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Dedifferentiation State Specific Increase of Trypsin- and Chymotrypsin-like Protease Activities during Urodele Limb Regeneration and Their Enhancement by Retinoic Acid Treatment

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Treatment of regenerating amphibian limbs with retinoic acid (RA) is known to induce pattern duplication, which is closely related to the extent of dedifferentiation. In the present study, the activities of trypsin- and chymotrypsin-like proteases are examined to delineate a possible role in the process of dedifferentiation in the regenerating limbs of urodeles, the Korean salamander (*Hynobius leechii*) and the Mexican axolotl (*Ambystoma mexicanum*). Specifically, we were interested to know if there is any correlation between trypsin- and chymotrypsin-like protease activities and the state of dedifferentiation which is augmented by RA treatment. We were also interested in expoloring if there is any species-specific difference in the profile of enzyme activities during limb regeneration. The results showed that the activities of these two enzymes reached a peak level at dedifferentiation stage, and RA treatment caused elevation of their activities, especially in the case of trypsin-like protease. The increase of trypsin-like protease activity after RA treatment was pronounced in the Korean salamander, which might reflect a species-specific responsiveness to RA. The present results imply that trypsin and chymotrypsin or similar proteases may play an active role in the process of dedifferentiation in regenerating limbs, and that trypsin or trypsin-like enzymes might be involved in the RA-evoked enhancement of dedifferentiation which precedes overt pattern duplication.

KEY WORDS: Urodele, Protease, Dedifferentiation, Regeneration, Retinoic Acid

One of the fundamental questions in developmental biology is how pattern is formed. Among many systems for the study of pattern formation, the regenerating limbs of amphibians have been used extensively due to their remarkable ability to restore missing parts. As in many developing systems, the basic question to be answered is how the undifferentiated regeneration blastemal cells which are provided by dedifferentiated cells of stump acquire position-specific properties which are in good harmony with those of the remaining structures.

In the study of regeneration, many attempts have been made to answer the questions of how the dedifferentiated cells retain the original position related properties and how the descendants of those cells acquire new, but more distalized positional properties. In this direction of research, retinoic acid (RA) has been introduced as a promising molecule to answer these questions because of its remarkable power to modify the positional properties of blastema cells in a

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predictable way which is manifested by the duplication of stump structures in the three cardinal axes.

RA evokes the duplication of digit structures in the anteroposterior (AP) axis in a dose-dependent and developmental stage-specific manner in the developing chick wingbuds (Summerbell, 1983). In the regenerating limbs of amphibians, RA also causes the duplication of stump structures. In the regenerating limbs of anuran tadpole, *Rana temporaria*, RA evokes duplication both in the proximodistal (PD) and the transverse axes (Maden, 1983). In the limbs of urodeles, *Ambystoma mexicanum* and *Hynobius leechii*, RA causes duplication mainly in the PD axis, but occasionally in the transverse axis in a dose- and stage-dependent manner (Kim and Stocum, 1986; Lee and Kim, 1990; Ju and Kim, 1994). Furthermore, at the tadpole stage, RA causes the homeotic transformation of a regenerating tail to hindlimbs in an anuran species, *Uperodon systoma* (Mohanty-Hegimadi *et al.*, 1992), and in another anuran species, *Rana temporaria* (Maden, 1993). On the other hand, RA, as a representative teratogen, has been shown to induce various types of abnormalities in many developing organs, such as heart, palatal shelf and limb in many systems including human (Alles and Sulik, 1989; Abbott and Birnbaum, 1990).

In the process of RA-induced pattern duplication of regenerating limbs, one of the interesting features is the augmented state of dedifferentiation, both in terms of its duration and intensity (Ju and Kim, 1994). Normally, at the dedifferentiation stage of urodele limb regeneration, stump tissues such as cartilages, bones, muscles, and other connective tissues are disintegrated and cells become morphologically dedifferentiated (Hinchliffe and Johnson, 1980). Also, corresponding with the histological changes at the dedifferentiation stage, activities of various hydrolytic enzymes, including acid phosphatase and matrix metalloproteinases (MMPs), are increased during the dedifferentiation period (Grillo *et al.*, 1968; Ju and Kim, 1994; Yang and Bryant, 1994). These hydrolytic enzymes whose activities are increased during the dedifferentiation period are believed to play roles in the dedifferentiation processes such as the demolition of tissues, the changes in cell morphology, and changes in ECM composition.

The cells involved in the dedifferentiation process, such as macrophages, are present in the distal region of the stump, especially at the early stage of limb regeneration (Hinchliffe and Johnson, 1980). In the mammalian skin wound, macrophages are known to contain inactive form of MMP's such as procollagenases (reviewed by Takemura and Werb, 1984), and mast cells are known to contain serine proteases such as tryptase and chymase (Saarinen *et al.*, 1994) and those enzymes are believed to be responsible for the demolition of tissues in the region of dedifferentiation (Grillo *et al.*, 1968). The serine proteases, trypsin and chymotrypsin, are known to have a broad range of substrate specificities (Gruber *et al.*, 1988; Saarinen *et al.*, 1994) and thus are expected to play roles in the change of ECM composition. In the present study, we were interested to know if there were any changes in trypsin and chymotrypsin activities at various stages of regeneration, and whether the enzyme activities are modified by RA treatment. The results showed that these enzyme activities increased at the stage of dedifferentiation in the process of urodele limb regeneration and that RA increased the enzyme activities.

Materials and Methods

Animals and maintenance

Korean salamanders, *Hynobius leechii*, and the Mexican axolotls, *Ambystoma maxicanum*, were used in the present study. The eggs of Korean salamanders were purchased from local dealers and maintained in dechlorinated tap water. After hatching, the larvae were fed newly hatched brine shrimp daily for 2 to 3 weeks. About 3 weeks after hatching, larvae were reared individually to prevent cannibalism and fed with finely chopped beef liver. At the time of amputation, the larvae were 7 weeks old and 25-32 mm in length. In the case of Mexican axolotls, larvae were obtained by mating of the stock animals and reared in the

same manner as in the case of Korean salamanders for 10 months after hatching. At the time of amputation, the axolotls were 120-160 mm in length.

Amputation, retinoic acid treatment, and tissue collection

Before amputation, animals were anesthetized in benzocaine solution (0.0067% for Korean salamanders; 0.02% for axolotls). Forelimbs were amputated at the level of the distal stylopodium and any protruding cartilages were trimmed to make the amputation surface flat. Retinoic acid (RA; all trans, type XX, Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) under dim light to minimize photo-isomerization of RA. Korean salamanders were injected intraperitoneally using a microliter syringe (Hamilton) with a dose of 200 μg RA/g body weight at 4 days after amputation (Lee and Kim, 1990). Control animals were injected with DMSO alone. Axolotls were injected intraperitoneally with 150 μg RA/g body weight at 4 days after amputation (Kim and Stocum, 1986). Since the previous study (Ju and Kim, 1994) showed that DMSO alone did not affect skeletal pattern or lysosomal acid phosphatase activity in the regenerating axolotl limbs, DMSO control group in axolotl was omitted in the present study. For the assay of trypsin- and chymotrypsin-like enzyme activities during the regeneration period, regenerating tissues were collected at 2 day intervals after amputation or at 2 day intervals after RA treatment. Usually, one hundred regenrates were collected for each sample in the Korean salamander and twenty regenerates were collected for each sample in the axolotl. At the time of tissue collection, care was taken to minimize the intact stump tissue in the collected tissues. Collected tissues were frozen at −70°C until use.

Assay of trypsin- and chymotrypsin-like enzymes activities in the regenerating tissues

The collected tissues were homogenized using a glass homogenizer in the buffer (50 mM Tris-HCl, 250 mM sucrose, 10 mM $CaCl_2$, 0.1% Triton X-100, pH 7.2) at 4°C. The homogenates were centrifuged for 10 min at 12000 × g, 4°C and the supernatants were used for the enzyme activity assay. Protein content in the supernatant was measured by the Bradford method (Bradford, 1976).

To assay the enzyme activities, two kinds of artificial fluorescent substrates, N-α-benzoyl-L-Arg-7-amido-4-methylcoumarin (BAAMC, Sigma) for trypsin-like enzyme activity assay and N-succinyl-Leu-Leu-Val- Tyr-7-amido-4-methylcoumarin (sucLLVY-AMC, Sigma) for chymotrypsin-like enzyme activity assay, were used. When these artificial substrates are cleaved enzymatically, the fluorescent product, 7-amino-4-methylcoumarin (AMC), is released from those substrates. The activities of trypsin- and chymotrypsin-like enzymes in each sample were measured according to the standard protocols with minor modification (Kanaoka, 1977; Zimmerman, *et al.*, 1976).

For the assay of trypsin- and chymotrypsin-like enzyme activities in the regenerating tissues with or without RA treatment, samples were diluted with homogenization buffer to a concentration of 250 μg protein/ml. After dilution, a 10 μl aliquot of each diluted sample was mixed with 290 μl reaction buffer I (50 mM Tris-HCl, 20 mM $CaCl_2$, 100 μM BAAMC, 1% DMSO, pH 8.0) for trypsin-like enzyme activity assay or reaction buffer II (50 mM Tris-HCl, 20 mM $CaCl_2$, 100 μM sucLLVY-AMC, 1% DMSO, pH 7.2) for chymotrypsin-like enzyme activity assay and incubated at 27°C for 30 min. The reaction was terminated by adding 1.7 ml of reaction termination buffer (50 mM Tris-HCl, 1 mM phenylmethanesulfonyl fluoride, 1% DMSO, pH 7.2), and the amount of released AMC was measured using a fluorometer (Model 450 digital flourometer, Sequoia-Turner) at 360 nm excitation and 430 nm emission. The assay for each sample was repeated three times. Prior to the assay of biological samples, it was determined that the production of AMC was proportional to the increase of the enzyme activity under the same reaction conditions.

After measurement of fluorescence in each sample, specific activities were calculated as follows. Because the final fluorescence product released after enzyme reaction was AMC, the measured fluorescence was converted into the

concentration (μM) of AMC by extrapolation to the standard fluorescence curve obtained from the fluorescence measurment of standard AMC (Sigma) solution. This value was divided by the amount (mg) of protein of sample and the reaction time (hour). Thus, the unit of specific activity of enzyme was expressed as μM AMC · mg protein^{-1} · hour^{-1}.

Results

Trypsin-like enzyme activity in Korean salamander

In the process of normal limb regeneration, trypsin-like enzyme activity increased after amputation and reached a peak value (41.3 μM AMC · mg protein^{-1} · hour^{-1}) at 4 days after amputation. Thereafter, the activity decreased continuously until 10 days after amputation, but increased again slightly after 10 days post-amputation (Fig. 1). Since the state of dedifferentiation is at its maximum level around 4 days postamputation as shown in previous histological studies, it can be concluded that trypsin-like enzyme activity correlates well with the state of dedifferentiation (Ju and Kim, 1994).

When RA was administered at the dedifferentiation stage (at 4 days after amputation), it caused a sharp elevation of trypsin-like enzyme activity with a peak value of 106.7 μM AMC · mg protein^{-1} · hour^{-1} at 8 days after RA treatment (12 days after amputation). Therefore, the peak value in the RA-treated regenerates was 2.6 times higher than that in the normal regenerates. Moreover, the lowest value of activity in the RA-treated regenerates was higher than the peak value in the normal limb regenerates, and the increased activity in the RA-treated regenerates extended over a more prolonged period. When DMSO, which was used as a vehicle for RA in the present study, was injected at 4 days after amputation, trypsin-like enzyme activity also increased. However, the degree of increase was not comparable to that in the RA-treated regenerates (Fig. 1). Therefore, the trypsin-like enzyme activity profile in RA-treated regenerates coincides well with the increased level and extended period of dedifferentiation after RA treatment as shown in our previous study (Ju and Kim, 1994).

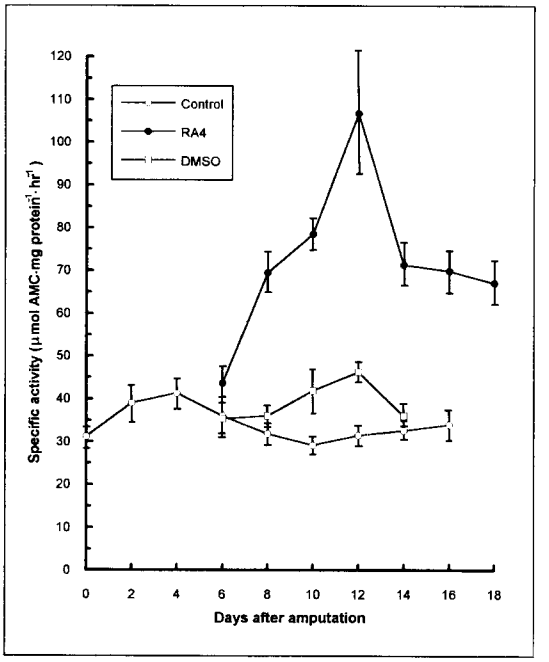

Fig. 1. Trypsin-like enzyme activity during limb regeneration of Korean salamander. Compare the trypsin activities in normal (CO: ○) and RA-treated (RA: ●) or DMSO-treated (DMSO: □) regenerates. RA or DMSO was injected at 4 days after amputation.

Chymotrypsin-like enzyme activity in Korean salamander

In the normal regenerates, the temporal expression pattern of chymotrypsin-like enzyme activity was similar to that of trypsin-like enzyme. Chymotrypsin-like enzyme activity in the normal regenerates increased slowly to reach a peak value of 50.9 μM AMC · mg protein^{-1} · hour^{-1} at 4 days after amputation, and it declined to the basal level thereafter (Fig. 2). Again, the increase in chymotrypsin-like enzyme activity correlates well with the state of dedifferentiation during the course of normal limb regeneration.

RA treatment at the dedifferentiation stage (at 4 days after amputation) caused the elevation of chymotrypsin-like enzyme activity to a peak value of 50.2 μM AMC · mg protein^{-1} · hour^{-1} at 6-8 days after RA injection (10-12 days after

amputation), at which time the state of dedifferentiation is most extensive after RA treatment as shown in our previous study (Ju and Kim, 1994). Thereafter, the activity slowly declined to the basal level over a prolonged period of time. However, the increase of chymotrypsin-like enzyme activity after RA treatment was not comparable to the increase of the trypsin-like enzyme activity. On the other hand, chymotrypsin-like enzyme activity was shown to be elevated slightly after DMSO treatment, but the extent of elevation was negligible (Fig. 2).

Trypsin-like enzyme activity in mexican axolotl

In the regenerating limbs of mexican axolotl, trypsin-like enzyme activity increased after amputation and reached a peak value (37.9 μM AMC · mg protein^{-1} · hour^{-1}) at 4 days after amputation. Thereafter, it declined slowly to the basal level. The trend of trypsin-like enzyme activity in the axolotl during the course of regeneration was similar to that in the Korean salamander, while the peak value of activity in the axolotl was lower than that in the Korean salamander (Fig. 3). The period when the activity is at its peak correlates well with the state of dedifferentiation as in the case of Korean salamander.

RA treatment at 4 days after amputation caused the elevation of trypsin-like enzyme activity, and the activity reached a peak value of 50.2 μM AMC · mg protein^{-1} · hour^{-1} at 4 days after RA injection (8 days after amputation). Therefore, the peak value in the RA-treated regenerates was 1.3 times higher than that in the normal regenerates, even though the effect of RA on trypsin-like enzyme activity in the axolotl was not comparable to that in the Korean salamander (Fig. 3). As in the case of Korean salamander, RA treatment caused an increase in trypsin-like enzyme activity both in terms of level and duration which corresponds well with the extensive

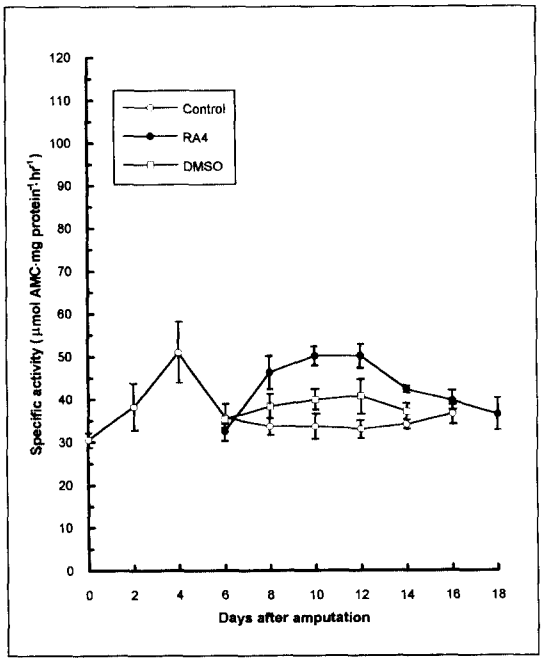

Fig. 2. Chymotrypsin-like enzyme activity during limb regeneration of Korean salamander. Compare the chymotrypsin activities in normal (CO: ○) and RA-treated (RA: ●) or DMSO-treated (DMSO: □) regenerates. RA or DMSO was injected at 4 days after amputation.

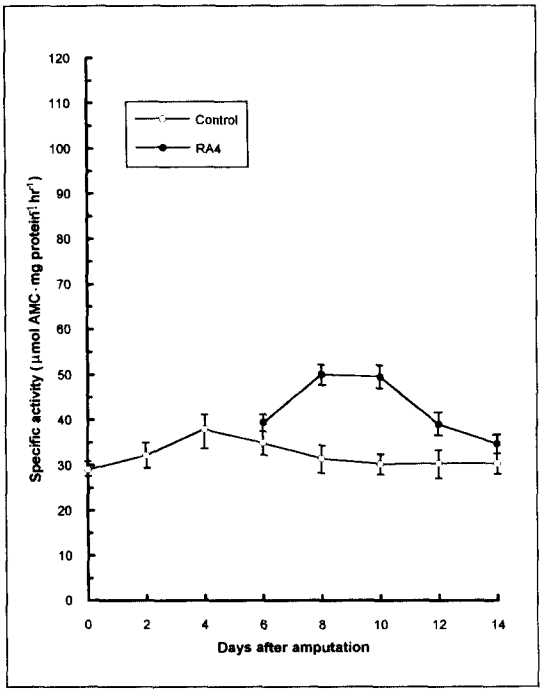

Fig. 3. Trypsin-like enzyme activity during limb regeneration of mexican axolotl. Compare the trypsin activities in normal (CO: ○) and RA-treated (RA: ●) regenerates. RA was injected at 4 days after amputation.

dedifferentiation observed after RA treatment.

Chymotrypsin-like enzyme activity in mexican axolotl

The profile of chymotrypsin-like enzyme activity in normal regenerates of the axolotl was similar to that of the Korean salamander. Chymotrypsin-like enzyme activity increased slowly and reached a peak value (43.2 μM AMC · mg protein^{-1} · hour^{-1}) at 4 days after amputation, when dedifferentiation is most extensive. Thereafter, it declined slowly to the basal level. However, the extent of the activity increase in the axolotl was lower than that in the Korean salamander (Fig. 4).

When RA was administered at 4 days after amputation, chymotrypsin-like enzyme activity increased again up to 42.2 μM AMC · mg protein^{-1} · hour^{-1} at 4 days after RA treatment (8 days after amputation) and declined more slowly compared to control (Fig. 4). The profile of chymotrypsin-like enzyme activity in normal and RA-treated regenerates of the axolotl was thus similar to that of the Korean salamander.

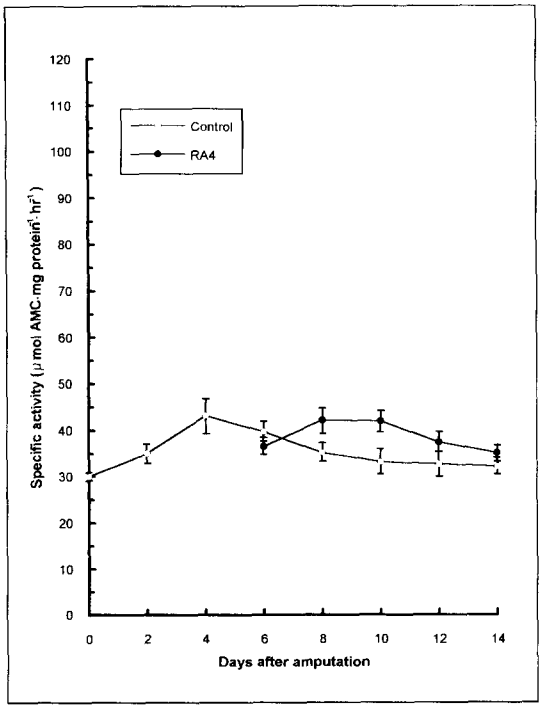

Fig. 4. Chymotrypsin-like enzyme activity during limb regeneration of mexican axolotl. Compare the chymotrypsin activities in normal (CO: ○) and RA-treated (RA: ●) regenerates. RA was injectedat 4 days after amputation.

Discussion

Administration of the regenerating urodele limbs with RA causes extensive dedifferentiation and pattern duplication (Ju and Kim, 1994). Since the degree of pattern duplication in the regenerating amphibian limb by RA treatment is closely related to the extent of dedifferentiation, Ju and Kim (1994) proposed that dedifferentiation is a very crucial event for the respecification of the positional property of future blastema cells.

Several kinds of hydrolytic enzymes are known to be involved in the process of dedifferentiation (reviewed in Stocum, 1995). Lysosomal acid phosphatase is enzyme whose activity has been shown to correlate with the extent of dedifferentiation (Grillo *et al.*, 1968; Ju and Kim, 1994). In the budding tunicates, RA treatment causes increased trypsin- and chymotrypsin-like enzyme activities (Kawamura, personal communication) prior to the formation of the secondary buds (Hara *et al.*, 1992), and it is speculated that these enzymes are involved in the dedifferentiation of mesenchymal cells. The above finding suggests that trypsin- and chymotrypsin-like enzymes might be involved in the dedifferentiation process of urodele limb regeneration, and we were interested to survey their activities over the course of the limb regeneration period. Especially, three questions were asked: (1) Is there any stage-specific difference in enzyme activities?, (2) Does RA modify enzyme activities?, and (3) Is there any species-specific difference in enzyme activities ?

In the regenerating limbs of the Korean salamander, trypsin-like enzyme activity increased after amputation and reached a peak value at 4 days after amputation when the activity of dedifferentiation was most extensive during the whole regeneration period as shown in previous studies (Lee and Kim, 1990; Ju and Kim, 1994). In the RA-treated regenerates, modification of trypsin-like enzyme activity in terms of both intensity and duration was observed. After RA

treatment, trypsin-like enzyme activity increased again steadily to reach a peak value at 8 days after RA injection, and the highest activity in the RA-treated regenerates was 2.6 times of the peak value in normal regenerates. Furthermore, higher than normal activity was maintained over a prolonged period of time, at least for 12 days after injection of RA. Therefore, the trypsin-like enzyme activity profile correlates well with the state of dedifferentiation, as in the case of acid phosphatase shown previously (Ju and Kim, 1994). In the normal regenerates of Korean salamander, the trend of chymotrypsin-like enzyme activity was similar to that of trypsin-like enzyme activity in which RA treatment caused the elevation of activity until 8 days after RA injection. However, after RA treatment, the peak value of chymotrypsin-like enzyme activity was similar to that of normal regenerates.

In the regenerating limbs of the axolotl, trypsin-like enzyme activity increased and reached a peak value at 4 days after amputation as in the normal regenerates of Korean salamanders. Also, the peak value of trypsin-like enzyme activity in normal regenerates of the axolotl was similar to that in normal regenerates of the Korean salamander. In the RA-treated regenerates of the axolotl, trypsin-like enzyme activity increased and the peak value was 1.3 times higher than that in the normal regenerates. However, the peak value of trypsin-like enzyme activity in RA-treated regenerates of the axolotl was much lower than that in RA-treated regenerates of the Korean salamander. Therefore, there was a clear difference between the two urodele species in trypsin-like enzyme activity after RA treatment. In normal regenerates of the axolotl, the chymotrypsin-like enzyme activity profile was similar to the trypsin-like enzyme activity profile. In the RA-treated regenerates, chymotrypsin-like enzyme activity increased and the peak value was similar to that in the normal regenerates. Therefore, as in the case of the Korean salamander, similarities and differences in the profile of trypsin- and chymotrypsin-like enzyme activities have been noted in the normal and RA-treated regenerates of axolotl.

From the data obtained in the normal regenerating limbs of Korean salamanders and axolotls, it is clear that trypsin- and chymotrypsin-like enzyme activities correlate well with the state of dedifferentiation. However, RA treatment caused somewhat different results; i.e., the peak level of enzyme activity was modified after RA treatment only in the case of trypsin-like enzyme, even though increases in both enzyme activities were maintained until 8-12 days after RA injection. The different enzyme activity profile after RA treatment might be due to the differential responsiveness of trypsin- and chymotrypsin-like enzyme genes to RA treatment. However, the above possibility remains to be tested using appropriate system.

Between the two urodele species, the profile of trypsin-like enzyme activity after RA treatment was somewhat different. Especially, the peak value of trypsin-like enzyme activity in the RA-treated regenerates of the Korean salamander was about 2-fold higher than that in the RA-treated regenerates of the axolotl. It is possible that this difference may reflect an inherent difference of RA-responsiveness between two species. In our previous study, pattern duplication in the transverse axes has been shown to be evoked more frequently in Korean salamander than in the axolotl, which might be the result of differential sensitivity to RA treatment (Ju and Kim, 1994). The other explanation for the difference might be found in the size difference between the two species of experimental animals. Since the size of axolotl used in the present study was far larger than that of Korean salamanders, more intact stump tissue could be included in the process of tissue sampling even though care was taken to minimize including intact stump tissues. Moreover, because the relative size of regenerates compared to stumps in larger animals is smaller than that in small animals as noted previously (personal observation), it is quite possible that the activity profile in axolotl may reflect the effect of dilution caused by intermixing of intact stump tissue and dedifferentiating tissue in the harvested samples. Therefore, a real enzyme activity in axolotl might be higher than the observed activity in the present study.

It is ambiguous whether the enzyme activities

observed in the present study faithfully reflect the enzyme activities of the *in vivo* condition. For example, it is possible that the present results reflect the activity of some other enzymes which have a substrate specificity similar to that of trypsin and chymotrypsin. Furthermore, because the assay condition in the present study was not the same as *in vivo* physiological state, the actual value of enzyme activity might be quite different from the present data. Therefore, further study using antibodies or riboprobes is required to resolve those ambiguities.

There are, at least, two possibilities for increases in trypsin- and chymotrypsin-like enzyme activities during the dedifferentiation period in the limb regeneration process, i. e., (1) activation and/or secretion of preformed enzymes, (2) *de novo* synthesis of enzymes. Tissue mast cells are known to contain tryptase and chymase (Irani *et al.*, 1986) which activate interstitial procollagenase, MMP-1 (Gruber *et al.*, 1988; Saarinen *et al.*, 1994). Also, the activation of macrophages related to tissue demolition has been shown to occur in the regressing tail of the metamorphosing *Xenopus* tadpole and in the remodeling process that takes place during mouse eye development (Weber, 1964; Lang and Bishop, 1993). In the regressing or remodeling tissues, activated macrophages have been shown to synthesize and secrete several proteases such as collagenase, elastase (Takemura and Werb, 1984). Interestingly, tryptase and chymase are known to be involved in the activation process of procollagenase (Gruber *et al.*, 1988; Saarinen *et al.*, 1994), and trypsin-like protease plasmin is speculated to be involved in the process of activation of precursor forms of MMP's (reviewed in Stocum, 1995).

RA treatment, especially in the case of trypsin-like enzyme, caused the elevation of enzyme activity. RA treatment has been known to destabilize the lysosomal membrane and cause the release of lysosomal enzymes (Lucy, 1969). Therefore, it is possible that proteases such as trypsin and chymotrypsin or other similar enzymes in secretory vesicles might be released by RA treatment. On the other hand, during dedifferentiation period in the regenerating urodele limbs, many kinds of proteins appear to be synthesized (Ju and Kim, manuscripts in preparation). Especially, protein synthetic pattern in the dedifferentiating normal regenerates appears very similar to that in the enhanced dedifferentiation period after RA treatment. Therefore, it is possible that RA causes activation of genes responsible for the synthesis of several enzymes such as trypsin- and chymotrypsin-like enzymes during dedifferentiation process.

Besides trypsin- and chymotrypsin-like enzymes, increases in other hydrolytic enzyme activities related to tissue demolition has been noted in the regenerates of urodele limbs and other systems. In the regenerating limbs of the urodeles *Ambystoma mexicanum* and *Nothopthalmus viridescens*, acid phosphatase and MMPs are shown to be activated in the early phase of regeneration, and the increase of enzyme activities coincides with changes in ECM components, such as hyaluronic acid and chondroitin surfate, during the early phase of limb regeneration (Mescher and Munaim, 1986; Yang and Bryant, 1994). Increased activities of hydrolytic enzymes such as trypsin, chymotrypsin, acid phosphatase and MMP's, are believed to play active roles in the modification of ECM structure during dedifferentiation process (Huhtala *et al.*, 1995; reviewed in Stocum, 1995).

Although the mechanism of the RA effect on pattern duplication during urodele limb regeneration are obscure, the dedifferentiation stage is the most effective stage for the RA-induced pattern duplication (Kim and Stocum, 1986; Lee and Kim, 1990). In regenerating limbs, the respecification of positional value by RA treatment may thus occur at the dedifferentiation stage. The adhesivity of blastema cells has been shown to be closely related with the positional values of blastema cells (Nardi and Stocum, 1983; Crawford and Stocum, 1988). In the case of the murine S91 melanoma cell, RA treatment causes the alteration of cell-surface glycoprotein which, in turn, modifies cellular adhesivity (Lotan *et al.*, 1981). Also, the inhibition of glycosylation by tunicamycin was observed to inhibit the proximalizing effects of RA in axolotl limbs (Johnson and Scadding, 1992). Those findings

suggest that positional value might be respecified by the modification of cell surface and/or ECM components in the RA-treated regenerate. In that sense, the increased activity of hydrolytic enzymes such as trypsin- and chymotrypsin-like enzymes, as shown in the present study, may be responsible for the modification of ECM composition and the change in positional memory induced by RA treatment.

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유미양서류 다리 재생 기간중 탈분화 시기 특이적 트립신, 키모트립신 유사 단백질 분해 효소의 활성도 증가

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재생중인 양서류의 다리에 레티노익산을 처리하면 패턴 복제가 유발되며 이러한 현상은 탈분화의 정도와 깊이 연관되어 있다. 본 연구에서는 한국산 도롱뇽(*Hynobius leechii*)과 멕시코산 도롱뇽의 일종인 엑소로틀(*Ambystoma mexicanum*)의 다리 재생 과정에서 탈분화 과정에 관여할 것으로 추정되는 트립신 및 키모트립신과 유사한 단백질 분해 효소의 활성도를 재생 단계에 따라 조사해 봄으로써 이들 단백질 분해 효소의 활성도와 레티노익산에 의해 증가되는 탈분화 상태의 정도사이에 상관 관계가 있는지를 알아 보고자 하였으며 다리 재생 기간중 상기 효소의 활성도 변화에 종 특이적 차이가 존재하는 지를 알아보고자 하였다. 연구 결과, 이들 효소의 활성도는 탈분화시기에서 최대에 도달함을 알 수 있었고 레티노익산 처리는 트립신 유사 단백질 분해효소의 활성도를 증가시키는 것으로 나타났다. 이러한 효과는 한국산 도롱뇽에서 두드러졌고 이는 레티노이산 처리에 의한 반응에 종 특이적 차이가 있음을 반영하는 것으로 해석되었다. 본 연구 결과는 트립신 및 키모트립신 유사 단백질 분해 효소가 재생중인 다리의 탈분화 과정에 중요한 작용을 하고 있으며, 이들 단백질 분해 효소가 레티노익산 처리에 의한 탈분화 과정의 강화에 관여하리라는 것을 암시한다.