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Temporal and spatial distribution of mast cells and steroidogenic enzymes in the human fetal adrenal

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ABSTRACT

Mast cells are present in the human adult adrenal with a potential role in the regulation of aldosterone secretion in both normal cortex and adrenocortical adenomas. We have investigated the human developing adrenal gland for the presence of mast cells in parallel with steroidogenic enzymes profile and serotonin signaling pathway. RT-QPCR and immunohistochemical studies were performed on adrenals at 16–41 weeks of gestation (WG). Tryptase-immunopositive mast cells were found from 18 WG in the adrenal subcapsular layer, close to 3βHSD- and CYP11B2-immunoreactive cells, firstly detected at 18 and 24 WG, respectively. Tryptophan hydroxylase and serotonin receptor type 4 expression increased at 30 WG before the CYP11B2 expression surge. In addition, HDL and LDL cholesterol receptors were expressed in the subcapsular zone from 24 WG. Altogether, our findings suggest the implication of mast cells and serotonin in the establishment of the mineralocorticoid synthesizing pathway during fetal adrenal development.

1. Introduction

The human fetal adrenal (HFA) cortex, which, like gonads, derives from the adreno-genital primordium, is organized by 7–8 weeks of gestation (WG) into two distinct parts: an inner fetal zone (FZ) accounting for 70–80% of the whole tissue and a thinner outer definitive zone (DZ) (Ishimoto and Jaffe, 2010). In addition, a third zone, called the transitional zone (TZ), is classically observed between the FZ and DZ during the second trimester of pregnancy. Quantification of steroid production during pregnancy and analysis of steroidogenic enzyme expression profile have showed that active steroidogenesis occurs very early, as soon as 8 WG for androgen production (Goto et al., 2002; Mesiano and Jaffe, 1997). Steroidogenesis requires cholesterol either locally synthesized or retrieved from the circulating lipoproteins LDL and HDL *via* their specific receptors (Bochem et al., 2013; Liu et al., 2000). In adults, the HDL receptor, also named SRB1 (Scavenger receptor class B type I), is crucial for adrenal cholesterol supply as demonstrated by altered corticosteroidogenesis in cases of *SRB1* mutations (Vergeer et al., 2011). Although some *in vitro* studies have indicated that cholesterol uptake mainly occurs *via* the LDL receptor pathway in the developing adrenal (Carr et al., 1980, 1982), the demonstration of high levels of *SRB1* mRNA in fetal glands suggest that HDL cholesterol could also be an important source of substrate for steroidogenesis in these tissues (Liu et al., 1997). Androgens, (dehydroepiandrosterone [DHEA] and its sulfate [DHEAS]), are the first steroids detected, owing to early expression of 17α-hydroxylase (17α-OH) in both the FZ and TZ (Narasaka et al., 2001). This androgen production is responsible for prenatal virilization of

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female fetuses suffering from congenital adrenal hyperplasia (CAH) (Speiser and White, 2003).

Synthesis of mineralo- and glucocorticoids also requires 21-hydroxylase (21-OH) and 3β-hydroxysteroid dehydrogenase (3βHSD). 21-OH immunoreactivity is visualized in the fetal adrenal as soon as 14 WG (Coulter and Jaffe, 1998; Narasaka et al., 2001). However, the accurate timing of 3βHSD expression is still controversial, since Narasaka et al. observed the presence of the enzyme in the TZ from 22 WG (Narasaka et al., 2001) while Folligan et al. detected 3βHSD from 16 WG (Folligan et al., 2005). In addition, Goto et al. reported an earlier 3βHSD expression from the first trimester of pregnancy with transient cortisol secretion at 8–10 WG (Goto, 2006), a finding which also suggests expression of 11β-hydroxylase (11β-OH) during this period. However, to our knowledge, the timing of 11β-OH expression in the fetal human adrenal has never been studied. Similarly, the fetal expression profile of aldosterone synthase, the key enzyme for aldosterone synthesis encoded by *CYP11B2*, has not been analyzed so that the capacity of the fetal adrenal to produce aldosterone remains a matter of debate.

It is now considered that cell-to-cell interactions are critical for organogenesis in mammals (Durdu et al., 2014). Recent studies have emphasized the role of immune cells in testis morphogenesis during which macrophages have been demonstrated to play a major role in the establishment of organ vascularization and architecture (DeFalco et al., 2014; Zhang et al., 2014). However, the involvement of adrenocortical cell microenvironment in adrenal development has not yet been investigated. In the human adult adrenal, adrenocortical cells interact with various types of immune cells which control their mitogenic and secretory activities (Kanczkowski et al., 2015). We have previously demonstrated that mast cells, located in the subcapsular region of the gland, stimulate aldosterone secretion through a paracrine mechanism involving local release of serotonin (5-hydroxytryptamine; 5-HT) and activation of 5-HT_4 receptors (Lefebvre et al., 2001). Consistent with this observation, incubation of the human adrenocortical cell line H295R with human mast cell line (LAD2)-conditioned medium provokes an increase in *CYP11B2* expression and aldosterone production (Duparc et al., 2015). Resident mast cells may also be involved in the pathophysiology of adrenal disorders. In fact, a positive correlation between adrenal mast cell density and plasma aldosterone levels was found in a subset of aldosterone-producing adenomas, arguing for a role of mast cells in aldosterone hypersecretion (Duparc et al., 2015). All these observations strongly suggest that mast cells may control mineralocorticoid synthesis and secretion in both physiological and pathophysiological conditions. As mast cells has been noticed in various fetal tissues (Chen et al., 2004; Noack et al., 2005; Raica et al., 2010), their presence in the human fetal adrenal gland may also be hypothesized with a particular role in the establishment of the mineralocorticoid production.

To test this hypothesis, we thus investigated the presence of mast cells in correlation with steroidogenic enzyme expression in the human developing adrenal gland.

2. Materials and methods

2.1. Tissue collection

Human fetal adrenal-kidney complexes (n = 33) from 16 to 41 WG were obtained at the time of autopsy performed either after medical terminations of the pregnancies for neurological or cardiac malformations (n = 12), spontaneous abortions (n = 10) or *in utero* death (n = 11) mainly due to chorioamniotitis. Medical termination of pregnancies had been approved by the local ethics committee of our Prenatal Diagnosis Multidisciplinary Center according to the French Law. In each case, a complete autopsy had been performed after informed consent of parents. All adrenal glands were macroscopically and microscopically free of any lesion.

Specimens for immunohistochemistry (n = 28) were formalin-fixed, embedded in paraffin and then cut into 5 μm sections. Adrenal samples for RNA extraction (n = 8, one sample for 18, 22, 29, 30, 33 and 41 WG, two samples for 24 WG) were immediately frozen for cryopreservation.

Normal adult adrenals from patients undergoing expanded nephrectomy for kidney cancer or brain-dead organ donors were used as positive controls for all immunohistochemistry experiments except for mast cell relative proteins immunohistochemistry for which human mastocytomas were employed as reference tissues. The kidney tissues present on the sections of fetal adrenal-kidney complexes were used as negative controls for the diverse immunostainings (Fig. S2A, B and C). The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committees and the French Agence Nationale de Biomédecine. Written informed consent from patient's relatives was obtained for all subjects.

2.2. Methods

2.2.1. Immunohistochemistry

Immunohistochemistry was performed as previously reported (Duparc et al., 2015). Briefly, after deparaffinization and rehydration, tissue sections were heated with either a trypsin solution at 37 °C for 15 min (SIGMA, St. Louis, USA) or 0.01 M Na citrate buffer solution (pH = 6) at 95 °C for 20 min, for antigen retrieval. They were then treated with peroxidase and alkaline phosphatase blocking solution (Dako, Trappes, France) for 5 min. Tissue sections were incubated with the different primary antibodies described in Table S1. The sections were then incubated with a streptavidin–biotin–peroxidase or alkaline phosphatase complex. Immunoreactivities were revealed using either EnVision + System-HRP kit or Envision TM G/2 doublestain kit (Dako). Sections were finally counterstained with hematoxylin, mounted in Eukitt (Kindler, Freiburg, Germany), then cover-slipped.

For each fetal stage, one to three adrenal tissue sections were analyzed as well as sections from adult adrenal gland as control tissues.

2.2.2. Analysis of immunohistochemical data

Digital images were captured using an Eclipse E-600 microscope equipped with a CCD DXC950 camera and recorded using the Nis Elements BR software 4.0 (Nikon Corporation, Tokyo, Japan). Immunoreactivity was semi-quantitatively assessed using McCarty's H-scoring system, in which the percentage of stained cells is multiplied by a number, 0–3, reflecting the intensity of the signal (McCarty et al., 1985). The relative intensities of immunoreactivity were characterized as not present (0), weak but detectable above control (1+), distinct (2+), and very strong (3+). Digital images of twenty separate square-shaped areas (total area 0.3 mm^2) within the definitive zone were analyzed to evaluate the H scores. This analysis was performed in duplicate by 2 independent observers with the assistance of two expert fetopathologists. Final H-scores were determined as the average of all the values obtained for each section analysis. An illustration of H-score determinations is presented in supplemental data (Fig. S3).

Mast cells density was evaluated using the Mercator image analysis software (ExploraNova, La Rochelle, France) on the Cell Imaging Platform of Normandy (PRIMACEN, University of Rouen). Mast cells were identified as immunopositive cells for the mast cell specific protease tryptase or/and CD117 (Duparc et al., 2015). In