Cellular Targets of TCDD-induced Immunotoxicity

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First I would like to thank all of you for providing me with the opportunity to be with you here today. I would like to talk about target cells of immunotoxicity induced by dioxin.

As you know, dioxins are derivatives of polychlorinated dibenzodioxin and dibenzofuran, and as Dr. van Loveren mentioned a little while ago, coplaner PCBs are dioxin-like compounds. Of particularly high toxicity is 2, 3, 7, 8-TCDD, a compound also said to be dioxin. Here we shall refer this simply as TCDD.

One of the mechanisms by which TCDD works is cross-talk with estrogens. Thus, TCDD is thought to be one of environmental endocrine disrupters. However, an obvious mechanism that has been studied in more detail concerns the direct effect arylhydrocarbon receptors, or Ah receptors (AhRs) have on cells when activated. By directly altering the function of immune cells, TCDD may disrupt endocrine system through immune-endocrine and neuro-endocrine interactions as Dr. Besedovsky mentioned a little while ago.

I will explain how AhRs work in somewhat simpler terms. When TCDD or dioxins enter cells, they bind with AhRs. This activates them and sends them to the nucleus. AhRs sent to the nucleus, together with another transcription factor called ARNTs, create a heterodimer and bind with special sequences called XREs in genes.

When they bind, the expression of gene which has the XRE sequence in its transcriptional control region is occurred, and sometimes expression is suppressed. I would like you to remember that what comes later is a drug metabolizing enzyme called CYP1A1, the gene of which is sensitively induced by dioxin.

Recently various research is being conducted, and various genes induced and proteins affected by AhRs have been discovered. Thus there are many factors to consider, but the problem is it is not well known how these relate to toxicity. There are various types of toxicity, but which cells will express toxicity by which pathways? We don't clearly know the linkage here.

When considering affect on health, we want to clarify this on the molecular basis. This is recently being done frequently in toxicogenomics. In order to be able to clearly draw the pathway, it is important for the target cells to be clarified. Especially in the case of immune system, various types of cells are involved. When some of these are activated, others are suppressed or enhanced by the activity, and sometimes have the exact opposite effect. It is important therefore to know where this will occur.

If you are wondering what the effect TCDD has on the immune system – this came up several time today – it is well known that it cause atrophy of the thymus gland. It is also known to suppress the important function of producing antibodies and suppressing cytotoxic T cell activity.

Today I would like to talk primarily about atrophy of the thymus gland and suppression of antibody production, which we are also studying. First is atrophy of the thymus gland. To explain briefly about the location, position and function of the thymus gland, when substances such as bacteria and toxins get into the body, the immune cells such as macrophages or B cells, differentiating into antibody production cells, work to eliminate them. The help of various cells is however required in order for these to actually work. Among them, CD4 T and CD8 T cells are particularly important because they function as a conning tower to govern the entire immune system.

The thymus gland contains precursor T cells that come from bone marrow and differentiate into CD4 or CD8 T cells. We would like to see what becomes of this a little more.

This is the thymus gland, and the precursor T cells that enter the thymus gland, especially as for surface antigens CD4/CD8, can be divided into the differentiation stage as you see here. From the stage of the most immature CD4-CD8-double negative cells, the cells multiply vigorously and become CD4+CD8+double positive cells. Further multiplication also occurs here. Most of them then die due to apoptosis, and the cells with the best function mature as CD4+ (single positive) or CD8+ (single positive) cells, go out into the periphery and function as T cells.

Because this CD4+8+double positive is a major population of more than 80% of the thymus gland, atrophy of the thymus gland generally occurs when the number of these cells decreases, and as a result appears to be atrophy of the thymus gland. This is currently disagreement on how TCDD causes atrophy of the thymus gland. The group of Dr. Kanno of Hiroshima University for example says double negative cells cause apoptosis and other groups think double positive cells cause cell cycle arrest by inducing p27kipl, or cause apoptosis here. Because of this, double positive cells decrease and atrophy of the thymus gland primarily appears, but this is still unsettled.

Another feature of TCDD we know is they increase the ratio of CD8T cells compared to CD4. In terms of this feature, one of the things we reported recently is that TCDD transiently activates and increases mitogen-activated protein kinase (MAPK), but a detailed study of the mechanism needs to be conducted in the future.

Several mechanisms such as this have been proposed, but I wonder if TCDD directly affects thymocytes, i.e., T cell precursors. Also, as Dr. van Loveren mentioned a little while ago, they work on stroma. As a result, it is possible that differentiation of such things could be inhibited. There is still disagreement concerning this as well.

I'll talk more about this later on, however, we created a certain transgenic mouse and obtained the results that the atrophy of the thymus gland occurs due to the fact that TCDD worked on the thymocytes. This is the current state of affairs concerning effect of TCDD on the thymus gland.

Next I would like to talk about the effect of TCDD on production of antibodies. This drawing shows up until the antibody forming cells are formed – this B cell receives antigenic stimulation, and differentiates as shown here. At this time help of T cells is required in all cases. CD4 T cells receive antigenic stimulation, and differentiate into Th2 cells. From here on help is absolutely necessary. These go on to become antibody forming cells. The theses presented so far state that TCDD suppresses production of antibodies. What was primarily studied however was the effect of TCDD on the production of antibodies, primarily IgM, by short-lived antibody forming cells.

On the other hand, a part of B cells multiply to an extreme degree in immune system organs such as the spleen, forming the germinal center where secretion occurs, producing cells with a strong affinity to produce antibodies, which then produce antibodies. These cells are extremely important for longer term immunological function. The affect on these has not been studied much, so we have recently conducted a study of these. As for the effect of TCDD on the short-lived AFC studied so far, it has been recently proposed that TCDD primarily acts on these at the stage where antibodies are finally produced. TCDD, for example, are said to act on the IgM enhancer area through the AhRs to suppress production. On the other hand, we studied the effect of TCDD on the differentiation of long-lived antibody forming cells and the entire area.

The protocol of the experiments we conducted consisted of immunizing mice with a commonly used antigen called OVA and giving them TCDD, and then traced the subsequent reaction. In this experiment, we studied production of IgM from short-lived AFC for the OVA which we used

for immunization and subsequent production of IgG1. Thus in our system as well we confirmed that TCDD suppresses production of antibodies.

We checked how the number of cells changes in the spleen where the cells that produce antibodies are produced. This is the total number of cells. The number of cells in the spleen increases as antibodies are produced. The majority of these are T and B cells, and they increase like this. When a mouse is exposed to dioxin, we have found that production of the cells is suppresses and the number of cells itself does not increase.

When long-lived AFC is produced, a place called the germinal center where the number of B cells can be multiplied is formed in the spleen. Here B cells in the best condition are selected. Our experiments clarified that the germinal center was insufficient in the groups exposed to dioxin. What is important is that in these cases it was observed that dioxin does not work on antibody production at the very end but rather inhibits differentiation as it occurs.

Here are the results of a study using cytokine as an index to see whether the decreased help from T cells caused the suppression of B cell increase. We prepared splenocytes from the spleen and cultured the same number of splenocytes from control group and TCDD-exposed group, and stimulated them once more with OVA to produce cytokine. We measured cytokines using ELISA. IL-2 from T cells works on activation of T cells. We found that, in control group, IL-2 production showed biphasic changes after immunization. It appeared significantly one time on the next day of immunization and then went down. Then it went back up. This is probably because the cells differentiated into a different type of T cell. If exposed to dioxin here, we have found that the initial increase drops swiftly without continuing to rise sufficiently.

IL-4 is a cytokine related to differentiation of B cells and T cells themselves. We have found that this appears to be suppressed by exposure to TCDD.

IL-5 and IL-6 from T cells are cytokines related to multiplication and differentiation of B cells. We have found this also is suppressed to an extreme degree in the groups exposed to TCDD.

The TCDD we used in the experiment was somewhat higher than usual (20 μg/kg), so we checked out from what dose the effect started using production of IL-5 as an index. By doing so, we discovered a tendency to drop beginning at a minimum dose of 0.2 μg/kg and a significant difference at 1 μg/kg. It was clearly suppressed in this experiment using five animals per group, but if the number of animals were increased, we might be able to detect suppression from a lower dose.

From the results obtained so far, we think that TCDD suppresses production of antibodies, but we assume that this suppresses T cells themselves from becoming activated, multiplying and differentiating. As a result, T cells do not provide B cells with enough help. Consequently, we think the cellular differentiation is suppressed resulting in a lack of antibodies being fully produced. From the results obtained so far, we have established a hypothesis that T cells are the primary target of immune suppression by TCDD.

To investigate this further, we created a transgenic mouse that specifically expresses constitutively activated AhR (CA-AhR) in T cells. Now, we are still creating them.

Another thing we did was to create a cell line of T cells transfected with CA-AhR to study changes in the genes. The primary T cell has AhRs, but all AhRs has disappeared from the cell line studied so far. So we made a system to observe the workings of various genes by reconstituting T cell line with CA-AhR once.

This is “wild type” AhR. This includes dymerization domain with ARNT, the domain that binds to DNA, the domain to which ligands such as TCDD bind, and transactivation domain, that is, the domain which activates gene expression. If you create a mutant for which the ligand-binding domain is cut as small as possible, even if it does not bind to the ligand, it is constantly activated and

sent to the nucleus, and by furthermore binding with ARNT, can induce gene expression. This was provided by Professor Fujii and Professor Mimura of the University of Tsukuba.

Using this CA-AhR, we first create a Jurkat T-cell line having CA-AhR to see what sort of phenotype would be expressed. In this case, GFP is connected to facilitate detecting CA-AhR expression. We also transfected the GFP only with Jurkat T-cells and studied how multiplication of cells changes.

Two days after transfecting cells with GFP or CA-AhR-GFP, we selected GFP positive cells with a FACS Vantage machine, and then spread the exact same number. When we culture this, those for which only GFP had been transfected on the second and fourth days, multiply just as normal Jurkat T-cells, but those containing CA-AhRs do not. In other words, when AhRs are activated in T-cells, it seems as though cell multiplication is suppressed.

Next we investigated why activated AhR suppresses cell multiplication, such as whether it is caused by cell cycle arrest or apoptosis.

Here are the results of our study of cell cycle. This is the original Jurkat cell cycle pattern. In the case of GFP only, there was no change. If transfected with CA-AhR-GFP, the G1 stage increased and the S stage decreased. Thus we found that G1 arrest appears to occur with CA-AhRs.

Next is apoptosis. Here is the result of studying cells for which apoptosis occurred using annexin V. Two days and four days after transfection, with GFP, about 10% of the cells were apoptic, so the proportion did not increase. The proportion of apoptic cells however increased to 24% and 26% for those transfected with CA-AhRs. Thus we currently think that AhR activation induces both cell cycle arrest and apoptosis in Jurkat T-cells.

Now I would like to talk about transgenic mice. For this, we used a promoter called VA2 that is T-cell specific and is used to induce CA-AhRs to be expressed in T-cells only. GFP is also injected to facilitate detection of cells containing transgenes. Since this is injected using the same promoter, in this case a mouse in which CA-AhRs and GFP usually express in the same cells is created. We injeced this in a fertilized ovum by the conventional method and created a transgenic mouse. This is currently backcrossed with C57BL/6, but today I will show results concerning second-generation heterozygotes.

The one line of the AhR transgenic mouse we created is called line A. Here are the results of studying gene expression in various organs of second-generation AF2 transgenic heterozygous mice. Our study confirmed the injected CA-AhRs are induced in the thymus gland and spleen as you see here. They are hardly induced anywhere else. The place where the next greatest number is induced is in the lungs. This is the sort of mouse that was created.

We also checked induction of CYP1A1 in order to confirm whether the CA-AhRs are really functioning. Whereas CYP1A1 is not induced in the thymus gland and spleen of the wild type, it was clearly observed to be induced in the transgenic type. We were therefore able to create a transgenic mouse whereby AhRs were activated at T-cells according to schedule.

We checked the phenotype of such mice. We then confirmed that there was no change in body weight and weight of the spleen in the AF2 wild type and transgenic type. However, the thymus gland of transgenic mice had become smaller. The number of cells of the thymus gland had also decreased.

When we investigated the cell population of the thymus gland for double negative (DN), double positive (DP), CD4+ and CD8+, just as is observed with dioxin exposure, an increase in CD8+ T cells was observed in this type of mouse. The decrease of double positive was observed with the phenotype as same as with TCDD exposure. The increase in double negative is the result of the

decrease in double positive, and we succeeded in creating a type of mouse in which the same phenotype is observed in the thymus gland as mice exposed to dioxin.

We have concluded from the these results that change occurs in the thymus gland due to activation of the AhRs of the thymocytes more than those of the stromal cells.

To summarize the results we have obtained so far, from our studies of transgenic mice, we have found TCDD directly affects thymocytes and suppresses thymocyte growth. Another thing is our work with Jurkat cell lines suggests that TCDD somehow suppresses the activation and proliferation of T-cells.

Because we haven't done it yet, I am not going to talk about this today, but we want to study whether or not immunization is subsequently suppressed in mice in which the AhRs of T-cells are activated using the transgenic mice we produced.

By focusing on the target cells and using toxicogenomics we hope to be able to determine the target genes with the cells and what the target molecules and proteins are.

This is the list of researchers who conducted this research jointly. In particular, we had the cooperation of the group at the University of Tsukuba in creation of transgenic mice and the constitutively active AhR.

Q&A

Q: I am really impressed with your accurate research of Ah receptors, Professor.

The fact that you said this effect starts at 0.2 μg/kg – I think Ah receptors will actually bind with lots of things. Could you tell us to what degree contaminants in the environment have an effect on the Ah receptors or through the receptors of either wild animals or human beings?

Nohara: That's a difficult question. Even in the case of human beings, if you study induction of CYP1A1 with human blood cells, it is extremely difficult to detect most, because you have to consider not only environmental factors, but various other factors such as smoking. I therefore think it would be very difficult to know to what degree people or wild animals in the environment are affected by AhR binding ligand.

In the case of an accident for example where contamination is extremely advanced, as Dr. van Loveren pointed out a little while ago, induction of CYP can be observed. What I want to say therefore is it would be very difficult to say how much contamination actually in the environment affects people and wild animals.

The amount currently in our bodies is several ng/kg. The figure of 0.2 μg/kg given a little while ago is 200 ng/kg and is tens of times the amount in our bodies. I think perhaps lower dose than that would have an effect. If we were to ingest say ten times the amount of that accumulated in our bodies, I think there would be an impact such was mentioned toady. I'm sorry I can't answer any more clearly than that.

Van Loveren: O.K. perhaps I can have a question. You state very pronounced, of course based on your results which are very clear that there is a direct interaction of thymocyte or T cells with dioxin and I do not think there is any doubt that it is true from the basis of your own results.
However, your results do not say anything about stromal cells; the fact that one can find direct effects on the thymocytes does not negate that something else may be true as well. The fact that the density of Ah receptors on the stromal cells is so high and the fact that morphologically, it is very easy to see this terminal differentiation of stromal cells, make me think that the idea of an interaction of TCDD and PCB's through the stromal cells is true, too. So I do not see why we need to think exclusively in one or the other direction. I think that things may be going on at the same time. Thymus involution may actually be a consequence of the effects that you described, but in part it may also be dependant on stromal cells. I think that the circle is not round yet, and I am not sure that we can discard either mechanism yet at this point. What do you think?

Nohara: Partly I agree with you, so I cannot exclude the possibility that these AhR also affects the stromal cells and caused a decrease of thymocyte proliferation.

But I want to mention about two previous papers. One is by Dr. Esser and colleagues, who showed the results using FTOC system. In their case, only stromal cell is the target of the TCDD-toxicity in the causation of thymus involution and they showed only stromal cells very important target. But on the other hand, Dr. Silverstone in the U.S.A. showed their results using chimeric mice's model with AhR knockout mice hematopoletic cells. They made chimeric mice with only AhR knockout or wild-type lymphocyte systems, and they showed that only hematopoletic cells are responsible for the thymus involution.

From their results, I feel that target cells or a primary target cells exist and depending on their results, we tried to determine which the real primary target cell is.

Van Loveren: I think the results show that they are a target and that have not fully been defined yet. For the primary target, you can only define it by doing the studies and see what first gets kicked out; that will be the key. The chimeric constructs that have been made provide important

information, of course, but you also need to mention the studies by Greenly of the *in vitro* exposure of stromal cells co cultured with normal pro-thymocytes, that lose their ability to sustain this effect if exposed to PCBs or TCDD, I should say.

Nohara: This time we made this kind of transgenic mice, so in our system thymocyte is one of the targets, and we want to identify the responsible gene which has caused the thymus involution. But if you use another system like transgenic mice with having activated HR in the stromal cells you may be able to identify other genes which are responsible for the thymus involution.

Van Loveren: That is exactly what I am saying: both possibilities should be addressed to come up with the full picture.

Are there any other questions? If not, then thank you very much.