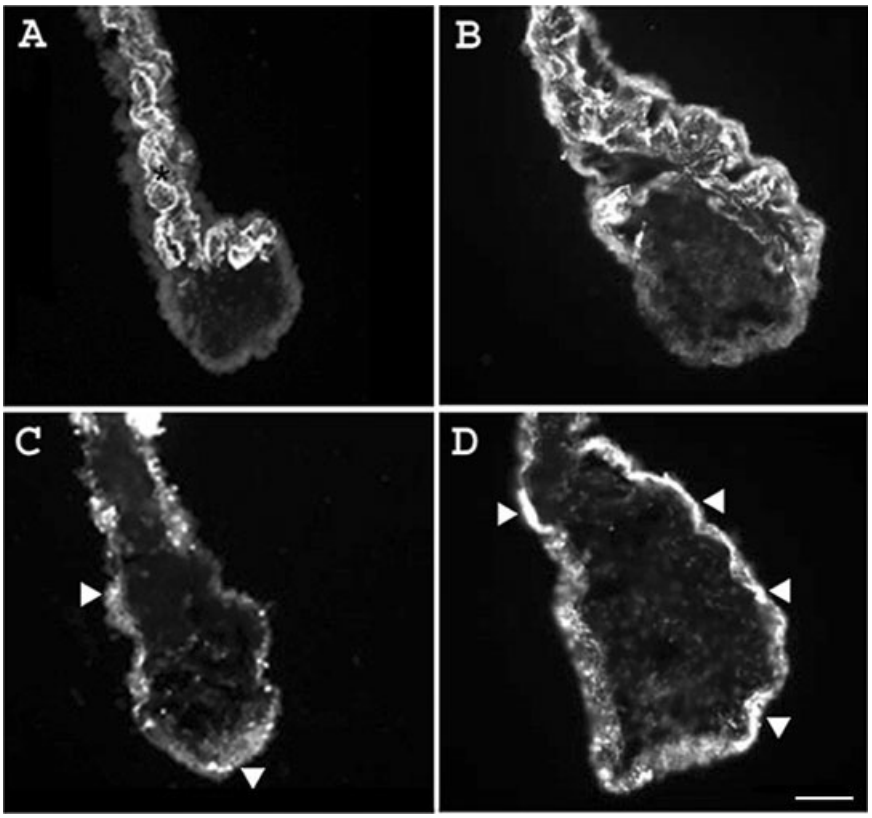

Fig. 3. Effects of RGDS and RGES injections on collagen expression and muscle formation. **A,B:** Collagen expression appears to be similar in animals injected with either RGDS (A) or RGES (B). **C,D:** In contrast, the formation of the muscle layer appears impaired in RGDS-injected animals (C) when compared with RGES-injected controls (D). Few muscle cells (arrowheads) are found within the mesenteric thickening of RGDS-injected animals, while in RGES-injected animals many more cells are present forming the initial muscle layer. Scale bar = 100 μm in D (applies to A–D).

RGES-injected animals, whereas this number decreased to approximately 12 in RGDS-injected animals. The number of spherule-containing cells within mesenteric edge thickening was also measured, and although a trend toward a smaller number of cells in RGDS-injected animals was found when compared with controls, no statistical differences were observed. This finding might be due to the large variability in the number of spherule-containing cells within the regenerating structure found among animals.

Fibronectin is one of the molecules that is known to interact with integrin receptors by means of its RGD epitope. In addition, fibronectin expression has been detected in the holothurian intestine during regeneration (Quinoñes et al., 2002). To determine whether there were changes in fibronectin expression in the injected animals, we performed an immunohistochemical analysis by using a commercial antibody against fibronectin. No significant difference in fibronectin expression was found among RGDS-, RGES-, or PBS-injected animals. In all groups, fibronectin was expressed in the connective tissue of the mesentery and within the regenerating structure (Fig. 5) in a pattern similar to that described previously (Quinoñes et al., 2002).

DISCUSSION

The overall effect of the RGD-containing peptides was an inhibition or retardation of the process of intestinal regeneration. The effect was evident in the decrease in size of the regenerating structure. However, to understand the extent of the effect of the RGD-containing peptides, it is necessary to describe more completely the process of intestinal regeneration in the holothurian model (for extensive descriptions, see García-Arrarás et al., 1998; García-Arrarás and Greenberg, 2001). In brief, intestinal regeneration starts soon after wound healing and reformation of the coelomic epithelia along the torn edge of the mesentery. One of the initial events during regeneration is a thickening of the mesenteric edge. This thickening is partly due to changes in ECM composition (Quinoñes et al., 2002) and partly due to an increase in the number of cells within the internal connective tissue (García-Arrarás et al., 1998). Little cellular division occurs during the first week of regeneration and this mainly takes place within the mesentery adjacent to the intestinal primordia and in the coelomic epithelia of the primordia itself. Unpublished results from our group suggest that a subpopulation of cells migrate through the mesentery to reach the regenerating structure. Migrating cells play various roles in the regeneration process, predominantly the degradation of the remaining collagen and eventually the deposition of a new ECM (Quinoñes et al., 2002). As ECM remodeling occurs, differentiation of muscle cells from precursors within the coelomic epithelia begins. These new muscle cells within the regenerating intestine appear to originate from the coelomic epithelia itself (Murray and García-Arrarás, manuscript submitted for publication).

Therefore, the decrease in the size of the regenerating structure observed after RGD-peptide injection could be due to or accompanied by changes in other cellular/molecular events. In this case, we observed an apparent lag in muscle layer formation. Moreover, an effect was also seen on the number of spherule-containing cells within the mesentery, suggesting that the process of cell migration into the regenerating structure was also affected. That the effect is specific to the RGD sequence was suggested by the lack of effect of RGES, which differs from the active molecule in only one amino acid. It is well known that peptides with the RGD motif compete for the binding sites of integrins, which are the cellular receptors for some types of ECM molecules (Ruo-

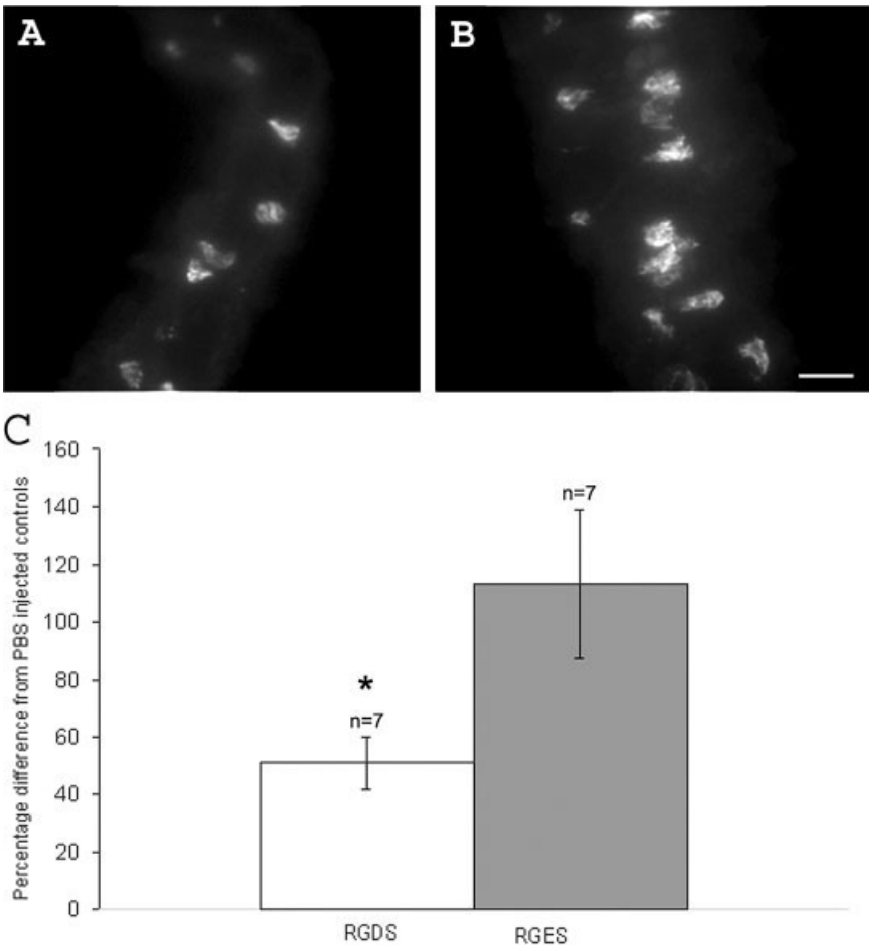

Fig. 4. Injections of RGDS reduce the number of spherule-containing cells in the regenerating mesentery. **A,B:** Immunohistochemical observations show fewer Sphe-2–labeled cells in the mesentery of RGDS-injected animals (A) at day 7 of regeneration when compared with those injected with RGES (B). **C:** Measurements of the number of spherule-containing cells in the mesentery of RGDS and RGES compared with that of PBS-injected controls show a 50% decrease in the number of cells in animals injected with RGDS. Animals injected with RGES have a similar number of cells as PBS-injected controls. Bars indicate the mean ± SEM. n, number of animals. The asterisk indicates a difference from RGES control, $P < 0.05$. Scale bar = 100 μm in B (applies to A,B).

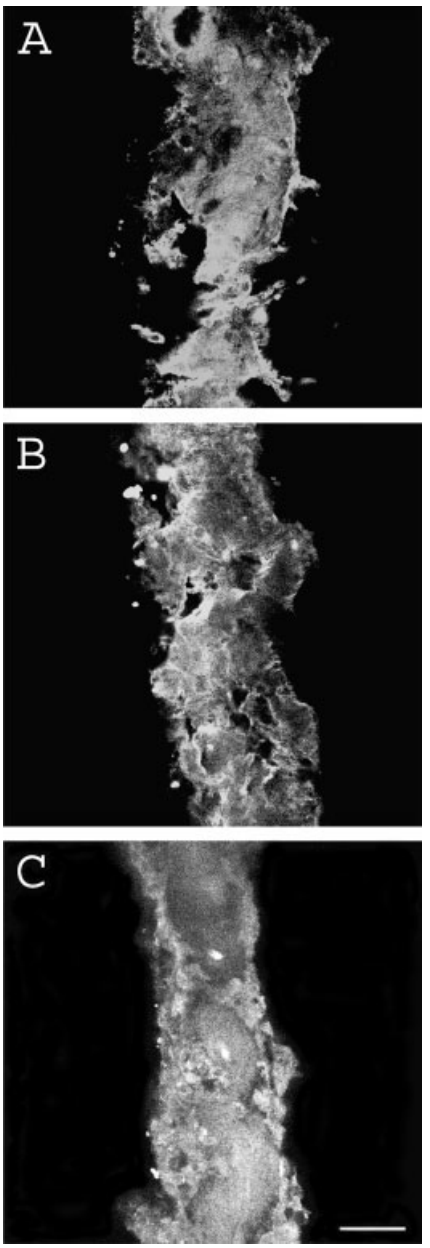

Fig. 5. Fibronectin expression in the mesentery of day 7 regenerating animals. **A–C:** PBS- (A), RGDS- (B), and RGES- (C) injected animals show a similar pattern of expression and similar localization of fibronectin. The molecule can be found within the connective tissue layer of the mesentery, forming a network of interstitial matrix fibers. Scale bar = 100 μm in C (applies to A–C).

slahti and Pierschbacher, 1987). In fact, many previous studies have used these RGD-containing peptides to define possible roles for ECM molecules in various cellular processes, including cell attachment and adhesion (Pierschbacher and Ruoslahti, 1984a,b; Ruoslahti and Pierschbacher, 1987), processes of tumor cell invasion (Humphries et al., 1986; Gehlsen et al., 1988), inhibition of platelet aggregation (Gartner and Bennet, 1985), and inhibition of neutrophil (Harler et al., 1999) and fibroblast (Greiling and Clark, 1997) migrations.

Although our results showed conclusively that the injections of RGD-containing peptides, particularly RGDS, inhibited or delayed intestinal regeneration, two important questions remain. First, which is the RGD-containing molecule whose association with cellular receptors is being affected by the RGD injections? Second, what regeneration process or processes are affected by the RGD injections resulting in a smaller regenerative structure, incomplete muscle layer formation, and fewer spherule-containing cells?

To answer the first question, one needs to focus on the ECM components that are known to contain RGD sequences. Among these, two ECM molecules stand out, fibronectin and collagen, both of which have been found within the ECM of the holothurian intestine and have been shown to undergo changes in their expression during intestinal regeneration (Quinoñes et al., 2002). The RGD sequence has been shown to play an important role in the binding of cells directly to type I collagen (Dedhar et al., 1987; Ruoslahti and

Pierschbacher, 1987), and in fact, a fibrous collagen, similar to vertebrate type I collagen, has been described in holothurians (Bailey, 1984). Although the labeling of this collagen disappears from the regenerating *H. glaberrima* intestine and mesentery possibly through breakdown by matrix metalloproteases, some collagen is still present during the first week of regeneration (Quinoñes et al., 2002). On the other hand, fibronectin expression can be found at all regenerative stages, with an apparent increase during the first 2 weeks of regeneration (Quinoñes et al., 2002). The importance of the RGD sequence found in fibronectin has been known for almost two decades (Pierschbacher and Ruoslahti, 1984a,b). Among the roles of the fibronectin molecule that depend on the RGD sequence are differentiation, motility, and metastasis (McCarthy and Furcht, 1984; Humphries et al., 1986; Singer et al., 1987; Ruoslahti and Pierschbacher, 1987; Gehlsen et al., 1988). Thus, fibronectin is also a possible candidate for the mediation of the RGDS effect. Nonetheless, we cannot rule out the possibility that the effect of the RGD-containing peptides is mediated by means of other molecules that contain the RGD epitope found in the ECM or in body fluids.

The answer to the second question might be found in two processes where ECM-integrin interactions appear to play important roles: the migration of cells by means of the mesentery into the regenerating intestine and the movement of precursor cells from the coelomic epithelium to form the new muscle layers. Thus, the principal effect of the RGD injections would be to block the ECM/cellular association and inhibit cellular migration. In experiments where RGD-containing peptides were used in mammalian models, cell migration or metastasis was inhibited and many of these effects were mimicked by using antibodies against fibronectin, suggesting that it is indeed the fibronectin molecule that is involved in the RGD-mediated processes (Humphries et al., 1986; Bilato et al., 1997; Brenner et al., 2000). In fact, RGD inhibition of cellular migration has been ascribed to fibronectin in many species, ranging from coelenterates to vertebrates (Donaldson et al., 1988; Perris et al., 1989; Stidwill and Christen, 1998; Brown, 2000). Thus, in the regenerating intestine, the fibronectin molecules with their RGD attachment sites could be available for cellular migration into the regenerating structure. These migrating cells play a key role in the thickening of the mesenteric edges and the formation of the blastema-like structure. Therefore, an inhibition of the migratory process would cause a delay in subsequent regenerative events. That RGD peptides caused a decrease in the number of cells within the mesentery of the regenerating intestine supports our hypothesis. Similarly, the process of muscle cell differentiation and layer formation could also be directly affected by the RGD injections. In effect, in other systems, it has been shown that RGD peptides interfere with smooth muscle cell migration after injury of blood vessels (Choi et al., 1994; Slepian et al., 1998).

Whatever the specific process inhibited by RGD peptides, the effects of RGD strengthen our contention that the ECM is playing an important role in the regenerative process. In a recent review, Davis and colleagues (2000) speculated that few RGD sequences are exposed in the normal tissue but that after tissue injury, the number of RGD sites increases. Part of this increase could be due to enzymatic degradation of ECM molecules that exposes matricryptic sites (active cryptic sites within ECM molecules that are revealed after structural or conformational alteration). They propose that these newly exposed RGD sequences are important in tissue repair. Our work fits beautifully in this context. We previously have shown ECM remodeling and metalloprotease (MMPs) activation to occur during intestinal regeneration (Quinoñes et al., 2002). The MMPs could be responsible for the ECM remodeling and the exposure of previously hidden RGD sites. In the current study, we have shown that RGD sequences are important for the regenerative process. More broadly, the ECM-MMP-RGD interaction might be common to many regenerative processes, including regeneration of ear holes in mice (Gourevitch et al., 2003), limb regeneration in newts (Kato et al., 2003), and even epithelial regeneration in hydra (Shimizu et al., 2002). Thus, the role of the ECM in regenerative processes may be highly conserved during evolution.

EXPERIMENTAL PROCEDURES

Animals

Holothuria glaberrima specimens were collected in the northeast coast of Puerto Rico and maintained in seawater aquaria at 22–24°C. Evisceration was induced by injecting 2–4 ml of 0.35 M KCl into the coelomic cavity. Before the dissections, the animals were anesthetized with 6% $MgCl_2$ for 1 hr.

Treatments

H. glaberrima specimens were eviscerated (day 0) and placed in aquaria. Animals were injected intracoelomically with peptides or vehicle twice during the first week of regeneration (day 2 and day 4), and dissected on day 6 or 7 for histological analyses. Peptides were dissolved in 0.1 M (pH 7.4) PBS. Injections were made in volumes of 200–500 μl at peptide concentrations ranging from 0.8 to 10 mg/ml. The volume of coelomic fluid is approximately 7 ml; therefore, the final concentration of the peptides ranged from 20 to 700 μg/ml of coelomic fluid. The peptides used were RGD (SIGMA), GRGDTP (SIGMA), and RGDS (American Peptide), p-EHP (SIGMA) and RGSE (American Peptide). Control injections were made with similar volumes of PBS, the drug vehicle.

Immunohistochemistry

Regenerating and noneviscerated intestines were fixed during 24 hr in Zamboni fixative and treated as described previously (García-Arrarás, 1993, 1998; Quinoñes et al., 2002). In brief, the following day, they were dehydrated with ethanol 80% (3 × 15 min), dimethyl sulfoxide (3 × 15 min) and rehydrated with 0.1 M PBS. They were then kept in PBS/sucrose

30% until use. Tissues were mounted in embedding medium (OTC; Miles, Inc., Elkhart, IN), and 20-μm transverse sections were obtained in a cryostat microtome (Leica CM 1900) at −30°C and recovered on polylysine–treated glass slides. The primary antibody was left in a humid chamber for 24 hr at room temperature. Then the slides were washed with 3 × 15 min PBS, and the secondary antibody was administered in a 1/50 dilution and was left for 1 hr in a humid chamber. In some cases, sections were immersed in a bath of Hoescht (1 μM) during 5 min, after rinsing off the primary antibody. The secondary antibody used was fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (GAM-FITC, BioSource Int., Camarillo, CA). The primary antibodies used for immunohistochemistry were (1) Hg-fCOL a monoclonal antibody against sea cucumber intestinal fibrous collagen (Quinoñes et al., 2002), (2) a polyclonal antibody against human plasma fibronectin (SIGMA #F3648), (3) HgM1, a monoclonal antibody that recognizes holothurian enteric muscle (García-Arrarás et al., 1998), and (4) Sph1 (IF3) and Sphe2 monoclonal antibodies for certain population of cells (Torres-Vazquez, 1993; García-Arrarás et al., manuscript in preparation). Sections were observed either on a Leitz Laborlux fluorescent microscope with N2, I2/3, and D filters or on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII, and 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI) filters. Measurements were made with the help of an optical micrometer. Fibronectin immunoreactivity was observed in a Zeiss LSM 510 Axiovert 100M confocal microscope.

Cell and Tissue Measurements and Analyses

Transverse sections of regenerating intestines from animals that had received the different injection treatments were analyzed for several parameters. For each analysis, at least three sections from each animal and at least three animals per treatment were used. The parameters studied were as follows.

Regenerating organ size.

In animals in which regeneration has been under way for 1 week, the intestinal primordia consists of a thickening of the edge of the mesentery that extends from the esophageal region to the cloaca (García-Arrarás et al., 1998). When observed in cross-sections under the microscope, the thickening of the mesenteric edge forms a blastema-like structure that can have a circular or oval morphology. The area of this structure was measured either with an optical micrometer to obtain the width times the length of the regenerating structure (see Fig. 1) or with a Metavue (Univ. Imaging Corp) image analysis system that traced the contours of the regenerating structure. Because both measurements provided relatively identical results, we opted for the simpler methodology of measuring the size of the regenerating intestine using the optical micrometer.

Muscle layer formation.

In 1-week regenerated intestine, a thin muscle layer forms just beneath the coleomic epithelium along most, if not all, of the mesenteric edge thickening (García-Arrarás et al., 1998). This muscle layer can be recognized immunohistochemically by using monoclonal antibody HgM1 (García-Arrarás et al., 1998) as well as other muscle markers (Murray and García-Arrarás, manuscript submitted for publication). HgM1 was used to label the muscle layer and to compare its degree of development among experimental and control animals. Although a subjective measurement, we quantified the collagen immunoreactivity in our samples assigning them a relative value of 0–3 (0, no muscle labeling; 1, labeling in less than 25% of the regenerating structure circumference; 2, labeling in 25 to 75%; and 3, labeling in 75–100%).

Collagen expression.

Previous studies from our group have shown, using immunohistochemistry and Western blots, the presence of fibrous collagen in the regenerating structure and its subsequent disappearance (Quinoñes et al., 2002). We also used a subjective analysis to determine the degree of collagen expression in the regenerating structure (0, no collagen labeling; 1, weak; 2, medium; 3, strong labeling).

Cellular migration.

We have observed labeling recently in a population of spherule-containing cells using monoclonal antibodies (Sphe1 and Sphe2) obtained against intestinal tissue homogenates (Torres-Vazquez, 1993; Schenk et al., manuscript in preparation). In this study, we used the antibody to quantify the same cell population in the mesentery and mesenteric edge thickening of experimental and control animals. In the mesentery, cells were counted in each microscope field of view (1.4-mm mesenteric length) moving sequentially from the junction with the regenerating structure to 4.2 mm toward the body wall. The number of cells from 1-week regenerates was compared with the PBS-injected controls to obtain the relative ratio of cells in RGDS- and RGES-injected animals compared with PBS-injected animals.

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