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# Developmental Regulation of a Matrix Metalloproteinase during Regeneration of Axolotl Appendages

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**Removal of specific extracellular matrix (ECM) components has been implicated in the initiation of salamander limb regeneration. Remodeling of the ECM at the distal stump is necessary for the release of cells that eventually contribute to the blastema. Several matrix metalloproteinases (MMPs) have been well characterized as important to various physiological and pathological processes, such as bone remodeling and tumor invasion. The goal of this study is to identify and characterize MMPs that modulate the ECM during appendage regeneration in the axolotl *Ambystoma mexicanum*. By analyzing axolotl tissue extracts using gelatin-substrate gels, we have identified a 90-kDa gelatinase/collagenase that is upregulated within hours after limb amputation. This gelatinase shows a dramatic elevation in activity during the dedifferentiation and blastema stages. Its activity declines by the palette stage and returns to its basal level by the digit stages. The increase in activity of the 90-kDa gelatinase in response to amputation is independent of the nerve supply and the wound epithelium but these factors affect its subsequent downregulation. In addition to the blastema, the 90-kDa gelatinase can be detected in the stump at least 4 mm proximal to the regenerate. Similar regulation of the 90-kDa gelatinolytic activity is observed during tail regeneration and flank wound healing. We suggest that this 90-kDa gelatinase/collagenase may play a role in the initiation and rapid growth phase of axolotl regeneration and wound healing.** © 1994 Academic Press, Inc.

## INTRODUCTION

Among urodele amphibians such as the axolotl (*Ambystoma mexicanum*), appendage regeneration can occur throughout life. Wound healing is complete within a few hours of amputation, and within a few days, cells have been mobilized from their connective tissue matrices and migrate toward the center of the amputation plane (Gardiner *et al.*, 1986). Cell division increases during the time that cells begin to contact normally distant neighbors, leading to the accumulation of the blastema (Kelly and Tassava, 1973). The events that take place immediately after amputation and that lead to the release of connective tissue cells from the matrix that surrounds them clearly create the conditions that enable the regeneration cascade to be initiated.

Evidence that axolotl limb cells produce matrix-degrading enzymes was obtained in a previous study (Groell *et al.*, 1993). In that study, it was discovered that blastema cells are difficult to culture as either monolayers or micromasses because they rapidly digest the matrix on which they are plated. This leads to loss of attachment to the substratum and release of the cells into the medium. Similar results are seen when axolotl limb bud cells are cultured (Gardiner and Bryant, unpublished), suggesting that this is an intrinsic property of axolotl limb cells, not just those engaged in regeneration. Since limb bud cells from other species (e.g., chick and mouse) do not show this enzymatic activity *in vitro*, it is conceivable that the unique matrix-degrading activity of axolotl cells is related to their unique ability to regenerate missing appendages.

Further evidence of matrix degradation during regeneration comes from studies of the distribution of extracellular matrix (ECM) components such as collagen, fibronectin, laminin, hyaluronic acid, and tenascin (Toole and Gross, 1971; Gulati *et al.*, 1983; Repesh *et al.*, 1982; Mescher and Munaim, 1986; Onda *et al.*, 1990, 1991; Arsanto *et al.*, 1990). In some studies it has been observed that ECM molecules are removed early in regeneration (Johnson and Schmidt, 1974; Grillo *et al.*, 1968; Mailman and Dresden, 1976; Yang *et al.*, 1992).

Matrix-degrading enzymes have been implicated in various physiological and pathological events in animals and humans. One family of such enzymes are matrix metalloproteinases (MMPs), consisting of gelatinases, collagenases, and stromelysins (Nagase *et al.*, 1992). MMPs have been shown to function in ECM remodeling during development, and they are expressed in concert with other proteinases. Their expression and activity are affected by oncogenes, cytokines, and specific inhibi-

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tors called tissue inhibitors of metalloproteinases (TIMPs) (Brenner *et al.*, 1989; Corcoran *et al.*, 1992; Alexander and Werb, 1989).

Although it has been shown that blastema cells are capable of matrix degradation (Groell *et al.*, 1993), the identity of the collagenolytic enzyme(s) is yet to be determined. The goal of the present study is to identify and characterize MMPs that are active during appendage regeneration in axolotls.

## MATERIALS AND METHODS

### *Surgical Procedures*

Axolotl (*A. mexicanum*) larvae were either spawned at UCI or were obtained from the Indiana University axolotl colony and were grown to 60–150 mm snout-tail tip length. The animals were maintained at 25°C and were fed Salmon pellets (Rangen Inc.) three times a week. All operations were performed while animals were anesthetized with neutralized 1:1000 MS222 (ethyl *m*-aminobenzoate methanesulfonate; Sigma). Axolotl limbs were amputated through the zeugopodia and were allowed to regenerate to various times and stages: 3.5 hr, one day (dedifferentiation), medium bud, palette, and digit stages (Tank *et al.*, 1976). Axolotl tails were amputated so as to remove two-thirds of the tail. The tails were allowed to regenerate for 1, 7, 14, or 21 days after amputation. Unless otherwise noted, regenerates were collected along with 1–2 mm of stump and extracted as described below. Limb and tail tissues removed by the initial amputation were used as unamputated control samples.

To investigate the involvement of more proximal tissues in the enzymatic response, limbs that had regenerated to the medium bud stage were removed at the shoulder. The blastema (region A) was separated from the stump, and the stump was divided further into three segments; distal zeugopod (region B; 2 mm region proximal to the base of the regenerate), proximal zeugopod (region C; region 2–4 mm proximal to the base of the regenerate), stylopod (region D; 4–8 mm region proximal to the regenerate).

### *Denervation of Amputated Limbs*

To examine the effects of nerve supply on the activity of collagenases during limb regeneration, we amputated right and left forelimbs through the mid radius/ulna, and then denervated the right limbs one day later by transecting the 3rd, 4th, and 5th spinal nerves at the brachial plexus (Singer, 1974). The left limbs served as innervated controls. Both forelimbs were collected for extraction and zymographic analysis when the control innervated limbs had regenerated to medium bud or palette stages.

### *Amputated Limbs Inserted into the Body Cavity*

To test the possible role of the wound epithelium in MMP regulation, we prevented wound epithelium formation as described by Goss (1956). Forelimbs were amputated through the zeugopodium, the protruding tips of the radius and ulna were trimmed level, and the limbs were skinned to the elbow. The amputated end of each right limb was then inserted into the body cavity through a slit cut through the skin and peritoneum. The limbs were secured in place by a single suture at the insertion site. Only limbs that remained inserted were sampled. The left limbs were not inserted and served as regenerating controls. Both forelimbs were collected for extraction when the control limbs had regenerated to medium bud or palette stages.

### *Flank Wounds*

In order to investigate whether collagenases function during wound healing, we extracted proteins from tissues during flank wound healing for zymography. Flank wounds were created by removing the skin in a 1-$cm^2$ area on one side of the animal. The musculature exposed by this procedure was then teased with sharp watchmakers' forceps. Flank wounds were allowed to heal for 4 hr, 1 day, 7 days, 14 days, and 21 days before the full thickness of the injured flank plus the adjacent (2 mm) flank tissues were sampled for extraction and analysis as described below. The flank tissue on the opposite side of the axolotl was sampled as uninjured control.

### *Extraction and Zymography of MMPs from Axolotl Tissues*

MMPs from axolotl tissues were extracted and analyzed using a protocol derived from Talhouk *et al.* (1991). After collection, tissues were frozen and pulverized in liquid $N_2$. The powder was suspended 1:5 (w/v) in 500 m*M* Tris-HCl, pH 7.6, containing 1% Triton X-100, 200 m*M* NaCl, and 10 m*M* $CaCl_2$. The suspension was frozen and thawed four times. After centrifugation, the supernatant (crude extract) was mixed with SDS-sample buffer (Laemmli, 1970) and the proteins were electrophoretically separated under nonreducing conditions in 6% polyacrylamide gels impregnated with 1.5 mg/ml gelatin. Equal amounts of protein were loaded into each lane as measured using the DC Protein Assay kit (Bio-Rad). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 and incubated in 50 m*M* Tris-HCl, pH 8.0 containing 5 m*M* $CaCl_2$, 0.02% $NaN_3$ (incubation buffer) overnight at 30°C. Bands containing collagen-

ases appear as clear bands in a dark blue background after staining with Coomassie brilliant blue R-250 (Bio-Rad). Zymograms were densitometrically analyzed using the SCAN analysis software (Biosoft) in order to demonstrate the relative changes in the level of the 90-kDa gelatinolytic activity during regeneration.

Some samples were treated with the organomercurial compound *p*-aminophenylmercuric acid (1 m*M;* APMA) at 37°C for 2 hr prior to zymographic analysis to distinguish the proenzyme from the active enzyme (Stetter-Stevenson *et al.*, 1989). Samples treated with distilled water alone at 37°C were used as negative controls.

To determine whether the enzymatic activity of interest is indeed an MMP, an inhibitor test was performed on the crude extracts. After electrophoretic separation of proteins as above, gels were incubated in incubation buffer plus 1,10-phenanthroline (10 m*M*), EDTA (10 m*M*), phenylmethylsulfonyl fluoride (PMSF; 10 m*M*), leupeptin (10 μg/ml), or pepstatin A (10 μg/ml) overnight at 30°C. All inhibitors were purchased from Sigma.

## RESULTS

### *MMPs during Limb Regeneration*

During regeneration, we can distinguish the presence of five separate gelatinases/collagenases with sizes of 90, 73, 60, 55, and 52 kDa (Fig. 1a). The same five bands can also be detected in unamputated limbs, but only at extremely low levels. The difference in enzyme activity in regenerating versus unamputated limbs is such that the two cannot be analyzed on the same gels, due to the disparity in incubation times needed for visualization. Although all are elevated in regenerating limbs in comparison to unamputated limbs, the change is most marked in the 90-kDa gelatinase. For this reason, we focus on the 90-kDa gelatinolytic activity during regeneration.

An increase in the activity of the 90-kDa gelatinase was seen as early as 3.5 hr after amputation (Fig. 1a; lane 3.5h). Densitometric analysis of the zymogram demonstrated a 2.6-fold increase in 90-kDa gelatinolytic activity in the 3.5-hr regenerate compared to unamputated limbs. Its activity is dramatically elevated 9-fold above basal level 1 day after amputation, when stump tissues are beginning to dedifferentiate prior to the formation of a blastema. At medium bud, activity of the 90-kDa gelatinase increased by approximately 10.3-fold above basal level (Fig. 1a; lane MB). By the palette stage (Fig. 1a, lane PAL), when proximal regions of the regenerate are redifferentiating, activity has declined in comparison to the medium bud stage. There is a 3.2-fold higher 90-kDa gelatinolytic activity in at medium bud stage than at palette stage, with activity at palette stage only 3.18-fold above that found in unamputated limbs. By the digit stages (Fig. 1a; lanes ED and LD), when the regenerate is almost fully differentiated, the activity of the 90-kDa enzyme has almost returned to its basal level.

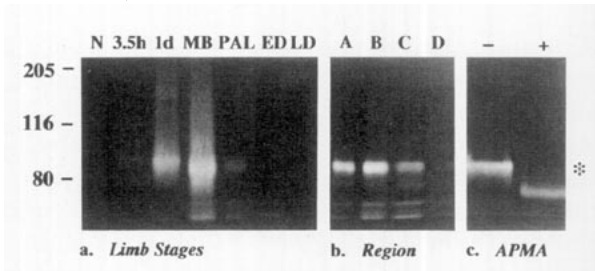


FIG. 1. Identification by zymography of gelatinolytic/collagenolytic enzymes in samples during axolotl limb regeneration. (a) 90-, 73-, 60-, 55-, and 52-kDa gelatinolytic enzymes are detectable at very low levels in unamputated limbs. In this gel, loaded with 50 μg total protein per lane, incubation times (overnight at 30°C) were too brief to clearly show the bands in unamputated limbs (N). By 3.5 hr after amputation (3.5h), the level of the 90-kDa gelatinase is significantly elevated over that in unamputated limbs. The level of the 90-kDa gelatinase further increases at 1 day after amputation (1d) and is most abundant at medium bud stage (MB). A decrease in the level of the 90-kDa enzyme is apparent by the palette stage (PAL) and returns close to the level prior to amputation by the digit stages (ED) and in the complete regenerate (LD). The four smaller gelatinases increase slightly in parallel with the 90-kDa gelatinase before decreasing during redifferentiation. (b) Zymographic analysis of segments along the length of a medium bud stage regenerating limb. Each lane contains 65 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. In the medium bud stage regenerating limb, the stump region within 2 mm from the regenerate showed the highest 90-kDa gelatinolytic activity (lane B). The regenerate itself (lane A) has almost as high a level of 90-kDa gelatinolytic activity. The region of the stump 2–4 mm from the regenerate (lane C) has elevated levels of 90-kDa gelatinolytic activity, but significantly lower levels than in the region closest to the amputation level. Extracts from the stylopodium (lane D) show a level of 90-kDa gelatinolytic activity that is not markedly different from unamputated limbs. This suggests that elevation of 90-kDa gelatinolytic activity is restricted to the regenerate and regions of the stump nearest the amputation level. (c) Determination of latent proenzyme(s) from active enzyme(s). Extracts from axolotl limbs 1 day after amputation were treated either with 1 m*M* APMA or an equal volume of distilled $H_2O$ for 2 hr at 37°C prior to zymography. Each lane contains 22 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. Treatment with APMA causes the activation and autoproteolytic cleavage of the N-terminal 80–84 amino acids from the latent proenzyme to convert it to the smaller, active enzyme. Incubation at 37°C for 2 hr alone (APMA–) does not cause autoactivation of the 90-kDa gelatinase. After treatment with 1 m*M* APMA (APMA+) the 90-kDa gelatinase completely disappears, while the activity at 72 kDa becomes intensified, suggesting that the former is the proenzyme (progelatinase) of the latter. The position of the 90-kDa gelatinase is marked by the asterisk. Molecular weight standards are indicated along the left margin.

### *Localization of 90-kDa Gelatinolytic Activity in the Regenerating Limb*

We examined 90-kDa gelatinolytic activity in segments along the length of the regenerating limb to de-

termine the location of the activity within the limb. Since the medium bud regenerate exhibited the highest 90-kDa gelatinolytic activity, we studied the localization of the 90-kDa gelatinase at this stage of regeneration (Fig. 1b). Of the different stump regions examined, the 2-mm section closest to the blastema (distal zeugopod) was found to have the highest activity (Fig. 1b; lane B). The blastema has the next highest activity (Fig. 1b; lane A), followed by the region of the stump 2–4 mm from the regenerate (proximal zeugopod) (Fig. 1b; lane C). The stylopodial region, 4–8 mm from the regenerate, does not exhibit elevated 90-kDa gelatinolytic activity (Fig. 1b; lane D). Densitometric analysis showed that the blastema has 2-fold, the distal zeugopod has 2.4-fold, and the proximal zeugopod has 1.7-fold more gelatinolytic activity than the stylopodial region. These results show that 90-kDa gelatinolytic activity is elevated in the regenerate and in the adjacent stump tissue in the region undergoing dedifferentiation (2–4 mm proximal to the amputation plane).

*Characteristics*

To determine whether the 90-kDa gelatinase possesses characteristics of an MMP (Nagase *et al.*, 1992), we examined the effects of APMA and various protease inhibitors.

APMA is an organomercurial compound that activates the latent form of MMPs by causing the autocatalytic removal of an 80- to 85-amino-acid propeptide (Stetler-Stevenson *et al.*, 1989; Okada *et al.*, 1992). Treatment of extracts from medium bud stage regenerates with 1 m*M* APMA at 37°C for 1 hr caused the disappearance of the activity at 90 kDa and an enhancement of activity at 72 kDa (Fig. 1c). Incubation at 37°C alone did not have this effect. We interpret this change in response to APMA to be a conversion of a latent proenzyme to a smaller, active form.

The activity of MMPs is inhibited by the chelators 1, 10-phenanthroline and EDTA but not by PMSF, leupeptin, or pepstatin A (see Nagase *et al.*, 1992). Incubation of gels in incubation buffer plus 10 m*M* 1, 10-phenanthroline or 10 m*M* EDTA inhibited the activity of the 90-kDa gelatinase. The protease inhibitors PMSF, leupeptin, or pepstatin A did not inhibit the 90-kDa gelatinolytic activity in these extracts (Table 1).

TABLE 1
INHIBITION TESTS ON CRUDE EXTRACTS FROM MEDIUM BUD REGENERATES

| Inhibitor | Inhibition |
|---|---|
| 10 m*M* 1,10-phenanthroline | + |
| 10 m*M* EDTA | + |
| 10 m*M* PMSF | − |
| 10 μg/ml leupeptin | − |
| 10 μg/ml pepstatin A | − |

*Denervation Effects on the Level of the 90-kDa Gelatinase during Limb Regeneration*

Urodele limb regeneration is known to require innervation (Singer, 1974). Without nerves, the early stages of regeneration are aborted. The level of activity of the 90-kDa gelatinase in denervated, amputated limbs (Fig. 2a; lane MB−), which are not regenerating, is indistinguishable by densitometric analysis from that of the nondenervated, medium bud control limbs (Fig. 2a; lane MB+). Hence, nonregenerating, denervated limbs have a markedly higher activity than unamputated control limbs. When control regenerating limbs reach the palette stage, 90-kDa gelatinolytic activity has almost returned to basal levels (Fig. 2a; lane PAL+). However, the contralateral denervated, amputated limb, which is not regenerating, continues to show high levels of activity (Fig. 2a; lane PAL−). Densitometric analysis revealed that innervated palette stage limbs had 1.5-fold less 90-kDa gelatinolytic activity than the contralateral denervated limbs. Denervation alone, without amputation, does not lead to elevation of 90-kDa gelatinolytic activity (data not shown).

*Wound Epithelial Effects on the Level of the 90-kDa Gelatinase during Limb Regeneration*

Limb regeneration does not occur in the absence of a wound epithelium (Carlson, 1974; Thornton, 1968; Singer and Salpeter, 1961). In order to examine the relationship between regulation of the 90-kDa gelatinolytic activity and the wound epithelium, we examined the level of 90-kDa gelatinolytic activity in amputated limbs that were prevented from forming a wound epithelium. This was achieved by insertion of the amputated limbs into the peritoneal cavity immediately after amputation (Goss, 1956). When the control limb reached medium bud, the inserted limb was removed from the body cavity and examined to ensure the absence of wound epithelium and then harvested. Densitometric analysis did not show any marked difference between medium bud regenerates (Fig. 2b; lane MB+) and the contralateral inserted, nonregenerating limb (Fig. 2b; lane MB−), indicating that the upregulation of 90-kDa gelatinolytic activity is independent of the wound epithelium. However, in the absence of a wound epithelium, the level of 90-kDa gelatinolytic activity is not downregulated to the same extent as in innervated limbs. As shown in Fig. 2b, the level of 90-kDa gelatinolytic activity in the inserted, nonregenerating limb (lane PAL−) is still 1.5-fold

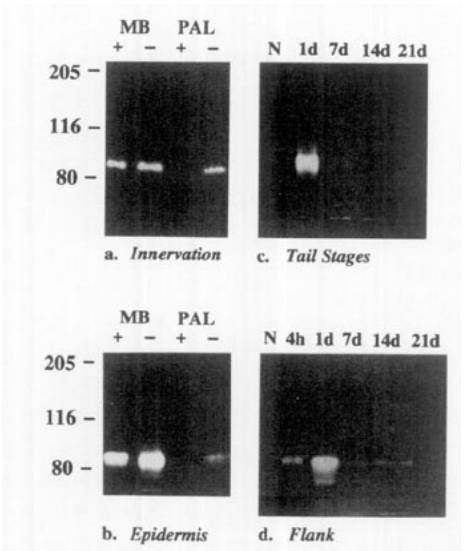


FIG. 2. (a) Zymographic analysis of denervated, amputated limbs. Each lane contains 36 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. The increase in activity of the 90-kDa gelatinase in response to amputation is independent of the nerve supply but prolonged denervation prevents its subsequent downregulation. Lanes: MB+, medium bud stage regenerating limb; MB−, contralateral denervated limb; PAL+, palette stage regenerating limb; PAL−, contralateral denervated limb. Denervation alone does not induce an increase in the activity of 90-kDa gelatinase (not shown). (b) Zymographic analysis of amputated limbs immediately inserted into the peritoneal cavity. Each lane contains 65 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. The upregulation of the 90-kDa gelatinolytic activity is not dependent on the presence of the wound epithelium. However, the absence of a wound epithelium impedes the gelatinolytic activity's timely downregulation. Lanes: MB+, medium bud stage regenerating limb; MB−, contralateral inserted limbs; PAL+, palette stage regenerating limb; PAL−, contralateral inserted limbs. (c) Zymographic analysis of samples from regenerating axolotl tails. Each lane contains 20 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. Similar increase in levels of the gelatinolytic species were observed during tail regeneration as in limb regeneration. The level of the 90-kDa gelatinase increases significantly during the early stages of tail regeneration and starts its decline by 7 days after amputation. Lanes: N, unamputated tail; 1d, 1 day after amputation; 7d, 7 days; 14d, 14 days; 21d, 21 days. (d) Zymographic analysis of gelatinases/collagenases during axolotl flank wound healing. Each lane contains 90 μg total protein and the gel was incubated overnight at 30°C after washing with 2.5% Triton X-100. Just as seen during limb regeneration, there is an apparent regulation of 90-kDa gelatinolytic activity that is observed during flank wound healing. The activity migrating at 90-kDa shows an upregulation from that seen in the uninjured flank (N) by 4 hr (4h) after injury. This activity shows a dramatic increase after 1 day (1d) of wound healing in the flank. By 7 (7d) and 14 (14d) days of flank wound healing, the level of the 90-kDa gelatinase has decreased and by 21 days of healing (21d) returns to its basal level. Molecular weight standards are indicated along the left margin.

higher than in the contralateral palette stage (lane PAL+) regenerate, in which the 90-kDa gelatinolytic activity is declining to the basal level.

### *MMPs during Tail Regeneration*

As in unamputated limbs, unamputated tails show very low levels of the same five MMPs. As in limb regeneration, developmental regulation of 90-kDa gelatinolytic activity in the regenerating tail is seen as a response to amputation. One day after amputation (Fig. 2c; lane 1d), the level of 90-kDa gelatinolytic activity is increased 4.6-fold compared to the level in the unamputated tail. In this gel, enzyme activity in unamputated tails (lane N) cannot be detected, and a longer incubation period is necessary to visualize the bands. Unlike that of the regenerating limb, this high level of activity, however, is not maintained for a long period of time. By 7 days after amputation (Fig. 2c; lane 7d), the level of 90-kDa gelatinolytic activity is drastically decreased to 2.6-fold above basal level. At 14 days (Fig. 2c; lane 14d), the activity of the 90-kDa gelatinase remains at this low level. 90-kDa gelatinolytic activity finally reaches its basal level by 21 days after amputation (Fig. 2c; lane 21d).

### *MMPs during Flank Wound Healing*

In order to examine a possible role of MMPs during wound healing, we used zymographic analysis on crude extracts from healing flank wounds (Fig. 2d). Uninjured flank tissues exhibit low levels of the same five collagenolytic/gelatinolytic enzymes as in the unamputated limb. As in the limb, 90-kDa gelatinolytic activity increases in the injured flank. 90-kDa gelatinolytic activity is increased 2.4-fold above the basal level by 4 hr after flank wounding (Fig. 2d; lane 4h). By 1 day after injury (Fig. 7; lane 1d), 90-kDa gelatinolytic activity has increased to 7-fold above basal level and is at the peak of its expression during flank wound healing. By 7 days (Fig. 2d; lane 7d), when the wound has shown substantial but yet incomplete healing, the level of 90-kDa gelatinolytic activity has decreased but not quite to the basal level. After 14 days of healing (Fig. 2d; lane 14d), the level of 90-kDa gelatinolytic activity remains comparable to that seen at 7 days. Since the wound at this time appears to have healed, the elevated level of 90-kDa gelatinolytic activity above basal may be associated with musculature that is still undergoing remodeling underneath the healed skin. By 21 days after injury (Fig. 2d; lane 21d), the level of 90-kDa gelatinolytic activity has returned to normal levels, suggesting that remodeling of the underlying tissues is complete.

## DISCUSSION

Mature axolotl appendages show low levels of activity of five distinct matrix-degrading enzymes. Elevation of these five gelatinases was observed during regeneration,

but because the 90-kDa gelatinolytic activity is upregulated markedly, we have focused this study on this particular activity. We can detect an increase in 90-kDa gelatinolytic activity in limbs within a few hours of amputation. Activity increases during dedifferentiation, reaches a peak at medium bud, and declines to basal levels during redifferentiation.

Previous studies have reported that regenerating limbs have collagenolytic activity. Gross and colleagues (Grillo *et al.*, 1968; Dresden and Gross, 1970) demonstrated the ability of whole blastemas to degrade collagenous substrates. More recently, Groell *et al.* (1993) demonstrated collagenolytic activity of dissociated and cultured blastema cells. Since we have observed a dramatic increase in activity of only one of the five enzymes, it is possible that the activity identified in these studies was the 90-kDa gelatinase.

The timing of the upregulation of the 90-kDa gelatinase, beginning within a few hours of amputation suggests a role very early in regeneration. This increase in 90-kDa gelatinolytic activity is one of the earliest changes reported to date and precedes wound healing. One of the obstacles to cells in the mature limb reentering a developmental pathway is their immobilization within their connective tissue matrices. The cells that are critical to pattern formation are fibroblasts of the connective tissue, especially those in the skin (Muneoka *et al.*, 1986). It has been shown that there is a lag time of a few days after amputation before cells begin to migrate from the dermis toward the center of the amputation plane (Gardiner *et al.*, 1986). It is likely that the delay represents the time required for the 90-kDa gelatinase to digest the matrix around the cells, freeing them for migration.

We do not know whether the 90-kDa gelatinase response to wounding is unique to urodeles. We do know that although fibroblasts in the dermis of mammals also begin to migrate in response to wounding, they do so while remaining attached to the matrix. In mammals this behavior leads to wound contraction and scar formation rather than to regeneration. It is possible that 90-kDa gelatinolytic activity in regenerating limbs is necessary not only to enable cells to migrate individually, but also to ensure that they are able to engage in cell-cell interactions free of intervening matrix. In addition, it is possible that as the matrix begins to be degraded, stored growth factors are released that would affect not only the cell cycle, but also the reactivation of positional properties in dedifferentiating cells. That proteinases may have a role in the release of sequestered growth factors is suggested by the observations of Saksela and Rifkin (1990) that the release of FGF from the ECM is mediated by plasminogen activator.

The timing of the down regulation of 90-kDa gelatinolytic activity later in regeneration provides a further link between 90-kDa gelatinolytic activity and dedifferentiation. Data presented by Tank (1977) make it possible to estimate the duration of the dedifferentiation phase of regeneration. These data indicate that dedifferentiation at the base of the blastema continues through medium bud and ceases by the palette stage. The period during which the 90-kDa gelatinase levels are elevated coincides with the period of dedifferentiation. Our observation that the stump piece within 2 mm of the amputation plane has elevated levels of the 90-kDa gelatinase is consistent with histological observations that the stump undergoes histolysis within a millimeter or two of the amputation plane (Carlson, 1974). Finally, our data regarding the localization of 90-kDa gelatinolytic activity is consistent with results of Grillo *et al.* (1968) indicating that the highest collagenolytic activity is in the stump tissues adjacent to the blastema, with the blastema exhibiting the next highest activity.

At this time, we do not know whether upregulation of 90-kDa gelatinolytic activity occurs at the transcriptional level or is post-transcriptional. Because of the rapid appearance of the 90-kDa gelatinolytic activity after injury it is possible that limb cells initially activate the latent proenzyme that is constitutively expressed throughout the limb. Although the *in vivo* activation of MMPs is not yet fully understood, recent evidence suggests tissue kallikrein as a candidate molecule for the *in vivo* activator of MMP-9 (Desrivières *et al.*, 1993). As the regenerative process continues, transcription of the 90-kDa gelatinase gene could be upregulated, leading to the observed increase in activity. Our results suggest that the 90-kDa gelatinase detected during regeneration belongs to the MMP family of enzymes. In addition, the apparent molecular weight of this gelatinase is similar to the 92-kDa type IV collagenase/gelatinase (MMP-9; gelatinase B; EC 3.4.24.35; Nagase *et al.*, 1992) implicated as a factor in matrix degradation in a variety of species of mammals. Attempts to isolate the genes of MMP-9 and other MMPs in the axolotl are in progress in order to better understand the upregulation of gelatinolytic/collagenolytic activity during axolotl appendage regeneration.

We are also interested in understanding how the 90-kDa gelatinolytic activity is downregulated at the end of regeneration. It is known that MMPs are involved in tumor metastasis, and understanding how these enzymes are naturally downregulated in regenerating limbs might lead to strategies to control metastasis. We have found that denervated limbs or limbs that are inhibited from forming a wound epithelium activate high levels of the 90-kDa gelatinase despite the fact that they are blocked from regenerating. This means that elevation of the 90-kDa gelatinase levels does not require in-

nervation or the wound epithelium. However, 90-kDa gelatinolytic activity remains high in these limbs, even after the contralateral limb has completed regeneration, suggesting a relationship between innervation, the wound epithelium and down regulation of 90-kDa gelatinolytic activity. Although there may be a direct involvement of nerves and wound epithelium in the regulation of the 90-kDa gelatinase, it is more likely that limbs lacking nerves or a wound epithelium are arrested in dedifferentiation and cannot progress through regeneration. The signals to downregulate the 90-kDa gelatinase may originate from the regenerate itself as it matures, a process for which neither nerves nor wound epithelium are required. Our observation that denervated, amputated limbs fail to downregulate 90-kDa gelatinolytic activity is consistent with previous observations (Mailman and Dresden, 1976) that denervation of larval axolotl limbs 1 day after amputation leads to the overexpression of collagenolytic activity and the subsequent regression of these limbs. The observation that transplantation of blastemas onto denervated limbs prevents their regression (Shotté *et al.*, 1941) suggests that blastemas may contain inhibitors of collagenases. In addition, we do not know whether prolonged denervations of amputated limbs completely inhibits the downregulation of 90-kDa gelatinolytic activity or whether it merely delays its timely decline. Longer denervation times need to be examined in order to distinguish these alternatives.

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