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**Differential roles of Annexin A1 (ANXA1/lipocortin-1/lipomodulin) and
thioredoxin binding protein-2 (TBP-2/VDUP1/TXNIP) in glucocorticoid signaling
of HTLV-I-transformed T cells**

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Summary

Glucocorticoid (GC) is widely used for therapeutic purposes in immunological and hematological disorders. Annexin A1 (ANXA1/lipocortin-1/lipomodulin), a GC-inducible molecule, was regarded as a vital anti-inflammatory mediator of GC. Thioredoxin binding protein-2 (TBP-2/VDUP1/TXNIP), a regulator of redox reactions, cell growth and lipid metabolism, was also reportedly induced by GC. HTLV-I infected T cells undergo the transition from the IL-2 dependent to IL-2 independent growth during the long-term culture in vitro. We found that these T cells responded to GC with growth arrest and apoptosis in the IL-2 dependent growth stage, whereas they failed to respond to GC after their growth had shifted into the IL-2 independent stage. Here we employed these T cell lines and studied the roles of ANXA1 and TBP-2 in mediating GC-induced apoptosis. In GC-sensitive T cells, ANXA1 expression was negligible and unaffected by GC treatment, whereas TBP-2 was expressed and induced by GC treatment. In GC-resistant T cells, however, ANXA1 was highly expressed regardless of GC treatment and promoted cellular proliferation. In contrast, TBP-2 expression was lost and could not mediate the GC-induced apoptosis. In conclusion, these results suggest that TBP-2, but not ANXA1, is directly involved in the switching of GC sensitivity and GC resistance in HTLV-I infected T cell lines, whereas ANXA1 may be

a biomarker indicative of the advanced stage of the transformation.

Keywords: Human T-cell leukemia virus type 1, Adult T cell leukemia,
Glucocorticoid, Annexin A1, Thioredoxin binding protein-2/Thioredoxin interacting
protein, Apoptosis

1. Introduction

As one of the most frequently prescribed drugs worldwide, glucocorticoid (GC) is a milestone in the history of drug discovery. The pleiomorphic effects of GC have been well described, including its effects on immune regulation, immune development, metabolism, and cell death[1,2]. Due to its potent apoptosis-inducing effects, GC has been commonly used in the treatment of lymphoid malignancies[3]. Extensive studies have demonstrated that GC response is mainly mediated through the ligand-bound glucocorticoid receptor (GR), which further regulates the expression of multiple downstream genes in the nucleus. It has been estimated that around 1% genes of the human genome can be regulated by GC[4]. It is now known that GC regulates the target genes in a cell-type-specific manner, which may explain the biological effects of GC in specific situations. Therefore, it is important to decipher how the molecules involved in the GC signaling pathways are functionally integrated in vivo.

Annexin A1 (ANXA1/lipocortin-1/lipomodulin), belonging to the Ca^{2+} -dependent phospholipid-binding superfamily of annexin proteins, can be induced by GC in different cell types. The expression of ANXA1 was regulated by the ligand-bound GR, due to the existence of glucocorticoid response element (GRE) in the promoter area of this gene[5,6]. Since ANXA1 was reported to inhibit the activity of phospholipase A2,

the GR/ANXA1 pathway is commonly considered to be an endogenous pathway mediating the anti-inflammatory actions of GC[4]. Furthermore, it has been reported that ANXA1 plays diverse roles in cell proliferation and differentiation, apoptosis, protein traffic, and membrane fusion[7]. Although many studies suggested the importance of ANXA1 in carcinogenesis, the conclusions were inconsistent[8-11]. Therefore, it has been suggested that ANXA1 may behave in a cell-type-specific manner when exerting its specific functions.

Thioredoxin binding protein-2 (TBP-2/VDUP1/TXNIP), identified as an endogenous binding partner and negative regulator of thioredoxin (TRX) [12], was reported to play a critical role in cell proliferation and lipid metabolism[13-15]. In separate studies, both TBP-2 and TRX were reported to be involved in the regulation of the immune responses in vivo[16-18]. Very recently, redox regulation by TBP-2/thioredoxin system was reported to play a key role in the activity of NLRP3 inflammasome, a regulator of innate immunity[19]. GC was reported to induce TBP-2 in murine thymocytes and murine T-cell lymphoma line WEHI7.1[20], although the significance of TBP-2 in the mediation of GC responses has not been widely recognized.

In order to explore the roles of GR/ANXA1 and GR/TBP-2 in mediating the effect

of GC-induced apoptosis, we utilized a unique model of cultured T cell lines infected by human T cell leukemia virus type I (HTLV-I) virus[21,22]. Adult T cell leukemia (ATL) and other autoimmune-like disorders are caused by HTLV-I infection and developed after decades of incubation periods[23-27]. Clinically, although GC has been included in the standard therapies for ATL, this reagent is normally effective only in the early stage of the disease, suggesting the impairment of GC signaling pathway in the late stage of ATL. HTLV-I-transformed T cell lines were cultured in the presence of IL-2 when established from ATL patients, although their growth tended to be free from the requirement of IL-2 during long-term cultures[21,22]. Previously, we reported the loss of TBP-2 in IL-2-independent HTLV-I infected T cells[13,28]. Therefore these cell lines may provide a suitable model for studying the dysregulation of GC responses.

In the present study, we found that T cells in the IL-2 dependent growth stage were sensitive to the GC-induced apoptosis, whereas the same T cells became to GC-resistant after their growth had shifted into the IL-2 independent stage. We found that TBP-2, but not ANXA1, was directly involved in GC responses in GC-sensitive ATL cells. In GC-resistant ATL cells, TBP-2 expression was lost and could not mediate the effect of GC. Furthermore, we found that ANXA1 was constitutively highly expressed in GC-resistant ATL cells, which suggested a role of ANXA1 in the proliferation of the

advanced stage of ATL cells.

2. Materials and methods

2.1. Cell cultures

HTLV-I-transformed T cells were cultured in RPMI 1640 medium (Sigma-Aldrich, MO) containing 10 % heat-inactivated fetal calf serum (Invitrogen, CA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Nacalai Tesque, Kyoto, Japan) at 37°C in a humid atmosphere of 5 % CO₂ in air. To maintain IL-2-dependent T cells, recombinant human IL-2 (1 U/ml; PeproTech EC, London, UK or Shionogi and Co., Osaka, Japan) was further added to the culture medium. Each set of IL-2-dependent and IL-2-independent cells has the same clonal origin, as confirmed by the T-cell receptor-β gene rearrangement and HTLV-I proviral integration sites[21,22,29].

2.2. Plasmids

The full-length cDNA of human TBP-2 was cloned in-frame into the pEGFP-C1 vector (Clontech, CA). Briefly, when constructing the pEGFP-C1-TBP-2 plasmid, the cDNA was first amplified by PCR, using two primers (Forward: 5'-GAATTCTATGGTGATGTTCAAGAAGAT, and Reverse: 5'-GTCGACTCACTGCACATTGTTGTTGA). The PCR product was then subcloned

into the pCR-BluntII-TOPO vector (Invitrogen, CA) to obtain pCR-BluntII-TOPO-TBP-2, which was further digested with EcoRI and SalI restriction enzymes (TOYOBO, Tokyo, Japan). The fragment was finally cloned into the pEGFP-C1 vector to obtain pEGFP-C1-TBP-2 plasmid. The plasmid was verified by DNA sequencing before it was used.

2.3. Transient transfection and RNA interference (RNAi) assay

Plasmids (TBP-2 expression vector or control vectors) were transfected into cells (1×10^6 cells) employing the Nucleofector II and AMAXA cell line kit V (Lonza Cologne, Cologne, Germany), according to the manufacturer's instruction. 2 μ g of DNA was used for each transfection. Expression of the target protein was verified by western blotting and by fluorescent microscopy. In the RNA interference assay, 100 pmol of duplex oligonucleotides (RNAi#1:UUAGUUAGAAUGUCAAGAUGGUUG, or RNAi#2: UAACCAUUAUGGCCUUAUGCAAGGC) (Invitrogen, CA) for selective silencing of the ANXA1 or control oligonucleotides (Stealth RNAi negative control) (Invitrogen, CA) were transfected respectively into cells (1×10^6 cells each time), employing the Nucleofector II and AMAXA cell line kit V.

2.4. Treatment with glucocorticoid or other reagents

IL-2 dependent ED40515 T cells (D-ED T cells) were first deprived of IL-2 for 24

hours to exclude the carry-over effect of IL-2 on growth proliferation. Then, viable cells were enriched using LSM Lymphocyte Separation Medium (MP Biomedicals, OH) according to the manufacturer's instructions. Dexamethasone (Nacalai Tesque, Kyoto, Japan) or GC antagonist RU486 (Sigma-Aldrich, CA) at the indicated concentration, were further added to the culture medium, while ethanol (0.004 % v/v) was added as a vehicle control. For IL-2 independent ED40515 T cells (I-ED T cells), dexamethasone (Nacalai Tesque, Kyoto, Japan) at the indicated concentration was added directly to the cells.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions. 1 µg total RNA was used as a template for cDNA synthesis employing the PrimeScript RT reagent Kit (TAKARA, Otsu, Japan) according to the manufacturer's instructions. A 1:20 fraction of each reverse transcriptase reaction mixture was further used as a template for semi-quantitative RT-PCR employing the KODPlus polymerase kit (TOYOBO, Tokyo, Japan) or real-time quantitative RT-PCR employing the SYBR Premix Ex TaqII kit (TAKARA, Otsu, Japan). The primers used for the amplifications were as follows: ANXA1, 5'-GCAGGCCTGGTTTATTGAAA (forward) and 5'-GCTGTGCATTGTTTCGCTTA (Reverse); β-actin,

5'-GGACTTCGAGCAAGAGATGG (forward) and
5'-AGCACTGTGTTGGCGTACAG (reverse); TBP-2,
5'-GCCACACTTACCTTGCCAAT-3' (forward) and
5'-GGAGGAGCTTCTGGGGTATC-3' (reverse), or GAPDH,
5'-ACCCACTCCTCCACCTTTG-3' (forward) and
5'-CTCTTGCTCTTGCTGGG-3' (reverse). Semi-quantitative RT-PCR was
performed employing under the following conditions: 25 cycles for ANXA1
(denaturing at 94°C for 15 s, annealing at 55°C for 30 min, and extension at 68°C for 1
min), 22 cycles for β -actin (denaturing at 94°C for 15 s, annealing at 55°C for 30 min,
and extension at 68°C for 1 min). The PCR products were visualized by electrophoresis
in 2 % agarose gel (Nacalai Tesque, Kyoto, Japan). For real-time quantitative RT-PCR,
fluorescent detection and analysis were performed on an ABI Prism 7000 Sequence
Detection System (Applied Biosystems, CA) under the conditions: 95°C for 30 s, 40
cycles of amplification (95°C for 5 s and 60°C for 31 s), followed by a dissociation
stage (95°C for 15 s, 60°C for 1 min and 95°C for 15 s).

2.6. Immunoblotting

After washing twice in cold PBS, cell pellets were resuspended in lysis buffer (0.5
% NP-40, 50 mM Tris-Cl pH7.2, 150 mM NaCl) supplemented with 1× protease

inhibitor cocktail (Roche, Tokyo, Japan) and 1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque, Kyoto, Japan). Cell lysates were fractionated by SDS-PAGE using 4-12 % Nupage Bis-Tris pre-cast gel (Invitrogen, CA) and further transferred to PVDF membranes (GE lifesciences, PA). For immunoblotting, primary anti-ANXA1 antibody (H-65, 1:200, Santa Cruz, CA), anti- β -actin (1:2000, Sigma-Aldrich, MO or ACTBD11B7, 1:200, Santa Cruz, CA), anti- α -tubulin (1:2000, Sigma-Aldrich, MO), anti-cleaved caspase-3 (1:1000, Cell Signaling, MA), anti-PARP (1:1000, Cell Signaling, MA), or anti-GFP (1:1000, Nacalai Tesque, Kyoto, Japan) were probed respectively. HRP-conjugated secondary anti-mouse-IgG or anti-rabbit-IgG (GE lifesciences, PA) was further applied according to the species of the primary antibodies. The whole process of immunoblotting is performed by SNAP i.d. protein detection system (Millipore, MA). Finally, luminescence was detected using a Chemi-Lumi One L kit (Nacalai Tesque, Kyoto, Japan).

2.7. Flow cytometry assay

After washing twice in PBS, cell pellets were resuspended in 1 $\times$ Annexin V binding buffer (BD Pharmingen, CA). For apoptosis analysis, cells were immediately stained using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, CA) and detected employing the BD FACSCanto II Flow Cytometry System (BD biosciences, CA). After

appropriate gating to exclude debris population, Annexin V-positive and propidium iodide (PI)-negative quadrant was assessed to represent the population undergoing early apoptosis, while the Annexin V-positive population was used to assess the overall cell apoptosis. Data in triplicate were analyzed using FlowJo software (Treestar, OR) and column graphs were made using Prism 5.0 software (Graphpad, La Jolla, CA). For PI staining assays, cells were first fixed with 70 % ethanol and then treated with 5 µg/ml RNaseA (Sigma-Aldrich, MO). Cells were stained with 50 µg/ml propidium iodide (Nacalai Tesque, Kyoto, Japan) and filtered through 40 µm nylon mesh (BD Falcon) before detection. Data in triplicate were analyzed using FlowJo software (Treestar, OR) and the Sub-G1 population shown in the histogram represents the cells undergoing apoptosis.

2.8. Cell proliferation assay

Cells ($0.5-1 \times 10^4$ cells) were seeded in 96-well flat-bottom microtitre culture plates (Corning, NY). Cell growth was monitored using SF cell count reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Briefly, colored substrate of formazan, derived from reduced tetrazolium salt (WST-8) in viable cells after SF reagent addition, was measured using a Microplate Reader (Molecular devices, CA) at OD450-650 nm wavelengths. In addition, a cell proliferation BrdU ELISA kit (Roche,

Tokyo) was also used in I-ED T cells according to the manufacturer's instructions.

2.9. Statistical analysis

Results obtained from the independent experiments (n=3-6) were shown as mean±SD. Statistical analysis was assessed using the unpaired Student's *t*-test (two-tailed). The results were considered statistically significant when $p < 0.05$.

3. Results

3.1. T cells were sensitive to GC in the IL-2 dependent growth stage of HTLV-I-induced transformation

Loss of GC efficacy is often observed in the treatment of the late stage ATL patients, while an in vivo model of multi-step transformation by HTLV-I has been postulated[30]. Consistently, HTLV-I infected T cell lines in vitro, go through the transition from the IL-2-dependent to IL-2-independent growth stage. Thus, we hypothesized that the early and late stages of HTLV-I infected T cell lines show differential sensitivities to GC.

To explore this hypothesis, we utilized ED40515 T-cell lines, which include the IL-2-dependent ED40515 T cell line (D-ED T cells) and the IL-2-independent ED40515 T cell line (I-ED T cells), and tested the sensitivity of T cells under dexamethasone treatment (1 μ M Dex). In accordance with the hypothesis, D-ED T cells showed growth

retardation after the GC treatment (Fig. 1A) and GC induced apoptosis in a dose-dependent manner (Fig. 1B). However, I-ED T cells were resistant to GC in both cell growth and apoptosis (Fig. 1C, 1D).

3.2. Dysregulated expressions of *ANXA1* and *TBP-2* in the GC-sensitive T cells

In view of the previous reports that *ANXA1* and *TBP-2* are involved in GC signaling, we first investigated the expressions of both genes in HTLV-I-transformed T cells. Unexpectedly, *ANXA1* expression, at both mRNA (Fig. 2A, 2B) and protein (Fig. 2C) levels, was constitutively high in four separate IL-2 independent T cell lines (“I” stage of ED40515, ATL43, ATL2, ATL35), all of which were resistant to GC treatment. However in IL-2 dependent T cell lines (“D” stage), which harbored intrinsic sensitivities to GC, the expression of *ANXA1* was negligible, as shown in Fig 2A, 2B, 2C.

TBP-2 expression was investigated in HTLV-I infected T cell lines (ED40515, ATL43, ATL2, Sez627) as a reasonable target molecule of GC in human T cells. In accordance with our previous report that *TBP-2* expression was lost in T cells during the transformation by HTLV-I, *TBP-2* was expressed only in IL-2-dependent T cell lines (Fig. 2D).

3.3. *TBP-2*, rather than *ANXA1*, played a role in mediating GC-induced apoptosis in the

GC-sensitive T cells

Due to the differential expression patterns of ANXA1 and TBP-2 in GC-sensitive T cells, we investigated whether either of the molecules could mediate GC effects, as reported by others[5,20]. In GC-sensitive ATL cells (D-ED T cells), ANXA1 remained unchanged after Dex treatment (21.5, 48, 72 hours), compared with the treatment using vehicle controls (Fig. 3A). On the contrary, at the same time, TBP-2 was significantly induced after Dex treatment (Fig. 3B). It seems more plausible that TBP-2, rather than ANXA1, mediated the GC-induced apoptosis in the D-ED T cells. To confirm that TBP-2 is directly regulated by GR, GR antagonist (RU486) was included in the D-ED cell cultures. RU486 treatment blocked the TBP-2 induction by GC(Fig. 3C), confirming that the expression of TBP-2 was regulated by GR. Inclusion of RU486 also blocked the GC-induced apoptosis, as shown by the inability to cleave apoptotic marker nuclear poly (ADP-ribose) polymerase (PARP) (Fig. 3C). Furthermore, in GC-resistant ATL cells (I-ED T cells), ANXA1 and TBP-2 remained unchanged after Dex treatment (72 hours), compared with treatment using vehicle controls (Fig. 3D and 3E). These data suggest the role of TBP-2, but not the ANXA1, in the response of the GC-sensitive ATL cells to GC, while the loss of TBP-2 is involved in the loss of GC sensitivity.

3.4. Constitutively high expression of ANXA1 promoted cell proliferation in the

GC-resistant T cells

Since the GC sensitivity of HTLV-I infected T cells and ANXA1 expression changed during the viral transformation, there may be a link between ANXA1 overexpression and GC resistance. To address this possibility, we investigated the knockdown effect of ANXA1 on cell proliferation in IL-2 independent ED40515 T cells (I-ED T cells). Even in the absence of GC treatment, I-ED cells knocked down by siRNAs targeted to ANXA1 (Fig.4A) showed a retardation of cell growth (Fig.4B) and enhanced cell apoptosis (Fig.4C and 4D), compared with the treatment of control siRNAs. These data suggest that the constitutively high expression of ANXA1 contributes to the promotion of cell proliferation in I-ED T cells, representing an advanced stage of leukemogenesis, during which T cells are multi-resistant to apoptosis induced by chemotherapies including GC.

Finally, we explored whether ectopic expression of TBP-2 in GC-resistant T cells could mediate apoptosis. As shown in Figure 4E and 4F, transfection of TBP-2 in I-ED cells inhibited cell growth and induced apoptosis at 72 hours after treatment. These data further suggest that TBP-2 is involved in GC-induced apoptosis, while impairment of the GR/TBP-2 pathway in an advanced stage of leukemogenesis leads to the GC resistance.

4. Discussion

The role of GC-induced apoptosis in regulating the immune system is important under physiological or pathological conditions. GC-induced apoptosis is implicated in the development of the immune repertoire and regulation of the immune responses. On the other hand, GC has been used for therapeutic purposes to induce apoptosis in hematological malignancies.

The HTLV-I infected T cell lines, established from ATL patients, were cultured in vitro initially in the presence of IL-2 with the spontaneous transition from the IL-2 dependent to IL-2 independent growth stage, which could mimic the multi-step transformation of T cells by HTLV-I virus. In this study we showed that the GC sensitivity of T cells changed during the in vitro transformation. IL-2 dependent ATL cells (D-ED T cells), which mimic the early stage of transformation are sensitive to GC, while IL-2 independent ATL cells (I-ED T cells), which mimic the late stage of transformation are resistant to GC. Therefore, these HTLV-I infected T cell lines may provide useful models for the study of the molecular mechanisms of GC responses. Our findings may also be useful in studying GC effects in other immunological disorders, such as allergy, asthma and autoimmune diseases.

The pleiomorphic functions of GC are mainly achieved through genomic

mechanisms, although minor non-genomic mechanisms have also been reported[31].

The genomic mechanism of GC function is the regulation of target gene expression, which is mediated by the ligand-bound glucocorticoid receptor (GR). It was considered that ANXA1, a GC-inducible gene, played a key role in mediating the anti-inflammatory and anti-proliferative effects of GC[4]. TBP-2 was reported to be induced by GC treatment in murine lymphoma cells WEHI7.2[20]. In our study, we proposed differential roles of ANXA1/TBP-2 in the mediation of GC-induced apoptosis in HTLV-I infected T cells. Unexpectedly, the classical GR/ANXA1 signal pathway was not effective in mediating GC-induced apoptosis in HTLV-I infected T cells.

Although the mechanism of this defect is still elusive, the significant suppression of ANXA1 in GC-sensitive ATL cells seems to be relevant to the impairment of the GR/ANXA1 pathway in these cells. It appeared that GC was unable to affect the expression of ANXA1. Interestingly, the classical way to induce ANXA1 expression by GC has been challenged in recent studies conducted on mice and humans[32,33]. On the contrary, we found that the GR/TBP-2 signal pathway was not impaired. TBP-2 was expressed and induced by GC in the GC-sensitive ATL cells. TBP-2 expression in response to GC was directly regulated through GR, since a GR antagonist blocked the TBP-2 induction. TBP-2 knockdown abrogated the GC-induced apoptosis in

GC-sensitive T cells (manuscript in preparation). Therefore, for GC-sensitive T cells representing the early stage of viral transformation, TBP-2 is more important than ANXA1 in mediating the effects of GC.

On the other hand, in IL-2-independent HTLV-I infected T cells, which are resistant to GC, the expression of TBP-2 was lost due to epigenetic modifications[28]. In fact, the expression of GR was also suppressed (data not shown), leading to the complete impairment of the GR/TBP-2 pathway. Here we showed that GC failed to replenish TBP-2 expression in this stage. ANXA1 expression was also unaffected by GC treatment. We have reported that stable transfectants of TBP-2 in this stage showed growth retardation in the G1 cell cycle[13]. Here we also found that transient expression of TBP-2 induced cell apoptosis. Thus the recovery of TBP-2 expression seems to be a promising strategy in treating ATL, particularly in a more advanced multi-drug resistant stage. The blockade of the GR/TBP-2 pathway is involved in the GC resistance in these cells.

Interestingly, our results showed a constitutively high expression of ANXA1 in the IL-2 independent and GC-resistant stage of HTLV-I infected T cells. ANXA1 seems to promote the growth of the leukemic cells, as ANXA1 knockdown inhibited cell proliferation and induced apoptosis. Contradictorily, in several types of cancers, the

expression of ANXA1 was reported to be significantly lower in a more advanced stage[9], leading to the assumption that it was a tumor suppressor. However it is now reconsidered that the expression of ANXA1 in cancers seems to be cell-type-specific[8,11]. Due to our findings, it may be promising to perform a large-scale clinical research to evaluate the significance of ANXA1 as a biomarker for disease progression in ATL as well as other lymphoproliferative disorders. Of note, the immunosuppressive status in ATL patients is well known[34]. As ANXA1 is reported to be an anti-inflammatory molecule, it is reasonable to hypothesize that ANXA1 overexpression is involved in the mechanism of immunosuppression in ATL patients. ANXA1, which is likely to be expressed and secreted constitutively in ATL cells, may contribute to the negative regulation of immune system in ATL patients. To address this hypothesis, further studies using fresh leukemic cells from ATL patients are scheduled.

Furthermore, there may be a possible link between ANXA1 overexpression and GC resistance in the more advanced stage of ATL. The expression of ANXA1 was reported to be constitutively higher in TNF- α resistant monocytic cells, rendering these cells resistant to TNF- α -induced apoptosis[35,36]. Moreover, we showed that the expression of ANXA1 was suppressed in IL-2 dependent and GC-sensitive T cells in an early stage of transformation by HTLV-I. However, at least in the case of ATL, more evidences are

required regarding the causative role of ANXA1 in the mediation of GC sensitivity.

Compared with ANXA1, TBP-2 is a more direct mediator of GC response since it was significantly induced in the GC-sensitive stage.

In conclusion our results suggest that TBP-2, but not ANXA1, is directly involved in the switching of GC sensitivity and GC resistance. GC resistance occurs when the expression of TBP-2 is lost. Furthermore, ANXA1 is constitutively highly expressed in GC-resistant ATL cell lines, suggesting a possible role of ANXA1 in promoting leukemogenesis and also in immunosuppression associated with HTLV-I infection.

It is also necessary to clarify the possible roles of TBP-2 in mediating the GC-dependent immunoregulatory mechanism for the control of allergy and autoimmune diseases using TBP-2 as a promising drug target.

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Figure Legends

Figure 1. T-cell is sensitive to GC in the IL-2 dependent stage of HTLV-I-induced transformation

(A, B) D-ED T cells (IL-2-dependent ED40515 T cells) were deprived of IL-2 for 24 hours to remove the carry-over effect of IL-2. Thereafter, cells were treated with different concentrations of dexamethasone (1nM, 10nM, 100nM, 1000nM) or the same amount (0.04% v/v) of empty ethanol vehicle. (A) Cell proliferation was monitored at 12, 24, 48 and 72 hours after treatment using SF cell count reagent, as described in the "Materials and methods" section. Untreated cells (-) were used as controls to further exclude any interference of growth due to ethanol. Data were shown as mean±SD (n=6 at each time point for each concentration). (B) At 48 hours after treatment, cell apoptosis was assessed by Annexin V-FITC staining. Representative histograms from a triplicate showed Annexin V-positive populations. (C, D) I-ED T cells (IL-2-independent ED40515 T cells) were treated directly with 1 μM dexamethasone or ethanol. (C) Cell proliferation was monitored before treatment or at 72 hours after treatment, using a cell proliferation ELISA BrdU (colorimetric) kit (n=3 at each time point). (D) Cell apoptosis was assessed as (B). Representative histograms from a triplicate set of experiments were shown. *P<0.05; **P<0.01; ***P<0.001.

Figure 2. Dysregulated expressions of ANXA1 and TBP-2 in T cells during the

transformation induced by HTLV-I

(A, B) Cell pellets from four paired HTLV-I infected T cell lines (ED40515, ATL2, ATL43, ATL35), undergoing IL-2 dependent or IL-2 independent growth, were used to detect the mRNA expression of Annexin A1. D: IL-2-dependent stage; I: IL-2-independent stage. (A) Semi-quantitative RT-PCR for detecting the expression of ANXA1 was performed as described in the “Materials and methods” section. β -actin served as a loading control. A representative gel image from a triplicate was shown here. (B) Quantitative RT-PCR was performed. Data of relative mRNA expressions of Annexin A1, which were normalized to the expressions of GAPDH, were shown as mean $\pm$ SD (n=3 for each cell lines). (C) Immunoblotting was performed to detect the protein expression of Annexin A1 in ED40515, ATL2, and ATL43 cell lines. α -tubulin served as a loading control. (D) Semi-quantitative RT-PCR for detecting the expression of TBP-2 was performed in ED40515, ATL2, ATL43 and Sez627 cell lines.

Figure 3. TBP-2, but not ANXA1, was a GC-regulated gene in GC-sensitive T cells

(A, B, C) D-ED T cells were treated as shown in Figure 1A. (A, B) At 21.5, 48, and 72 hours after treatment, pellets of cells treated with 1 μ M Dex or ethanol vehicle, were collected respectively. The relative mRNA expressions of ANXA1 (A) or TBP-2 (B), normalized to the expressions of GAPDH, were detected, using real-time quantitative

RT-PCR (n=4 for each sample). ***P<0.001. (C) D-ED T cells were treated with 10nM Dex, 10nM Dex and 100nM RU486, or 100nM RU486, respectively, for 48 hours. Ethanol vehicle was employed as a control. Immunoblotting was performed to detect the apoptosis markers PARP and TBP-2. α -tubulin was used as a loading control. (D, E) I-ED T cells were treated as shown in Figure 1C. At 0 and 72 hours after treatment, pellets of cells treated with 1 μ M Dex or ethanol vehicle were collected respectively. Real-time quantitative RT-PCR was performed. Relative mRNA expressions of ANXA1 (D) or TBP-2 (E), normalized to the expressions of GAPDH, were detected. TBP-2 remained undetected (“0”) in I-ED T cells within the detection cycles (n=40).

Figure 4. Constitutively high expression of ANXA1 promoted cell proliferation in GC-resistant T cells

(A, B, C, D) Two sets of specific oligonucleotides targeting ANXA1 or the negative control siRNA were transfected respectively into I-ED T cells, as described in the "Materials and methods" section. (A) At 48 hours after the transfection, knockdown efficiency was assessed using immunoblotting, and α -tubulin served as a loading control. (B) At 12, 24, and 48 hours after transfection, cell proliferations were assessed using SF cell count reagent. Data were shown as mean $\pm$ SD (n=3 at each time point for each sample) (C) At 48 hours after siRNA, cell apoptosis was assessed using double

staining of Annexin V and PI. A representative dot-blot from a triplicate of experiments was shown here. Early apoptosis proportions, shown as Annexin V⁺ and PI⁻, were plotted in the column graph. Data were shown as mean $\pm$ SD (n=3). (D) At 48 hours after siRNA, cell pellets were collected and immunoblotting was performed to detect the apoptotic marker protein, cleaved caspase-3, and α -tubulin served as a loading control. (E, F) pEGFP-C1 or pEGFP-C1-TBP-2 plasmids were transfected respectively into I-ED T cells, as described in the "Materials and methods" section. (E) At 72 hours after transfection, transfection efficiency was assessed using immunoblotting, and β -actin served as a loading control. (F) PI staining for DNA to detect apoptotic sub-G1 populations in fixed cells was employed at 72 hours after transfection, as described in "Materials and methods". A representative histogram from a triplicate of experiments was shown here. Sub-G1 proportions were plotted in a column graph.

Figure 5. A simplified scheme for the mechanism of switching GC-sensitivity and GC-resistance in the HTLV-I-transformed T cells

HTLV-I transformed T cells go through the transition from the IL-2 dependent to IL-2 independent growth stage. In IL-2 dependent growth stage, TBP-2 was expressed while ANXA1 expression was negligible. In IL-2 independent growth stage of the same cells, ANXA1 was highly expressed and TBP-2 expression was lost. As distinct from

ANXA1, TBP-2 was induced and directly involved in the growth arrest and apoptosis by GC treatment, while loss of TBP-2 induction by GC occurred in the GC-resistant T cells during the transition. ANXA1 may play a role in promoting leukemogenesis and immunosuppression in HTLV-I infection.

A

B

Cell count

C

D

A

C

B

D

IL-2 dependent growth stage -----> IL-2 independent growth stage

GC Sensitive

TBP-2 ↑
caspase activation ↑
Apoptosis ↑

GC Resistant

~~TBP-2 ↑~~
~~Apoptosis ↑~~

HTLV-I transformed T cells