Studies on the Regeneration of Epidermis in Fresh-water Planarian, *Dugesia japonica*

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**Introduction**

In fresh-water Planarians it has been shown previously that diverse tissues are differentiated from neoblasts, but with nerve, epidermis, and intestine there have been some uncertainties about their neoblast origin. Uchida (unpublished) and Sugino and Uchida (1964) observed that when fresh-water planarians were cut transversely, or when a patch of their epidermis was removed, the epidermis of the adjoining regions became thinner, covering the wound to form the epithelioid; under which differentiating cells of neoblast origin lined up, ultimately to become the new epidermis after the falling off of the epithelioid. Kido (1966), however, insisted that in the regeneration of epidermis the epithelioid did not fall off, but thickened and itself became the new epidermis; in his opinion neoblasts formed only the basal membrane. Uchida followed the regeneration process in detail for 24 hours after cutting, but he did not follow the further process of formation of new epidermis. Sakamoto and Tsutsumi (unpublished) also tried to find evidence of the neoblast origin of regenerated epidermis, but has not succeeded in establishing the neoblast origin of new epidermis, in the face of strong opposition by some authors. The purpose of the present study was to clarify these uncertainties, by elucidating the origin of regenerated epidermis, as well as the process of regeneration of epidermis and basal membrane. Experiments were carried out between March 1967 and February 1968.

**Materials and Methods**

The fresh-water planarians *Dugesia japonica* ICHIKAWA et KAWAKATSU,
used in this study, were collected from the following districts. 1) Nagano prefecture (Otaki-mura), September 1966 ~ October 1967. 2) Osaka prefecture (Ushitaki), November 1966 ~ July 1967. They were kept in glass baths containing filtered tap water (pH 7.0 ~ 7.2) at temperatures between 18° ~ 25°C in the dark. They were given with chicken liver or crushed crawfish once a week, and after each feeding the water changed twice.

For experiments healthy animals that has not divided recently, of about 20mm in length, were selected and starved in separate baths for over 1 week prior to operation. Operations were performed upon filter paper soaked with 1/50 Ringer solution, placed upon an ice box, with a knife for ophthalmic use.

1. Stripping experiment or delamination.

Animals were anesthetized with 0.2% aqueous solution of chloretone. The epidermis of prepharyngeal region was removed in a patch of 1.5mm long by 1mm wide, as in Fig. 1, A. After operation, the animal was placed in a Petri-dish together with the filter paper; 1/50 Ringer solution was added to the Petri-dish, to provide appropriate moisture, with care being taken that it does not touch the wound. The operated animal was kept in this condition at 17° ~ 21°C during the regeneration process, until fixed for observation. In order to observe cell division, some of the operated animals were treated for 48 hours with 1/3000M colchicine, after they had been kept in a refrigerator (9.7°C) for 24 hours following operation in 1/50 Ringer solution. After the colchicine treatment they were kept in 1/50 Ringer solution at 20°C for 1 ~ 10 days.

2. Transverse section.

Two types of transverse section experiments were performed. In one type, the animal was cut in the prepharyngeal as well as in the postpharyngeal region, as in Fig. 1, B. In the other type of experiment, the animal was cut in the prepharyngeal region as in Fig. 1, C. The operated animal was kept during the regeneration process in a Petri-dish containing 1/50 Ringer solution at 20° ~ 22°C until they are fixed for observation. Some of the operated animals were treated with 1/3000 M colchicine for 24 hours immediately following the operation, and after the treatment were kept at 20°C in 1/50 Ringer solution.
The animals were anesthetized by cooling to \(-2^\circ\text{C}\), and were fixed with either Bouin's solution, Carnoy's solution or 2\% glutaraldehyde. Serial sections 5 ~ 7\(\mu\) thick were stained for observation with Delafield's haematoxylin and eosin, or Giemsa's solution.

**Observations**

1. Falling off of epithelioid. The wound surface shrank in some minutes after a transverse cut, and in 30 minutes a thin membrane had extended from the edge of the wound to cover the wound surface. This thin membrane designated epithelioid (Lindh 1957; Kido 1965) consisted of cells containing flat nuclei and crushed rhabdites. The epithelioid could be still observed at 19 hours after cutting (Pl. I Fig. 1). From this time neoblasts gradually accumulated under the epithelioid. Neoblasts are large spindle-shaped or pyriform cells with a large nucleus, in which one nucleolus is observed.

Their cytoplasm stains blue by the Giemsa's solution, whereas in the nucleus only the chromatin is stained blue.

After 20 hours of cutting the epithelioid fell off, pushed up by the increasing number of differentiating cells derived from neoblasts. In some instances the edge of the fallen off epithelioid had thickened (Pl. I Fig. 2, 3). In other cases the edge of the epithelioid was wedge-shaped (Fig. 2; Pl. I Fig. 4), while still in other cases the epithelioid fell off from the wound in many small fragments. At this time the differentiating cells that had accumulated under the epithelioid were lining up in two layers (Fig. 3; Pl. I Fig. 5).

2. Differentiation of neoblasts into epidermis and basal membrane.

![Fig. 2. 20 hours after cutting. \(\times 400\). Note that the end of epithelioid is wedge-shaped, rh, rhabdite; ep, epithelioid.](image)

![Fig. 3. 25.5 hours after cutting. \(\times 200\). The edge of burst epithelioid are wedge-shaped.](image)
The two layers of neoblast cells under the epithelioid were still uniform in shape 24 hours after cutting. Usually the underlying layer was still sparse at this period. After cutting thirty hours, the upper layer cells had thickened but still spindle-shaped. As the neoblast layer gained in thickness the epithelioid was pushed up, finally forming a fissure, and in it differentiating cells were exposed to the surface. It was often observed that near the cut end of the old epidermis differentiating cells entered in the epithelioid, side by side with old epidermis cells (Fig. 4, 5; Pl. II Fig. 7, 8). The underlying layer had not shown any change, except that its cell number had increased.

Thirty five hours after cutting, the upper neoblast layer had increased further in thickness, and its cells became cubic. The underlying layer began to flatten around this time, and was clearly distinguishable from the upper layer. In cells of the upper layer, small and round bodies called rhabdites began to appear also around this time (Fig. 6; Pl. II Fig. 9).

Forty five hours after cutting, the cells of the upper layer grew a little taller, and rhabdites had become clearer (Pl. II Fig. 10).

Fig. 4. 36 hours after cutting. ×400. Neoblasts have entered in the epithelioid, as well as at the end of the wound.

Fig. 5. 39 hours after cutting. ×1000. Neoblasts have entered in the epithelioid. Neoblasts in the underlying layer have somewhat flattened.
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Fig. 6. 45 hours after cutting. ×400. Neoblasts have burst the epithelioid. Neoblasts in the left have features approaching those of epidermis cells. rh, rhabdite.

Fifty hours after cutting, intercellular spaces had formed between cells of the upper layer. At this stage, the new epidermis was almost completed, but cilia had not yet appeared. Scaly fragments of the thin membrane or epithelioid had fallen off, but old and new epidermis could be clearly distinguished by the difference in the degree of staining with the Giemsa’s solution. The underlying layer had become more and more flat, and connection with the adjacent cells had formed in some places. Their nuclei could still be clearly distinguished also by haematoxylin staining (Fig. 7; Pl. II Fig. 11).

Sixty hours after cutting, both the epidermis and the basal membrane were almost complete. The new epidermis could still be distinguished from the old epidermis by the difference in the degree of staining with Giemsa’s solution, and by the absence of cilia in the ventral epidermis; but the shape and magnitude of cells were almost indistinguishable (Fig. 8; Pl. II Fig. 12).

3. Division of neoblast.

During this study, special attention was paid to division of neoblasts under
Considerations and Conclusions

1. On epithelioid There are some opinions on the origin of regenerated epidermis of fresh-water planarians. It is generally agreed, that the first step of regeneration consists in the formation of a thin membrane, by the extension end of the damaged epidermis. This thin membrane is called either epithelioid or the primary epidermis. This origin of epithelioid is also clear from the fact that it has cilia, and also that it contains rhabdites and complete flat nuclei. It seems that rhabdites plays some part in the formation of the epithelioid.

When the animal is cut transversely, it has been observed that the epithelioid formed will fall off within 20 hours (Uchida unpublished; Sugino and Uchida 1964). The present investigation confirmed this observation. Thus, at 17 hours after cutting, most of the animals still retain the epithelioid; later than this, however, neoblasts that have accumulated under the epithelioid push it upward, until a fissure is made and the epithelioid fall off. Three types are observed in the mode of falling off of the epithelioid. In the first type, the edge of the epithelioid becomes wedge-shaped, rolls off to fall off. In the second type, the edge of the epithelioid contracts and looks as if it had thickened. In the third type, the epithelioid falls off in small scurfy fragments torn by invading neoblasts. These different modes of falling off of the epithelioid seem to be conditioned by the shape of the wound at the tissue of falling off. Thus the first type described above was observed when the section was parrallel to the fissure in the epithelioid; whereas the second type was observed when the direction of section was at right angle to the fissure. Sometimes the epithelioid was still observed at 40 hours after cutting.
2. Origin of new epidermis and stages in regeneration

There are some opinions on the origin of the new epidermis. Thus,

1) the epithelioid that is formed by the extension of the neighboring old epidermis grows in thickness to form the new epidermis (Kido 1966).

2) After the falling off the epithelioid, the edge of the old epidermis is rejuvenated to grow, ultimately covering the wound surface (Kido 1965).

3) After the falling off of the epithelioid, the upper layer of neoblasts accumulated under it differentiates into the new epidermis, while the underlying layer secretes the basal membrane (Lindh 1957; Sugino and Uchida 1964).

The first theory is based upon the assumption that the epithelioid remains and does not fall off. It was clearly observed to fall off. Moreover, at no time after cutting was observed a state in which the transition of epithelioid to epidermis was evident. Kido insists that the falling off of epithelioid is due to artefact of fixation, but the fixative used in our study, glutaraldehyde, is identical with the one used by Kido. From this it is clear that his assumption is erroneous. If the epithelioid became the new epidermis, the upper layer of neoblasts should form the basal membrane, which is inconsistent with the fact that it grows in thickness with time.

The second theory enumerated above would require that the cells of the old epidermis must divide for it to cover the wound, resulting in increased mitosis at the edge of the wound. Observations with colchicine-treated specimens revealed no mitotic figure at the periphery of the old epidermis, in spite of a significant increase in mitosis in other types of cells, in comparison with untreated samples. Moreover, this second theory would require that the regeneration proceeds only from the periphery of the wound surface, contrary to the observed fact that regenerated epidermis also appears in the center of the wound, which joins with the regenerating tissue from the periphery. Also the great difference in the degree of staining by Giemsa's solution between the new and the old epidermis at the junction speaks against a growth of the old epidermis. The two theories discussed above require that the upper layer of neoblasts form the basal membrane, which is contrary to the observed fact.

According to the third hypotheses, the epithelioid falls off, and the upper layer of the neoblasts that have accumulated under the epithelioid goes to form the new epidermis, while the basal membrane formed from the underlying layer. Our observations revealed that the upper layer of the neoblasts
accumulated under the epithelioid gradually gained in thickness at about 20 hours after cutting, forming a fissure in the epithelioid; at 45 hours after cutting, the differentiating cells were cube-shaped, containing well-developed rhabdites; at 50 hours after cutting, intercellular space had been formed, and the differentiating cells had almost differentiated into the epidermis. It requires some more time for cilia to become visible, and 70 hours seems to be required in all for the completion of the new epidermis. It is clear that the origin of the new epidermis cells is the neoblasts, since, until the time when the differentiating cells have become cube-shaped, the epithelioid still remains, and there is a difference in the degree of staining by the Giemsa's solution. At 30 hours after cutting, the cells of the sparse underlying layer of neoblasts gradually become flat, and at 50 hours they are interconnected. At 60 hours after cutting, they have already secreted the basal membrane, and nuclei are no longer visible.

The variation observed in the time required for differentiation from the neoblasts seems to be conditioned by the variation in temperature.

From all the above considerations, we suggest the third theory described above. It may be added that observations with living animals undergoing regeneration decisively support the above conclusion. The cause of the differentiation of the neoblasts remains investigated.

3. Growth of the new epidermis For the growth of the new epidermis with the growth of the regenerating region of the animal, two possible methods are conceivable, namely, 1) by division of the epidermis cells, or 2) the migration of neoblasts through the basal membrane. The first possibility is excluded by the result of colchicine treatment, in which mitotic figures could not be detected. On the contrary, the second possibility is supported by frequent observations of neoblasts passing through the basal membrane, to form epidermis cells. It is known in the young of Polycelis tenuis that the neoblasts form the epidermis passing through the basal membrane (Skear 1965). Our conclusion is that it is also the case with regenerated epidermis.

4. Growth of the old epidermis With the growth of the animal the area of the old epidermis also increases, which means that the cell number of old epidermis must also increase by some means. For this two different possibilities are conceivable just as in the case of the new epidermis; namely, 1) division of epidermis cells, or 2) migration of neoblasts. According to a personal communication from Teshirogi, mitosis was observed also in the
epidermis. In our study, however, no mitosis could be observed in the old epidermis. Therefore, division of old epidermis cells, if any, does not seem to be sufficient to account for all the growth of the old epidermis. In the old epidermis, sometimes, was observed cells that stained well with Giemsa's solution, showing characteristics of the neoblasts. Although in our study observations of migration of neoblasts through the basal membrane to form epidermis were not very numerous, still we consider that also in the old epidermis growth is effected by the migration of neoblasts.

**Summary**

1. This study was performed in order to elucidate the origin of regenerated epidermis, as well as the regeneration process, in the Japanese fresh-water planarian, *Dugesia japonica* Ichikawa et Kawakatsu. For this purpose, regeneration of epidermis after transverse cutting of the animal at the prepharyngeal or postpharyngeal region, and after removal of a small patch of dorsal epidermis, was observed.

2. In thirty minutes after operation, an epithelioid has formed by the extension of the adjacent old epidermis, to cover the wound surface, at about 17 hours after cutting, accumulation of neoblasts begins, resulting in the falling off of the epithelioid. The upper layer of the neoblasts differentiates into the new epidermis within 50 hours after cutting.

3. With the growth of the upper layer, the differentiating cells in the underlying layer become flat, clearly distinguishable from those of the upper layer, at about 40 hours after cutting. These flat cells secrete the basal membrane, which is complete in about 60 hours after cutting.

4. The distinction between the new and the old epidermis was made by difference in the degree of staining with the Giemsa's solution, and by the absence or presence of the basal membrane, which was revealed by haematoxylin staining.

5. From these observations it is concluded that the new epidermis originates from the neoblasts.

6. The growth of the new epidermis is considered to be effected by the migration of additional neoblasts passing through the basal membrane, because, first, few mitotic figure was detected in the new epidermis even in colchicine-treated samples, and, second, neoblasts were after observed passing
through the basal membrane. We believe that similar conclusion holds also for old epidermis, but this point still requires further confirmation.

References


PLATE I

Fig. 1. 19 hours 40 minutes after cutting. ×400. Bouin fix., haematoxylin stain. Epithelioid formed. ep, epithelioid
Fig. 2. 19 hours after cutting. ×400. Guuteraldehyde fix., Giemsa stain. Epithelioid burst and retracted. ep, edge of burst epithelioid
Fig. 3. 19 hours after cutting. ×400. Carnoy fix., haematoxylin stain. Epithelioid burst, the edge of which contracted. ep, edge of burst epithelioid
Fig. 4. 20 hours after cutting. ×400. Bouin fix., Giemsa stain. The burst edge of epithelioid is wedge-shaped. Same as in Textfig. 2.
Fig. 5. 25 hours 30 minutes after cutting. ×400. Bouin fix., haematoxylin stain. The edges of burst epithelioid are wedge-shaped. Same specimen as in Textfig. 3. ep, edge of epithelioid
Fig. 6. 22 hours 40 minutes after cutting. ×400. Bouin fix., Giemsa stain. Epithelioid is just falling off in scurfy fragments, neoblasts are accumulating.

PLATE II

Fig. 7. 36 hours after cutting. ×400. Bouin fix., Giemsa stain. Differentiating cells have entered in the epithelioid, as well as at the end of the wound. Same as in
PLATE II

7

8 dc

9

10

11 ec

12

13 m2

m1
Textfig. 4.

Fig. 8. 39 hours after cutting. $\times 1000$. Bouin fix., Giemsa stain. Differentiating cells have entered in the epithelioid. Differentiating cells in the underlying layer have somewhat flattened. Same as in Textfig. 5. dc, differentiating cell entered in the epithelioid.

Fig. 9. 45 hours after cutting. $\times 400$. Bouin fix., Giemsa stain. Differentiating cells have burst the epithelioid. Differentiating cells in the left have feature approaching those of epidermis cells. Same as in Textfig. 6.

Fig. 10. 45 hours after cutting. $\times 800$. Bouin fix., Giemsa stain. Boundary of old epidermis and wound.

Fig. 11. 52 hours after cutting. $\times 400$. Bouin fix., Giemsa stain. Differentiating cells have formed epidermis. Cells of the underlying layer that will secrete the basal membrane have become flat. The old epidermis in the right stain differently. Same as in Textfig. 7. ec, almost complete epidermis cell.

Fig. 12. 57 hours after cutting. $\times 1000$. Bouin fix., haematoxylin stain. New epidermis is completely formed. Nuclei still visible in the basal membrane forming cells. Same as in Textfig. 8.

Fig. 13. 22 hours after cutting. $\times 400$. Bouin fix., haematoxylin stain. Mitosis figures of differentiating cells just under the fallen off epithelioid are seen. $m_1$, mitosis of differentiating cell $m_2$, telophase of newly formed epidermis.