Development of a Test Method for Risk Assessment of Endocrine Disrupting Chemicals Using DNA Microarray Technology

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My name is Akihiro Kondo of Takara Shuzo, and I am honored to have the opportunity to talk about our research.

Just as was mentioned in the introduction a little while ago, we are working on development of a method of testing activity of endocrine disrupting chemicals, or EDCs, and assessing risk of those chemicals using DNA microarrays and DNA chips. As has been mentioned here over and again today, it has been reported that activity of substances that act like so-called "environmental hormones" and nonylphenol has been observed in fish. These compounds are listed here. As shown here, they enter directly from the outside to act naturally, which is through natural hormone receptors. Or, the metabolized compounds disrupt the endocrine system of the person or animal that ingests the chemical. This reaction is called "disruption" and the compounds that cause it are called "endocrine disrupters."

As was previously described, *in vitro* assays of this effect include the receptor binding assay, the reporter gene assay, and the cell proliferation assay, which is a well-known E screen method. *In vivo* assays include the uterotrophic assay, the Herschberger assay, and the so-called "toxicity study."

Just as was previously reported, these test methods are not always sufficient for testing endocrine disrupting effect. As for the reason – as shown here, the main problems for example are low level exposure and effects such as low dose effect. This is a long-term exposure. In some cases, they may effect fetus in the mother's uterus or newborn infant through the mother's milk.

I think you see what I mean when I say these test methods may not always be sufficient to solve these problems.

Because we are therefore looking at binding subsequent to the reaction, we wanted to see the signal after binding in the genetic flow. This for instance shows the part we were talking about a little while ago in a little more detail. Although it is not particularly limited to estrogen receptors, when ligand enters from the outside as a signal through the receptors, there may be points where the signals branch into endocrine disrupting effects that are not normal hormone effects. If we investigated these genes, we thought we might be able to check the endocrine disrupting effect before it caused abnormalities of the organs and abnormal behavior.

This is a map of a DNA chip. As for the advantages of the DNA chip itself, or DNA microarray, it is capable of high throughput screening such as you see at the very top. With conventional high throughput, you can test lots of compounds all at once, but in this case, high throughput means you can see suppression and expression of the effect of various types of genes for a certain compound. When we add the dose and the fact that the time course was altered, we can then analyze the pathway. If we can identify the substance transcribed to the protein of the discovered key gene, it could serve as a biomarker.

What we have listed here is the group of genes placed on the DNA chip we checked. These include nuclear receptors, oncogenes, signal transduction genes, plus co-factors and gonad differentiation factors. The system used for the test includes *in vitro* and *in vivo*. *In vitro* tests involved biological cells. The data was placed in a database using cells expressed by ERAR. With the *in vivo* tests, intrauterine exposure was first conducted. We also studied the function of genes in the brains of the fetuses. The results of this study were presented at a recent conference on environmental hormones, so I will limit my talk to the cells of this part today.

Although I am repeating myself, our goal is to create a database of gene expression profiles. A database is to be created for each compound and for each gene. When the data is accumulated, the information can be accessed by various people working on various aspects of this research. We then identify key genes discovered to be involved in disruption of the endocrine system and biomarkers.

Here are the cells used for the experiment this time. The cells are the MCF component that is frequently used for this sort of experiment and the T-47D type used for similar experiments. Both are breast cancer cells, but subsequent data indicates that they behave differently.

Next we have androgen-responsive genes. The two types of cells are cultivated and pre-incubated. For so-called "estrogenic response" is suppressed, time before introducing EDCs varies from 96 to 120 hours according to the type of cell. Those to which EDCs were introduced following pre-incubation and those to which they were not introduced are then incubated for six hours. The respective messengers are then recovered and labeled. Thus we obtain labeled cDNA. We then scan using the 2-color method. With this method, the test specimen is dyed red, so, when the compounds are introduced, many red spots appearing means it is expressed a lot. Green only appears in controls. It does not appear in the test specimens. In other words, it is suppressed. During this time they appear as numbers such as an even number for example.

This is an actual spot, here red is clearly expressed and green is suppressed to an extreme degree. If you are wondering what happens to this spot during this time, for example this is control and this is test. If you find out the ratio, you will know how many times greater one is than the other.

Thus we convert the data into a ratio, conduct normalization, compile the data, and then cluster the data for each compound for example. The next figure gives the results of clustering for bisphenol A, diethylstilbestrol (DES), estradiol (E2) and genistein. The cells at this time are MFC-7 cells, but which genes possess the cluster. This shows the clusters for each compound. Today I would like to talk about the cluster for each compound in particular.

With this data, number 2 and 3, DES and E2 behave similarly. Genistein and bisphenol A behave quite differently from DES and E2. Numbers 1 though 7 are data for compounds suspected of being EDCs, and for which the previously described assays were conducted. We know the compounds of numbers 2, 3, 4 and 5 exhibit estrogenic activity. For example, this is clearly expressed in PS-2 protein, but is suppressed in number 1.

We know this, so if we pick these up and show them – this is the diagram of MCF-7 I described a little while ago – T-47D is like you see here, but if E2 were placed in the center, I think you would get quite different pattern.

If we however dye this estrogenic compound red, just because this cluster moves here, the correlation of EE (= E2) and DES is different. This grouping therefore has not collapsed at all. If you want to know for example why it is collapsed, concerning expression of ER, there is not a huge difference between the MCF component and T-47D for any of the compounds. Concerning the reaction of PS2 however, whereas the MCF component is expressed in estrogenic compounds to an extreme degree, it is not expressed to a great extent in T-47D. Concerning estrogen receptors, oppositely, the results for NP and DBP are as you see here. That means there is a difference in the clustering we talked about a little while ago.

You can say almost the same thing of the factor group. The expression pattern is very similar to that of P300. Concerning SMAD-3 or ACTR however, the MCF component is suppressed when an estrogenic compound is administered. This is not originally expressed with T-47D. The difference in clustering I mentioned a little while ago is due to this difference.

This is the pattern of Sertoli cells and Leydig cells, which are testicular cells. Concerning Leydig cells, estrogenic compounds tend to form clusters in a similar manner to breast cancer cells that have an estrogenic reaction as was previously described. Sertoli cells are divided as you see here. I can explain about each respective gene, but time does not permit, so I will leave it out.

The shape you see here now is the compound clustering. We have narrowed down to the key gene here by combining with gene clustering on the side. If you are wondering how this data will be used in the future, it was used for cancer cells in the case of the human we are using. If used for normal cells with a little better response – this is a cell from a mammary gland and this is a cell from smooth muscles of the uterus. Primary culture cells were used for both Sertoli cells and Leydig cells. After conducting the same kind of assay for these, we will compare the data with the data we have obtained so far.

Concerning in vivo experiments as well, we plan to compare the behavior of genes in the uterotrophic assay often used to measure actual estrogen activity with a data in a database of such gene behavior.

I would like to take this opportunity to introduce one more theme here. This is something new we wanted to try. In this experiment we try to pick up cells with laser radiation called “laser capture micro-dissection” and we are currently planning and attempt to see the reaction for each cell with a DNA chip. After picking up the cells from the section prior to differentiation, because the number of cells is about ten to the sixth power, even if we use several, it would still take at least several hundred. In the experiment we are planning, this is amplified by the anti-sense amplified RNA method and we then attempt to transfer it to the DNA chip.

These experiments were conducted in cooperation with the groups of Dr. Takeda of the Science University of Tokyo and Dr. Iguchi of Okazaki National Research Institute. Thank you for your attention.