Proliferation and differentiation of organoid hair follicle cells co-cultured with fat cells in collagen gel matrix culture

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Summary
Using rat skin, we studied the influence of fat cells on the proliferation and differentiation of organoid hair follicle cells in a three-dimensional collagen gel matrix culture system. We cultured organoid hair follicles embedded in collagen gel under each of the following three conditions: cell-free collagen gel for control experiments (condition 1); co-culture with fat cells in close apposition (condition 2); and co-culture with fat cells in spatial separation (condition 3). Outgrowths of epithelial cells from the organoid hair follicles associated with perifollicular proliferation of fibroblasts were observed under conditions 1 and 3. Under condition 2, proliferation of both organoid hair follicle cells and fibroblasts was inhibited, but differentiation of the hair follicle cells appeared to be accelerated. Fat cells are considered to have an inhibitory effect on the proliferation of perifollicular fibroblasts, which might have resulted in the inhibition of hair follicle cell proliferation and also in the better maintenance of normal follicular structure and integrity, allowing for hair-type differentiation to proceed. A direct accelerating effect of fat cells on hair follicle differentiation may also have been responsible. In a physiological state (co-culture with keratinocytes on the collagen gel), similar results were observed under conditions 1 and 2. The different findings under conditions 2 and 3 may be due to either of two possibilities: either the concentration gradient of the solute factors released from fat cells, acting either on the hair follicle cells or the perifollicular fibroblasts as an inhibitor of proliferation, caused the difference in the results, or direct contact between the organoid hair follicle cells and fat cells may have influenced the accelerating effect of fat cells on the differentiation of hair follicle cells.

Development and homeostasis of the skin depend on epithelial–mesenchymal interactions. Several studies on this issue have been conducted in vivo using the nude mouse grafting model and in vitro using three-dimensional collagen gel matrix culture. In most instances, these studies have focused on keratinocytes or hair follicle cells as the epithelial components and dermal fibroblasts or dermal papilla cells as the mesenchymal components. Although the hypodermis, which is composed of mature fat cells and situated around the deep portion of the hair follicle, is one of the most important mesenchymal elements, little attention has been given to the role of the hypodermis in epithelial–mesenchymal interactions. In contrast, adipose tissue has recently received attention as an endocrine organ producing physiologically active, secretory proteins.

We have demonstrated that a three-dimensional collagen gel matrix culture is suitable for the study of structural differentiation and the influence of the fibroblasts–extracellular matrix on various types of epithelial cells. We recently reported that fat cells influence the epidermal organization in collagen gel culture. In the present study, using rat skin, we studied the influence of fat cells on the proliferation and differentiation of organoid hair follicle cells in a three-dimensional collagen gel matrix culture system. Also, we investigated the influence of the physiological condition by co-culturing keratinocytes at the air–liquid interface on collagen gel containing organoid hair follicle cells and fat cells.

Materials and methods
Cell isolation and preparation of collagen gel
Skin was obtained from 3- to 4-day-old Wistar rats (supplied by Biotech, Toso, Japan) and treated with...
0.15% collagenase at 4°C overnight to separate epidermis and dermis. The dermis, containing its hair follicles, was digested by incubation in 0.35% collagenase at 37°C for 30 min. After filtration and centrifugation (500 g, 5 min, three times) of the digested fluid, hair follicles representing various stages of maturation (immature and mature hair follicles) were obtained as a pellet. The epidermis was digested with 0.25% trypsin solution at 37°C for 15 min, then separated into interfollicular keratinocytes and hair follicle buds (a pellet) by low-speed centrifugation (300 g, twice).1 Interfollicular keratinocytes were obtained by removing the pellet (hair follicle buds). The dermis-derived hair follicles and the epidermis-derived interfollicular keratinocytes were used for culture in this study. Fat tissue was obtained from the lower abdominal subcutaneous fat tissue of 4- to 8-day-old Wistar rats. Unilocular fat cells were obtained by digestion of the fat tissue with 0.2% collagenase solution according to Rodbell’s method.29

We prepared a collagen gel matrix by modifying the method described by Eldadah and Bard.30 Briefly, eight volumes of acid-soluble type I collagen solution were mixed with one volume of 10× concentrated minimum essential medium and one volume of reconstruction buffer (2.2 g NaHCO3 and 477 g HEPES in 100 mL 0.05 mol/L NaOH).

Culture and co-culture

The procedures essentially followed the method previously reported.14,19,22 To investigate the influence of fat cells on the proliferation and differentiation of organoid hair follicle cells embedded in collagen gel, we employed the following three culture conditions: cell-free collagen gel for control experiments, co-culture with fat cells in close apposition; and co-culture with fat cells in spatial separation. Two millilitres of collagen solution containing hair follicles obtained from the skin of one rat, embedded in 2 mL of collagen gel, was placed into a culture dish (35 mm) and immediately warmed to 37°C to form a gel. Each of the gels was overlaid with 2 mL of the culture medium (minimum essential medium supplemented with 10% fetal bovine serum and antibiotics) containing no growth factors. To produce spatial separation between the two cell types, we cultured the hair follicles obtained from the skin of one rat, embedded in 2 mL of collagen gel in an inner dish placed in an outer dish underlaid with 3 mL of collagen gel containing 2 × 10^5 unilocular fat cells. Both the inner and outer dishes were filled with the culture medium.

To investigate the influence of fat cells on organoid hair follicle cells under more physiological conditions, we also co-cultured epidermal keratinocytes at the air-liquid interface on collagen gel containing organoid hair follicle or fat cells. We prepared two types of collagen gels as the dermal equivalents, containing either organoid hair follicles alone for the control experiments or organoid hair follicles and fat cells in close apposition, according to the procedure described above. Each of the two types of collagen gel (2 mL) was poured into a dish (20 mm in diameter), the bottom of which was composed of nitrocellulose membrane (Millipore CM, Millipore Corp., Bedford, MA, U.S.A.), and immediately warmed to 37°C to form gel in the dish. Epidermis-derived interfollicular keratinocytes (3 × 10^5) were seeded on the surface of each of the two types of collagen gels. This culture dish (the inner dish) was then placed in a larger outer dish. Both the inner and outer dishes were filled with the culture medium. Submerged keratinocytes were cultured for 4 or 5 days to a confluent monolayer. An air-liquid interface was subsequently formed by withdrawal of all of the medium over the keratinocytes in the inner dish and some of that in the outer dish, until the keratinocytes were completely exposed to air. The experiments were performed for each of these conditions in five procedures, using five different Wistar rats in both of the investigations.

Examination of cultured cells

We examined the morphological features, as indicators of proliferation and differentiation, of the cultured or co-cultured organoid hair follicle cells using phase contrast microscopy, histological observation of paraffin-embedded sections stained with haematoxylin and eosin and periodic acid-Schiff (PAS), and immunohistochemical study of paraffin-embedded sections by the avidin–biotin–peroxidase complex method. For haematoxylin and eosin staining, the three-dimensional collagen gels containing cultured cells were fixed with 10% formalin and routinely processed, embedded in paraffin and cut both vertically and horizontally. Antibody to proliferating cell nuclear antigen (PCNA) (Dako, Japan) was used in the immunohistochemical study to examine proliferation. Antibody to vimentin (Dako) were also used. An ultrastructural study was performed by standard methods. The cultured cells and the collagen gel detached from the dish were fixed in 2.5% glutaraldehyde for this procedure.
Figure 1. Phase contrast microscopic images (a,c,e) and histological appearance (b,d,f) of organoid hair follicles in collagen gel. (a,b) On day 3, organoid hair follicles show outgrowths of epithelial cells in an amoeboid configuration associated with perifollicular dendritic cells. (c,d) On day 5, the outgrowth of epithelial cells from organoid hair follicles is more accelerated in association with many dendritic cells surrounding hair follicles. (e,f) Vertical section reveals that long pseudopodium-like structures from hair follicles elongate in the horizontal direction and that these structures are positive for polyclonal keratin, which shows no staining in the dendritic cells (haematoxylin and eosin; horizontal section: (b) original magnification × 80; (d) original magnification × 65; vertical section: (c) original magnification × 65; immunohistochemical stain: vertical section: (f) original magnification × 65).

Figure 2. Phase contrast microscopic images (a,c,e) and histological appearance (b,d,f) of organoid hair follicles co-cultured with fat cells in close apposition in collagen gel. (a,b) On day 3, the hair follicles show outgrowths of epithelial cells with an amoeboid configuration associated with dendritic cells. (c,d) On day 5, no acceleration of the outgrowths from hair follicles is apparent, and the hair follicles show rounded contours. (e,f) On day 7, most of the inner portion of the hair follicles shows a dark colour change in phase contrast microscopy, and this portion contains eosinophilic material, suggesting keratinisation of the hair follicles (haematoxylin and eosin; horizontal section: original magnification × 65).

Statistical analysis
To evaluate the proliferation of the hair follicles embedded in collagen gel under each of the three conditions in the culture procedure without keratinocytes, we counted the number of pseudopodium-like cell growths from hair follicles visible under a phase contrast microscope, and the number of PCNA-positive cells in the immunostained sections. Under the phase contrast microscope, we chose at random 10 hair follicles for measurement of pseudopodium-like cell growths in each condition. The number of each under condition 1 was compared with the numbers under conditions 2 and 3. The differences were analysed using the Bonferroni/Dunn procedure (Dunn’s procedure as a multiple comparison). Scheffe’s F-test and Fisher’s least significant difference, with P < 0.05 regarded as significant.

Results
Phase contrast microscopic and histological examinations
The results of our investigation of the influence of fat cells on organoid hair follicles in collagen gel culture were as follows: in cell-free collagen gel (control experiments), after culture for 2–3 days, the hair follicles, especially immature ones (hair follicles with incomplete hair formation), showed outgrowths of epithelial cells around their outer root sheaths. They formed amoeboid-like structures with associated perifollicular dendritic cell proliferation (Fig. 1a,b). After 5–6 days, the outgrowth of epithelial cells was more accelerated, and pseudopodium-like structures radiated from the hair follicles (Fig. 1c,d). Many associated dendritic cells surrounded the hair follicles. Vertical sections showed that long, fibril pseudopodium-like structures from hair follicles elongated in the horizontal direction (Fig. 1e). The cytoplasm of most of the epithelial cells growing out from the hair follicles contained FNS-positive, diastase-digestible deposits of glycogen. The pseudopodium-like structures, but not the perifollicular dendritic cells, were immunohistochemically positive for polyclonal keratin (Fig. 1f). The perifollicular dendritic cells were positively stained with vimentin.

The hair follicles that were co-cultured with fat cells in close apposition in collagen gel, also showed outgrowths of epithelial cells with amoeboid appearance after co-culture for 2–3 days (Fig. 2a,b). Some dendritic cells surrounding hair follicles and cytoplasmic extensions from unilobar fat cells were observed. After co-culture for 4–5 days, no acceleration of the outgrowth of epithelial cells from the hair follicles was seen, and hair follicles showed a rounded contour like that of the hair in the skin. In contrast to the streaming outgrowth observed in the culture of hair follicles only (Fig. 2c,d). After co-culture for 7 days, most of the inner portion of the hair follicles darkened under phase contrast microscopy, and these portions contained eosinophilic material, suggesting keratinisation of the hair follicles (Fig. 2e,f). No proliferation of perifollicular dendritic cells was observed, and some unilobar fat cells showed transition to multilobar or fibroblast-like fat cells, as described previously.14,21

In hair follicles that were co-cultured with fat cells in spatial separation in collagen gels, the morphological findings were essentially identical to those when hair follicles only were cultured in collagen gel. However, the degree of epithelial cell growth was slightly greater than that in control experiments.

The results of the investigation of the influence of fat cells on organoid hair follicles under more physiological conditions (co-culture with keratinocytes) were as follows: in the culture in which the dermal equivalents contained organoid hair follicles alone (the control experiments), the keratinocytes proliferated and reached a confluent state on days 4 or 5, when the organoid hair follicles showed epithelial cell growth with apparent pseudopodium-like structures. Two or 3 days after air-exposure of the epidermal keratinocytes, the hair follicle cells, superficially located in the collagen gel, elongated and contacted the keratinocytes (Fig. 3a). On day 12, when the surface had been exposed to air for 7 days, the epidermis showed an increase in the number of cell layers, and keratohyaline granules appeared in the upper portion of the epidermal cell layer (Fig. 3b). However, the upper portion of the epidermis consisted mostly of clear cells, which suggested that epidermal differentiation either was not complete or was influenced by the contacted hair follicles. The epithelial cells derived from hair follicles completely connected with the reconstructed epidermis, and some of the hair follicles were incorporated into the epidermis (Fig. 3c).

In the culture in which the dermal equivalents contained organoid hair follicles and fat cells in close apposition, the keratinocytes proliferated and reached a confluent state on days 4 or 5, and after air-exposure, on day 12 the reconstructed epidermis was well keratinised, although it contained fewer layers. The hair follicles
did not elongate and contact the reconstructed epidermis. The hair follicles surrounded by unicellular fat cells contained eosinophilic material, suggesting keratinization (Fig. 5d).

**Immunohistochemical examination and statistical analysis**

In the hair follicle cells cultured in the collagen gel, the nuclei of the cells situated in the periphery of the organised hair follicles were positive for PCNA. The number of the positively stained hair follicle cells was increased in culture containing hair follicles alone compared with that in co-culture with fat cells in close apposition (Fig. 4a,b).

Statistical analysis revealed significantly (P<0.0001) fewer pseudopodium-like cell growths detected by phase contrast microscopy and significantly (P<0.0001) fewer PCNA-positive hair follicle cells on days 3 and 7 in the co-culture with fat cells in close apposition compared with those in the culture without fat cells (Tables 1 and 2). These values were not significantly different between the conditions of co-culture with fat cells in spatial separation and of culture without fat cells.

**Ultrastructural examination**

The morphological findings from culture of the organised hair follicle cells are described in detail in the previous section. The presence of a well-developed cytoskeleton and a defined subcellular organisation is evident. The thin filaments are organized into a network of filament bundles, and the presence of the keratin intermediate filaments is evident. The presence of the cytoskeletal elements is evident in the morphological organisation of the hair follicle cells in culture.
Discussion

In our study, the morphological characteristics of hair follicle cells as organoids in three-dimensional collagen matrix culture were identical to the observations reported by other investigators. Specifically, the outgrowth of epithelial cells, which were considered to be outer root sheath cells, arose from hair follicles having an amoeboid appearance. However, in our study, the outgrowth of epithelial cells from the organoids, forming fibre structures, was evident without these growth factors. In our method, the proliferation of dermal cells surrounding the organoids was associated with the proliferation of organoids. These dermal cells are considered to be fibroblasts derived from perifollicular connective tissue, and they are assumed to participate in the proliferation of epithelial cells of the organoids. It has been reported that the growth of hair follicle epithelial cells is enhanced by co-culture with dermal fibroblasts or NIH 3T3 cells. In the observation in this study that proliferating cells in organoid hair follicles, which were probably outer root sheath cells, were limited to the periphery of the follicles is in accord with a previous report.

The results of our study revealed that the outgrowth of epithelial cells from organoid hair follicles was distinctly inhibited by co-culture with fat cells in close apposition, and that the organoid hair follicles morphologically showed a rounded contour like that of hair follicles in the skin, rather than an amoeboid appearance. Another distinguishing characteristic of this co-culture with the two cell types in close apposition was the absence of proliferation of perifollicular fibroblasts. Therefore, we suggest that fat cells have an inhibitory effect on the proliferation of perifollicular fibroblasts in this co-culture system, which resulted in no outgrowth of epithelial cells of hair follicle organoids.

In addition, in histological and electron microscopic studies, differentiation in hair follicles in close contact with fat cells appeared to have been promoted, although we could not confirm this finding by immunohistochemical staining because there is no reliable marker for hair follicle differentiation in rats. If this promotion did occur, it may have been due to fat cells having inhibited the proliferation and outgrowth of the perifollicular fibroblasts, thereby causing normal follicular structure and integrity to be better maintained, which allowed hair-type differentiation to proceed further; that is to say, this condition (close contact of the two cell types) represents an organ culture system, while the system changes to a cell culture model under other conditions (i.e. in the absence of inhibitory fat cell influence).

Another possible cause of this promotion may be a direct accelerating effect by fat cells on hair follicle differentiation. There is an inverse relationship between proliferation and differentiation in all types of epithelial cells. Thus, this direct accelerating effect on hair follicle differentiation might reflect the inhibition of proliferation of organoid hair follicle cells by fat cells.

Recently, it has become evident that fat cells produce physiologically active, secretory proteins, such as tumour necrosis factor-α, EGF, leptin, plasminogen activator inhibitor, and other yet unidentified cytokines. It is possible that these soluble factors released from fat cells acted on either hair follicle cells or perifollicular dermal cells through the paracrine system.

This inhibitory effect on proliferation in organoid hair follicle cells was not seen when the fat cells and hair follicles were spatially separated, indicating that close apposition of the two cell types is necessary for the expression of the inhibitory effect of fat cells. There are two possible explanations for this finding, as the soluble factors released from fat cells act on either hair follicle cells or perifollicular dermal cells as an inhibitor of proliferation, the concentration gradient of the soluble factors might have caused the difference in results. The inhibition of trichohyaline granule formation and hair growth by treatment with a mitotic inhibitor. By contrast, the relation between organoid hair follicles and the fat cells may be an important factor in the accelerating effect on the differentiation of hair follicle cells. Under the physiological conditions (co-culture with keratinocytes on the collagen gel), when the culture was free of fat cells, the epithelial cells outgrown from the organoid hair follicles had a tendency to elongate and contact the reconstructed epidermis. Some of the hair follicles were incorporated into the reconstructed epidermis, suggesting that hair follicle cells (probably outer root sheet cells) have a affinity for keratinocytes. However, when the fat cells were co-cultured with organoid hair follicles in close apposition in the collagen gel, there was no outgrowth of epithelial cells from organoids forming fibre structures, and there was little cell-to-cell contact between keratinocytes and hair follicle epithelial cells. Instead, the organoid hair follicle cells were well keratinized. Under the physiological conditions in the presence of keratinocytes, the fat cells both inhibited proliferation and promoted differentiation of organoid hair follicles.

It seems physiologically reasonable that the fat cells would have the capability of inhibiting the proliferation of hair follicles penetrating deep into the dermis with the development of the hair or hair cycle: in addition, in the early anagen stage of the hair cycle, after downward growth of the hair follicle (the secondary hair germ) into the subcutaneous fat tissue, a new hair bulb is formed with subsequent differentiation in terms of convincing physiological evidence that fat cells possess the capacity to accelerate the differentiation of hair follicles.

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References


