Immunohistochemical Localisation of Epidermal Growth Factor, Transforming Growth Factor α and EGF Receptor during Organogenesis of the Murine Hypophysis in vivo

By
Yasutomo IWAI-LIAO, Shunji KUMABE, Masaki TAKEUCHI and Yoshikage HIGASHI

Department of Oral Anatomy, Osaka Dental University, 8-1 Kuzuhanazono-cho, Hirakata-shi, Osaka 573–1121, Japan

Key Words: EGF, TGFα, EGFR, Pituitary gland, Foetus, Mouse

Summary: In previous study of melanocyte-stimulating hormone (MSH) cell development in the proliferating pars intermedia, which is in close apposition to the presumptive pars nervosa, no direct cell-to-cell contact was found between the boundary neurohypophyseal pituicytes (PIC), adenohypophyseal precursor stem cells (PSC) and the related diencephalic mesenchymal cells. Here, we have used immunohistochemistry to examine cytokine expression in the development of the hypophysis during foetal stages II–IV. Light and confocal laser scanning microscopy indicated diffuse expression of both TGFα and EGF in the hypophysis at different foetal stages. While no findings indicative for temporal changes of TGFα and EGF patterns were found in the foetal hypophysis, a temporary increment of EGF molecules was distinct in the diencephalic mesenchyme at stages III and IV. On the other hand, light microscopy intensively immunolocalised EGFR in the adenohypophysis and neurohypophysis at different developmental stages. Immunoreactivity of EGFR in the cytoplasm and nucleus suggested active proliferative events in the PIC and PSC of stages II–IV mouse pituitaries.

We previously detected active cell turnover with programmed cell death in the presumptive adenohypophysis (AP) and neurohypophysis (NP) and their associated cephalic mesenchyme during murine hypophysis development. The transmission electron microscopic (TEM) study suggested that signal transduction between the AP and NP via the extracellular matrix (ECM) might initiate the differentiation of melanocyte-stimulating hormone (MSH) cells in the presumptive pars intermedia at the late mid-foetal stage (Han et al., 1998).

Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), which both act via the EGF receptor (EGFR), are multivalent cytokines which control cell proliferation, differentiation and ECM biosynthesis of both the epithelium and mesenchymal cells (Nexo et al., 1980; Todaro et al., 1980; Adamson et al., 1981; Yoneda & Pratt, 1981; Cohen & Pratt, 1982; Adamson, 1983; Cohen, 1986; Abbott et al., 1988). Recently, the EGFR was also localised to various normal human epithelial tissues, which were categorised according to the intensity and pattern of EGFR expression (Damjanov et al., 1996).

Apoptosis of medial edge epithelial (MEE) cells is presumed to be essential for the fusion of the bilateral secondary palatine shelves (Taniguchi et al., 1995). A study on murine palatogenesis found that excess exogenous EGF/TGFα caused inhibition of cell death that induces cleft palate due to down-regulation of the EGFR (Adam & Warshaw, 1982). Another study observed that loss of expression of EGF, TGFα and their related receptors may initiate cell death, while epithelia which continuously produce TGFα differentiates stratified squamous keratinising epithelia (Pratt, 1987). Strong TGFα expression was co-localised with sparse EGF expression in the developing palate of murine foetuses, and strong TGFα and EGFR expression was observed at the degenerative medial MEE remnants during palatogenesis (Abbott et al., 1988; Dixon et al., 1991). Therefore, TGFα appears to play an important role in the degeneration and epithelial-mesenchymal transformation of the MEE region (Ferguson, 1988; Dixon et al., 1991). Several studies implicated TGFα as the embryonic homo-
logue of EGF, since it was usually found in the same locations as EGFR, however a more recent study has demonstrated similar patterns of EGF, TGFα and EGFR expression during morphogenesis of the murine primary palate (Nexo et al., 1980; Twardzik et al., 1982; Marquardt et al., 1984; Twardzik, 1985; Freemark & Comer, 1987; Iamaroon et al., 1996).

Direct contact occurring between the Rathke's pouch stomodeal epithelium, diencephalic down-growth neuroepithelium and surrounding mesenchyme are thought to be important factors in the induction of the pituitary organogenesis (Thompson & Trimble, 1976; Ikeda et al., 1988; Stefanovic et al., 1993). In the present study, we localised EGF, TGFα and EGFR expression in the developing murine hypophysis by immunohistochemistry to determine their temporo-spatial expression during determination and differentiation of this structure.

Materials and Methods

Foetuses of Slc: ICR SPF mice (SLC, Tokyo, Japan) were removed from the uterus at 9 to 15 days from insemination following the Guidelines for Animal Experimentation at Osaka Dental University. The day the vaginal plug was observed was designated to be the gestational day 0. The development of the foetal hypophysis was divided into four stages (stages I–IV) paralleling other previous studies (Thompson & Trimble, 1976; Stoëckel et al., 1979; Ikeda & Yoshimoto, 1991; Kaufman, 1992; Han et al., 1998; Hashimoto et al., 1998; Sasaki & Nishioka, 1988). In the present study, stage II (gestational days 9–10) foetuses and the head portions of stage III (gestational days 11–12) and stage IV (gestational days 13–15) samples were trimmed into blocks and fixed by immersion with Carnoy's solution and embedded in paraffine following conventional methods. Serial sagittal 2–5 μm sections of the middle portion of the head were cut using stainless steel knives mounted on an AS-500 Universal Microtome (Anglia Scientific, Cambridge, UK).

Light Microscopy (LM)

The specimens were deparaffinised and examined mainly with Ultra Vision Mouse Tissue Detection System Anti-mouse HRP/DAD (Ready-To-Use) kit (Lab Vision, CA, USA) and anti-mouse anti-EGF monoclonal antibody (Monosan, Uden, The Netherlands) for detection of EGF. Endogenous peroxidase and background non-specific reactions were blocked with Hydrogen Peroxide Block and Ultra V Block following the manufacturer's instructions, respectively. Reactive Igs were blocked using Rodent Block, and the endogenous biotin was blocked with Avidin D blocking solution and Biotin blocking solution using a Blocking Kit (Vector Lab, CA, USA). The specimens were reacted with the anti-mouse anti-EGF monoclonal antibody (primary antibody; 5 μg/ml, 2h, room temp), biotinylated goat anti-mouse antibody (secondary antibody; 15 min, room temp) and then treated with streptavidin peroxide (15 min, room temp). Subsequently, the DAB substrate-treated specimens were stained with DAB chromogen and counter-stained with methylgreen. The specimens were mounted on slide glasses, embedded in Permount (Fisher Scientific, New Jersey, USA), examined and photographed under an Olympus Vanox-S light microscope (Olympus, Tokyo, Japan).

Expression of the TGFα and EGFR of the developing hypophysis was examined with an anti-rat anti-TGFα polyclonal antibody (primary antibody; Biogenesis, Poole, UK) and anti-EGF polyclonal antibody (primary antibody; Santa Cruz Biotech, CA, USA) using the ImmunoCruz Staining System (Santa Cruz Biotech, CA, USA). The endogenous peroxidase and non-specific reactions were blocked by peroxide block and serum block, respectively. After blockage, the specimens were further treated with Avidin D blocking solution and Biotin blocking solution, and were then reacted with the anti-TGFα and anti-EGFR antibodies. The specimens were reacted with biotinylated secondary antibody and HRP-streptavidin complex, stained by a mixture of DAB Chromogen, peroxidase substrate and substrate buffer, and then were counter-stained by methylgreen and prepared for LM.

Confocal Laser Scanning Microscopy (CLSM)

For localisation of EGF and TGFα molecules using CLSM, some specimens were treated with an anti-EGF antibody (primary antibody; Monasan, Uden, The Netherlands) and an anti-TGFα antibody (primary antibody; Biogenesis, Poole, UK), respectively. The specimens were subsequently treated with biotinylated goat anti-mouse antibody (secondary antibody) for detection of EGF using an UltraVision Mouse Tissue Detection System Anti-mouse HRP/DAD (Ready-To-Use) kit (Lab Vision, CA, USA), as well as with goat anti-rabbit antibody (secondary antibody) for detection of the TGFα using the ImmunoCruz Staining System (Santa Cruz Biotech, CA, USA). Then specimens were reacted with streptavidin, Alexa 488 conjugate (fluorescence wavelength 519 nm; Molecular Probes, Oregon, USA), and the RNA in the tissue was digested by RNase A (Amresco, Ohio, USA). Subsequently, the specimens were counter-stained
with Propidium Iodide DNA staining (PI; fluorescence wavelength 615 nm; Molecular Probes, Oregon, USA) for identification of the cell nucleus. They were mounted in Vectashield Mounting Medium (Vector Lab, CA, USA), and were then analysed using an Olympus personal Fluoview CLSM (Argon ion laser, excitation wavelength 488 nm (CH1); Green HeNe ion laser, excitation wavelength 543 nm (CH2); using CH1: BA510IF & BA530IF, CH2: BA590 & BA565IF filters; Fluoview ver. 2.0.32; OS: MS Windows NT Workstation Ver. 4.0; Olympus, Tokyo, Japan).

Results

The pattern of expression of TGFα in the developing hypophysis was relatively unchanged at all stages (stages II–IV) by LM. TGFα was diffusely found in the developing AP, NP and in the associated diencephalic mesenchyme, and there were no apparent temporal changes in TGFα expression (Figs. 1 and 2). EGF immunoreactivity in the developing hypophysis was weak but also relatively unchanged at all stages. Weak reactivity was seen on the basement membrane and cells in the NP and AP, with strong expression evident in the associated diencephalic mesenchyme (Figs. 3 and 4). EGF expression increased with time in the diencephalic mesenchyme (Fig. 4). EGFR immunoreactivity was seen in the developing hypophysis and surrounding mesenchyme at all stages examined (Figs. 5 and 6). Specific EGFR reactivity was seen in the pars distalis at stage IV, and also in the nucleus of actively proliferating NP and AP cells (Figs. 5 and 6).

CLSM of specimens labelled with streptavidin, Alexa 488 conjugate and PI using the LsAB method showed intense fluorescence (CH1) specific for EGF and TGFα in the presumptive NP and AP at proliferative stage II (Figs. 7a–8). Similar patterns of expression of TGFα and EGF was seen in the pituitary at stages III–IV (Figs. 8–14). Consistent with LM, CLSM showed no marked temporal changes in TGFα or EGF expression in the proliferating hypophysis. Histology of the developing hypophysis showed that the NP and AP at stages III–IV were separated with the surrounding diencephalic mesenchyme and capillaries (Figs. 8–10). In addition, specific cell-to-cell contacts across a thin immunoreactive layer were seen between the developing NP and pars intermedia at stage IV (Figs. 10 and 13).

Discussion

A previous study observed that the diencephalic mesenchymal tissue invaded and thereby cleaved the developing AP into many lobules (Hashimoto et al., 1998). Subsequent penetration of vascular elements from the hypophyseal portal system into the foetal pars distalis was suggested to be an important determinant of all secretory cell types in the hypophysis (Thompson & Trimble, 1976). In addition, a three-dimensional histological study of the developing human pituitary showed that AP cells with closer physical contact to the developing NP have increased epithelial characteristics, while cells in the pars distalis which are less proximal to the NP differentiate into endocrine cells earlier (Ikeda et al., 1988). In contrast, one study found that ACTH-positive cells have different staining patterns in the foetal and adult pars distalis and pars intermedia, and have specific temporal patterns of expression in different sites of the pituitary (Perry et al., 1985). However, no selective increase in ACTH-like activity in the anterior pituitary at late gestation was observed using bioassay and radioimmunoassay methods (Fora et al., 1996). Another study found that isolated ACTH-cells first appeared at the boundaries where pars intermedia cells migrated towards the pituitary anterior lobe (Altukhova et al., 1983). Other studies have stressed that differentiation of MSH cells is evident before vascularisation and innervation, or folliculation of the pars intermedia (Chatterjee, 1975; Perry et al., 1981; 1982). In a previous TEM study, we observed that the proliferating AP was heavily lobulated, but did not differentiate into endocrine cell types in the mid-foetal stage. In addition, we identified the first appearance of MSH cells in the presumptive pars intermedia (Han et al., 1998).

A recent study reported that several transcription factors that regulate the determination of specific types and organs were expressed in a ventral-to-dorsal gradient at different stages in pituitary organogenesis (Treier et al., 1998). Various factors in the pituitary-specific transcriptional cascade that regulates cell proliferation, differentiation and maturation have been identified (Rodriguez & Jolin, 1993; Kimura et al., 1996; Parks et al., 1997; Sheng et al., 1997; de la Hoya et al., 1998; Watkins-Chow & Camper, 1998). In situ hybridisation showed a decrease in p75 nerve growth factor receptor (NGFR) expression in the Rathke’s pouch and pars intermedia during the differentiation of pituitary cell types (Ramaekers et al., 1997). The S-100 acidic calcium-binding protein is co-localised with vimentin in melanotropes coinciding with the onset of dopaminergic innervation and morpho-
logical changes in glial-like cells of the rat pars intermedia (Sands et al., 1995). Furthermore, morphological changes in glial-like cells of the pars intermedia is associated with subsequent changes in protein expression (Gary et al., 1995).

The growth and functional differentiation of the adrenal gland is induced by EGF, and inhibited by a glucocorticoid negative feedback mechanism (Coulter et al., 1996). In addition, both EGF and fibroblast growth factor-4 (FGF-4) inhibit apoptosis and thereby regulate proliferation and differentiation of dental tissues (Vaahatokari et al., 1996). In the present study, we localised weak EGF but intense TGFα immunoreactivity in the mouse pituitary at different developmental stages by LM. CLSM with fluorescent antibodies clearly mapped similar distribution of EGF and TGFα expression in the foetal pituitary at different stages. However, neither the LM nor CLSM analyses showed evidence of any temporal changes in EGF or TGFα (the embryonic homologue of EGF) expression in the NP and AP (Nexo et al., 1980; Twardzik et al., 1982; Marquardt et al., 1984; Twardzik, 1985; Freemark & Comer, 1987). In contrast, EGFR was differentially expressed in the pituitary at different stages; intense immunoreactivity was found in the pars distalis at stage IV. EGFR expression in the cytoplasm and nucleus seemed to indicate down-regulation by an internalisation mechanism of EGFR and EGF-receptor complexes in the actively proliferating NP and AP cells. The results of the present study are consistent with those of our previous histological study, which indicated that the mid-foetal murine pituitary is at the active proliferation stage (Han et al., 1998).

TEM of the proliferating AP showed junctional complex in folliculo-stellate cells facing the hypophysial cleft and zonula adherens-type junctions between the AP cells (Herbert, 1979; Han et al., 1998). A recent TEM study which showed substance P immunoreactivity in the anterior pituitary suggested that the presence of synaptoid contacts is responsible for cross-talk between the AP glandular cells of the rat (Liu & Ju, 1998). Observation of intracellular diffusion and freeze-fracture TEM of the cell surface in the pituitary gland have shown a dense cellular network connected by functional gap junction-mediated cell-to-cell communication among agranular folliculo-stellate cells which differentiate into granular and chromophilic cells (Kurono, 1996; Morand et al., 1996; Munari-Silem & Rousset, 1996; Guerineau et al., 1997; Soji et al., 1997). However, the development of gap junctions has been reported to be induced by certain paracrine interactions in the pituitary gland (Kurono, 1996; Schwartz et al., 1998). We previously found that the close cell-to-cell contact sites between the proliferating NP and AP were actually interposed with a thin layer of ECM (Stefanović et al., 1993; Han et al., 1998). To elucidate intercellular communication and signal transduction events which initiate the differentiation of MSH cells in the pars intermedia, further immunohistochemical study is needed to localise structures and inducers related to cell-to-cell contacts.

Acknowledgements

The study was performed using the Laboratory Animal and Morphological Research Facilities in the Institute of Dental Research, Osaka Dental University. The study was supported by a grant-in-aid from the Osaka Dental University Research Funds.

References

12) de la Hoya M, Vila V, Jimenez O and Castrillo JL. Ante-

13) Fora MA, Valego NK, Lively MO, Castro MI and Rose JC. ACTH-like bioactivity and immunoactivity in fetal lamb pituitaries at 0.65 and 0.95 gestation. Reproduction, Fertility, & Development 1996; 8:195–201.


**Abbreviations used in Figs. 1–14**

<table>
<thead>
<tr>
<th>A</th>
<th>Presumptive adenohypophysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>N</td>
<td>Presumptive neurohypophysis</td>
</tr>
<tr>
<td>PD</td>
<td>Presumptive pars distalis</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor α</td>
</tr>
<tr>
<td>S2</td>
<td>Developmental stage II</td>
</tr>
<tr>
<td>S3</td>
<td>Developmental stage III</td>
</tr>
<tr>
<td>S4</td>
<td>Developmental stage IV</td>
</tr>
</tbody>
</table>

**Explanation of Figures**

**Plate I**

Fig. 1. Light microscopy (LM) showing immuno localization of TGFα in stage II (S2) developing murine hypophysis composed of the diencephalic downgrowth (the presumptive neurohypophysis: N) and Rathke’s pouch (the presumptive adenohypophysis: A).

Fig. 2. TGFα expression at stage IV (S4) hypophysis observed by LM. No temporal change of TGFα expression is evident. The proliferating pars distalis (PD) of A is deeply invaginated with the diencephalic mesenchyme and its associated capillaries (arrows).

Fig. 3. LM shows weak EGF expression in the N and A of the S2 developing hypophysis.

Fig. 4. Intense EGF expression is found in the diencephalic mesenchyme of the stage III (S3) hypophysis, while weak EGF reactivity is observed in the proliferating hypophysis, in particular at the basement membrane and cell membrane facing the hypophyseal cleft.
Plate II

Fig. 5. LM mapped EGFR to the presumptive N and A at the S2 developmental stage. The EGFR is also immunolocalised in cell nuclei of the proliferating hypophysis.

Fig. 6. EGFR is expressed in proliferating cells of the N, A and PD of a developing S3 hypophysis (LM).

Fig. 7. Confocal laser scanning microscopy (CLSM) of TGFα expression in an S2 specimen. 7a. TGFα expression in both the N, A and the related tissues. 7b. Higher magnification of the same specimen clearly shows specific TGFα expression in the proliferating hypophysis.

Fig. 8. CLSM showing similar EGF and TGFα expressions (Fig. 7b) in the foetal S2 hypophysis. Arrows indicate that the N and A are separated by a thin layer of diencephalic mesenchyme containing capillaries.
Plate III

Fig. 9. While LM only showed weak EGF immunoreactivity (Fig. 4), CLSM clearly identifies EGF expression in a proliferating S3 hypophysis.

Fig. 10. EGF expression in cells of N, A and proliferating PD of an S4 hypophysis by CLSM. Arrowheads indicate that projections from A is very closely associated with N, but they are actually separated by the basement membrane, which shows EGF immunoreactivity.

Fig. 11. CLSM of another S4 specimen. Photo also demonstrates that the A bulged with a large projection, which extends towards the proliferating N. However, the N and A are separated by a thin layer of diencephalic mesenchyme (arrowheads).

Fig. 12. TGFα expression in an S3 specimen viewed under CLSM. TGFα molecules are widely and strongly expressed in the N, A and surrounding tissues.

Fig. 13. Similar to findings in the S3 hypophysis (Fig. 12), TGFα expression is distinctly localised in the N, A and PD in S4 specimens using CLSM. Arrowheads indicate projections from the presumptive pars intermedia.

Fig. 14. CLSM of TGFα expression in another S4 specimen. An arrowhead also shows the intimate relationship between the N and A.