2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) STIMULATES THE POSITIVE SELECTION OF $CD4^+CD8^+$ THYMOCYTES THROUGH THE ACTIVATION OF MKK1/2 AND PKC

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Introduction

It has been reported that TCDD has a profound effect on thymocytes in mice, such as inhibition of proliferation, stimulation of positive selection, and skewing toward $CD4^-CD8^+$ cells[1-3].

The mitogen-activated protein kinase (MAPK) pathways including ras, raf-1 and MKK1/2 have been generally implicated in differentiation and proliferation of cells. This pathway is also necessary for positive selection of thymocytes. Dominant negative forms of Ras, Raf-1, or MKK1 inhibited positive selection. Constitutive expression of active Raf-1 enhanced positive selection, confirming the importance of this pathway during T cell maturation[4-6].

T cell receptor ligation induces phosphatidylinositol-4,5-bisphosphate hydrolysis through the activation of phospholipase Cγ1 to form two products: inositol 1,4,5-triphosphate and diacylglycerol. A major role of inositol 1,4,5-triphosphate is the elevation of intracellular calcium levels. Diacylglycerol, on the other hand, is involved in the activation of protein kinase C (PKC). PKC is suggested to be involved in positive and negative selection of thymocytes[7,8], while other worker reported that the inhibition of PKC had no effect on either positive or negative selection[9].

In this reports, we examined the role of MKK1/2 and PKC in the TCDD-stimulated positive selection of thymocytes using specific inhibitors of these kinases.

Methods and Materials

Chemicals: 2,3,7,8-TCDD was purchased from Cambridge Isotope Laboratory. The purity was higher than 99.5 %. The solvent DMSO was from E. Merck. U0126, Gö6976 and PD98059 were from Calbiochem.

Fetal thymus organ culture: Lobes of the thymus were randomly collected aseptically from 15.5-day-old C57BL/6N embryos with a precautious measure of avoiding connective tissue. Three lobes were placed on a nitorocellulose filter of 45-μm pore size, which was set in a 12-well culture plate with 1 ml culture medium (RPMI 1640 supplemented with 10 % fetal calf serum, 5×10^{-4} M β-mercaptoethanol, 100 U/ml penicillin and 0.1 mg/ml streptomycin). 2,3,7,8-TCDD dissolved in DMSO was added to a final concentration of 10 nM in 0.05 % DMSO. Control lobes were cultivated in the medium plus DMSO. The thymus lobes were incubated at 37 °C and 5 % CO_2 in a water-saturated atmosphere. Every 3 days, half the culture medium in each well was replaced by fresh medium containing the same concentration of chemicals. On day 1, 2, 6 and 9 single-cell suspensions were prepared by gently homogenizing the thymus lobes in phosphate-buffered saline

(PBS) between slide glasses.

Flow cytometric staining and analysis: Portions of thymocytes were stained in a volume of 100μl with titrated amounts of fluorescence-conjugated anti-mouse antibodies, for 30 min on ice in the dark. The following antibodies and reagents were used: phycoerythrin (PE)-conjugated anti-mouse CD4 (clone GK1.5), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 (clone 53-6.7), biotin-conjugated anti-mouse CD3ε (clone 145-2C11), and streptavidin-APC, all from PharMingen (San Diego, CA).

Cell permeabilization and Bcl-2 staining: For Bcl-2 staining, cells were permeabilized as described previously[10]. Briefly, single cell suspensions were stained with anti-mouse Bcl-2 (clone 3F11, PharMingen) in staining buffer (SB, 1 % FCS in PBS) containing 0.03 % saponin. After washing, the cells were incubated with biotinylated goat anti-hamster serum in SB containing saponin. The cells were washed and stained with streptavidin-APC in saponin-containing SB. After washing, cells were stained with anti-CD4 and anti-CD8 mAb in SB without saponin.

Results and Discussion

On day 6 and 9 after the start of fetal thymus organ culture (FTOC) from gestation day 15.5 fetuses, we observed a significant increase in the expression of CD3, CD69 and Bcl-2 in the $CD4^+CD8^+$ thymocytes in-the presence of 10 nM TCDD as well as the skewing to $CD4^-CD8^+$ cells. Particularly, the up regulation of CD3 was remarkable. The up regulation of these proteins in the $CD4^+CD8^+$ cells indicated the enhancement of positive selection by TCDD.

A molecular mechanism by which TCDD stimulates the positive selection is unknown. MKK1/2 is reported to be involved in the differentiation of thymocytes, especially to $CD4^+CD8^-$ cells. We added an inhibitor of MKK1/2 (PD98059 or U0126) in the FTOC in the presence of or absence from TCDD to test the role of MAPK signaling pathway in the TCDD-stimulated positive selection. As a result, these inhibitors suppressed the TCDD-stimulated CD3 up-regulation as well as the percentage of $CD4^+CD8^-$ cells, but did not suppress the differentiation to $CD4^-CD8^+$ cells. These results indicated that TCDD-stimulated positive selection depended on the activation of MKK1/2 as found in the normal differentiation.

In primary-cultured hepatocytes, TCDD was reported to enhance phospholylation of Shc protein and to stimulate the complex formation of Shc-Grb2-Sos[11]. The complex is thought to activate Ras protein followed by the activation of MAPK pathway. If TCDD enhances phospholylation of Shc in thymocytesn as well, it is thought to stimulate MAPK pathway and positive selection as observed in this study.

Although PKC is essential for the activation initiated by TCR in preipheral T cell, the role of PKC in the differentiation of thymocytes is not clear, which led us to utilize a PKC inhibitor Gö6976. We found that Gö6976 inhibited the TCDD-stimulated up-regulation of CD3 among $CD4^+CD8^+$ cell populations. On the contrary, Gö6976 did not have any effects on the population defined by CD4 and CD8. Higher concentration of Gö6976 repressed the CD3 expression of $CD4^+CD8^+$ cell populations in the absence of TCDD. Therefore, the results indicated that PKC was involved in normal and TCDD-stimulated CD3 expression.

In conclusion, TCDD enhances the CD3 up regulation of $CD4^+CD8^+$ double positive thymocytes (i.e. positive selection) through the activation of MKK1/2 and PKC.

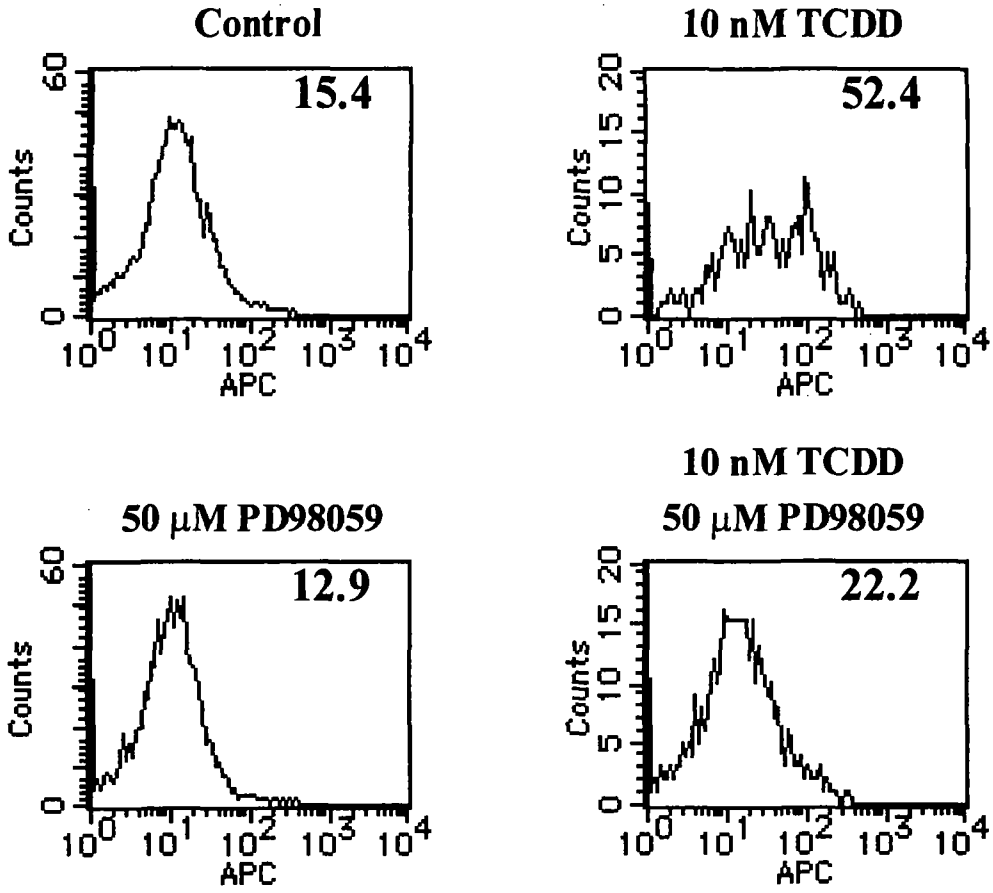

Figure 1. The effects of MKK1/2 inhibitor PD98059 on the CD3 expression of $CD4^+CD8^+$ thymocytes. Mean fluorescence intensity is indicated in the right-upper corner of each figure.

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