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Epithelial morphogenesis in hydra requires de novo expression of extracellular matrix components and matrix metalloproteinases

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SUMMARY

As a member of the phylum Cnidaria, the body wall of hydra is organized as an epithelium bilayer (ectoderm and endoderm) with an intervening extracellular matrix (ECM). Previous studies have established the general molecular structure of hydra ECM and indicate that it is organized as two subepithelial zones that contain basement membrane components such as laminin and a central fibrous zone that contains interstitial matrix components such as a unique type I fibrillar collagen. Because of its simple structure and high regenerative capacity, hydra has been used as a developmental model to study cell-ECM interaction during epithelial morphogenesis. The current study extends previous studies by focusing on the relationship of ECM biogenesis to epithelial morphogenesis in hydra, as monitored during head regeneration or after simple incision of the epithelium. Histological studies indicated that decapitation or incision of the body column resulted in an immediate retraction of the ECM at the wound site followed by a re-fusion of the bilayer within 1 hour. After changes in the morphology of epithelial cells at the regenerating pole, initiation of de novo biogenesis of an ECM began within hours while full reformation of the mature matrix required approximately 2 days. These processes were monitored using probes to three matrix or matrix-associated components: basement membrane-associated hydra laminin β1 chain (HLM-β1), interstitial matrix-associated hydra fibrillar collagen (Hcol-I) and hydra matrix metalloproteinase (HMMP). While upregulation of mRNA for both HLM-β1 and Hcol-I occurred by 3 hours, expression of the former was restricted to the endoderm and expression of the latter was restricted to the ectoderm. Upregulation of HMMP mRNA was also associated with the endoderm and its expression paralleled that for HLM-β1. As monitored by immunofluorescence, HLM-β1 protein first appeared in each of the two subepithelial zones (basal lamina) at about 7 hours, while Hcol-I protein was first observed in the central fibrous zone (interstitial matrix) between 15 and 24 hours. The same temporal and spatial expression pattern for these matrix and matrix-associated components was observed during incision of the body column, thus indicating that these processes are a common feature of the epithelium in hydra. The correlation of loss of the ECM, cell shape changes and subsequent de novo biogenesis of matrix and matrix-associated components were all functionally coupled by antisense experiments in which translation of HLM-β1 and HMMP was blocked and head regeneration was reversibly inhibited. In addition, inhibition of translation of HLM-β1 caused an inhibition in the appearance of Hcol-I into the ECM, thus suggesting that binding of HLM-β1 to the basal plasma membrane of ectodermal cells signaled the subsequent discharge of Hcol-I from this cell layer into the newly forming matrix. Given the early divergence of hydra, these studies point to the fundamental importance of cell-ECM interactions during epithelial morphogenesis.

Key words: Hydra, Morphogenesis, ECM, Laminin, Collagen, Matrix metalloproteinase

INTRODUCTION

The extracellular matrix (ECM) functions as both a structural entity and as a signaling entity that has been reported to modulate the differentiation of cells associated with it, and the shape of cells and organs during early embryogenesis and later histogenesis (Chen and Ingber 1999; Davis et al., 2000; Relan and Schuger 1999; Streuli, 1999). It is now accepted that cell-ECM interactions are fundamental to a wide variety of developmental processes to include epithelial morphogenesis (Aumailley and Gayraud, 1998; Colognata and Yurchenco, 2000; Darribere et al., 2000; Relan and Schuger, 1999). From

a structural standpoint, ECM exists as a complex polymerized lattice that is typically organized as either an epithelial-associated basal lamina [i.e. basement membrane (BM)] or a connective tissue-associated interstitial matrix (IM). Tissues of metazoans commonly have these two forms of ECM adjacent to one another (e.g. skin, gut, etc.) and this organizational structure has been described in the most ancient animal groups. For example, in hydra (a member of Cnidaria, the second oldest phylum of the animal kingdom), the structure of ECM has been shown to resemble that observed in more complicated metazoans. Previous studies have shown that the ECM of hydra resides between the epithelial bilayer (outer ectoderm and inner endoderm) and that this matrix is organized as two subepithelial zones (basement membranes associated with both the ectoderm and endoderm) with a central fibrous zone (interstitial matrix) (Sarras et al., 1993; Sarras and Deutzmann, 2001). Because of the high regenerative capacity of hydra (e.g. regeneration of the complete adult form from tissue representing as little as 1/50th that of the adult polyp) (Shimizu et al., 1993), several bioassays have been developed to analyze the role of cell-ECM interactions during morphogenesis and development in this simple epithelial organism. These studies have shown that the ECM of hydra functions in a broad range of developmental processes such as: cell proliferation, cell migration, cell differentiation and morphogenesis (Gonzalez Agosti and Stidwill, 1991; Sarras et al., 1993; Sarras et al., 1994; Stidwill and Christen 1998; Zhang et al., 1994; Zhang and Sarras, 1994; Ziegler and Stidwill 1992).

Given the fundamental relationship of ECM biogenesis to epithelial morphogenesis, this study was designed to examine the structural and functional relationship between ECM biogenesis and epithelial morphogenesis in hydra. The study monitored these events during head regeneration and other regenerative processes involving the epithelium. The study also examined whether the biosynthesis of specific components of the ECM is required for epithelial morphogenesis to occur.

MATERIALS AND METHODS

Culture of hydra

Hydra vulgaris and *Hydra magnipapilatta* were used in all experiments. Animals were cultured in hydra medium (1 mM $CaCl_2$, 0.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.08 mM $MgSO_4$ and 0.03 mM KNO_3) at 18°C. in glass or polystyrene dishes. Hydra polyps were fed with freshly hatched Brine Shrimp larvae one to three times a week and were starved at least 48 hours before use.

Initiation of head regeneration, epithelial repair and grafting

Hydra regeneration was performed as previously described (Shimizu et al., 1993). Animals that had the first bud protrusion were collected from a cultured population. The head was amputated at the 1/4 position between the head pole and the bud protrusion using a surgical blade. Decapitated hydra were kept in fresh culture medium in a 50 mm plastic dish.

Epithelial repair was initiated by making a transverse incision on the side of body at the 2/4 position between the head pole and bud protrusion.

Grafting was performed according to the procedures of Shimizu et al. (Shimizu et al., 1993). Briefly, animals collected as mentioned above were cut transversely into two pieces and then grafted back by threading a nylon fish line of 230 μm in diameter into the gut of the two tissues and pressing them from both ends using parafilm sections for 2-3 hours. The fish line was then carefully removed and the graft was allowed to heal in the culture solution.

Immunofluorescence

Regenerating, wound healing or grafted animals were allowed to relax, elongate and be immobilized in 2% urethane solution in culture medium for 2-3 minutes. Specimens were then fixed in Lavdowsky's fixative for 30-60 minutes, washed three or four times with phosphate-buffered saline (PBS) and stored in a blocking solution. Fixed animals were incubated with either the monoclonal antibody, m52 (raised against the hydra laminin β1 chain, HLM-β1) or m39 (raised against hydra fibrillar collagen, Hcol-I) for 30 minutes, washed and then incubated with FITC secondary antibody for 30 minutes, washed again before being mounted on glass slides with 25% glycerol in PBS. The specimens were then examined and photographed using an epifluorescent microscope.

Northern blot analysis

Northern blot analysis was performed as previously described (Sarras et al., 1994). Hydra total RNA was isolated using the RNeasy Mini Kit (Qiagen, California). [^{32}P]dCTP (NEN Life Science, MA) random labeled DNA probes were generated and used according to standard procedures (Sambrook et al., 1989). Probes were generated using a 3 kb clone corresponding to the 5′ ORF of the HLM-β1 chain (Sarras et al., 1994) and a 2.1 kb clone corresponding to the 5′ ORF of Hcol-I (Deutzmann et al., 2000). After hybridization and washing, filters were exposed to a phosphorus plate for 24 hours. The plate was then scanned with a phosphorus imager and analyzed using software provided with the instrument (Cyclone, Parkard BioSciences, Meriden, CT).

In situ hybridization

Whole-mount in situ localization of mRNA was performed using digoxigenin-labeled RNA probes generated as described previously (Deutzmann et al., 2000; Leontovich et al., 2000). Probes for HLM-β1 chain and Hcol-I matched those used for northern blot analysis. Probes for HMMP were identical to those previously described and used by Leontovich et al. (Leontovich et al., 2000). Fixation, processing, hybridization and visualization of the riboprobe in whole-mount preparations was performed as previously described (Grens et al., 1995; Martinez et al., 1997; Grens et al., 1999). Briefly, hydra polyps were fixed with 4% paraformaldehyde after relaxation in 2% urethane. Specimens were subsequently treated with ethanol and proteinase K to facilitate diffusion of the probes into the epithelial bilayer. To stabilize digested tissues, specimens were re-fixed with 4% paraformaldehyde and then prehybridized in hybridization solution (50% formamide, 5× SSC, 1× Denhardt's, 200 mg/ml tRNA, 0.1% Tween 20, 0.1% Chaps, and 100 mg/ml heparin) to block nonspecific hybridization sites. This was followed by a 48 hour hybridization with the digoxygenin-labeled RNA probe and a subsequent wash in hybridization solution and SSC. Specimens were washed in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) and pre-blocked in MAB with 20% sheep serum and 1% BSA. This was followed by a 16 hour incubation at 40°C with anti-digoxigenin antibody, which was diluted 1:2000 in the blocking solution. Animals were then washed eight times with MAB and briefly in alkaline phosphatase buffer (100 mM Tris HCl pH 9.5, 50 mM MgCl, 100 mM NaCl, and 0.1% Tween-20). Specimens were put in BM Purple AP substrate solution (Boehringer Mannheim) for generation of a color reaction. Specimens were lastly dehydrated with ethanol and mounted in Euparol (Asco Laboratories).

Use of localized electroporation (LEP) for introduction of antisense oligonucleotides into hydra

For functional analysis studies, we have developed a procedure to test specifically the effect of antisense oligonucleotides on head or foot

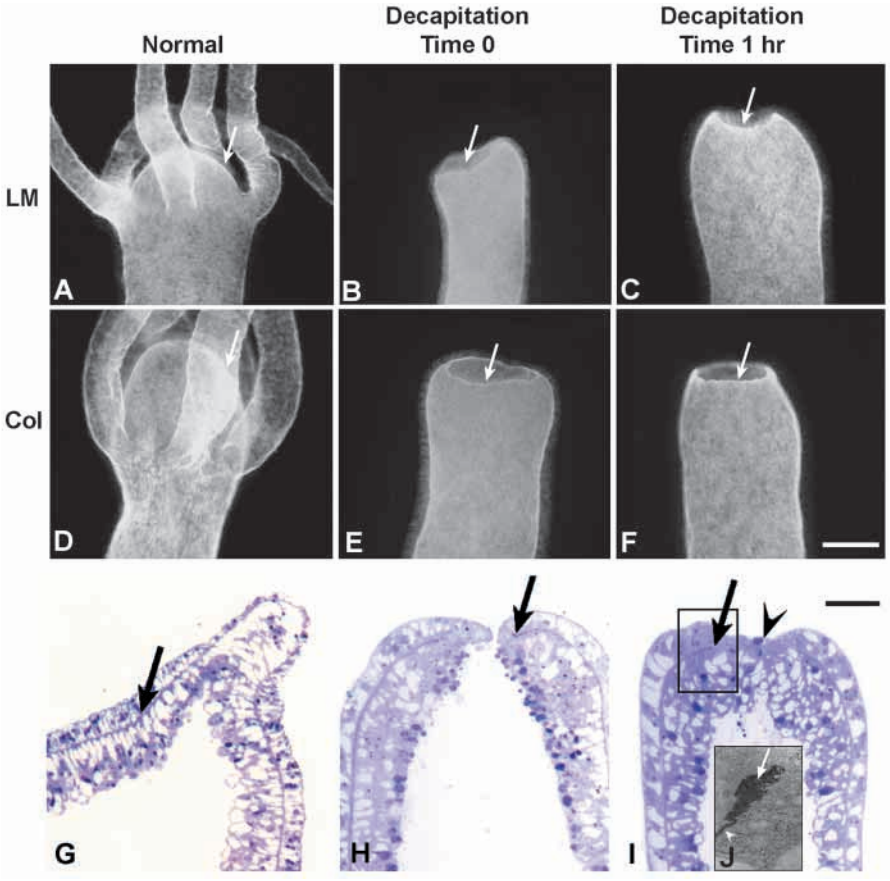

Fig. 1. Initial morphological events within 1 hour of decapitation as monitored by whole-mount immunofluorescence using antibody to hydra laminin β1 chain (LM) (A-C) and hydra type I collagen (Col) (D-F), light microscopy (G-I) and transmission electron microscopy (TEM) (J). As shown in A,D,G, the ECM (arrow) is continuous along the head pole. Immediately after decapitation, the epithelial bilayer is separated into two halves and as indicated by the arrows in B,D,H, the ECM is contained within each half. The cut edge of the ECM can be visualized in whole mounts by immunofluorescent staining of both LM (localized to basal lamina) (B) and Col (localized to the interstitial matrix) (E). One hour after decapitation, the two separated halves of the bilayer have fused (I) creating a closed head pole that lacks the morphological features of an adult polyp (no hypostome or tentacles). The arrow in I indicates that the ECMs of each epithelial bilayer half are still not fused at this time as shown by TEM analysis in J (region indicated by the box in I). As also shown in J, the cut edge of the ECM is thickened (white arrow in J) when compared with the normal thickness of the ECM more distal to the cut edge (white arrowhead in J). The thickened cut edge of the ECM 1 hour after decapitation is seen in whole-mount immunofluorescence as a bright circular signal (white arrows in C and F) at the apical pole of the body column as monitored by staining for LM (C) or Col (F). The epithelium at the apical pole that has fused, but lacks an ECM, is flattened (arrowhead in I) when compared with the epithelium that is associated with an ECM (epithelium in the left half of the box shown in I). Scale bars: in F, 250 μm for A-F; in I, 100 μm for G-I.

regeneration in hydra. This approach uses a localized electroporation technique (LEP) in order to introduce antisense thio-oligonucleotides into the head or foot pole of hydra. This procedure has been applied to the functional analysis of a number of hydra genes and has been described in detail previously (Deutzmann et al., 2000; Yan et al., 2000a; Yan et al., 2000b). Applying the LEP procedure, we tested the hypothesis that de novo biosynthesis of the HLM-β1 chain and HMMP are each required for normal head morphogenesis after decapitation. Based on the work of Dr Richard W. Wagner (Flanagan et al., 1996; Wagner 1994; Wagner 1995) a series of 20-mer oligonucleotides with phosphorothioate linkages were designed. Five oligonucleotides were synthesized for the HLM-β1 chain and six oligonucleotides were synthesized for HMMP. These oligonucleotides included antisense, mismatch (randomized), and sense sequences. The antisense oligonucleotides were as follows.

HLM-β1: 5′UTR 5′-TTGCCCAAAACATAAT-3′; initiation, 5′-TTTTGCGTCCGACCAT-3′; coding, 5′-CTTAATTTATCTTTGT-3′; 3′UTR, 5′-AGAAAAAATATCAAAT-3′.

HMMP: initiation, 5′-AACCCTAACGAAATGAACAT-3′; coding, 5′-TGGTTCCCTGCAGCGTATAT-3′.

Because HLM-β1 chains and HMMP are both expressed in the endoderm layer of cells at the head and foot pool, LEP was performed on the inner gastric surface of the head region according to procedures previously described (Yan et al., 2000a; Yan et al., 2000b; Zhang et al., 2001). Electroporated hydra were observed every 24 hours and the degree of regeneration was compared with mock-electroporated controls over a period of 72 hours. For initiation of head regeneration, animals were cut in the neck region just inferior to the mouth and tentacle ring. The degree of head regeneration was monitored by (1) observing the morphology of the head process under a dissecting microscope and determining the degree of tentacle eruption and hypostome formation, and (2) analyzing the cellular morphology of cells of the hypostome and tentacles using Nomarski optics. In controls, head regeneration is normally completed within 72 hours and therefore, experimental groups in which inhibition was observed were monitored for an additional 5 days to determine if recovery from inhibition had taken place. As previously stated, control animals were treated with mismatched oligos (randomized sequence) or sense oligos if a particular antisense thio-oligo was found to block morphogenesis. Inhibition of protein translation was monitored using antibodies to the gene product of interest in conjunction with whole-mount immunofluorescent techniques (Deutzmann et al., 2000). In addition, rescue experiments were performed using isolated hydra laminin or recombinant HMMP. In these experiments, soluble ECM extracts containing hydra laminin were isolated using modification (X. Z. and M. P. S., unpublished) of procedures developed for isolation of hydra collagens (Deutzmann et al., 2000) and recombinant HMMP was expressed and folded to an active structure as described by Leontovich et al. (Leontovich et al., 2000). Protein fractions were dialyzed into DMSO-loading buffer (Zhang and Sarras, 1994). Hydra laminin (total protein 50 μg/ml) or HMMP (total protein 200 μg/ml) were introduced into polyps by 12 hours post LEP using the DMSO loading procedure previously described by Zhang and Sarras (Zhang and Sarras, 1994). Four groups were analyzed for these head regeneration experiments according to the basic LEP protocols described above. These four regeneration groups were (1) antisense, (2) antisense + DMSO loading, (3) DMSO loading without LEP and (4) regeneration with no treatment. Ten hydra were analyzed per group and the experiment was

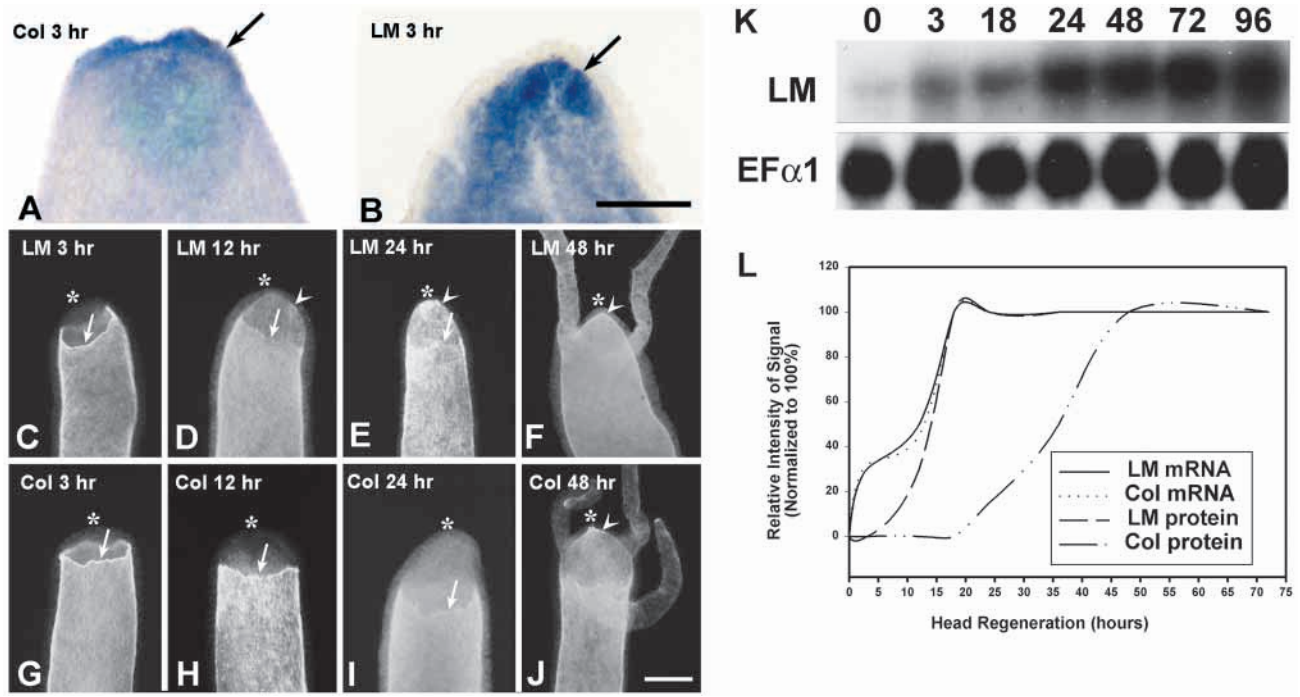

Fig. 2. Morphological and biosynthetic events occurring within 3 to 96 hours after decapitation as monitored by whole-mount in situ hybridization for hydra collagen I (Col) (A) and hydra laminin β1 chain (LM) (B), whole-mount immunofluorescence for LM (C-F) and Col (G-J) and Northern blot analysis (K). As shown in A,B, upregulation 3 hours after decapitation of hydra collagen is associated with the ectoderm (A, arrow) while upregulation of hydra laminin is associated with the endoderm (B, arrow). While the epithelial bilayer has already fused at the apical pole (asterisk in C-J), a hiatus in the ECM still exists 3 hours after decapitation (C,G). The original cut edge of the ECM can still be detected up to 24 hours after decapitation, as monitored with antibodies to LM (C-E) and Col (G-I). Reformation of a continuous ECM at the regenerating head pole is first observed with antibodies for LM between 7 and 12 hours after decapitation (D, arrowhead) and this signal continues for 24-48 hours of regeneration (E,F, respectively; arrowhead). By contrast, an ECM-associated signal for hydra Col is only weakly detected by 15-24 hours (not evident at the magnification shown in I), while an easily observed signal is seen between 24 and 48 hours at this same magnification (J, arrowhead). Upregulation of mRNA for LM (K) and Col (data not shown) precedes the appearance of immunofluorescent signals for proteins associated with the reforming ECM. Elongation factor α1 (Efα1) is used as a loading control for northern blot analysis of the mRNA lanes shown in K; rimes above each lane are in hours. The relative fluorescent and northern blot signals for LM and Col over 72 hours after decapitation are shown in L. Scale bars: in B, 200 μm for A,B; in J, 250 μm for C-J.

repeated three times. Groups were monitored as described above every 24 hours. Control and experimental groups were statistically compared using a Chi squared test and an ANOVA test.

RESULTS

Initiation of head regeneration involves a loss of ECM at the site of decapitation

The ECM of hydra is normally continuous at the head pole as well as the rest of the animal (Fig. 1G) as monitored using antibody to either basement membrane components (HLM-β1 chain; Fig. 1A) or interstitial matrix components (Hcol-I, Fig. 1D). Decapitation resulted in a loss of ECM at the head pole as monitored using these same antibodies to the HLM-β1 chain (Fig. 1B) or Hcol-I (Fig. 1E). Immediately after decapitation, the cut edges of the epithelium were free and had not fused (Fig. 1H). This loss of ECM was still observed 1 hour after decapitation (Fig. 1C,F), even though the epithelium at the cut edges had fused by this time (Fig. 1I). At this same time (arrowhead in Fig.1I), cells of the ectoderm that had no underlying ECM at the regenerating head pole were flattened (low cuboidal morphology) when compared with adjacent body column cells that had a high cuboidal morphology and were in contact with their ECM. This flattening effect is not apparent in the endoderm cell layer possibly owing to the fact that the endoderm is organized with longitudinal ridges called taeniolae that give the layer a more complicated histological morphology (Campbell and Bode, 1983). Ultrastructural analysis of the head pole 1 hour after decapitation indicated that the ECM was indeed absent between the ectoderm and endoderm cell layers. The brighter immunofluorescent signal observed at the cut edge of the ECM (see Fig. 1B,C,E,F) was seen as a thickening of the cut edge of the ECM (presumably owing to a retraction of the matrix at the time of decapitation), as observed by ultrastructural analysis (Fig. 1J). These observations indicate that while hydra epithelial cells with no underlying ECM can survive for a limited time (Sarras et al., 1993), the loss of a matrix causes cell shape changes. It is not clear from these studies, however, to what degree the retraction of the ECM after decapitation was due to (1) the inherent tensile biophysical properties of the matrix and/or (2) to the action of the epithelium on the ECM via alterations in cell-ECM adhesions.

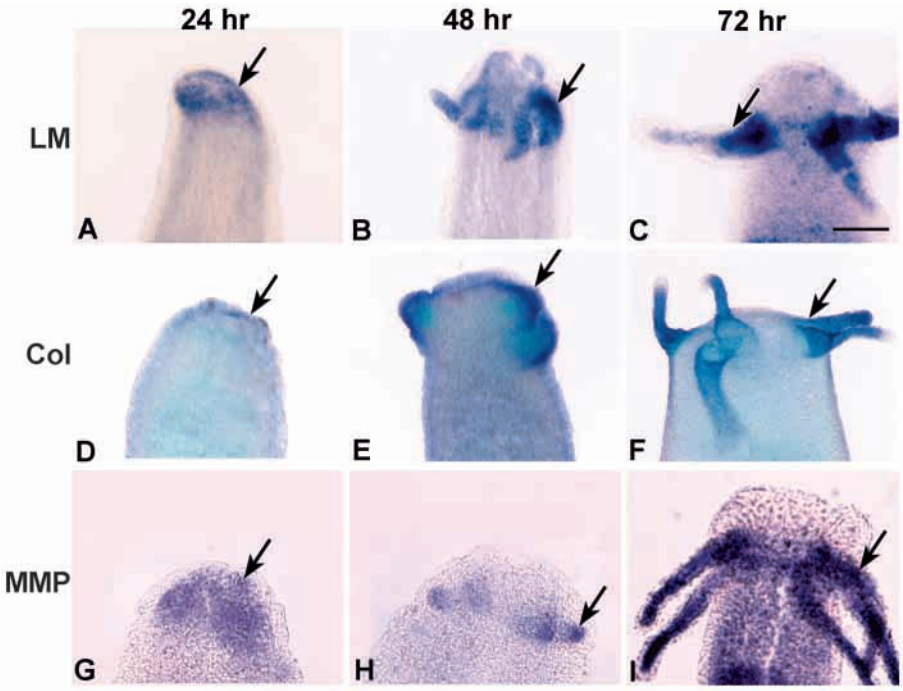

Fig. 3. Whole-mount in situ hybridization for hydra laminin β1 chain (LM), hydra type I collagen (Col), and hydra matrix metalloproteinase (MMP) mRNA monitored at 24, 48 and 72 hours after decapitation. The progression of the in situ signal (arrows) for LM, Col and MMP mRNA over this time frame changes from a general signal along the apical pole at 24 hours (A,D,G) to one associated with erupting tentacles at 48 hours (B,E,H) and 72 hours (C,F,I). Scale bar: 250 μm.

Head regeneration involves an up-regulation of ECM components as monitored at the mRNA and protein level

As an extension of previous studies, concomitant in situ whole-mount analysis of both Hcol-I (Fig. 2A) and HLM-β1 chain (Fig. 2B), indicated that both of these genes were upregulated 3 hours after decapitation. In agreement with previous observations (Leontovich et al., 2000), expression of HLM-β1 chain mRNA was associated with the endoderm (Fig. 2B), while expression of Hcol-I was associated with the ectoderm (Fig. 2A). Upregulation of ECM mRNA for both basement membrane and interstitial matrix components was confirmed as monitored by northern blot analysis for HLM-β1 chain (Fig. 2K) and Hcol-I (data not shown) of regenerating head pole segments after decapitation. Upregulation of ECM mRNA continued for 96 hours after the time of decapitation (Fig. 2K). Whole-mount immunofluorescent analysis of head regeneration after decapitation indicated that de novo biogenesis and polymerization of hydra matrix components into a newly formed ECM occurred over a period of about 48 hours (Fig. 2C-J). The basement membrane component HLM-β1 chain was not observed at the regeneration head pole 3 hours after decapitation (Fig. 2C), but was detected 7-12 hours after decapitation (Fig. 2D). By contrast, an initial (but weak) signal for the interstitial matrix component, fibrillar collagen, was initially observed about 15-24 hours after decapitation, although this is not evident at the magnification shown in Fig. 2I. A stronger and clearly discernable signal was observed between 24 and 48 hours (compare Fig. 2I and Fig. 2J). Comparative qualitative analysis of (1) the immunofluorescent signal obtained from antibody to HLM-β1 chain and Hcol-I, and (2) the Northern blot signal obtained from ^{32}P-labelled probes for these matrix components reflected a temporal disconnection between transcriptional and translational events. As shown in Fig. 2L, while mRNA for both HLM-β1 chain and Hcol-I upregulated in parallel after decapitation (reaching a maximum by about 20 hours); at the protein level, the appearance of basement membrane-associated laminin β1 chain preceded the appearance of interstitial matrix-associated fibrillar collagen about 8 hours after decapitation. The immunofluorescent signal for the HLM-β1 chain reached a relative maximum at about 16 hours, while the immunofluorescent signal for Hcol-I did not reach a relative maximum until about 48 hours.

Head regeneration involves coordinated expression of hydra ECM components and hydra MMP

Comparative in situ whole-mount analysis of HLM-β1 chain (Fig. 3A-C), Hcol-I (Fig. 3D-F) and HMMP (Fig. 3G-I) indicated a concomitant expression of all three of these gene products when monitored 24, 48 and 72 hours after decapitation. As shown in Fig. 3, 24 hours after decapitation, expression of HLM-β1 and HMMP was associated with the endoderm (Fig. 3A,G, respectively) while expression of Hcol-I was associated with the ectoderm (Fig. 3D), indicating that while hydra ECM is organized as a symmetrical structure (two peripheral basement membranes with an intervening interstitial matrix), its components are synthesized in a non-symmetrical manner. Expression of HMMP (Fig. 3G-I) was also restricted to the endoderm, as was observed with HLM-β1. By 48 hours, expression of each of these genes was associated with developing tentacles (Fig. 3B,E,H), and this continued for at least 72 hours after decapitation (Fig. 3C,F,I).

Hydra ECM components are also upregulated after simple incision of the epithelial along the body column and during grafting

In order to determine if upregulation of ECM components was restricted to head (current study) or foot regeneration (Leontovich et al., 2000), experiments were initiated to monitor these processes using intact hydra that had been surgically injured in the body column. In parallel with the spatial

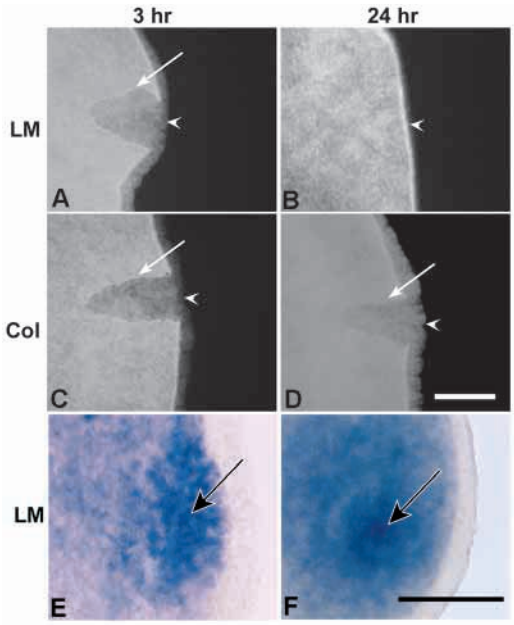

Fig. 4. Reformation of an ECM is also associated with wound healing along the body column. An incision along the body column (at 90° to the longitudinal axis) results in creation of a gap in the ECM (A,C) that is still present 3 hours after wounding, as monitored by whole-mount immunofluorescent staining for laminin (LM) and collagen (Col). The cut edge of the ECM at the wound site is indicated by the arrows in A,C. As observed during head regeneration, the epithelium has already fused by this time (arrowheads in A,C). Upregulation of mRNA occurs 3 hours after wounding (LM shown in E; a similar signal for Col and MMP also occurs, data not shown). Twenty-four hours after wounding, reformation of the ECM has occurred, as monitored with antibody to LM. At this time, a signal for Col is not apparent at this magnification. As with head regeneration, a signal for Col is more easily observed between 24 and 48 hours (data not shown). Upregulation of ECM mRNA continues for 24 hours after wound healing, as monitored for LM (F). Upregulation of Col and MMP mRNA also continues through this time (data not shown). Scale bars: in D, 100 μm for A-D; in F, 200 μm for E,F.

and temporal expression patterns observed during head regeneration, a transverse incision (90° to the head-foot axis) made in the body column resulted in a gap in the ECM (0 to at least 3 hours) after injury as monitored by immunofluorescent staining for either laminin β1 chain (Fig. 4A) or fibrillar collagen (Fig. 4C) accompanied by an upregulation of mRNA for matrix components such as HLM-β1 as monitored by whole-mount in situ analysis (Fig. 4E). A similar upregulation of mRNA for Hcol-I and HMMP was observed 3 hours after injury (data not shown). Again, in parallel with that observed during head regeneration, the appearance of the HLM-β1 chain protein was seen in the ECM at the incision site 24 hours after injury (Fig. 4B), while a signal for Hcol-I was not as easily detected at this time (Fig. 4D) [during head regeneration, an easily detectable signal for hydra fibrillar collagen is seen between 24 and 48 hours after decapitation (see Fig. 2I,J,L)]. A maximal signal for Hcol-I was observed 48 hours after injury (data not shown), as was observed during head regeneration (Fig. 2J,L).

Consistent with the simple incision studies described above, ECM biogenesis was also observed whenever grafting was initiated in hydra (Fig. 5). In some cases, the loss of an ECM induced by bisection of the body column followed by re-fusion of the two body halves resulted in a deformation (indicated by arrowheads in Fig. 5) of the epithelium at the grafting site (Fig. 5A-C). This deformation was observed as either a bulging or narrowing of the body column along the longitudinal axis. These experiments followed the procedures of Shimizu et al. (Shimizu et al., 1993) and simply involved cutting hydra into an upper and lower half, and grafting them back together using nylon fish line. The original cut edge of the ECM is marked with an arrow (stained with mAb to hydra laminin). Arrowheads mark the region in each specimen where the gap in the matrix exists and de novo biogenesis of a new ECM is occurring.

As also shown in Fig. 5, after decapitation, morphogenesis of a new hypostome and tentacles always occurred apical to the plane where the original ECM was cut (indicated by the arrows in Fig. 5D-F, mAb to hydra collagen). Biogenesis in this case involves synthesis of basement membrane components, interstitial matrix components and hydra MMP. Therefore, morphogenesis spatially correlates with that region of the epithelial bilayer where de novo biogenesis of a new ECM was occurring.

Antisense thio-oligonucleotides to hydra laminin β1 chain or to hydra matrix metalloproteinase reversibly block head regeneration and affect subsequent deposition of hydra fibrillar collagen in the newly forming ECM

To determine if inhibition of the translation of HLM-β1 chain or HMMP could cause an inhibition of head regeneration, antisense experiments used the localized electroporation (LEP) techniques previously described for hydra (Deutzmann et al., 2000; Fowler et al., 2000; Yan et al., 2000b; Zhang et al., 2001). This technique uses a drawn glass capillary tube in combination with electroporation to introduce antisense oligonucleotides into specific regions of hydra such as the head or foot pole. The technique allows introduction of oligonucleotides (or other exogenous material) into either the endodermal or ectodermal cell layer, depending on the expression pattern of any given mRNA. As shown in Table 1, a significant inhibition of head regeneration was observed with antisense thio-oligonucleotide to the coding region of HLM-β1 when compared with mismatch and sense controls. In these experiments multiple antisense thio-oligonucleotides were designed because without knowing the secondary structure of a particular mRNA, it is impossible to predict which antisense molecule will be effective in inhibiting translation (Flanagan et al., 1996; Wagner, 1994; Wagner, 1995). Therefore, for any given set of antisense oligonucleotides tested, one or more may be found to be effective. In the context of the current studies, the number of effective antisense oligonucleotides was found to differ between HLM-β1 and HMMP. Additional controls involved an attempt to rescue antisense-blocked hydra with isolated hydra laminin introduced into the regenerating head pole using a DMSO-loading procedure (Zhang and Sarras, 1994). As described in the Materials and Methods, four groups were analyzed: (1) antisense, (2) antisense + DMSO loading, (3) DMSO loading without LEP and (4) regeneration with no treatment. As described in the Materials and Methods 10 hydra

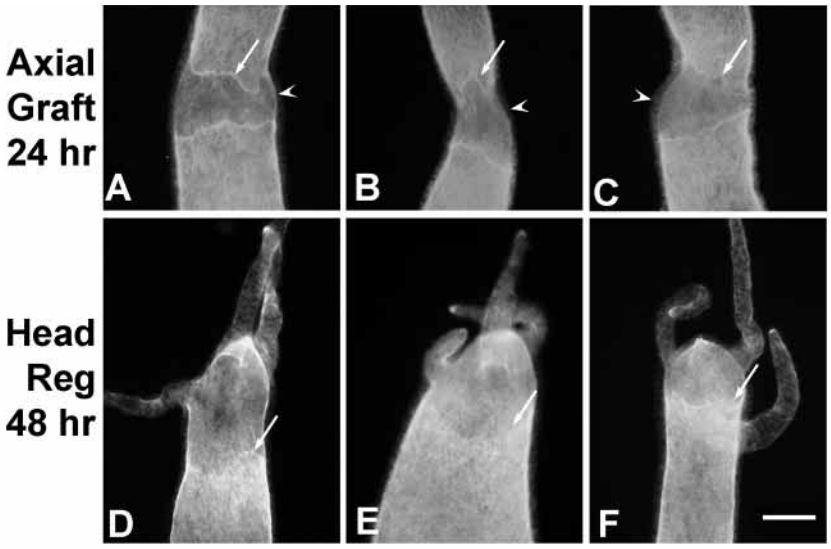

Fig. 5. Reformation of an ECM is also observed at the incision site during grafting experiments (A-C) and with morphogenesis during head regeneration (D-F), as monitored by whole-mount immunofluorescence. (A-C) Staining with mAb for hydra laminin (m52). (D-F) Staining for hydra fibrillar collagen (m39). As observed 24 hours after grafting of body segments, the region between the graft halves is the site of ECM biogenesis (region indicated by the arrowheads in A-C). The cut edges of the ECM from each graft appear as two transverse signals at the graft site (only one edge of the ECM is indicated by the arrows in A-C). The region of fusion of the epithelium at the graft site where ECM biogenesis is occurring correlates with deformation in the bilayer. This deformation may be observed as a narrowing or bulging in the body wall at the graft site (indicated by arrowheads in A-C). After decapitation, head regeneration always occurs apical to the original cut edge of the ECM (arrows in D-F), where matrix biogenesis is occurring. Scale bar: 250 μm.

were included per group and the experiment was repeated three times. As shown in Table 1, DMSO-loading of intact trimeric hydra laminin into antisense-treated animals could significantly rescue inhibition. In addition, recovery from antisense treatment was observed 5 days after the occurrence of inhibition (Table 1). As shown in the immunofluorescent images of Fig. 6, no HLM-β1 chain protein was observed 24 hours after decapitation and LEP with the coding region antisense oligonucleotide to this matrix component (Fig. 6A). A clear immunofluorescent signal for the protein was observed at 24 hours in the case of LEP, using the sense control oligonucleotide to HLM-β1 chain (Fig. 6B). Because of the temporal delay between the appearance of HLM-β1 chain and the appearance of Hcol-I in the ECM at the head pole of regenerating hydra (see Fig. 2), we also monitored the appearance of Hcol-I after LEP of antisense to HLM-β1 chain. As shown in Fig. 6, inhibition of the translation and appearance of HLM-β1 chain caused a subsequent inhibition in the appearance of Hcol-I in the ECM (Fig. 6C). Because appearance of Hcol-I lags behind that of HLM-β1, staining for hydra collagen was performed as early as 36 hours and as late as 48 hours post-LEP, to allow sufficient time for the protein to accumulate in the ECM (if it was indeed secreted after inhibition of hydra laminin). The fact that hydra fibrillar collagen was not observed in the ECM after inhibition of HLM-β1 indicates that the binding of HLM-β1 to the basal membrane of the ectoderm may play a role in this process. Such a role could involve signaling events associated with the secretion of Hcol-I from the ectodermal layer of cells or its normal processing for proper polymerization in the ECM after discharge. In parallel experiments, LEP of antisense to HMMP was also observed to cause reversible inhibition of head regeneration, when compared with mismatch and sense controls (Table 2). As with HLM-β1, recovery from HMMP antisense-induced inhibition was observed within 5 days (Table 2). We also attempted to rescue the inhibitory effect of antisense to HMMP using DMSO-loading of recombinant HMMP. As before, four groups were analyzed: (1) antisense, (2) antisense + DMSO loading, (3) DMSO loading without LEP and (4) regeneration with no treatment. As shown in Table 2, DMSO loading of an active form of recombinant HMMP could rescue inhibition. The lack of an antibody to HMMP prevented our evaluation using an immunofluorescent approach; however, the sense controls, recovery from inhibition and the ability to rescue the inhibitory effect by DMSO-loading recombinant HMMP support the specificity of the antisense experiments. The antisense experiments therefore indicate that biogenesis and polymerization of ECM during head regeneration is associated with the simultaneous expression of a matrix-degrading enzyme.

DISCUSSION

Surgical decapitation induces a loss of ECM in hydra at the incision site

Hydra is organized as a gastric tube with a mouth and tentacle ring at its head pole and a basal disc and peduncle at its foot pole. In addition, the body wall of hydra has a simple morphology that is organized as an epithelial bilayer (ectodermal and endodermal cell layer) with an intervening ECM. Biochemical and cloning studies have established that the ECM of hydra has similar matrix components to that seen in vertebrate species (Sarras and Deutzmann, 2001). As shown in the present study, hydra is unique among metazoans in that surgical excision of the head pole (or foot pole, data not shown) gives rise to a significant area of tissue not underlain by the ECM, even after the cut edges of the bilayer has fused and the wound site is sealed. In this process, the mechanisms that underlie loss of the ECM probably involve a combination of factors related to (1) the intrinsic flexibility of hydra matrix (Sarras and Deutzmann, 2001) and (2) alterations in ECM-epithelial adhesions that result in subsequent changes in the relative position of epithelial cells to the underlying ECM (Campbell, 1967). Loss of the ECM at the regenerating pole triggers rapid morphological changes in cells of the head pole that is most evident in the ectoderm cell layer.

Rapid changes in epithelial morphology after loss of the ECM at the wound site may relate to cell-ECM signaling processes

The rapid changes in the structure of cells that lose an ECM

Table 1. Effect of antisense oligonucleotides to HLM-β1 on head regeneration and rescue using isolated hydra laminin*

Antisense experiments†		
Oligonucleotide	(*n*)	Percent inhibition of head regeneration
5′ UTR (antisense)	31	0
Intiation (antisense)	33	0
Coding (antisense)	37	33 (0% at 5 days post inhibition)‡
Mismatch (antisense)	55	0
Coding (sense)	36	6
Rescue experiments§		
Oligonucleotide and treatment	(*n*)	Percent inhibition of head regeneration
Coding (antisense)	30	30
Coding (antisense) + HLM¶	30	10
DMSO loading of HLM without LEP**	30	5
Head regeneration alone††	30	5

*The conditions for LEP and initiation of head regeneration are described in the Materials and Methods. Isolated intact trimeric hydra laminin was used for the rescue experiments.

†The sequence of thio-oligonucleotides is given in the Materials and Methods.

‡The coding antisense group was statistically different from the other groups. Hydra observed to be inhibited at 72 hours in the coding group were monitored for an additional 5 days and were found to recover from inhibition and to complete head regeneration.

§The conditions for rescue are described in the Materials and Methods. Only the antisense coding oligonucleotide was tested for recovery based on the results shown above.

¶DMSO loading of isolated intact trimeric hydra laminin (HLM) after LEP introduction of the antisense-coding oligonucleotide.

**Hydra were DMSO loaded with HML but no LEP was performed.

††Hydra were decapitated and monitored for head regeneration with no further treatment conditions.

association (from a high to a low cuboidal morphology in the case of the ectoderm) may be related to signaling processes involving tensegrity mechanisms as previously discussed by Ingber and associates (Chen et al., 1997; Ingber, 1997; Wang et al., 1993). Tensegrity mechanisms involve structures that are mechanically stable not because of the strength of individual members, but because of the way the entire structure distributes and balances mechanical stresses. In the case of cell-ECM interactions, these structural balances involve the inter-relation of the ECM, plasma membrane ECM receptors and cytoskeletal systems within the cell that are linked to the ECM via cell surface receptor systems (Chen et al., 1997). Such alterations in cell morphology resulting from the presence or absence of an ECM have commonly been reported for cells under in vitro conditions (e.g. cell culture studies) (Madden and Sarras, 1988), but have been less frequently reported for cells under in vivo conditions. One notable exception to this is the process of wound healing in mammals. As recently reviewed (Nedelec et al., 2000), an incision to the skin results in a migration of fibroblasts through the ECM at the site of the wound. Subsequent wound healing involves re-epithelialization and reformation of an intact basement membrane associated with the reformed epidermis. With regard to the epithelial components, this mimics what is observed in hydra and suggests that these processes have been highly conserved during evolution.

ECM biogenesis in hydra involves sequential upregulation of ECM components that appears to involve both cell-cell and cell-ECM signaling events

Within 3 hours of decapitation, when the ECM is no longer in contact with the head pole bilayer, epithelial cells of the ectoderm and endoderm layer upregulate genes for ECM components of both the basement membrane (HLM-β1) and interstitial matrix (Hcol-I). The spatial and temporal pattern for expression of ECM components in hydra is more complicated than originally envisioned. While the symmetrical organization of hydra ECM [two peripheral subepithelial-associated basement membranes and one central interstitial matrix (Sarras and Deutzmann, 2001)] might suggest that both epithelial layers would be involved with secretion of all basement membrane components, it is clear from this study that at least one basement membrane components, HLM-β1, is produced solely by the endoderm, while at least one interstitial matrix component, Hcol-I, is produced solely by the ectoderm. Like Hcol-I, hydra collagen type IV is also produced by the ectoderm (Fowler et al., 2000), but lack of an antibody to this matrix component has prevented our ability to assign it to any particular region within hydra ECM. Based on other vertebrate and invertebrate systems, however, we propose that collagen type IV protein is localized to both epithelial-associated basement membranes in hydra ECM. Although not anticipated, hydra matrix metalloproteinase (HMMP) is also expressed during ECM biogenesis and as with laminin, is produced by the endoderm. The temporal pattern observed for the secretion of matrix components implies that a coordinated cross-epithelial signaling process is occurring during the biogenesis of hydra ECM. This cross-signaling would occur in two phases. The first phase would occur immediately after decapitation when the ECM retracts, and the ectoderm and endoderm directly contact one another along their basal plasma membrane surfaces. The combination of ECM loss and the subsequent contact of the basal plasma membrane surfaces of the bilayer results in an upregulation of mRNA for matrix components and HMMP 3 hours after decapitation. In the second phase, laminin is secreted from the endoderm into the inter-basal plasma membrane compartment of the bilayer. Laminin seeds to the basal plasma membrane of both the ectoderm and endoderm. This directly supports the work of Colognato and Yurchenco (Colognato and Yurchenco, 2000), who have proposed that similar laminin seeding processes occur in mammalian systems. Binding of laminin to the bilayer then stimulates ectodermal cells (Deutzmann et al., 2000) to begin to secrete Hcol-I (between 15 and 24 hours after decapitation) that then polymerizes in the central fibrous zone of hydra ECM. This polymerizing interstitial matrix is seen as an easily detectable fluorescent signal between 24 and 48 hours. An alternative explanation is that the delay in the appearance of Hcol-I relates to some changes in the normal processing of Hcol-I after its secretion and this altered processing prevents recognition by our monoclonal antibody to Hcol-I. This seems unlikely, however, because use of a polyclonal antibody raised to mature Hcol-I also gives the same results (data not shown). In addition, a causal relationship between laminin binding and the discharge of Hcol-I follows from the antisense studies described in the current study. These studies clearly indicated that inhibition of the discharge of laminin resulted in the lack of appearance of Hcol-I at the site

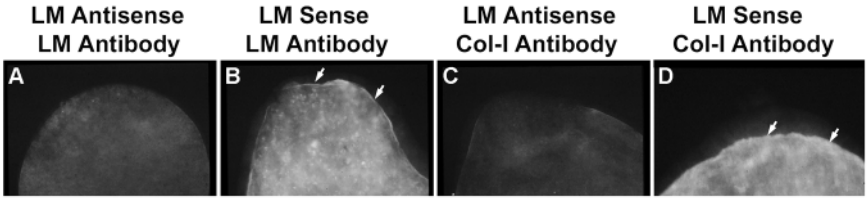

Fig. 6. Localized electroporation (LEP) of antisense thio-oligonucleotides to hydra laminin β1 chain (LM) or hydra matrix metalloproteinase (MMP) into the apical pole of decapitated polyps results in a reversible inhibition of head regeneration. As monitored by whole-mount immunofluorescence, antisense oligos to LM blocked the appearance of laminin protein at the apical pole at 24 hours after LEP and decapitation (A). Sense oligos did not block the appearance of LM protein at the regenerating pole (B, arrows indicate reforming ECM). Antisense oligos to LM also blocks the appearance of hydra type I collagen (Col-I), as monitored as early as 36 hours and as late as 48 hours after decapitation with antibody to Col-I (C). Sense oligos to LM had no effect on the appearance of Col-I (D, arrows indicate reforming ECM). The area between the two arrows in B and D represents 100 μm.

of ECM formation. Previous studies (Agbas and Sarras, 1994; Sarras et al., 1994), as well as more recent studies (X. Z. and M. P. S., unpublished) have shown that hydra has ECM receptors for laminin and some of these receptors appear to be of the integrin class. Taken in total, these studies indicate that while the body wall of hydra is structurally reduced to an epithelial bilayer with an intervening ECM, matrix biogenesis by this bilayer is complicated and involves signaling events between both the ectoderm and endoderm. These signaling events function to coordinate the expression and final polymerization of both basement membrane and interstitial matrix components and also involve the simultaneous expression of matrix metalloproteinases.

ECM biogenesis is induced whenever epithelium repair is initiated along the body column

The sequence of events after excision of the head pole (i.e. fusion of the epithelium, retraction of the ECM resulting in an epithelial bilayer with no underlying matrix, shape changes of epithelial cells and subsequent biosynthesis of a new ECM), is not unique to regeneration processes in hydra. Epithelial repair experiments in which a transverse incision of the body column was inflicted, resulted in the same ECM-associated events observed during head regeneration: retraction of the ECM at the incision site and subsequent up-regulation of basement membrane components, interstitial matrix components and hydra matrix metalloproteinase. This coincidence of cell-ECM events strongly suggests that the de-novo biogenesis of a matrix after injury to the epithelium is a fundamental process of the bilayer that is not unique to head or foot regeneration. As shown in Fig. 5, grafting of hydra also induces ECM biogenesis. In some cases, grafting results in abnormalities in the cylindrical shape of the body column. Like decapitation, surgical bisection of hydra results in a loss of the ECM at the graft site followed by a de novo biogenesis of a new matrix between the two grafted halves. The shape abnormalities induced in the body wall in some grafts could result from a number of variables such as poor adhesion of the two cut epithelial surfaces (Shimizu and Sawada, 1987), in combination with a loss of the ECM at the time of grafting. Because all grafts have a retraction and reformation of the ECM, but not all grafts show deformation of the body column, it is not likely that cell-ECM interactions are the sole factor contributing to body abnormalities after grafting. These studies do indicate, however, that ECM biogenesis does occur whenever grafting is performed and therefore cell-ECM interactions are one of a number of factors that must be considered when evaluating such body shape abnormalities.

It should be noted that a certain degree of ECM biogenesis and turnover is always occurring along the body column of hydra. This has been shown for laminin (Leontovich et al., 2000), fibrillar collagen (Deutzmann et al., 2000), type IV collagen (Fowler et al., 2000) and hydra matrix metalloproteinase (Leontovich et al., 2000). Levels of ECM biogenesis and turnover vary along the longitudinal axis. Higher levels are associated with positions of cell transdifferentiation (e.g. base of tentacles and basal disc region). Therefore, when we indicate that incision of the epithelium induces ECM biogenesis to occur, this is a relative statement and means that incision induces a significant increase in the expression of ECM components over the normal background levels. This increase is clearly related to the fact that incision results in a loss of ECM at the wound site. Loss of an ECM then results in a complete de novo biogenesis of matrix components.

Epithelial morphogenesis in hydra is dependent on ECM biogenesis as monitored during head regeneration and epithelial repair after surgical incision of the bilayer

Previous studies with hydra cell aggregates (morphogenesis of intact hydra from a pellet formed from dissociated hydra cells) have shown that reagents that perturb cell-ECM interactions, such as antibodies to matrix components or fragments of matrix components, can block epithelial morphogenesis (Sarras et al., 1993; Zhang et al., 1994). The current study extends these earlier studies by showing that cell-ECM interactions are inherent to a wide variety of morphogenetic processes in hydra, including simple incision of the body column. In the context of this study, we use the term epithelial morphogenesis in a broad sense to include a spectrum of developmental processes such as cell differentiation, cell shape and the establishment of three-dimensional form, etc. Cell-ECM interactions may affect one or more of these processes and our studies do not point to any one of these processes. As shown in Fig. 5, the region of the epithelial bilayer undergoing head morphogenesis is always located apical to the original cut edge of the ECM. Therefore, head morphogenesis in hydra always involves de novo biogenesis and polymerization of ECM. This is consistent with that reported for other epithelial systems, such as the pulmonary system, salivary glands, the mammary gland and renal system of vertebrates; however, these later studies used organ cultures (Relan and Schuger, 1999), while the current studies with hydra represent in vivo conditions. This further supports the idea that ECM biogenesis is closely coupled to epithelial morphogenesis and that this fundamental relationship has been maintained throughout evolution.

In hydra, the importance of ECM biogenesis to head morphogenesis is functionally confirmed by antisense experiments that involved inhibition of the translation of the HLM-β1 chain. Recent studies (Deutzmann et al., 2000) indicate that inhibition of the translation of an interstitial matrix component such as Hcol-I can also inhibit head regeneration. Additionally, Fowler et al. (Fowler et al., 2000) have indicated that inhibition of hydra collagen type IV (localization in the hydra ECM unknown) also causes inhibition of head regeneration. Therefore, inhibition in the translation of either basement membrane or interstitial matrix components will lead to inhibition in head regeneration. These cell-ECM interactions are likely multifaceted and involve (1) the role of ECM as an extracellular structural entity whose assembly and presence affects the three-dimensional shape of tissues; (2) the role of ECM as a polymerized network of macromolecules that have endogenous signaling sequences such as RGD or YIGSR (either open or cryptic) that can interact with cell surface matrix receptors; and (3) the role of ECM as a scaffolding for the attachment of signaling molecules such as growth factors, small peptides or other signaling compounds. There is evidence for each of these processes occurring in hydra (Sarras and Deutzmann, 2001). In these studies, inhibition of the translation of laminin is likely to initially affect head morphogenesis because of perturbations in the normal polymerization of the basal lamina associated with the ectoderm and endoderm. Parallel studies (X. Z. and M. P. S., unpublished) indicate that inhibition of the hydra laminin α chain also results in an inhibition of head regeneration. The fact that these two convergent studies yield the same result strengthens the proposal that laminin biosynthesis is essential for normal head regeneration to occur. As discussed previously, laminin has also been reported to be a seeding molecule that promotes basement membrane assembly (Colgnato and Yurchenco, 2000). The lack of incorporation of laminin into the polymerizing matrix would be expected to have profound effects on the basic structure of the ECM that would in turn affect the overlying epithelium. Structural changes in hydra ECM have been observed at the ultrastructural level when matrix polymerization has been perturbed (Zhang et al., 1994). In addition, the lack of incorporation of laminin into the ECM would prevent the presentation of cell binding domains to epithelial cells such as the FTGTQ sequence of the laminin β1 chain. This sequence has been shown to be important for cell-ECM interactions in hydra (Sarras et al., 1994) and recent studies have shown that it can bind to an integrin-class protein in hydra (X. Z. and M. P. S., unpublished). The absence of this sequence could potentially prevent epithelial signaling cascades that normally occur during head regeneration. As discussed in the section on ECM biogenesis, it should be noted that that inhibition of the translation of laminin also prevents subsequent incorporation of hydra fibrillar collagen into the ECM. Therefore inhibition of the translation of laminin would have the added effect of further perturbing ECM structure by affecting interstitial matrix assembly. We do not know if the incorporation of hydra type IV collagen in ECM is also affected in this case. Besides being an important structural component of the hydra ECM (Zhang et al., 1994), hydra type IV collagen is known to contain RGD sequences (Fowler et al., 2000); however, it is not known if they are involved in cell signaling events during regeneration. Finally, it should be pointed out that biogenesis of ECM is not restricted to regeneration and epithelial repair events in hydra. In situ studies during bud formation indicate that upregulation of ECM components and HMMP also occurs (data not shown). This is of interest, because in the case of budding no loss of ECM occurs before the time of bud emergence. Rather, the ECM is continuous at the sites of bud formation and what occurs is simply an increase in the expression of HLM-β1, Hcol-I and HMMP as evagination of the bud progresses. In situ analysis at the earliest times of bud formation (placode stage), before evagination of the bud occurs, indicates that upregulation of at least HMMP has already occurred. High expression of both basement membrane and interstitial matrix components occurs throughout all stages of bud formation.

Table 2. Effect of antisense oligonucleotides to HMMP on head regeneration and rescue using recombinant hydra MMP*

Antisense experiments†		
Oligonucleotide	(*n*)	Percent inhibition of head regeneration
5′ UTR (antisense)	77	5
Intiation (antisense)	32	16
Coding (antisense)	61	10
3′UTR (antisense)	107	21 (2% at 5 days post inhibition)‡
Mismatch (antisense)	53	0
3′UTR (sense)	18	5
Rescue experiments§		
Oligonucleotide or treatment	(*n*)	Percent inhibition of head regeneration
3′UTR (antisense)	30	25
3′UTR (antisense) +HMMP¶	30	5
DMSO loading of HMMP without LEP**	30	3
Head regeneration alone††	30	5

*The conditions for LEP and initiation of head regeneration are described in the Materials and Methods. Recombinant and refolded HMMP (active form) was used for the rescue experiments.

†The sequence of thio-oligonucleotides is given in the Materials and Methods.

‡The 3′ UTR antisense group was statistically different from the other groups. Hydra observed to be inhibited at 72 hours in the 3′ UTR group were monitored for an additional 5 days and were found to recover from inhibition and complete head regeneration.

§The conditions for rescue are described in the Materials and Methods. Only the antisense 3′ UTR oligonucleotide was tested for recovery based on the results shown above.

¶DMSO loading of recombinant and re-folded (active form) hydra matrix metalloproteinase (HMMP) after LEP introduction of the antisense 3′ UTR oligonucleotide.

**Hydra were DMSO loaded with HMMP but no LEP was performed.

††Hydra were decapitated and monitored for head regeneration with no further treatment conditions.

De novo biogenesis of ECM in hydra is functionally coupled to the expression of matrix degrading enzymes (HMMP)

Interestingly, ECM biogenesis in hydra is always accompanied by the expression of hydra matrix metalloproteinase (HMMP). Antisense studies indicate that inhibition of the translation of this proteinase also results in an inhibition of head regeneration. HMMP has been shown to degrade a broad

spectrum of hydra ECM components (Leontovich et al., 2000) and therefore its upregulation during ECM biosynthetic events raises the obvious question of why a matrix degrading enzyme should be expressed at time when formation of an intact ECM is occurring. Insight into the answer may come from the recent finding that HMMP activity is required for foot regeneration and maintenance of the differentiated state of basal disc cells of the foot process (Leontovich et al., 2000). As discussed by these investigators, HMMP may function at multiple levels: (1) to assist in the assembly of hydra ECM, (2) in the exposure of cryptic ECM signaling sites (e.g. RGD-like sequences) or (3) in the exposure of ECM-associated latent growth factor-like molecules that are involved in signaling pathways during head morphogenesis and differentiation (Leontovich et al., 2000; Yan et al., 1995; Yan et al., 2000a). Thus, the function of HMMP could be more complex than originally suggested by its name. Further studies are now under way to elucidate the mechanisms that underlie cell-ECM signal transduction during regeneration and cell differentiation in hydra.

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