Korean J Biol Sci 6: 301-304, 2002

Effect of Retinoic Acid on *Fgf-8* Expression in Regenerating Urodele Amphibian Limbs

Man-Jong Han† and Won-Sun Kim*

Department of Life Science, College of Natural Sciences, Sogang University, Seoul 121-742, Korea

Key Words:
Limb regeneration
Fgf-8 (fibroblast growth factor-8)
RA (retinoic acid)
Salamander
Axolotl

In our previous study, we have shown that *Fgf-8* is expressed in the basal layer of the apical epithelial cap (AEC) and in the underlying thin layer of mesenchymal tissue of the regenerating limbs of Mexican axolotl, *Ambystoma mexicanum*. Our present RT-PCR data also demonstrate that *Fgf-8* transcript is localized both in the mesenchymal and epidermal tissues. To understand the effect of retinoic acid (RA) on the expression of *Fgf-8* in the regenerating axolotl limbs, RA was injected intraperitoneally at the dedifferentiation stage of limb regeneration. The RA treatment caused a change in the *Fgf-8* expression profile of the regenerating limbs. In RA-treated limbs, duration of *Fgf-8* expression was prolonged and a high level of expression was maintained during dedifferentiation and blastema formation stages. These results suggest that *Fgf-8* is an important molecule in the process of pattern duplication of regenerating salamander limbs evoked by RA treatment.

Local application of RA to the anterior margin of chick wing buds induces mirror-imaged pattern duplication of digits in the AP (anteroposterior) axis (Tickle *et al.*, 1982; Han and Kim, 1988). A similar result is induced by implantation of ZPA (zone of polarizing activity) to the anterior margin of the wing bud (Saunder and Gasseling, 1968). The induction of a duplicated AP structure in the developing wing bud is the result of formation of ectopic ZPA that expresses *Shh* (Riddle *et al.*, 1993). However, whether endogenous RA initiates a similar signaling network at the posterior limb bud margin is still controversial.

In regenerating urodele limbs, RA causes pattern duplication in the PD (proximodistal) axis and pattern completion in the transverse axes (Maden, 1982; Ju and Kim, 1994). In the process of RA-induced pattern duplication of regenerating limbs, RA coordinately proximalizes level-specific blastema cell adhesivity (Crawford and Stocum, 1988). The modification of cell adhesivity might be related with changes of cell surface glycoconjugates (Gudas *et al.*, 1994). Moreover, one of the interesting features of the effects of RA is the elevated state of dedifferentiation, both in terms of duration and level, as has been demonstrated by the elevated and extended activities of lysosomal acid phosphatase (LAP), cathepsin D and gelatinase in limb regeneration of Korean salamander after RA treatment (Ju and Kim, 1994; 1998; 2000; Park and Kim, 1999).

It is intriguing to study the effect of RA on *Fgf-8* expression during limb regeneration because both are associated with patterning in PD axis of regenerating and developing limbs. In the present study, we examined whether RA treatment would affect the expression pattern of *Fgf-8* in the regenerating limbs of Mexican axolotl.

Materials and Methods

Experimental animals and manipulation

Mexican axolotl (*Ambystoma mexicanum*) larvae were obtained from the Sogang University Amphibian Colony and were maintained according to standard laboratory procedures (Kim, 1996). Regenerating limbs of larvae were staged referring to Stocum (1979). Animals were anesthetized in 0.02% (w/v) ethyl *p*-aminobenzoate (benzocaine, Sigma, E1501) prior to limb amputation, retinoic acid injection, or collection of limb regenerates. To initiate the limb regeneration, both forelimbs were amputated at elbow level. After amputation, protruding cartilage was trimmed to create a flat amputation surface to avoid abnormal regeneration. Animals were then returned to water and allowed to regenerate to the desired stage. Regenerates were collected at 0.5 d, 1 d, 2 d (wound healing stage), 4 d (dedifferentiation stage), 7 d (early bud stage), 10 d (medium bud stage), 13 d (late bud stage), 16 d (palette stage), and 20 d (digital outgrowth stage) after amputation.

* To whom correspondence should be addressed.
Tel: 82-2-705-8453 Fax: 82-2-704-3601
E-mail : wskim@ccs.sogang.ac.kr
† Present address:
Dept. of Cell and Molecular Biology,
Tulane University, New Orleans, LA 70118, USA

Expression pattern of Fgf-8 by RT-PCR

To compare the expression of *Fgf-8* in mesenchymal and epidermal tissues by RT-PCR, tissues of 7 d-old regenerates (blastema formation-early bud stage, Table 1) were separated by treatment with 0.2% EDTA in Ca^{+2}-free Holtfreter solution for 30 min for loosening of the epidermis and then the epidermis were peeled off with watchmaker's forceps (Stocum and Dearlove, 1972). Total RNAs were isolated from the mesenchymal and epidermal tissues, and single strand cDNAs were synthesized from each RNA using Expand reverse transcriptase (Roche) and oligo$(dT)_{12\text{-}18}$ primer. 35 cycles of PCR was performed with sense (5′-TGC AGA ATT CAA AGG TAA TGT TCA GTC C) and antisense (5′-CCT AGT CGA CTG TTT TGG TTC CTA TCG C) primers. For normalization, 20 cycles of PCR was performed at 57℃ of annealing temperature for axolotl glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with sense (5′-AAG TGA AGG CTG AGG ACG GT) and antisense (5′-TGC CAG TGA GTT TCC ATT GA) primers.

Administration of retinoic acid

Retinoic acid (RA; all trans; Sigma, R2625) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 40 mg/mL (w/v). The solution was freshly prepared under dim light to minimize photo-oxidation. At 4 d after amputation when the RA effect is most pronounced, animals were injected intraperitoneally via microliter syringe (Hamilton Co., USA) with 150 μg of RA per gram of body weight (Kim and Stocum, 1986).

Probe preparation and whole-mount in situ hybridization

To prepare DIG-labeled *Fgf-8* riboprobe for *in situ* hybridization, the phagemid DNA containing axolotl *Fgf-8* cDNA was linearized with *EcoR*I, and antisense RNA was synthesized and labeled using T7 RNA polymerase and RNA labeling Mix containing digoxigenin-11-UTP (Roche). Whole-mount *in situ* hybridization was performed essentially as described by Han et al. (2001).

Results and Discussion

Expression pattern of Fgf-8 in regenerating limbs

In the developing limbs of higher vertebrates such as chicks and mice, the transcripts of *Fgf-8* have been known to be localized only in the AER. Regenerating limbs and developing limb buds of vertebrates utilize a similar set of signals that regulate pattern formation (Muneoka and Sassoon, 1992). However, it was previously reported that the expression pattern of *Fgf-8* in regenerating limbs of amphibians was different from developing limbs of amniotes (Han et al., 2001). To examine specific expression pattern of *Fgf-8* in regenerating urodele limbs, we carried out RT-PCR with RNAs obtained from the mesenchymal and epidermal tissues. The *Fgf-8* expression was observed in both tissues (Fig. 1). This is consistent with our previous results showing that *Fgf-8* is expressed in the basal layer of the AEC and the underlying thin layer of mesenchymal tissue of regenerating limbs (Han et al., 2001).

Effect of retinoic acid (RA) on expression of Fgf-8 in regenerating limbs

In normal regenerating limbs not treated with RA, *Fgf-8* began to be expressed in the distal mesenchymal tissue of stump at 12 h after amputation (wound healing stage; Fig. 2A). At 2 d after amputation (dedifferentiation stage), its expression was detected in the muscle bundle of the stump (Figs. 2B, 2C). At the blastema formation stage (10 d after amputation), *Fgf-8* transcripts were localized at the distal mesenchymal tissue (Fig. 2D). As limbs were redifferentiated (16 d after amputation), *Fgf-8* expression was profiles gradually decreased (Fig. 2E), and disappeared when the original

Table 1. Comparison of expression level and domains of *Fgf-8* in the normal and RA-treated limb regenerates of axolotl. Samples were harvested at the days indicated by bold numbers

Normal	Day*	0.5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
	Stage	Wound healing			Dedifferentiation			Early bud			Mid bud			Late bud			Palette				Digit				Completion of regeneration				
								Blastema formation									Redifferentiation													
	Expression domain	Mesenchymal tissue of distal stump						Epidermal and mesenchymal tissues of blastema																						
	Intensity	+++		++	++			++			+++			+			-				-				-					
RA	Intensity							++			++			++			+++				++				-				-	
	Expression domain							Distal cartilage			Blastema and proximoposterior region																			
	Stage	Wound healing			Dedifferentiation							Blastema formation													Redifferentiation					
	Day*	0.5	1	2	3	RA 4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28

Day*: Days after amputation. Intensities: +++ (high), ++ (moderate), + (low), - (not expressed)

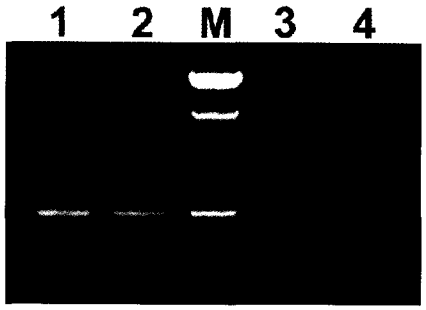

Fig. 1. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of *Fgf-8* in the mesenchymal and epidermal tissues of 7-d old regenerates (blastema formation stage) of 7-9 cm axolotl. 35 cycles of PCR were performed for *Fgf-8* and 20 cycles for GAPDH. 1 and 2. *Fgf-8* expression in the mesenchymal and epidermal tissues, respectively. M. 100 bp DNA ladder marker. 3 and 4. GAPDH expression in the mesenchymal and epidermal tissues, respectively.

limb pattern was almost restored (Fig. 2F; 20 d afer amputation). On the other hand, with RA treatment at 4 d post-amputation, *Fgf-8* expression profiles appeared to be modified in the regenerating axolotl limbs. As com- pared with the normal regenerating limb, at 7 d post- amputation (3 d after RA-treatment) when the regeneration was still in the dedifferentiation stage, the *Fgf-8* signal in RA-treated regenerates was detected in

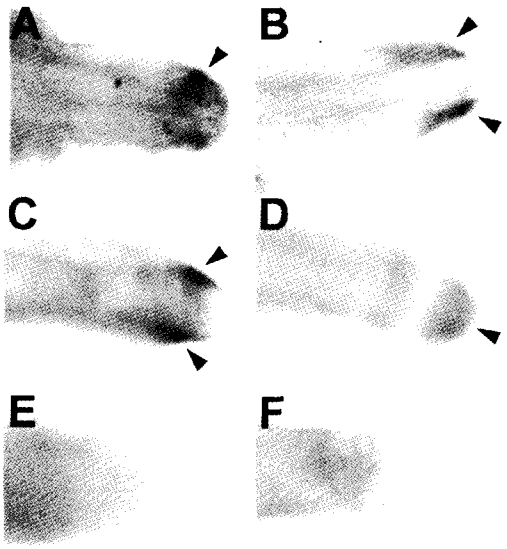

Fig. 2. Expression profiles of *Fgf-8* in normal regenerating limbs of axolotl amputated at the elbow level. A, 12 h after amputation (wound healing stage, early). Strong *Fgf-8* expression is notable in the distal stump tissue near the amputation site (arrowhead). B, 2 d after amputation (dedifferentiation stage, early). Note the high level of *Fgf-8* expression in the stump tissue undergoing dedifferentiation, probably the muscle bundle in the stump (arrowheads). C, 4 d after amputation (dedifferentiation stage, late). The expression pattern is similar to the previous stage but with somewhat stronger intensity (arrowheads). D, 10 d after amputation (medium bud stage). The *Fgf-8* signal is present in the mesenchymal tissue of the limb regenerate (arrowhead). E, 16 d (palette stage). The *Fgf-8* signal is virtually absent. F, 20 d (early digit stage) after amputation.

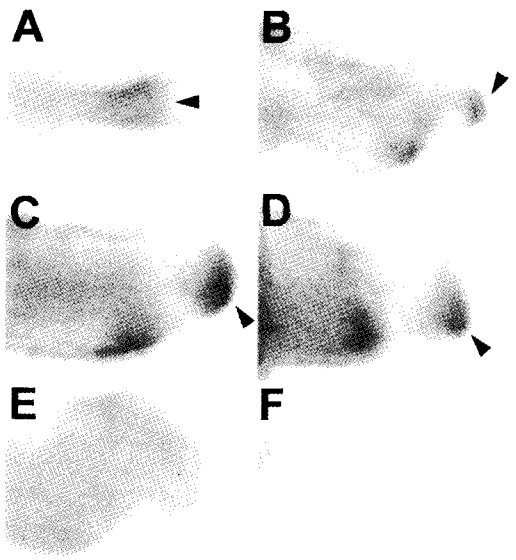

Fig. 3. Expression profiles of *Fgf-8* in retinoic acid (RA)-treated regenerating axolotl forelimbs amputated at the elbow level. A, 7 d after amputation (3 d after RA treatment). Note the strong expression of *Fgf-8* in the distal cartilage and around it (arrowhead). B, 10 d after amputation (6 d after RA treatment). *Fgf-8* signal can be seen in the mesenchymal tissue of blastema (arrowhead). C, 13 d after amputation (9 d after RA treatment). The expression pattern is similar to the previous stage. D, 20 d after amputation (16 d after RA treatment). Note the expression of *Fgf-8* in the mesenchymal tissue of blastema (arrowhead). E, 24 d after amputation (20 d after RA treatment). *Fgf-8* expression begins to disappear in blastema as redifferentiation proceeds. F, 28 d after amputation (24 d after RA treatment). The *Fgf-8* signal is virtually absent in the regenerate.

the distal tip of the amputated cartilage of the stump (Fig. 3A). At blastema formation stage (10-20 d after amputation), *Fgf-8* was expressed in the distal mesenchymal tissue of blastema (Figs. 3B-D). In the RA-treated regenerates, *Fgf-8* expression began to disappear 24 d after amputaion (Fig. 3E). At 28 d after amputation with RA treatment, the signal of *Fgf-8* was virtually absent in the regenerates (Fig. 3F). Overall, regeneration processes were delayed by about 1 week by RA treatment, and the expression pattern of *Fgf-8* paralleled to the delayed regeneration stages. The expression profile of *Fgf-8* in the normal and RA-treated axolotl forelimbs are summarized in Table 1.

RA induces pattern duplication in regenerating urodele limbs (Maden, 1983). In addition, RA affects the expression of several genes including *HoxA13*, *MMP9*, and *Cathepsin D* which are implicated in the dedifferentiation process or pattern regulation process (Gardiner et al., 1995; Ju and Kim, 1994; 1998). Moreover, both RA and FGFs have been known to play a role in differentiation and proliferation of various cell types (Buxton et al., 1997; Voigt et al., 2000). Considering that FGF-8 is a potent signaling molecule for limb development and regeneration, we were interested to find out if RA affects *Fgf-8* expression as well in the regenerating axolotl limb. Our results showed that RA caused extended period of *Fgf-8* expression in limb

regenerates, and that it coincided well with the extended dedifferentiation period by RA treatment as has been reported previously (Ju and Kim, 1994).

Recently, two closely related homeobox genes, *Meis1* and *Meis2*, have been identified as determinants that define the proximal compartment in PD axis of limb (Mercader et al., 1999). Both *Meis1* and *Meis2* are positively regulated by RA and endogenous RA signaling is required for the maintenance of *Meis* expression in the proximal domain of limb (Mercader et al., 2000). Interestingly, expression of *Meis* genes are repressed by FGF-8 bead implantation in developing chick limb bud. Since RA modifies the pattern of PD axis by proximalizing the cells in regenerating limb, it is suggested that RA and FGF-8 signalling might play antagonistic roles in limb regeneration. On the other hand, it has been suggested that the binding of FGFs to low-affinity heparan sulfate proteoglycans (HSPG) present in the ECM is a prerequisite step for its binding to the high-affinity receptor (Vlodavsky et al., 1987; Klagsbrun and Baird, 1991). Binding of FGFs to HSPG provides the cells with a growth factor reservoir (Flaumenhaft et al., 1989). RA is known to cause the release of hydrolytic lysosomal enzymes such as LAP by stimulating exocytosis of lysosomes in mouse liver (Wang et al., 1976), and to increase the activity of LAP during dedifferentiation stage of urodele limb regeneration (Ju and Kim, 1994). Thus, in limb regeneration, exogenously treated RA may also stimulate the release of FGFs including FGF-8 from ECM by discharging them from HSPG by lysosomal enzymes at the dedifferentiation phase. Analysis of amount and distribution of FGF-8 in terms of protein level after RA treatment will elucidate possible causal relationship between the extended dedifferentiation period by RA and FGF-8 localization in blastema.

Acknowledgements

This work was supported in part by the Sogang University Research Grants in 2002 to W.-S. Kim.

References

Buxton PG, Kostakopoulou K, Brickell P, Thorogood P, and Ferretti P (1997) Expression of the transcription factor slug correlates with growth of the limb bud and is regulated by FGF-4 and retinoic acid. *Int J Dev Biol* 41: 559-568.

Crawford K and Stocum DL (1988) Retinoic acid coordinately proximalizes regenerate pattern and blastema differential affinity in axolotl limbs. *Development* 102 :687-698.

Flaumenhaft R, Moscatelli D, Saksela O, and Rifkin DB (1989) Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long-term stimulation of plasminogen activator production and DNA systhesis. *J Cell Physiol* 140: 75-81.

Gardiner DM, Blumberg B, Komine Y, and Bryant SV (1995) Regulation of *HoxA* expression in developing and regenerating axolotl limbs. *Development* 121: 1731-1741.

Gudas LJ, Sporn MB, and Roberts AB (1994) Cellular biology and biochemistry of the retinoids. In: Sporn MB, Roberts AB, and Goodman DS (eds), The Retinoids: Biology, Chemistry and Medicine, 2nd Ed., Raven Press, New York, pp 443-525.

Han MJ and Kim WS (1988) The effects of retinoic acid on the developing chick wingbuds. *Environ Mutag Carrcinog* 8: 47-55.

Han MJ, An JY, and Kim WS (2001) Expression patterns of *Fgf-8* during development and limb regeneration of the Axolotl. *Dev Dyn* 220: 40-48.

Ju BG and Kim WS (1994) Pattern duplication by retinoic acid treatment in the regenerating limbs of Korean salamander larvae, *Hynobius leechii*, correlates well with the extent of dedifferentiation. *Dev Dyn* 199: 253-267.

Ju BG and Kim WS (1998) Upregulation of cathepsin D expression in the dedifferentiating salamander limb regenerates and enhancement of its expression by retinoic acid. *Wound Repair Regen* 6: 349-357.

Ju BG and Kim WS (2000) Cloning of a cDNA encoding cathepsin D from salamander, *Hynobius leechii*, and its expression in the limb regenerates. *DNA Seq* 11: 21-28.

Kim. WS (1996) Amphibian maintenance facilities at Sogang University, Korea. *Axolotl Newsletter* 25: 11-13.

Kim WS and Stocum DL (1986) Retinoic acid modifies positional memory in the anteroposterior axis of regenerating axolotl limbs. *Dev Biol* 114: 170-179.

Klagsbrun M and Baird A (1991) A dual receptor system is required for basic fibroblast growth factor activity. *Cell* 67: 229-231.

Maden M (1982) Vitamin A and pattern formation in the regenerating limb. *Nature* 295: 672-675.

Maden M (1983) The effect of vitamin A on the regenerating axolotl limb. *J Embryol Exp Morphol* 77: 273-295.

Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, and Torres M (1999) Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* 402: 425-429.

Mercader N, Leonardo E, Piedra ME, Martinez-A C, Ros MA, and Torres M (2000) Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of *Meis* genes. *Development* 127: 3961-3970.

Muneoka K and Sassoon D (1992) Molecular aspects of regeneration in developing vertebrate limbs. *Dev Biol* 152: 37-49.

Park IS and Kim WS (1999) Modulation of gelatinase activity correlates with the dedifferentiation profile of regenerating salamander limbs. *Mol Cells* 9: 119-126.

Riddle RD, Johnson RL, Laufer Ed, and Tabin C (1993) *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* 75: 1401-1416.

Saunders JW and Gasseling MT (1968) Ectodermal-mesenchymal interactions in the origin of limb symmetry. In: Fleischmajer R and Billinghams RE (eds), Epithelial-mesenchymal interactions, Willams and Wilkins, Baltimore, pp 78-97.

Stocum DL (1979) Stages of forelimb regeneration in *Ambystoma maculatum*. *J Exp Zool* 209: 395-416.

Tickle C, Alberts B, Wolpert L, and Lee L (1982) Local application of retinoic acid to the limb bond mimics the action of the polarizing region. *Nature* 296: 564-566.

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, and Klagsbrun M (1987) Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. *Proc Natl Acad Sci USA* 84: 2292-2296.

Voigt A, Hartmann P, and Zintl F (2000) Differentiation, proliferation and adhesion of human neuroblastoma cells after treatment with retinoic acid. *Cell Adhes Commun* 7: 423-440.

Wang CC, Straight S, and Hill DL (1976) Destabilization of mouse liver lysosomes by vitamin A compounds and analogues. *Biochem Pharmacol* 25: 471-475.

[Received October 9, 2002; accepted November 8, 2002]