Histologic Study of the Regeneration Process of Human Hair Follicles Grafted onto SCID Mice after Bulb Amputation

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This study examines histologically the degeneration and subsequent regeneration processes of human hair follicles whose bulb is severely damaged. Human scalp hair follicles were isolated and grafted onto immunodeficient mice after their bulb was amputated. On day 14, thickening and corrugation of the vitreous membrane, apoptosis of follicular keratinocytes, and regression of the lower portion of the follicles were observed. By day 20, mesenchymal cells had accumulated around the lower end of the follicles. From day 14 through 50, the follicular regression and apoptosis continued, and between days 30 and 40 the follicles became maximally shortened, and the vitreous membrane disappeared. By day 50 the lower end of the follicles had become cup-shaped, and the cup surrounded an aggregate of mesenchymal cells that corresponded to the dermal papilla. By day 60, all the grafted follicles had developed into anagen VI follicles, and the apoptosis had ceased. These results indicate that human scalp hair follicles whose bulb is completely destroyed enter into dystrophic telogen after restoration of the dermal papilla, then into anagen, and that the duration of the dystrophic telogen is shorter than that of the normal hair cycle. ***Key words: apoptosis/dermal papilla.***
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The hair bulb is an essential site for the production of a hair shaft. It is also a major site for injuries in hair loss disorders such as chemotherapy-induced alopecia (Cece *et al*, 1996) and alopecia areata (Messenger *et al*, 1986). When the insult ceases, hair production usually recovers completely. These clinical findings suggest that human hair follicles have the potential to regenerate a severely damaged bulb. Transplantation experiments with nude mice show that human hair follicles whose bulb is amputated can regenerate (Jahoda *et al*, 1996); however, the histology of the regeneration of the grafted follicles has not been sufficiently investigated. There seem to be some common histologic findings in the regeneration process of both grafted hair follicles whose bulb is amputated and pathologic hair follicles whose bulb is severely injured. Therefore, we think that this histologic study of the amputated hair follicle contributes significantly to our understanding of the regeneration process in the severely injured hair follicles in hair loss disorders.

Recently, we reported an animal model that is useful for the study of the regeneration process, in which human scalp hair follicles are isolated and grafted onto severe combined immunodeficient (SCID) mice (Hashimoto *et al*, 2000). In this model, histologic information about the grafted hair follicles can be precisely evaluated. In this study, we used this model to investigate histologically the degeneration and subsequent recovery processes of human scalp hair follicles whose bulb was amputated.

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Abbreviation: SCID, severe combined immunodeficient.

MATERIALS AND METHODS

Isolation and transplantation of hair follicles Human terminal hair follicles were isolated from scalp specimens obtained surgically from young Japanese people whose hair was well-pigmented. The specimens were stored at 4°C in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 2.5 mg amphotericin B per ml, 100 U penicillin per ml, and 100 mg streptomycin per ml (Gibco BRL, Grand Island, NY) until the isolation, which was performed in the culture medium at room temperature. The isolation was performed within 24 h of the surgery.

The scalp samples were cut with scissors under a dissecting microscope into small pieces containing a bundle of hair follicles. Most of the dermal and subcutaneous tissue was abraded with scalpels and forceps. To preserve the integrity of the connective tissue sheath, the dermal tissue closely attached to the hair follicles was not removed; this precaution avoided injury to the follicular epithelium and the dermal papilla. The arrector pili muscle was difficult to identify and therefore could not be left uninjured. Most of the sebaceous glands, the interfollicular epidermis, and the upper part of the infundibular epithelium were removed. The bundled hair follicles were divided into single follicles with a scalpel. The isolated single hair follicles were stored in the culture medium at 37°C in 95% air/5% CO_2 until they were grafted.

Only anagen hair follicles were selected, and their base was cut off just above the bulb under the dissecting microscope (**Fig 1**). The follicles without their bulb were transplanted onto BALB/cA-SCID mice aged 4–8 wk, within 96 h of the isolation.

Histologic examination Biopsies of the grafted follicles were taken on days 14, 20, 30, 40, 50, and 60 after transplantation. The specimens were fixed in 10% neutralized buffered formalin and embedded in

Figure 1. A macrograph of an isolated human scalp hair follicle before bulb amputation. Anagen hair follicles were mechanically isolated from scalp skin specimens. *Arrows* show the level of bulb amputation. The upper part of the amputated hair follicle, which should include the bulge, was grafted onto the back skin of SCID mice.

paraffin. Deparaffinized sections were examined histologically with hematoxylin and eosin staining.

RESULTS

All the hair shafts were shed before day 14. By day 14 the lower part of the follicles was atrophied (**Fig 2*a***), and many shrunken, eosinophilic keratinocytes that corresponded histologically to apoptotic cells (**Fig 2*b***) and vacuolated keratinocytes (**Fig 2*c***) were observed within the atrophic area. Differentiation into a hair cortex and an inner root sheath was not observed. The connective tissue sheath was thickened, and the vitreous membrane was thickened and corrugated (**Fig 2*a*–*c***). The regression of the lower part of the follicles continued from day 14 through day 50, and during this period scattered apoptotic cells were observed. By day 20, the lower part of the follicles developed an epithelial rod (**Fig 2*d***), and the basal cell layer of the rod was organized in palisades (**Fig 2*e***). Mesenchymal cells had accumulated beneath the lower end of the follicles (**Fig 2*e***). Although the vitreous membrane was almost completely preserved, it was disconnected at the tip of the epithelial rod, and some mesenchymal cells were in contact with the epithelial cells. Several epithelial protuberances appeared at the middle portion of the follicle (**Fig 2*d***), which has been identified to be the bulge in this experimental model (Hashimoto *et al*, 2000). Between days 30 and 40, the follicles were maximally shortened, and their lower end was localized just below the epithelial protuberances (**Fig 2*f***). The vitreous membrane disappeared by these time points. The aggregate of the mesenchymal cells was localized adjacent to the lower end of the follicles, and the cells were larger than on day 20 (**Fig 2*g***). By day 50, the lower end of the follicles had become cup-shaped and the cup surrounded the aggregated mesenchymal cells (**Fig 2*h***). The epithelial and mesenchymal structures correspond with the secondary germ and the dermal papilla, respectively. By day 60, differentiation of the inner root sheath and hair shaft had commenced, and apoptosis had ceased (**Fig 3*a***, ***b***). The grafted follicles reproduced a well-pigmented hair shaft and were morphologically identical with a normal anagen VI follicle. Even in these follicles the sebaceous glands were small or absent, and the arrector pili muscle was not found.

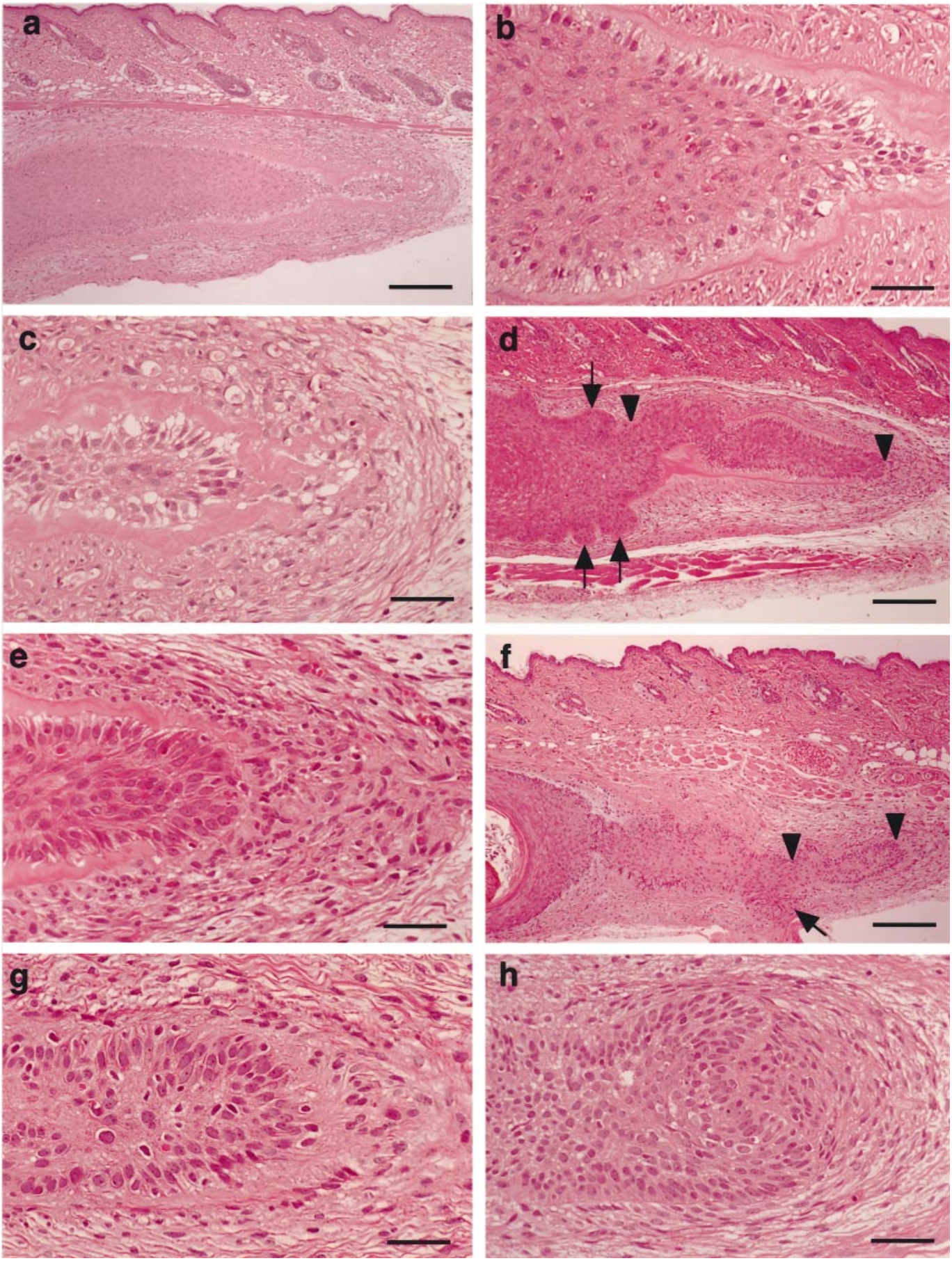
DISCUSSION

Recently we showed that isolated human hair follicles grafted onto SCID mice are superior to a full-thickness scalp graft for histologic study of the grafted human hair follicle (Hashimoto *et al*, 2000). In the latter graft, the hair follicles are in various stages of the hair cycle, and they are usually bent in random directions by shrinkage of the dermal connective tissue after transplantation. In our method, anagen hair follicles can be specifically selected for grafting, and their bulb can be amputated at a consistent level. Because the follicles are not bent, longitudinal tissue sections are easily obtained, as shown in this study. Although the arrector pili muscle, which is a marker of the bulge (Abell, 1994), is removed during isolation of the follicles, epithelial protuberances can be used as a substitute marker in this model. These advantages enable us to evaluate precisely the histology of the regenerating process of grafted hair follicles whose bulb is amputated.

When the isolated hair follicles are grafted without amputation of their bulb, characteristic degeneration and subsequent regeneration occur (Hashimoto *et al*, 2000). The grafted follicles enter into dystrophic catagen immediately after transplantation, and subsequently into dystrophic telogen. The follicles then enter anagen, and they all advance to anagen VI before day 70. This process corresponds to the "secondary recovery" that has been observed in chemotherapy-induced alopecia of the mouse (Paus *et al*, 1994). In this study, the lower part of the hair follicles whose bulb was amputated also regressed soon after transplantation, and then regenerated after the formation of the secondary germ. The regressing hair follicles and the maximally shortened ones correspond morphologically to dystrophic catagen and dystrophic telogen follicles, respectively. These histologic findings indicate that the bulb-amputated hair follicles regenerated through the "secondary recovery" process; however, there are two differences in the degeneration and regeneration processes between the hair follicles without bulb amputation (Hashimoto *et al*, 2000) and the bulb-amputated ones: the fate of the dermal papilla and the length of time from the transplantation to the beginning of anagen. When the hair follicles were grafted without bulb amputation, their dermal papilla was preserved with the cup-shaped follicular end through the degeneration stage. Because the hair follicles were grafted after bulb amputation in this study, neither the dermal papilla nor the bulb was observed on day 14. Early in the degeneration stage, an aggregate of mesenchymal cells was organized beneath the follicular base. At the end of this stage, the mesenchymal aggregate developed into the dermal papilla, and an epithelial bud, which corresponded to the secondary germ, formed at the lower end of the maximally shortened hair follicle. In addition, the regeneration of the amputated hair follicles commenced earlier than did that of the follicles without amputation: all the former follicles entered into anagen VI by day 60, whereas the latter ones did so by day 70.

When the bulb is amputated, the regeneration of the dermal papilla is the first key event in the regeneration of human scalp hair follicles as well as of rodent vibrissae (Oliver, 1966; Jahoda *et al*, 1996). In this study, an aggregate of mesenchymal cells, which would develop into the dermal papilla, appeared beneath the lower end of the follicles by day 20. The connective tissue sheath cells have the potential to form the hair-inductive dermal papilla in rat vibrissae (Matsuzaki *et al*, 1996; Horne and Jahoda, 1992) and human scalp hair follicles (Reynolds *et al*, 1999). These data suggest that the mesenchymal cell aggregate derives from the lower connective tissue sheath, although the possibility that mouse fibroblasts were recruited to this region cannot be denied (Jahoda *et al*, 1996).

The mechanism that induces the aggregation of the mesenchymal cells remains unclear; however, factors released from the follicular epithelium may participate in its induction. Platelet derived growth factor (PDGF) and Sonic hedgehog (Shh) are likely candidate molecules for these factors. During hair follicle embryogenesis, the epithelial cells produce these two molecules, and the mesenchymal cells express their receptors (Orr-Urtreger and Lonai, 1992; Pontén *et al*, 1994; Akiyama *et al*, 1996; Iseki *et al*, 1996; Takakura *et al*, 1996; Chiang *et al*, 1999; Karlsson *et al*, 1999). The number of dermal papilla cells and connective tissue sheath cells in the follicles are decreased in PDGF knockout mice,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suggesting that PDGF plays a role in the development of the dermal papilla and the connective tissue sheath (Karlsson *et al*, 1999). PDGF potentiates the growth of dermal papilla cells and stimulates chemotaxis of skin fibroblasts *in vitro* (Seppa *et al*, 1982; Goodman and Ledbetter, 1992). Shh is involved in the maturation of the mesenchymal condensate into the dermal papilla in the hair germ stage of embryonic hair development and in the transition of hair follicles from telogen to anagen in postnatal skin (Chiang *et al*, 1999; St-Jacques *et al*, 1998; Karlsson *et al*, 1999; Sato *et al*, 1999; Wang *et al*, 2000). The Wnts, a family of secreted glycoproteins expressed in the matrix cells, are involved in the activation of the dermal papilla (Kishimoto *et al*, 2000). These data suggest that PDGF, Shh, and Wnts are synthesized by the epithelial cells in the base of the grafted follicle: PDGF stimulates the connective tissue sheath cells to proliferate and migrate toward the follicular bottom, and Shh and Wnt induce the development of functional dermal papilla cells from the connective tissue sheath cells.

In this study, the dermal papilla matured and was surrounded by the secondary germ by day 50, and then the follicle entered anagen. In the normal hair cycle, the dermal papilla has been thought to induce the proliferation of the keratinocytes in the secondary germ at the onset of anagen (Cotsarelis *et al*, 1990). Dermal papilla cells synthesize keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), whose receptors are expressed in hair bulb keratinocytes adjacent to the dermal papilla (Shimaoka *et al*, 1994; LaRochelle *et al*, 1995; Rosenquist and Martin, 1996; Lindner *et al*, 2000). HGF stimulates the proliferation of hair follicle keratinocytes in cell culture (Shimaoka *et al*, 1995) and hair follicle elongation in organ culture (Jindo *et al*, 1994a, b, 1995; Shimaoka *et al*, 1995). HGF also enlarges the size of hair follicles in newborn mice (Jindo *et al*, 1998). KGF stimulates proliferation of hair matrix cells and hair growth *in vivo* (Pierce *et al*, 1994; Danilenko *et al*, 1995). These data suggest that HGF and/or KGF secreted by the mature dermal papilla play roles in the proliferation of bulb keratinocytes, inducing anagen. Insulin-like growth factor-1 (IGF-1), which is produced by dermal papilla cells (Itami *et al*, 1995; Messenger, 1989; Little *et al*, 1994; Rudman *et al*, 1997), also has a mitogenic effect on keratinocytes (Ristow and Messmer, 1988). Because its receptor is not expressed in the proliferating matrix cells adjacent to the dermal papilla, it appears not to contribute to the proliferation of bulb keratinocytes (Rudman *et al*, 1997).

In the secondary recovery of chemotherapy-induced alopecia, the mouse pelage regenerates rapidly after passing through dystrophic telogen, of which the duration is much shorter than that of normal telogen. The present and our previous data showed that whether the bulb is amputated or not, the grafted human hair follicles also recover after passing through a short dystrophic telogen. Injured human hair follicles seem to use the same strategy for their rapid and complete regeneration as do follicles of the mouse pelage. Our data also show that anagen commenced earlier in the amputated hair follicles than in the nonamputated ones. Factors released from the dermal papilla have been shown to protect follicular keratinocytes from some damage or from apoptosis. KGF protects hair follicles from radiation damage (Booth and Potten, 2000), and reduces hair loss in chemotherapy-induced alopecia (Pierce *et al*, 1994). HGF retards the development of catagen in the spontaneous hair cycle, perhaps by suppressing follicular keratinocyte apoptosis (Lindner *et al*, 2000). IGF-1 acts as an antiapoptotic factor in many cell types and blocks the transition of hair follicles from anagen to catagen (Philpott *et al*, 1994; Stewart and Rotwein, 1996). These data suggest that these growth factors are secreted from the remaining dermal papilla, and delay the follicular regression and thus the onset of their recovery process.

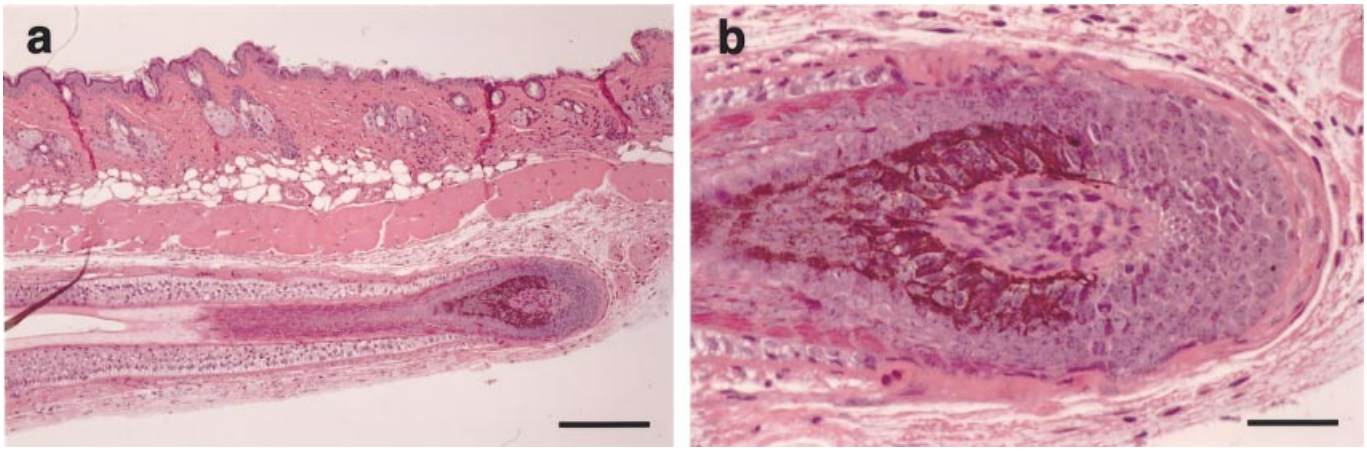

Figure 3. Histologic findings of a regenerated hair follicle on day 60. Hematoxylin and eosin stain. The regenerated hair follicle is histologically the same as a normal anagen hair follicle. A well-pigmented hair shaft and inner root sheath have formed (*a*) The regenerated hair bulb has a hair matrix, pigmented dendritic melanocytes, and a dermal papilla. No apoptotic cells are found in the hair bulb (*b*) *Scale bars*: (*a*) 50 μm; (*b*) 200 μm.

Figure 2. Histologic findings of the regeneration process of grafted hair follicles. Hematoxylin and eosin stain. (*a*, *b*, *c*) Day 14. The follicle shows a tapered end surrounded by a thickened connective tissue sheath and a vitreous membrane (*a*). Apoptotic cells are observed within the lower follicle (*b*). Hair matrix and inner root sheath are not observed in the follicular base, and many mesenchymal cells are present around the vitreous membrane (*c*). (*d*, *e*) Day 20. A few epithelial protuberances (*arrows*) are observed above an epithelial rod (between *arrowheads*) (*d*). The proximal end of the follicle is round, not enclosed by the vitreous membrane, and in contact with an aggregate of mesenchymal cells (*e*). Apoptotic cells are observed in the lower follicle. (*f*, *g*) Day 30. The epithelial rod (between *arrowheads*) below the epithelial protuberance (*arrow*) is more shortened than at day 20 (*f*). The vitreous membrane disappears and apoptotic cells are observed in the lower follicle (*g*). (*h*) Day 50. The cup-shaped end of the follicle is contiguous with the dermal papilla-like aggregate of mesenchymal cells. Apoptotic cells are still observed in the proximal end of the follicle. No inner root sheath or hair shaft has developed. *Scale bars*: (*a*, *d*, *f*) 50 μm; (*b*, *c*, *e*, *g*, *h*) 200 μm.

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