Clobetasol synergistically diminishes Ciz1 expression with genistein in U937 cells

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Abstract. Cip-interacting zinc finger protein 1 (Ciz1) stimulates DNA replication and has been implicated in the tumorigenesis of breast cancer cells. In order to investigate the possibility of using medicinal glucocorticoids against breast cancer, we studied whether certain glucocorticoids affect the expression of Ciz1. The *in vitro* effect of clobetasol treatment on the reduction of Ciz1 expression was detected by reverse transcriptase-polymerase chain reaction. Western blotting also confirmed the down-regulation of the protein in a dose-dependent manner upon clobetasol treatment in U937 monocytoid cells. Furthermore, we found that Ciz1 protein expression was decreased after pre-treatment of the cells with clobetasol and genistein. An extract of *Lens culinaris* also had a synergistic effect on the repression of Ciz1 protein expression.

Introduction

The pathways by which cell proliferation is regulated during differentiation have yet to be established, therefore proteins that play a role in DNA replication are of considerable interest. Cip-interacting zinc finger protein 1 (Ciz1) stimulates DNA replication depending on the estrogen receptor (ER) and participates in the regulation of the cell cycle by increasing cdk2 kinase activity and inducing G1-S transition (1). Ciz1 protein regulates the ER by enhancing its transactivation activity and recruitment to target gene chromatin. Hypersensitivity to estrogen has been observed in patients with breast cancer (2). Ciz1 induces such a hypersensitivity to estrogen in breast cancer cells and induces the expression of cyclin D1, a target gene of ER. Overexpression of Ciz1 promoted the cell growth rate, anchorage independence and tumorigenesis of breast cancer cells (1). Conversely, repression of Ciz1 expression is expected to have interference effects on breast cancer progression. In fact, depletion of Ciz1 from cancer cells restrains entry to the S phase and inhibits cell proliferation. Ciz1 has also been found to be a glucocorticoid receptor (GR)-regulated gene (3).

Glucocorticoids play an essential role in embryonic and cancer development (4,5). Due to their wide spectrum of activity and pro-apoptotic properties, glucocorticoids are some of the most commonly used drugs in hematological malignancies and are also used as chemotherapy regimens for, among others, solid cancer treatment. GR is a member of the nuclear hormone receptor family. Hormone-activated GR binds to glucocorticoid responsive elements (GRE) near target genes. In breast cancer, glucocorticoids, through their receptor, may interact with ER in a feedback loop regulating the activities of each other (6). It is known that glucocorticoids are capable of playing a complex role in breast cancer epidemiology, biology and treatment. We recently found the dose-dependent down-regulation of the Ciz1 protein upon genistein treatment in Daudi lymphoid cells (7). We, therefore, hypothesized that glucocorticoids and certain isoflavones may synergistically affect the expression of Ciz1.

Materials and methods

Cell culture. The human cell lines U937, Daudi and K562 were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2.

Preparation of extracts and reagents. Ground bean powders were purchased at a food market in Japan. The powders were dissolved in 80% ethanol and subsequently diluted in 40% ethanol at a stock concentration of 150 mg/ml. The mixtures were vortexed rigorously for 3 min followed by a 3-min sonication. Following centrifugation (1,500 x g, 5 min), the supernatants were collected and stored at -20˚C until use. Other reagents used were basically dissolved in ethanol and subsequently diluted at a stock concentration of 10 mM. They were stored at -20˚C until use. For the cell treatments, a range of 0.5-10.0 μl was added to 1 ml of the cell culture medium.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Ciz1 and GAPDH mRNAs were analyzed by semi-quantitative RT-PCR. Total RNA was extracted using the RNA isolation kit

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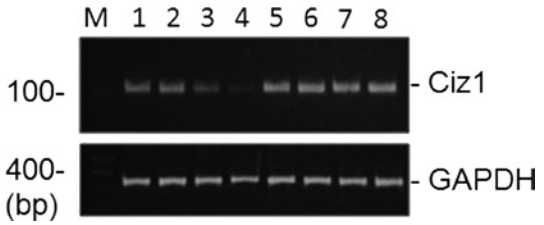

Figure 1. Ciz1 mRNA was analyzed by semi-quantitative RT-PCR. The semi-quantitative RT-PCR was performed using primers specific to Ciz1 on RNA prepared from U937 cells treated without (lanes 1 and 5) or with clobetasol propionate at the final concentration of 10^{-7} M (lane 2), 10^{-6} M (lane 3), 10^{-5} M (lane 4) or with dexamethasone at the final concentration of 10^{-7} M (lane 6), 10^{-6} M (lane 7) and 10^{-5} M (lane 8) for 24 h. Specific expression was determined in relation to the expression of the housekeeping gene GAPDH used as an internal loading control. At least four independent experiments were performed, and typical two-paired results were documented.

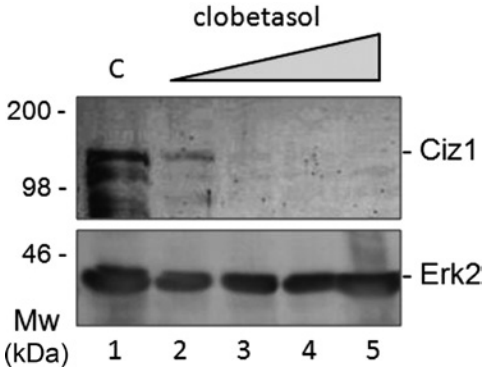

Figure 2. Dose-dependent repression of Ciz1 protein expression by clobetasol. U937 cells were treated without (lane 1) or with clobetasol propionate at the final concentration of 10^{-7} M (lane 2), 10^{-6} M (lane 3), 10^{-5} M (lane 4) and 10^{-4} M (lane 5) for 48 h. The levels of Ciz1 protein were detected by Western blot analysis using anti-Ciz1. Western blotting with the anti-Erk2 antibody was also shown as equal levels of protein loading.

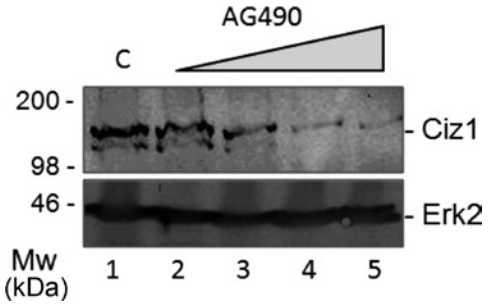

Figure 3. Dose-dependent repression of Ciz1 protein expression by AG490. U937 cells were treated without (lane 1) or with AG490 at the final concentration of 10^{-7} M (lane 2), 10^{-6} M (lane 3), $3x10^{-6}$ M (lane 4) and 10^{-5} M (lane 5) for 48 h. The levels of Ciz1 protein were detected by Western blot analysis using anti-Ciz1 antibody, as shown in Fig. 2. Western blotting with the anti-Erk2 antibody was shown as equal levels of protein loading.

(Takara Bio Inc., Shiga, Japan). Total RNA (2 μg) was reverse-transcribed using Phusion RT-PCR kit (NEB Inc., Ipswich, MA, USA), according to the manufacturer's protocol. Cycle-based PCR was used to semi-quantitate the ubiquilin1 and presenilin1 gene level. GADPH was also used as an internal loading control. All of the samples were analyzed within 3 months following collection. The primers used for the PCR were designed as follows, Ciz1 Fw 5'-ACATATCCACAGGTCCACACAC-3', Ciz1 Rv 5'-CTGCTCATGGGTCTGCTCTG-3' (expected size 102 bp); GAPDH Fw 5'-TCCCATCACCATCTTCCA-3', GAPDH Rv 5'-CATCACGCCACAGTTTCC-3' (expected size 376 bp). Thermo-cycling was carried out according to the manufacturer's instructions, at a 55˚C annealing temperature in a final volume of 10 μl, including *Taq* DNA polymerase.

Western blot analysis. Equal amounts of protein samples were used for Western blot analysis with anti-Ciz1 (Cosmo Bio Co. Ltd., Tokyo, Japan) and anti-Erk2 (BD Bioscience, San Jose, CA, USA) antibodies and quantified by densitometry. All Western blots were repeated at least three times, and the representative data are shown.

Results and Discussion

In order to investigate the possibility of using certain glucocorticoids to affect Ciz1 expression, clobetasol as well as dexamethasone (Sigma-Aldrich, Co. Ltd., St. Louis, MO, USA) were added to a cell culture medium of U937, K562 or Daudi cells, and the levels of Ciz1 expression were examined. We employed RT-PCR analysis to quantify the expression level of the Ciz1 gene. Total RNA was isolated 24 h after treatment to detect Ciz1, and the levels of mRNA were determined using conventional RT-PCR. As shown in Fig. 1, the Ciz1 gene expression level greatly decreased after treatment of clobetasol at the final concentration of 10^{-5} M, compared to the untreated ethanol vehicle and dexamethasone, in the U937 monocytoid cells. By contrast, the expression of the housekeeping gene GAPDH was unaltered. A larger amount of dexamethasone (10^{-4} M) reduced the Ciz1 gene expression (data not shown). There was almost no difference in the results of gene expressional profiles between U937 and Daudi cells, and no reduction in Ciz1 expression was noted in K562 cells. To exclude the possibility of carry-over contamination, reactions containing all RT-PCR reagents, including primers without sample RNA, were preformed as negative controls. No such RNA contamination was detected (data not shown). To further confirm the reduced expression level of Ciz1 by clobetasol, Western blotting was also performed to analyze the level of Ciz1 protein in the U937 cells. As shown in Fig. 2, after pre-treatment of the cells with a set of different doses of clobetasol, we found that Ciz1 protein expression was decreased with increasing concentrations of genistein. A final concentration 10^{-6} M of clobetasol diminished Ciz1 expression by more than 90% in the U937 cells after 48 h of clobetasol stimulation. This protein expression profile closely agreed with the result of RT-PCR as shown in Fig. 1.

We recently reported the *in vitro* effects of genistein and isoflavone treatment on the reduction of Ciz1 expression in Daudi lymphoid cells (7). Genistein is one of the known isoflavones, which is a group of compounds found in soybeans. Genistein inhibits the oncogenic signal mediated by tyrosine kinases, such as EGF receptor, ErbB2 and Src family kinases (8). We speculated that other tyrosine kinase inhibitors could also reduce Ciz1 expression. As expected, after pre-treatment of the cells with a set of different doses of AG490, a tyrosine kinase inhibitor, we found that Ciz1 protein expression was decreased with increasing concentrations of AG490, as shown

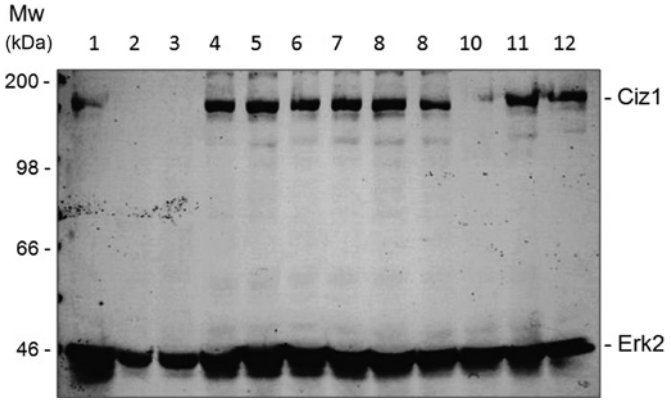

Figure 4. Genistein, AG490 and an extract of *Lens culinaris* synergistically diminish the expression of Ciz1 with a low concentration of clobetasol. U937 cells were first pre-treated with clobetasol propionate at the final concentration of 10^{-7} M for 24 h. The U937 cells were then treated without (lane 1) or with 25 μg/ml genistein (lane 2), 10^{-6} M AG490 (lane 3), 50 μg/ml green soybeans (lane 4), 50 μg/ml soybeans (lane 5), 50 μg/ml Azuki soybeans (lane 6), 50 μg/ml black soybeans (lane 7), 50 μg/ml white soybeans (lane 8), 50 μg/ml red kidney beans (lane 9), 50 μg/ml lentils (*Lens culinaris*) (lane 10), 50 μg/ml garbanzo beans (lane 11) and 50 μg/ml pinto beans (lane 12) for 48 h. Following treatment, cell lysates were isolated and the levels of Ciz1 protein were detected by Western blot analysis. Western blotting with anti-Erk2 antibody was also shown as equal levels of protein loading.

in Fig. 3. We then addressed the question whether clobetasol synergistically reduces Ciz1 expression with isoflavones, genistein or AG490. We found that Ciz1 protein expression was decreased by more than 90% after pre-treatment of the cells with clobetasol and genistein or AG490 in the U937 cells, as shown in Fig. 4. Notably, an ethanol extract of lentils (*Lens culinaris*), which is rich in genistein, also exhibited a synergistic effect with clobetasol among the extracts of beans used in the experiment.

GR are specific cytoplasmic transcription factors that mediate the biological actions of corticosteroids. On ligand binding, GR translocates into the nucleus and binds to DNA at GRE in the promoter region of responsive genes that induce transcription. In the present study, we showed that clobetasol, a strong corticosteroid, is a potent transcriptional repressor for the Ciz1 gene. In addition, extracts of *Lens culinaris*, genistein and AG490 synergistically reduced Ciz1 expression with clobetasol in U937 cells. Genistein affects cellular function via inhibition of tyrosine protein kinases, cycloxygenase 2 and cytochrome p450 enzymes (9,10). Genistein was also found to modulate ER levels, the activity of topoisomerase II, enzymes involved in phospho-inositide turnover, mitogen-activated protein kinases and NF-κB signaling pathways (11,12). Other studies have shown that genistein decreased mammary tumor growth compared to vehicle (13). Ciz1 promotes initiation of mammalian DNA replication and is present within nuclear matrix-associated DNA replication machineries (14). The precise mechanism of transcriptional regulation of Ciz1 remains unclear. The Ciz1 gene may be complexly regulated by various transcription factors. As body exercise and stress induce glucocorticoids (15), consuming beans, including lentils, and exercising may have preventative effects for certain types of breast cancer. More studies, including *in vivo* experiments, need to be undertaken to elucidate the molecular mechanisms of glucocorticoids and tyrosine kinase inhibitors.

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