最終試験の結果の要旨

報告番号	総研第 341 号		学位申請者	Muflihatul Muniroh
審査委員	主査	堀 内 正 久	学位	博士（医学・歯学・学術）
	副査	出 雲 周 二	副査	橋 口 照 人
	副査	武 田 泰 生 印	副査	宮 田 篤 郎

主査および副査の５名は、平成27年 7月24日、学位申請者 Muflihatul Muniroh 君に面接し、学位申請論文の内容について説明を求めると共に、関連事項について試問を行った。具体的には、以下のような質疑応答がなされ、いずれについても満足すべき回答を得ることができた。

Q1: Why did you use sub-toxic doses of MeHg in your experiments?
A: The mechanisms of cell death caused by high-dose MeHg exposure has already been examined by many studies, but the effect of low dose MeHg is not well understood. Therefore, we studied the molecular and cellular effects of MeHg exposure at doses which did not cause cell death.

Q2: You examined the effects of MeHg only for a 6 hour-treatment. Did you try to examine longer periods of MeHg exposure? For example, 1 week or more? Why didn't you examine chronic effects?
A: The mRNA levels of cytokine expressions reached a peak at around 6 hours after the addition of MeHg to the culture medium, and it declined after 12 hours, and reached almost zero levels after 24 hour-exposure. On the basis of these results, we concluded that MeHg caused inflammatory cytokine expressions in early stages of exposure. Therefore, we did not conduct further experiments to examine the effects of longer exposure periods.

Q3: Dr. Eto and his colleagues conducted an animal experiment using marmosets, and examined the effects of 24-day treatment of MeHg. Why didn't you examine such long-term effects?
A: Our experiments focused on a short-term and early-stage effect of MeHg, and its direct effects *in vitro*. In the animal experiment, it is possible to examine both acute and chronic effects. They reported that MeHg-exposed marmosets did not show any severe symptoms. However, in their pathological analysis, cell death and gliosis were observed. In a long term-exposure, MeHg will be accumulated in tissue and in cells. After MeHg concentration reached a threshold level for inducing inflammation in the tissue, similar phenomenon to what was observed in our experiments may occur *in vivo*.

Q4: Is the amount of reactive oxygen species (ROS) induction affected by MeHg doses? Even in the low concentration of MeHg, will MeHg generate ROS and affect cell viability?
A: The ROS induction may be dependent on the dose of MeHg. Previous studies showed the large amount of ROS induction and cell death by high dose of MeHg. In this study, however, our main purpose was to examine the mechanism of low-dose MeHg-induced inflammation. We also confirmed the ROS induction even at low-dose of MeHg. Since we intentionally applied a non-toxic dose of MeHg, it was hard to examine the effect of MeHg-induced ROS on cell viability at low dose.

Q5: What kind of roles did IL-6 and MCP-1 play in your experiments? Isn't there a possibility that IL-6 and MCP-1 have protective roles to cells?
A: The protective roles of cytokine including IL-6 and MCP-1 *in vivo* are reported. In our experiment, however, it is impossible to clarify the protective role of excreted cytokines because *in vitro* experiments using a single cell line cannot examine the interaction among different cells.

Q6: Do astrocytes have a high affinity to MeHg? Why did you examine the effect of MeHg on astrocytes?
A: Because of the location of astrocytes, MeHg is transferred from blood to neuronal cells through astrocytes. And thus, the function of astrocytes may be affected by MeHg exposre even though MeHg does not cause cell death of astrocytes.

Q7: Are there any good health effects of Hg on humans?
A: As far as we know, there are no good health effects of mercury exposure on humans.

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Q8: Why did the low-level exposure of MeHg increase cell viability by up to 20%?
A: We used the WST-8 assay kit to determine the cell viability since it is useful to know the threshold of MeHg toxicity in the cells easily. The WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. The amount of the formazan dye generated by cellular dehydrogenases is proportional to the number of living cells, but it is also known that some cellular stresses activate dehydrogenase activities. The observed increase of cell viability in our experiments would reflect the stimulated dehydrogenase activities.

Q9: As same as the experiment using MCP-1 knockout mice, the application of IL-6- knockout experiments will be useful to know the role of IL-6 in MeHg toxicity.
A: That would be a worthy experiment, and will be very useful to clarify the contribution of IL-6 in MeHg toxicity.

Q10: Why did you emphasize extracellular chelation of MeHg by NAC, and ignored intracellular chelation?
A: MeHg-induced cytokine expressions were the most effectively suppressed in the co-experiment with NAC among three experiments which indicated an effective extracellular suppression by NAC. Because this extracellular NAC effect can be explained by only chelation, we described the extracellular chelation. Although NAC can chelate metals not only extracellular but also intra-cellular, we did not prove the intracellular chelation of MeHg by NAC.

Q11: Do you know any agents other than NAC that chelate MeHg?
A: Selenomethionine also works as a chelator for Hg.

Q12: What are the basic pathological changes caused by MeHg?
A: MeHg causes cell death and gliosis, and these changes are observed in mainly cerebrum and cerebellum in the brain of Minamata disease patients, suggesting that these regions are vulnerable to MeHg exposure.

Q13: What is the definition of inflammation?
A: Inflammation is biological reaction caused by infection or non-infection such as metal exposure, and lymphocytes and macrophages play main roles to induce various cytokines and interactions among related cells.

Q14: What is the protective effect of IL-6?
A: We cannot identify the role of IL-6 in our experimental condition, and *in vivo* experiments will be useful to clarify it. Inflammation such as IL-6 expression will have dual roles in early and chronic stages of MeHg exposure.

Q15: Microglia is important in inflammation. Did you examine the inflammation by MeHg using microglia?
A: Thank you for pointing it out. Although we did not, it is worthy to examine the effects of MeHg in microglia.

Q16: How is Hg methylated in the environment?
A: Methylation of inorganic mercury is considered to be mediated by bacteria in aquatic environment.

Q17: Can you explain the difference in stability of MeHg from other organic Hg?
A: MeHg is more stable than other organic Hg such as ethylmerury (thimerosal) or phenylmercury (pesticide). As far as we know, only MeHg was detected as organic mercury from natural organisms.

Q18: Why did Iraq accident occur whereas the MeHg toxicity became well-known after Minamata disease?
A: MeHg was used as a fungicide for wheat, which was supposed to use for farming in the next spring. However, they ate MeHg-treated wheat to make bread because of the lack of food.

Q19: A malignant cell line was used in your experiments. What is the difference between malignant cell lines and primary cultured astrocytes?
A: Malignant cells could have higher metabolic rates. Sensitivity to MeHg exposure may be higher in primary cultured astrocytes.

Q20: Did you obtain direct evidence for mercury chelation by NAC?
A: No, we did not examine the chelation of MeHg directly. Chelation can be confirmed by LC/ICP-MS or LC/ESI-TOF-MS by showing the conjugated MeHg with NAC.

Q21: How does H_2O_2 production was cancelled by NAC?
A: The NAC is a precursor of glutathione, which is a natural antioxidant. As we showed in our experiments, NAC would eliminate the production of H_2O_2 through chelation of MeHg and anti-oxidative stress.

以上の結果から、５名の審査委員は申請者が大学院博士課程修了者としての学力・識見を有しているものと認め、博士（医学）の学位を与えるに足る資格を有するものと認定した。