Development 122, 1243-1251 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV3341

Hepatocyte growth factor is involved in the morphogenesis of tooth germ in murine molars

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SUMMARY

The patterns of gene expression for hepatocyte growth factor (HGF) and its receptor, c-Met, were revealed in the tooth germ of rat mandibular molars using RT-PCR. In situ hybridization demonstrated that the HGF gene was expressed only in the cells of the dental papilla of the tooth germ in vivo. The characteristic temporospatial distribution of HGF and c-Met during germ development was revealed using immunohistochemical studies in vivo. In order to demonstrate the functional role played by HGF in tooth development, HGF translation arrest by antisense phosphorothioate oligodeoxynucleotide (ODN) was carried out in vitro. In the control experiment, explants of tooth germs from embryonic 14 day mice were cultured in a modification of Trowell's system under serum-free and chemically defined conditions for two weeks. Other explants were cultured with 15mer antisense or sense ODN targeted to the HGF mRNA. Both the control and the sense-treated explants showed normal histological structure, as observed in vivo. On the other hand, antisense-treated explants exhibited an abnormal structure in which the enamel organs were surrounded by a thin layer of dentin and dental papilla, appearing 'inside-out' compared to the control and sense-treated explants, although the cytodifferentiation of ameloblasts and odontoblasts was not inhibited. The explants treated with recombinant human HGF combined with antisense ODN showed normal development, indicating that exogenous HGF rescued the explants from the abnormal structure caused by antisense ODN. The findings of a BrdU incorporation experiment suggested that the imbalance between the proliferation activity of the inner enamel epithelium and that of the dental papilla caused by HGF translation arrest results in the abnormal structure of the tooth germ. These results indicate that HGF is involved in the morphogenesis of the murine molar.

Key words: murine tooth development, hepatocyte growth factor (HGF/SF), c-*met*, antisense oligodeoxynucleotide, organ culture of tooth germ

INTRODUCTION

Hepatocyte growth factor (HGF) was originally identified in the serum and platelets of partially hepatectomized rats and was found to be a potent mitogen for normal adult rat hepatocytes in culture (Nakamura et al., 1984; Russell et al., 1984). It was subsequently cloned from both rats and humans (Nakamura et al., 1989). HGF is a heterodimeric molecule composed of a 69 kDa α-subunit and a 34 kDa β-subunit (Nakamura et al., 1986; Gohda et al., 1988), and the molecular cloning of HGF has revealed that it has four kringle domains in the α-chain and a serine protease-like domain in the β-chain (Nakamura et al., 1989; Tashiro et al., 1990). Several studies have established the c-*met* proto-oncogene product as the cellular receptor for HGF (Bottaro et al., 1991; Naldini et al., 1991).

Recently, it has been shown that HGF has a mitogenic effect on various types of epithelial and endothelial cells (Rubin et al., 1991; Nakamura, 1991; Matsumoto et al., 1992) and chondrocytes (Takebayashi et al., 1995). HGF also enhances the motility of various type of cells, acting as 'scatter factor', a molecule capable of causing the dissociation and migration of epithelial cells (Stoker et al., 1987; Weidner et al., 1990). HGF is also considered to be a humoral mediator of morphogenic epithelial-mesenchymal interactions; it induces branching tubule formation by renal tubular epithelial cells (Montesano et al., 1991a) and hepatic bile duct epithelial cells (Johnson et al., 1993) when the cells are cultured in a three-dimensional collagen matrix. These observations suggest that HGF plays a crucial role as a mitogen, a motogen, and a morphogen in the organization of multicellular structures.

There are indications that tooth development is controlled by a series of interactions between apparently undifferentiated oral epithelium and neural crest-derived mesenchyme, resulting in the differentiation of ameloblasts and odontoblasts, respectively, and the secretion of enamel and dentin matrices (Kollar and Baird, 1970; Slavkin and Bringas, 1976; Mina and Kollar, 1987; Karavanova et al., 1992; Vainio et al., 1993; Joseph et al., 1994). Although the importance of epithelial-mesenchymal interactions in the development of the tooth is well established, the actual mechanisms involved in the mediation of inductive signals are only now beginning to be elucidated. For example, the cell surface proteoglycan, syndecan-1, and the extracellular matrix glycoprotein, tenascin, have been shown to be involved in epithelial-mesenchymal interactions in tooth development (Vainio et al., 1989, 1991; Vainio and Thesleff, 1992). Soluble diffusion factors, such as BMP-4 and TGF-β1 (Vaahtokari et al., 1991; Vainio et al., 1993), growth hormone (Zhang et al., 1992), IGF-1 (Joseph et al., 1993), FGF (Gonzales et al., 1990; Cam et al., 1992), NGF (Byers et al., 1990) and transcription factors, such as Egr-1, Msx-1 and Msx-2 (Karavanova et al., 1992; Jowett et al., 1993), have also been shown to be involved. Recently, Sonnenberg et al. (1993) reported the gene expression of HGF and c-*met* in the rat tooth germ, suggesting that HGF is involved in the epithelial-mesenchymal interactions that take place during tooth development. However, to our knowledge, no report has been made on the distribution of HGF and c-Met in tooth germ and its role in tooth development.

In this study, we demonstrate the gene expression of HGF and c-*met* in tooth germ. We also show the characteristic temporospatial distribution of HGF and c-Met in the dental epithelium and mesenchyme, which correlates with the histogenesis and cytodifferentiation in the tooth germ of first molars (M_1) of rat mandible, suggesting the involvement of HGF in tooth development. Further, we show that HGF antisense treatment on mouse tooth germ cultured in chemically-defined and serum-free medium produced an abnormal histostructure in which the enamel organ was surrounded by a thin layer of dentin and dental papilla, appearing 'inside-out' compared to control and sense-treated explants. Exogenous HGF provided a recovery to the normal structure in the antisense-treated explants. The results of BrdU incorporation suggested that an imbalance between the cell proliferation activity of the inner enamel epithelium and that of the dental papilla caused by antisense treatment resulted in the abnormal structure of the tooth germ. These findings suggest that HGF is involved in the morphogenesis of the murine molar.

MATERIALS AND METHODS

Animals

Pregnant time-mated and newborn Sprague-Dawley rats were purchased from Nihon Doubutsu (Osaka, Japan). Pregnant time-mated ICR mice were purchased from SLC Ltd (Shizuoka, Japan). The day a vaginal plug appeared was designated as E0 (embryonic day 0) and the day of birth was designated as P0 (postnatal day 0).

Total RNA preparation and RT-PCR

Total RNA was prepared by the method of Smale and Sasse (1992), with minor modifications. Ten to 20 freshly isolated tooth germs of mandibular M_1 were homogenized in 2 ml of 4 M guanidine isothiocyanate (GITC), 0.1 M Tris-HCl (pH 7.5), and 1% 2-mercaptoethanol (4 M GITC solution). The homogenate was mixed with 100 μl of 10% lauryl sarcosine solution, and spun for 5 minutes in a microcentrifuge; then 2 ml of supernatant was overlaid on an equal volume of density gradient (ρ=1.6) of cesium trifluoroacetate, containing 1 mM EDTA (pH 8.0), in a Beckman polyallomer centrifuge tube (13 mm × 51 mm). The samples were then centrifuged at 35,000 rpm (147,000 ***g***) for 20 hours at 18°C. After removal of the supernatant by aspiration, the precipitate was dissolved in 200 μl of 4 M GITC solution and then extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The extract was mixed with 20 μl of 3 M sodium acetate (pH 4.8) and precipitated with 2 vols (440 μl) of ethanol. The pellet was dissolved in 20-25 μl DEPC-treated water.

First strand cDNA synthesis from 0.5 μg total RNA was performed with Superscript reverse transcriptase (Gibco-BRL, Grand Island, NY) and random hexanucleotide primers. Subsequent amplification was carried out for 35 cycles under the following conditions: 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1.5 minutes. Primers for the detection of rat HGF were 5′-GCCATGAATTTGACCTC-TATGAA-3′ and 5′-TTTAATTGCACAATACTCCCACG-3′, generating a 518 bp fragment (Tashiro et al., 1990). Primer sequences for the detection of rat c-*met* were: 5′-CAGT(A/G)ATGATCT-CAATGGGCAAT-3′ and 5′-AATGCCCTCTTCCTATGACTTC-3′, generating a 725 bp fragment (Park et al., 1987; Chan et al., 1988). Primers for the detection of rat glyceraldehyde-3-phosphate dehydrogenase, which was the control internal marker were: 5′-AAGCAA-CATAGACGTTGTCGC-3′ and 5′-AATCAACACCTTCTTCG-CACC-3′, generating a 286 bp fragment (Tso et al., 1985). The amplified products were analyzed by 1.5% agarose gel electrophoresis.

In situ hybridization

A 1.4 kb *Eco*RI fragment of a rat HGF/SF cDNA (RBC1 clone) (Tashiro et al., 1990) was subcloned into the pGEM7 vector to synthesize both antisense and sense run-off transcripts labeled with digoxigenin-UTP (Boehringer Mannheim GmbH Biochemical, Germany). The labeled transcripts were alkaline-hydrolyzed to 300-500 nucleotides for use as riboprobes.

In situ hybridization was performed as described previously (Noji et al., 1990). Briefly, samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated in ethanol, cleared with toluene and embedded in paraffin. Sections 10 μm thick were then mounted in poly-L-lysine-coated glass slides. The sections were deparaffinized, treated with glycine and acetic anhydride, and hybridized with probes at 50°C for 16 hours. After hybridization, the slides were washed with 2× SSC at 50°C for 1 hour, treated with RNase A (20 μg/ml) at 37°C for 30 minutes, and then washed twice with 0.1× SSC at 50°C for 1 hour. Immunological detection of digoxigenin-UDP with alkaline phosphatase-conjugated anti-digoxigenin polyclonal antibody (Boehringer Mannheim GmbH Biochemical, Germany) was carried out using exactly the same procedure as described previously (De Block and Debrouwer, 1993).

Recombinant HGF and antibodies

Recombinant human and rat HGF (rhHGF and rrHGF) were purified from the culture medium of Chinese hamster ovary (CHO) cells transfected with expression vector containing rat HGF cDNA (Seki et al., 1990; Tashiro et al., 1990). Polyclonal antibody against rrHGF was raised in rabbits, as described previously (Montesano et al., 1991a,b). Monospecific anti-rat HGF antibody was purified using highly purified recombinant rat HGF immobilized on Sepharose beads. This antibody recognized both the α- and β-chains of mouse and rat HGF, and did not recognize the following proteins: human HGF, plasmin, insulin, EGF, TGF-α, TGF-β, tissue plasminogen activator, acidic FGF, and basic FGF. Western blotting analysis using this antibody on the mandibular M_1 of E20 rat also revealed the 69 kDa HGF-α chain and the 34 kDa HGF-β chain without significant noise. A polyclonal

antibody against mouse c-Met, raised in a rabbit, was purchased from Santa Cruz Biochemistry (Santa Cruz, CA), and also used for mouse and rat. Western blotting analysis using this antibody on the mandibular M1 of E20 rat revealed the 50 kDa c-Met-α chain and the 145 kDa c-Met-β chain without significant noise.

Immunohistochemistry

Rat mandibular M1 tooth germ with surrounding tissues was embedded in OTC compound (Miles Lab. Inc., Elkhart, IN), frozen quickly, cryo-sectioned (10 μm), mounted on glass slides and fixed with neutralized buffered 10% formalin or PLP fixative for 30 minutes. Procedures for immunostaining were carried out as described previously (Tabata et al., 1992) but 3% (v/v) normal goat serum in PBS was used as a blocking reagent and solutions of anti-rrHGF (1:200) or anti-c-Met antibody (1:200) were used as the first antibody, and a combination of the biotin-streptavidin amplification method and a β-galactosidase-enzymatic reaction was applied. Color was generated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranose (X-gal) as the substrate for β-galactosidase, which was visualized as a light blue. After immunostaining, some sections were counterstained with hematoxylin and eosin.

Preliminary, nonspecific staining was examined by employing preimmune sera, and specific staining using antisera was examined for Kupffer cells in rat liver.

Organ culture

Bud stage tooth germs of mandibular M1 were removed surgically from E14 mice and cultured for periods of up to 14 days using chemically-defined medium in a modification of Trowell's system (Yamada et al., 1980; Bringas et al., 1987; Shum et al., 1993); the explants were cultured using BGJb medium (Fitton-Jackson's modified BGJ; Gibco) freshly supplemented with 100 μg/ml ascorbic acid and 100 U/ml penicillin-streptomycin (Gibco) under conditions of 100% humidity in an atmosphere containing 5% CO_2 and 95% air at 37°C. The initial pH was adjusted to 7.4 and the medium was changed every 2 days.

Synthetic antisense phosphorothioate oligodeoxynucleotides

We designed two phosphorothioate oligodeoxynucleotides (ODNs) for the cDNA sequences of mouse HGF (Liu et al., 1994), one of which was a 15mer directly upstream from the initiation codon, the other being a 15mer directly downstream from the codon. In the preliminary experiment, however, the former affected the histology of the tooth germs in vitro, while the latter had no effect. We, therefore, used the latter (5′CAT GCT TGC AGT TCG3′) in the present study. The corresponding control sense ODN was 5′CGA ACT GCA AGC ATG3′. ODNs were prepared by Sawaday Ltd (Tokyo, Japan): they were synthesized using a PCR Mate EP 394 DNA Synthesizer (Applied Biosystems, Foster City, CA) and subsequently purified by HPLC (Zon et al., 1985). Both the antisense and sense ODNs were dissolved in double distilled water and quantitated by absorbance (*A*) at 260 nm.

HGF translation arrest by antisense ODN

To determine the role played by HGF in tooth morphogenesis, we examined the effect of translation arrest of HGF gene by antisense ODN in developing mouse tooth germ in an organ culture system. In this system, mouse M1 germs were used rather than rat ones, because the mouse tooth germs could be cultured more stably than rat ones owing to their small size. Antisense or sense ODN at 30 μM was added to the culture medium and changed every other day together with the culture medium. In control cultures, an equal volume of culture medium was added instead of ODN. In the rescue experiment, 30 μM antisense ODN and 10 μg/ml rhHGF were added to the cultures simultaneously. Eleven explants were used in each experimental group, cultured for 14 days, and used for histological analysis: fixation with 4% paraformaldehyde overnight, paraffin-sectioning and hematoxylin-eosin (HE) or Van Gieson staining was done. In Van Gieson staining, the dentin matrix between the dental papilla and the inner enamel epithelium was stained vivid pink.

Analysis of incorporation of antisense ODN

To evaluate the efficiency of synthetic ODN diffusion into the cultured explants, we added FITC-conjugated antisense ODN at a concentration of 30 μM to the culture medium and the explants were cultured for 5 days. The FITC-conjugated antisense ODN and medium was changed every other day. Explants were then embedded in OTC compound (Miles Laboratories Inc.) and frozen quickly. Cryostat sections (10 μm thick) mounted on glass slides, were fixed with neutralized buffered formalin for 10 minutes, and rinsed with PBS. To reduce fluorescence fading, the specimens were sealed with PBS-buffered glycerol containing *p*-phenylenediamine. The specimens were then examined using a fluorescent microscope (VANOX, Olympus, Tokyo Japan).

Cell proliferation assay

Explants in culture for 9 days were pulsed with the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) (cell proliferation kit, Amersham Life Science) for 4 hours, chased with PBS for 5 minutes, fixed with 10% neutralized buffered formalin, and cryosectioned (10 μm sections) as described above. Sections were immersed in 3%

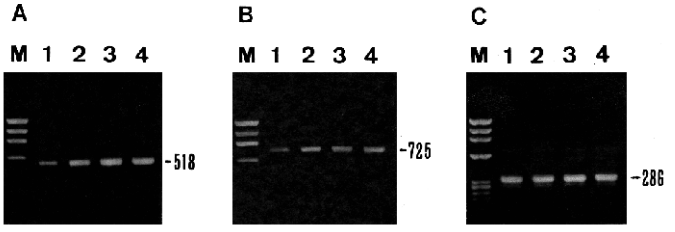


Fig. 1. HGF and c-*met* gene expression in the tooth germs of rat mandibular first molars, determined by RT-PCR. The expression of HGF and c-*met* transcripts is shown in (A) and (B), respectively. In both panels, total mRNA was isolated from tooth germs at E16.5 (lane 1), E17.5 (lane 2), E18.5 (lane 3), and E19.5 (lane 4). The RNA was subjected to RT-PCR, utilizing HGF (A) and c-*met* (B) primers. The gene expression of G3PDH as a control transcripts also is shown in (C). The RT-PCR products were resolved to size (indicated in bp) by electrophoresis through a 4% agarose gel and stained with ethidium bromide. Lane M, molecular mass standards (λ*Hin*dIII digest).

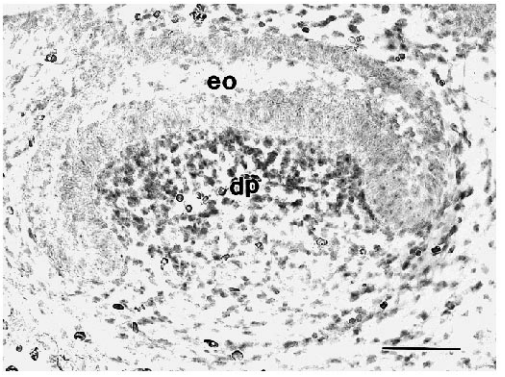


Fig. 2. HGF gene expression in the tooth germ of E18 rat mandibular first molar, determined by in situ hybridization. Note that positive expression is observed only in the cells of the dental papilla (dp). eo, enamel organ. Bar, 100 μm.

normal goat serum for 30 minutes to block non-specific binding of antibody, then nuclease/anti-BrdU antibody solution was applied for 2 hours, followed by horseradish peroxidase-conjugated anti-mouse IgG solution and, finally, polymerizing diaminobenzidine in the presence of cobalt and nickel, giving blue-black staining at the sites of BrdU incorporation. At the end of each step, sections were soaked in TPBS for 5 minutes. The sections were counterstained with eosin and the percentage of labelled cells counted.

RESULTS

HGF and c-*met* gene expression in rat mandibular M1 in vivo

To examine the gene expression of HGF and its receptor, c-Met, in tooth germs, we isolated mRNA from the intact tooth germs of rat mandibular M_1 at various stages and subjected them to RT-PCR. Amplified products corresponding to HGF and c-*met* transcripts were detected in RT-PCR samples derived from the tooth germs of mandibular M_1 from rat embryos at E16.5 (early cap stage) to E19.5 (mid-bell stage) (Fig. 1A,B), but these products were not detected in the samples at E15.5 (bud stage) and P2 (late bell stage) (data not shown), and also significant change is not detected in the gene expression of glyceraldehyde-3-phosphate dehydrogenase used as an internal marker control (Fig. 1C). These findings indicate that the gene expression of HGF and c-*met* show characteristic temporospatial patterning in tooth germ from the early cap stage through the mid-bell stage.

In situ hybridization analysis for HGF gene expression

HGF gene expression in the tooth germ of rat mandibular M_1 in E18 was examined by in situ hybridization. The cells of the dental papilla gave a positive signal, while those of the enamel organ gave a negative signal (Fig. 2). This finding coincides with the finding of HGF gene expression in mice mandibular molars, previously reported by Sonnenberg et al. (1993).

Immunohistochemical analysis for HGF

Positive staining for HGF was first detected in the mesenchymal cells of the dental papilla underlying the enamel organ of mandibular M_1 tooth germs at the cap stage (Fig. 3A), during which the epithelium gradually invaginated to form a cap and the mesenchymal cells condensed to form the dental papilla. At the early bell stage, during which the enamel organ showed the beginnings of cusp formation and was composed of the inner enamel epithelium, the outer enamel epithelium, the stellate reticulum, and the stratum intermedium, the staining increased in the mesenchymal cells of the dental papilla (Fig. 3B). At the bell stage, in which the outlines of the cusps are formed and the cells of the inner enamel epithelium differentiate to preameloblasts, or presecretory ameloblasts, downward from the tip of the cusp, intense staining spread out in each cusp and weak staining was also observed in the enamel organ (Fig. 3C). In the dental papilla, the staining was detected in the cytoplasm of the cells, while in the enamel organ, including the outer enamel epithelium, the stellate reticulum and the stratum intermedium, and in preameloblasts, the staining was observed mainly in the plasma membranes of the cells (Fig. 3D). At the advanced bell stage, in which functional odontoblasts secreting dentin matrix were present in all cusps and the dental papilla was composed of distinct peripheral odontoblasts and pulp cells, the staining began to disappear from the dental papilla of the cusp tip, while positive staining remained in the dental papilla of the deep portion of the intercuspal groove (Fig. 3E). The staining had almost entirely disappeared in the late bell stage of the tooth germs (data not shown). Control sections using PBS or preimmune serum instead of

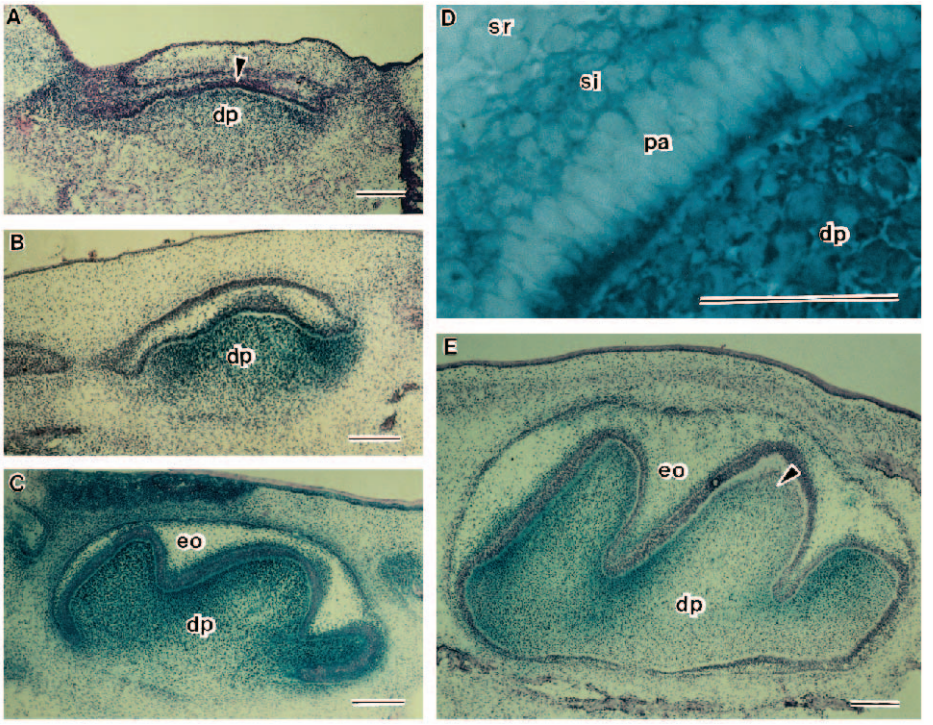


Fig. 3. HGF expression in the tooth germs at various developmental stages. Sagittal cryosection of tooth germs of rat mandibular first molars immunostained for HGF and counterstained with HE. Light blue color demonstrates the localization of HGF. (A) Cap stage of E17 rat. Positive staining is observed in the mesenchymal cells of the dental papilla (dp) underlying the inner enamel epithelium (arrowhead). (B) Early bell stage of E18 rat. The staining is increased in the mesenchymal cells of the dental papilla (dp). (C) Bell stage of E19 rat. Positive staining spreads out in the dental papilla and weak staining also appears in the enamel organ. (D) Higher magnification of (C). Positive staining is observed in the cytoplasm of the cells of the dental papilla (dp), and in the plasma membrane of preameloblasts (pa), cells of the stellate reticulum (sr), and cells of the stratum intermedium (si). Some cells in the stratum intermedium also seems to show positive staining in the cytoplasm. (E) Advanced bell stage of E20 rat. Positive staining has disappeared at the cusp tip of the dental papilla (arrowhead), while the positive staining remains in the deep portion of the intercuspal groove of the dental papilla. eo, enamel organ; dp, dental papilla; sr, stellate reticulum. Bar, 200 μm.

primary antiserum showed negative staining (data not shown). These results indicate that the immunohistochemical staining pattern described above definitely demonstrates the HGF localization in the tissue.

Immunohistochemical analysis for c-Met

Positive staining for c-Met was first detected at the inner enamel epithelium of the tooth germ of the E17 rat at the cap stage, when HGF was also first detected (Fig. 4A). At the advanced bell stage of the E21 rat, the staining was observed in preameloblasts and in some cells of the stellate reticulum (Fig. 4B).

Antisense treatment in organ culture

At first, to examine whether exogenous antisense ODN was taken up by the cells of tooth explants in vitro, the explants were cultured for 5 days in medium containing FITC-conjugated ODN. The FITC labeled cells were uniformly distributed throughout the explants, suggesting that ODN was uniformly incorporated by the cells of the explants (Fig. 5A).

Then the experiments were designed to examine the effects of antisense ODN on mouse E14 M_1 explants cultured for 14 days in chemically defined and serum-free medium. All of the control explants cultured without ODN (Fig. 5B), and sense-treated explants (Fig. 5C) showed a normal structure, as observed in vivo (Table 1). However, the antisense-treated explants showed an abnormal structure, in which the enamel organ was surrounded by a thin layer of dentin and dental papilla, appearing 'inside-out' compared to the control and sense-treated explants, and also in which a size-reduction of the tooth germ was observed, indicating that normal development of the explant was inhibited (Fig. 5D). Although morphology of the tooth germ was abnormal, the cytodifferentiation of ameloblasts and odontoblasts was not inhibited (Fig. 5E) in the case of HGF translation arrest by antisense ODN. Of 11 antisense-treated explants 10 explants showed abnormal structure of the tooth germ (Table 1).

The explants treated with rhHGF in combination with antisense ODN showed a normal tooth germ structure, indicating that exogenous HGF overcame the antisense effect (Fig. 5F).

Finally, to elucidate the mechanism acting in the formation of a characteristic abnormal structure in antisense-treated explants, we examined the cell proliferation activity in the explants by a BrdU-uptake experiment. The tooth germ was partitioned into three areas: inner enamel epithelium (containing ameloblasts), cells of the enamel pulp (containing cells of the stellate reticulum and stratum intermedium), and cells of the dental papilla. BrdU labelling indices of these three areas were determined (Fig. 6). Labeling indices in the cells of the tooth germ were decreased by antisense treatment except in the cells of the enamel organ. The decrease in the labeling index was much greater in the inner enamel epithelium than in the dental papilla, i.e. the ratios of the mean labeling index of the inner enamel epithelium to that of the dental papilla were 2.0 and 1.1 for control and antisense-treated explants, respectively. These findings suggest that HGF translation arrest causes an imbalance between the cell proliferation of the enamel organ and that of the dental papilla, which results in abnormal tooth germ structure.

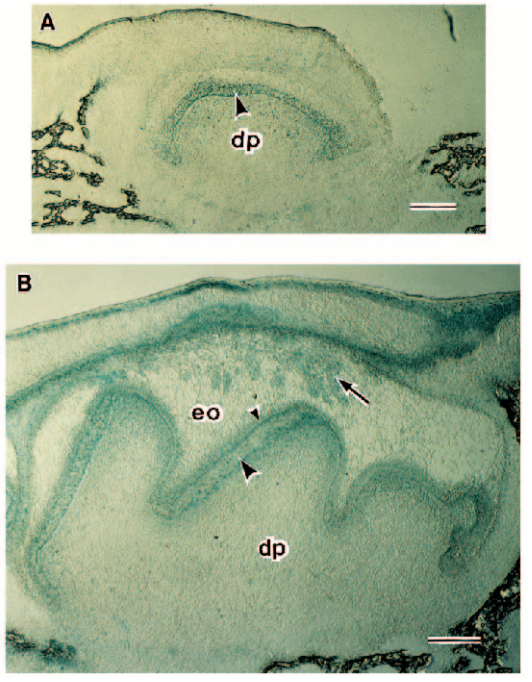


Fig. 4. c-Met expression in rat tooth germs. Sagittal cryosection of tooth germs of rat mandibular first molars immunostained for c-Met and counterstained with HE. Light blue color demonstrates the localization of c-Met. (A) Cap stage of E17 rat. Positive staining is observed in the inner enamel epithelium (arrowhead). (B) Advanced bell stage of E21 rat. Positive staining is observed in preameloblasts (large arrowhead), in cells of the stratum intermedium (small arrowhead) and stellate reticulum (arrow). eo, enamel organ; dp, dental papilla. Bar, 200 μm.

DISCUSSION

In antisense experiments, the sequence of the antisense probe and the culture conditions employed are important experimental factors (Toulmé, 1992). In this study, first, we used the 15mer sequence directly upstream from the initiation codon for mouse HGF, since previous study has demonstrated that the most efficient translational arrest is produced when the antisense oligodeoxynucleotide (ODN) is designed to hybridize with the 5′ region of the mRNA, including the AUG initiation codon (Shakin-Eshleman and Liebhaber, 1988). Secondly, we used the phosphorothioate ODN to maintain their stability (Marcus-Sekura, 1988), e.g. phosphorothioate ODNs for human HOXB genes were stable for at least 72 hours in culture medium and did not show the obvious toxic effects on the cultured cells even at a final concentration of 100 μM (Faiella et al., 1994). Thirdly, we decided to add phosphorothioate

Table 1. Effect of antisense or sense oligonucleotides on the development of mouse mandibular molars in vitro

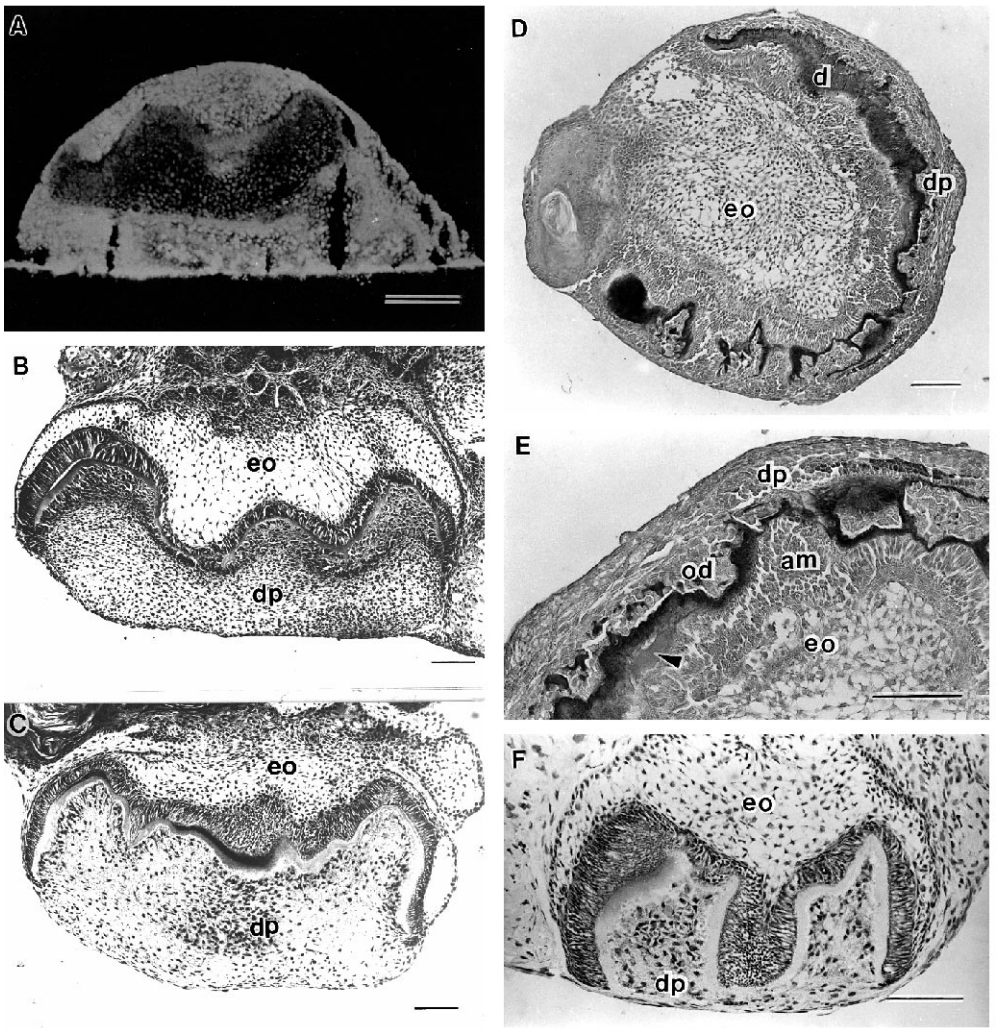
	Control	Sense	Antisense
Total number of explants	11	11	11
Normal structure	11	11	1
Abnormal structure	0	0	10*

*Incidence is significantly different from that of control as well as from sense group at $P<0.001$ (χ^2 analysis).

ODNs at a final concentration of 30 μM and to replace them after 48 hours, according to the translation arrest experiment by ODN onto the tooth germs in vitro (Diekwisch et al., 1993; Chai et al., 1994; Shum et al., 1993). Although sense-treated explants showed normal histogenesis, HGF antisense phosphorothioate ODN blocked the normal histogenesis without toxic effect (Fig. 5). It has been reported that antisense ODNs for EGF (Kronmiller et al., 1991; Shum et al., 1993), amelogenin (Diekwisch et al., 1993), and TGF-β2 (Chai et al., 1994) affect the development of tooth germ in vitro. Our findings and these previous reports demonstrate that antisense ODNs selectively suppress gene expression when an appropriate sequence for antisense ODNs and appropriate experimental conditions are selected.

The diffusion of soluble factors such as bone morphogenetic protein 4 (BMP-4) mediates epithelial-mesenchymal interactions during early tooth development (Vainio et al., 1993). Other soluble factors, such as growth hormone (Zhang et al., 1992), IGF-1 (Joseph et al., 1993), aFGF, bFGF (Gonzales et al., 1990; Cam et al., 1992) and NGF (Byers et al., 1990; Mitsiadis et al., 1992, 1993) have been reported to be involved in odontoblast terminal differentiation. Recently, an in situ hybridization study on the gene expression of HGF and c-*met* in tooth germ has suggested that HGF is one of the soluble factors mediating epithelial-mesenchymal interactions during tooth development (Sonnenberg et al., 1993).

HGF stimulates DNA synthesis and/or the proliferation of



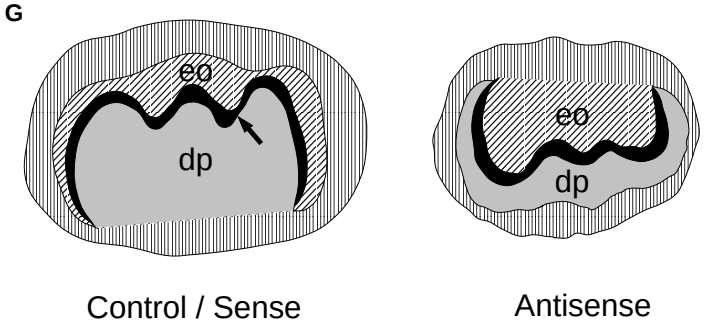


Fig. 5. Effects of oligodeoxynucleotides (ODN) on the development of mouse tooth germs in vitro. (A) Cryosection of explant from E14 mouse cultured for 7 days in medium containing FITC-conjugated antisense ODN. Note that labeled antisense ODN is uniformly distributed throughout the cells of the explant, indicating that the antisense ODN has diffused into all cells. (B) Section of control explant cultured without ODN. The tooth germ shows normal histological structure, as seen in vivo. (C) Section of explant cultured with sense ODN. The histological structure is normal, as in the control explant. (D) Section of explant cultured with antisense ODN. Note the abnormal structure of the tooth germ, in which the enamel organ (eo) is surrounded by dentin (d) and dental papilla (dp). A reduction in the size of the explant is also observed. (E) Higher magnification of (D). The differentiation of ameloblasts (am) and odontoblasts (od) and the production of enamel matrix (arrow) are almost normal. (F) Section of explant cultured with antisense ODN plus recombinant HGF. Note that the tooth germ shows normal structure, as observed in the control (B) and sense-treated (C) explants. (G) Schematic representation of the control, and sense- and antisense-treated explants. In the antisense-treated explant, the enamel organ is surrounded by a thin layer of dentin (arrow) and dental papilla, appearing 'inside-out' compared with the control and sense-treated explants. A reduction in explant size is also observed. (B,C and F), HE staining; (D and E), Van Gieson staining; am, ameloblasts; eo, enamel organ; dp, dental papilla; od, odontoblasts. Bar, 100 μm.

epithelial and endothelial cells (Rubin et al., 1991; Nakamura, 1991; Matsumoto et al., 1992), and it promotes the motility of epithelial and endothelial cells (Stoker et al., 1987; Gherardi and Stoker, 1990; Furlong et al., 1991). In addition to being a mitogen and a motogen, HGF can be also a morphogenesis inducer under the appropriate conditions. Montesano et al. (1991a) observed that MDCK cells formed tubular structures when grown in collagen gels in the presence of fibroblast-conditioned medium or when grown with fibroblasts under conditions that prevented heterocellular contact. Using HGF-specific neutralizing antiserum and recombinant HGF, the same group established that HGF was responsible for this effect (Montesano et al., 1991b). Subsequently, others have reported a similar influence of HGF on the formation of lumen-like structures by human epithelial carcinoma cell lines in vitro (Tsarfaty et al., 1992), observing HGF receptors on the luminal surface of cells forming these structures both in vitro and in sections of whole breast tissues.

In the present study, we have demonstrated that HGF and its receptor, c-Met, showed a temporospatial distribution during the development of tooth germ (Figs 3, 4) and that HGF translation arrest caused the formation of abnormal tooth structure in which the enamel organs are surrounded by a thin layer of dentin and dental papilla, appearing 'inside-out' compared with that of the normal tooth germ (Fig. 5). Results from BrdU incorporation suggested that the balance between the cell proliferation of the inner enamel epithelium and that of the dental papilla disturbed by HGF translation arrest resulted in the abnormal structure (Fig. 6). These findings indicate that HGF is an essential factor for the formation of a normal tooth structure.

Our HGF translation arrest experiment indicated that HGF also has an effect on the cell proliferation of dental papilla as well as dental epithelium (Fig. 6). Since the localization of c-Met was limited in dental epithelium except in enamel pulp, the effect of HGF on mesenchyme was considered to be caused indirectly. One possible explanation for this is that HGF-stimulated inner enamel epithelium may not only increase proliferation activity but may also increase or begin to synthesize an unknown factor which can regulate the cell proliferation of the dental papilla. Further examinations are needed to elucidate the mechanism functioning in this process.

Sonnenberg et al. (1993) suggested that the HGF-mediated signal may lead to growth arrest and to degeneration of the outer enamel epithelium in tooth germs rather than to cell proliferation, since the HGF transcript was detected only in the dental mesenchyme at the advanced bell stage, and the c-*met* transcript, which was found in the outer enamel epithelium, subsequently disappeared. However, our cell proliferation assay in vitro suggested that the signal lead to cell proliferation of enamel epithelium, primarily. Furthermore, our immunohistochemical findings in vivo also suggested that HGF appeared in the mesenchymal cells of dental papilla adjacent to the epithelium, which has high proliferation activity in the early stage of their differentiation. Therefore, at least in the early stages of the tooth development, the HGF-mediated signal may lead to cell proliferation rather than to growth arrest.

Previous studies indicated that HGF acts in a paracrine fashion, since it was found that many cells derived from the mesenchyme secreted HGF and that cells of epithelial origin that have the HGF receptor, c-Met, responded to it (Stoker et al., 1987; Gherardi et al., 1989). Sonnenberg et al. (1993) have reported that, in mouse tooth germs, the HGF gene was expressed in the cells of the dental mesenchyme and the c-*met* gene was expressed in the cells of the outer enamel epithelium. We also observed HGF gene expression only in the cells of dental mesenchyme (Fig. 2). However, we detected the immunohistochemical signal for HGF in the dental mesenchyme as well as in the enamel organ (Fig. 3C,D). In the enamel organ, positive staining was observed on the plasma membrane or at the intercellular space of the cells. Therefore, it is thought that the positive staining at ameloblasts represents HGF moving from the dental mesenchyme to the enamel organ.

The promoter region of the murine HGF gene is very large and complex (Liu et al., 1994; Rosen et al., 1994). This region contains IL-6-responsive elements, binding sites for NF-IL6, a TGF-β inhibitory element, a cAMP response element, estrogen-responsive elements, cell type-specific transcription factor binding sites, and multiple positive and negative regulatory elements for which transcription factors have not been identified. TGF-β1, TGF-β2, and TGF-β3 subtypes are present in odontogenic tissues during mouse tooth development (D'Souza et al., 1990; Thesleff et al., 1992; Ruch et al., 1983, 1995), however, the specific functions of these regulatory molecules have not been determined. Recently, Chai et al. (1994) suggested that TGF-β2 functions specifically to

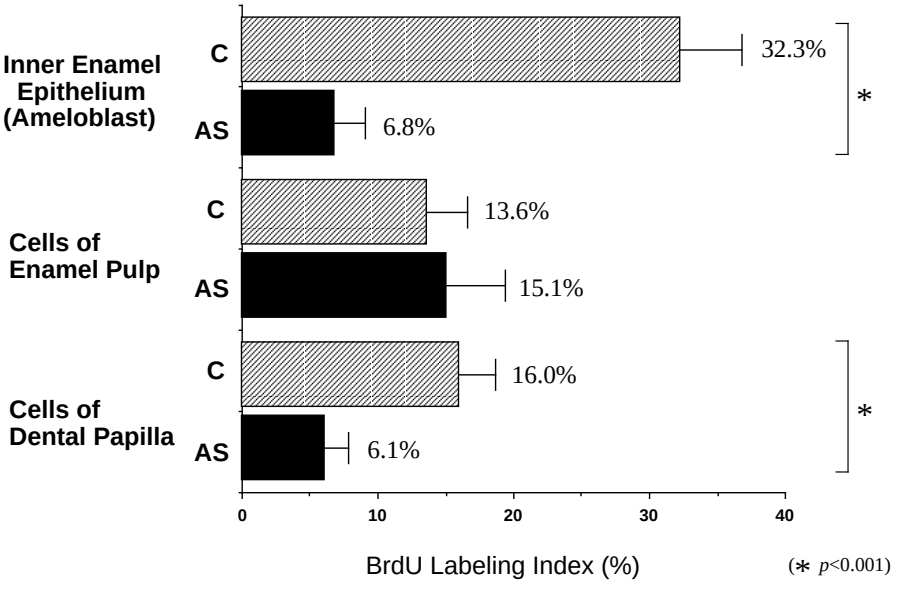


Fig. 6. BrdU labeling index in tooth germ in vitro. Shaded column indicates the index of the control explant. Solid column indicates the index of the antisense-treated explant. Bars mean confidence interval at 95%. *There is significant difference between control and antisense group at $P<0.001$ (χ^2 analysis).

regulate tooth development. This function of TGF-β2 in tooth development may be mediated by HGF. TGF-β1 is the most potent suppressor for the gene expression of HGF (Matsumoto et al., 1992). The identification of HGF regulators, their receptors, and the relevant transcription factors will undoubtedly enhance the understanding of mesenchyme-epithelial communication during tooth development.

In conclusion, HGF is involved in the morphogenesis of tooth germ in murine molars, and it is the essential soluble factor mediating epithelial-mesenchymal interactions by regulation of the cell proliferation activity in tooth germ.

We are indebted to Dr S. Noji, Faculty of Technology, Tokushima University, for his suggestions during the course of this study. We thank Dr T. Shioda, MGH Cancer Center, for valuable advice. This study was supported by a Grant for Scientific Research from the Japanese Ministry of Education, Science, and Culture (Project 06454509).

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(Accepted 8 January 1996)