

signaling pathways that are involved in tumorigenesis and proliferation. We isolated EVs from the conditioned media of human wild-type hPheo1 cells and hPheo1 cells with shRNA *SDHB* knockdown. The EVs from three separate preparations of each group were characterized by nanoparticle tracking analysis, transmission electron microscopy, and Western blotting using antibodies against different types of EV and one non-EV marker. Our results show small EVs from the *SDHB* knockdown hPheo1 cells increased the activation of phosphotyrosine residues in wild-type cells compared to cells treated with control EVs from the same cell type. Additionally, our data show these EVs increase phospho-STAT3 compared to the control EVs (3843.10 \pm 1138.89 vs. 213.65 \pm 40.75; $p < 0.05$; $n = 3$) in cultured wild-type hPheo1 cells. Protein tyrosine kinases (PTKs) control various cellular processes including growth, differentiation, and metabolism by activating various signaling pathways including STAT3. The significance of these findings is that in some cancers, elevated succinate from a *SDHx* mutation has been shown to activate STAT3 which may explain a possible pathway for tumorigenesis. Studies from other investigators have shown that STAT3 expression is elevated in malignant PPGL tissues. Through enriched EV analysis our findings have confirmed the role of STAT3 in *SDHB* deficient cells. Additional studies are needed to identify other metabolites that are enriched in EVs that regulate phosphorylation of tyrosine residues and STAT3 activation.

Adrenal

ADRENAL - BASIC AND TRANSLATIONAL ASPECTS

Human Adrenal Cortical Zone Changes With Aging

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Background: Previous adrenal morphological studies have shown that the zona reticularis (ZR) and the zona glomerulosa (ZG) decrease in size with aging. Although several lines of evidence indicate that the hypothalamic-pituitary-adrenal axis becomes hyperactive in elderly, little is known about age-related transformations of the adrenal zona fasciculata (ZF). **Objectives:** To investigate the morphological and functional changes of the adrenal cortex across the adult life span, with emphasis on: 1) the understudied ZF, and 2) potential sexual dimorphisms. **Methods:** We used immunohistochemistry to evaluate the expression of several cortical proteins: aldosterone synthase (CYP11B2), visinin-like protein 1 (VSNL1), β -hydroxysteroid dehydrogenase type II (HSD3B2), 11β -hydroxylase (CYP11B1) and cytochrome b_5 type A (CYB5A). The ZF area was estimated by subtracting the VSNL1-positive (a ZG marker) area from the HSD3B2-expressing area (ZG and ZF). All captured images were quantitated by ImageJ. In addition, we employed liquid chromatography-tandem mass spectrometry to quantify the morning serum concentrations of 6 steroids: cortisol, 11-deoxycortisol (11dF), 17α -hydroxyprogesterone

(17OHP4), 11-deoxycorticosterone (DOC), corticosterone, and androstenedione (A4). The Mann-Whitney *U* test and Spearman's rank correlation coefficients were used for statistical analysis, as appropriate. **Results:** We included 60 adrenal glands from 30 men and 30 women, with ages between 18–86 years. The total cortical area was positively correlated with age ($r = 0.34$, $p = 0.008$), and this association was significant only in men ($p = 0.02$). Both the total (VSNL1-positive) and functional ZG (CYP11B2-positive) areas declined abruptly with aging in men ($r = -0.57$ and -0.76 , $p = 0.001$ and $p < 0.0001$, respectively), but not women ($p = 0.06$ and 0.27 , respectively). The CYB5A-positive area, marking the ZR, correlated negatively with age ($r = -0.76$, $p < 0.0001$) in both sexes. In contrast, the estimated ZF area showed a strong positive correlation with age both in men ($r = 0.59$, $p = 0.0006$) and women ($r = 0.49$, $p = 0.007$), while CYP11B1-positive area remained stable across ages ($p = 0.86$). Finally, we measured morning levels of 6 steroids in 149 men and 149 women, with ages between 21–95 years, matched for age and body mass index. Serum cortisol, corticosterone, and DOC levels remained relatively stable across ages ($p = 0.38$, 0.64 and 0.25 , respectively), while 11dF levels increased slightly with age ($r = 0.16$ and $p = 0.007$), particularly so in men ($p = 0.005$). Expectedly, 17OHP4 and A4 declined with aging ($r = -0.37$ and -0.37 , $p < 0.0001$ for both). **Conclusions:** In contrast with the ZG and ZR, the ZF and the total adrenal cortex area enlarge with aging. An abrupt decline of the ZG occurs with age in men, but not in women, possibly contributing to sexual dimorphism in cardiovascular risk.

Adrenal

ADRENAL - BASIC AND TRANSLATIONAL ASPECTS

Intracellular Cholesterol Metabolism in Aldosterone-Producing Adenoma. ~A Possible Association With Cellular Morphometry and Genotype~

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Introduction: Primary aldosteronism (PA) is the most common cause of secondary hypertension. More than 70% of APAs have been reported to have KCNJ5 somatic mutation in Asian countries. Patients with KCNJ5 mutated APAs generally harbor high plasma aldosterone concentration (PAC), and are mainly composed of clear tumor cells containing abundant lipid droplets. However, an association among intracellular cholesterol metabolism, morphological features and genotypes in tumor cells has

remained virtually unknown. [Materials and methods] Three cholesterol receptors (SR-B1, LDL-R, VLDL-R) were immunolocalized in 25 APA cases (KCNJ5 mt: n= 15, WT: n=10) who underwent adrenalectomy at Tohoku University Hospital. Image analysis software (HALO, India Labs) was used to quantitatively analyze the intracellular localization and immunoreactivity and exposed their correlation with genotype and clinical factors. [Results] LDL-R immunoreactivity was significantly lower in KCNJ5 mt group than WT group (P = 0.0369). In KCNJ5 mt group, a significant correlation was detected between LDL-R immunoreactivity and CYP11B2 (Aldosterone synthase), (P = 0.0271, $\rho = 0.5684$) but not in WT group. In addition, LDL-R immunoreactivity was significantly inversely correlated with tumor size (P = 0.0142, $\rho = -0.6176$) and PAC (P = <0.001, $\rho = -0.7179$) in mt group as well as in whole APA cases. [Discussion] This is the first study to compare cholesterol receptor expression profiles with morphological tumor cell subtype, genotype, and clinical data in APAs. Results indicated that KCNJ5 mt APA abundantly stored cholesterol ester in their cytoplasm and cholesterol uptake was less activated, resulting in rather efficient aldosterone biosynthesis in tumor cells. In addition, a significant correlation was detected between LDL-R and CYP11B2 with the abundant localization of LDL-R in tumor cells. Therefore, LDL-R could be a predominant resource of plasma lipoprotein uptake in aldosterone-producing tumor cells, especially for KCNJ5mt APAs.

Adrenal

ADRENAL - BASIC AND TRANSLATIONAL ASPECTS

Investigating the Role of Cholesterol and Lipid Trafficking in Mitotane Resistance in Adrenocortical Carcinoma

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Introduction: Adrenocortical Carcinoma (ACC) is a rare aggressive cancer which carries a poor prognosis. Adjuvant mitotane improves survival but is limited by poor response rates and resistance following tumour recurrence. Mitotane's efficacy has been attributed to intracellular accumulation of toxic free cholesterol (FC) predominantly through inhibition of cholesterol storage through SOAT1. Yet SOAT1 specific inhibitors demonstrate inferior efficacy to mitotane in inducing ACC cell death. We hypothesize that mitotane's efficacy to induce toxic FC accumulation in ACC cells is also mediated through enhanced breakdown of stored cholesterol within intracellular lipid droplets (LDs). **Methodology:** ATCC-H295R (mitotane sensitive) and MUC-1 (mitotane resistant) ACC cells were evaluated for neutral lipid content using BODIPY493/503 under baseline and cholesterol loaded conditions using Amnis ImageStream, additionally cells were treated with mitotane (H295R - 20, 40, 50 μ M; MUC1 - 50, 100, 200 μ M) for 6hr. Analysis of LDs using CE-BODIPY and FA-BODIPY identified cholesterol ester (CE) and

triacylglycerol (TAG)-containing LDs, respectively. Lipid droplet-associated proteins (LDAPs) Perilipin (PLIN) 1–4 and hormone sensitive lipase (HSL) were evaluated using western blotting and PCR. Lipid uptake receptors; SRB1, LDLR, LRP1 and CD36 were measured by flow cytometry. **Results:** Mitotane treatment, within therapeutic range, decreased staining for LDs significantly in H295R. This was also reflected by decreased expression of LDAPs, PLIN1 and PLIN3. The decrease in H295R LDs was associated with increased activation of HSL (pHSL and LIPE). However, this effect was only evident in MUC-1 at supratherapeutic mitotane (200 μ M). H295R and MUC-1 demonstrated similar overall LD numbers at baseline and under cholesterol supplementation. Expression of PLIN3 was high in both cell lines, while PLIN1, PLIN2 and PLIN4 demonstrated distinct LD profiles in each. Investigation of LD content showed that H295R preferentially store CEs while MUC-1 store only TAG, irrespective of cholesterol-loading. Mitotane treatment significantly reduces both CE and TAG LDs in H295R and MUC-1. Expression of lipid uptake receptors also demonstrated significant variability between cell lines including SRB1 and LRP1. **Conclusion:** We highlight that lipolysis through LD breakdown and activation of HSL represents a putative additional mechanism for mitotane induced FC cytotoxicity in ACC. We also demonstrate significant differences in cholesterol handling and LDAPs between mitotane sensitive and mitotane resistant models, in particular, the absence of CE LDs in MUC-1. We therefore propose a mechanism of resistance to mitotane through absent CE storage. Further understanding of cholesterol and lipid handling in ACC offers novel therapeutic exploitation, especially in the setting of mitotane resistant disease.

Adrenal

ADRENAL - BASIC AND TRANSLATIONAL ASPECTS

Involvement of JAK-STAT-SOCS3 Pathway in the Regression of Mice X-Zone of Adrenal Cortex

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Previously, we showed that the histological markers of the mice X-zone of adrenal cortex were still present in adult male and female postpartum SF1/SOCS3KO mice. Abnormal distribution of lipid droplets along the adrenal cortex and reduced ACTH-induced corticosterone secretion were observed in SF1/SOCS3KO mice (1). Here we have examined the adrenals of the SF1/SOCS3KO male and virgin female at 3, 8, 15 and 30 weeks through morphological and molecular analysis. Hematoxylin-eosin stains showed X-zone retention in the SF1/SOCS3KO mice adrenals regardless of the postnatal age analyzed. CYP17A1-positive cells were immunolocalized in the X-zone of SF1/SOCS3KO mice that were confirmed by immunoblotting. A fetal adrenal enhancer (FAdE) and Pik3c2g and 20 α HSD genes expression were analyzed by RT-PCR, and these genes were present in the male SF1/SOCS3KO mice up to the age of 8 and 15 weeks, but not in the control mice. Therefore,