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Protein Synthesis Pattern Analysis in the Regenerating Salamander Limb

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Retinoic acid (RA) evokes pattern duplication in the regenerating salamander limb. Interestingly, it also enhances dedifferentiation in the regenerate by the morphological, histological and biochemical criteria. To examine whether there is any correlation between the RA-evoked pattern duplication and *de novo* protein synthetic profile in the regenerating salamander limb, especially during dedifferentiation, we analyzed stage-specific protein synthesis pattern in the normal and RA-treated regenerating limbs by metabolic labeling followed by two-dimensional gel electrophoresis. In the regenerating limbs without RA treatment, a few hundred kinds of proteins were found to be synthesized at the stage of wound healing and the total number of protein synthesized increased greatly as regeneration proceeded. The same trend was also observed in the RA-treated regenerating limbs. Interestingly, some protein spots were noted to be either newly synthesized or highly expressed by the RA treatment especially at the stage of dedifferentiation. The results shows that the enhancement of dedifferentiation state after the RA treatment correlates well with the protein synthesis profile, and suggest that those proteins are important for the RA-evoked pattern duplication in the regenerating limbs of salamander.

In vertebrates, urodeles including salamanders and newts are well-known for their remarkable ability to regenerate even in the adult (reviewed in Stocum, 1995). If a salamander limb is amputated, a faithful replicaed of the lost part is developed from the remaining stump. Therefore, the regenerating limb of urodele has frequently been a subject to study how original structural pattern is restored. Limb regeneration of urodeles occurs through an epimorphic process and blastema cells are supplied from the stump cells, which undergo dedifferentiation after the amputation. In the process of limb regeneration, only the part distal to the amputation level is restored. This phenomenon, called distal transformation, implies that position-related properties or positional values in the blastema cells can only be distalized in the regeneration process (Rose, 1970). However, this rule can be violated by the treatments with vitamin A and its metabolites retinoids since retinoids cause duplication of stump structures in the regenerating amphibian limbs when properly administered (Niazi and Saxena, 1978; Maden, 1982; Thoms and Stocum, 1984). Therefore, RA seems to respecify the positional values probably by modifying the position related memory of the blastema cells to a more proximal position (Maden, 1982). Subsequently, it was found that RA can also respecify the positional values of limb blastema cells to more posterior and ventral directions along these axes (Kim and Stocum, 1986; Ludolph et al., 1990).

In the RA-evoked pattern duplication of regenerating salamander limbs, there are two interesting phenomena related to the dedifferentiation. First, the most sensitive stage in the RA treatment for the duplication of stump structure in the regenerating limb is the stage of dedifferentiation. In the regenerating salamander limb, administration of RA either before or after the stage of dedifferentiation results in little or no pattern duplication (Thoms and Stocum, 1984). However, it is still not clear why the most sensitive stage is dedifferentiation. Second, the limb stump treated with RA exhibits an extensive degree of dedifferentiation compared to that of untreated stump (Ju and Kim, 1994). Not only histological observation but also biochemical studies suggest that the stage-specific response to RA is related to the differential state of differentiation of preblastemal cells in the stump after the amputation, and that dedifferentiated cellular state might be the prerequisite condition for the RA-evoked pattern duplication (Ju and Kim, 1994).

During dedifferentiation, the cells in the stump tissues including muscle, bone or cartilage, nerve sheath, and connective tissues lose their characteristics and become mononucleated, undifferentiated and enter cell cycling to form the blastema. If protein synthesis is inhibited by actinomycin D treatment in the regenerating newt limb, tissue dedifferentiation and finally limb regenera-

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tion are blocked even though wound healing is not inhibited (Carlson, 1967). With [^{35}S] methionine labeling at various stages of the limb regeneration, it was shown that quite an amount of label was shown to be taken up during dedifferentiation (Bodemer and Everett, 1959). This indicates that protein synthesis is very active while the stump tissue is histolysed. Certainly, many of the proteins that are synthesized during dedifferentiation should be remodeling enzymes of the extracellular matrix, muscle and cartilage. In fact, it has been reported that many kinds of lysosomal enzymes and matrix degrading enzymes are present in the dedifferentiating tissues of urodele limbs (reviewed in Stocum, 1995).

To understand the relationship between dedifferentiation and RA-evoked pattern duplication, it is necessary to identify gene products that are differentially expressed during dedifferentiation and by RA treatment. Therefore, protein synthetic profiles in the normal and RA-treated regenerating limbs of salamander were examined by two-dimensional gel electrophoresis after metabolic labeling. The results show that dedifferentiation-specific proteins are also synthesized during the extended period of dedifferentiation in the RA-treated limb regenerates and a few proteins are synthesized only in the RA-treated limb regenerates, especially during dedifferentiation.

Materials and Methods

Animals and maintenance

The larvae of Korean salamander *Hynobius leechii* were used in this study. Naturally spawned eggs were maintained in dechlorinated tap water. After hatching, the larvae were fed with freshly hatched brineshrimp daily. About a month later after hatching, the larvae were reared individually in the latticed acryl container to prevent cannibalism and fed with finely chopped beef heart. At the time of limb amputation, the animals ranged from 20 mm to 30 mm in length.

Amputation levels

The forelimbs of each animal were amputated bilaterally through the distal stylopodium (upper arm). Immediately after the amputation, any protruding limb cartilage from the surface was trimmed to give a flat surface.

Preparation and administration of retinoic acid

Retinoic acid (all trans, type XX, Sigma) was dissolved in dimethyl sulfoxide to make a stock solution of 30 mg/ml, prepared under subdued light to minimize photoisomerization. A microliter syringe (Hamilton) was used for intraperitoneal injection of RA at 200 μg RA/g body weight at 4 d after the amputation. Before the injection, the animals were anesthetized in 0.02% benzocaine and weighed on a top-loading balance to determine the amount of RA to be injected.

Metabolic labeling of regenerating limb tissues

Metabolic labeling of regenerating limb tissue was performed according to Slack's protocol (1982). To study the *de novo* protein synthesis in the normal and RA-treated regenerating larval limbs, normal regenerates were collected at 2 d intervals from 2 d to 12 d after the amputation and RA-treated regenerates were collected at 2 d intervals from 2 d to 14 d after the RA injection. Soon after the collection, the regenerates were minced into small pieces in normal amphibian medium containing 20 mM glucose (Mohun et al., 1980). The minced tissue was rinsed in 70% Eagle's minimal essential medium and was incubated in 50 μCi [^{35}S]- methionine (>1000 Ci/m mole, Amersham) for 10 h at 25℃. The samples were then rinsed twice in normal amphibian medium containing 1 mM unlabelled methionine.

Two-dimensional gel electrophoresis and fluorography

To compare the *de novo* protein synthesis between the normal and RA-treated regenerating larval limbs, two-dimensional (2D; IEF and SDS-PAGE) gel electrophoresis was performed following O'Farrell's method (O'Farrell, 1975). The samples were homogenized in lysis buffer (9.5 M urea, 2% ampholyte mix, NP-40 and 5% β-mercaptoethanol) containing 2 mM PMSF (phenylmethanesulphonyl fluoride) as a protease inhibitor. Ampholyte mixture was prepared by mixing one part of Pharmalyte 3-10 (Pharmacia) with two parts of Pharmalyte 5-8 (Pharmacia). The homogenates were centrifuged at 15,000 × g for 10 min and the supernatants were used as protein samples for the 2D gel electrophoresis. Protein concentration in each supernatant was determined by Ramagli and Rodriguez's method, since lysis buffer contained urea (1985). For isoelectric focusing (IEF), the precleaned glass tubes 1.5 mm in i.d. and 13.5 cm in length were filled with 3% polyacrylamide solution containing 2% ampholyte mix. After polymerization, the gel was loaded with 40 μg of protein sample. The gels were placed in a tube gel electrophoresis chamber of which the lower and upper reservoirs were filled with 0.1 M NaOH and 0.1 M H_3PO_4, respectively. After prerunning at 200 V for 15 min, at 300 V for 30 min and at 400 V for 30 min, isoelectric focusing was carried out at 700 V for 12 h, and finally at 800 V for 1 h. The gels were taken out of the glass tubes and equilibrated in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, and 62.5 mM Tris-HCl) for 10 min before transferring into second dimension gel.

The second dimension gel was a 1.5 mm thick 10% polyacrylamide gel containing 10% SDS without a stacking gel. After polymerization, the equilibrated IEF gel was fixed onto the second dimensional gel with hot

agar solution containing bromophenol blue. The gel was set in an electrophoresis apparatus with buffer (25 mM Tris-HCl, 0.1% SDS, and 0.192 M glycine) and was allowed to run at 40 mA/gel until the dye front reached 10 mm from the bottom of the gel. To determine the isoelectric point (pI) and molecular weight (MW) of protein, IEF Mix 3.5-9.3 (Sigma) and molecular weight marker for SDS-PAGE (Sigma) were used, respectively. After the 2-D gel electrophoresis, the slab gel was processed for fluorography by the method of Bonner and Laskey (1974). Dried gel was exposed to preflashed Agfa Curix RP1 X-ray film for 1 wk at 70℃.

Results

To obtain information on stage-dependent protein expression profile, protein synthesis patterns in the regenerating limbs of salamander (*Hynobius leechii*) with or without RA treatment were examined by two-dimensional gel electrophoresis after metabolic labeling. About 700 protein spots were resolved under the present experimental condition. From the initial examination of the 2 D electrophoretograms, 24 protein spots were found whose expression or abundance were characterisic in the two representative stages during the normal limb regeneration such as the stages of dedifferentiation (6 d after amputation) and redifferentiation (12 d after amputation). Therefore, those protein spots could be divided into 2 groups, i.e., i) dedifferentiation stage-specific protein spots and ii) redifferentiation stage-specific protein spots. In addition, some protein spots were found to be specific in the RA-treated regenerates.

Dedifferentiation specific protein synthetic profile

As shown in Fig. 1A and summarized in Table 1, about 160 protein spots were noted to appear at the initial stage of wound healing (12 h after amputation). At the stage of dedifferentiation (6 d after the amputation), the number of newly synthesized protein spots increased remarkably (Fig. 1B). At least 12 protein spots (spots 1-12) were noted to be newly synthesized or expressed highly at the stage of dedifferentiation. However, their expression except for spot 1 declined noticeably at the stage of redifferentiation (Fig. 1C). Therefore, those 12 protein spots were considered as 'dedifferentiation-specific proteins'. Interestingly, most of the dedifferentiation-specific proteins were acidic and their molecular weights were low. In the RA-treated limb regenerate, the expression levels of these proteins except for one (Spot 12) were either comparable to or even higher than those of the control at the stage of dedifferentiation (Fig. 1D). These results coincide well with the previous studies that RA treatment was shown to maintain and enhance the extent of dedifferentiation during salamander limb regeneration.

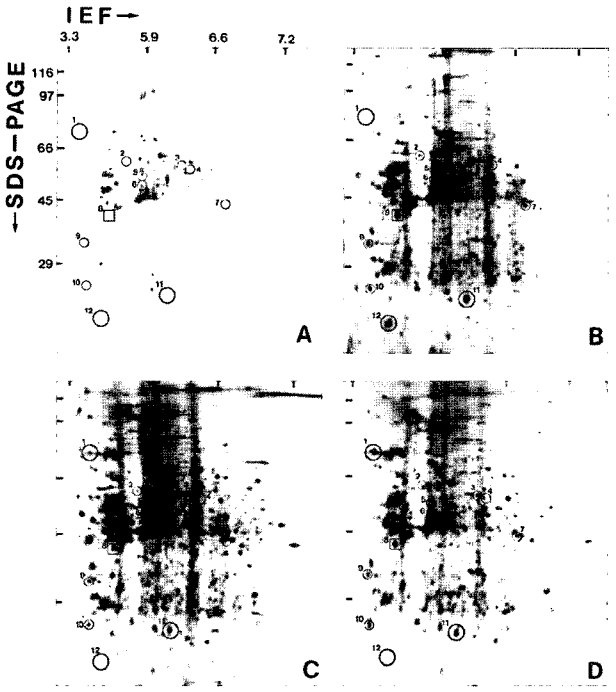

Fig. 1. Dedifferentiation-specific protein synthesis profile in the limb regenerates of Korean salamander, *Hynobius leechii*. Protein spots in the circles or the square represent dedifferentiation specific proteins. Numbers correspond to the identification number given to various protein spots shown as in Table 1. A, B, C; Normal limb regenerates. D, RA-treated limb regenerate. A, at the stage of wound healing (at 12 h after amputation). B, at the stage of dedifferentiation (at 6 d after amputation). C, at the stage of redifferentiation (at 12 d after amputation). D, at 6 d after RA treatment (at 10 d after amputation).

Redifferentiation specific protein synthetic profile

As shown in Fig. 2 and summarized in Table 1, 12 protein spots (spots 13-24) were noted to be newly synthesized or increased in their expression level at the stage of redifferentiation. Among them, protein spots 16, 17 and 24 were expressed from the stage of dedifferentiation at a basal level, but they were highly expressed at the stage of redifferentiation. Other protein spots (spots 13, 14, 15, 18, 19, 20, 21, 22 and 23) were newly synthesized at a high level during the stage of redifferentiation. However, in the RA-treated limb regenerates, most of the redifferentiation-specific protein spots except for the protein spots 15 and 19 were found to be decreased or disappeared at 6 d after the RA treatment, when the dedifferentiation activity is at its peak level, as noted by the previous studies.

RA treatment specific protein synthetic profile

Interestingly, three protein spots (spots 25, 26, and 27) were noted to be distinctively synthesized in response to the RA treatment. Among them, protein spots 25 and 27 were found to be expressed at 6 days (Fig. 3, Table 1).

Table 1. Summary of the protein synthetic profiles in the normal limb regenerates (N; without RA treatment) and RA-treated limb regenerates (R) of the salamander larvae, *Hynobius* leechii

Spot No	Strength of Intensity*														PI	M. W. (kDa)	Grade**
	N0	N2	N4	N6	N8	N10	N12	R2	R4	R6	R8	R10	R12	R14			
1	0	1	2	2	1	1	2	3	4	5	3	2	2	2	3.8	72	A
2	0	0	1	3	1	0	1	0	3	4	4	1	1	1	5.3	60	C
3	0	0	3	3	2	0	0	0	2	4	5	3	4	3	6.3	57	C
4	0	0	1	3	2	0	0	0	1	3	5	1	2	1	6.4	56	C
5	2	1	5	5	4	3	2	1	3	3	5	2	3	2	5.7	55	B
6	2	1	5	5	4	3	3	1	3	3	5	2	3	2	5.7	53	B
7	0	0	0	3	0	2	1	1	2	4	3	2	2	1	6.9	45	C
8	2	1	4	5	3	3	2	2	5	5	4	2	2	3	4.2	42	C
9	1	1	3	4	2	3	3	1	2	4	5	2	2	2	3.6	35	B
10	1	1	1	3	2	2	2	1	2	5	5	3	3	2	3.8	29	B
11	2	2	5	5	1	3	3	2	3	4	4	2	2	2	6.1	25	A
12	1	1	3	4	3	1	0	0	1	1	2	1	0	0	3.9	22	A
13	0	0	0	0	0	3	3	0	0	0	0	0	0	0	5.2-5.4	107	C
14	0	0	0	0	0	3	4	0	0	0	0	0	1	1	6.0-6.2	102	C
15	0	0	0	0	0	3	3	0	0	2	1	3	3	3	6.8	65	C
16	0	0	2	2	1	3	4	0	1	1	1	1	1	1	3.7	53	B
17	0	0	2	2	1	4	4	0	1	1	2	1	2	2	3.9	49	B
18	0	0	0	0	0	3	3	0	0	0	0	1	1	1	5.4	47	C
19	0	0	0	0	0	3	3	0	0	0	0	1	3	3	6.3	47	B
20	0	0	0	0	0	4	3	0	0	0	0	0	0	1	7.0	47	C
21	0	0	0	0	0	4	3	0	0	0	0	0	0	1	7.1	47	C
22	0	0	0	0	0	3	3	0	0	1	0	2	2	2	6.4	46	B
23	0	0	0	0	0	3	3	0	0	0	1	1	2	2	6.6	46	C
24	0	0	1	1	2	5	5	0	0	0	1	0	2	1	3.8	44	A
25	0	0	0	0	0	0	0	0	0	3	1	1	1	1	5.1	117	C
26	0	0	0	0	0	0	0	0	0	3	3	4	5	4	6.1	55	B
27	0	0	0	0	0	0	0	1	3	4	4	1	1	1	6.3	36	B

* Numbers indicate the varying spot intensity in a given spot of protein (1; lowest, 5; highest)

** Grades ranging from A to C (decreasing intensity) represent comparative intensities of protein spots

Discussion

In our previous study, morphological, histological and biochemical data clearly showed a close relationship between dedifferentiation state and RA-evoked pattern duplication in the regenerating limbs of salamander (Ju and Kim, 1994). Here, we wanted to know if there exists a correlationship in the protein synthetic profiles and regeneration stages in the normal and the RA-treated limb regenerates. Using high-resolution two-dimensional gel electrophoresis after metabolic labeling, at least 27 proteins were noted whose expression levels were variable during the course of limb regeneration with or without RA treatment. Of these, 12 proteins were newly synthesized or highly expressed during dedifferentiation (6-8 days after amputation). Interestingly, those dedifferentiation specific proteins were highly expressed or maintained in the RA-treated limb regenerates. Therefore, there also exists strong correlation between the extent of dedifferentiation and the protein expression profile in the RA treated limb regenerate.

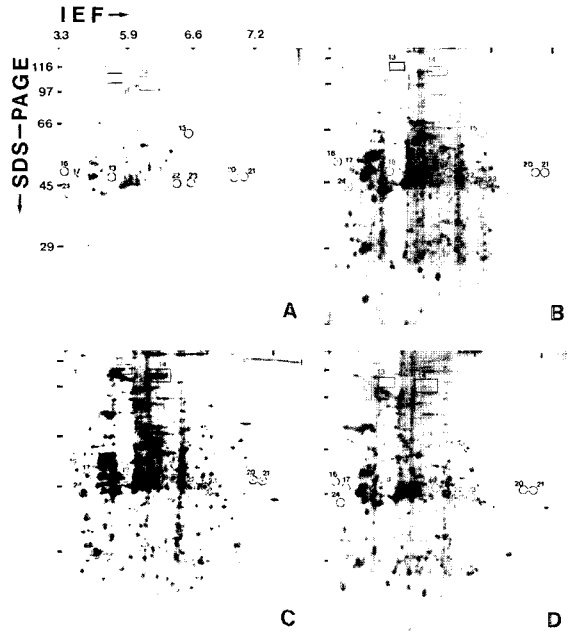

Fig. 2. Redifferentiation-specific protein synthesis profile in the limb regenerates of Korean salamander, *Hynobius leechii* (same autoradiograms as in Fig. 1). Protein spots in the circles or the square represent redifferentiation specific proteins. Numbers correspond to the identification number given to various protein spots shown as in Table 1. A, B, C; Normal limb regenerates. D, RA-treated limb regenerate. A, at the stage of wound healing (at 12 h after amputation). B, at the stage of dedifferentiation (at 6 d after amputation). C, at the stage of redifferentiation (at 12 d after amputation). D, at 6 d after RA treatment (at 10 d after amputation).

In this study, we could not identify the nature of the stage-specific proteins both in the normal and RA-treated regenerates. However, some of the dedifferentiation specific spots are believed to be matrix remodeling enzymes and lysosomal enzymes. The initiation of dedifferentiation is dependent on de novo protein synthesis (Carlson, 1967), and protein synthesis is very active in the stump tissue during dedifferentiation (Bodemer and Everette, 1959). Among the newly synthesized proteins some of them are certainly remodeling enzymes of the stump tissues. In fact, various lysosomal acid hydrolases such as acid phosphatase, cathepsins, carboxylic ester hydrolase, and β-glucuronidase had been found in the dedifferentiating limbs of

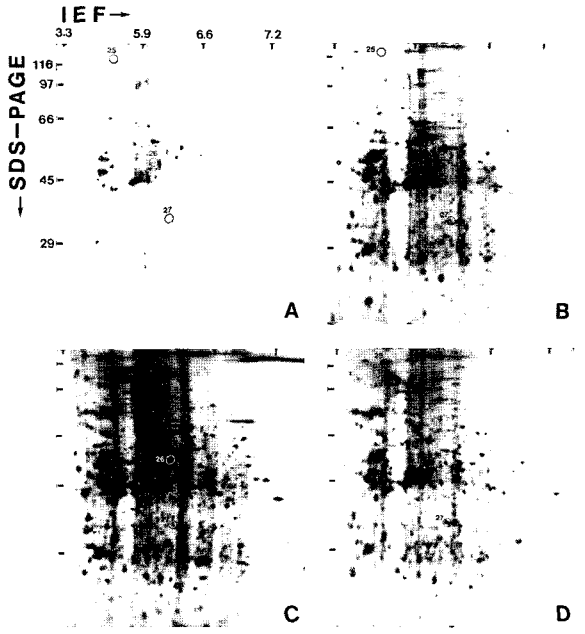

Fig. 3. RA treatment specific protein synthesis profile limb regenerates of Korean salamander, *Hynobius leechii* (same autoradiograms as in Fig. 1). Protein spots in the circles or the square represent the RA treatment specific proteins. Numbers correspond to the identification number given to various protein spots shown as in Table 1. A, B, C; Normal limb regenerates. D, RA-treated limb regenerate. A, at the stage of wound healing (at 12 h after amputation). B, at the stage of dedifferentiation (at 6 d after amputation). C, at the stage of redifferentiation (at 12 d after amputation). D, at 6 d after RA treatment (at 10 d after amputation).

urodele (Dukiet and Niwelinski, 1960; Schmidt and Weidman, 1964; Schmidt and Norman, 1965; Slattery and Schmidt, 1975; Ju and Kim, 1994, 1998). Also, serine proteases (trypsin- and chymotrypsin-like enzymes) have been found to increase in the regenerating salamander limb (Lee and Kim, 1996), and the activities of MMPs (matrix metalloproteinases) and collagenases have also been noted to increase in the regenerating limbs of urodele (Grillo et al., 1968; Park and Kim, 1999) during dedifferentiation. Also, expression of cathepsin D gene was found to increase in the regenerating salamander limb (Ju and Kim, 1998). These trends correlate well with the results obtained in this study. However, further studies are needed to verify that a large number of those dedifferentiation specific proteins correspond to tissue remodeling enzymes.

Interestingly, those dedifferentiation specific enzymes are upregulated even further in the RA-treated limb regenerates of salamander (Ju and Kim, 1994; Lee and Kim, 1996; Park and Kim, 1998). However, the mechanism of this is not well known. The presence of RARs (retinoic acid receptors) in the regenerating limbs suggests that RARs mediate directly or indirectly the effects of RA on the expression of those protein (Giguere et al., 1989; Ragsdale et al., 1989, 1992). In that sense, it is worthwhile to note that a high level of RAR1 mRNA is present in the regenerating newt limb and expression of RAR1 is increased during dedifferentiation and blastema formation in the regenerating newt limb (Giguere et al., 1989; Ragsdale et al., 1992).

As redifferentiation proceeded, a number of protein spots were also found to be newly synthesized. In fact, many kinds of cellular and ECM components such as collagen, laminin, integrins, catenin are reported to be newly synthesized in the limb regenerates (reviewed in Stocum, 1995). Therefore, some of the redifferentiation specific spots might represent those cellular and ECM molecules. On the other hand, a part of the newly synthesized proteins by RA treatment (spots 25, 26, and 27) might be stress proteins. In the regenerating limbs of newt (*Notophthalmus viridescens*), RA induces synthesis of the stress or heat shock proteins (Carlone et al., 1993).

RA treatment at the dose of pattern duplication causes elevation and prolongation of the dedifferentiation state with respect to protein expression in the regenerating salamander limbs. These results support the hypothesis that pattern duplication by RA is feasible due to enhanced dedifferentiation state in the regenerating salamander limb. However, to understand the RA-evoked pattern duplication more completely, the molecular nature of the dedifferentiation specific proteins must be characterized, and their role in the patterning process should be elucidated in the future.

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