Hepatocyte growth factor is a predominant chemoattractant for gingival epithelial cells produced by radicular cyst-derived fibroblast-like cells

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Abstract: To clarify whether fibroblasts could be involved in the pathogenesis of periodontal pocket formation, the chemotactic activity of radicular cyst-derived fibroblast-like cell (RCF)-conditioned medium (RCF-CM) for gingival epithelial cells was examined using a modified Boyden chamber assay. RCF-CM possessed significant chemotactic activity, which was decreased markedly by treatment with anti-human hepatocyte growth factor (HGF) antibody. Furthermore, the chemotactic activity of RCF-CM was well correlated with HGF content. These results show that the RCF secrete an HGF-like factor, and suggest that such a factor derived from periodontal fibroblasts might play a role in epithelial apical migration in periodontitis. (J. Oral Sci. 42, 101-106, 2000)

Key words: radicular cyst-derived fibroblast-like cell; chemotactic activity; gingival epithelial cell; hepatocyte growth factor.

fibers become detached from the cementum. For the development of epithelial down-growth, collagen fibers between root cementum and alveolar bone have to be broken down, so that epithelial tissues invade the space occupied by connective tissue.

Although the epithelial cell itself may secrete some matrix-degrading enzymes such as collagenase (2,3), as we have reported previously, epithelial cells also secrete a substance that stimulates collagenase production by surrounding fibroblasts, suggesting that this co-relationship may cause periodontal disease (4,5).

From another viewpoint, as several cytokines have been reported to act as chemoattractants for migrating epithelial cells (6-13), it is possible that epithelial down-growth could be induced by some factor secreted from surrounding fibroblasts. A fibroblast-derived chemotactic factor for epithelial cells in the oral region, however, has scarcely been tested. Therefore, in this preliminary study, we examined whether radicular cyst-derived fibroblast-like cells secrete some chemoattractant, such as hepatocyte growth factor, for a gingival epithelial cell line, Ca9-22.

Introduction

The loss of connective tissue attachment followed by epithelial down-growth is the most complicated problem in periodontitis. According to a histological view of pocket formation, Aisenberg and Aisenberg (1) hypothesised that epithelial pegs migrate in an apical direction and extend between the periodontal ligament fiber bundles before the

Materials and Methods

Gingival epithelial cells

A well-characterized gingival epithelial cell line (14-17), Ca9-22 (JCRB 0625), provided by the Health Science Research Resources Bank (Osaka), was used in this study. The cells were cultured in Dulbecco's modified Eagle medium (DMEM, Iwaki) supplemented with 10 % fetal bovine serum (FBS, Biowhittaker) and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin, Sigma) at 37°C in a humidified atmosphere with 5 % CO₂/95 % air. The

cells were passed using 0.5 % trypsin/5.3 mM EDTA solution (Gibco), and used as gingival epithelial cells in this study.

Culture of radicular cyst-derived fibroblast-like cells (RCF)

Connective tissues from 5 surgically removed radicular cyst walls were rinsed and dissected. All the patients gave informed consent before providing the samples. The fragments were placed on the bottom of 6-well plates (Iwaki) and covered with coverslips; each cyst tissue was separately treated. The tissue fragments were incubated for 2 weeks under the same conditions as described above. The cells derived from the explants were collected, and subcultured up to the fifth passage.

Sample preparation for chemotaxis assay

Confluent RCF cultures from 5 individual cysts were incubated in fresh DMEM supplemented with 10 % FBS for a further 48 h under the same conditions, and the medium was separately harvested as RCF-conditioned medium (RCF-CM) to use for the epithelial cell chemotaxis assay.

Several cytokines having chemotactic activity for epithelial cells (6-13) were also used in this study; 10 ng/ml each sample of cytokine-containing fresh DMEM supplemented with 10 % FBS was used for testing epithelial cell chemotaxis. The cytokines used were as follows: recombinant human transforming growth factor- α (6) (TGF- α , Gibco), recombinant human epidermal growth factor (6) (EGF, Gibco), recombinant human keratinocyte growth factor (11,13) (KGF, Strathmann Biotech), recombinant human hepatocyte growth factor (13) (HGF, R&D) and recombinant human interleukin-8 (10) (IL-8, R&D).

Epithelial cell chemotaxis assay

The chemotactic activity of each sample of RCF-CM was determined by modified Boyden chamber assay (10,18) without matrix coating on the filter. Five hundred microliters of RCF-CM or cytokine-containing fresh medium was put into the lower chamber of a 24-well plate (Sumitomo Bakelite), and then the chemotaxis (upper) chamber, which had an 8- μ m pore filter (Chemotaxicell, Kurabo), was placed to allow application of 250 μ l of the epithelial cell suspension (4×10^5 cells/ml). The epithelial cells were allowed to migrate for 40 h in the same atmosphere as described above. After the incubation, the upper chambers and media were discarded, and the cells that had migrated and attached to the bottom of the well were stained with crystal violet solution [2 % (w/v) crystal violet, 0.04 %

(w/v) ammonium oxalate, 20 % (v/v) methanol] and counted under an inverted microscope. Four assays per specimen were performed, and chemotactic activity was calculated as means and standard deviations.

Effect of anti-hHGF neutralizing antibody

A sample of RCF-CM (case 1) was treated with 10 μ g/ml goat anti-human HGF neutralizing antibody (R&D) or normal goat IgG (R&D) for 10 min at room temperature. The chemotactic activity of the antibody-treated RCF-CM was determined as described above.

Correlation between the HGF content and chemotactic activity

In another experiment, the HGF content of the RCF-CM was determined with a commercially available ELISA kit (R&D). Simultaneously, the chemotactic activities of RCF-CM for epithelial cells were determined, as described above. Correlation between chemotactic activity (mean of the four assays) and the HGF quantity (mean of the duplicate assays) was calculated using Pearson's correlation coefficient.

Results

Culture of RCF

A photomicrograph of the primary culture of fibroblast-like cells derived from the connective tissue of the radicular cyst wall is shown in Fig. 1. These cells were spindle-shaped, with a so-called fibroblast-like appearance. In this study, these cells were used as RCF.

Fig. 1 Phase-contrast photomicrograph of primary culture of radicular cyst wall connective tissue-derived fibroblast-like cells. The bar represents 200 μ m.

Fig. 2 Effects of cytokines on gingival epithelial cell chemotaxis. Ten ng/ml of each cytokine-containing fresh DMEM sample supplemented with 10% FBS was placed into the lower compartment of the chemotaxis chamber. After the cells were allowed to migrate for 40 h, the cells attached to the bottom of the well were stained with crystal violet and counted. The results are means and standard deviations (SD) from four determinations.

Fig. 3 Chemotactic activity of radicular cyst-derived fibroblast-like cell-conditioned medium (RCF-CM) for gingival epithelial cells. RCF-CM from 5 specimens was placed into the lower compartment of the chemotaxis chamber. After the epithelial cells were allowed to migrate for 40 h, the cells attached to the bottom of the well were stained and counted. The results are means and SD from four determinations.

(a)

(b)

Fig. 4 Photomicrographs of migrated gingival epithelial cells. Fresh DMEM supplemented with 10 % FBS (a) or RCF-CM (b) was placed into the lower compartment of the chemotaxis chamber. After the chemotaxis assays were performed, the cells attached to the bottom of the well were stained and photographed under an inverted microscope. Original magnification $\times 40$.

Effects of cytokines on epithelial cell chemotaxis

Among the cytokines used, only HGF had chemotactic activity (approximately threefold the activity of the control) for gingival epithelial cells (Fig. 2).

Chemotactic activity of RCF-CM

The chemotactic activity for gingival epithelial cells

was induced by RCF-CM from 5 specimens (Fig. 3). The chemotactic activity of each specimen of RCF-CM varied, and the activity was about 5- to 20-fold that of the control.

Photomicrographs of migrated and attached cells on the bottom of the well are shown in Fig. 4. Numerous cells were attached to the bottom of the well in a test sample (b) compared with the control (a).

Effect of anti-hHGF antibody treatment

RCF-CM treated with anti-hHGF antibody show significant decrease in chemotactic activity for epithelial cells (case 1, Fig. 5). The activity decreased to approximately a quarter of the activity of RCF-CM without antibody treatment. Similar results were obtained for the other four RCF-CM (cases 2-5, data not shown).

Correlation between the chemotactic activity and HGF content

Correlation between the chemotactic activity of 5 RCF-CM samples and HGF content is shown in Fig. 6. The chemotactic activity was well correlated to HGF content (correlation coefficient, $r = 0.983$).

Discussion

HGF acts as a mitogen, motogen, morphogen, and anti-apoptotic factor for various kinds of epithelial cells (19-21), and also acts as a scatter factor (SF). It is a protein secreted by fibroblasts and promotes motility and matrix invasion of epithelial cells (9). For medical use, HGF is expected to treat disorders such as hepatitis owing to its regenerative action; however, it activates some types of tumor cell invasion and metastasis with its characteristics as an SF (21). For the above reasons, we assume that epithelial down-growth following the loss of connective

tissue attachment could be induced by HGF/SF or by an HGF-like factor. Our preliminary but obvious results clearly demonstrate that RCF secrete some chemotactic factor for gingival epithelial cells. Furthermore, the chemotactic activity of RCF-CM was significantly decreased by the treatment with anti-hHGF neutralizing antibody, suggesting that the major chemotactic factor existing in RCF-CM is HGF itself or a closely related factor. Neaud et al. (18) reported a similar observation, that chemotactic (invasive) activity for hepatocellular carcinoma cells by a myofibroblast-derived factor was completely blocked by anti-hHGF antibody. However, the chemotactic activity of RCF-CM was not completely inhibited by anti-hHGF treatment in this study, indicating that RCF-CM contains some other chemoattractants.

HGF expression in periodontal stromal cells has been little reported (22,23). Sugiyama et al. (23) reported that lipoteichoic acid and interleukin-1 synergistically stimulate HGF production by gingival fibroblasts in culture. They also demonstrated that recombinant hHGF stimulates DNA synthesis by epithelial cells, but they did not test HGF action as a chemoattractant. Further investigation is needed to clarify which factor, such as HGF/SF, is actually involved in the apical migration of epithelial cells in periodontitis. Now we are investigating whether gingival or periodontal ligament fibroblasts secrete some chemoattractant for gingival epithelial cells.

Fig. 5 Effect of anti-human HGF neutralizing antibody. A sample of RCF-CM was treated with 10 μ g/ml of anti-human HGF neutralizing antibody or normal goat IgG for 10 min at room temperature. Then, gingival epithelial cell chemotaxis assays were performed as described in Fig. 3.

Fig. 6 Correlation between the HGF content and chemotactic activity. Correlation between the HGF content in RCF-CM was measured by ELISA and its chemotactic activity was calculated. They were well-correlated (correlation coefficient, $r = 0.983$).

We used connective tissue-derived fibroblast-like cells from radicular cyst walls as a source of fibroblast-conditioned media. One of the reasons arose from an observation reported by Gao et al. (24). Their study revealed that stromal cells expressing keratinocyte growth factor (KGF) exist just beneath the proliferating antral lining of epithelial cells of apical lesions. As KGF is a stroma-derived, potent mitogen for epithelial cells (11,13,25), we assumed that KGF-expressing cells simultaneously express HGF, as reported by Li and Tseng (26).

Receptors for HGF (c-Met protein) mRNA are coded as *c-met* (9,20,21). We have shown by reverse transcriptase polymerase chain reaction that not only the gingival epithelial cell line (Ca9-22), but also the radicular cyst lining epithelium, express mRNA for *c-met* (submitted). The epithelial lining of a radicular cyst is thought to originate from the epithelial cell rests of Malassez (27), and these cells are likely to respond some kinds of growth factors (28-30). Spouge (31-33) has suggested repeatedly that there is a relationship between the epithelial cell rests of Malassez and chronic marginal periodontitis. If the epithelial cell rests of Malassez express c-met, and respond to HGF/SF produced by periodontal stromal fibroblasts, it would not be surprising if they facilitate apical migration of the junctional epithelium during the pathogenesis of periodontal pocket formation.

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