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Title of research project	Studies of a new screening method for compounds with teratogenicity through changing retinoic acid concentration and the molecular mechanism
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【Abstract】

In this study, to identify compounds affecting retinoic acid concentration in organ tissues, we first established an adenovirus expression system of retinaldehyde dehydrogenase (RALDH) and cytochrome P450 (CYP) 26, which are enzymes that synthesize and metabolize retinoic acid, respectively. Because HepG2 cells possess some degree of intrinsic RALDH and CYP26 expression, we used HeLa cells, which express very low levels of these endogenous enzymes, to measure these enzyme activities. The metabolites were measured using ultra-performance liquid chromatography-tandem mass spectrometry, and the detection limit for the substrate was found to be 5 μM. Here, we investigated azole-compounds including agricultural chemicals and typical azole-antimicrobial drugs.

Some compounds strongly inhibited the catalytic activity of the drug-metabolizing enzyme CYP used in the control experiment. In particular, the inhibition constants of the azole-antimicrobial drugs were similar to those reported previously. However, only talarozole strongly inhibited CYP26A1 while the other compounds did not. In addition, the inhibition constant of talarozole against CYP26A1 activity was 2 μM, which was significantly different from the previously reported value of 5 μM. Moreover, ketoconazole, an antimicrobial azole-drug, is also reported to be an inhibitor of CYP26A1 activity with a half-maximal inhibitory concentration (IC_{50}) of 0.55 μM, but no inhibition was observed in this experiment even at 10 μM. In planning this research study, we predicted that numerous azole-agricultural chemicals would inhibit CYP26 activity because azole antimicrobial drugs efficiently inhibit microbial CYP51 activity. In addition, azole antimicrobial drugs strongly inhibit the drug-metabolizing enzyme CYP. Therefore, we attributed the induction of teratogenicity by azole-compounds to the inhibition of CYP26 activity which leads to a higher concentration of retinoic acid, that is subsequently reduced by the induction of the CYP26 enzyme in the organ tissues. We thought that if the tissue concentrations could be measured, this assumption could be proved. On the other hand, in this experiment, we confirmed the expression of the CYP26 and RALDH enzymes by performing detailed western blot analysis and enzyme assays. Furthermore, the inhibition studies of the drug-metabolizing CYP enzyme activity were simultaneously performed as a control experiment, and the results supported our assumption. Accordingly, we considered the experiment technique and recombinant enzymes are satisfactory. Although the inhibition experiment was performed with a low concentration of the substrate relevant to the levels found in organ tissues, the inhibition constant should be comparable in the enzymology experiments. The inhibition constant against CYP26A1 activity reported for talarozole is 4-5 μM, suggesting that a very small amount of talarozole has the potential to develop teratogenicity. In addition, the enzyme mRNA expression level was

studied to examine the influence of azole compounds on the gene expression of the enzymes. Although it was not comparable to that of retinoic acid, the transcriptional activation of the CYP26A1 gene was increased by some azole-compounds, among which the effect of talarozole was remarkable. Conversely, the transcriptional activation of RALDH gene was decreased by some azole-compounds by approximately 50%, and the retinoic acid concentration of the organ tissues might have been affected by the change in the expression amount enzymes involve in the synthesis and metabolism rather than by the inhibition of those activities.

Since it is difficult to measure the retinoic acid concentration in tissues even by using ULPC-MC/MC, we proposed the use of a reporter assaying method for measuring it in this experiment. For the said method, we isolated the promoter genes of HoxA, HoxA2, and CYP26A1 whose transcriptional activation was enhanced by retinoic acid, and generated their reporter plasmids. A strong induction was observed in the reporter plasmid of CYP26A1 but not in those of HoxA1 and HoxA2. Furthermore, to ensure a high sensitivity, an artificial retinoid X receptor (RXR) binding sequence was inserted into this plasmid. As a result, the reporter activity obtained was approximately 10 times stronger than that without the plasmid. At the same time, a reporter plasmid expressing a fluorescence protein fused with the luciferase (chemiluminescence enzyme of a firefly) was prepared. After this reporter plasmid had been constructed into an adenovirus, detectability of the reporter activity in mouse tissues was examined. As the result, the reporter activity was not detectable in the mouse liver in a preliminary experiment, and therefore we needed to modify the experimental conditions to obtain the activity in the mouse organs.

When a pregnant ICR mouse was administered talarozole (5 mg/kg) or WIN18446 (4 mg/body), the number of malformed fetuses increased significantly. In particular, a hypoplastic defect and cleft lip of the nose were observed at a high frequency following both treatments. In addition, sporadic eyelid twitching, eye lens dysplasia, polydactyly and short-tail/bending-tail were also observed following both treatments.