Effects of Stromal Fibroblasts and Fat Cells and an Environmental Factor Air Exposure on Invasion of Laryngeal Carcinoma (HEp-2) Cells in a Collagen Gel Invasion Assay System

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Objective: To clarify the invasion mechanism of laryngeal carcinoma (HEp-2) cells.

Design: Human HEp-2 cells were cultured on a collagen gel containing fibroblasts and/or fat cells. The HEp-2 cells were also treated with air exposure as the local environment of the laryngeal epithelial mucosa. A collagen gel invasion assay was conducted under these culture conditions.

Results: No invasion of HEp-2 cells was found in the stromal cell–free collagen gel, but a slight invasion was observed in the fibroblast-embedded gel. A deeper invasion of HEp-2 cells occurred in the fibroblast- and fat cell–coembedded gel and in the fibroblast-embedded gel with air exposure. The most extensive invasion of HEp-2 cells was observed under the fibroblast- and fat cell–coembedded gel in combination with air exposure.

Conclusions: Fat cells and air exposure clearly increase the invasive effect of fibroblasts in squamous cell carcinoma of the larynx. The combined effect of these 3 factors (ie, fat cells, fibroblasts, and air exposure) plays a very important role in the invasive growth of the carcinoma cells. This observation suggests that both tumor cell–stromal cell interaction and tumor cell–local environmental factor interaction should be taken into account in an investigation of the invasive and proliferative mechanisms of laryngeal carcinoma.


Cancer is characterized by local invasion and distant metastasis.1,2 In invasive and metastatic cascades, tumor cell–stromal cell or tumor cell–extracellular matrix interaction plays a crucial role.1,2 In fact, several studies have shown that fibroblasts, as stromal component cells, promote invasion of carcinoma cells originating from the mouth or esophagus.3,4 No investigation to determine what stromal or locally environmental factors actually affect the invasive growth of laryngeal carcinoma cells has yet been carried out. In terms of the histoanatomical structure of the larynx, the laryngeal tumor cells as well as those of other organs are considered to intimately interact with stromal fibroblasts or extracellular matrix at an early invasive stage of this carcinoma.3 In the subsequent deeply invasive growth of the tumor, neoplastic cells would be expected to interact with fat cells, because fat cells exist in the deep layer of the larynx, and were recently suggested to affect many cell types by their production of various cytokines.6 Also, the laryngeal mucosal epithelium is physiologically exposed to air.3 We have therefore hypothesized that an interaction between tumor cells and fibroblasts, fat cells, and/or air exposure might be involved in the invasive growth of laryngeal carcinoma. To elucidate this hypothesis, we performed a collagen gel invasion assay,3 using a laryngeal squamous cell carcinoma cell line (HEp-2)7 and isolated fibroblasts and fat cells.8 In this study, HEp-2 cells formed a stratified epithelial layer on collagen gel, resembling the epithelial stratification in a reconstruction culture of normal laryngeal mucosa previously described by us,7 and showed no invasive growth into the gel without stromal fibroblasts, fat cells, or air exposure. We conclude that the combination of fibroblasts, fat cells, and air exposure induces the greatest invasive growth of HEp-2 cells.

RESULTS

To examine the effects of stromal component cells, ie, fibroblasts and fat cells, and of a local environment, ie, air exposure,
on the invasion of HEp-2 cells, we performed a collagen gel invasion assay. The results are summarized in Table 1.

EXPERIMENT 1 (STROMAL CELL–FREE SYSTEM)

To investigate whether the HEp-2 cells cultured on stromal cell–free collagen gel invaded the gel, we conducted experiment 1-1 as a control counterpart. The HEp-2 cells on stromal cell–free collagen gel were elliptical or cuboidal at the longitudinal cross section. Their cellular stratification on the collagen gel was observed during the 4-week observation. In the first week, the HEp-2 cells formed a stratified layer of 1 or 2 cells, and then the thickness of the cell layer gradually increased. By the fourth week of the cell culture, a cellular layer of 5 to 6 cells was formed (Figure 2). The proliferation of HEp-2 cells was evaluated at various times by the method of BrdU uptake, and the results are shown in Table 2. There was no significant difference in the indices among these culture stages. The HEp-2 cells were demarcated and arranged in a linear fashion at the side where they were in contact with the gel, but they did not invade the gel. This borderline at the contact point between the cells and the gel corresponded to a distinct basal membrane organized by the HEp-2 cells.

This membrane structure was clearly demonstrated on reticulin silver impregnation staining (Figure 2). The results indicated that the HEp-2 cells did not invade the stromal cell–free gel at any culture stage during the observation.

To examine effects of the local environmental factor of air exposure alone on invasion of HEp-2 cells, we conducted experiment 1-2. The HEp-2 cells formed a stratified layer of 5 to 8 cells in the first week of culture and thereafter gradually increased the thickness of the cell layer. They formed a stratified layer of 18 to 22 cells by the fourth week of culture. In fact, the BrdU intake of the cells with air exposure was higher than that of the cells without air exposure (Table 2). Interestingly, we found that HEp-2 cells invaded the collagen gel with a stimulation of air exposure alone (Figure 3, E, and Figure 4). This invasion indicated that air exposure alone promoted an invasive growth of HEp-2 cells in the gel.

EXPERIMENT 2 (STROMAL FIBROBLAST–CONTAINING SYSTEM)

To examine the effects of stromal fibroblasts alone on the invasion of HEp-2 cells, we conducted experiment 2-1 (Figure 1). The cellular stratification was nearly the same

MATERIALS AND METHODS

CARCINOMA CELL LINES AND ISOLATION OF FIBROBLASTS AND FAT CELLS

In this study, we used 2 types of squamous cell carcinoma cell lines, HEp-2 (ATCC, CCL 23) and KB (ATCC, CCL 17), which were derived from human laryngeal and oral carcinomas, respectively. The KB cells were used as a reference to avoid the possibility of HEp-2 cell–limited phenomena. Human fibroblasts and rat fat cells were used as stromal cells. Fibroblasts were collected from human laryngeal specimens; tissue tips were cultured; and migrating fibroblasts were subcultured for 3 to 10 passages and used for assay. Fat cells were collected from the abdominal hypodermis of rats, and aseptically excised adipose tissue was minced, digested with a collagenase solution at 37°C for 30 minutes, and filtered through a 70-µm mesh sieve, as described previously. The filtered cells in suspension were dispersed in minimum essential medium supplemented with 50% fetal calf serum. After centrifugation, the fat cells floating on top were collected with a Pasteur pipette. This suspension-centrifugation course was repeated 3 times to dilute the collagenase solution, and dissociated fat cells were obtained.

COLLAGEN GEL INVASION ASSAY

To conduct the collagen gel invasion assay, we used a 3-dimensional collagen gel culture, as described previously.8,9

Experiment 1

Eight volumes of acid-soluble type I collagen solution (pH 3), 1 volume of ×10 concentrated minimum essential medium, and 1 volume of reconstruction buffer (2.2 g of sodium bicarbonate and 4.77 g of HEPES dissolved in 100 mL of 0.05N sodium hydroxide) were mixed. The mixture, without addition of fibroblasts or fat cells, was poured into a 30-mm dish with a nitrocellulose bottom and incubated at 37°C for 30 minutes to solidify the gel. This inner dish was placed into a 90-mm outer dish, and medium was added to both dishes. The collagen gel layer prepared in the inner dish corresponded to the lamina propria in vivo. Then, 2 mL of HEp-2 cell suspension in culture medium at a concentration of 1 × 10⁶ cells per milliliter was spread onto the reconstructed lamina propria (experiment 1-1). The HEp-2 cells became confluent on the reconstructed lamina propria within 2 days. At this stage, the culture medium on the HEp-2 cells was removed for exposure of 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 HEp-2 cells. Since the humidity inside the incubator was higher than 95%, the surface of the HEp-2 cells was not completely dry (experiment 1-2). This was the standard procedure for our collagen gel invasion assay (Figure 1). To examine the interaction between the HEp-2 cells and the stromal cells, the following 3 systems were prepared, and a collagen gel invasion assay was performed in a similar manner to that of experiment 1-1.

Experiment 2

Fibroblasts were added to the collagen solution mixture at a concentration of 10 × 10⁶ cells per milliliter, and 2 mL of the mixture was placed in the inner dish. After gelation, the HEp-2 cell suspension was spread on the gel, the lamina propria substrate (Figure 1).

Experiment 3

Fat cells were added to the collagen solution mixture at a concentration of 3 × 10⁶ cells per milliliter; the solution was

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mixed; and 2 mL of the mixture was poured into the inner dish. After gelation, the HEp-2 cell suspension was spread on the gel (Figure 1).

Experiment 4
One and a half milliliters of a collagen solution mixture containing fat cells was poured into an inner dish. This fat cell-containing layer was compatible with the submucosal layer of the larynx. Then, after gelation, 0.5 mL of collagen solution mixture containing fibroblasts was overlaid on the gel. After second gelation, a HEp-2 cell suspension was spread on the gel; therefore, these artificial conditions very closely simulated the epithelial-to-submucosal layer of the larynx (Figure 1).

Finally, air exposure treatment also was conducted in experiments 2 through 4 using the same method as that used in experiment 1-2.

EXAMINATION OF CULTURE CELLS
Culture cells in this study were examined by the following methods: (1) The cells were observed with a phase contrast microscope. (2) Collagen gel containing cultured cells was fixed with 10% formalin, embedded in paraffin, thin-sectioned vertically, deparaffinized, and stained with hema-toxylin-eosin. To observe basement membrane formed by cultured Hep-2 cells, deparaffinized sections were subjected to reticulin silver impregnation staining. The results of this staining agreed with those of the immunohistochemical technique using type IV collagen. (3) Cancer cells and fibroblasts were identified by immunostaining with cytokeratin, a marker of epithelial cells, and vimentin, a marker of mesenchymal cells, respectively, as described previously.9 Fat cells were identified morphologically with oil red O lipid stain. (4) Bromodeoxyuridine (BrdU, 2 μL, 30 mg/mL) was added to 2 mL of culture medium for 12 hours. Deparaffinized sections from gels fixed with acetic acid-ethanol were immunostained with anti-BrdU antibody using a commercially obtained BrdU kit (Amersham, Arlington Heights, Ill). To obtain the labeling index of nuclear BrdU uptake, 1000 cells were counted, and the percentage of BrdU-positive nuclei was calculated, as previously described.10

MORPHOLOGICAL AND MORPHOMETRIC ANALYSIS OF HEp-2 CELL INVASION

In this study, we assayed an invasion of HEp-2 cells into gel as follows. The HEp-2 cells cultured on stromal cell-free gel formed a linear borderline at the contact points between the cells and the gel, and the cells had no downgrowth into the gel. This borderline, corresponding to a basement membrane–like structure, which was visualized using a reticulin silver impregnation stain, made the HEp-2 cells linearly arrange parallel to the gel layer. Therefore, we judged both the smoothly linear borderline and the nondowngrowth of the cells into the gel as a non-invasion (Figure 2). In contrast, we judged both the irregular borderline and the downgrowth as an invasion (Figure 3).

To semiquantitatively examine the invasion of the HEp-2 cells into the gel, we measured the invasive degree by the following formula: the sum of the lengths of the invasive parts at the level of the basement membrane divided by the sum of the lengths of the basement membrane.

STATISTICAL ANALYSIS

The data obtained through 4 independent experiments were statistically analyzed by 2-way analysis of variance. Values represent the mean ± SD; P<.05 was considered significant.

as in experiment 1-1. The HEp-2 cells formed a stratified layer of 1 or 2 cells in the first week of culture, and then the thickness of the cell layer gradually increased. By the fourth week of cell culture, the cells organized a stratified layer of 5 to 6 cells. The proliferation of the HEp-2 cells was almost the same as that of the cells in the fibroblast-free gel without air exposure (Table 2). We also found that HEp-2 cells invaded the collagen gel (Figure 3, A, and Table 3).

To analyze the coeffects of fat cells and air exposure on HEp-2 cells, we conducted experiment 3-2. The HEp-2 cells clearly demonstrated thicker stratification in the presence of air exposure than in the absence of air exposure (Figure 3, B, and Table 3). This finding indicated that air exposure promoted the invasive effect of fibroblasts on HEp-2 cells.

EXPERIMENT 3 (STROMAL FAT CELL–CONTAINING SYSTEM)

To examine effects of stromal fat cells alone on invasion of HEp-2 cells, we conducted experiment 3-1 (Figure 1). The cellular stratification of HEp-2 cells was thicker in experiment 3-1 than in experiment 1-1. The HEp-2 cells formed a stratified layer of 2 to 4 cells in the first week of culture, and then the thickness of the cell layer gradually increased. By the fourth week of cell culture, the cells organized a stratified layer of 8 to 12 cells. However, we also found that the HEp-2 cells never invaded the collagen gel (Figure 3, C, and Table 3).

To analyze the coeffects of fat cells and air exposure on HEp-2 cells, we conducted experiment 3-2. The HEp-2 cells clearly demonstrated thicker stratification in the presence of air exposure than in the absence of air exposure (Figure 3, D). Interestingly, the HEp-2 cells invaded the fat cell–embedded gel with air exposure. This finding indicated that the combination of fat cells and air exposure induced an invasion of HEp-2 cells, whereas fat cells alone did not.

EXPERIMENT 4 (STROMAL FIBROBLAST–AND FAT CELL–CONTAINING SYSTEM)

To examine the combined effect of stromal fibroblasts and fat cells on the invasion of HEp-2 cells, we conducted experiment 4-1. The HEp-2 cells on the gel containing both fibroblasts and fat cells (experiment 4-1) showed deeper and more extensive invasive growth in the gel than those on a fibroblast-only–embedded gel (ex-
The invasive degree was almost the same as that in experiment 1-2 at any stage of culture.

To examine the combined effect of fibroblasts, fat cells, and air exposure on HEp-2 cells, we conducted experiment 4-2. The HEp-2 cells on the gel containing fibroblasts and fat cells along with air exposure invaded the gel most deeply and showed thicker stratification than those on the collagen gel without stromal component cells (Figure 3, F and G, and Figure 5). The indices of BrdU uptake were about 6.6% at any stage of culture. Figure 4 shows the invasive degree. These results indicate that the combination of stromal fibroblasts and fat cells and of a local environmental factor, ie, air exposure, induced the most invasive growth of HEp-2 cells.

Finally, the results obtained from the collagen gel invasion assay of the KB cells under the culture conditions examined in this study were almost similar to those of the HEp-2 cells.

COMMENT

To examine the invasive mechanism of laryngeal carcinoma, we performed a collagen gel invasion assay with a squamous cell carcinoma cell line (HEp-2) derived from a human laryngeal carcinoma. Some researchers conducting studies on the invasive mechanism of carcinoma cells with a collagen gel invasion assay have reported that there is an interaction between carcinoma cells and stromal cells, such as fibroblasts.3,11 A recent study demonstrated that, in addition to adipose storage, fat cells may be involved in the production of cytokines.6 However, to our knowledge, the interaction of fat cells and carcinoma cells has not been studied. In the present study, we observed the influence of stromal fibroblasts and fat cells on the invasion of a laryngeal carcinoma cell type in a collagen gel matrix culture. Also, the invasive growth of HEp-2 cells was examined with the additional factor of air exposure, because the laryngeal mucosa is exposed to air under physiological conditions. Interestingly, we have demonstrated, for the first time to our knowledge, that the combination of all 3 factors (stromal fibroblasts, stromal fat cells, and exposure to air) induces the greatest invasion and proliferation of the cells. This observation suggests that tumor cell–local environment factor interaction as well as tumor cell–stromal cell interaction should be taken into consideration when the
invasive and proliferative mechanisms of laryngeal carcinoma are being investigated.

In studies on the invasion of carcinoma cells of the head and neck regions, some investigators have conducted a collagen gel invasion assay using oral and esophageal squamous carcinoma cells. The invasion of carcinoma cells occurred in the collagen gel containing fibroblasts as well as in the fibroblast-conditioned medium, although the invasive degree of the tumor cells was greater in the fibroblast-containing gel than in the fibroblast-conditioned medium. This invasion was not found in the fibroblast-free collagen gel in their study. Another study showed that the hepatocyte growth factor/scatter factor (HGF/SF) mediates the invasion of the carcinoma cells. Some studies suggest that carcinoma cells could produce several kinds of soluble factors, such as platelet-derived growth factor, basic fibroblast growth factor, and interleukin 1. Furthermore, these cytokine-affected fibroblasts actively produce HGF/SF and thereby induce invasion of carcinoma cells. Our study demonstrated that the interaction between the carcinoma cells and their stromal cells plays an important role in the invasion of carcinoma cells. This result allows us to consider HGF/SF as a soluble growth factor related to the invasion of HEp-2 cells.

In the present study, we examined an interaction between fat cells, a stromal component cell type of the larynx, and carcinoma cells. Although no invasion of HEp-2 cells was found in the collagen gel containing only the fat cells, a markedly stratifying growth of the carcinoma cells was shown. In the reconstruction culture of the skin, our previous study showed that fat cells clearly promote the epidermal stratification without an invasive downgrowth of epidermal cells into the gel. These results suggest that fat cell stimulation alone may play a more important role in the proliferation and stratification of HEp-2 cells than their invasion. Also, our current study has shown that the stimulation of air exposure alone enhances both the invasion and the proliferation of HEp-2 cells. In a reconstruction culture of the normal larynx and cornea, our

Figure 2. Paraffin-embedded vertical cross section of collagen gel invasion assay system of laryngeal carcinoma (HEp-2) cells. A and B, The HEp-2 cells form a stratified layer of 1 or 2 cells during the first week. C and D, The HEp-2 cells form a stratified layer of 2 or 3 cells in the second week. E and F, By the fourth week of cell culture, 5 or 6 cellular layers are shown on the cell-free collagen gel. During all culture stages, the HEp-2 cells contacting the cell-free collagen gel are linearly arranged on the gel with a distinct basement membrane–like structure (arrowheads), which is positive for the reticulin silver impregnation stain. The HEp-2 cells never undergo an invasive growth into the gel (A, C, and E, hematoxylin-eosin; B, D, and F, reticulin silver impregnation stain; bars = 40 µm).
Figure 3. Paraffin-embedded vertical cross section of collagen gel invasion assay system of laryngeal carcinoma (HEp-2) cells at 3 weeks of culture. A, The HEp-2 cells on the fibroblast-embedded gel slightly invade the gel. B, The HEp-2 cells on the fibroblast-embedded gel with air exposure invade the gel more deeply and show thicker stratification than cells without air exposure. C, The HEp-2 cells on the fat cell-embedded gel do not invade the gel, although they show thicker stratification than those on the collagen gel without stromal component cells. D, The HEp-2 cells on the fat cell-embedded gel with air exposure invade the gel more deeply than those on the fibroblast-embedded gel and show remarkable stratification. E, The HEp-2 cells on the cell-free collagen gel with air exposure invade the gel more deeply than those on the fibroblast-embedded gel and show thicker stratification than those on the collagen gel without stromal component cells. F and G, The HEp-2 cells on the gel containing both fibroblasts and fat cells in combination with air exposure show the deepest invasion of the gel and the thickest stratification. Arrowheads indicate invasive lesions of HEp-2 cells; arrows, fibroblasts; and asterisks, fat cells (A-F, hematoxylin-eosin; G, reticulin silver impregnation stain; bar = 40 μm).
and cornea.\textsuperscript{15,16} Our previous studies demonstrated that air exposure alone does not induce the invasive down-growth of these normal epithelial cells into gel, although it clearly promotes their proliferation. These results suggest that air exposure may make HEp-2 cells themselves, but not normal epithelial cells, produce some soluble factors and that these factors may generate an invasive growth of the cells in an autocrine manner. Further studies are needed to explain the exact mechanism of HEp-2 cell invasion under the condition of air exposure.

We did not find any invasion of HEp-2 cells in the collagen gel that contained only fat cells, although Hep-2 cells showed proliferative ability on the gel. However, this invasion of the HEp-2 cells was more remarkable in the collagen gel that contained both fibroblasts and fat cells than in the collagen gel that contained only fibroblasts. These results appear to indicate that fibroblasts directly promote the invasion of carcinoma cells, while fat cells indirectly enhance this invasive growth through improving the proliferation of carcinoma cells.

Finally, oral squamous carcinoma–derived KB cells underwent invasive growth similar to that of HEp-2 cells under our culture conditions. This suggests that fibroblasts, fat cells, and air exposure, alone or in combination, may play an important role in the invasive growth of squamous cell carcinoma that originates in the upper airway.

In conclusion, an outstanding outcome of our present experiment is that a combination of fibroblasts, fat cells, and air exposure induced the highest invasive growth of laryngeal carcinoma cells (HEp-2) under all culture conditions tested. Our collagen gel invasion assay system appears to provide carcinoma cells with a physiological environment in which carcinoma cells can in-
teract with laryngeal stromal component cells, fibroblasts, and fat cells, and be exposed to air, a laryngeal local factor. This system is very valuable not only to clarify the mechanism of the proliferation and invasion of laryngeal carcinoma cells, but also to pioneer a biological approach for the treatment of laryngeal carcinoma. Also, this method will probably be useful in the selection of chemotherapy, cytokine therapy, or radiotherapy for the treatment of laryngeal carcinoma. To clarify these invasive mechanisms in more detail and to develop new strategies for the treatment of laryngeal carcinoma, further studies are in order.

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