The effects of retinoic acid and its receptors on the function of Nur77 in T-lymphocytes

Thesis by
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Abstract

The Effects of retinoic acid and its receptors on the function of Nur77 in T-lymphocytes

Apoptosis in T-lymphocytes is an important mechanism for maintaining immune tolerance and cellular homeostasis in T-cell development. Nur77 is an orphan nuclear receptor that has a major role in T-cell receptor-mediated apoptosis. Retinoids, potent immune modulators, are known to inhibit the activation-induced apoptosis of immature thymocytes and T-cell hybridomas. To illustrate the mechanism of the retinoid-induced inhibition of T-cell apoptosis, the effects of retinoic acid (RA) on the expression and transactivation function of Nur77 were examined in the present investigation. All-*trans*-RA inhibited the anti-CD3 antibody- and PMA/Ionomycin-induced apoptosis in the human peripheral blood mononuclear cells and the human T-cell leukemia cell line, Jurkat. It also repressed the expression of Fas ligand that was induced by PMA/Ionomycin in Jurkat. All-*trans*-RA dramatically repressed the DNA binding of Nur77 protein, and the transcriptional induction of Nur77. Among the two potential *trans*-acting factors that activate Nur77 gene promoter, *i.e.*, AP-1 and related serum response factor (RSRF), all-*trans*-RA repressed the DNA binding and reporter gene activity of AP-1, but not that of RSRF, suggested that the inhibition may be mediate through AP-1. Furthermore, Nur77 bound retinoid X receptor (RXR) as well as RA receptor (RAR) in the mammalian two-hybrid test. Co-transfection of RAR α or RXR α together with Nur77 into CV-1 cells, transcriptional activity of Nur77 was significantly inhibited, which showed that the transcriptional function of Nur77 was regulated by retinoid receptors. These results demonstrated that transactivation

function of Nur77 was post-transcriptionally regulated by retinoid receptors. Taken all together, RA repressed Nur77 function through a multiple mechanism that may provide basis for RA inhibition on the apoptosis of activated T-lymphocytes.

Key Words : Nur77, retinoic acid, T-cell receptor (TCR)-mediated apoptosis

The effects of retinoic acid and its receptors on the function of Nur77 in T-lymphocytes

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I. Introduction

Apoptosis is a form of cell death in which cell activates an internal death program. In particular, it has an essential role in shaping the T-lymphocyte repertoire in the body. Thymocytes that undergo productive T-cell receptor (TCR) gene rearrangement must be selected in some way, so that only those thymocytes whose receptors exhibit self-major histocompatibility complex (MHC) restriction are permitted to mature. Positive selection ensures that the TCRs expressed in a given individual T-cell will bind to self-MHC. Cells that fail positive selection are eliminated within the thymus by apoptosis. Also eliminated, by negative selection, are thymocyte bearing a high affinity receptor for self-MHC molecules alone or self-antigen plus self-MHC

molecules. About 95% of all thymocyte progeny die by apoptosis within the thymus.[1] Also of importance is the activation-induced T-cells apoptosis which plays a critical role in the deletion of activated T-cells at the termination of an immune response.[2] Stimulation of the TCR complex in resting T-cells leads to activation, but antigenic stimulation of activated T-cells results in apoptosis. Such activation-induced cell death is defined as apoptosis of T-cells by any signal that results in lymphocyte activation in particular, by stimulation of the TCR-CD3 complex with antigens or antibodies.

Stimulation of the TCR-CD3 complex results in the activation of signaling pathways, including activation of inositol-lipid-specific phospholipase C gamma (PLCγ), hydrolysis of plasma membrane inositol phospholipids, increase in intracellular Ca^{2+} level and activation of various protein kinases that subsequently phosphorylate a number of proteins involved in the activation of the signal. Phosphorylation of PLCγ allows it to hydrolze phosphatidylinositol 4,5-bisphosphate (PIP_2), into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG).[3] Once formed, IP_3 and DAG initiate two necessary signaling pathways. In one pathway, IP_3 triggers an increase intracellular Ca^{2+} and the subsequent activation of a calmolulin-dependent phosphatase, calcineurin.[4,5] In the other pathway, DAG activates protein kinase C (PKC), which then phosphorylates various cellular substrates.[1] Thus, TCR signaling consists of two pathways, PKC signaling and Ca^{2+} signaling. The signaling pathways lead expression of genes which mediate apoptotic process of T-cells.

Several genes which are required for activation-induced apoptosis in T-cell hybridomas have been identified. One of the genes is Nur77 (NGFI-B, N10, Nak1), which is induced rapidly in the course of the TCR-mediated apoptosis.[6-9] Nur77 is classified as a member of steroid/thyroid hormone receptor superfamily, but is an orphan receptor of which ligand is not known.[10] The Nur77 protein has the typical steroid receptor structure, composed of an N-terminal transactivation domain, a central DNA binding domain containing two zinc fingers, and a C-terminal with homology to hormone-binding domain.[11,12] Unlike most other hormone receptors which bind as dimers, Nur77 can bind as a monomer to the Nur77-DNA binding element (NBRE); 5'-AAAGGTCA-3'.[13] It also bind as a homodimer to a palindromic NurRE motif (5'-TGATATTTACCTCCAAATGCCA-3'), which was shown to be activated during T-cell apoptosis.[14] Nur77 forms heterodimer with the retinoid X receptor (RXR) and to confer 9-*cis*-retinoic acid (RA)-dependent transcription to RA response element (RARE) and in some case the NBRE sequence.[15-17] Thymocytes from transgenic mice constitutively expressing the full-length Nur77 protein showed massive apoptosis.[18] On the other hand, the expression of dominant negative and antisense Nur77 mutants inhibited apoptosis in T-cell hybridomas.[19,20] Protein binding to the NBRE motif correlated well with the onset of apoptosis, while cyclosprin A (CsA), an immunosuppressive drug, inhibited the NBRE binding in T-cell hybridomas. Further, expression of full-length Nur77 induced the expression of Fas Ligand (FasL) protein in transgenic mice.[21] All together, the existing data evidenced that high level induction of Nur77 is a key step in TCR-mediated apoptosis.[22] However, the precise mechanism of Nur77 induction as well as the downstream target genes have not been clarified.

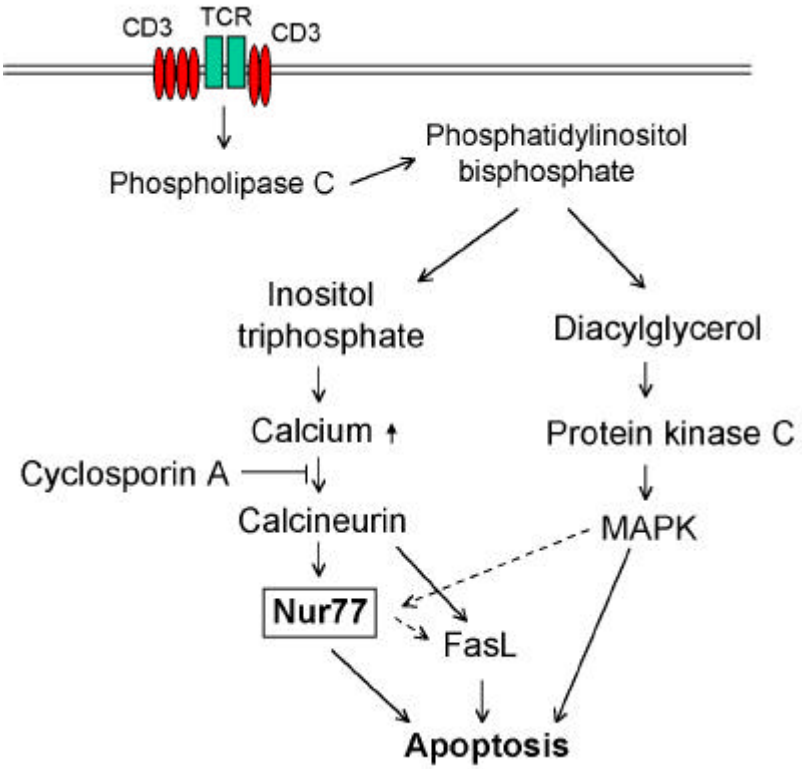

Figure 1. Signaling pathway following TCR-mediated apoptosis.[23] Both activation of PKC and increasing of intracellular Ca^{2+} are required for the TCR signaling pathway. Nur77 and FasL are regulated by both PKC and Ca^{2+} signaling pathways.

Retinoids are a group of natural and synthetic vitamin A derivatives, which are known to regulate a broad range of biological processes, including growth, differentiation of a variety of cell types, and development of vertebrate. Cellular actions of RA are mediated by retinoid receptors that belong to the steroid/thyroid hormone receptor superfamily. The nuclear retinoid receptors are divided into RA receptor (RAR) and RXR subfamilies, each composed of three subtypes, α, β and γ. RAR has high affinity for both all-*trans*-RA and 9-*cis*-RA, while RXR has high affinity only for 9-*cis*-RA. Retinoid receptors function in the form of RAR/RXR heterodimers or RXR/RXR homodimers in the presence of RAs. RXR has been shown to form heterodimers with several members of the nuclear receptor superfamily, including RAR, thyroid hormone receptor (TR), vitamin D receptor (VDR) and peroxisome proliferator-activated receptor (PPAR).[24] All-*trans*-RA and 9-*cis*-RA have been shown to inhibit activation-induced apoptosis of immature thymocytes and T-cell hybridomas.[25,26] Although the mechanism of the inhibition is not well understood, RA may block activation-induced apoptosis through its ability to inhibit expression of FasL following activation.[27-29]

In the present study, the possibility that RA and its receptors modulate the expression and transactivation function of Nur77 was investigated as a possible mechanism for the inhibition of TCR-mediated apoptosis by RA.

II. Materials and Methods

1. Cell culture

Jurkat, a human T-cell leukemia cell line (ATCC CRL 1990), was maintained at 37 ℃ and 5% CO_2, in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco BRL). CV-1, a green monkey kidney cell line (ATCC CCL 70), was grown at 37 ℃ and 5% CO_2, in Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL) supplemented with 10% FBS (Gibco BRL).

When RA was treated, cells were incubated with medium containing activated charcoal-treated FBS (CT-FBS), to remove residual retinoids present in serum. To make CT-FBS, activated charcoal (1.2 g) was mixed with 100 ml FBS, set at 4 ℃ for 10 min, and filtered using bottle top filter (Nalgene, Lund, Sweden).

2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected from two healthy donors. Ten milliliter blood was collected with heparinized syringe and transfered to a tube containing 10 ml of RMPI 1640 medium. Ten milliliter of diluted blood was carefully transfered to a tube containing 5 ml of Ficoll/Hypaque (Sigma, St. Louis, MO, USA) and centrifuged at 2,000 rpm for 20 min at room temperature (RT). The PBMCs

in intermediated phase were collected with a Pasteur pipet and transfered to a tube containing 10 ml RPMI 1640 medium. The tube was centrifuged at 1,500 rpm for 15 min at RT. The supernatant was removed and the cell pellet was resuspended in 10 ml RPMI 1640 medium. After centrifugation at 1,000 rpm for 10 min at RT, the resulting cell pellet was resuspended in RPMI 1640 containing 10% FBS. Cells were counted and seeded in 100 cm^2 dishes.

3. Reagents and antibodies

All-*trans*-RA, 9-*cis*-RA and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Ionomycin was obtained from Calbiochem (La Jolla, CA, USA). All-*trans*-RA and 9-*cis*-RA were dissolved in DMSO:ethanol (1:1). PMA and Ionomycin were dissolved in DMSO. Anti-Fas antibody (CH 11) was purchased from Medical and Biology Laboratories (Watertown, MA, USA). A mouse antibody to human CD3 (OKT3) which were prepared from the ascites of hybridoma-injected athymic mice, were kindly provided by Dr. Jong-Sun Kim (Yonsei University, Seoul, Korea). A anti-Flag antibody was obtained from Upstate Biotech. (Lake Placid, NY, USA). Anti-c-Fos and anti-c-Jun antibodies were obtained from Santa Cruz Biotech. LTD. (Santa Cruz Bio Technology, Santa Cruz, CA, USA). The purified GST-Flag-Nur77 protein was kindly provided by Dr. Xiao-Kun Zhang (The Burnham Institute, CA, USA).

When anti-CD3 antibody was used to stimulate PBMCs, anti-CD3 antibody-coated dish was employed. A 100 cm^2 dish was coated by covering the dish

with 10 ml anti-CD3 antibody solution (1:400 dilution in 1 M Tris, pH 9.5) at 4 ℃ for overnight. The next day, the antibody-coated dish was washed with PBS for 3 times and used for stimulation.

4. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

Jurkat cells (5,000 to 7,500) or PBMCs (10,000 to 20,000) were seeded in a 96-well culture plate. Cells were pre-incubated with all-*trans*-RA (0.1 μM to 1.0 μM) for 24 hr. Then cells were stimulated with PMA (10 ng/ml)/Ionomycin (0.5 μM), anti-CD3 antibody (1:400 dilution) or anti-Fas antibody (250 ng/ml) for 24 hr. MTT solution (Sigma) was made up 2 mg/ml in sterile PBS. At the end of treatment, 50 μℓ of the MTT solution was added into the 96-well culture plate and incubated for 4 hr at 37 ℃. When the incubation was completed, the plate was centrifuged at 3,000 rpm for 10 min. Supernatant was carefully removed and remaining adhered cells were dissolved in 50 μℓ DMSO. The plate was read at 570 nm using ELISA reader.

5. RNA isolation, cDNA synthesis and reverse transcriptase-polymerase chain reaction (RT-PCR)

Jurkat cells ($2x10^6$) were seeded in a 100 cm^2 dish. Cells were incubated

with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr in the absence or presence of 0.01 μM to 1.0 μM all-*trans*-RA pre-treatment for 24 hr. At the end of treatment, total RNA was prepared using RNeasy Kit (Qiagen Inc., Chatsworth, CA, USA), according to the manufacture's instructions. To synthesize cDNA, 4 μg of total RNA was mixed with 100 ng random hexamer (Phamacia, Uppsala, Sweden), boiled at 70 ℃ for 10 min, and then quickly chilled on ice. Three microliter of 5X first strand buffer, 1.5 μℓ of 0.1 M DTT, 2 μℓ of 10 mM dNTPs and 200 units of Murine Molony Leukemia Virus-Reverse Transcriptase (MMLV-RT) (GibcoBRL) were added into the reaction mixture and incubated at 37 ℃ for 1 hr. The reaction mixture was boiled at 95 ℃ for 5 min, quickly chilled on ice, and added 20 μℓ of distilled water.

PCR reaction was performed using specific primers for FasL. PCR reaction mixture was prepared with 4 μℓ of cDNA, 4 μℓ of 1.25 mM dNTPs, 1 μℓ of 10 pmol up-stream primer, 1 μℓ of 10 pmol down-stream primer, 2.5 μℓ of 10X PCR buffer, 13.25 μℓ of distilled water and 1.25 unit of Taq polymerase (Perkin Elmer, Norwalk, CT, USA). PCR reaction was performed by the following cycling condition : 94 ℃ for 5 min: 52 ℃ for 5 min: 72 ℃ for 5 min for 28 cycles. To determine that the equal amount of RNA was used, the expression level of β-actin mRNA was also examined. The primers used are: FasL, forward: 5'-ATGTTTCAGCTCTTCACCTACAGAAGGA-3', reverse: 5'-CA GAGAGAGCTCAGATACGTTGAC-3' and β-actin, forward: 5'-CGTGGGCCG CCCTAGGCACCA-3', reverse: 5'-TTGGCCTTAGGGTTCAGGGGGG-3'.

6. Northern blot analysis

PBMCs were placed in a 100 cm^2 anti-CD3 antibody-coated dish for 4 hr. Junket cells ($2x10^6$) were seeded in the 100 cm^2 dish, pre-incubated with or without 1.0 μM all-*trans*-RA for 24 hr, and stimulated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr. At the end of treatment, total RNA was prepared from PBMCs and Jurkat using RNeasy Kit (Qiagen Inc.). Twenty microgram of total RNA was separated on a 1% agarose gel contaning 6% formaldehyde/1X MOPS, and then transferred to a Hybond TM-N nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England). A *Pst*I-*Eco*RI fragment (1.5 Kb) of Nur77 cDNA was used to make probe. The DNA fragment (25 ng) was denatured by heating at 95 ℃ for 5 min, followed by quick chilling on ice. The DNA fragment was labeled with [α^{32}P]dATP using a random primer labeling kit (Promega, Madison, WI, USA). The DNA fragment was incubated with 2 μℓ of BSA, 2 μℓ of dNTPs (without dATP), 10 μℓ of 5X Klenow buffer, 5 μℓ of [α^{32}P]dATP and 1 unit of Klenow for 3 hr at RT. The labeled fragment was purified with Sephadex G-50 and used as probe when specific activity was measured greater than $2x10^7$ cpm/μg DNA. Hybridization was carried out in a buffer containing 50% formaldehyde, 0.25 M sodium phosphate buffer, 1 mM EDTA, 0.25 M NaCl, 7% SDS and 100 μg/ml salmon sperm DNA at 42 ℃ for overnight. After hybridization, the membrane was washed with 0.2X SSC containing 0.5% SDS two times for 5 min at RT. Wash with 0.2X SSC containing 0.5% SDS for 10 min at 55 ℃ was repeated for several times. Autoradiography was performed with an intensifying screen at -70 ℃ for several days. To determine that the equal

amount of RNA was used, the expression of β-actin was examined.

7. Gel shift assay

Treatment of cells with anti-CD3 antibody or PMA/Ionomycin was the same as described in the above section. At the end of treatment, cells were collected by centrifugation at 1,500 rpm for 15 min. Cell pellet was resuspended in 1 ml Lysis buffer containing 10 mM Tris-Cl (pH 7.4), 3 mM $CaCl_2$, and 2 mM $MgCl_2$, and then centrifuged again. Supernatant was removed and cell pellet was resuspeded in 1 ml NP-40 lysis buffer containing 10 mM Tris-Cl (pH 7.4), 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 1% NP-40. The cell suspension was transferred into an ice-cold Dounce homogenizer and the cells were broken by fifty to hundred strokes. Nuclei was harvested by centrifugation and washed in Buffer A containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF. Nuclear proteins were extracted with 30 μℓ Buffer C containing 20 mM HEPES-KOH (pH 7.9), 25% Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF for 40 min at 4 ℃ in cold room using an orbital shaker. Nuclear extract was obtained after centrifugation at 4 ℃ at 14,000 rpm for 30 min. Probe was prepared with 7 pmole of oligonucleotide, 5 μℓ of 2.5 mM dNTPs (without dATP), 2.5 μℓ of 10X Klenow buffer, 5 μℓ of [α-^{32}P]dATP and 1 unit of Klenow (Gibco BRL). Reaction mixture was incubated for 1 hr at 37 ℃ and the resulting labeled probe was purified in a 100 μℓ STE buffer by passing with a Sephadex G-25 column. Usually,

radioactivity of probe was approximately $1x10^6$ cpm/μℓ.

For DNA binding reaction, 5 μg of nuclear extract was incubated with probe in a 20 μℓ binding buffer containing 10 mM Tris-Cl (pH 7.5), 100 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 1 mg/ml BSA and 5% glycerol at RT for 20 min. When antibodies were used, 5 μg of nuclear extract was incubated with antibodies at RT for 30 min before probe was added. The reaction mixture was analyzed on a 5% polyacrylamide gel. Electrophoresis was carried out at RT for 3 hr at 150V. After running, gel was dried at 80 ℃ for 1 hr. Autoradiography was performed with an intensifying screen at -70 ℃ for overnight. To determine that the equal amount of nuclear extract was used, the DNA binding of SP-1 was examined. The sequences of oligonucleotides used as probe in the experiments were: NBRE, 5'-TCAGGTC ACTGTGACCTGA-3'; NurRE, 5'-GTGATATTTACCTCCAAATGCCAG-3'; AP-1-like, 5'-GATCTCCATGCGTCACGG-3'; consensus AP-1, 5'-AGCTTGAT GAGTC-3'; RSRF, 5'-GATCTACTATATTTAGCCGG-3'; and SP-1, 5'-GATCG ATCGGGGCGGGGCGAG-3'.

8. Plasmids

The reporter genes, Nur77 promoter (-454 +57)-Luc, AP-1-RE-Luc, RSRF-RE-Luc, Gal4-*tk*-Luc, NBRE-Luc and NurRE-Luc, and the receptor expression vectors, pECE-Nur77, pECE-RARα, pECE-RXRα, pVP16-RARα, pVP16-RXRα and pM-$Nur77_{Hga}$ were used for the present study.

The Nur77 promoter-Luc reporter plasmid was constructed as follows. Nur77

promoter (-454 +57) fragment was amplified by PCR using Nur77 promoter-CAT as template.[22] The primers used were : upsteam 5'-GATC AGCTGTTGGCACCA-3' and downstream 5'-AGCTCCGCAGTCCTTCTA-3'. The PCR product was subcloned into the T-vector (Novagen, Darmstadt, Germany). For ligation, reaction mixture was prepared with 5 ㎕ of PCR product, 1 ㎕ of 10X ligase buffer, 1 ㎕ of 10 mM ATP and 4 units of ligase and 2 ㎕ H_2O and reaction was carried out at 16 ℃ for overnight. After transformation, bacteria colony expressing the ligated plasmid was seleted on an agar plate containing 50 ㎍/ml ampicillin, and confirmed by restriction digestion. To construct reporter gene encoding luciferase, a *Bam*HI-*Hind*III fragment was obtained from the T-vector containing the promoter fragment by restriction digestion, purified by Gene Clean Kit (Bio-Rad, Vista, CA, USA), and inserted into the corresponding sites of pGL2-basic (Promega).

To construct RSRF-RE-Luc, the oligonucleotide encoding RSRF binding sequence and *Bgl*II overhang was annealed into double-stranded DNA and phosphorylated. For annealing, a mixture of 1 nmol sense-strand and 1 nmol anti-sense-strand was boiled at 100 ℃ for 5 min, and then slowly chilled at 30 ℃. The resulting double-stranded DNA was phosphorylated in a mixture containing 4 ㎍ of double-stranded DNA, 1 ㎕ of 10X kinase buffer, 1 ㎕ of 10 mM ATP and 1 unit of T4 kinase at 37 ℃ for 1 hr. An equal volume of phenol/chloroform was added into the reaction mixture, and centrifuged at 12,000 rpm for 5 min. Supernatant was transferred into a new tube, two volumes of 100% ethanol was added, and placed at -70 ℃ for 30 min. DNA was recovered by centrifugation at 12,000 rpm for 15 min at 4 ℃. The resulting DNA pellet was dissolved in 10 ㎕ distilled water. pGL2-promoter

plasmid which was digested with *Bgl*II was purified by Gene Clean Kit and dephosphorylated by incubating with 1 μℓ of 10X buffer and 1 unit of calf-intestinal alkaline phosphatase (CIAP) at 37 ℃ for 1 hr. The double-stranded DNA and the dephosphorylated pGL2-promoter vector was ligated, transformed, and selected. The obtained RSRF-RE-Luc construct was confirmed by sequencing (Yonsei University Clinical Research Center).

For mammalian two-hybrid assay, $Nur77_{Hga}$ was subcloned into pM vector (Clontech Laboratory, Inc. Palo Alto, CA, USA) which contained the Gal4-DNA binding domain. $Nur77_{Hga}$, 1 Kb fragment, restricted with *Eco*RI/*Bam*HI from pBlueScript/$Nur77_{Hga}$ was subcloned into the corresponding site of pM.

9. Reporter gene analysis and mammalian two-hybrid assay

Jurkat cells ($1 \sim 2 \times 10^7$) were harvested by centrifugation at 1,000 rpm for 5 min and resuspeded in 500 μℓ sterile PBS. Resuspended cells were transferred into a Gene Pulser Cuvette (Bio-Rad) and were added the β-gal expression vector (2.5 μg) and reporter gene (7.5 μg), *i.e*. Nur77 promoter-Luc, AP-1-RE-Luc or RSRF-RE-Luc. Then the cuvette was incubated on ice for 10 min and electroporated with Gene Pulser (Bio-Rad) at 250 V, 960 μF. After transfection, cells were aliquoted and incubated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr with or without pre-treatment of 1 μM all-*trans*-RA for 24 hr. After treatment, cells were harvested by centrifugation at 1,500 rpm for 5 min. To prepare cell lysate, cells were resuspended in 100 μℓ of 1X reporter lysis buffer (Promega), freezed at -70 ℃ for 10 min, and

thawed at 37 ℃. After vortexing, cell lysate was obtained by removing cell debris using by centrifugation at 12,000 rpm for 5 min.

For transfection of CV-1 cells, calcium phosphate precipitation method was used. Cells ($5x10^4$) were seeded in a 24-well culture plate and incubated for overnight. Culture medium was changed at 4 hr earlier transfection. DNA mixture (1 μg) contained reporter gene (0.1 μg), β-gal expression vector (0.15 μg) and various combinations of receptors, *i.e.*, pECE-Nur77, pECE-RARα, pECE-RXRα, pVP16-RAR_{full}, pVP16-RXR_{full}, and pM-$Nur77_{Hga}$ and carrier DNA. Calcium chloride (2.5 M, 2.5 μℓ) was added into the DNA mixture and was incubated with an equal volume of 2X BES for 15 min at RT. The DNA solution was added into a well drop-wise and the plate was incubated at 3% CO_2 incubator for overnight. At the end of incubation, medium was replaced with DMEM containing 10% CT-FBS and treated with all-*trans*-RA or 9-*cis*-RA for 24 hr. Cell was lysed with 200 μℓ of 1X reporter lysis buffer.

Luciferase activity was measured by adding 50 μℓ luciferin (Promega) into 10 μℓ of cell lysate using an Analytical luminescence luminometer according to the manufacturers instructions. To measure β-gal activity, 50 μℓ of cell lysate was incubated with 142.5 μℓ of 0.1 M sodium phosphate buffer (pH 7.4), 55 μℓ of O-Nitrophenyl β-D-Galactopyranoside solution (ONPG) (Sigma) and 2.5 μℓ of $MgCl_2$ mixture at 37 ℃ in 96-well plate. ONPG solution was prepared with 0.4 g ONPG in 100 ml of 0.1 M sodium phosphate buffer (pH 7.4). $MgCl_2$ mixture was composed of 0.1 M $MgCl_2$ and 5 M β-mercaptoethanol. 96-well plate was read in 405 nm using ELSIA reader. Luciferase activitiy was normalized for transfection efficiency by the corresponding β-gal activity.

10. Statistics

One-way analysis of variance was performed using GraphPad Instat® (GraphPad Software, San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

III. Results

1. All-*trans*-RA inhibited cell death of PBMCs and Jurkat.

In order to confirm the inhibition of activation-induced cell death of T-cells by all-*trans*-RA, we examined the cell death of Jurkat cells and PBMCs obtained from two heathy donors using MTT assays (Figure 2A). Human PBMCs and Jurkat cells were pre-incubated with or without all-*trans*-RA for 24 hr and then treated with PMA/Ionomycin, anti-CD3 antibody or anti-Fas antibody, that are known to generate apoptotic signal in T-cells. The incubation with PMA/Ionomycin alone and anti-CD3 antibody alone induced 40-45% and 30-35% cell death, respectively, in PBMCs. Similarly, treatment with PMA/Ionomycin and anti-CD3 antibody induced 20-25% and treatment with anti-Fas antibody induced 15% cell death of Jurkat cells. When the cells were pre-incubated with all-*trans*-RA, the percentage of apoptotic cells decreased significantly in a dose-dependent manner. Incubation with 0.1 μM and 1.0 μM all-*trans*-RA reduced approximately 45% and 70% of PMA/ Ionomycin- and anti-CD3 antibody-induced cell death of PBMCs. Pre-treatment of 0.1 μM and 1.0 μM all-*trans*-RA decreased cell death of Jurkat cells approximately to 35% and 50% (Figure 2A).

To know whether all-*trans*-RA repressed the FasL expression in PBMCs and Jurkat cells, the expression of FasL transcripts was examined using RT-PCR (Figure 2B). Jurkat was treated with PMA/Ionomycin for 6 hr, with or without 1.0 μM all-*trans*-RA pre-treatment for 24 hr. As shown in Figure 2B, FasL

expression was significantly induced by PMA/Ionomycin, and repressed by all-*trans*-RA dose-dependently. Thus, the results demonstrated that all-*trans*-RA repressed TCR-mediated apoptosis, and FasL expression in PBMCs and Jurkat.

2. All-*trans*-RA decreased DNA binding of Nur77.

To understand the mechanism of RA-induced inhibition of TCR-mediated apoptosis, we tested whether all-*trans*-RA regulated DNA binding of Nur77 (Figure 3). Active Nur77 that bound DNA was monitored using gel shift assay with NBRE or NurRE as probe.

When PBMCs were stimulated with anti-CD3 antibody for 4 hr, DNA binding of Nur77 was significantly induced on both NBRE and NurRE (Figure 3). When the cells were pre-treated with all-*trans*-RA, the basal and anti-CD3 antibody-induced DNA binding of Nur77 was repressed. To confirm that Nur77 protein was indeed present in the NBRE and NurRE DNA binding complex, we performed a competetion assay and an antibody supershift assay. A 100-fold excess of unlabeled probe completely abolished the NBRE and NurRE binding of Nur77. Bacteria expressed GST-Flag-Nur77 protein which strongly bound on both NBRE and NurRE, was supershifted by additional anti-Flag antibody on both NBRE and NurRE (Figure 3).

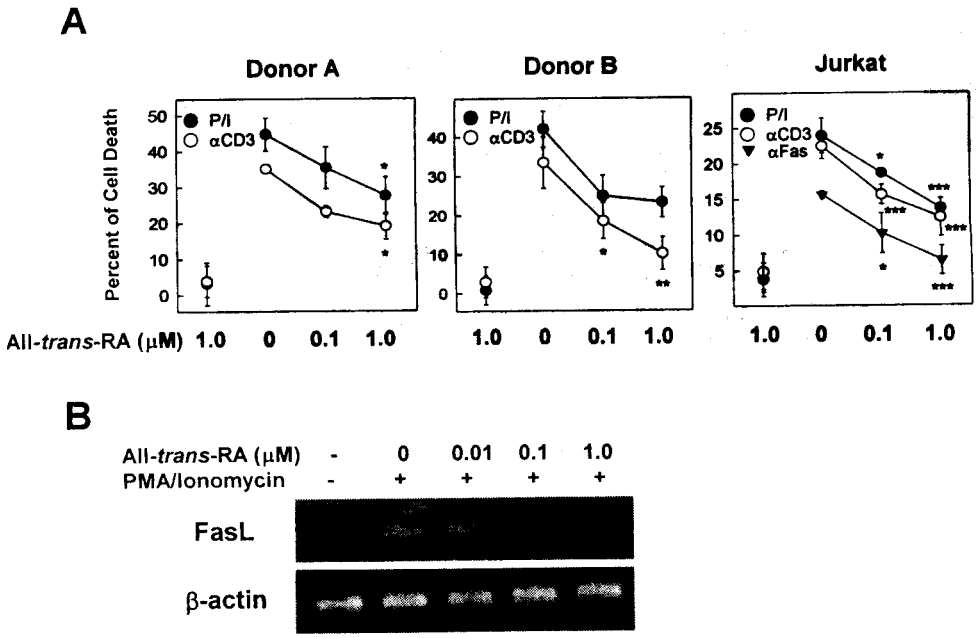

Figure 2. Effect of all-*trans*-RA on the TCR-mediated apoptosis of human PBMCs and Jurkat. (A) PBMCs obtained from two healthy donors and Jurkat cells were pre-incubated with 0.1 μM to 1.0 μM all-*trans*-RA for 24 hr. The cells were stimulated with PMA (10 ng/ml)/Ionomycin (0.5 μM), anti-CD3 antibodies (400:1 dilution of ascites) or anti-Fas antibodies (250 ng/ml) for 24 hr as indicated. Cell death was determined by MTT assays. Results are expressed as percent of cell death compared with control. Data shown are the mean $\pm$ SD of four independent determinations. *, $p<0.05$ and ***, $p<0.001$ *vs* anti-CD3 antibodies, PMA/Ionomycin or anti-Fas antibodies alone treated. (B) Effect of all-*trans*-RA on the FasL mRNA expression. Jurkat cells were pre-incubated with the indicated concentration of all-*trans*-RA for 24 hr, and then stimulated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr. The expression of FasL transcripts was detected by RT-PCR. The expression of β-actin mRNA was also monitored as a control.

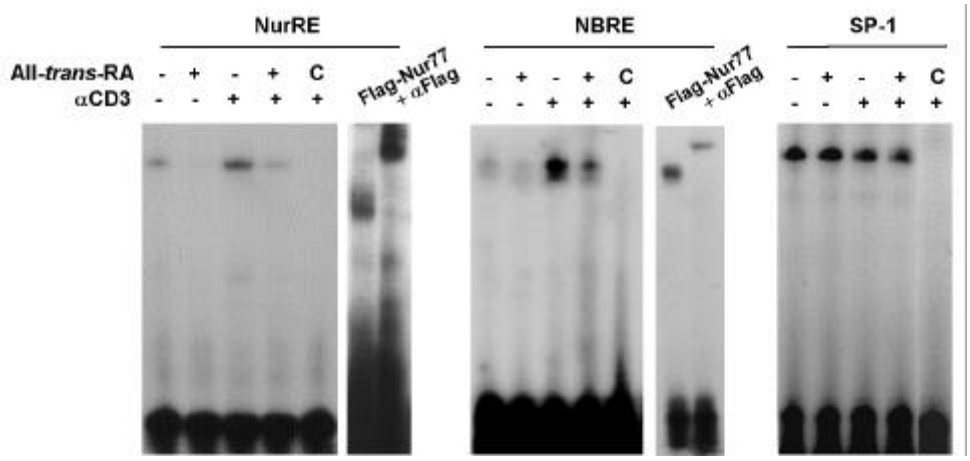

Figure 3. All-*trans*-RA repressed DNA binding of Nur77 in PBMCs. PBMCs ($7x10^6$ cells) from a healthy donor were stimulated in a 100 cm^2 dished pre-coated with anti-CD3 antibodies for 4 hr with or without 1.0 μM all-*trans*-RA pre-treatment for 24 hr, as indicated. The reaction mixture containing 5 μg nuclear extract or purified GST-Flag-Nur77 (3 μℓ) were incubated with ^{32}P-labeled oligonucleotide *i.e.* NBRE and NurRE, and analyzed by gel shift assays. Anti-Flag antibodies were used to show specific binding of Nur77 on the indicated oligonucleotide probe. C indicates 100-fold excess amount of unlabeled oligonucleotide used for competition. The binding of SP-1 was shown as a control.

Treatment of PMA/Ionomycin also increased DNA binding on NBRE in Jurkat. All-*trans*-RA repressed the NBRE binding of Nur77 dose-dependently (Figure 4). The inhibition was observed with as low as 0.1 nM all-*trans*-RA treatment and the maximal inhibition was obtained with 0.1 μM all-*trans*-RA treatment (Figure 4). To determine that the equal amount of nuclear extract was used, the DNA binding of SP-1 was examined. The DNA binding of SP-1 remained same regardless of the treatment. Therefore, the results showed that all-*trans*-RA significantly repressed both basal and the apoptotic stimuli-induced DNA binding of Nur77.

3. All-*trans*-RA repressed transcriptional expression of Nur77.

To test whether or not the repression of DNA binding of Nur77 resulted from a reduction of the transcriptional expression of Nur77, we examined the effect of all-*trans*-RA on Nur77 transcriptional level using Northern blot analysis (Figure 5A). PBMCs that were pre-treated with or without all-*trans*-RA were seeded in an anti-CD3 antibody pre-coated dish for 4 hr. When PBMCs were stimulated with anti-CD3 antibody, the level of Nur77 transcripts increased significantly. Similarly, when Jurkat cells were treated with PMA/Ionomycin, Nur77 transcripts were increased remarkably (Figure 5A). The Nur77 induction reached its maximal level after 6 hr of PMA/ Ionomycin stimulation and diminished with time in Jurkat (data not shown). When cells were pre-treated with 1.0 μM all-*trans*-RA, the induction of Nur77 transcripts was abolished in both PBMCs and Jurkat (Figure 5A).

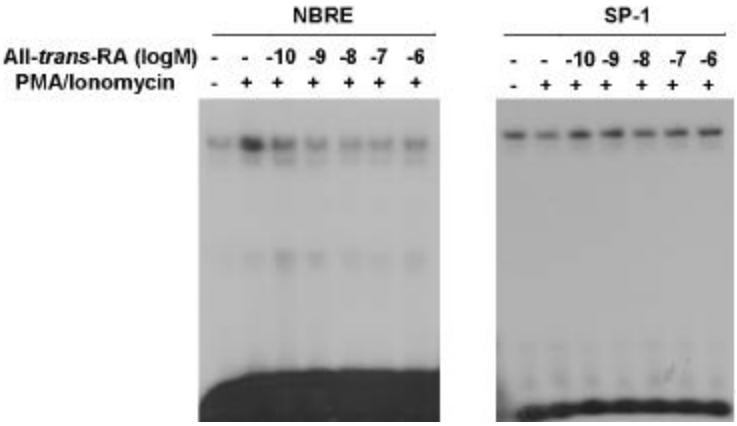

Figure 4. All-*trans*-RA repressed DNA binding of Nur77 in Jurkat cells. Jurkat cells ($3x10^6$ cells) were treated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr with or without of the indicated concentration of all-*trans*-RA pre-treatment for 24 hr as indicated. The reaction mixture containing 5 μg nuclear extract was incubated with ^{32}P-labeled oligonucleotide *i.e.* NBRE, and analyzed by gel shift assay. The binding of SP-1 was shown as a control.

To confirm these inhibitory effects of all-*trans*-RA on Nur77 gene expression, a Nur77 promoter-Luc reporter containing 511 bp (-454 to +57) of genomic DNA sequences was constructed. Transient transfection of the reporter gene into Jurkat cells showed about an 8.5-fold activation of the reporter by PMA/Ionomycin treatment. When the cells were pre-incubated with 1.0 μM all-*trans*-RA, the reporter gene activity was repressed approximately 40% (Figure 5B). The results obtained from both Northern blotting and reporter gene analysis clearly showed that all-*trans*-RA decreased the transcriptional expression of Nur77.

4. The AP-1-like motif in Nur77 promoter conferred responsiveness to RA.

The Nur77 promoter (-454 to +57) contains several potential *cis*-acting regulatory elements; three SP-1 binding sequences, four AP-1-like binding sequences and two related serum response factor (RSRF). However, there are no consensus retinoid response elements present in the region, suggesting that retinoid receptors may not bind directly to this portion of the Nur77 promoter.

To identify potential *trans*-acting factors that are responsible for RA-induced down-regulation of Nur77 promoter, the DNA binding of AP-1, RSRF and SP-1 was examined. PMA/Ionomycin treatment significantly induced DNA binding on AP-1-like sequences in nuclear extract obtained from Jurkat cells. An oligonucleotide encoding consensus AP-1 as well as antibodies against c-Jun and c-Fos effectively abolished the binding indicating that the binding

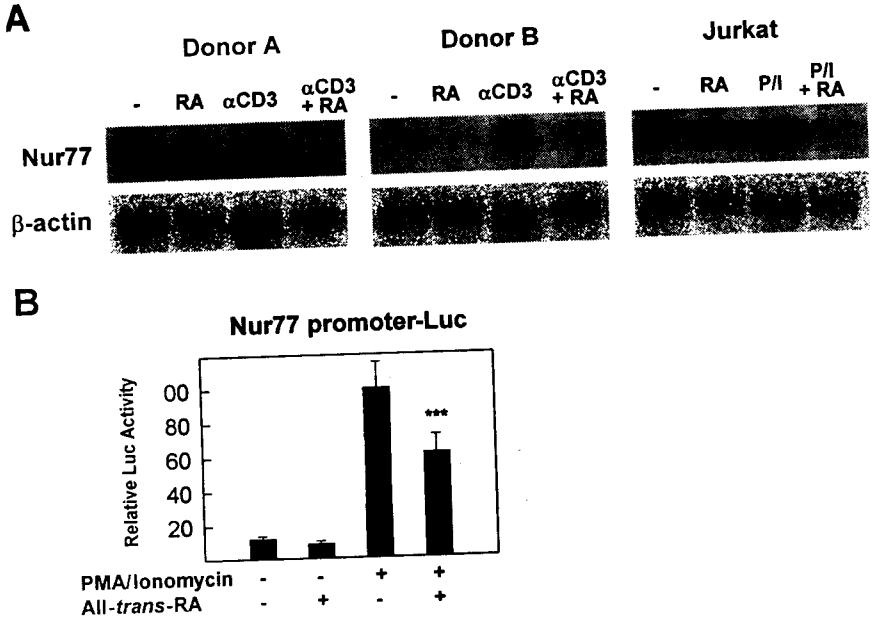

Figure 5. All-*trans*-RA repressed transcriptional expression of Nur77. (A) Effect of all-*trans*-RA on the expression of Nur77 transcripts was examined by Northern blot analysis. PBMCs ($7x10^6$ cells) obtained from a healthy donor was stimulated in a 100 cm^2 dishes pre-coated with anti-CD3 antibodies for 4 hr with or without 24 hr of 1.0 μM all-*trans*-RA pre-treatment. Jurkat cells ($3x10^6$ cells) were treated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr with or without 24 hr of 0.1 μM all-*trans*-RA pre-treatment. The expression of β-actin was monitored as a control. (B) All-*trans*-RA down-regulated transcriptional activity of Nur77 promoter. The Nur77 promoter (-454 to +57)-Luc reporter together with β-gal expression vector was transiently transfected into Jurkat cells. Transfected cells were treated with or without 1.0 μM all-*trans*-RA for 24 hr and further incubated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr. After incubation, luciferase activity was measured and normalized by β-gal activity. Data represent mean $\pm$ SD of four independent experiments. ***, $p<0.001$ *vs* PMA/Ionomycin alone treated.

complex consisted of AP-1 (Figure 6). All-*trans*-RA and 9-*cis*-RA significantly reduced both the basal and PMA/Ionomycin-induced DNA binding on AP-1-like sequences (Figure 6). In contrast, any significant changes on RSRF binding in nuclear extract obtained from Jurkat after treatment with PMA/Ionomycin in the presence or absence of RA was not observed the activities (Figure 6).

To confirm the DNA binding of AP-1 and RSRF, reporter genes encoding AP-1 and RSRF were measured. Consistent with the DNA binding results, AP-1-RE-Luc reporter gene activity induced by PMA/Ionomycin stimulation was repressed by all-*trans*-RA treatment in Jurkat (Figure 7). The RSRF-RE-Luc activity-induced by PMA/Ionomycin stimulation was unchanged by all-*trans*-RA treatment, while CsA, which blocks the Ca^{2+} signaling pathway, completely abolished the reporter activity (Figure 7). Thus, the data indicated that all-*trans*-RA preferentially inhibited the PKC signaling pathway rather than the calcium signaling pathway.

5. Retinoid receptors repressed transcriptional activity of Nur77 response elements *via* direct protein-protein interaction.

The cross-talk between Nur77 and retinoid receptors could take place at different levels during the transactivation of Nur77 *in vivo*. To test whether RARα binds to Nur77 in mammalian cells, mammalian two-hybrid assay that reproduces the heterodimeric receptor-receptor interactions was performed. As shown in Figure 8, co-transfection of pVP16-RARα into CV-1 cells increased

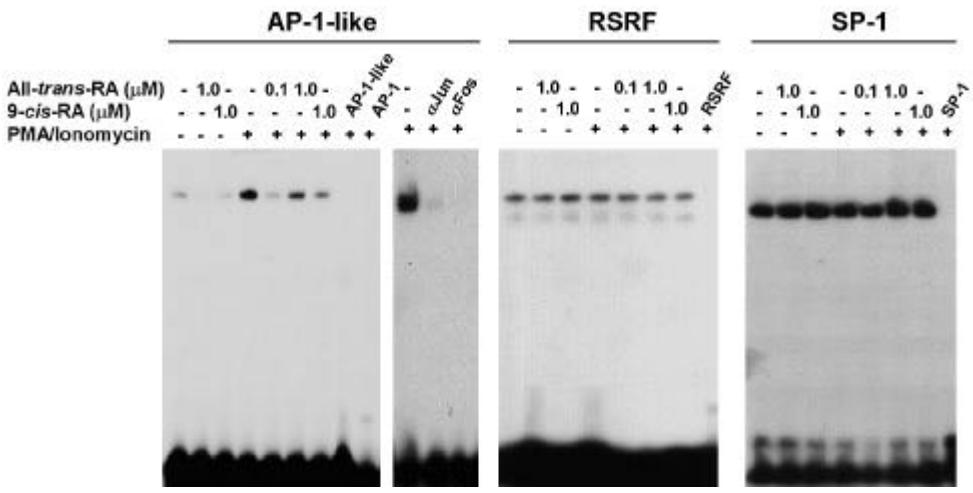

Figure 6. AP-1-like binding motif in Nur77 promoter conferred responsiveness to all-*trans*-RA. Jurkat cells were treated with PMA (10 ng/ml)/Ionomycin (0.5 μM) with or without 24 hr of all-*trans*-RA or 9-*cis*-RA pre-treatment as indicated. The reaction mixtures containing 5 μg of nuclear extract were incubated with ^{32}P-labeled oligonucleotide, *i.e.* AP-1-like or RSRF, and analyzed by gel shift assay. AP-1-like, AP-1, RSRF and SP-1 indicate 100-fold excess amount of each unlabeled probe used for competition to show specificity of the binding. Anti-c-Jun antibody (αJun) and anti-c-Fos antibody (αFos) were used to show specific binding of AP-1.

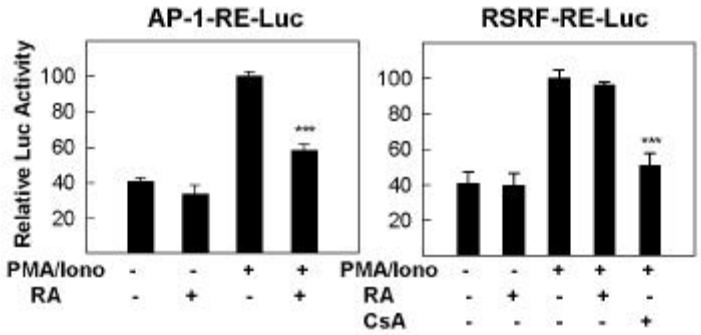

Figure 7. Effects of all-*trans*-RA on the transcriptional activities of AP-1-RE and RSRF-RE reporter genes. The indicated reporter gene together with β-gal expression vector was transiently transfected into Jurkat. Transfected cells were treated with or without 1.0 μM all-*trans*-RA for 24 hr and further incubated with PMA (10 ng/ml)/Ionomycin (0.5 μM) for 6 hr as indicated. After incubation, luciferase activity was measured and normalized by β-gal activity. Data represent mean ± SD of four independent experiments. ***, $p<0.001$ *vs* PMA/Ionomycin

the Gal4/Nur77$_{Hga}$-mediated induction of the Gal4-*tk*-Luc reporter gene activity in a dose-dependent manner, whereas pVP16 alone did not. Addition of all-*trans*-RA further enhanced the reporter gene activity. Co-transfection of pVP16-RXRα induced a strong activation, which was consistent with the previous result.[16] These results demonstrated the physical interaction of Nur77 with retinoid receptors. To assess the functional consequences of these interactions, an increasing amount of RARα or RXRα was co-transfected with a human Nur77 expression vector and the reporter constructs encoding NurRE or NBRE into CV-1 cells. As shown in Figure 9, RARα effectively repressed the transcriptional activity of both NurRE-*pomc*-Luc and NBRE-*tk*-Luc. When all-*trans*-RA was present, the inhibition by RARα was further enhanced on both reporters. RXRα repressed the activities of NurRE-*pomc*-Luc, but not that of NBRE-*tk*-Luc (Figure 9), indicating different nature of receptor complex activating each response element. The results also showed that the inhibition by retinoid receptors was not due to non-specific squelching effects resulted from overexpression of receptors. These results suggests that retinoid receptors antagonized the transcriptional function of Nur77 at the post-transcriptional level, possibly through mechanisms including direct protein-protein interactions.

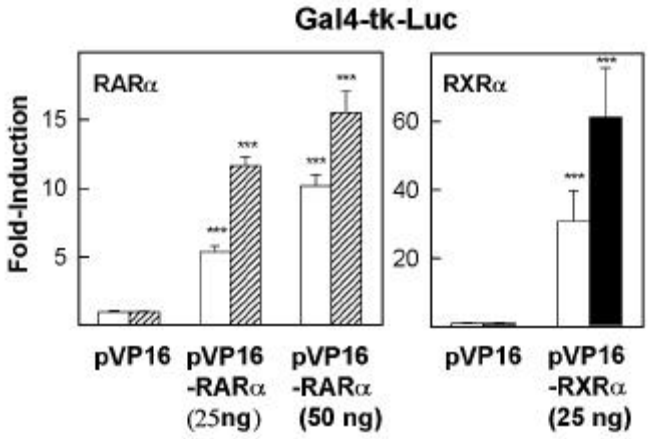

Figure 8. Interaction between Nur77 and retinoid receptors in mammalian cells. CV-1 cells were transfected with Gal4-*tk*-Luc (0.1 μg) and pM-Nur77$_{Hga}$ (50 ng) along with pVP16 (25 ng), pVP16-RARα (25 and 50 ng) or pVP16-RXRα (25 ng). Transfected cells were incubated for 24 hr in the absence (empty bar) or presence of 1.0 μM all-*trans*-RA (hatched bar) or 9-*cis*-RA (filled bar) and then assayed for luciferase activity. The corresponding β-gal activity was used to normalize luciferase activity. Data represent mean ± SD of three independent experiments. ***, $p<0.001$ *vs* pVP16 transfected.

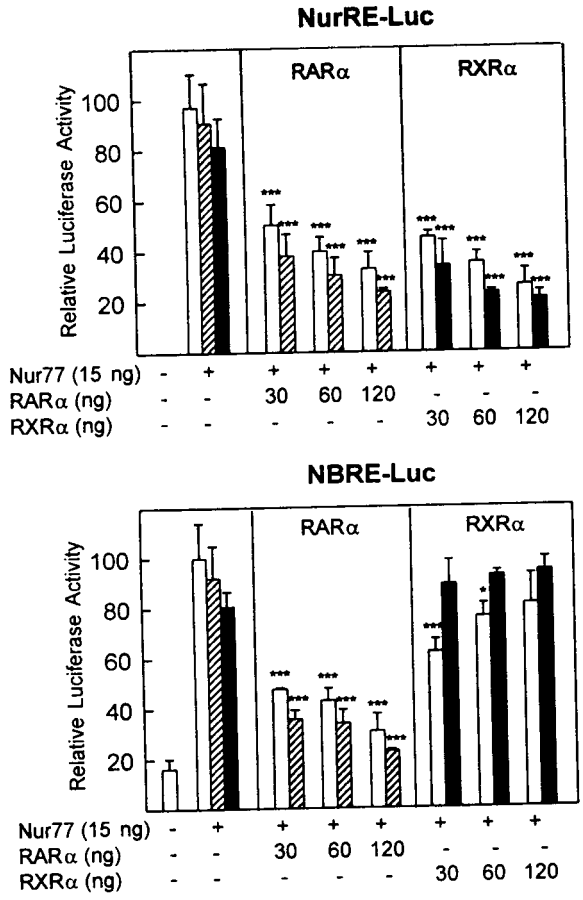

Figure 9. Retinoid receptors repressed transcriptional activity of the Nur77 response elements. NurRE-*pomc*-Luc (0.1 μg) or NBRE-*tk*-Luc (0.1 μg) was cotransfected with the indicated combinations of receptor expression vectors of pECE-Nur77, pECE-RAR α and pECE-RXR α into CV-1 cells. Transfected cells were incubated for 24 hr in the absence (empty bar) or presence of 1.0 μM all-*trans*-RA (hatched bar) or 9-*cis*-RA (filled bar) and then assayed for luciferase activity. The corresponding β-gal activity was used to normalize luciferase activity. Data represent mean $\pm$ SD of four independent experiments. *, $p<0.05$ and ***, $p<0.001$ *vs* Nur77 alone transfected.

IV. Discussion

Transcriptional activation as well as a high-level induction of Nur77 has been thought as a key step in the activation-induced apoptosis of T-lymphocytes.[19-23] RA has been shown to inhibit the activation-induced apoptosis of immature thymocytes and T-cell hybridomas.[25-27] However, the mechanism of the inhibition has not been clearly understood. In the present investigation, the molecular mechanism of the inhibition of T-cell apoptosis by RA was studied. The results obtained from this study showed that all-*trans*-RA and its receptors repressed both the transcriptional induction and transactivation function of Nur77.

The expression of Nur77 is mainly controlled by two *cis*-acting elements, RSRF and AP-1-like binding sequences. The transcriptional activity of RSRF was known to be modulated by increasing intracellular Ca^{2+} concentration.[22] Recently, Youn *et al*,[30] reported a mechanism of RSRF activation in T-cells. They showed that RSRF was normally sequestered by Cabin1, a calcineurin inhibitor, in a transcriptionally inactive state. In contrast, when Ca^{2+} concentration was increased by TCR-mediated signaling, RSRF was dissociated from Cabin1 and activated. The other *cis*-acting element, AP-1-like sequence, was repeated four times in the promoter of Nur77. JunD, one of the AP-1 family, may bind these sites to induce transcriptional activation of Nur77.[31] In the present study, the repression of Nur77 gene transcription by RA/RAR was mediated by the AP-1-like elements (Figure 6, and 7). These results were consistent with previous data that RAR antagonized AP-1 through prevention of c-Jun phosphorylation and inhibition of c-Fos expression.[32,33] In this study,

RA significantly repressed transcriptional activation of AP-1 but not that of RSRF, suggesting that RA preferentially inhibits the PKC signaling rather than Ca^{2+} signaling in T-lymphocytes. However, interestingly, a preliminary data showed that RA also inhibited transcriptional activity of NFAT, which was activated by increased intracellular Ca^{2+} concentration. It is not known how RA influences differentially on the activity of two transcriptional factors which are regulated by Ca^{2+} signaling, and it needs to be further studied.

Possible roles for Nur77 in the induction of apoptosis in T-lymphocytes may include regulation in the expression of modulators of apoptosis. It has been shown that Fas/FasL-mediated killing was the major pathway of TCR-mediated apoptosis of T-cell hybridomas.[34,35] Transcriptional activation of Nur77 and FasL in T cell apoptosis occurred simultaneously and were similarly repressed by all-*trans*-RA.[27,28] These reports imply that Nur77 may lead apoptosis *via* the induction of FasL expression. Recently, several laboratories have reported that the FasL promoter contained the NF-*k*B, AP-1, ATF2, and NFAT-binding motives that possibly contribute to the transcriptional activation of the gene.[36,37] However, the direct binding site for Nur77 was not found in the promoter. This fact indicated that Nur77 may activate FasL by an indirect mechanism. RA significantly repressed transcriptional activity of AP-1 (Figure 6, and 7) and NFAT (data not shown), suggesting that all-*trans*-RA controls FasL expression either directly by repression on FasL promoter and/or indirectly through down-regulation of the transactivation function of Nur77.

The effects of RA are shown to be mediated through two classes of nuclear receptors, RARs and RXRs, which are ligand-dependent transcriptional factors that belong to the steroid/thyroid hormone receptor superfamily. RARα and

RXRα have been shown to mediate the inhibition of activation-induced apoptosis of thymocytes and T-cell hybridomas by retinoids.[25,26] Recently, Szondy *et al*.[26] have shown that RARα stimulation inhibited the activation-induced apoptosis, while RARγ enhanced. This result suggests that the balance between stimulation by RARα and RARγ would decide whether RA would enhance or inhibit TCR-mediated apoptosis. It would be interesting to know whether each retinoid subtype differentially modulates Nur77 function. RARα, β and γ and RXRα, were expressed in Jurkat when the transcripts were measured by RT-PCR (data not shown). However, the relative contribution of each subtype on the inhibition of TCR-induced apoptosis in theses cells is not known. Since, the TCR-mediated apoptosis was blocked by RA, the modulation of Nur77 function by RA may be mediated by RARα rather than RARγ *in vivo* in the cell.

In addition to Nur77, two other family members of, *i.e*., Nurr1 (Not 1) and Nor-1 (MINOR) have been identified.[38,39] Stimulation through the TCR leads to a prolonged and high level expression of Nur77 and Nor-1. Nurr1, on the other hand, is only expressed transiently and at a low level upon TCR engagement.[40] Interestingly, RA markedly suppressed Nur77 mRNA level but increased the Nor-1 mRNA and slightly increased Nurr1 mRNA in breast cancer cell line, MCF7, which indicated distinctive roles for each subfamily member.[41] In this study, RA dramatically repressed Nur77 mRNA level (Figure 5), but mRNA level of Nor-1 and Nurr1 was not examined. Since Nor-1 and Nur77 transactivate through the same DNA elements as Nur77, some results obtained from these experiments may represent the sum of RA effects on the Nur77 family. It should be of interest for further study as to

how RA generates differential modulation on the transcription of closely related genes in function, and the consequences of differential modulation in Nur77-mediated biological signaling in target cells and tissues.

Recently, in addition to its role in activation-induced T cell apoptosis, a large volume of data has implicated the physiological importance of Nur77. Nur77 is widely expressed in tissues, particularly in brain. Nur77 mRNA was induced corticotropin-releasing hormone (CRH) neurons in response to stress stimuli,[42] and it was also induced by neuron growth factor (NGF).[7] These reports suggest that Nur77 may have a role in neuronal signal transduction in brain. In addition, the role of Nur77 has been implicated in viral pathogenesis; the viral 40 kDa regulatory phosphoprotein Tax, which provides the basis of human T-lymphotropic virus type 1 (HTLV-1) pathogenesis, regulates Nur77 gene expression,[43] and the transcriptional activation of Nur77 was mediated by Tax through AP-1-like elements, and JunD.[44] The role of Nur77 in HTLV-1 infection may be the up-regulation of FasL which induce intensive apoptosis of the infected cells. Since RA and its receptors efficiently antagonize the function of Nur77, a diverse physiological pathways involving Nur77 may be also modulated by RA.

V. Conclusion

1. All-*trans*-RA reduced the cell death of PBMCs and Jurkat cells induced by PMA/Ionomycin, anti-CD3 antibody or anti-Fas antibody. Also, all-*trans*-RA decreased the expression of FasL which was induced by PMA/Ionomycin in Jurkat.

2. All-*trans*-RA significantly repressed the induction of DNA binding of Nur77 on NBRE and NurRE that was induced by TCR-stimulation. Also, all-*trans*-RA decreased the transcriptional induction of Nur77 during TCR-mediated apoptosis in PBMCs and Jurkat.

3. The DNA binding and reporter gene activity of AP-1-like motif in Nur77 promoter, but not RSRF, was reduced by all-*trans*-RA, suggesting that all-*trans*-RA preferentially inhibited the PKC signaling pathway rather than the calcium signaling pathway.

4. A Mammalian two-hybrid test demonstrated that Nur77 directly interact with retinoid receptors, RAR α and RXR α.

5. Co-transfection of RAR α/RXR α together with Nur77 repressed the transcriptional activity of both NBRE and NurRE reporter gene. These data suggested that RA antagonized the transcriptional function of Nur77 at the post-transcriptional level, possibly through mechanisms including direct protein-protein interactions.

In conclusion, RA repressed Nur77 function through multiple mechanism that may provide the basis for RA inhibition on the apoptosis of activated T-lymphocytes.

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국문요약

T 임파구에서 레티노이드와 그 수용체가 Nur77의 전사 기능에 미치는 영향

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T-세포에서 세포 고사(apoptosis)는 T-세포의 발달 과정, 면역 반응의 정도와 지속 기간을 조절하는 중요한 기전이다. Nur77은 고아 핵 수용체(orphan nuclear receptor)로서 T-세포 수용체 매개 세포 고사(TCR-mediated apoptosis)에서 빠르게 그 발현에 증가됨으로써 세포 고사 과정에서 중요한 역할을 하며, 한편 레틴산(Retinoic acid;RA)은 미성숙 가슴선 세포(immature thymocyte)와 T-세포 하이브리도마에서 T-세포 수용체 매개 세포 고사를 억제한다고 보고되고 있다. 이에 본 연구 논문에서는 레티노이드에 의한 T-세포 수용체 매개 세포 고사의 억제 기전을 알아보기 위해, 사람 말초 혈액 단핵 세포구와 T-세포 백혈병 세포주인 Jurkat에서 Nur77의 발현과 전사활성이 레티노이드에 의해 조절되는 지 살펴보았다. All-*trans*-RA는 이들 T-임파구에서 T-세포 수용체 매개 세포 고사를 억제하였으며, 동시에 Fas ligand의 발현 유도를 감소시켰다. All-*trans*-RA는 NBRE와 NurRE 모두에서 Nur77의 DNA 결합능력을 현저히 감소시켰으며, 또한 Nur77의 발현도 감소시켰다. 레티노이드는 Nur77에 존재하는 *cis*-acting element 중 AP-1-like의 DNA 결합능력과 전사활성을 감소시킨 반면 RSRF에는 아무런 영향을 주지 않았다. 따라서 레틴산은 Nur77 promoter의 AP-1-like site를 통해

Nur77의 발현을 조절하는 것이라고 생각된다. 다음으로, 레티노이드 수용체를 통해 Nur77의 전사 활성이 조절되는 지를 알아보고자 하였다. Mammalian two-hybrid test을 통해 Nur77은 retinoid X 수용체 뿐만 아니라 RA 수용체 (RAR)와도 결합할 수 있음을 알 수 있었다. Nur77과 RAR/RXR co-transfection assay를 수행한 결과 NBRE-Luc와 NurRE-Luc 모두에서 레티노이드 수용체에 의해 Nur77의 전사활성이 억제되는 것을 확인할 수 있었으며, 이는 레티노이드가 Nur77의 기능을 전사 과정 뿐만 아니라 전사 후의 조절에 의해 억제하고 있음을 제시한다. 이러한 결과들로써 레티노이드가 세포 고사에서 중요한 기능을 하고 있는 Nur77를 다양한 분자생물학적 기전을 통하여 조절함이 증명되었으며, 이는 레티노이드가 T-세포 수용체 매개 세포 고사를 억제하는 주요 기전으로 사료된다.

핵심되는 말 : Nur77, 레티노이드, T-세포 수용체 매개 세포 고사