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

By

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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 TGF α and EGF in the hypophysis at different foetal stages. While no findings indicative for temporary changes of TGF α 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 (TGF α), 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 *et al.*, 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/TGF α caused inhibition of cell death that induces cleft palate due to down-regulation of the EGFR (Adams & Warshaw, 1982). Another study observed that loss of expression of EGF, TGF α and their related receptors may initiate cell death, while epithelia which continuously produce TGF α differentiates stratified squamous keratinising epithelia (Pratt, 1987). Strong TGF α expression was co-localised with sparse EGF expression in the developing palate of murine foetuses, and strong TGF α and EGFR expression was observed at the degenerative medial MEE remnants during palatogenesis (Abbott *et al.*, 1988; Dixon *et al.*, 1991). Therefore, TGF α 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 TGF α 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, TGF α and EGFR expression during morphogenesis of the murine primary palate (Nexo *et al.*, 1980; Twardzik *et al.*, 1982; Marquardt *et al.*, 1984; Twardzik, 1985; Freemerk & Comer, 1987; Iamaroon *et al.*, 1996).

Direct contact occurring between the Rathke's pouch stomodaeal 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; Stefanović *et al.*, 1993). In the present study, we localised EGF, TGF α 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; Stoeckel *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 μ m 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 in-

structions, 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 μ g/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 TGF α and EGFR of the developing hypophysis was examined with an anti-rat anti-TGF α polyclonal antibody (primary antibody; Biogenesis, Poole, UK) and anti-EGFR 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-TGF α 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 TGF α molecules using CLSM, some specimens were treated with an anti-EGF antibody (primary antibody; Monosan, Uden, The Netherlands) and an anti-TGF α 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 TGF α 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 wave length 488 nm (CH1); Green HeNe ion laser, excitation wave length 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 TGF α in the developing hypophysis was relatively unchanged at all stages (stages II–IV) by LM. TGF α was diffusely found in the developing AP, NP and in the associated diencephalic mesenchyme, and there were no apparent temporal changes in TGF α 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 TGF α in the presumptive NP and AP at proliferative stage II (Figs. 7a–8). Similar patterns of expression of TGF α and EGF was seen in the pituitary at stages III–IV (Figs. 8–14). Consistent with LM, CLSM showed no marked temporal changes in TGF α 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 pituitary (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 (Rodríguez & 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 (Vaahatoki *et al.*, 1996). In the present study, we localised weak EGF but intense TGF α immunoreactivity in the mouse pituitary at different developmental stages by LM. CLSM with fluorescent antibodies clearly mapped similar distribution of EGF and TGF α expression in the foetal pituitary at different stages. However, neither the LM nor CLSM analyses showed evidence of any temporal changes in EGF or TGF α (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; Free-mark & 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 hypophyseal 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 (Stefanovi'c *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.

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Abbreviations used in Figs. 1–14

A	Presumptive adenohypophysis	PD	Presumptive pars distalis
CLSM	Confocal laser scanning microscopy	TGF α	Transforming growth factor α
EGF	Epidermal growth factor	S2	Developmental stage II
LM	Light microscopy	S3	Developmental stage III
N	Presumptive neurohypophysis	S4	Developmental stage IV

Explanation of Figures

Plate I

- Fig. 1. Light microscopy (LM) showing immunolocalisation of TGF α 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. TGF α expression at stage IV (S4) hypophysis observed by LM. No temporal change of TGF α 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 TGF α expression in an S2 specimen. 7a. TGF α expression in both the N, A and the related tissues. 7b. Higher magnification of the same specimen clearly shows specific TGF α expression in the proliferating hypophysis.
- Fig. 8. CLSM showing similar EGF and TGF α 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. TGF α expression in an S3 specimen viewed under CLSM. TGF α molecules are widely and strongly expressed in the N, A and surrounding tissues.
- Fig. 13. Similar to findings in the S3 hypophysis (Fig. 12), TGF α 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 TGF α expression in another S4 specimen. An arrowhead also shows the intimate relationship between the N and A.