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SUMMARY OF DOCTORAL THESIS

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Title: Study on the novel effects of indole-3-acetic acid on colorectal cancer cells
（大腸癌細胞に対するインドール-3-酢酸の新規作用に関する研究）

Colorectal cancer (CRC) has gained much attention recently because it is one of the most common cancer worldwide, and about 1.9 million new cases are diagnosed with CRC globally. In CRC tissues, elevated expression of tumor necrosis factor α (TNFα), an inflammatory cytokine, is positively correlated with the growth of CRC as well as involved in the induction of the epithelial-mesenchymal transition in promoting CRC invasion and metastasis. Moreover, increased TNFα expression is associated with tumor recurrence in patients with metastases of CRC. Furthermore, TNFα expression is increased in serum from patients with CRC. On the other hand, cytochrome P450 1A1 (CYP1A1), one of P450 inducible phase I metabolizing enzyme, is involved in the process of metabolic activation of the xenobiotics like polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) in the carcinogenesis. Furthermore, researchers found a relationship between the progression of CRC and the increased CYP1A1 activity by single nucleotide polymorphisms in its gene previously. However, other researchers showed a loss of metabolic clearance of carcinogenic DNA-adduct in CYP1A1(-/-) mice. Therefore, the present study attempted to unveil the effects of indole-3-acetic acid (IAA) derived from dietary tryptophan produced by the intestinal microbiota and the mechanism of IAA involved in the regulation of TNFα and CYP1A1 expressions. Initially, I studied the influence of treating human epithelial colorectal adenocarcinoma (Caco-2) cells with IAA on the expression of CYP1A1, a typical target gene for aryl hydrocarbon receptor (AhR), and TNFα. Since the moisture content of normal feces is 60%-80%, and the maximum fecal concentration of skatole, an IAA metabolite synthesized by intestinal microbiota, is 1000-1200 μM, the maximum fecal IAA concentration might be similar. Therefore, I treated the Caco-2 cells with 1000 μM IAA. I found significant effects of IAA on the Caco-2 cells, as evident with the substantial increase in the mRNA levels of CYP1A1 expression for 1-8 days, suggesting that IAA activates AhR. Conversely, I also found that IAA downregulated the mRNA expression of TNFα for 2-8 days. Besides, the present immunoblotting study also showed a decreased TNFα protein levels after incubation of Caco-2 cells for 8 days with 1000 μM IAA. Since most studies of indole derivatives such as IAA have been conducted based on the premise that such compounds attenuate the inflammatory response through activating AhR, I conducted the tests with CH223191 (10 μM), a specific AhR antagonist, to check if AhR regulates CYP1A1 and TNFα expression levels or not. Results from my antagonist experiments demonstrated that CH223191 significantly repressed the IAA-induced expression levels of CYP1A1 mRNA at 2 and 8 days after stimulation, whereas CH223191 had no antagonistic action on the IAA-induced suppression of TNFα expression in Caco-2 cells on any day. These results suggested that AhR is not involved in suppressing TNFα expression induced by IAA in Caco-2 cells rather than via AhR-independent pathways. These findings also indicate that the IAA also binds to receptors other than AhR. Thereafter, I intended to identify a new receptor for IAA. To do that, I checked the CYP1A1 expression by dose- and

time-dependently. I found that the mRNA expression of CYP1A1 started to increase significantly from 3 hours after stimulation with 1000 μM IAA, and gradually increased up to 4 days and then started decreasing from 4 days, but continued for 3 hours-8 days. Moreover, the current results also indicated that the abundance of CYP1A1 expression was similar at 3 hours after stimulation with 50-1000 μM IAA, while expression of CYP1A1 at 8 days after stimulation with IAA increased dose-dependently. Next, I planned to examine whether Toll-like receptor 4 (TLR4) is involved in the regulation of CYP1A1 expression from 3 hours to 8 days after stimulation with IAA. I got that after treating the cells with TAK242 (1 μM), a specific TLR4 antagonist, it did not affect the increased CYP1A1 expression induced by IAA at 3 hours, although, on 8 days, it promoted the IAA-induced CYP1A1 expression. Based on the results of 8 days, I examined whether any changes of AhR and AhRR mRNA expression levels at 8 days in TAK242 treated Caco-2 cells and found no significant changes for both the genes. The AhR protein level also remains unchanged, indicating that neither increased AhR mRNA nor protein expression was involved in promoting the increase in expression CYP1A1 induced by TAK-242 at 8 days after stimulation with IAA. Since researchers reported the c-Jun-N-terminal kinase (JNK) activation in the TLR4 pathway, the present study confirmed by the immunoblotting results that the TAK-242 suppressed IAA-induced JNK activation. Furthermore, I used SP600125 (5 μM), a specific JNK inhibitor, and found that after treating the Caco-2 cells with SP600125 followed by IAA resulted in the promotion of IAA-induced increased CYP1A1 expression levels at 8 days after stimulation. These two data suggest that activation of TLR4/JNK signaling pathway induced by IAA may have partially attenuated increased CYP1A1 expression at 8 days after stimulation with IAA. Finally, I assessed the effects of TAK-242 on the suppression of TNFα expression induced by IAA and found that TAK-242 alleviated the suppression of TNFα expression induced by IAA at 2 days, but not at 8 days after stimulation. Thus, the present findings suggest that TLR4 is a receptor for IAA in addition to AhR and IAA-activated TLR4 regulates CYP1A1 and TNFα expression depending on the culture stage of Caco-2 cells. Furthermore, my findings at 8 days after stimulation with IAA indicated that even more unidentified receptors for IAA regulate TNFα expression. Therefore, more extensive studies are required for the identification of new receptor(s) involving in the regulation of TNFα expression by IAA in CRC cells. Thus, the results of my current study may be useful to understand the benefits of IAA derived from dietary tryptophan produced by the intestinal microbiota on the pathophysiology of gastrointestinal cells. Moreover, these results can help to offer new approaches in CRC management. On the other hand, increased CYP1A1 expression has been shown in the production of toxic metabolites, though others relate this with metabolic clearance of carcinogenic compounds. Besides, there is no such information about the impacts of IAA on phase II metabolizing enzymes, which are involving in the conjugation reactions to make the metabolites more hydrophilic and helps them out from the body. Therefore, the balance between a high amount of protein intake and substrates for the CYP1A1 may provide a better alternative for the protective and delayed effects on the initiation and progression of CRC. In conclusion, my studies provide novel findings to understand how indole derivative IAA produced by intestinal flora affects the host physiology.