Planarian regeneration in the absence of a blastema

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ABSTRACT. During planarian regeneration, a new tissue called the blastema is formed after amputation and is thought to play an important role in pattern formation. To investigate its role, posterior fragments with irradiated stumps were generated by regional X-irradiation, followed by amputation. No blastema formation occurred in the irradiated fragments until 3 days after amputation, because all the neoblasts had been depleted from the stump area by irradiation. However, regeneration of the pharynx occurred in the predicted region of the fragment, even in the absence of a blastema. These results suggest that the blastema is not essential for pattern formation during planarian regeneration.

KEY WORDS: pattern formation, polarity, intercalary regeneration, neoblasts, platyhelminthes

INTRODUCTION

Freshwater planarians possess many organs, including a central nervous system, a complicated branched gut, a pharynx and protonephridial ducts. Planarians have a defined body pattern and each of these organs is located in a specific position (Hyman, 1951). Planarians also exhibit remarkable regeneration abilities; the head and tail can both regenerate if the anterior or posterior parts are cut off, respectively. Complete animals with body patterns the same as that of the original animal can be regenerated from tiny body fragments derived from any region posterior to the eyes, except the pharynx. When and how does body pattern formation occur during planarian regeneration? Regeneration is divided into several processes (Baguña, 1998). Following amputation, (1) the wound is closed by extension of the epithelial cell layer, (2) a blastema is generated at the wound through the migration and proliferation of neoblasts in the stump below the wound, (3) the blastema grows by subsequent proliferation and migration of the neoblasts, and (4) cell differentiation occurs and new tissue is formed. What is a role of the blastema in these processes? Based on intercalary regeneration observed in grafting experiments, Agata et al. (2003) speculated that intercalation between the blastema and the old stump induced rearrangement of the body regions. According to a previous model, pattern determination occurs in a narrow strip (200–300 μm) of the stump below the wound, as a result of a distal-proximal sequence of induction during the first day of regeneration. The pre-patterning is subsequently amplified and translated into morphological patterning through cell proliferation and differentiation as the blastema grows (Baguña, 1998). Thus, the blastema is thought to play an important role in pattern formation during planarian regeneration.

In this study, regional X-irradiation was used to produce regenerating fragments with no blastema. Regeneration of the pharynx was observed in the same position as in un-irradiated fragments. These results suggest that the blastema does not play an important role in pattern formation during planarian regeneration.

MATERIALS AND METHODS

The isogenetic asexual strain HI of the freshwater planarian *Dugesia japonica* was used in this study (Kato et al., 1999). Animals were maintained at room temperature (about 22°C) in autoclaved tap water and fed with chicken liver once a week. Worms 1.5–2 cm in length were selected and starved for at least 4 days before use. Regional irradiation was performed as described previously (Ito et al., 2001; Orii et al., 2005). Briefly, worms were placed on wet filter paper on ice. The un-irradiated region was covered with a lead sheet, while the remainder was irradiated using a SOFTEX B-4 X-ray generator (Softex) (Fig. 1).

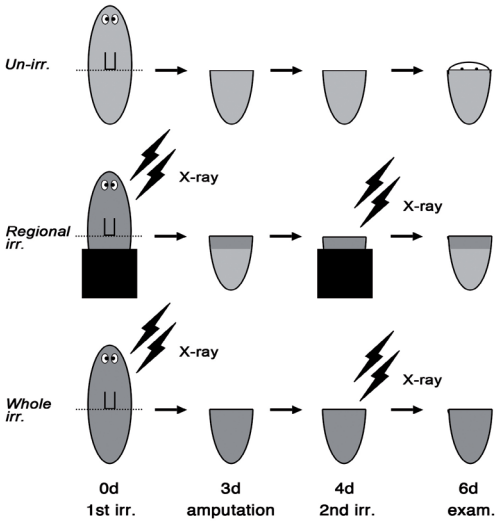

Fig. 1. – Diagram showing irradiation and amputation experiments. The posterior fragments from un-irradiated (upper), regionally irradiated (middle), and completely irradiated planarians (lower) were allowed to regenerate for 3 days. Blastemas developed in un-irradiated fragments, but not in completely or regionally irradiated fragments.

The radiation dose was about 55 R, which is the minimum dose required for complete inhibition of regeneration in this planarian strain (Kato et al., 2001). Three days after irradiation, the planarian was amputated, as shown in Fig. 1, and the posterior fragment was allowed to regenerate at room temperature. On the next day, the stump region was re-irradiated in the same way. The second regional irradiation was performed to deplete the neoblasts in the stump region completely, which resulted in many regenerating fragments with no blastema. Three days after amputation, the fragment was observed under a binocular microscope to confirm the absence of a blastema. The fragment was then fixed, embedded in paraffin, and sectioned, according to the standard protocol (Kobayashi et al., 1998). Sagittal sections were subjected to in situ hybridization using the myosin heavy chain-A *(Djimch-A)*, as a marker gene for the regenerating pharynx, as described previously (Kobayashi et al., 1998; Sakai et al., 2002).

RESULTS AND DISCUSSION

Freshwater planarians possess many pluripotent stem cells, called neoblasts, which account for about 30% of the total cell number (Baguna & Romero, 1981; Orii et al., 2005). Neoblasts are distributed in the mesenchyme throughout the body, except in the pharynx and the region anterior to the eyes (Orii et al., 2005). Neoblasts are sensitive to X-rays, and regional irradiation of planarians results in depletion of the neoblasts in the irradiated part. Regional irradiation was used about 50 years ago to investigate the contribution of neoblasts to planarian regeneration (Wolff, 1962). Using this method, we also previously demonstrated that the distal part of the pharynx was able to regenerate in an epimorphic manner (Ito et al., 2001).

In this study, posterior fragments with an irradiated stump were prepared by regional irradiation followed by amputation. The region where the pharynx would regenerate was not irradiated. Three days after amputation, a small pair of eyes was observable between the blastema and the stump in the fragments derived from un-irradiated planarians (Fig. 2A). As predicted, some fragments with an irradiated stump had no blastema (Fig. 2B). These were similar to fragments from completely irradiated planarians (Fig. 2C). Pharynx regeneration in the fragment with no blastema was examined by in situ hybridization using *Djmhc-A* gene. This gene is strongly expressed in pharynx muscle cells and in pharynx-anchoring muscles, and therefore provides a good marker of pharynx regeneration (Kobayashi et al., 1998; Orii et al., 2002). A rudimentary pharynx generally appears in the tail fragment 2–2.5 days after amputation, and then increases in size (Sakai et al., 2002). Surprisingly, a regenerating pharynx was observed at the normal position in the fragments with no blastema (Figs. 2B and E). In addition, the timing of the regeneration process appeared to be similar, or only slightly delayed, in comparison with that in un-irradiated fragments (Figs. 2D and E). No sign of pharynx regeneration was observed in completely irradiated fragments (Fig. 2F). These results indicate that the blastema is not required for normal pharynx regeneration in posterior planarian fragments.

Until now, the blastema has been thought to play a central role in pattern formation. This theory is based on the results of numerous grafting experiments; a graft from a different region induces new tissue between the graft and the surrounding tissue (Agata et al., 2003). It is possible that such induction occurs in a narrow stump below the wound (Baguna, 1998). However, there is no evidence that pattern formation during regeneration after amputation occurs in same way as intercalary regeneration after grafting.

Hox genes, which are expressed along the anteroposterior axis and are thought to play a role in maintaining body pattern, are also expressed in the posterior blastema. Their expression is concentrated in the posterior part of the posterior fragment after amputation (Orii et al., 1999). The

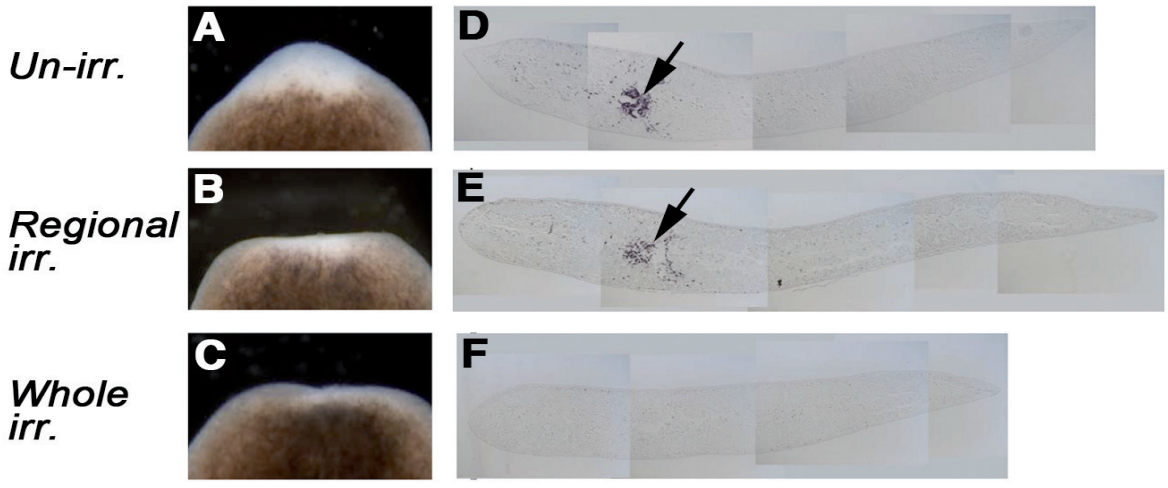

Fig. 2. – Blastema formation and regeneration of the pharynx in the irradiated posterior fragments 3 days after amputation. (A-C), dorsal view of the wound region of the regenerating fragment. (D-F), in situ hybridization of sagittal sections with *Djmhc-A*. Arrowheads indicate regenerating pharynx. (A) and (D), un-irradiated fragment. (B) and (E), fragment with irradiated stump. (C) and (F), completely irradiated fragment.

BMP gene, a key player in dorsoventral patterning, is also expressed in non-regenerating animals (Orii et al., 1998). Thus it is difficult to distinguish between non-regenerating and regenerating animals on the basis of expression of the genes involved in pattern formation.

Wound closure, rather than the blastema, may play an important role in pattern formation. Wound closure and pattern formation occurred even in regenerating fragments with no blastema (Figs. 2B and E). Kato et al. (1999) demonstrated that interactions between dorsal and ventral tissues at the wound could act as a trigger for regeneration and had an important role in blastema formation. Chandebois (1979 & 1980) suggested that contact between the dorsal and ventral epidermis stimulated regeneration, and that the manner of the wound closure played a key role in determining the anteroposterior pattern. It is possible that wound closure acts as an organizer and plays a key role in pattern formation. Based on the results of the present study, I propose the hypothesis that pattern formation occurs in the whole fragment after amputation (Fig. 3). The original pattern is cancelled following amputation and new pattern formation occurs throughout the whole fragment after wound closure. The new pattern may not be formed as the result of an induction sequence, such as that seen during intercalary regeneration between a graft and the surrounding tissue. Thus, the new pattern is not restricted to the stump and blastema. The pluripotent neoblasts then differentiate according to the new pattern. The formation and growth of the blastema is independent of pattern formation.

It has recently been reported that silencing of Wnt signaling by RNA interference for the β-catenin gene resulted in transformation of tail to head characteristics (Gurley et al., 2008; Iglesias et al., 2008; Petersen & Reddien, 2008), while up-regulation of Wnt signaling conversely resulted in transformation of head to tail characteristics (Gurley et al., 2008). These results suggest that Wnt signaling is involved in pattern formation along the anteroposterior axis. However, the temporal and spatial regulation of this signaling mechanism remain to be determined. It is also needed to compare the bases of pattern formation between planarians and other animals with good regeneration abilities, such as hydra and newts.

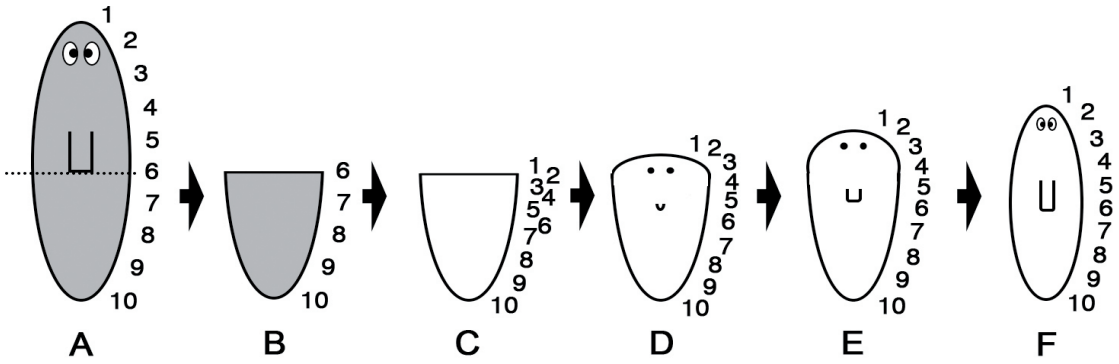

Fig. 3. – A model for pattern formation during regeneration of the posterior fragment. (A) An intact planarian grows according to this pattern. The dotted line indicates the level of amputation. (B) A posterior fragment isolated by amputation. (C) A new pattern is reformed throughout the fragment after wound closure, but before blastema formation. (D) Blastema formation with cell differentiation according to the new pattern. (E) Regeneration including reformation of old tissues proceeds as the blastema grows. (F) Finally, the fragment becomes a small animal with the same shape as the original animal. The pattern is maintained. The regionality of the intact body is indicated by the numbers. 1, 5-6 and 10 indicate the anterior tip, middle part and the posterior tip, respectively.

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REFERENCES

Agata K, Tanaka T, Kobayashi C, Kato K & Saitoh Y (2003). Intercalary regeneration in planarians. Dev. Dyn., 226: 308-316.

Baguna J & Romero R (1981). Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina.* Hydrobiologia, 84: 181-194.

Baguna J (1998). Planarians. In: Ferretti P & Geraudie J (eds), Cellular and molecular basis of regeneration: from invertebrates to human, John Wiley & Sons Ltd: 135-165.

Chandebois R (1979). The dynamics of wound closure and its role in the programming of planarian regeneration. I. Blastema emergence. Dev. Growth Differ., 21: 195-204.

Chandebois R (1980). The dynamics of wound closure and its role in the programming of planarian regeneration. II. Distalization. Dev. Growth Differ., 22: 693-704.

Gurley KA, Rink JC & Sanchez Alvarado A (2008). β-Catenin defines head versus tail identity during planarian regeneration and homeostasis. Science, 319: 323-327.

Hyman LH (1951). The Invertebrates: Platyhelminthes and Rhynchocoela. McGraw-Hill Inc., New York.

Iglesias M, Gomez-Skarmeta JL, Salo E & Adell T (2008). Silencing of *Smed-βcatenin1* generates radial-like hypercephalized planarians. Development, 135: 1215-1221.

Ito H, Saito Y, Watanabe K & Orii H (2001). Epimorphic regeneration of the distal part of the planarian pharynx. Dev. Genes Evol., 211: 2-9.

Kato K, Orii H, Watanabe K & Agata K (1999). The role of dorsoventral interaction in the onset of planarian regeneration. Development,126: 1031-1040.

Kato K, Orii H, Watanabe K & Agata K (2001). Dorsal and ventral positional cues required for the onset of planarian regeneration may reside in differentiated cells. Dev. Biol., 233: 109-121.

Kobayashi C, Kobayashi S, Orii H, Watanabe K & Agata K (1998). Identification of two distinct muscles in the planarian *Dugesia japonica* by their expression of myosin heavy chain genes. Zool. Sci., 15: 855-863.

Orii H, Kato K, Agata K & Watanabe K (1998). Molecular cloning of bone morphogenetic protein (BMP) gene from the planarian *Dugesia japonica,* Zool. Sci., 15: 871-877.

Orii H, Kato K, Umesono Y, Sakurai T, Agata K & Watanabe K (1999). The planarian HOM/HOX homeobox genes *(Plox)* expressed along the anteroposterior axis. Dev. Biol., 210: 456-68. Orii H, Ito H & Watanabe K (2002). Anatomy of the planarian Dugesia japonica. I. The muscular system revealed by antisera against myosin heavy chains. Zool. Sci., 19: 1123-1131.

Orii H, Sakurai T & Watanabe K (2005). Distribution of the stem cells (neoblasts) in the planarian *Dugesia japonica.* Dev. Genes Evol., 215: 143-157.

Petersen CP & Reddien PW (2008). *Smed-β catenin*-1 is required for anteroposterior blastema polarity in planarian regeneration. Science*,* 319: 327-330.

Sakai T, Kato K, Watanabe K & Orii H (2002). Planarian pharynx regeneration revealed by the expression of myosin heavy chain-A. Int. J. Dev. Biol., 46: 329-332.

Wolff E (1962). Recent researches on the regeneration of planaria. In: Rudnick D (ed), Regeneration. 20th growth symposium, Ronald Press, New York: 53-84.