Immunolocalization of acidic and basic fibroblast growth factors during mouse odontogenesis

YVES CAM1*, MARIE-ROSE NEUMANN1, LISA OLIVER2, DANIEL RAULAIS2, THIERRY JANET3# and JEAN-VICTOR RUCH1

1Institut de Biologie Médicale, INSERM CJF 88-08, Université Louis Pasteur, Faculté de Médecine, Strasbourg, 2INSERM Unité 118, Paris and 3Laboratoire de Neurobiologie Ontogénique CNRS UPR 417 - Centre de Neurochimie, Strasbourg, France

ABSTRACT Acidic and basic fibroblast growth factors (aFGF and bFGF), are both known to bind to extracellular matrix components, particularly proteoheparan sulfates, and to regulate in vitro proliferation, differentiation and morphology of cells of neuroectodermal and mesodermal origins. Their patterns of distribution were studied during mouse odontogenesis by means of indirect immunofluorescence and immunoperoxidase histochemistry on frozen fixed sections and after Bouin’s fixative and paraffin embedding. Localization of aFGF on frozen fixed sections was observed in the oral epithelium, dental lamina and oral mesenchyme (day-12 of gestation), the stellate reticulum and oral epithelium (day-14), the stratum intermediate and at the basal and apical poles of preameloblasts at bell stage. After birth aFGF epitopes were localized within the predentin-dentin area, the stratum intermediate and at the secretory pole of ameloblasts. There was no staining with anti-aFGF antibodies after Bouin’s fixative and paraffin embedding. In contrast, using this protocol, intense stainings were found with anti-bFGF antibodies predominantly within dental and peridontal basement membranes and mesenchyme: staining of the dental basement membranes was transient (bud and cap stage) and discontinuous; a preferential concentration of bFGF epitopes in the condensed dental mesenchyme of incisors (cap stage) and the dental papillae mesenchymal cells of molars (bell stage) was observed in the posterior and the cervical part of tooth germs. An intense immunostaining of the stellate reticulum with anti-bFGF antibodies was also found on paraffin sections from bud to bell stage. Localization of bFGF on frozen fixed sections was observed in the dental lamina, stellate reticulum and dental basement membranes at bud and cap stage, the stratum intermediate and at the secretory pole of ameloblasts after birth. Treatment of sections with NaCl (2-3M) solutions and heparinase diminished, but did not abolish, specific immunostaining obtained with bFGF antibodies. Our results suggest that, among growth factors, a- and bFGFs might intervene in different ways during odontogenesis, particularly through binding to the cellular and/or extracellular matrix heparan sulfate-containing molecules, and may participate in the control of proliferation, determination and terminal differentiation of preodontoblasts and preameloblasts.

KEY WORDS: immunohistochemistry, acidic and basic fibroblast growth factors, mouse odontogenesis, tissue fixation

Introduction

Odontogenesis is a well studied example of cell kinetic-dependent developmental processes that are mediated through homotypic and heterotypic cell interactions. Most of these interactions involve the temporal and spatial action of extracellular matrices first as solid substrata that interact with plasma membrane receptors and ligands and second as potential reservoirs for diffusible molecules such as growth factors (Cam et al., 1987; Ruch, 1987, 1990; Siavlin, 1988; Thesleff et al., 1990; Lesot et al., 1990; Mark et al., 1990; Kronmiller et al., 1991a, b; Vainio et al., 1991; Hall and Ekanayake, 1991).

Abbreviations used in this paper: BSA, bovine serum albumin; DEAE, diethylaminoethyl; EDTA, ethylene diamine tetra acetate; EGF, epidermal growth factor; HBSS, Hank’s balanced salts solution; Ig, immunoglobulin; IGF, insulin like growth factor; Kd, dissociation constant; KGF, keratinocyte growth factor; NGF, nerve growth factor; PBS, phosphate buffered saline; RNA, ribonucleic acid; TGF, transforming growth factor; Tris, tris (hydroxymethyl) aminomethane.

*Address for reprints: INSERM CJF 88-08, Faculté de Médecine, Institut de Biologie Médicale, 11, rue Humann, 67085 Strasbourg-Cedex, France. FAX: 33-88242005.
#Present address: Department of Anatomy and Cell Biology, University of Marburg, D-3550 Marburg, Germany.
Several circulating and tissue growth factors and their receptors have been detected either at the mRNA and/or the protein level in murine dental tissues, especially pro-pro EGF (Sneed et al., 1989), EGF (Kronmiller et al., 1991a), TGFα (Dixon et al., 1991), NGF (Mitsiadis et al., 1992), TGFβ1-3, α-SM-Actin (Haenel et al., 1987; Lehnerth and Akhurst, 1988; Cam et al., 1990; D’Souza et al., 1990; Pelton et al., 1990; Vaathokari et al., 1991), EGFR receptor (Partanen and Thesleff, 1987; Abbott and Pratt, 1988; Cam et al., 1990; Wise et al., 1990) and NGF receptors (Byers et al., 1990). Growth hormone receptors (Zhang et al., 1992) have also been localized in these tissues.

Acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) are monomeric proteins sharing 55% structural homology that have no signal sequence in contrast to the other members of the GFB family, i.e., the protooncogene int-2, hst/FGF, FGF-5, FGF-6 and KGF (reviewed by Goldfarb, 1990). Besides the in vitro growth-promoting activity towards fibroblasts originally described, different properties have been assigned to FGFs as have possible in vitro and in vivo roles such as chemotaxis, induction of proteases, including collagenases, synthesis and secretion of hormones, modulation of cell morphology and differentiation.

Expression of FGF genes at the RNA and the protein levels has been reported during chick, mouse and rat development: both mRNAs and proteins have been localized in tissues of mesodermal and neuroectodermal origins (Risau et al., 1986; Seed et al., 1988; Wilkinson et al., 1989; Gonzalez et al., 1990: Fu et al., 1991; Haub and Goldfarb, 1991).

Two types of receptors interacting both with aFGF and bFGF have been described so far. High affinity (Kd ≈ 2 to 15 x 10^-11) binding sites are membrane glycoproteins that have an external immunoglobulin-like domain and tyrosine kinase activity within their intracytoplasmic domain (Dionne et al., 1990). Low affinity (Kd ≈ 2 x 10^-9) binding sites for bFGF have also been described (Baird and Ling, 1987; Vigny et al., 1988; Bernfield and Sanderson, 1990; Kiefer et al., 1990; Fayein et al., 1990): these are heparan sulfate-containing proteoglycans located either within the extracellular matrix, especially basement membranes, or as constituents of plasma membranes, like syndecan. Other possible interactions or binding of FGFs have been evoked that involve type IV collagen, laminin and fibronectin (Thompson et al., 1988; Feige et al., 1989). The differential expression of two high affinity receptors, FGF-R1 (the lig gene product) and FGF-R2 (the bek gene product), in mesenchyme and epithelium respectively, has been recently demonstrated in rat and mouse embryos (Wanan et al., 1991; Orr-Urtreger et al., 1991; Peters et al., 1992).

In the present investigations, we used monospecific polyclonal antibodies to immunolocalize aFGF and bFGF during mouse molar and incisor development and incisor formation, demonstrating that both factors are present in dental tissues at different stages of morphogenesis and during functional odontodifferentiation.

**Results**

**Immunolocalization of aFGF**

No significant staining of Bouin's fixed sections was found in the presence of specific anti-aFGF antibodies (not shown). In contrast, frozen fixed sections showed positive immunoreactivity within the oral epithelium, the dental lamina and the enamel organ of molars (Fig. 1A-B). Accumulation of aFGF was observed at the apical and the basal poles of preameloblasts (Fig. 1C-D) and ameloblasts (Fig. 1E) while a strong scattered specific immunostaining appeared at the predentin-dentin level (Fig. 1E). At bell stage and after birth cells of the stratum intermedium were also stained (Fig. 1C, D, G).

**Immunolocalization of bFGF**

On frozen sections fixed according to protocols 1, 2a and 3a (Figs. 2C, H and 3C) and paraffin-embedded sections after Bouin's fixative (Figs. 2B, 3A and B), the distal of bFGF was similar in the oral epithelium and the stellate reticulum (compare Fig. 2B-C), in some dental basement membranes (compare Figs. 3A and B) and between ameloblasts (compare Figs. 2D and 3E). Intense immunoreactivity was observed after Bouin's fixative in oral, dental and peridontal mesenchymes; positive reactivity in the dental mesenchyme was found during dental lamina formation and at the bud stage (Fig. 2A-B); this reactivity was progressively restricted to clusters of cells in the incisors (Fig. 3B and D) and to some areas of the dental papilla in the posterior part of the molars (Figs. 2D-G and 3E). The stellate reticulum was also intensely stained after Bouin's fixative (Figs. 2G, 3D and 3E).

**bFGF immunostaining following treatment with NaCl (2M-3M) or digestion with heparitinase**

When acetone-fixed sections were treated with increasing concentrations of NaCl (2M and 3M) (Protocol 2b), bFGF immunostaining progressively decreased except in the stratum intermedium (not shown). Digestion of acetone-fixed sections by heparitinase (Protocol 2c) resulted in slight but significant decrease of staining especially in the basement membranes and the epithelial tissues (not shown).

**Discussion**

Despite some similarities between immunolocalizations of aFGF and bFGF, for instance at the secretory pole of ameloblasts and within the stratum intermedium after birth, our results stress that the stainings of aFGF and bFGF appear different. In particular the
intense mesenchymal staining observed with bFGF antibodies is not observed with aFGF antibodies, nor is the very intense staining in stellate reticulum. Simultaneous expression of aFGF and bFGF genes has been reported by Hebert et al. (1990) in mouse embryos ranging from day-11 to day-17 of gestation; however bFGF was expressed more abundantly than aFGF in facial tissues and limbs. Fu et al. (1991) have also demonstrated the simultaneous presence of aFGF and bFGF within extracts of diverse tissues of rat embryos and stressed the similar pattern of immunostaining of both factors.

The specific localization of aFGF epitopes found in the predentin-dentin zone after birth leads to different hypotheses. Given that odontoblastic cell processes and nervous fibers are known to cross this matrix, such a localization might be related to the neurotropic or neurotrophic activities of FGFs previously reported (reviewed by Goldfarb, 1990). The fact that aFGF is an acidic protein might also contribute to the process of mineralization of the dentin matrix (Weiner and Addadi, 1990). The fact that aFGF is an acidic protein might also contribute to the process of mineralization of the dentin matrix (Weiner and Addadi, 1990).

Our investigations stress the importance of tissue fixation and paraffin embedding with regard to immunostaining with monospecific polyclonal antibodies. The distribution of bFGF epitopes on Bouin’s fixed sections in dental and peridental mesenchyme and in association with oral and dental basement membranes is consistent with observations by Gonzalez et al. (1990).

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Fig. 2. Immunolocalization of bFGF by means of the indirect immunofluorescence method (A-G) and the immunoperoxidase technique (H) on frontal sections of molars fixed and incubated as follows: A-B, D-G (Protocol 3b; anti b antibody); C (Protocol 2a; anti-b antibody); H (Protocol 1; anti b antibody). (A) Section of a day-12 mandible; immunostaining was predominant in the oral mesenchyme (om) and the basement membranes (bm); the dental lamina (dl) was faintly stained. \(x \times 270\). (B) Section through day-14 upper and lower molars (cap stage) showing intense immunoreactivity of the peri-odontal mesenchyme and the basement membranes (bm) bordering the outer dental (ode) and oral (oe) epithelia (arrows); the stellate reticulum (sr) and the dental mesenchyme (dm) exhibited pronounced immunostaining; the (oe) stained positively. \(x \times 150\). (C) Section of a day-14 lower molar fixed differently from that shown in B; the (oe) and the (sr) still stained positively while the basal lamina and the mesenchyme remained negative (see Discussion). \(x \times 260\). (D-G) Sections of day-18 molars (bell stage) at different levels (D-E: anterior and posterior parts of lower molar, respectively; x115; F: posterior part of upper molar (x115); G: enlargement of F (x235)). (D) Section showing intense immunoreactivity of the (om) and the (sr); the dental sac (ds) and part of the dental pulp (dp) exhibited pronounced staining. (E) Section of a day-21 molar; immunostaining was primarily seen at the secretory pole of ameloblasts (s); positive staining was found between and at the non-secretory pole of (a), in (sl) and (ode). (x125).
Fig. 3. Immunofluorescent localization of bFGF on frontal sections of incisors fixed and incubated as follows: A, B, D and E (Protocol 3b; anti-b antibody); C (Protocol 3a; antib1-24 antibody). (A) Section of the anterior part of a 14-day lower incisor showing intense immunostaining in the stellate reticulum (sr) and the dental basement membranes (bm) (arrows); the dental and oral mesenchymes and the lip furrow (lf) exhibited positive staining; the oral epithelium (oe) was faintly stained (x210). (B) In the posterior part of the same incisor as shown in A, the dental mesenchyme (dm) and the oral mesenchyme (om) exhibited bright immunostaining; the sr remained positively stained while the bm facing the dental mesenchyme was not stained. (x210). (C) Section of a 14-day lower incisor fixed differently from that in A and B; positive staining of the sr and bm was still observed (see Discussion). (x265). (D) Section of a day-16 incisor; immunostaining predominated in the sr and dm while some cells of the dental pulpal (dp) were faintly stained. (x200). (E) Section of a day-18 incisor showing intense immunoreactivity of the sr; the dental sac (ds) was positively stained while staining remained in clusters of cells in the dp and some staining appeared between ameloblasts (al). (x170). The open arrows in D and E point to the lingual side of the incisor.

Sources of specific antibodies

First we used polyclonal antibodies raised in rabbits against the synthetic (1-24 amino terminal) fragment of bovine bFGF—conjugated to BSA (Sigma, St-Louis, MO, USA) — and prepared as described by Baird and Ling (1987) and Gonzalez et al. (1990); these antibodies detected bFGF and did not recognize less than 1% of aFGF; they were designated “anti-b1-24”.

Polyclonal antibodies specific for bFGF were also prepared by immunizing mice with bFGF previously purified from bovine brain by heparin-sepharose chromatography, as described by Pettmann et al. (1985) and Janet et al. (1987). These antibodies were designated “anti-bbb”.

Rabbit polyclonal antisera directed against human recombinant aFGF and bFGF were purified by affinity chromatography. Briefly, to purify antibodies to aFGF, 8 ml of sera were dialyzed against 35 mM NaCl (Merck, Darmstadt, Germany) in 25 mM Tris (Sigma), pH 8.8 at room temperature for 3 h, then passed over a DEAE Sepharose (Kabi-Pharmacia, Paris, France) column; the IgG fraction was dialyzed against PBS, then loaded onto a bFGF Aminolink (Pierce, Eurochimie BV, The Netherlands) column to remove any cross reacting antibodies. The IgG fraction was cycled over an aFGF-Aminolink column several times over-night at 4°C; the column was washed extensively with PBS and the bound antibody was eluted in 0.1M glycine (Sigma), pH 2.8 and immediately buffered in Tris, pH 8.0. To purify antibodies to bFGF, a similar procedure was used, i.e. an IgG fraction was
Developmental stages | Dental lamina | Bud stage | Cap stage | Bell stage
---|---|---|---|---
Molecules | Epi | Mes | Epi | Mes | Epi | Mes | Epi | Mes
N-myc | +(m) | - | - | -(m) | - | +(m) | -(m) | -
c-myc | - | +(m) | - | +(m) | - | +(m) | - | +(m)
Int-2 | -(p) | - | +(p) | + | -(p) | + | -(p) | +
aFGF | ++(p) | + | +(p) | + | +(p) | + | +(p) | +
bFGF | - | +(p) | - | +(p) | - | +(p) | - | +(p)
FGFR1 | - | - | +(m) | + | - | + | - | +
FGFR2 | - | - | +(m) | + | - | + | - | +
Syndecan | + | +(p) | + | +(p) | + | +(p) | + | +(p)
prepro EGF | + | - | + | - | + | - | + | -
TGFα | + | - | + | - | + | - | + | -
EGFR | + | - | + | - | + | - | + | -
NGF | + | - | + | - | + | - | + | -
NGFR | +(m,p) | + | +(m,p) | + | +(m,p) | + | +(m,p) | +
TGFβ1 | - | - | +(m) | - | -(m) | + | -(m) | -
TGFβ2 | - | - | +(m) | - | -(m) | + | -(m) | -
TGFβ3 | - | - | +(m) | - | -(m) | + | -(m) | -

Epi: tissue components of enamel organ; Mes: dental mesenchyme. The intensities of immunostaining (p) and in situ hybridization (m) were evaluated from the descriptions by Abbott and Pratt (1988); Byers et al., (1990); Cameron et al., (1990); Dixon et al., (1991); D'Souza et al., (1990); Gonzalez et al., (1991); Heine et al., (1987); Hirning et al., (1991); Lehnert and Akhurst (1988); Mitsiadis et al., (1992), Pelton et al., (1990, 1991); Sneed et al. (1989); Vaithaniva et al., (1991); Vainio et al., (1991); Wilkinson et al., (1989). (t) means discrepancies between authors.

passed over an aFGF Aminolink column and purified by cycling over a bFGF-Aminolink column. Specificity of affinity purified antibodies for their respective antigen, either aFGF or bFGF, was assessed by ELISA and Western-blot experiments; these preparations were designated "anti-a," and "anti-b," respectively.

**Immunohistochemical procedures**

The procedures of fixation, permeabilization and enzymatic digestion of sections that resulted in reproducible positive or negative immunostaining were as follows (see Table 2).

**Protocol 1**

Frozen sections were fixed in 4% (w/v) paraformaldehyde (Serva, Heidelberg, Germany) freshly prepared in PBS pH 7.2, and rinsed in PBS containing 0.1% (v/v) BSA and merthiolate (50 mg/ml); then sections were permeabilized with methanol alone or methanol containing 0.6% (v/v) (final concentration) H₂O₂ (Prolabo) when peroxidase-conjugated secondary antibodies were to be used. Sections were subsequently incubated with bovine testes hyaluronidase (Sigma), diluted at 1 mg/ml in 0.1M sodium acetate (Merck) buffer pH 5.5 for 30 min at 37°C.

**Protocol 2**

Frozen sections were fixed and permeabilized with cold acetone for 3 min and then incubated with pepsin (Sigma) diluted to 100 ng/ml in 0.5 M acetic acid (Merck) for 15 min at room temperature (protocol 2a). Some sections were further incubated either in 2M and 3M NaCl solutions in PBS, pH 7.2 for 2 h at room temperature (protocol 2b) or in the presence of heparitinase from Flavobacterium heparinum (Seikagaku Kogyo, Tokyo, Japan) diluted (40 μg/ml) in 50 mM Tris, pH 7.5 containing 5 mM EDTA (Merck) for 45 min at 37°C (protocol 2c).

**Protocol 3**

Frozen sections were fixed with methanol and digested with hyaluronidase as described in protocol 1 (protocol 3a). Once deparaffinized in toluene and rehydrated in decreasing concentrations of

**TABLE 2**

**HISTOCHEMICAL PROTOCOLS USED FOR IMMUNOLOCALIZATION OF FGFS**

<table>
<thead>
<tr>
<th>Protocol Number</th>
<th>Type of section</th>
<th>Fixation</th>
<th>Permeabilization</th>
<th>Enzymatic and/or chemical treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Frozen</td>
<td>Parafomaldehyde</td>
<td>Methanol or Methanol+H₂O₂</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>2a</td>
<td>Frozen</td>
<td>Cold acetone</td>
<td>Cold acetone</td>
<td>Pepsin</td>
</tr>
<tr>
<td>2b</td>
<td>Frozen</td>
<td>Cold acetone</td>
<td>Cold acetone</td>
<td>Pepsin + NaCl</td>
</tr>
<tr>
<td>2c</td>
<td>Frozen</td>
<td>Cold acetone</td>
<td>Cold acetone</td>
<td>Pepsin + heparitinase</td>
</tr>
<tr>
<td>3a</td>
<td>Frozen</td>
<td>Methanol</td>
<td>Methanol</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>3b</td>
<td>Frozen</td>
<td>Bouin's</td>
<td>Methanol</td>
<td>Hyaluronidase</td>
</tr>
</tbody>
</table>
ethanol, Bouin’s fixed sections were also permeabilized with methanol and digested with hyaluronidase (protocol 3b).

All types of sections were subsequently rinsed in PBS containing 0.1% (w/v) BSA and non-specific binding was prevented by blocking epithopes with PBS containing 0.5% (w/v) BSA and 1.5% (v/v) normal goat serum (NGS) (Gibco) for 15 min at room temperature.

Depending on the size of sections, 5 to 50 μl of primary antibodies diluted in PBS containing 1% BSA were incubated on the slides overnight at 4°C (see Table 3).

Sections were rinsed in PBS containing 0.1% BSA and incubated with either fluorescein isothiocyanate (FITC) or peroxidase conjugated anti-rabbit (or mouse according to primary antibody) IgGs (Cappel Labs, Cochranville, USA) diluted 1:40 in PBS containing 1% BSA for 1 h at room temperature. Sections that had been treated with FITC-conjugated secondary antibodies were mounted in glycerol: PBS (9:1, v/v), viewed in a Leitz Orthoplan microscope equipped with epifluorescence and photographed with an Orthomat automatic camera. Sections processed for immunoperoxidase staining were rinsed with PBS alone and incubated with 4-chloro-1-naphthol (Merck) diluted first to 14% (w/v) in absolute ethanol and then to 1:400 (v/v) in PBS containing 0.02% (v/v) final concentration H2O2. These sections were finally rinsed, mounted in glycerol-gelatin (Sigma), viewed and photographed.

Control sections were obtained by using either rabbit/mouse preimmune or commercial IgGs (Cappel Labs) instead of primary antibodies.

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References


TABLE 3

| SOURCES AND DILUTIONS OF ANTIBODIES AND PROTOCOLS USED FOR IMMUNOLOCALIZATION OF FGFs |
|---------------------------------|---------------------------------|--------------------------------|---------------------------------|---------------------------------|
| Antibodies to FGFS | Anti-b-1,2 | Anti-b-3 | Anti-b | Anti-a-1,2 |
| Dilutions | 1:40 | 1:30 | 1:40 | 1:100 |
| Protocols | 1, 2a-b-c, 3a | 1, 2a | 2a-b, 3b | 2a-b-c, 3a-b |


