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Angiogenesis in the intermediate lobe of the pituitary gland alters its structure and function

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ABSTRACT

The pars distalis (PD) and the pars intermedia (PI) have the same embryonic origin, but their morphological and functional characteristics diverge during development. The PD is highly vascularized, whereas the highly innervated PI is essentially non-vascularized. Based on our previous finding that vascular endothelial growth factor-A (VEGF-A) is involved in vascularization of the rat PD, attempt was made to generate transgenic *Xenopus* expressing VEGF-A specifically in the melanotrope cells of the PI as a model system for studying the significance of vascularization or avascularization for the functional differentiation of the pituitary. The PI of the transgenic frogs, examined after metamorphosis, were distinctly vascularized but poorly innervated. The experimentally induced vascularization in the PI resulted in a marked increase in tissue volume and a decrease in the expression of both alpha-melanophore-stimulating hormone (α-MSH) and prohormone convertase 2, a cleavage enzyme essential for generating α-MSH. The transgenic animals had low plasma α-MSH concentrations and displayed incomplete adaptation to a black background. To our knowledge, this is the first report indicating that experimentally induced angiogenesis in the PI may bring about functional as well as structural alterations in this tissue.

1. Introduction

Amphibians have the ability to camouflage themselves by changing their color to match that of their surroundings. This color change response is under the control of alpha-melanophore-stimulating hormone (α-MSH), which is released from melanotrope cells in the pituitary pars intermedia (PI) (Baker, 1981). The primordium of the epithelial pituitary originates at the neural ridge, migrates underneath the brain to form the so-called Rathke's pouch, and differentiates into the pars distalis (PD) and PI after making contact with the brain (Kawamura and Kikuyama, 1992; Kouki et al., 2001). In mammals, vascular endothelial growth factor (VEGF)-A is known to be involved in the formation of the hypothalamic-pituitary vascular system (Ferrara and Henzel, 1989; Nakakura et al., 2006), which is a capillary network that connects the pituitary PD with the median eminence (ME) through the hypophyseal portal vessels (Daikoku et al., 1981; Murakami et al., 1987; Szabo and Csanyi, 1982). Both the close contact of the adenohypophysis with the ME during development and the acquisition of sensitivity of the ME tissue to stimulation by thyroid hormone have been shown to be prerequisites for the development of the ME with abundant capillaries (Etkin et al., 1965/1966). Various neurohormones synthesized in the hypothalamus are supplied to the PD through the hypothalamic-pituitary vascular system, where they regulate the secretion of PD hormones. In contrast, the PI is essentially non-vascular (Daikoku et al., 1981; Hadley et al., 1977; Szabo and Csanyi, 1982) and does not express

Abbreviations: PD, pars distalis; PI, pars intermedia; VEGF, vascular endothelial growth factor; ME, median eminence; α-MSH, alpha-melanophore-stimulating hormone; PC, prohormone convertase; POMC, proopiomelanocortin; GFP, green fluorescent protein; MS222, 3-aminobenzoic acid ethyl ester; MMR, Mark's modified Ringer's solution; TRITC, tetramethylrhodamine isothiocyanate; PFA, paraformaldehyde; PB, phosphate buffer; PBS, phosphate buffered saline; BSA, bovine serum albumin; TH, tyrosine hydroxylase; DAPI, 4′,6-diamidino-2-phenylindole; DW, distilled water; RT, reverse transcription; TR-FIA, time-resolved fluorescence immunoassay; NIL, neurointermediate lobe; PN, pars nervosa; ACTH, adrenocorticotropic hormone; MI, melanophore index; ANOVA, analysis of variance; LSD, least significant difference.

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VEGF-A (Jabbour et al., 1997; Jakeman et al., 1992; Nakakura et al., 2006). Despite the poor vascularization of the pars intermedia, systemically injected horse radish peroxidase (HRP) has been shown to rapidly penetrate this tissue (Perryman and Bagnara, 1978), demonstrating good communication between the blood and the extracellular space of the lobe. Thus the presence of a network of capillaries at the border between the neural lobe and the intermediate lobe (Landsmeer, 1963) has been considered sufficient to allow the secretory products released by the intermediate lobe to reach the blood vessels of the neural lobe (Lamacz et al., 1991). Secretion of α-MSH is negatively regulated by direct innervation of dopamine neurons that originate from the suprachiasmatic nucleus of the hypothalamus and subsequently distribute themselves among the melanotropes of the PI (Hadley et al., 1977; Ubink et al., 1998; van Strien et al., 1991).

When *Xenopus laevis* are placed on a black background, the melanotropes synthesize large amounts of both the precursor protein proopiomelanocortin (POMC) and prohormone convertase 2 (PC2) that is required for generating α-MSH from POMC (Braks et al., 1992; Jenks et al., 1993; Seidah and Chretien, 1992). The released α-MSH causes pigment dispersion in dermal melanophores. In contrast, α-MSH release is inhibited in *Xenopus* placed on a white background, and the melanotropes are biosynthetically inactive. Thus, the secretory activity of melanotropes can be physiologically manipulated by placing the frogs on a black or a white background (Jenks et al., 1993).

The stable expression of transgenes has been demonstrated in *Xenopus laevis* (Kroll and Amaya, 1996; Sparrow et al., 2000). More recently, we have developed a refined technique that enables a transgene to be overexpressed specifically in the melanotropes of the PI in black-adapted *Xenopus* by using a POMC gene promoter fragment (Bouw et al., 2004; Jansen et al., 2002). The endogenous expression of POMC mRNA is higher in animals adapted to a black background (Martens et al., 1987) and, in keeping with this observation, it has been shown in transgenic *Xenopus* that the POMC promoter can drive expression of gene constructs in a physiologically regulated way (*i.e.* high expression in animals on black background, low expression in animals on white background) (Bouw et al., 2004; Jansen et al., 2002). This expression is specific to the pituitary, a finding consistent with the fact that a different promoter is responsible for expression of POMC in the brain (Jenks, 2009). Using this approach, we have previously shown that transgenic manipulation of expression in the PI melanotrope cells may modulate α-MSH physiology (Bouw et al., 2004; de Groot et al., 2006).

In the study reported here, transgenic *Xenopus* that specifically express the green fluorescent protein (GFP)-VEGF-A-122 fusion protein under the control of the POMC gene promoter fragment in the melanotropes were generated. Our aim was to determine if the experimentally induced vascularization would alter the function as well as structure of the PI, thus highlighting possible effects of VEGF-A and vascularization in the developmental processes leading to the formation of the hypophyseal system.

2. Materials and methods

2.1. Animals

Both wild-type male and female frogs (*Xenopus laevis* Daudin) aged 3 years were used for generating the F0 strain. Transgenic male frogs of the F0 strain reared approximately 6 months after metamorphosis and wild-type females aged 3 years were used to obtain F1 offspring. Both transgenic and wild-type animals used for the experiment were kept under standard laboratory conditions and fed a commercial trout-based feed (Nitto Fuji Flour Milling, Tokyo, Japan) on a regular regimen. The embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). The transgenic F1 frogs aged approximately 3–4 months were mostly used for analyzing the function and morphology of the intermediate lobes unless otherwise mentioned. Wild-type frogs from the same cohort as transgenic frogs served as controls. Both wild type and transgenic frogs were comparable in age and reared in a similar condition. For the morphological and histological studies, 4–6 frogs for each group were used. A representative photograph is presented in the figures when histological or morphological features were confirmed to be almost similar among the specimens of each group. All animal experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals of Shizuoka University and approved by the university's ethics committee.

2.2. Generation of the DNA construct encoding VEGF-A122 amino-terminally fused to green fluorescent protein (GFP)

Xenopus VEGF-A122 cDNA was prepared by amplifying by PCR an ortholog of mouse VEGF-A120 (Cleaver et al., 1997), which is the cDNA fragment encoding a sequence of the VEGF-A122 mature protein (Accession number AF008593, +231 to +593) that is devoid of the signal peptide region, using primers containing the *Eco*RI and *Xho*I recognition sites at their 5′ ends (xVEGF-SPEGFP-*Eco*RI: 5′-GGGGAATTCATGCCAGGAGAGGGAGACC-3′; xVEGF-SPEGFP-*Xho*I: 5′-GGGGCTGAGTCACCGTCGTGGCTTT-3′). This step resulted in a 363-bp PCR product. The VEGF-A122 PCR fragments were digested with *Eco*RI and *Xho*I and subcloned into the pPOMC(A)$^{2+}$-SP-GFP vector containing a 529-bp *Xenopus* POMC gene promoter fragment, a sequence encoding the signal peptide of the *Xenopus* secretory pathway component Ac45 (Holthuis et al., 1999), a GFP sequence, and a cytomegalovirus poly(A) signal. The resulting construct was pPOMC(A)$^{2+}$-GFP-VEGF-A122.

2.3. Generation of transgenic Xenopus embryos with GFP-VEGF-A122

A 2009-bp *Sal*I/*Not*I fragment that contained the SV40 poly(A) signal behind the pPOMC-GFP-VEGF-A122 fragment (Fig. 1A) (50 ng/μl) was mixed with sperm nuclei (2.5×10^5 in a total volume of 2.5 μl) and sperm dilution buffer, and approximately 10 nl of the mixture was injected into each egg using a Harvard syringe pump (Harvard Apparatus, Holliston, MA). Sperm nuclei and fresh eggs were prepared as described previously (Jansen et al., 2002; Sparrow et al., 2000). Normally cleaving eggs were selected at the four-cell stage and cultured in 0.1× Mark's modified Ringer's solution (MMR) (10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.5 mM HEPES) containing 6% Ficoll-400 (pH 7.5) at 18 °C until gastrulation (stage 12), at which time the embryos were transferred into 0.1× MMR with 50 μg/μl gentamicin (Invitrogen, Carlsbad, CA) at 22 °C. When the embryos reached stage 45, they were anesthetized with 0.25 mg/ml MS222 (3-aminobenzoic acid ethyl ester; Nacalai Tesque, Kyoto, Japan), and the pituitary was screened for GFP fluorescence using a fluorescent stereomicroscope equipped with a Leica DC200 colour camera (Leica, Wetzlar, Germany). The photographs were examined using Leica DC Viewer software.

2.4. In vitro fertilization of wild-type Xenopus eggs with transgenic sperm cells to generate F1 offspring

The testes of male transgenic frogs were isolated and gently pulled apart prior to use. Pieces of the testes were brought into physical contact with unfertilized eggs harvested from wild-type females. After 10 min, the eggs were incubated in 0.1× MMR. The embryos were reared and then screened for fluorescence in the pituitary following the procedures described in the previous section. Throughout the larval stage and until 3 months after metamorphosis, the transgenic F1 off-springs were kept in