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研究課題名（和文）Development of a high-throughout drug recovery assay of stress for natural compounds screening

研究課題名（英文）Development of a high-throughout drug recovery assay of stress for natural compounds screening

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：慢性ストレス下での免疫系を通じた腸内細菌・脳・腸軸について検討した。免疫系と腸内細菌が慢性ストレス下のマウスで変化することを発見した。次に慢性ストレスのバイオマーカーとしてIL-6とIL-10を選択し、それぞれの相対発現量をリアルタイムで検出するため、定常発現レポータを導入したRAW264.7を作成した。LPS刺激後の細胞に52種類の天然物をそれぞれ負荷した結果、ナンキョウに含まれる1S-1-アセトキシチャビコールでIL-6を下げIL-10を上げる変化を測定出来た。以上の事から、これら2つの定常発現細胞を用いて抗炎症活性のスクリーニングを行い、抗ストレスの新薬候補探索が出来ると考えられる。

研究成果の学術的意義や社会的意義
In the future, we may reduce animal experiments by this assay for screening compounds or drugs. However, this finding is meaningful not only for academics, but it may lead the people concern that stress could make immune system change, and cause the disease, and herb to reboot immune system.

研究成果の概要（英文）：In this study, I studied microbiota-brain-gut axis via immune system in chronic mild stress. I found that immune system and gut microbiota were changed in PAWW stress mice. Finally, I selected IL6 and IL10 to be bio-marker of PAWW stress mice. I developed new and stable RAW 264.7 derived dual-color IL-6/gapdh and IL-10/gapdh reporters. This assay allowed us to easily determine relative IL-6 and IL-10 levels with 96-well plate within one step. I evaluated the relative IL-6 and IL-10 levels in the LPS-induced stable cells testing 52 natural products by real-time bioluminescence monitoring. By this screening assay, I found that 1`S-1`-acetoxychavicol from greater galangal could reduce IL-6 level and increase IL-10 level. Finally, I can suggest that these stable cells by real-time monitoring could serve as a screening assay for anti-inflammatory activity and may be used to discover new drugs against stress.

研究分野：immune system

キーワード：stress　IL-6　IL-10　bioluminescence

１．研究開始当初の背景

Despite the induction of many diseases due to stress, there is almost no model stress assay method to evaluate chronic psychological stress, so development of stress relieving methods and medicine has been very lagging behind. Meanwhile, we succeeded in developing a model stress system reflecting chronic mental stress by the Perpetual Avoidance of Water on a Wheel (PAWW) which is an AIST (産総研) original mice stress model (Miyazaki, 2013). Comparison of PAWW stress with other conventional stress systems including chronic mild stress, social defeat stress and total sleep deprivation, only our continuous PAWW stress model can use for chronic mild stress that is similar to human stress situation. Therefore, it became possible to promote the development of relaxation methods and drugs. Whereas, the assay system in animals has extreme limitations in screening drugs that are cost effective and time effective.

To develop cell-based assay for replacement of mice model, we need to clearly understand mechanism of stress. Recently, microbiota-brain-gut axis has gradually become an important focus in research related to disorders such as depression, stress and anxiety (Foster et al., 2016). The exact mechanisms through which microbiota can exert an effect on the brain have been proposed several putative mechanisms including modulation of the immune system, the hypothalamic-pituitary-adrenal axis and tryptophan metabolism (Kelly et al., 2016). Recently, mechanism via immune system is become an important route of communication between gut microbes and the brain. Whereas, microbiota-brain-gut axis via immune system has not yet been extensively studied especially in chronic mild stress. Therefore, microbiota-brain-axis via immune system in PAWW stress mice will be studied.

２．研究の目的

There are NO high-throughput drug/compounds screening assay in vitro for psychological stress evaluation so far. Therefore, our research goal is establishment of cell-based stress evaluation assay for natural compounds screening. For this purpose, I will incorporate own unique cell-based screening system by multi-bioluminescence. This multi-bioluminescence is an AIST original technique which can simultaneously monitor more than 2 gene expressions in a single cell. This assay saves time and money, and can analyze a huge number of samples. Therefore, this assay will be suitable for screening assay. As a result, our novel assay could serve as a replacement of chronic stress mice model for compounds screening. I can use this novel assay system to screen stress relaxation agents that no one could ever carry out. This success makes it possible to screen many compounds in a short period of time by a high-throughput manner, and

is expected to dramatically promote the development of compounds to against stress and methods. By this novel system, I am convinced that not only can we suppress many diseases, but we also expected reduction of medical expenses, contribute to support insurance system in the future.

３．研究の方法

1) Determination of microbiota-brain-gut axis via immune system in PAWW stress mice

As I mentioned above, I make hypothesis of this study as shown in Fig. 2. To elucidate this hypothesis, I determined immune-related cytokines level such as IL1ra, IL1a, IL1b, IL-6, IL-10, in lymphocyte and spleen by Quantitative real-time PCR. These finding will elucidate brain-immune interaction in PAWW stress model (Fig. 2 (1)). Moreover, I determined immune related cytokines level in Mesenteric Lymph Node and Peyer`s Patch from intestine. This determination will elucidate immune-gut interaction (Fig. 2 (2)). More specifically, I will employ a metataxonomic approach - that is, high-throughput 16S rRNA gene amplicon sequencing - to comprehensively profile the gut microbiota in PAWW stress mice. This method is also an AIST originality technique. we will utilize synthetic spike-in controls previously developed by AIST (Tourlousse et al., 2017) as a means to quantitatively compare microbiota found in stressed and control mice. Using these quantification standards, we will be able to accurately identify which microorganisms are differentially abundant among mice. This finding will elucidate gut-microbiota interaction (Fig. 2 (3)).

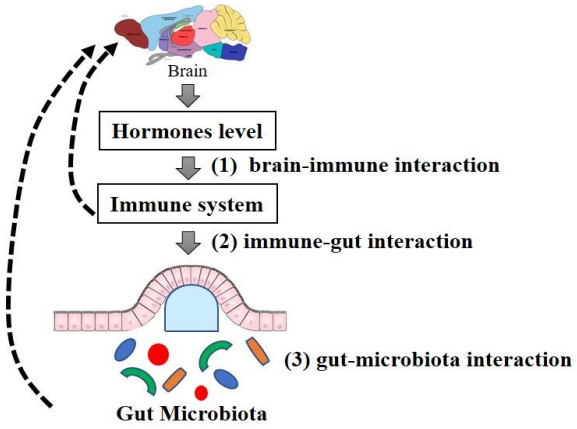

Fig. 2 Route of microbiota-brain-gut axis via immune system

2) Novel gene reporter assay for stress evaluation *in vitro* with multi-color bioluminescence.

Based on the first-year study, macrophage cells (RAW 264.7 cells) and IL-6 and IL-10 genes were selected and developed to cell-based assay. I created novel IL-6 and IL-10 gene promoters. Then, I used novel promoters and developed cell-based assay by multicolor bioluminescence motoring in Real-time. Comparing with other methods, only this method can use two kinds of gene promoters (target gene promoter and reference gene promoter) in single cell. While, other methods can use only single promoter in single cell. Moreover, I developed this method for novel stable cell derived dual reporters. As a result, we established a novel unique gene reporter assay system for stress evaluation which can evaluate compounds in Realtime.

3) Screening of natural compounds from Thailand by the novel developed cell-based assay

As I am a Thai people, we will screen natural compounds from Thailand which were reported for stress relaxation by this novel developed assay. Whereas, Japan is recently concerning The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising (ABS) to the Convention on Biological Diversity (CBD). Now, I have collaboration with Thai institute and got ABS permission of Thai herbs from Thai government. Therefore, I screened Thai herds with this new assay.

４．研究成果

1) Determination of microbiota-brain-gut axis via immune system in PAWW stress mice

PAWW stress mice were stimulated for 7 weeks. The feces were collected every week. After 7 weeks, blood, spleen, brain, Peyer's patches and Mesenteric lymph nodes were collected. IL-1ra, IL-1a, IL-1b, IL-6 and IL-10 in lymphocyte, spleen, Peyer's patches and Mesenteric lymph nodes were determined by Quantitative real-time PCR. The results showed that IL6 level of PAWW stress mice was increased significantly in lymphocyte and Peyer's patches, and IL-10 level was decreased in lymphocyte. Moreover, the levels of IL-1ra, IL-1a and IL-1b in PAWW stress mice were increased. The gut microbiota from feces in PAWW stress mice were characterized by high-throughput 16S-seq. In PAWW stress mice, Proteobacteria (Xanthomonadales, Pseudomonadales, Enterobacteriales) were increased significantly, but Actinobacteria was decreased significantly at 7 weeks. These results suggested that PAWW stress mice has changing of immune system and microbiota.

2) Novel gene reporter assay for stress evaluation *in vitro* with multi-color bioluminescence.

Based on mouse experiment, IL-6 and IL-10 cytokines were selected for biomarkers for this study. I established IL-6 and IL-10 promoter assay which can monitor with reference gene as Glyceraldehyde 3-phosphate dehydrogenase (gapdh) promoter in living single cell. It could determine IL-6 and IL-10 levels continuously in real-time within two steps. We evaluated IL-6 and IL-10 reporter expression in LPS-induced RAW 264.7 cells with well-known anti-inflammatory compounds such as quercetin, xanthones, b-D-glucan and dexamethasone. As the results, the expression of IL-6 and IL-10 reporters were strongly induced by LPS. The

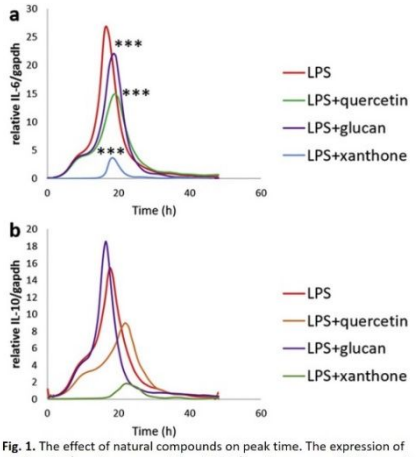

Fig. 1. The effect of natural compounds on peak time. The expression of relative IL-6/gapdh reporters (a) and IL-10/gapdh reporters (b). The results represent the mean of four representative experiments. ***p < 0.01 versus the LPS positive control (peak time).

expression of IL-6 reporter was inhibited by all anti-inflammation compounds in LPS-induced RAW 264.7 cells. The expression of IL-10 reporter was inhibited by quercetin, xanthones and dexamethasone in LPS-induced RAW 264.7 cells. While, expression of IL-10 reporter was induced by b-D-glucan. These results indicated that this assay could use for determination of IL-6 and IL-10 reporter expression in LPS induced RAW 264.7 cells for anti-inflammation activity. Moreover, the results showed that natural compounds have an effect on the time course of IL-6 and IL-10 expressions (Fig. 1). Therefore, real-time monitoring has a merit for natural compounds screening. These results suggested that this assay could serve as a compound screening assay for anti-inflammation activity.

3) Screening of natural compounds from Thailand by the novel developed cell-based assay

Finally, I developed and established new and stable RAW 264.7 derived dual-color IL-6/gapdh and IL-10/gapdh reporters. This assay allowed us to easily determine relative IL-6 and IL-10 levels with 96-well plate within one step. I evaluated the relative IL-6 and IL-10 levels in the LPS-induced stable cells testing 52 natural products by real-time bioluminescence monitoring and time-point determination using a microplate luminometer. By this new assay, I found that the relative IL-6 and IL-6/IL-10 values decreased by 1`S-1`-acetoxychavicol (ACA) from greater galangal using real-time bioluminescence monitoring. At the same time, the relative IL-10 was induced. The relative IL-6 and IL-6/IL-10 decreased by 1`S-1`-acetoxychavicol acetate at 6 h (Fig. 2). This finding suggested that the use of these stable cells by real-time monitoring could serve as a screening assay for anti-inflammatory activity and may be used to discover new drugs against stress.

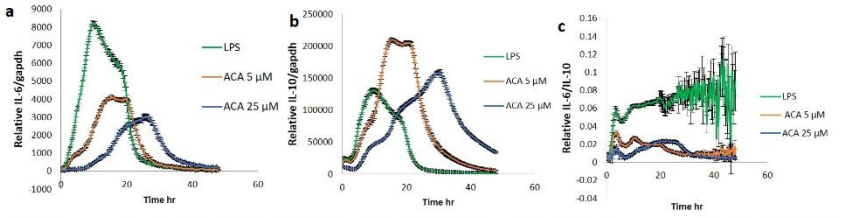

Fig. 2 The expression of relative IL-6/gapdh, IL-10/gapdh and IL-6/IL-10 by real-time bioluminescence recording for 48 h. Stable IL-6/gapdh RAW264.7 cells were treated with 1'S-1'-Acetoxychavicol acetate (ACA) at 5 and 25 μM and LPS at 100 ng/ml. Each value represents the mean ± SD (n = 4).

＜引用文献＞

Miyazaki, K., et al. PloS one, 8(1), e55452, (2013)

Foster, Jane A., et al. International Journal of Neuropsychopharmacology, 19(5), (2016)

Kelly, John R., et al. Annals of epidemiology, 26(5), 366-372, (2016)

Tourlousse, Dieter M., et al. Nucleic acids research, 45(4), e23-e23, (2017)

Tian, Rui, et al. The Scientific World Journal, (2014)

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　1件）

１．著者名 Saiki Papawee、Nakajima Yoshihiro、Van Griensven Leo J.L.D.、Miyazaki Koyomi	４．巻 505
２．論文標題 Real-time monitoring of IL-6 and IL-10 reporter expression for anti-inflammation activity in live RAW 264.7 cells	５．発行年 2018年
３．雑誌名 Biochemical and Biophysical Research Communications	６．最初と最後の頁 885～890
掲載論文のDOI（デジタルオブジェクト識別子） https://doi.org/10.1016/j.bbrc.2018.09.173	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 Saiki Papawee、Kawano Yasuhiro、Nakajima Yoshihiro、Van Griensven Leo J. L. D.、Miyazaki Koyomi	４．巻 20
２．論文標題 Novel and Stable Dual-Color IL-6 and IL-10 Reporters Derived from RAW 264.7 for Anti-Inflammation Screening of Natural Products	５．発行年 2019年
３．雑誌名 International Journal of Molecular Sciences	６．最初と最後の頁 4620～4620
掲載論文のDOI（デジタルオブジェクト識別子） https://doi.org/10.3390/ijms20184620	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 Saiki Papawee
２．発表標題 Real-time monitoring of IL-6 and IL-10 reporter expression for anti-inflammation activity in live RAW 264.7 cells
３．学会等名 第35回学術講演会
４．発表年 2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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