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Original Article

Ameloblast-lineage cells of rat tooth germs proliferate and scatter in response to hepatocyte growth factor in culture

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ABSTRACT Hepatocyte growth factor (HGF) is considered to be one of the mediators of epithelial-mesenchymal interactions during early organogenesis and to be involved in the development of murine molars. In this study, the immunohistochemical localization of HGF and of its receptor, c-Met, revealed that HGF was distributed in the proliferating mesenchymal cells in the dental papillae and that c-Met was continuously expressed in the epithelial cells during the development of rat incisors. These observations confirmed the involvement of HGF in the development of rat incisors, as demonstrated previously in molars. We then used a primary culture of ameloblast-lineage cells, prepared from mandibular incisors of young rats, to examine the direct effects of HGF on the growth and differentiation of ameloblasts. We found that HGF at 2-20 ng/ml induced a marked increase in the number of ameloblast-lineage cells and in the scattering of such cells. Our results suggest that HGF promotes the proliferation and scattering of ameloblast-lineage cells simultaneously.

KEY WORDS: *hepatocyte growth factor, c-Met, serum-free culture, ameloblast-lineage cell, scattering, cell proliferation*

Introduction

Many growth factors have been reported to participate in the morphogenesis of teeth (Thesleff and Sahlberg, 1996), for example, nerve growth factor (Byers *et al.*, 1990), fibroblast growth factors (Gonzalez *et al.*, 1990), bone morphogenetic proteins (Vainio *et al.*, 1993) and insulin-like growth factor (Joseph *et al.*, 1994). In a recent study *in vitro*, we demonstrated that hepatocyte growth factor (HGF) also participates in the morphogenesis of murine molars (Tabata *et al.*, 1996a). We showed that blockage of the translation of the mRNA for HGF by an antisense oligodeoxynucleotide resulted in formation of abnormal teeth, in which the enamel organ was surrounded by a thin layer of dentin and dental papillae. The appearance of these teeth was "inside-out", as compared with normal tooth germs. It seems likely that this abnormal structure was the result of an imbalance between the proliferative activity of the cells of the inner enamel epithelium and that of the dental papillae. However, the mechanism remains to be clarified.

Hepatocyte growth factor (HGF; Nakamura *et al.*, 1989), known also as scatter factor (SF; Weidner *et al.*, 1990), is synthesized mainly in mesenchymal cells. It has the unique ability to stimulate mitosis of hepatocytes (Michalopoulos, 1990) and many types of epithelial cell (Igawa *et al.*, 1991; Matsumoto *et al.*, 1991a,b; Rubin *et al.*, 1991). HGF also has dissociation- and migration-promoting activities in epithelial cells (Stoker *et al.*, 1987) and endothelial cells (Rosen *et al.*, 1994). c-Met tyrosine kinase, encoded by the *MET* protooncogene, is a high-affinity receptor for HGF (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). Expression of this protein has been detected in various fetal tissues, such as the lung, pancreas, submandibular salivary gland and nasal cavity, as well as teeth (Sonnenberg *et al.*, 1993). These observations suggest that HGF might be a humoral mediator of epithelial-mesenchymal interactions in developing organs.

In this study, we examined the direct effects of HGF on the growth and differentiation of ameloblasts using a primary culture of ameloblast-lineage cells that consisted of inner enamel epithelial cells, preameloblasts and ameloblasts, which we prepared from the mandibular incisors of young rats. Prior to our study of cells in

Abbreviations used in this paper: HGF, hepatocyte growth factor; SF, scatter factor; PBS, phosphate-buffered saline; CV, crystal violet; OD, optical density; BrdU, 5-bromo-2'-deoxyuridine; E-, embryonic day; P-, postnatal day.

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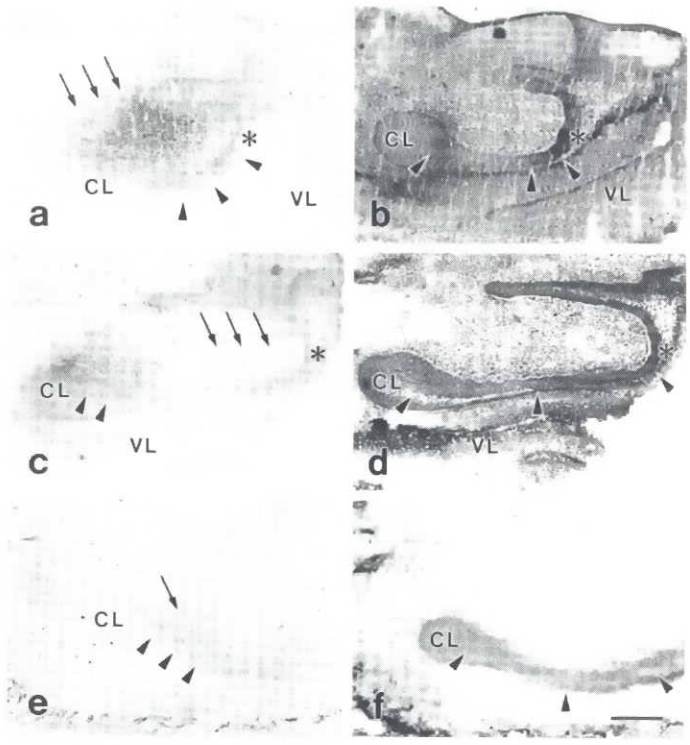

Fig. 1. Expression of HGF and c-Met in incisor tooth germs at various stages of development. *Sagittal cryosections of rat mandibles (E18 to P0) were immunostained for HGF (a,c,e) and c-Met (b,d,f).* **(a)** *HGF at E18. Intense staining is detectable in dental papillae (arrows), and weak staining is also detectable in the inner enamel epithelium (arrowheads).* **(b)** *c-Met at E18. Positive staining is visible throughout the enamel organ. Strong signals are visible especially in the inner enamel epithelium (arrowheads), the stellate reticulum and the stratum intermedium.* **(c)** *HGF at E19. Positive staining has disappeared at the tip of the dental papillae (arrows), but weak signals can be detected in the inner enamel epithelium of the cervical area (arrowheads).* **(d)** *c-Met at E19. Positive signals are still detectable throughout the enamel organ, including the inner enamel epithelium (arrowheads), the stellate reticulum and the stratum intermedium.* **(e)** *HGF at P0. Weak staining is still detectable in the dental papillae near the cervical loop (arrow), facing the proliferation zone (arrowheads) of the inner enamel epithelium.* **(f)** *c-Met at P0. Positive signals are visible in the inner enamel epithelium (arrowheads), the stellate reticulum and the stratum intermedium. Asterisk, the tip of the incisor; CL, cervical loop; VL, vestibular lamina. Bar, 200 μm.*

culture, we performed an analysis of intact tissue to localize HGF and c-Met in rat incisors in order to confirm the involvement of HGF in their development.

Results

Distribution of HGF and c-Met in the rat incisor

We detected immunoreactivity specific for HGF in the entire area of dental papillae at E18 (Fig. 1a). However, immunoreactivity decreased from the tip of the dental papillae towards the inner area of that and was present at a limited level only in the dental papillae adjacent to the cervical loop at E19 (Fig. 1c). By P0, the immunoreactivity has decreased still further, and signals were detected only in the dental papillae that faced the proliferation zone (Smith, 1980) of the inner enamel epithelium (Fig. 1e). At all stages analyzed, HGF was also detected at distal end of the inner enamel epithelium cells (Fig. 1a,c,e).

We detected the expression of c-Met in both the tooth germ and the surrounding mesenchyme at an early stage. In the tooth germ, we detected immunoreactivity throughout the enamel organ and immunostaining was particularly intense in the inner enamel epithelium at the tip and the cervical loop at E18 (Fig. 1b). Such staining was also observed at E19 and P0. However, the signals were weak, with the exception of those in the cervical loop (Fig. 1d,f).

Expression of c-Met in cultured ameloblast-lineage cells

We detected immunoreactivity specific for c-Met in all the cell types (Fig. 2a). The percentage of cells that were immunopositive for c-Met tended to increase during the culture period, being 78% on day 1 and more than 99% from day 4 onwards. In the presence of HGF at 6 ng/ml, the percentage did not change after day 4 (over 99%) but the relative number of scattering cells was much greater than that in the absence of HGF (Fig. 2b).

Effect of HGF on the proliferation of cells

To determine the optimum effective concentration of HGF in cultures of ameloblast-lineage cells, we included HGF at 0 to 20 ng/ml in the culture medium for either 4 days or 6 days (Fig. 3a). HGF at concentrations of 2 ng/ml and 6 ng/ml had an obvious stimulatory

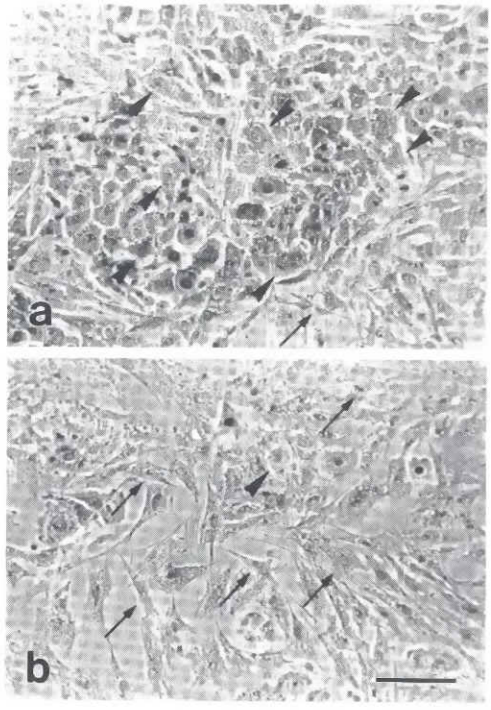

Fig. 2. Immunoreactivity specific for c-Met and morphology of primary cultured ameloblast-lineage cells on day 4 after inoculation, with culture in the absence (a) and presence (b) of 6 ng/ml HGF. *Positive staining is visible in almost all cells, namely, both scattering (arrows) and clustered (arrowheads) cells.* **(a)** *Most cells have formed clusters, but scattered cells can also be seen (arrows).* **(b)** *There are a few clusters of cells, but mainly scattering cells (arrows) can be seen. Bar, 100 μm.*

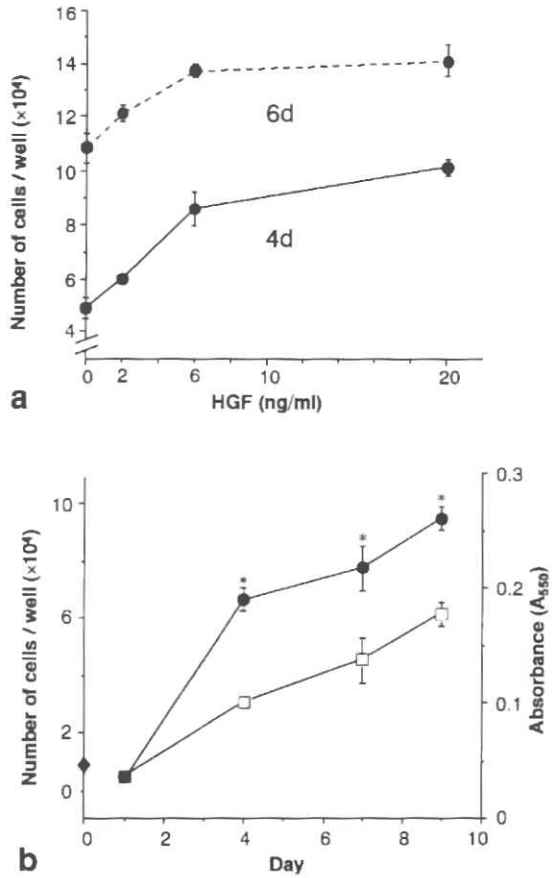

Fig. 3. Effects of HGF on cell growth. (a) *Dose-dependent stimulation by HGF of the growth of ameloblast-lineage cells. Ameloblast-lineage cells were cultured with HGF at 0, 2, 6 and 20 ng/ml for 4 days (solid line) or 6 days (dotted line). Cell numbers were estimated by counting cells in ten 0.25-mm² fields. Each point and bar represent the mean of results from 4 samples ±SE.* **(b)** *Time course of growth of primary cultured ameloblast-lineage cells in the presence (closed circles) and absence (open squares) of HGF. HGF was added to the medium at 6 ng/ml every second day, when the medium was changed. Each point and bar represent the mean of results from 4 samples ±SE. * A significant difference compared with the control ($p < 0.05$). Right ordinate represents A_{550} after staining with CV, and left ordinate represents cell number, as estimated from staining with CV. The rhombus on the left ordinate represents the initial number of ameloblast-lineage cells (1×10⁴ cells/well).*

effect on the proliferation of ameloblast-lineage cells, as compared with proliferation in the absence of added HGF. The effect of HGF at 20 ng/ml was similar to that at 6 ng/ml. Therefore, 6 ng/ml appeared to be the optimum effective concentration of HGF in cultures of ameloblast-lineage cells. The time courses of proliferation of ameloblast-lineage cells cultured with or without 6 ng/ml HGF showed that HGF had a stimulatory effect on cell growth (Fig. 3b).

Effect of HGF on the scattering of cells

HGF at 6 ng/ml promoted the scattering of ameloblast-lineage cells. It increased the number of single cells as compared with numbers in cultures without HGF (Fig. 2). In the presence of HGF, the scattering index of ameloblast-lineage cells increased from 8.0% to 13% during the first 4 days of culture and then remained constant until day 9 (Fig. 4). By contrast, in the absence of HGF, the index decreased to 3% during the first 4 days. Furthermore, removal of HGF after 4 days of culture in the presence of HGF resulted in a decrease in the index from 13% to 6% over the next 3 days. These findings indicate that ameloblast-lineage cells scatter in the presence of HGF and form clusters in its absence.

Distribution of proliferating cells

BrdU was incorporated predominantly by scattering ameloblast-lineage cells and by the peripheral cells in cell clusters and little was recognized in the inner cells of cell clusters (Fig. 5a,b). Thus, the former two types of cell had higher proliferative activity than the latter. In a quantitative analysis of the effects of HGF on the distribution of BrdU-labeled cells, we found that HGF had no obvious effect on their distribution. Thus, the labeling indices of single cells were close to 39% both in the presence and in the absence of HGF, while those of clustered cells were about 7% and 8% in the presence and in the absence of HGF, respectively (Fig. 5c). In other words, the respective rates of proliferation remained unchanged. Together with the increase of sacattering cells in the presence of HGF (Fig. 4), the increase of the cells (Fig. 3) was thought to be caused by the high proliferation rate of scattering cells.

Discussion

Immunohistochemical analysis demonstrated the characteristic temporal and spatial distribution of HGF, as well as the continuous expression of c-Met, in rat incisor tooth germs (Fig. 1). HGF was localized in the dental papillae that faced the proliferation zone of the inner enamel epithelium (Fig. 1a,c,e) and c-Met was expressed continuously in the cells of the enamel organ (Fig. 1b,d,f). The limited expression of HGF in restricted areas of mesenchyme is thought to determine the site of an epithelial response. These findings are essentially consistent with data obtained from murine molars (Tabata *et al.*, 1996a), that is, HGF is expressed in the mesenchymal area near the cervical loop in both molars and incisors, as well as in the intercuspal groove in molars (Fig. 6). These areas face the proliferation zone of the inner enamel epithelial cells (Smith, 1980), and thus, HGF appears to be involved in the proliferation of epithelial cells. Our study in culture also showed that HGF has a strong effect to the proliferation of ameloblast-lineage cells (Fig. 3). These results all indicate that HGF stimulates the proliferation of ameloblast-lineage cells.

The expression of HGF was still detectable in the dental papillae near the cervical loop in incisors after birth (Fig. 1e), but not in molars (Tabata *et al.*, 1996a). This difference in the timing of expression is probably based on the specific nature of the development of murine incisors. Murine incisors erupt to form a crown continuously throughout the animal's life and formation of a crown is never terminated. Moreover, incisors do not have a root and have their own special morphology. If HGF is absent from molar tooth germs, formation of the crown is confounded by the imbalance in rates of proliferation between ameloblast-lineage cells (i.e., the epithelial cells in the enamel organ) and dental papilla cells (i.e., the mesenchymal cells that face the enamel organ) (Tabata *et al.*, 1996a). In this study, we demonstrated that HGF stimulates the proliferation of ameloblast-lineage cells. Therefore, one possible role of HGF is to promote the proliferation of ameloblast-lineage

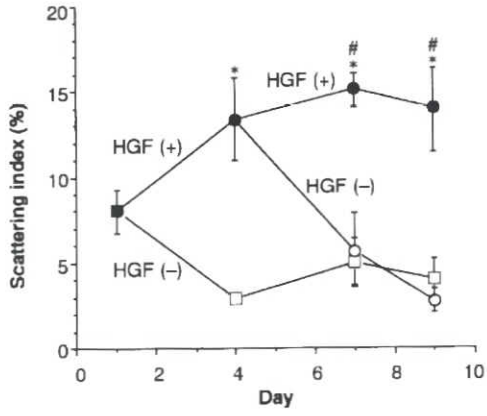

Fig. 4. Time course of the scattering effect of HGF on primary cultured ameloblast-lineage cells. *An index of scattering activity was estimated as the percentage of single cells relative to the total number of cells (see Materials and Methods). HGF was added to the medium at 6 ng/ml for 9 days (closed circles) or for the first 4 days only (open circles). Control cultures (open squares) were incubated without HGF. Each point and bar represent the mean of result from 3 samples ±SE. Symbols indicate a significant difference as compared to the control culture (*) or to the culture treated with HGF for the first 4 days only (#) ($p < 0.05$ in both cases).*

cells. This promotion should raise the ameloblast sheet sufficiently to cover the mass of the dental papilla and allow normal crown formation.

The proliferation of cells seemed to be coupled with the scattering of cells (Fig. 4). The respective rates of proliferation of scattering cells and clustered cells were unchanged by HGF (Fig. 5c). When HGF was present in the medium, clusters of cells disappeared and many scattered cells were observed (Fig. 5a,b). This effect was temporary (Fig. 4). However, no obvious scattering is observed *in vivo*. Some mechanisms to overcome this effect of HGF might exist, for example, binding via extracellular matrices between ameloblast-lineage cells and dental papillae.

Separation of mesenchymal cells from epithelial cells is important in the establishment of primary cultures of epithelial cells. We used complete MCDB 153 medium, which is a serum-free culture medium that was originally used for the culture of keratinocytes (Boyce and Ham, 1983). This medium contains a low concentration of Ca^{2+} ions, which prevents the terminal differentiation of epithelial cells, and includes hydrocortisone to prevent the growth of mesenchymal cells that might contaminate the preparation during its isolation from intact tissue. In a preliminary study, cultures of ameloblast-lineage cells for 10 days were intentionally contaminated with dental papilla cells (at ratios of ameloblast-lineage cells to dental papilla cells of 10:0, 9:1, 7:3, 5:5 and 0:10). Only ameloblast-lineage cells were able to proliferate in the culture system (data not shown). Preparation of cells was facilitated by the fact that the ameloblast layer is located on the dentin or on the cheesy enamel in the mandibular incisor at postnatal stages, so it can easily be stripped off with minimum contamination by dental mesenchymal cells.

We cannot disregard the possibility of contamination by other types of epithelial cells, namely, cells of the stratum intermedium, stellate reticulum, outer enamel epithelium and oral epithelium. Although we could not distinguish these epithelial cells from ameloblast-lineage cells in culture, the extent of contamination by these cells was probably limited, as judged from the results of analysis with amelogenin-specific monoclonal antibody (Tabata *et al.*, 1996b). The contamination by oral epithelium was considered to be minimal because the layer of ameloblast-lineage cells is separated by alveolar bone from the oral epithelium in the rat incisor. Although the medium had to be supplemented with bovine pituitary extract, in which the main effector is regarded to be basic fibroblast growth factor (bFGF), preliminary experiments revealed that bFGF did not modify the effects of HGF on ameloblast-lineage cells.

Treatment of ameloblast-lineage cells with 6 ng/ml HGF induced scattering of cells, which was accompanied by a change in

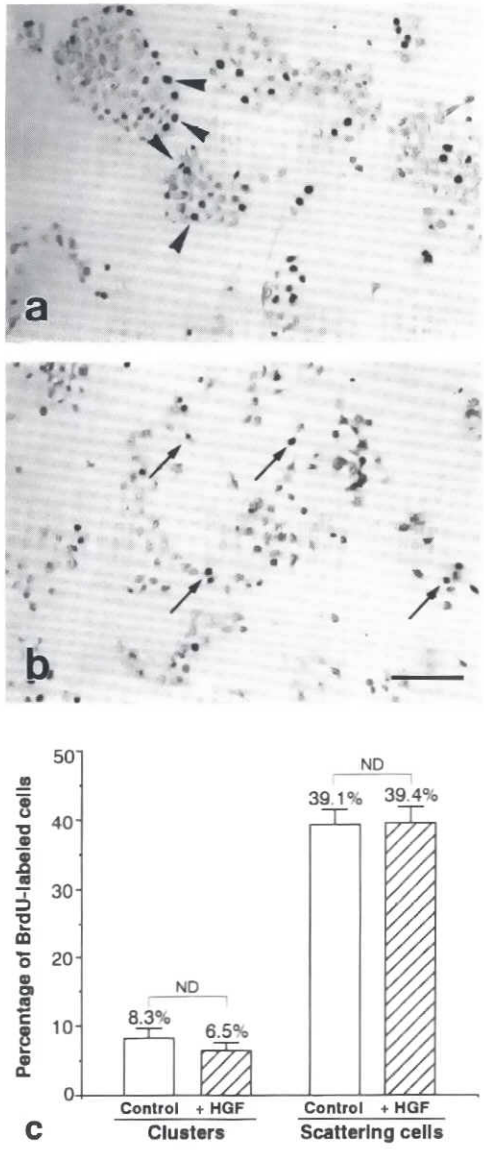

Fig. 5. Labeling of ameloblast-lineage cells with BrdU. *Ameloblast-lineage cells were cultured in the absence* **(a)** *or presence* **(b)** *of HGF at 6 ng/ml for 4 days. BrdU was incorporated by scattering cells (arrows) and by peripheral cells in cell clusters (arrowheads). Bar, 150 μm.* **(c)** *Comparison of BrdU labeling indices of clustered and single cells in the absence (open columns) and in the presence (shaded columns) of 6 ng/ml HGF. Bars represent SE for results from 4 samples. ND, No significant difference.*

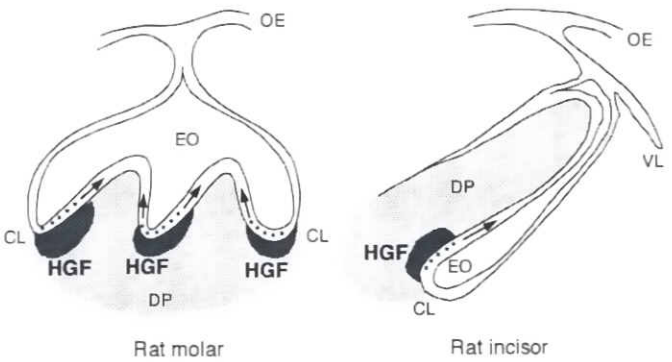

Fig. 6. A schematic representation of the expression of HGF (densely shaded area) and the differentiation of cells in developing tooth germs. *Inner enamel epithelial cells proliferate in response to stimulation by HGF and differentiate in the direction of the future cusp tip of the molar or the tip of the incisor (arrows). OE, Oral epithelium; CL, cervical loop; VL, vestibular lamina; DP, dental papilla; EO, enamel organ; asterisks, proliferation zones of ameloblast-lineage cells (taken from Smith, 1980). The distal-proximal direction in these teeth corresponds to the left-right direction in the Figure.*

the morphology of the cells to a more fibroblastic type (Fig. 2). This finding is consistent with previous reports of studies with keratinocytes (Matsumoto *et al., 1991a*), Madin-Darby canine kidney (MDCK) cells (Weidner *et al.*, 1991), and corneal epithelial cells (Wilson *et al.*, 1994). HGF inhibits intercellular communication via gap junctions in murine keratinocytes. This effect is rapid, being apparent 5 to 10 min after the addition of HGF, and it is also transient (Moorby *et al.*, 1995). The dissociation or loosening of intercellular contacts among epithelial cells is also thought to be important in morphogenesis during epithelial-mesenchymal interactions (Birchmeier and Birchmeier, 1993). The scattering of cells in monolayer cultures is also considered to indicate the ability, *in vivo*, of such cells to undergo complex cytoskeletal rearrangement and to participate in intercellular interactions, as demonstrated by the induction of tubulogenesis and scattering of cells in a murine line of inner medullary collecting-duct epithelial cells (mIMCD-3) by HGF (Cantley *et al.*, 1994). Thus, the scattering of ameloblast-lineage cells in the presence of HGF might be an event that occurs prior to cell proliferation and tooth morphogenesis.

In this study, we monitored the expression of HGF in dental papillae and observed the continuous expression of c-Met in ameloblast-lineage cells in rat incisor tooth germs. HGF stimulated the proliferation of ameloblast-lineage cells in culture and such proliferation was coupled with the scattering of cells. Taken together with our previous results (Tabata *et al.*, 1996a), these results indicate that HGF is an essential factor in crown formation, promoting the proliferation of ameloblast-lineage cells.

Materials and Methods

Animals

Newborn Sprague-Dawley rats were purchased from Nihon Dobutsu (Osaka, Japan). Rats on embryonic day-18 (E18), E19, and postnatal day 0 (P0) were used for immunohistochemical analysis, and P7 rats were used for preparation of ameloblast-lineage cells.

Immunohistochemical staining

Mandibles, surgically removed from E18, E19, and P0 rats, were embedded in OCT compound (Miles Lab., Elkhart, IN) by quick freezing without pre-fixation and decalcification, and they were cut into serial sections (14 μm) on a cryostat. The specimens were mounted on glass slides, air-dried, fixed with neutralized buffered 10% formalin for 30 min, and used for immunohistochemical analysis. Immunostaining using biotin-streptavidin-conjugated β-galactosidase was performed as described previously (Tabata *et al.*, 1992, 1996a). As primary antibodies, we used affinity-purified rabbit antibodies against mouse c-Met (SP260; 1:200; #sc-162; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit antibodies against rat HGF (1:200; a generous gift from Dr. T. Nakamura, Osaka University Medical School). As second antibodies, we used biotinylated goat antibodies against rabbit IgG (1:200; Zymed Laboratories, San Francisco, CA). Cultured ameloblast-lineage cells were immunostained by the same procedures, after removal of the culture medium.

Antiserum against rat HGF had been raised in rabbits immunized with recombinant rat HGF, and antibodies were purified by use of highly purified recombinant rat HGF that had been immobilized on Sepharose beads. The antibodies recognized both the α- and β- chains of murine and rat HGF (Tabata *et al.*, 1996a). Non-specific staining was examined by use of preimmune sera, and specific staining with the antibodies was examined in Kupffer cells of rat liver.

Primary culture of ameloblast-lineage cells

Ameloblast-lineage cells were prepared from P7 rat mandibular incisors as described by Tabata *et al.* (1996b). The cells were resuspended in complete MCDB 153 medium, inoculated at a density of 1×10^4 cells/100 ml/well on type I collagen-coated 96-well plates (#4860-010; Iwaki, Tokyo, Japan) and then cultured in an atmosphere of 5% CO_2 in air at 37°C. The day of inoculation was designated day 0. Since these cells consisted of ameloblasts, preameloblasts and inner enamel epithelial cells, they were designated ameloblast-lineage cells. During culture, the medium was changed every second day. Recombinant human HGF (a generous gift from Dr. T. Nakamura, Osaka University Medical School, Japan) was added to the medium as indicated in the Results section.

Determination of cell growth

Cells that had been cultured on 96-well plates were rinsed with PBS and stained with 50 μl/well of 0.4% crystal violet (CV) in methanol for 30 min at room temperature (Kueng *et al.*, 1989; Saotome *et al.*, 1989). Stained specimens were rinsed with water to remove excess dye and then air-dried. The optical density (OD) of the specimens was measured at 550 nm with a microplate reader (model 450; Bio-Rad Laboratories, Richmond, CA). The number of cells was correlated with the absorbance by reference to a blank control, and the cell number was calculated from the following the formula: cell number (cells/well)= $4.1051 \times 10^5 \times A_{550} - 8799.5$. The linear relationship between OD and actual cell number was determined by counting the number of cells per 0.01-cm^2 field in 96 trials.

Identification of proliferating cells

Proliferating cells were identified by monitoring incorporation of BrdU with a Cell Proliferation kit (#RPN 20; Amersham International plc, Buckinghamshire, UK). Cells were pulse-labeled for 2 h with BrdU in complete MCDB, rinsed with PBS, and fixed with neutralized buffered 10% formalin for 30 min.

After treatment with 3% normal goat serum in PBS for 30 min to block non-specific binding of antibodies, the cells that had incorporated BrdU were stained blue-black upon immunostaining with horseradish peroxidase-conjugated BrdU-specific antibodies and subsequent treatment with the appropriate substrates. When maximum staining had developed, cells were rinsed with water to stop the reaction.

Scattering index

To quantify the scattering of cells incubated with or without HGF, a scattering index, namely, the percentage of obviously scattering cells, was estimated by counting individual cells under a light microscope after staining with CV, as described above. Those cells with growing pseudopods and a fibroblast-like shape, which made no contact with any surrounding

cells, were regarded as scattering cells. The index was calculated from the mean percent of scattering cells in ten 0.25-mm^2 fields per well.

Acknowledgments

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