Subcutaneous Adipocytes Promote the Differentiation of Squamous Cell Carcinoma Cell Line (DJM-1) in Collagen Gel Matrix Culture

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Cancer cell–stromal cell interaction plays a crucial role in the malignant growth of cancer cells. In the skin, the main stromal cell types consist of dermal fibroblasts and subcutaneous adipocytes. Fibroblasts are shown to promote the invasive growth of various cancer cell types. The interaction between cancer cells and stromal adipocytes, however, has not been sufficiently studied even in cutaneous carcinoma. To address the effects of adipocytes on the biologic behavior of cancer cells, we examined the growth and differentiation of a squamous cell carcinoma cell line of the skin (DJM-1), using a three-dimensional collagen gel matrix culture with a cutaneous environmental factor, air exposure. The growth was estimated by the uptake of bromodeoxy-uridine (BrdU) for 24 h. The BrdU indices of DJM-1 cells in stromal-cell-free, fibroblast-containing, and adipocyte-containing conditions were 19.7 ± 1.9%, 19.8 ± 2.8%, and 4.7 ± 1.4%, respectively, whereas the BrdU index on the gel containing both fibroblasts and adipocytes was 10.4 ± 3.3%. In terms of differentiation, DJM-1 cells cocultured with adipocytes constructed the best-organized stratified layer with a cornified-like structure in all conditions above. The differentiation markers involucrin and cytokeratin 10 were immunohistochemically detected in this structure of DJM-1 cells. Adipocyte-induced phenomena were not affected distinctively by air exposure. These results indicate that adipocytes, but not fibroblasts, promote the differentiation of squamous cell carcinoma cells (DJM-1) and inhibit their growth. These adipocyte-induced phenomena were not completely inhibited by fibroblasts. In conclusion, we suggest that stromal adipocytes may be involved in the differentiating mechanisms of cutaneous carcinoma cells. Key words: air exposure/cancer cell–stromal cell interaction/cutaneous carcinoma/subcutaneous adipose tissue. J Invest Dermatol 117:244–250, 2001

Local invasion and metastasis characterize the biologic behavior of cancer cells (Liotta, 1992; Steeg, 1992). In the invasive and metastatic cascade, cancer cell–stromal cell or cancer cell–extracellular matrix interactions play a crucial role (Fusenig et al., 1991a; Liotta, 1992; Steeg, 1992). In fact, recent studies have shown that fibroblasts, as a stromal cell type, promote the local invasion of squamous cell carcinoma into the neighboring tissues (Matsumoto et al., 1989; Kawahara et al., 1993; Iwazawa et al., 1996; Yamada et al., 1999). In terms of the histoanatomical structure of the skin, the growth of cutaneous squamous cell carcinoma is considered to intimately interact with dermal fibroblasts and subcutaneous adipocytes. The relationship between the carcinoma cells and dermal fibroblasts has been fully studied, but the carcinoma cell–adipocyte interaction remains to be investigated.

Recently, the production and secretion of cytokines as well as the storage of triacyl glycerol have been reported as the essential function of the adipocytes (Maeda et al., 1996; 1997). In connection with these reports, our studies have reported that adipocytes influenced the proliferation and differentiation of epidermal keratinocytes (Sugihara et al., 1991) and organoid hair follicle cells (Misago et al., 1998) in collagen gel matrix culture.

In general, the skin is physiologically exposed to air. Several experiments have shown that air exposure is an important factor in the proliferation and differentiation of keratinocytes (Regnier and Darmon, 1989; Sugihara et al., 1991; Boelsma et al., 1999). Few studies about the influence of air exposure on carcinoma cells have been performed, however, even for cutaneous squamous cell carcinoma (Boukamp et al., 1985; Suter et al., 1991; Turksen et al., 1991).

We hypothesized that subcutaneous adipocytes as a stromal cell type and air exposure as an environmental factor of the skin might play crucial roles in the proliferation and differentiation of cutaneous carcinoma cells. As a result, we herein describe for the first time that adipocytes induced both the promotion of differentiation and the relative inhibition of proliferation in a squamous cell carcinoma cell line of the skin (DJM-1). In addition, the local environmental factor air exposure promoted the proliferation and stratification of the carcinoma cells. These results indicate that subcutaneous adipocytes have a crucial role in the biologic behavior of cutaneous carcinoma cells. It is widely accepted that malignant tumor cells express an altered and incomplete version of the differentiation program of their tissue of origin (Fusenig et al., 1991b). Therefore, to investigate the role of the subcutaneous adipocyte on the DJM-1 cells will help to understand the cancer cell–stromal cell interaction in the skin. This study may suggest the
possibility of designing a novel therapeutic strategy for cutaneous squamous cell carcinoma.

MATERIALS AND METHODS

Carcinoma cell line, fibroblast cell line, and isolation of unilocular adipocytes

We used a squamous cell carcinoma cell line (DJM-1) that had been derived from human skin (Kitajima et al., 1987) (Fig 1A). For stromal cell types, we used mouse 3T3-Swiss albino fibroblasts (American Tissue Culture Collection CCL-92, Rockville, MD) and unilocular adipocytes, i.e., mature adipocytes, derived from the subcutaneous adipose tissue of rats. Adipocytes were collected from the abdominal subcutaneous adipose tissue of rats and the human with the informed consent. Aseptically excised adipose tissue was minced, digested with a collagenase solution at 37°C for 30 min, and filtered through a 70 μm mesh sieve, as described previously (Sugihara et al., 1988). The filtered cells in suspension were dispersed in complete medium: Ham's F-12 supplemented with 50% newborn calf serum. After centrifugation, floating adipocytes on the top were collected with a Pasteur pipette. We repeated this suspension–centrifugation process three times to dilute the collagenase solution, and finally collected the unilocular adipocytes (Fig 1B).

Three-dimensional collagen gel matrix culture

To study the effects of stromal cells and air exposure on the proliferation, invasion, and differentiation of DJM-1 cells, we used a three-dimensional collagen gel matrix culture, as described previously (Sugihara et al., 1988; Yamada et al., 1999) (Fig 2).

Experiment 1: Eight volumes of acid-soluble type I collagen solution (pH 3) was mixed with 1 volume of Ham’s F-12 medium concentrated 10 times and 1 volume of reconstruction buffer (2.2 g of NaHCO₃ and 4.77 g HEPES in 100 ml 0.05 N NaOH). The mixture without addition of fibroblasts or adipocytes was poured into a 30 mm dish with a nitrocellulose bottom (Millicell-CM, Millipore, Bedford, MA) and incubated at 37°C for 30 min to solidify the gel. This inner dish was placed into a 90 mm outer dish (Terumo, Tokyo, Japan), and the medium was added to both dishes. This collagen gel layer prepared in the inner dish corresponded to the in vitro dermis. Then, 1.0 × 10⁶ DJM-1 cells were poured onto the reconstructed dermis (Experiment 1-1). DJM-1 cells became confluent on the gel within 2 d. At this stage, the culture medium on the DJM-1 cells was removed to expose the cells to air. At the same time, the culture medium in the outer dish was withdrawn to the level of the cells in the inner dish. Accordingly, there was no seepage of culture medium over the DJM-1 cells. The humidity inside the incubator was above 95%, the surface of the DJM-1 cells was not completely dry (Experiment 1-2). The methods of Experiments 1-1 and 1-2 were the standard procedure for our study. In order to examine the interaction between DJM-1 cells and stromal cells, the following three conditions were prepared.

Experiment 2: Fibroblasts were added to the collagen solution mixture at a concentration of 5.0 × 10⁵ cells per ml, and 1.5 ml of the mixture was placed in the inner dish. After gelation, the DJM-1 cells were poured onto the gel, as the dermis substrate.

Experiment 3: Adipocytes were added to the collagen solution mixture at a concentration of 1.0 × 10⁵ cells per ml, and 1.5 ml of the mixture...
was placed in the inner dish. After gelation, the DJM-1 cells were poured onto the gel, as the subcutaneous adipose tissue substrate.

**Experiment 4** One milliliter of a collagen solution mixture containing adipocytes was placed in the inner dish. This adipocyte-containing layer was compatible with the subcutaneous adipose tissue. After gelation, 0.5 ml of collagen solution mixture containing fibroblasts was overlaid on top of the gel. After the second gelation, the DJM-1 cells were seeded onto the gel. This artificial condition was similar to the normal skin structure.

Finally, air exposure treatment was also conducted in Experiments 2–4, according to the method used in Experiment 1–2.

To further examine the interaction between DJM-1 cells and adipocytes, the following condition was prepared. Adipocytes were added to the collagen solution mixture at a concentration of 1.0 \( \times 10^5 \) cells per ml, and 1.5 ml of the mixture was placed in the inner dish. After the gelation, 0.5 ml of collagen solution mixture without addition of fibroblasts or adipocytes was overlaid on top of the gel. After the second gelation, the DJM-1 cells were seeded onto the gel. In this condition, DJM-1 cells were in contact with adipocytes. In addition, we also performed these experiments on HeLa cells and conducted a preliminary experiment on T3M-1 cells (derived from human lower jaw squamous cell carcinoma) (Sato et al., 1989).

**Chemicals added to culture cells** The chemicals added to the culture cells were as follows: (i) 10–100 \( \mu \)g per ml leuplin (Linco Research, St. Charles, MO); (ii) 10–30 \( \mu \)g per ml tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) (Genzyme, Cambridge, MA); (iii) 10–30 \( \mu \)g per ml insulin-like growth factor II (IGF-\( \alpha \)) (Sigma RoI, Natick, MA). These cytokines are known to be produced from adipocytes, as “the adipocytokines” (Maida et al., 1996, 1997).

**Examination of culture cells**

**Histologic method** Collagen gel containing cultured cells was fixed with 10% formalin in paraflin-embedded vertically, deparaffinized, and then stained with hematoxylin and eosin. To observe the basement membrane formed by cultured DJM-1 cells, deparaffinized sections were subjected to reticulin silver impregnation staining. The results of this staining agreed to some extent with those of immunohistochemistry with type IV collagen (DAKO Japan, Kyoto, Japan). The culture cells were also examined by electron microscopy after glutaraldehyde and osmic acid fixation and the standard treatment.

**Immunohistochemistry** DJM-1 cells and fibroblasts were identified by immunostaining with cytokeratin (monoclonal antibody, which covered a spectrum of molecular masses of 40, 45, 46, and 56 kDa; Nichirei, lipid stain. Immunostaining with cytokeratin 10 (monoclonal antibody; DAKO Japan, Tokyo, Japan), a suprabasal marker of differentiation in stratified squamous epithelia, was expressed in the upper layers of the stratified layer (Fig 6C). Also, cytokeratin 10, which is a suprabasal marker of differentiation in stratified squamous epithelia, was expressed in the upper layer, but was not expressed at the basal layer of the stratified layer of DJM-1 cells (Fig 6G). In contrast, involucrin and cytokeratin 10 were scantily expressed in the stratified layer under the stromal-cell-free condition (Fig 6D, E).

To examine the coeffects of adipocytes and air exposure on DJM-1 cells, we conducted Experiment 3–2 (Fig 3F). DJM-1 cells clearly underwent a thicker stratification and lost the flattened cells containing granular materials, but maintained the high organization and keratinization at the upper layers containing differentiated “swollen” cells with degenerated nuclei. The BrdU index of stratified DJM-1 cells was 15.5 ± 5.4% for 24 h (Fig 4). This result was significantly lower than that under the stromal-cell-free condition with air exposure. Involucrin and cytokeratin 10 were expressed in the upper layers of the stratified layer (Fig 6D, H). We also used human subcutaneous adipocytes in Experiment 3–1. The result was similar to that of Experiment 3–1 with rat subcutaneous adipocytes (data not shown).

**RESULTS**

**Monolayer culture** Human squamous cell carcinoma cell line cell line of the skin (DJM-1) cells grew directly on plastic dishes in Ham’s F-12 medium containing 15% newborn calf serum. The cells made a monolayer sheet with a cobblestone appearance (Fig 1A).

**Experiment 1 (Stromal-cell-free system)** We conducted Experiment 1–1 as a control counterpart to other stromal-cell-containing systems (Fig 3A). On the stromal-cell-free collagen gel, DJM-1 cells formed an unpolarized stratified layer of five to six cells within 2 wk. Invasive growth was scarcely visible. The BrdU index of stratified DJM-1 cells was 19.7 ± 1.9% for 24 h (Fig 4). BrdU-positive cells were randomly scattered in every layer of the structure. To examine the effects of air exposure on the behavior of DJM-1 cells, we conducted Experiment 1–2 (Fig 3B). The air exposure caused enhanced stratification of DJM-1 cells and formed an unpolared stratified layer of eight to 10 cells within 2 wk. The BrdU index of DJM-1 cells under the air-exposed condition was 24.2 ± 6.7% for 24 h. Furthermore, under the air-exposed condition, DJM-1 cells underwent cellular hypertrophy with enlarged cytoplasm and nucleus.

**Experiment 2 (Dermal-fibroblast-containing system)** To examine the effects of dermal fibroblasts on DJM-1 cells, we conducted Experiment 2–1 (Fig 3C). The stratification and BrdU index (19.8 ± 2.8%) of DJM-1 cells were almost the same as those of the cells on fibroblast-free gel without air exposure (Fig 4), but we found that DJM-1 cells minimally invaded the fibroblast-embedded gel within 2 wk. To examine the coeffects of the fibroblasts and air exposure on proliferation and invasion of DJM-1 cells, we conducted Experiment 2–2 (Fig 3D). DJM-1 cells clearly underwent an enhanced stratification and invaded deeper into the fibroblast-embedded gel with air exposure than without it. The BrdU index of DJM-1 cells was 20.8 ± 3.3% for 24 h (Fig 4).

**Experiment 3 (Subcutaneous-adipocyte-containing system)** To examine the effects of adipocytes on the proliferation and differentiation of DJM-1 cells, we conducted Experiment 3–1 (Fig 3E). Under this condition, DJM-1 cells formed a well-organized stratified layer within 2 wk. flattened and cuboidal cells were aligned at the upper and lower layers, respectively, of the structure. The flattened cells contained granular materials and were overlaid by an amorphous cornified-like structure. The flat cornified-like structure at the uppermost layer and cuboidal basal cell-like cells in the basal layer were also clearly observed by electron microscopy (Fig 5). The BrdU index of stratified DJM-1 cells was 4.7 ± 1.4% for 24 h (Fig 4). This result was significantly lower than that under the stromal-cell-free condition. Involucrin, which is a marker of terminal differentiation of the epidermis, was expressed in the upper layer, but was not expressed at the basal layer of the stratified layer of DJM-1 cells (Fig 6C). Also, cytokeratin 10, which is a suprabasal marker of differentiation in stratified squamous epithelia, was expressed in the upper layer, but was not expressed at the basal layer of the stratified layer of DJM-1 cells (Fig 6G). In contrast, involucrin and cytokeratin 10 were scantily expressed in the stratified layer under the stromal-cell-free condition (Fig 6D, E).

To examine the coeffects of adipocytes and air exposure on DJM-1 cells, we conducted Experiment 3–2 (Fig 3F). DJM-1 cells clearly underwent a thicker stratification and lost the flattened cells containing granular materials, but maintained the high organization and keratinization at the upper layers containing differentiated “swollen” cells with degenerated nuclei. The BrdU index of stratified DJM-1 cells was 15.5 ± 5.4% for 24 h (Fig 4). This result was significantly lower than that under the stromal-cell-free condition with air exposure. Involucrin and cytokeratin 10 were expressed in the upper layers of the stratified layer (Fig 6D, H). We also used human subcutaneous adipocytes in Experiment 3–1. The result was similar to that of Experiment 3–1 with rat subcutaneous adipocytes (data not shown).

**Experiment 4 (Stromal-fibroblast- and adipocyte-containing system)** To examine the effects of fibroblasts and adipocytes together on DJM-1 cells, we conducted Experiment 4–1 (Fig 3G). DJM-1 cells on the gel containing both fibroblasts and adipocytes made a well-organized stratified layer, similar to that of Experiment 3–1. The flattened cell with granular materials and the amorphous cornified-like structure, however, were not seen in the stratified layer. Moreover, the DJM-1 cells showed almost the same invasive growth into the gel as those on the merely fibroblast-embedded gel (Experiment 2–1). The BrdU index of stratified DJM-1 cells was 10.4 ± 3.3% for 24 h (Fig 4). To examine the total coeffects of fibroblasts, adipocytes, and air exposure on DJM-1 cells, we
conducted Experiment 4-2 (Fig 3H). DJM-1 cells stratified into eight to 10 cell layers and invaded deeper into the fibroblast- and adipocyte-containing collagen gel with air exposure than without air exposure. The degree of invasion of DJM-1 cells was similar to that observed in Experiment 2-2. The BrdU index of stratified DJM-1 cells was 19.4 ± 4.0% for 24 h (Fig 4).
Deposition of extracellular matrix components. Reticulin silver staining was localized in a linear pattern beneath the stratified layer of the DJM-1 cells in all the experiments. Type IV collagen was immunolocalized in a linear pattern similar to the reticulin silver staining in all the experiments. The type IV collagen was immunolocalized more intensely in Experiment 2 (fibroblast-containing system) and Experiment 4 (fibroblast- and adipocyte-containing system), however, than in Experiment 1 (stromal-cell-free system) and Experiment 3 (adipocyte-containing system). Moreover, invaded DJM-1 cells also expressed type IV collagen (Fig 3, insert).

The expression of MMP-2. The DJM-1 cells did not express MMP-2 in any experiments of our study. Only fibroblasts in Experiments 2 and 4 expressed MMP-2 (data not shown).

Adhesion of cytokines. We examined the effects of leptin, TNF-α, and IGF-II on DJM-1 cells. These cytokines did not replicate the above adipocyte-induced phenomena in DJM-1 cells. As a positive control, we administered 10 ng per ml TNF-α to culture adipocytes. TNF-α clearly induced lipolysis of adipocytes (data not shown). We also administered leptin to culture Madin–Darby canine kidney (MDCK) cells. Leptin influenced tubulogenesis of the cells in a dose-dependent manner: 100 ng per ml leptin caused MDCK cells to form smaller tubules than 10 or 500 ng per ml leptin (data not shown). These results indicate that the TNF-α and leptin examined in this study have biologic effects on the target cell types.

DISCUSSION

In this report, we have described that mesenchymal stromal cell types of dermal fibroblasts and subcutaneous adipocytes affect the proliferation and differentiation of a squamous cell carcinoma cell line of the skin (DJM-1).

In epidermal keratinocytes, cellular maturation is characterized by the appearance of keratohyalin granules in the stratum granulosum, the formation of cornified envelopes in the stratum corneum, and the transition from synthesis of low to high molecular weight keratin polypeptides (Knight et al., 1984; Fuchs, 1990). Malignant transformation disrupts this normal process and expression of terminal differentiation markers. The DJM-1 cells cultured alone on the gel as a control experiment formed an unpolarized stratified layer. In the adipocyte-containing system (Experiment 3-1), however, the DJM-1 cells formed a well-organized stratified layer. The well-organized stratified layer in vitro expressed differentiation markers of involucrin and cytokeratin 10. This expression pattern resembled that of normal epidermis in vivo. This phenomenon means that adipocytes promote differentiation of DJM-1 cells. Moreover, adipocytes exerted a differentiation-promoting effect on other cell lines, HeLa cells and lower jaw squamous cell carcinoma-T3M-1 cells, as well (data not shown).

This phenomenon must be regulated mostly by paracrine or matricrine mechanisms, as the effects are observed in the absence of direct cell–cell contact (data not shown) and thus are most probably mediated by adipocyte-derived cytokines, “adipocytokines”. These cytokines, including heparin-binding epidermal growth factor, IGF-II, TGF-α, leptin, adiponectin, transforming growth factor β, plasminogen activator inhibitor I, and undetected factors (Maeda et al., 1996, 1997), were recently suggested to affect normal keratinocytes (Sugihara et al., 1991), hair follicle cells (Misago et al., 1998), human lung squamous cell line (Tsuchiya et al., 1999), and also laryngeal squamous cell carcinoma cells, as described in Yamada’s report. These effects of adipocytes on cancer cells are tumor cell type-specific to some degree. For example, adipocytes promoted the invasive effects of fibroblasts or air exposure in squamous cell carcinoma of the larynx (Yamada et al., 1999). We tried to examine whether “adipocytokines” such as TNF-α, IGF-II, or leptin affect DJM-1 cells, but we have not obtained any positive results so far. Further examination of other cytokines is recommended.

The fibroblasts in the gel facilitated the invasive growth of DJM-1 cells. Fibroblast-induced invasion of cancer cells has been reported in many studies (Matsumoto et al., 1989; Kawahara et al., 1993; Iwazawa et al., 1996). In our experiments, fibroblasts promoted both type IV collagen secretion and invasive growth of DJM-1 cells. This phenomenon was also reported in a previous study (Kawahara et al., 1993). These results suggest that DJM-1 cell-produced type IV collagen does not suppress their invasive growth. Conversely, DJM-1 cells may actively utilize type IV collagen secreted by the tumor cells themselves for undergoing invasive growth (Chen et al., 2000). In the invasive cascades of carcinoma cells, MMPs, i.e., 72 kDa gelatinase (MMP-2) and 92 kDa gelatinase (MMP-9), play a crucial role (Pyke et al., 1992, Kawahara et al., 1993; Dumas et al., 1999). In these experiments, we also confirmed that fibroblasts, but not DJM-1 cells, expressed MMP-2 (data not shown). Thus, DJM-1 cells may make use of fibroblast-derived MMP-2 for degrading extracellular matrix molecules and performing invasive growth (Pyke et al., 1992, Sato et al., 1999).

Interestingly, the fibroblasts lessened the facilitating effect of adipocytes on the differentiation of DJM-1 cells. Conversely, the adipocytes did not suppress the facilitating effect of fibroblasts on the invasive growth of DJM-1 cells, with or without air exposure (Experiments 4-1 and 4-2). We believe that adipocyte-derived cytokines have a promoting effect on differentiation of DJM-1 cells, but do not have an inhibitory effect on invasion of DJM-1 cells in vitro. Clinically, we know many cases of advanced cutaneous squamous cell carcinomas that invade into subcutaneous adipose tissue (Friedman et al., 1985; North et al., 1997). The metastatic rate significantly increases in proportion to the invasive depth into cutaneous tissue (Breuninger et al., 1990). Because many factors are involved in a complex way in cancer cell invasion, the effects of...
adipocyte-produced cytokines on cancer cells may be masked by the other factors in vivo. Taken together, our findings suggest that subcutaneous adipocytes have a crucial role in the biologic behavior of cancer cells.

With regard to basement membrane formation, type IV collagen expression of DJM-1 cells was more intense in Experiment 2 (fibroblast-containing system) and Experiment 4 (fibroblast- and adipocyte-containing system) than in Experiment 1 (stromal-cell-free system) and Experiment 3 (adipocyte-containing system). This suggests that differentiation-promoting and proliferation-inhibiting effects of subcutaneous adipocytes on DJM-1 cells may not depend on basement-membrane-associated molecules, i.e., type IV collagen.

Several studies have shown that cancer cell–stromal cell, cancer cell–air exposure interactions play crucial roles in the proliferation, differentiation, and invasive growth of cancer cells (Fusenig et al, 1995). In our study, air exposure promoted the proliferation of DJM-1 cells. This condition of cell culture has been applied to keratinocytes and cancer cells in previous studies (Boukamp et al, 1985; Sugihara et al, 1991; Boelsma et al, 1999; Yamada et al, 1999). In the adipocyte-containing system, air exposure also stimulated the proliferation and stratification of the DJM-1 cells. Even under this air-exposed condition, DJM-1 cells maintained a well-organized stratified layer with intense expressions of involucrin and cytokeratin 10. Our results suggest that the effects of adipocytes on DJM-1 cells are not disrupted completely by a cutaneous environmental factor, air exposure. Although we cannot sufficiently clarify the mechanism of the adipocyte-induced phenomena above, further studies about this adipocyte-related phenomenon would advance cutaneous cancer research.

In conclusion, we have shown for the first time that subcutaneous adipocytes facilitate the differentiation of squamous cell carcinoma cells of the skin in vitro. Greater knowledge of the effects of adipocytes, induction of differentiation, and the reduction of proliferation of cancer cells will be helpful in the designation of differentiation therapy.

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Figure 6. Localization of differentiation markers of each experiment in the vertical sections of DJM-1 cells cultured on the collagen gels. The expressions of involucrin and keratin were seen only sporadically in the carcinomatous layer on the cell-free collagen (A, Experiment 1-1) and in the enhanced stratified layer on the cell-free collagen with air exposure (B, Experiment 1-2). Note the high expressions of involucrin confined to the suprabasal layers of the carcinomatous layer on adipocyte-embedded gel (C, Experiment 3-1) and in the enhanced stratified layer on adipocyte-embedded gel with air exposure (D, Experiment 3-2). The expressions of cytokeratin 10 in each condition were similar to that of involucrin (E, Experiment 1-1; F, Experiment 1-2; G, Experiment 3-1; H, Experiment 3-2). *Unilocular adipocytes. Scale bar: 100 μm.