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MODULATION OF DEXAMETHASONE-INDUCED THYMOCYTE APOPTOSIS BY HEAT-SHOCK PROTEIN 90-BINDING AGENTS

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Abstract

Heat-shock protein 90 (HSP90) is known to affect a variety of cellular activities. The present study showed that the HSP90-binding agents, geldanamycin, herbimycin A and radicicol, inhibited the murine thymocyte apoptosis induced by dexamethasone and was accompanied by the inhibition of the reduction of the mitochondrial transmembrane potential ($\Delta \Psi m$). HSP90-binding agents did not inhibit etoposide-induced apoptosis. The inhibition of dexamethasone-induced apoptosis was in part due to the interference of HSP90 with the glucocorticoid receptor, resulting in the inhibition of nuclear translocation of the receptor. The expression of inositol 1,4,5-triphosphate receptors, which were shown to be involved in dexamethasone-induced apoptosis, did not participate in the inhibition of apoptosis.

Key words: Thymocytes—Apoptosis—Glucocorticoid—HSP90—Geldanamycin

INTRODUCTION

Thymocyte apoptosis is involved in the clonal deletion of self-reactive T cells in the thymus during development and is physiologically regulated by cross-talk involving intracellular signal transduction through stimulation of T cell receptors or associated molecules such as CD3, glucocorticoids, and cytokines. Glucocorticoid is an important regulator of thymocyte apoptosis and elicits its effects by binding to its cognate receptor, which functions as a ligand-dependent transcription factor. In proliferating cells, glucocorticoid treatment induces changes in cell cycle components including p53, Rb, and c-myc, cdk and cyclines, followed by the induction of apoptosis. In contrast, the signaling pathway leading to apoptosis does not appear to involve changes cell cycle proteins in non-proliferating cells. However, glucocorticoids may induce the transcription of genes involved in carrying out apoptosis, because glucocorticoid-induced thymocyte apoptosis is inhibited by the inhibitors of RNA and protein synthesis, actinomycin D and cycloheximide, respectively. In addition, the transactivation domain of the glucocorticoid
receptor is required for apoptosis induction\(^3\). Two genes that mediate apoptosis in thymocytes by glucocorticoids have been reported; one is a purinergic receptor P2X\(_n\), which functions as an ATP-gated calcium channel\(^27\), and the other is the inositol 1,4,5-triphosphate receptor (IP\(_3\)R), which regulates intracellular calcium homeostasis\(^6\). The latter plays important roles in the homeostasis of mitochondrial functions\(^11\). The mitochondrial transmembrane potential (\\(\Delta \Psi_m\\)) results from an asymmetric distribution of protons and other ions on the two sides of the inner mitochondrial membrane and maintains various mitochondrial functions\(^8\). The mitochondrial permeability transition with the reduction of $\Delta \Psi_m$ regulates the opening of a large, nonspecific pore in the inner mitochondrial membrane; it has been shown to be a critical event in thymocyte apoptosis and to be regulated in part by calcium homeostasis\(^8,10\).

Thymocyte apoptosis induced by dexamethasone in the rat is specifically inhibited by herbimycin A, a potent inhibitor of protein tyrosine kinase\(^9\), although herbimycin A itself induces apoptosis in thymocytes after a longer incubation time\(^2\). Herbimycin A is now thought to exert tyrosine kinase inhibitor activity indirectly by somehow destabilizing the tyrosine kinases through interfering with the binding of these proteins to HSP90\(^19\). In the present study, we examined the effects of the HSP90-binding agents, benzoquinoid ansamycins, geldanamycin and herbimycin A, and a structurally unrelated agent, radicicol, on DNA fragmentation, the mitochondrial permeability transition, and the expression of IP\(_3\)Rs in dexamethasone- and etoposide-treated thymocytes.

**MATERIALS AND METHODS**

1. **Materials**

Herbimycin A, geldanamycin, radicicol, etoposide, dexamethasone, and 3,3'-dihexyloxacarbocyanide iodide (DiOC\(_6\)(3)) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-glucocorticoid receptor antibody (M-20) and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture materials were purchased from IWAI Kagaku Co. (Tokyo, Japan).

2. **Thymocyte culture**

Thymocyte suspensions (4–8 $\times 10^6$ cells/ml) were prepared from the thymus glands of male, 5- to 6-week-old, BALB/c mice in RPMI 1640 media supplemented with 10% fetal bovine serum, 50 $\mu$M 2-mercaptoethanol, 10 mM HEPES, and antibiotics\(^2\).

3. **Assay of DNA fragmentation**

DNA fragmentation was determined as described previously\(^2\). The percentage of DNA fragmented was defined as the ratio of DNA in the detergent-soluble supernatant (fragmented DNA) obtained by centrifugation at $27,000 \times g$ to that in the lysate (total DNA).

4. **Fluorometric analysis of mitochondrial transmembrane potential**

After incubation, thymocytes were collected, and the $\Delta \Psi_m$ was analyzed by a flowcytometer, FACSCalibur (Becton Dickinson, San Jose, CA) as described previously\(^18\).

5. **Western blotting of glucocorticoid receptor**

Cells were collected, washed with phosphate-buffered saline, and then incubated in the lysis buffer (10 mM Tris-HCl buffer, pH 7.4, containing 10 mM NaCl, 3 mM MgCl\(_2\), 0.5% Nonidet P-40, and complete protease inhibitor cocktail (Roshe Diagnostics, Germany)) for 5 min at 4°C. Nuclei were isolated by centrifugation at $2,000 \times g$ for 5 min. Nuclear proteins were separated on 10% SDS-polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA). The membrane was blocked with 5% skim milk, incubated with anti-glucocorticoid receptor antibody, and detected with horseradish peroxidase-conjugated anti-rabbit IgG antibody and the ECL system (Amersham Bioscience, Tokyo, Japan).
MODULATION OF APOPTOSIS BY HSP90 INHIBITORS

Fig. 1 Time courses of DNA fragmentation induced by geldanamycin of mouse thymocytes. Thymocytes were incubated with (closed circle) or without (open circle) 0.2 μM geldanamycin, and DNA fragmentation was analyzed at the indicated times. Data are shown as means ± standard deviation (S.D.) of three experiments.

6. Quantitative RT-PCR

Quantification of IP₃R mRNA expression was performed using real-time RT-PCR with ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and SYBR Green chemistry. Total RNA isolated from the thymocytes using the RNeasy kit (Qiagen, Valencia, CA) was used as a template for cDNA synthesis using MMLV reverse transcriptase (Toyobo, Osaka, Japan) and oligo dT primers. All samples were analyzed according to the manufacturer’s procedures, and the data were normalized using the GAPDH expression as an internal control. The PCR primers specific for the IP₃R type I, type II, and type III and GAPDH were as follows:

5'GAGACTGCCTCTTTAAGCTATGTCC-3' and 5'-CAGTTGACGGAGTTGACCTCATTGC-3' for IP₃R I,
5'-GCAGTGCGGCGCATCTGCAGCTG-3' and 5'-TCGGTTTCCCACAAAACTCACCAG-3' for IP₃R II,
5'-CAGGGAGCTGCCACATTTATGGGC-3' and 5'-TCCTCAGTCGTGGTTTCATGACGG-3' for IP₃R III and
5'-TGCCCGAGACATCATCCCTG-3' and 5'-TCAGATCCACGACGGACACA-3' for GAPDH.

Fig. 2 Effects of HSP90-binding agents on dexamethasone- and etoposide-induced DNA fragmentation. Thymocytes were cultured with dexamethasone (Dex, A) or etoposide (Etop, B) for 6 hr in the presence or absence of geldanamycin (GA), radicicol (RAD), or herbimycin A (HMA) at the indicated concentrations, and DNA fragmentation was analyzed. Data are shown as means ± S.D. of three experiments. *p<0.01 vs. Dex by Student’s t test.

RESULTS

1. HSP90-binding agents inhibited the DNA fragmentation induced by dexamethasone, but not by etoposide

Geldanamycin did not induce DNA fragmentation at 6 hr of incubation, but it induced DNA fragmentation after 12 hr of incubation as observed in herbimycin A-treated thymocytes; there was about 54% DNA fragmentation at 24 hr (Fig. 1). Both dexamethasone- and etoposide-induced DNA fragmentation were time- and dose-dependently . Geldanamycin
inhibited the DNA fragmentation induced by dexamethasone dose-dependently (Fig. 2A), but did not inhibit that induced by etoposide (Fig. 2B). Other agents binding to HSP90, herbimycin A and a structurally unrelated agent, radicicol, also did not induce DNA fragmentation at 6 hr incubation. However, they inhibited the DNA fragmentation induced by dexamethasone (Fig. 2A) but not that induced by etoposide (Fig. 2B).

2. HSP90-binding agents inhibited the dexamethasone-induced ΔΨm reduction

The reduction of the ΔΨm is a prerequisite for dexamethasone- and etoposide-induced thymocyte apoptosis. To determine whether HSP90-binding agents affect the ΔΨm, we analyzed the ΔΨm with a flow cytometer using DiOC6(3) (Fig. 3). The low ΔΨm fraction in the dexamethasone-treated cells was about 47%. Neither geldanamycin nor radicicol alone affected the ΔΨm, but it inhibited the reduction of ΔΨm induced by dexamethasone; the low ΔΨm fraction was then about 7%. Herbimycin A also inhibited the dexamethasone-induced reduction of the ΔΨm. The etoposide-induced reduction of ΔΨm was not inhibited by geldanamycin, herbimycin A, or radicicol (Fig. 3). Because herbimycin A is an inhibitor of tyrosine kinases, we examined the effect of other tyrosine kinase inhibitors on dexamethasone-induced DNA fragmentation and ΔΨm reduction. PP2 and AG1478, inhibitors of src family tyrosine kinases and EGF receptor-tyrosine

Fig. 3 Effects of HSP90-binding agents on ΔΨm reduction induced by dexamethasone or etoposide. Thymocytes were incubated with 1μM dexamethasone (Dex) or 10μM etoposide (Etop) for 4 hr in the presence or absence of 0.16μM herbimycin A (HMA), 0.2μM geldanamycin (GA), or 0.5μM radicicol (RAD). The ΔΨm was determined by DiOC6(3) labeling, and results are typical of three independent experiments.

Fig. 4 Effects of protein tyrosine kinase inhibitors on ΔΨm reduction induced by dexamethasone. Thymocytes were cultured with 1μM dexamethasone (Dex) for 4 hr in the presence or absence of 5μM PP2 or 5μM AG1478. The ΔΨm was determined by DiOC6(3) labeling, and results are typical of three independent experiments.
kinases, respectively, inhibited neither the DNA fragmentation (data not shown) nor the ΔΨm reduction induced by dexamethasone (Fig. 4). These results suggest that herbimycin A and a related compound, geldanamycin, acted as HSP90 interfering agents, as did radicicol, rather than as inhibitors of tyrosine kinases.

3. HSP90-binding agents inhibited the nuclear localization of glucocorticoid receptors

The glucocorticoid receptor forms a complex with HSP90, which is essential to the glucocorticoid action. To confirm whether the effect of the HSP90-binding agents is due to their interference in the interaction between the glucocorticoid receptor and HSP90, the localization of the receptors in the cells was examined by Western blotting. The receptors were almost absent from the nuclear fraction in untreated thymocytes, but they were clearly detected in the nuclear fraction after dexamethasone-treatment (Fig. 5). Treatment of herbimycin A or geldanamycin slightly induced a translocation of the receptor into the nuclear fraction, but no DNA fragmentation was observed above the control level at the incubation time. Treatment with herbimycin A or geldanamycin inhibited the translocation of the receptors into the nuclear fraction that should have been induced by dexamethasone. Thus, the prevention of apoptosis by HSP90-binding agents is in part due to the inhibition of the translocation of the receptor to the nucleus.

4. Effects of herbimycin A on the expression of IP,Rs

The inhibition of the translocation of the receptors by HSP90-binding agents indicates inhibition of the gene expression involved in apoptosis. Because the involvement of IP,R-III expression in glucocorticoid-induced thymocyte apoptosis in rat has been reported, we examined the effect of herbimycin A on the expression of IP,Rs by quantitative real-time RT-PCR (Fig. 6). In mouse thymocytes, IP,R-I, -II, and -III are constitutively and equally expressed. Treatment with herbimycin
A increased the expression of the type I receptor about 3-fold, but reduced the other types by less than 50%. Note that no apoptosis was induced at the incubation time with herbimycin A. Dexamethasone treatment enhanced the expressions of IP₃R-I and -II about 1.5-fold and 2.5-fold, respectively. The level of IP₃R-III was not altered. The expression of IP₃Rs mRNA changed by dexamethasone treatment was slightly affected by co-treatment with herbimycin A. These results suggest that the expression of IP₃Rs is not directly involved in dexamethasone-induced apoptosis in mouse thymocytes.

**DISCUSSION**

Glucocorticoid hormones are physiological regulators of thymocyte apoptosis and are also potent inhibitors of T cell proliferation. Thus, glucocorticoids are frequently used as immunosuppressants and for treatment of lymphomas and lymphocytic leukemias. However, the precise mechanisms of glucocorticoid action on T cells are not known. The effects of glucocorticoids on T cells are mediated through the glucocorticoid receptor, a ligand-activated transcription factor, which induces or represses the transcription of individual genes and gene networks. The glucocorticoid receptors form a complex of ligand binding proteins such as HSP90, HSP70, and immunophilins, and, upon binding of glucocorticoids to their receptors, the complex moves to the nucleus. HSP90 is one of the most abundant cytosolic proteins in eukaryotes and interacts with a variety of intracellular proteins. A functional antagonism between HSP90-binding agents and glucocorticoids should be taken into account in the mechanism of thymocyte apoptosis.

The present study showed that HSP90-binding agents inhibited the dexamethasone-induced apoptosis, but not that induced by etoposide, although long incubation of thymocytes with geldanamycin did induce apoptosis. It has been reported that geldanamycin is more toxic to rapidly proliferating cells including activated splenic T cells and malignant cells, the effect on the latter cells is referred to as the tumoricidal activity of geldanamycin. Because the majority of thymocytes are CD4⁺, CD8⁺ double-positive cells and less proliferative, geldanamycin failed to induce apoptosis at 6 hr incubation (Fig. 1). However, incubation above 12 hr did induce apoptosis, presumably by other HSP90 activities including protein tyrosine kinase inhibition, because HSP90 has a variety of functions other than glucocorticoid receptor-chaperone. The specific inhibitory effect of the HSP90-binding agents on the dexamethasone-induced apoptosis indicated an interaction between HSP90 and the glucocorticoid receptors. In fact, treatment with herbimycin A or geldanamycin inhibited the translocation of the receptors into the nuclear fraction (Fig. 5). This reduction in nuclear translocation of the receptors may result in the inhibition of the glucocorticoid-mediated gene expression involved in apoptosis. T lymphocytes undergoing apoptosis in response to dexamethasone were found to have increased amounts of mRNA and protein for IP₃R-III. However, they did not distinguish each IP₃R expression because the primer set they used was not specific for IP₃R-III. We observed no alteration of IP₃R-III expression in dexamethasone-treated thymocytes by using a specific primer set (Fig. 6). In IP₃R-I deficient Jurkat cells, the monoclonal antibody to CD3, glucocorticoids, irradiation, and Fas ligand, which activate multiple apoptotic pathways, did not induce apoptosis, whereas the parent Jurkat cells with IP₃R-I did undergo apoptosis. However, Hirota et al. showed that IP₃R-I is not essential for T cell development and function. We showed that herbimycin A itself induced high level expression of IP₃R-I, but did not induce apoptosis. The alteration of IP₃Rs induced by dexamethasone was not restored by herbimycin A in spite of the complete inhibition of apoptosis. The present study suggests that the expression of IP₃Rs might not participate in the glucocorticoid-induced apoptosis.
Recently, two genes that mediate apoptosis in lymphoid cell lines by glucocorticoids have been reported; one encoding Bim\(^{20}\), which is a pro-apoptotic Bcl-2 family protein, and the other, \(\text{dig}^2\), encoding an unknown function protein\(^{21}\). The latter gene is one of the stress-responsive genes and is also induced by etoposide. The genes whose expression are inhibited by HSP90-binding agents in dexamethasone-treated cells remain to be elucidated.

HSP90 blocks the activation of caspase-9 by inhibiting the formation of cytochrome c-mediated oligomerization of Apaf-1 and procaspase-9\(^{15}\). The reduction of \(\Delta\Psi_m\) may lead to the release of cytochrome c, resulting in the activation of caspase-9 in thymocytes. HSP90-binding agents inhibited the reduction of \(\Delta\Psi_m\) induced by dexamethasone, but not that induced by etoposide (Fig. 3). Thus the effect of HSP90 on the oligomerization may not directly participate in the geldanamycin-induced process of the dexamethasone-induced apoptosis. Note that the oligomerization of Apaf-1 and procaspase-9 is downstream of the mitochondrial process.

Geldanamycin and herbimycin A, derivatives of benzoquinone, exert tyrosine kinase inhibitor activity indirectly by somehow destabilizing these proteins through interfering with the binding of these proteins to HSP90\(^{19}\). The tyrosine phosphorylation has been shown to participate in the regulation of the glucocorticoid receptor function. Herbimycin A decreased the number of high-affinity binding sites of glucocorticoids in the cytosolic fraction and increased the equilibrium dissociation constant in cultured rat hepatocytes\(^{12}\). In addition, tyrosine phosphorylation of the glucocorticoid receptor has been shown to be involved in the regulation of its nuclear export to the cytoplasm in an in vitro system\(^{25}\). However, radicicol, with a different structure, exhibited the same effect on the dexamethasone-induced apoptosis, and specific inhibitors of tyrosine kinases, AG1478 and PP2, did not inhibit either DNA fragmentation or \(\Delta\Psi_m\) reduction. Hence these HSP90-binding agents inhibited the interaction between HSP90 and the glucocorticoid receptors, resulting in the prevention of apoptosis. However, the expression of IP\(_3\)Rs is not directly involved in dexamethasone-induced apoptosis in mouse thymocytes. What gene expression is involved in glucocorticoid-treated thymocyte still remains to be elucidated.

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