Molecular Analysis of the Inhibition of Chemokine Gene Expression by Xenoestrogens

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I gave my presentation entitled, "Molecular Analysis of the Inhibition of Chemokine Gene Expression by Xenoestrogens." I would like to talk about the following three topics.

First of all, I would like to talk about the effect of estrogen imitators, or xenoestrogens, which exist among the chemical substances currently present in the natural environment have on chemokine production and its associated molecular mechanism.

Next, I picked up dioxin from among the endocrine disrupters in the environment as a substance that has a marked effect on the immune system, and studied the degree to which gene expression varies in certain organ and cells due to the influence of dioxin using Serial Analysis of Gene Expression (SAGE). I would like to give you an overview of the results of that study.

Finally – this is an ongoing job – we would like to establish a system to study the effect of certain chemicals on the immune system or on certain organs using a cDNA microarray. This work involves toxicogenomics. I would like to give a brief description of this work.

As you see here, there are various chemicals in the environment that imitate the effect of estrogen. These are called "xenoestrogens." We cannot avoid being exposed to these xenoestrogens. Some of these are artificially synthesized chemicals, but others such as phytoestrogen and mycoestrogen naturally exist in the environment. Some are concentrated in the body, and there is concern about what effect they have on the body. Somebody asked a question a little while ago about whether such chemicals affect the immune system. I don't think it is yet clear whether or not they do.

On the other hand, we have known for a long time that estrogens do have an effect on the immune system. We know for example that the immune systems of females tend to respond better than those of males. There are reports that children born to females administered diethylstilbestrol (DES), a synthetic female hormone, tend to contract autoimmune disease. Also well known is the fact that the incidence of disease concerned with autoimmunity is overwhelmingly higher for females such as rheumatism, SLE, primary biliary cirrhosis and diseases of the thyroid. It is a definite fact that sex hormones affect the immune system.

When considering the possible effect of environmental chemicals on the immune system, we must think the possibilities of direct effect on the immune system and also indirect effect on the immune system due to its effect on the reproductive system and cerebral nerves system. Using an *in vitro* culture system, we studied xenoestrogen, a chemical substance found in the environment, to see if it has a direct effect on the immune system. As a target, we used a type of cytokine called chemokine. "Chemokine" is a made-up contraction formed from chemotactic cytokine or chemoattractant cytokine. Chemokine is known to play an important role in immunoreaction.

The immune system consists of various types of cells that express receptors for chemokine, and it has already been proven that chemokine and the chemokine receptor system play an important role when the cells of the immune system accumulate locally at spots of inflammation and are activated. We direct our attention to MCP-1. The MCP-1 I am using here is an acronym for Monocyte Chemoattractant Protein-1. Noting that MCP-1 is known to play an important role in migration and activation of monocytes, we studied the molecular mechanism and effect of xenoestrogens on production of MCP-1.

The cell strain used here is MCF-7 cells of the human breast cancer cell line. When MCF-7

cells are stimulated by IL-1, MCP-1 protein can be detected in the culture supernatant. The protein was quantified using ELISA. We also conducted the Northern Blot using cDNA concerning the expression of MCP-1 message. We also created various reporter genes containing the promoter region of human MCP-1 genes and studied the molecular mechanism of estrogen on production of MCP-1.

When MCF-7 cells of the human breast cancer cell line are stimulated by an inflammatory cytokine such as IL - 1α or TNF, production of MCP-1 on the ng/ml order is observed in the culture supernatant. When stimulated with IL - 1, MCP-1 protein can be detected in the culture supernatant after 24 hours, and continues to rise constantly for at least 72 hours after being stimulated.

Eight hours after stimulation, induction of MCP-1 message that peaks at the 24th hour due to stimulation is observed. Using this system therefore, we studied what effect 17β estradiol (E2), which is natural estrogen, would have on production of MCP-1.

Along with IL-1 stimulation, we added E2 to the culture supernatant and measured the amount of MPC-1 protein at the 72nd hour. We then found that MPC-1 protein, which increased as a result of IL-1 stimulation, was inhibited proportional to the amount of E2 added (dose-dependent). We also found that it was completely blocked when estrogen receptor antagonist ICI-182,780 was added simultaneously. Thus we proved that estrogen inhibits production of MCP-1 through estrogen receptors. Next we studied the effect of environmental estrogen such as bisphenol A (BPA) and nonylphenol (NP).

Just like the E2 shown a little while ago, we proved that BPA and NP inhibit production of MCP-1 proportional to dose (dose-dependent). When an ICI-182,780 is added simultaneously, inhibition is completely blocked. This proves that BPA and NP inhibit production of MCP-1 through the estrogen receptors.

When checked these results by Northern Blot method at RNA level, when stimulated by IL-1, MCP-1 message is observed in MCF-7 cells. When E2, BPA or NP is added, we found that MCP-1 message is inhibited dose-dependently. We therefore studied the molecular mechanism of how estrogen inhibits MCP-1 production.

No consensus sequence of the so-called “estrogen-responsive element” is observed in the promoter region of human MCP-1 genes. Two NF- κB consensus sequences of binding region are, however, observed near the –2,600 in the promoter region of human MCP-1 genes. In order to find out if the two NF- κB consensus sequences are functional, we created reporter genes that link the enhancer region with the nearby promoter region including the SP-1 site. First we checked response to IL-1. We named the reporter gene “ENH.” When this wild type ENH is stimulated with IL-1, activity of the reporter gene increases about 10 times. When we introduced mutations at two NF- κB sites called A1 and A2, responsibility by IL-1 is completely blocked. This proves that two NF- κB sites, A1 and A2 are functional. We then checked effect of E2, BPA and NP on activity of reporter genes using the wild type ENH reporter gene.

This shows the results of activity of reporter genes when E2, BPA and NP are added along with stimulating with IL-1. Although partial, we found that activity of the reporter gene is blocked significantly when estrogen is added. To further confirm this, we conduced a Gel Shift Assay by using probes of two NF- κB sites A1 and A2.

When stimulated with IL-1, we found the binding of the NF- κB for A1 increases, while uses nuclear protein treated with estrogen, we found that the binding is blocked proportional to the dose. We also found that binding of the NF- κB similarly decreases when treated with BPA and NP.

Next are the results of the gel shift assay by using the probe of the A2 site.

Just like the A1 site, when we used nuclear protein to which BPA and NP were added, we

found that binding decreased for the NF- κB A2 site proportional to the dose.

To summarize the findings we have obtained thus far, we found that these chemicals inhibit production of MCP-1, which is a chemokine that plays an important role in the immune system. The concentration effect in the system was anywhere from 1/1,000 to 1/10,000 lower than that of E2. As a result of studying the molecular mechanism, we found that at least partly the activity was inhibited through the NF - κB site in the promoter region of human MCP-1 genes.

It is widely known that NF - κB is a transcription factor that plays an important role in response of the immune system. It is also widely known that it plays an important role in inhibiting expression of not only chemokine, but cytokine, cytokine receptors and adhesion molecules. These results suggest the possibility that chemical substances in the environment affect not only expression of MCP-1, but expression of other cytokines or adhesion molecules through NF - κB.

As for the second topic, I would like to talk about the analysis of dioxin (TCDD) responsive genes using the SAGE method.

TCDDs are known to have various effects on the living body. Besides having an effect on the immune system, particularly thymus atrophy, which is the theme of today's symposium, TCDDs induce various types of drug metabolizing enzymes, effect on the reproductive system, teratogenicity and hepatotoxicity. As a target organ prior to studying the effect on the immune system, we studied expression of what genes in liver fluctuated.

That is to say, it is known that lipophilic TCDDs mostly accumulate in the liver or fat tissue. There have been reports of tumor in the livers of rats chronically administered TCDDs. The liver is unquestionably the main target of TCDDs. This toxicity is clearly induced primarily through the AhR and ARNT system, so we studied to what degree of gene expression in the liver moved through this system. We studied response to TCDDs orally administered a single time to C57/B6 mice using the SAGE method.

This shows an overview of the SAGE method. SAGE is an acronym for Serial Analysis of Gene Expression. Reported by the group of Vogelstein and Kinzler in "Science" in 1995, SAGE is a method of comprehensively analyzing information concerning genes expressed in certain cells and organs. In other words, the method attempts to represent genetic information for expression of certain genes with 10 base pair on downstream from the CATG site near the 3prime site, the site nearest poly (A) tail of the messenger RNA. Even if a mere 10 base pair, there are 4 types of bases, C, A, T and G. Because 4^{10} is about 1 million, there are about a million types even for a mere 10 base pair nucleic acid sequence. Although how many there are for human genes has yet to be ascertained, if there were 35,000, it would still be a vastly superior number. Using the tag of the 10 base pair sequence, the method attempts to comprehensively analyze expression of genes expressed in certain cells. The 10 base pair downstream from the CATG site nearest poly (A) tail is called "tag." Two connected ones are called "di-tag." These are con-catemarized. In other words it is a method that efficiently analyzes information of genes expressed in a single cell. Using this method, we can obtain information for 10,000 genes expressed in a certain cell or organ for sequences about 200 times. We can also identify certain genes from the sequence of the tag, and by checking out expression incidence of the tag, we can obtain information about the expression incidence.

In addition to the SAGE method, methods of obtaining information about genes expressed in certain cells and organs include cDNA microarrays and oligoarrays. In our experience, with the current measurement sensitivity, by using cDNA microarrays, it is hard to directly measure when the amount of genes expressed is low. In other words, if you use a cDNA microarray for genes functioning in a place where there is a small amount of expression, in our experience, it is difficult to find a difference with good sensitivity. Using the SAGE method, the genes are expressed as

sequences called “tags.” If the amount of genes expressed is low, even if there is little difference, it can be detected with good sensitivity by SAGE. This is why we used this method for analysis.

This slide shows the top 10 genes expressed in the livers of mice by the result of SAGE. Abundance is shown by the figure on the left indicating expression in percentage, and on the right it shows what types of genes are expressed. Albumin, apoprotein and major urinary protein in the livers of mice are mouse-specific proteins in the blood. We have found that these proteins in the blood are expressed at an extremely high incidence in the livers of mice.

From the livers of mice exposed to TCDDs, we likewise studied abundance of genes in a control group and a group exposed to TCDDs using the SAGE method. The statistical analysis showed that response of genes depends upon whether totaled by 0.05 or 0.01. If totaled by more severe figure, response of genes decreases. It was found that there are 346 genes if we chose 0.05 as a cut-off value and 56 genes if we chose a cut-off value at 0.01 in the livers of mice. We also found that some of the genes increase and some are down-regulated.

This slide shows an arrangement of genes for which expression increases significantly. I will summarize this and show it to you later. As for this tag, we studied approximately 55,000 tag sequences for normal and TCDDs respectively, and there were about 240 incidences of expression for the group not exposed to TCDDs. Just take note that these are the genes for which expression had increased to about 490 when exposed to TCDDs.

This is a list of genes for which expression decreased.

Here we have selected the main ones. As is already known, we found that drug metabolizing enzymes such as CYP1A2 are up-regulated and expressions of metallothionein, albumin, and heat-shock protein are induced by exposure to TCDDs. As for down-regulated genes, we found that apolipoprotein and complement genes.

If we put these in the functional category, we found that various transcription factors and plasma protein (I spoke briefly about albumin and apoprotein a little while ago) genes increase as well as drug metabolizing enzymes. We found that not only drug metabolizing enzymes and stress response genes change, but expression of various other kinds of genes as well.

Based on the results of SAGE, shown here, we established a system of cDNA microarray to see if certain chemicals have an effect on the liver from change in gene expression. Now I will show you the results of the cDNA microarray concerning the liver of mice.

A total of 352 genes are spotted on the cDNA microarray system. So it does not always contain a large amount of genes. We have selected genes that have been confirmed to be well expressed in the livers of mice. In other words genes with a low amount of expression or genes not expressed at all are omitted on this cDNA microarray system. It is characteristic that we narrowed the genes which are spotted depending the result of SAGE. Drug metabolizing enzymes, cytokine, and chemokine are also spotted on the array. Concerning the inflammation model whereby leukocytes migrate, cytokine, chemokines and those receptors have been expressed in the liver as well, so they are spotted for such inflammatory mediators as well. As a positive control, GAPDH and β-actin are spotted and yeast genes are spotted as a negative control.

In order to confirm if the cDNA microarray we established can actually be useful, we first studied the effect on gene expression in mouse livers using carbon tetrachloride (classic substance for causing hepatotoxicity). Although 10 ml/kg is a massive dose, it was administered intraperitoneally. Specimens were sacrificed after 4, 8 and 24 hours. The livers were then removed and analyzed by cDNA microarray.

When analyzed by our cDNA microarray system, we found that the group of genes can be separated into 4 clusters. The first cluster is the one whose expression was increased after 4, 8 and 24

hours, the second and the third cluster are the ones whose expression increased transiently after 4 or 8 hours, respectively, and the last cluster is the one of down-regulated time-dependently.

Thus we feel that our cDNA microarray system can be used for screening substances that cause hepatotoxicity. We are currently conducting the same study for other substances.

I will now summarize what I have talked about today. First we studied the effect of environmental estrogen on production of chemokine, which is a mediator of the immune system. This is an *in vitro* system, but we found that estrogen imitators in the environment such as NP and BPA inhibit production of MCP-1. We also found that one molecular mechanism was it acted through NF - κB, which exists in the promoter region of human MCP-1 genes.

Next, dioxin is known to exhibit immunotoxicity. Today I described for the first time the results of analysis of to what degree gene expression in certain organs is affected by dioxin by the SAGE method. As a result, we found that not only known drug metabolizing enzymes and genes related to stress response, but other genes as well exhibited a wide response. In other words, along with considering TCDD's suppression of the immune system, we must consider the direct effect of TCDD on the immune system. However, our findings also suggest that in some cases, there may be an indirect effect through proteins in the blood and other genes which respond to TCDD in the liver.

Next, we established a system of screening that uses a cDNA microarray to study the effect of certain chemical substances on the liver, and today I reported these findings as the third topic. We are currently studying the possibility of using such an array system for studying the effect on immune system.

Finally I would like to mention that this research was conducted jointly by the Environmental Science Center to which I belong, the Department of Molecular Preventative Medicine of the graduate school of Medicine, the University of Tokyo, the National Institute of Environmental Studies, Sanwa Chemical and Kaken Geneqs.

Thank you for your attention.

Q&A

Van Loveren: Thank you very much. This is open for discussion. Any questions?

Becker: Thank you. Richard Becker from ACC. Very nice discussion. I have a question about the studies looking at MCP-1 suppression. With the xenoestrogen, nonylphenol, bisphenol A, you showed a response. I think you said that 1,000 times or 10,000 times lower, are that right?

Inadera: That is right, yes.

Becker: In the dose response was a normal dose response. No unusual low dose response, no U-shape curves?

Inadera: In this *in vitro* system, no low-dose effect can be observed. But I do not know if the low dose effects can occur in an *in vivo* system.

Becker: But in this system, no.

Inadera: Not in this system.

Becker: O.K. Thank you. And I look very forward, I think the stage technology, and your development of that has great promise, so I look forward to that research being reported in the near future.

Inadera: Thank you

Sakabe: Sakabe, Kitasato Research Center. I have one extremely basic question concerning MCP-1. I understand that expression of chemokine MCP-1 was inhibited, but this MCF-7 contains a growth signal by estrogens. I think almost all cell cycles go to the S stage by treatment with estrogens. What do you think about expression of MCP-1 here?

Inadera: I have not studied the linkage with cell cycle, so I cannot answer your question. But if you want to know why we selected MCF-7 cells, the reason is as follows. MCP-1 is expressed abundantly in monocyte, macrophage and fibroblast by stimulation with IL-1, however, the suppressive effect on estrogen is not so strong. When we used MCF-7 cells, the estrogen effect was well pronounced. That is why we think that MCF-7 cells would be good for studying its molecular mechanism.

Perhaps this is not what you had in mind, but I'm afraid I can't answer your question.

Sakabe: Thank you.

Q: Thank you for your valuable presentation. I analyze BPA and NP at an environmental monitoring laboratory. I have checked quite a bit of tap water, so I was really interested in your presentation.

What was the concentration of the BPA and NP used in the experiment?

Inadera: This was *in vitro* system, but from other literature we found the chemicals bound with the estrogen receptors at 10^{-6} mol. We had a question a little while ago from one of the foreign visitors, but at any rate, we started from a low concentration and began to get an effect at a little less than 10^{-7}. A significant change occurred at 10^{-6}.

Q: In the future do you plan to report on substances other than plastic additives and raw materials, such as pesticides?

Inadera: This study is complete, but as you pointed out, we think we can use the system to study other environmental xenoestrogens. We believe this assay system can be used to study the effect of certain chemicals on the immune system and to screen the effect on the immune system. If possible, I would like to do that.

Q: Thank you very much.

Van Loveren: One last question from me, maybe. You seem to find more genes regulated by TCDDs than you had expected.

What is it that you are going to do with this information? How are you going to use it?

Inadera: That is a difficult question to answer. The role of the responsive gene to affect on, for example, liver toxicity or liver carcinogenesis is not known. And also it is not known whether these gene expressions are directly regulated by Ah receptor or indirectly regulated by another system. But first we want to obtain the transcriptome of a normal mouse liver, then we want to obtain which kind of gene was regulated by TCDD because we want to obtain some markers for TCDD exposure. That is why we started performing this analysis.

Van Loveren: I understand.

Inadera: You see, this is not a good answer.

Van Loveren: Are there no more questions? I guess then thank you very much to the audience for being so long with us. Thank you to the speakers for nice lectures. This concludes our session. Thank you very much.