Research Paper

Contribution of Subcutaneous Connective Tissues to the Epithelialization and Cyst Formation by the Skin Transplanted Subcutaneously

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KEY WORDS

skin, epithelialization, transplantation, GFP, epidermis, cyst

ABSTRACT

Skins and hollow organs have been shown to form epithelialized cysts when transplanted into subcutaneous tissue of a recipient animal, expanding their surface areas. This system seems to offer a good potential for regenerating organs. We investigated the functional and structural contribution of epithelia and connective tissue compartments in this regeneration system with two experimental systems.

Disperse-separated epidermis often forms epithelialized cysts when combined with dermal connective tissue whereas disperse-separated epidermis alone does not form cysts or epithelialize, indicating the functional importance of the dermal connective tissue in the regeneration process.

When GFP rats were used as donors for the skin, the donor-derived tissue was composed of whole epidermis and parts of the connective tissue cells and blood vessels under the newly epithelialized portion of the cyst wall. Small capillaries of granulation tissues were shown to be of recipient origin, but some large vessels were of donor origin. These results showed the significant functional and structural contribution of dermal connective tissue in the regeneration of the skin in subdermal transplant.

INTRODUCTION

Induction of regeneration of various organs is an important and promising area for future medicine. Many attempts have been made to this end using stem cells and cells in culture, and this is one of the most thriving areas of current medicine.¹⁻⁵

We have shown that, when implanted in the subcutaneous tissue⁶ or other locations of the host,⁷ many hollow organs and the skin can produce cysts whose inner surfaces are covered with the epithelia. In these cases, the area of the epithelia is almost doubled, indicating its usefulness of this method of implantation in expanding the surface area of these organs.

Cyst formation and epithelialization comprise several processes; mobilization of the epithelia, formation of granulation tissues as a scaffold for the epithelia, movement of the epithelia, growth of cells to fill the expanded surface area and other processes not discussed further here.

The process of regeneration is often viewed as being analogous to the process of development.^{8,9} In the developmental process including the movement of the epithelium, the epithelium often crosstalks with the underlying layers i.e., mesenchymal tissues, in a process called epithelial-mesenchymal interaction. The mutual interaction between the epithelium and the mesenchyme is often indispensable for many phenomena to occur.¹⁰⁻¹²

In this report we investigated the relationship between the epithelial and mesenchymal compartments in the cyst formation and epithelialization processes. We took two approaches: reconstruction of skin tissues after dissociating the epidermis and the dermis by enzymatic means to see the influence of the mesenchymal compartment on epithelial migration; and grafts of GFP-transgenic rats to show the contribution of the donor epithelium and mesenchyme in the epithelialized cysts. The results indicated a significant functional and structural role played by the dermal connective tissue of the donor tissues.

MATERIALS AND METHODS

Tissues and operation. Skins of 0–4 day old Wistar rats were cut to approximately 5 mm x 5 mm in size and digested with 0.2% dispase (Godo Shusei, Tokyo, Japan) in PBS (137 mM NaCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄) at 37°C for 2 hrs. After digestion, the epidermis and the dermis which were almost separated, were peeled off with fine forceps. As controls, skins which had been

Figure 1. AUTHOR please provide figure legend.

incubated only with PBS, and skins which had been incubated with enzymes but not peeled off were used. We used perinatal rats, in which the skin appendages are not well-developed, allowing contamination of dermal tissues into the epidermal fraction to be minimized.

Donor tissues thus prepared were transplanted between the back skin and back muscles of adult Wistar rats as described previously (Fig. 1).⁶ All the operations were done under pentobarbital anesthesia.

GFP-transgenic Wistar rats were produced as described previously.¹³⁻¹⁴ The skins of the 4 day-old GFP-rats were dissected out and used as donor tissues as above.

Light microscopy. Ten days after the operation, the recipient rats were killed with overdoses of ether anesthesia, and the donor tissues with adjacent recipient tissues were dissected out and fixed with PLP fixative¹⁵ for 6 hrs at room temperature or overnight at 4°C. The tissues were immersed in 30% sucrose in PBS overnight, embedded in OCT compound and frozen with isopentane cooled with liquid nitrogen. Cryostat sections, 10 micrometers in thickness, were made from frozen tissues, and subjected to Hematoxylin-Eosin staining or immunofluorescence microscopy. After staining or immunolabeling, the sections were observed and photographed on an Olympus BH2 microscope equipped with an epifluorescence illumination device. Rabbit anti-laminin antibody was raised in our laboratory,¹⁶ and rabbit anti-von Willebrand factor antibodies were obtained from DAKO (Glostrup, Denmark) and Sigma (St. Louis, MO). Alexa 546 anti-rabbit IgG was purchased from Molecular Probes Inc. (Eugene, OR).

The efficiency of cyst formation and epithelialization was evaluated 10 days after the operation as shown in Table 1. O indicates that transplants had formed cysts or were forming cysts larger than the original transplants. Δ indicates that transplants persisted but had not formed cysts. × indicates that transplants had disappeared or did not seem viable.

Semithin epon sections and electron microscopy. Some of the PLP fixed specimens were refixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 overnight at 4°C, rinsed with phosphate buffer, postfixed with 1% osmium tetroxide in phosphate buffer, block stained with 2% uranyl acetate for 20 min at room temperature, dehydrated with a graded series of ethanol, and embedded in Epon 812.

Figure 2. AUTHOR please provide figure legend.

Table 1 **Effect of disperse treatment and separation of epidermis and dermis**

	PBS-treated Skin	Disperse-treated Skin	Epidermis + Dermis	Epidermis only
○	6	4	4	0
Δ	0	1	6	4
×	0	1	2	8
Total	6	6	12	12

Semithin sections were cut on a Reichert Ultracut N ultramicrotome, stained with toluidine blue, and observed and photographed on a BH2 microscope. Ultrathin sections were also cut on the ultramicrotome, stained with uranyl acetate and then with lead citrate, and observed on a Hitachi H7500 transmission electron microscope (Hitachi Co. Ltd., Tokyo, Japan) operated at 80 kV.

RESULTS

By disperse digestion, the epidermis and dermis were completely separated. Electron microscopy showed that the tissues were separated at the epidermal basal lamina, and the resulting epidermis and the dermis seemed to remain almost intact (Fig. 2).

In disperse-treated skins, if the epidermis and dermis had not been separated (Fig. 3B), the rate of cyst formation and epithelialization was fairly high (4 of 6 cases) as with intact skin (6 of 6 cases) (Fig. 3A), showing the low toxicity of disperse (Table 1). After separating the disperse-treated skins into dermis and epidermis and rejoining them, cyst formation and epithelialization often occurred although the rate of success was lower (4 of 12 cases) than in unseparated specimens (Fig. 3C). Occasionally unsuccessful cases with disorganized many cysts corresponding to hair follicles were observed (Fig. 3D). They were composed of concentric layers of epidermal cells sometimes seen continuous to the epidermis, and often contain remnants of hair shafts. When the epidermis alone was transplanted, none of 12 cases showed cyst formation or epithelialization during the 10 days experiment (Fig. 3E), indicating the significant importance of the existence of donor dermal tissues in these regenerative processes.

Figure 3 (Above). AUTHOR please provide figure legend.

Figure 4 (Above). AUTHOR please provide figure legend.

With the skin of GFP-transgenic rats, we could trace the contribution of donor tissues. We did not use adult GFP-rats, since the expression of GFP often attenuates with age¹⁴ and adult GFP-rats may not show sufficient GFP activity. The donor-derived tissue can be easily recognized under a fluorescence microscope. The epithelialized epidermis was shown to be of donor origin as expected (Figs. 4 and 5). Several structures also showed GFP fluorescence. In the newly formed dermis, short irregular elongated structures

probably indicating dermal fibroblasts showed GFP fluorescence (Fig. 5B). They made loose clusters in only a limited area of the dermis (Fig. 4, arrowheads). Probably the rest of the newly formed dermis was derived from the recipients. Another structure positive for GFP fluorescence was circular structures in the dermis. In order to investigate the possibility of blood vessels, we stained the sections with anti-von Willebrand factor or with anti-laminin antibodies. The latter gave a more clear result. In the dermal connective tissue, circular immunofluorescence with anti-laminin antibodies of two different sizes was observed. The smaller ones did not show GFP fluorescence, whereas the larger ones sometimes showed GFP fluorescence. We considered that smaller circular structures were blood capillaries of granulation tissues derived from the recipient, and the larger ones showing GFP reaction were continuation of donor derived blood vessels larger than the capillaries of granulation tissues. We could not obtain evidence to determine whether or not the two kinds of blood vessels were connected with each other.

DISCUSSION

Transplantation of portions of organs in appropriate areas in the body often induces regenerative reactions of the organs.^{6,7} This activity seems to be stronger in young animals,¹⁷ but still remains in the adult tissues.^{6,7} Although these regenerative processes are not the same as the organogenesis in embryo, and the present system is a simple expansion of preexisting parts without complex phenotypic changes in the cells,

there is also a possibility that these processes may use the same machineries, such as epithelial-mesenchymal interaction.

In the first set of experiments, dispase was shown to be an appropriate means of dissociating the epidermis and dermis of perinatal rats. Significant cyst formation and epithelialization occurred in dispase-treated, separated, and reconstituted skin. Some of the unsuccessful cases seemed to be due to the inappropriate orientation of the tissues during reconstruction (see Fig. 3D). The results for dispase-treated, separated, epidermis alone, however, were very poor. Many of the epidermis grafts disappeared during the 10 day experiment, and at best, persistent existence of the original tissue without significant expansion was observed. This means that the dermal connective tissue plays a very important role in this regeneration process. We could not exclude, however, the possibility that in the long run the epidermis alone can form cysts and epithelialize. In this context, we sometimes observed that even in whole skin transplantation, epidermis alone moves far beyond the donor-derived dermal tissues, and in culture systems, epidermal cells can move on a culture dish in the absence of mesenchymal cells.¹⁸ These facts may indicate that epithelial tissue might have the ability to form cysts without mesenchymal connective tissues, but its ability was very much strengthened by the influence of the mesenchymal compartment. The nature of mesenchymal influence was not clear but fibroblasts accelerate reepithelialization of the epidermis,¹⁹ and dermal connective tissue influence epithelial tissue to differentiate into epidermal appendages in a similar system.²⁰ Various growth factors and cytokines are secreted from dermal connective tissues, and members of the FGF and EGF families are known to induce the movement of

Figure 4 (Above). AUTHOR please provide figure legend.

epidermal cells.²¹⁻²⁴ GM-CSF and other factors also show positive effects on reepithelialization.^{25,26} Some of these factors may be the nature of the influence from the dermal connective tissues in the present system.

With GFP transgenic skins, the structural contribution of donor tissues was observed. The whole epidermis and part of dermal tissues were of donor origin. Around 10 days after the operation, rich granulation tissues were observed in the recipient area. They seemed to be of recipient origin since the capillaries in these areas were of recipient origin.²⁷ It is known that recipient-derived blood vessels often invade into donor tissues to supply blood.²⁸ On the other

hand, the donor-derived larger blood vessels were located closer to the epidermis than the recipient-derived capillaries of granulation tissues. There are reports showing the contribution of donor vessels or the chimeric nature of blood vessels in grafts.^{29,30}

In conclusion, an extensive functional and structural contribution of the connective tissue compartment of donor tissue in this cyst formation and epithelialization process was shown in the present regeneration system for the skin.

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