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学位授与の日付	２０２２年３月３１日
論文名	Ephrin-B1 and EphB4 as novel markers for steroidogenic cells in naturally cycling mouse ovary and adrenal gland (自然発情周期下のマウスの卵巣と副腎のステロイド産生細胞における新規マーカー分子 ephrin-B1 と EphB4)
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論文要旨

Introduction

Erythropoietin-producing hepatocellular (Eph) receptors and their ligands, ephrins, are expressed in many tissues and organs. These membrane proteins serve as a cell-cell communication system with a variety of roles in normal development, physiology, and disease pathogenesis. In mammals, Eph receptors are divided into EphA (A1–A8 and A10) and EphB (B1–B4 and B6), which promiscuously bind to ligands of ephrin-A (A1–A5) and ephrin-B (B1–B3), respectively. Eph/ephrin signalling has been implicated in tissue homeostasis via cell positioning, proliferation, migration, adhesion, and/or differentiation in several epithelial tissues. Recent studies showed that not only adult Leydig cells but also foetal Leydig cells co-expressed ephrin-B1 and EphB4 in the mouse testis. Based on this, the author hypothesised that ephrin-B1 and EphB4 are co-expressed ubiquitously in steroid-producing cells in gonads and adrenal glands. However, expression and localisation of these molecules have not been sufficiently examined in normal cycling mouse ovaries and adrenal glands. To test this hypothesis, therefore, the author examined ephrin-B1 and EphB4 expression and localisation in the naturally cycling mouse ovary as well as adrenal glands of male and female mice.

Chapter 1: Identification of developmental/regressing stages of corpora lutea in the naturally cycling mouse ovary

Corpora lutea up to 2 or 3 generations appear in a section of all phases in naturally cycling ovaries (proestrus, oestrus, metoestrus, dioestrus). Thus, the author first examined the properties of corpora lutea by immunofluorescence staining using serial sections with 3β-HSD as a marker of steroidogenic cells and CD31 as a marker of vascular endothelial cells. By a combination of the 3β-HSD immunoreactivity in luteal cells and CD31-positive vascular densities, corpora lutea could be classified into four: CD31-positive thick blood vessels were sparsely distributed and ran towards the centre in some corpora lutea filled with strongly 3β-HSD-positive small luteal cells, and thus identified as developing corpora lutea (CLd) that frequently appeared in metoestrus, while CD31-positive blood vessels were generally thin/fine in other corpora lutea. The characteristic corpora lutea were composed of blood vessels of a prominently high density and strongly 3β-HSD-positive large luteal cells that frequently appeared in dioestrus and proestrus, and thus were identified as temporally mature corpora lutea (CLm) of the current oestrous cycle. Corpora lutea other than CLd and CLm, which composed of strongly 3β-HSD-positive luteal cells and CD31-positive blood vessels of a low density as well as composed of weakly 3β-HSD-positive luteal cells and CD31-positive blood vessels at a low or high density, frequently appeared in all four phases, and were thus identified likely as regressing corpora lutea of the previous oestrous cycle and/or corpora lutea unable to undergo full differentiation into CLm (CLs). Moreover, characteristic small corpora lutea composed of strongly 3β-HSD-positive cells distributed sparsely and CD31-positive blood vessels of a prominently low density that were frequently appeared in oestrus, and thus considered as regressing corpora lutea (CLrl) of a late phase.

Chapter 2: Expression and localisation of ephrin-B1 and EphB4 in the naturally cycling mouse ovary

Efnb1 and *Ephb4* were detected by RT-PCR in all oestrous phases in the naturally cycling mouse ovary. The relative expression levels of *Efnb1* and *Ephb4* were similar among the four phases in the mouse ovaries. This finding indicates that *Efnb1* and *Ephb4* expression levels do not change in the whole ovary according to the oestrous cycle.

Ephrin-B1 immunoreactivity was detected prominently in corpora lutea and theca folliculi in the naturally cycling mouse ovary. Ephrin-B1 immunoreactivity was similar among theca folliculi in four oestrous cycle phases. Thus, ephrin-B1 immunoreactivity similar to or greater than that observed in the theca folliculi was defined as ‘strong’, and immunoreactivity clearly less than that in theca folliculi as

‘weak’ or ‘faint’. In contrast, ephrin-B1 immunoreactivity was appreciably different among corpora lutea in a section of ovaries. Therefore, ephrin-B1 immunoreactivity was examined thoroughly in corpora lutea by double immunofluorescence staining with ephrin-B1 and 3β-HSD as well as with ephrin-B1 and CD31 in serial sections. Ephrin-B1 immunoreactivity was weak in CLd that was frequently present in metoestrus. Moreover, ephrin-B1 immunoreactivity was also weak in CLm that were frequently present in dioestrus and proestrus. By contrast, ephrin-B1 immunoreactivity was strong in CLs. Moreover, ephrin-B1 immunoreactivity varied (strong and weak/faint) in CLrl that were frequently present in oestrus. Ephrin-B1 immunoreactivity was also detected weakly in 3β-HSD positive interstitial gland cells. Ephrin-B1 immunoreactivity was faint but substantial in granulosa cells in follicles. Moreover, Ephrin-B1 was expressed strongly in CYP17A1-positive steroidogenic theca interna cells. These findings indicate that ephrin-B1 is substantially expressed in all steroidogenic cells in the ovaries, while their expression levels vary especially in/among corpora lutea.

EphB4 immunoreactivity was detected in vasculature in the stroma and follicles as well as in corpora lutea where EphB4 immunoreactivity and expression patterns varied. In CLd, EphB4 immunoreactivity was relatively strong in CD31-positive vascular endothelial cells and weak in CD31-negative small round cells, i.e. luteal cells. In CLm, EphB4 expression varied in CD31-positive vascular endothelial cells: it was strong in thick/relatively large blood vessels and weak in thin blood vessels. EphB4 immunoreactivity was also detected weakly in CD31-negative large round/polygonal cells, i.e. luteal cells, in CLm. By contrast in CLs, EphB4 immunoreactivity was weakly detected in blood vessels but was not apparent in CD31-negative cells. EphB4 immunoreactivity was prominent/strong in granulosa cells in follicles. Moreover, EphB4 was expressed weakly/faintly in CYP17A1-positive steroidogenic theca interna cells in follicles. EphB4 immunoreactivity was also detected weakly in interstitial gland cells of naturally cycling mouse ovaries. These findings indicate that EphB4 is substantially expressed in all steroidogenic cells in the ovaries, where ephrin-B1/EphB4 signalling may occur, while their expression levels vary especially in luteal cells of corpora lutea.

Chapter 3. Expression and localisation of ephrin-B1 and EphB4 in adrenal glands of male and female mice

Efnb1 and *Ephb4* were detected by RT-PCR in the adrenal glands of the adult mouse. The relative expression levels of *Efnb1* and *Ephb4* were similar between males and females.

The mouse adrenal cortex is composed of outer zona glomerulosa (zG) and inner zona fasciculata (zF) except zona reticularis that is located at the bottom of the cortex in many mammals. In young virgin female mice, the x-zone (xZ) appears between zF and the medulla. Ephrin-B1 immunoreactivity was relatively strong in the adrenal cortex and faint in the adrenal medulla of both male and female mice. Moreover, ephrin-B1 immunoreactivity was strong and similar in CD31-positive vascular endothelial cells distributed in zF beneath zG in male and female. Thus, ephrin-B1 immunoreactivity similar to or greater than that in those endothelial cells was defined as ‘strong’ and less than that as ‘weak’ or ‘faint’. Ephrin-B1 immunoreactivity was strong in 3β-HSD-positive cortical parenchymal cells in zG and bottom region of zF, weak/faint in middle region of zF in male, while strong in cortical parenchymal cells in zG, weak/faint in zF and xZ in females. These findings indicate that ephrin-B1 was substantially expressed in all steroidogenic cells in the adrenal cortex.

EphB4 immunoreactivity was detected in the adrenal cortex and medulla similarly in male and female mice. EphB4 immunoreactivity was strong and similar in CD31-positive vascular endothelial cells distributed in medulla. Thus, EphB4 immunoreactivity similar to that in those endothelial cells defined as ‘strong’ and less than as ‘weak’ or ‘faint’. EphB4 immunoreactivity was substantial but weak in 3β-HSD-positive cortical parenchymal cells in zG, weak/faint in zF of male and female mice. Moreover, EphB4 immunoreactivity was weak in cortical parenchymal cells of xZ in female mice. These findings indicate that EphB4 and ephrin-B1 are co-expressed and their signalling possibly occurs in steroidogenic cells of the cortex.

Conclusions

1. Ephrin-B1 and EphB4 were substantially co-expressed in all of the 3β-HSD-positive steroidogenic cells in the ovaries, i.e. luteal cells, granulosa cells, steroidogenic theca cells, and interstitial gland cells.

2. Ephrin-B1 and EphB4 are co-expressed in all of the 3β-HSD-positive steroidogenic cells of the adrenal cortex.

3. Co-expression of ephrin-B1 and EphB4 is likely a good marker to identify steroidogenic cells located in diverse tissues and organs.

審査結果の要旨

本研究の対象分子 Eph 受容体と ephrin リガンドは膜タンパクで，各々A, B サブクラスに別れ，同じサブクラスであれば結合する。Eph 発現細胞と ephrin 発現細胞が接触すると，両細胞にシグナルが発生し，細胞の接着・遊走，増殖と分化，組織の境界形成，分泌制御など多様な働きが報告されている。最近の研究で，成体と胎性のライディッヒ細胞は ephrin-B1 と EphB4 を発現していることが明らかになった。本研究では，この研究報告をもとに「ステロイド産生細胞は ephrin-B1 と EphB4 を共発現する。」と仮説を立てた。ステロイドを産生する代表的器官は精巣，卵巣，副腎であるが，卵巣と副腎における ephrin-B1 と EphB4 の発現と局在を調べた報告はほとんどなく，詳細は不明であった。

卵巣では，内卵胞膜細胞で合成されたアンドロジェンは顆粒層細胞でエストロジェンに変換され，黄体細胞ではプロジェステロンが合成される。顆粒層細胞と内卵胞膜細胞は排卵後に形質転換して黄体細胞になるが，ステロイド合成酵素 HSD3B はこれら細胞のマーカーになる。HSD3B にはアイソザイムが存在し，HSD3B1 はヒトの胎盤と末梢組織，HSD3B2 は卵巣・精巣と副腎に発現する。マウスには 6 種類のアイソザイムが見られ，HSD3B1 は精巣と副腎，HSD3B2 は卵巣に発現するなど，動物種間で統一性はない。一方，副腎皮質では，球状帯 (zG)で鉱質コルチコイド，束状帯 (zF)で糖質コルチコイド，網状帯 (zR)で性ステロイドが合成されるが，成体マウスには zR は存在しない。代わりに，x 帯 (zX；胎生期の副腎皮質に由来)が発育期に見られるが，雄では 5 週齢迄に，雌では妊娠後に消失する。また，zG 上層に幹細胞が局在し，分化・形質転換し zG，zF，zR へと移動することが分かっている。本研究では，自然発情周期下のマウスの卵巣および副腎を対象に ephrin-B1 と EphB4 の発現を調べ，仮説を検証した。

自然発情周期下のマウスの卵巣では，発育・成熟黄体と退行黄体が混在するが，HE 染色で識別することは難しい。Ephrin-B1 と EphB4 の免疫反応性は黄体間で異なっていたため，第 1 章では HSD3B 免疫反応性 (黄体細胞)と CD31 陽性毛細血管密度の組み合わせで黄体の分類を試み，4 種に識別できることを明示した：(1) 発達期の黄体 (HSD3B 中等度陽性の黄体細胞と低密度の血管で構成；現周期の黄体で発情後期に出現)；(2) 成熟期の黄体 (HSD3B 強陽性の黄体細胞と高密度の血管で構成；現周期の黄体で発情間期と発情前期に出現)；(3) 退行期の黄体 (不完全な黄体：HSD3B 強陽性または弱陽性の黄体細胞と低密度の血管，および，HSD3B 弱陽性の黄体細胞と高密度の血管で構成；全発情周期に出現；1 周期前の黄体と推察)；(4) 退行期末期の黄体 (HSD3B 強陽性と陰性細胞が混在し低密度の血管で構成；退行期末期の小型黄体で発情期に出現；2 周期前の黄体と推察)。

第 2 章では，自然発情周期下のマウスの卵巣を材料に ephrin-B1 と EphB4 の免疫反応性を検討した。その結果，顆粒層細胞は ephrin-B1 微弱陽性／EphB4 強陽性，内卵胞膜細胞は ephrin-B1 強陽性／EphB4 弱陽性，間質腺細胞は ephrin-B1 弱陽性／EphB4 弱陽性で，これらの免疫反応性は発情周期の影響を受けないことが明らかになった。黄体細

胞の免疫反応性は，HSD3B/CD31 免疫染色で黄体のステージを特定し，連続切片で解析した。黄体細胞は，(1) 発達期の黄体では ephrin-B1 弱陽性／EphB4 強陽性・弱陽性，(2) 成熟期では ephrin-B1 弱陽性／EphB4 弱陽性，(3) 1 周期前の退行黄体では ephrin-B1 強陽性／EphB4 微弱陽性，(4) 退行期末期では，ephrin-B1 強陽性と陰性細胞が混在し，EphB4 は微弱陽性または陰性であった。以上の結果から，卵巣の全ての種類のステロイド産生細胞は ephrin-B1 と EphB4 を共発現すること，黄体細胞の ephrin-B1 と EphB4 の免疫反応性は黄体のステージで異なり，ephrin-B1 の免疫反応性は排卵を境に増強し，反対に EphB4 の免疫反応性は排卵を境に減弱することが示唆された。

第 3 章では，雌雄の成体マウスの副腎を材料に ephrin-B1 と EphB4 の免疫反応性を検討した。その結果，ephrin-B1 と EphB4 の免疫反応性は主に皮質に限局することが判明した。ステロイド産生細胞の免疫反応性は皮質の領域で異なり，雌雄共通して，zG では ephrin-B1 強陽性／EphB4 弱陽性，zF の中央部では ephrin-B1 と EphB4 は共に弱・微弱陽性であった。また，雄の副腎では zF の底部 (髄質の皮質と境界部)のステロイド産生細胞は ephrin-B1 強陽性／EphB4 弱・微弱陽性であり，雌の副腎では zX の皮質細胞は ephrin-B1 微弱陽性／EphB4 弱陽性であった。以上の結果から，副腎の全ての種類のステロイド産生細胞は ephrin-B1 と EphB4 を共発現すること，ephrin-B1/EphB4 の免疫反応性は皮質の各領域間で異なることが明らかになった。

卵巣と副腎の全ての種類のステロイド産生細胞が ephrin-B1 と EphB4 を共発現することを明示した研究成果から，両分子の共発現はステロイド産生細胞の新規マーカーになると考えられる。黄体細胞においては発達・退行過程で ephrin-B1 と EphB4 の発現レベルが変遷すること，副腎皮質の区画により皮質細胞の ephrin-B1 と EphB4 の発現レベルが異なることから，両分子の発現レベルは黄体細胞の分化・退行と副腎皮質の区画化に関与する可能性も示唆された。本研究は，ステロイド産生細胞を同定する指標として新たな情報を提供する点から，基礎獣医学ならびに基礎医学の発展・展開に貢献するものと考えられる。従って，本論文の審査ならびに最終試験の結果と併せて，博士(獣医学)の学位を授与することを適当と認める。