論文の内容の要旨

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論文題目

Functional analysis of sex steroid hormone receptors in osteocytes

(骨細胞における性ステロイドホルモン受容体の高次機能解析)

1. Introduction

Sex steroid hormones, such as estrogen and androgen, have various functions in maintenance of homeostasis: development of reproductive organs and mammary gland, potentiation of muscles, and osteoprotection. Osteoprotective actions of sex steroid hormones have been illustrated that deficiency of sex steroid hormones cause the reduction of bone mass, particularly in post-menopausal women and aged men. However, it is largely unknown how sex steroid hormones exert their osteoprotective effects. The effects of sex steroid hormones on bone tissue can be considered as the sum of the direct effects to bone cells and the indirect effect to other tissue. While the various mechanisms of indirect effects have been proved like the effects through cytokines produced by immune cells and the increased levels of pituitary gland hormones, the direct effects are not fully understood.

Estrogen and androgen exert their effects by binding to its own nuclear receptors, such as Estrogen Receptor (ER) α, β and Androgen Receptor (AR), which work as transcription factors. Since it is known that ERβ has limited function for maintenance of bone metabolism among these receptors, we focused on the analyses of functions of ERα and AR in this study. The analyses of conventional ERα or AR knockout mice failed to address the direct functions of these receptors in bone because these mice exhibited endocrine disturbances. From these backgrounds, generations and analyses of cell type specific knockout (KO) mice are required to clarify the functions of ERα and AR in bone.

Osteoclastic ERα KO mice were generated and it was proved that osteoclastic ERα shorten life span of osteoclasts by promoting their apoptosis to date. Also in my master thesis, it was shown that osteoblastic ERα might be important for osteoprotection in male. Unpublished data in our lab indicated that AR also plays important roles for bone remodeling in osteoblasts and osteoclasts. These results indicate that ERα and AR in both osteoblasts and osteoclasts have osteoprotective function. However, the

functions of ERα and AR in osteocyte, which is the other cell type in bone, are more ambiguous than in osteoblasts and osteoclasts. Osteocyte is embedded in extracellular matrix of bone and represents more than 90% of cells existing in bone. Recently it is postulated that osteocyte can orchestrate bone metabolisms by regulating number and activity of osteoblasts and osteoclasts through secreting various kinds of proteins. Thus, we presumed that the functions of sex steroid hormone receptors in osteocytes play possible significant role in bone metabolism.

From these backgrounds, in this study, we tried to elucidate the functions of ERα and AR in osteocytes by generating and analyzing the mice lacking ERα or AR in osteocytes.

2. Results

A. Function of Estrogen Receptor α in osteocytes

To investigate the role of ERα in osteocytes, we generated the mice lacking ERα in late-osteoblasts/osteocytes by crossing ERα floxed mice with Dmp1 Cre mice, which express Cre recombinase driven by the Dmp1 promoter. The mice harboring the genotypes of $Dmp1^{Tg/0}$; $ER\alpha^{L2/L2}$ and $ER\alpha^{L2/L2}$ were analyzed as $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ mice, respectively. As the results from bone radiological analyses, 12-week-old female $ER\alpha^{\Delta Ocy/\Delta Ocy}$ mice, not male mice, exhibited significantly decreased BMD at the proximal tibia when compared to $ER\alpha^{flox/flox}$. To further explore the cellular basis for the bone loss observed in the $ER\alpha^{\Delta Ocy/\Delta Ocy}$, bone histomorphometry was performed. As the results, in $ER\alpha^{\Delta Ocy/\Delta Ocy}$, the bone formation parameters, such as the number of osteoblasts, osteoblast surface and bone formation rate as well as the number of osteocytes/area were significantly decreased, when compared to the $ER\alpha^{flox/flox}$. On the other hand, the bone resorption parameters such as osteoclast numbers or surfaces were not altered in $ER\alpha^{\Delta Ocy/\Delta Ocy}$. Also, the number of TUNEL staining positive osteoblasts/osteocytes was not significantly different between $ER\alpha^{flox/flox}$ and $ER\alpha^{\Delta Ocy/\Delta Ocy}$. Also, to verify whether osteocytic ERα mediates osteoprotective estrogen actions, the mice were ovariectomized (ovx) at 8 weeks old with or without E2 replacement treatment at 10 weeks old. As expected, the recovery of BMD by E2 treatment in the ovx $ER\alpha^{\Delta Ocy/\Delta Ocy}$ was significantly less than that in the ovx $ER\alpha^{flox/flox}$, suggesting that a part of osteoprotective estrogen action indeed mediated through osteocytic ERα. These data suggest that osteocytic ERα play a role in bone metabolism by positive regulation of osteoblastic bone formation.

Moreover, estrogen/ ERα signaling is known to be involved in mechano-sensing and increasing bone formation under over loading conditions. Also, it is reported that tail suspension induced bone loss is significantly enhanced by ovariectomy. To determine whether ERα in osteocytes plays a role in unloading induced bone loss, tail suspension experiments were performed starting at 8 weeks of age for 4 weeks. As the results, tail suspension induced bone loss in the femoral diaphysis of $ER\alpha^{\Delta Ocy/\Delta Ocy}$ female mice was significantly greater than that of $ER\alpha^{flox/flox}$. This result indicates that ostecytic ERα exerts its function for resistance against unloading induced osteopenia.

B. Trial to explore the molecular basis of ERα function in osteocytes

To explore the molecular basis of bone loss in $ER\alpha^{\Delta Ocy/\Delta Ocy}$, osteocyte isolation method was established and gene expression profiles of isolated osteocytes obtained from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ were compared. To isolate highly purified population of osteocytes, the mice with GFP labeled osteocytes by crossing $ER\alpha^{\Delta Ocy/\Delta Ocy}$ or $ER\alpha^{flox/flox}$ line with a GFP expressing mouse line driven by the Dmp1 promoter were generated. GFP+ and GFP- cell population were sorted by FACS (Fluorescence-activating cell sorter) from cells obtained by sequential enzymatic digestion of calvariae of mice at the age of 10 days. It was confirmed that GFP+ population represented osteocytes and GFP- population represented osteoblasts by the expression of marker genes in each population. Then, gene expression profiles of osteocytes were compared between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ by a GeneChip analysis. As the results, 276 genes were found to be significantly at least 2-fold differentially expressed between $ER\alpha^{\Delta Ocy/\Delta Ocy}$ and $ER\alpha^{flox/flox}$ (p<0.01). The differentially expressed genes were further classified according to biological processes and molecular functions using the Functional Annotation Clustering tool of the DAVID Bioinfomatics Resources. In regard to Biological Processes, categories of cell differentiation, developmental process, and cellular developmental process were highly ranked. In addition, the category of “extra-cellular component” in Cellular Component and “binding” in Molecular Function was highly ranked. Furthermore, ‘Secreted’ was listed top in the Keyword analysis when sorted by p value. In terms of GO analysis, Sostdc1 (Sclerostin domain containing 1), reported as one of the antagonists of Wnt signaling, was raised as a possible candidate gene of ostecytic ERα. The expression level of Sostdc1 was increased more than 3-fold in osteocytes sorted from $ER\alpha^{\Delta Ocy/\Delta Ocy}$ calvariae. In fact, there is a report implying that ERα is necessary for contribution of Wnt/β-catenin signaling, which plays pivotal roles in bone formation, in bone mechano-sensing.

Taken together, it was suggested that osteocytic ERα regulates gene expression of secreted proteins, which can be involved in the regulation of osteoblastic bone formation.

C. Function of Androgen Receptor in osteocytes

From the data mentioned above, osteocytes seem to transmit positive signals to osteoblasts through ERα in osteocytes in female mice. However, the osteoprotective actions of estrogen through osteocytes were unlikely from the similar type of analyses in male $ER\alpha^{\Delta Ocy/\Delta Ocy}$. This fact led us to raise an idea that the androgenic actions are dominant in male skeletal health. To address this idea, mice selectively deleted AR in osteocytes were generated as same as did for $ER\alpha^{\Delta Ocy/\Delta Ocy}$. BMD of 12-week-old male $AR^{\Delta Ocy/Y}$ mice, not in female mice, was significantly decreased in both metaphysis and diaphysis of femurs when compared to those of the control mice, showing that male $AR^{\Delta Ocy/Y}$ exhibited greater decrease of bone mass than that of female $ER\alpha^{\Delta Ocy/\Delta Ocy}$. To further explore the cellular basis for the bone loss observed in the $AR^{\Delta Ocy/Y}$, bone histomorphometry was performed. As the results, the number of osteoclasts, osteoclast surface, the number of osteoblasts, osteoblast surface, bone formation rate and mineral apposition rate

were significantly increased in $AR^{\Delta Ocy/Y}$ compared to $AR^{flox/Y}$. Thus, $AR^{\Delta Ocy/Y}$ exhibited osteoporotic bone phenotype with high bone turnover. Also, bone strength was evaluated by three-point bending test using femurs, showing that maximum load of $AR^{\Delta Ocy/Y}$ was 37% lower than that of $AR^{flox/Y}$. These results suggest that AR in osteocytes contributes to maintaining bone strength by repressing bone resorption in male.

3. Discussion

On the basis of the reported evidences on the functions of sex steroid hormone receptors in bone, it is considered that estrogen is osteoprotective by regulating life span of osteoclasts through osteoclastic and osteoblastic ERα. However, little is known about the functions of osteocyte in osteoprotective sex steroid hormone actions for skeletal homeostasis. To decipher the direct functions of ERα and AR in osteocytes, the mice lacking ERα and AR in osteocytes were genetically generated and their bone phenotypes were analyzed in this study. ERα in osteocytes was found to play a significant role for maintenance of bone mass by regulating osteoblastic bone formation only in female. It was further revealed that ERα in osteocytes is supportive for maintaining bone mass not only under normal loading condition but also under tail suspension induced unloading condition, which can be considered as experimental recapitulation of recumbency or space flight. These results are consistent with a previous report, in which bone mass adaptation induced by over mechanical loading was impaired in ERα total KO mice. Furthermore, to investigate possible molecular basis underlying ERα function in osteocytes, we established osteocyte isolation technique from the conditional knockout mice by FACS. The results obtained from the Functional Annotation Clustering of differentially expressed genes suggested that osteocytic ERα might regulate transcription of the genes related to secrete proteins, which may regulate osteoblastic bone formation and contribute to maintenance of bone homeostasis. In fact, Sostdc1, an antagonist for Wnt signal, was raised as a candidate target gene of osteocytic ERα. These observations indicate that osteocytic ERα might play a role in estrogen's osteoprotective action by regulating osteoblastic bone formation through facilitating Wnt signaling pathway.

The results from the analyses of $AR^{\Delta Ocy/Y}$ revealed that AR in osteocytes mediates osteoprotective function through inhibition of osteoclastic bone resorption in male. Since the previous report revealed that male AR total KO exhibited increased bone resorption with the increased expression levels of RANKL, which is a crucial factor in osteoclastogenesis, RANKL might be a possible target gene of AR in osteocytes, which recently reported to be a major source for RANKL production.

Taken together, this study uncovered that sex steroid hormones play essential roles to maintain skeletal homeostasis through their receptors' action in osteocytes.

1) Kitase Y, Barragan L, Qing H, Kondoh S, Jiang JX, Johnson ML, Bonewald LF. *J Bone Miner Res.*,12,2657-68, (2010)