Involvement of Tiam1 in Apoptosis Induced by Bufalin in HeLa Cells

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Abstract. Background: It has been previously demonstrated that bufalin, an active agent in the Chinese medicine chan’su, induces apoptosis in human leukemia cells by altering the expression of apoptosis-related genes, such as bcl-2 and c-myc. Tiam1 was also found to play a critical role in bufalin-induced apoptosis through the activation of the Rac1, PAK and JNK pathway in human leukemia cell lines. In the present study, the involvement of the Tiam1 gene products in bufalin-induced apoptosis in human solid tumor HeLa cells was examined. Materials and Methods: HeLa cells were treated with 10–8 M bufalin and apoptosis was measured by ELISA quantification of nucleosomes. Tiam1 mRNA levels were quantified by real-time PCR analysis and inhibited by transfected siRNA specific for Tiam1. Results: Apoptosis was induced in HeLa cells by treatment with 10–8 M bufalin. Expression of both Tiam1 mRNA and its protein was induced 0.5 h after the start of the bufalin treatment. Transfection of Tiam1-specific siRNA into HeLa cells markedly inhibited bufalin-induced apoptosis. Conclusion: Our results suggest that Tiam1 is a downstream mediator of bufalin-induced apoptosis in the human solid tumor HeLa cell line, as well as in leukemia cell lines.

Apoptosis, also known as programmed cell death, is an active process and plays an important role in maintaining homeostasis in multicellular organisms (1, 2). The importance of apoptosis has been emphasized by recent demonstrations that various chemotherapeutic anti-cancer agents, such as cisplatin (3, 4), doxorubicin (5) and taxol (6), induce apoptosis in cancer cells. Apoptosis-inducing agents specific for cancer cells might be expected to be ideal antitumor drugs, since apoptotic cell death does not induce an inflammatory response. Several differentiation-inducing or apoptosis-inducing compounds were identified by our group (7-12), and bufalin, a component of the traditional Chinese medicine chan’su, was found to act as a potent differentiation- and apoptosis-inducing agent in human leukemia cells (7, 13).

Recently, the mechanisms of induction of apoptosis, especially gene expression and intracellular signaling, have been extensively studied. In a previous report, genes involved in bufalin-induced apoptosis were investigated using the differential display technique (14) and Tiam1 was identified as a gene involved in bufalin-induced apoptosis (15). Tiam1 was originally identified in T-lymphoma cells as the product of a gene that confers invasive and metastatic properties (16). The predicted Tiam1 protein contains a Dbl homology (DH) domain (17) and two pleckstrin homology domains (18, 19). The DH domain is considered to be catalytic and is present in GDP-dissociation stimulator (GDS) proteins for Rho-like GTPases, such as Rac1 and Cdc42, and is considered to be a catalytic domain (16). Tiam1 is a GDS protein for Rac1, like Vav (20) and PIX (21) and Tiam1 specifically activates Rac in vitro, as well as in vivo (22).

The results of our previous study have demonstrated that bufalin at concentrations from 10–9 to 10–8 M strongly induced apoptosis in human leukemia cells, and that the Tiam1 gene was associated with the bufalin-induced apoptosis. The aim of present study was to investigate the ability of bufalin to induce apoptosis in a human solid tumor HeLa cell line and to study the involvement of the Tiam1 gene in apoptosis.

Bufalin induced apoptosis in HeLa cells in a dose-dependent manner. Moreover, transfection of HeLa cells with Tiam1-specific siRNA inhibited the induction of apoptosis by bufalin. These results suggest that Tiam1 plays an important role in the regulation of bufalin-induced apoptosis, not only in human leukemia cells, but also in human solid tumor HeLa cells.

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Materials and Methods

Reagents, cell culture and cell death detection. Bufalin, purchased from Sigma Chemical Co. (St. Louis, MO, USA), was dissolved in ethanol at a concentration of 10\(^{-2}\) M and stored at 4°C. All other chemicals were of reagent grade. Rabbit polyclonal antibody against Tiam1 (C-16) and mouse monoclonal antibody against actin (C-2) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HeLa cells were purchased from HSRRB (Human Science Research Resource Bank, Osaka, Japan). The cells were maintained in Eagle’s minimum essential medium (GIBCO, Glasgow, UK), supplemented with 10% heat-inactivated fetal calf serum (FCS), in an atmosphere of 5% CO\(_2\) at 37°C. Cells were seeded at a concentration of 2-3x10\(^5\) cells/ml and maintained in logarithmic growth by passage every 2-3 days. Cell death was detected by the cell death detection ELISA plus kit (Boehringer Mannheim, Mannheim, Germany), which quantifies nucleosomal formation.

Preparation of cell lysates. HeLa cells were washed twice with PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 5 µg/ml antipain, 50 mM NaF, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaCl, 2 mM sodium orthovanadate, 1% Triton X-100, and 0.5 mM PMSF]. The lysate was centrifuged at 15,000 xg for 15 min and the supernatant was subjected to Western blotting analysis.

Western blotting analysis. A cell lysate containing 50 µg of protein was fractionated by SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was first rinsed with TBST [20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.05% Tween 20] and then blocked with Block Ace (Yukijirusi Co. Ltd., Tokyo, Japan) for 2 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with a 1:1000 dilution of primary antibodies in blocking buffer. After the membrane had been washed three times with TBST, it was incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG. The membrane was washed with TBST and bands of protein on the membrane were visualized with an ECL Western blotting detection kit (PerkinElmer Life Sciences, Inc., Boston, MA, USA).

Real-time PCR. Total RNA was extracted with Isogen (Nippon gene Co. Ltd., Tokyo, Japan). One µg of total RNA was reverse transcribed using Omniscript reverse transcriptase (Qiagen, Inc., Chatsworth, CA, USA). Using the Tiam1 forward primer 5’ - CTGAGACCCACCTTCTA-3’ and reverse primer 5’ - CAGGG ACTGCTCACATAC-3’. The expression level of Tiam1 mRNA was determined by iCycler (Bio-Rad Lab. Inc., Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Lab., Inc.). For standardization, actin cDNA was also amplified in iQ SYBR Green Supermix.

The siRNAs and transfection of cells. Tiam1 siRNAs was produced by QIAGEN, Inc. (Chatsworth, CA, USA) with the sequence: 5’ - AATAGTGTGAAGGAAAAACCGGT-3’. HeLa cells were treated with siRNA according to the instructions provided with the RNAiFect™ transfection reagent (QIAGEN, Inc.) with slight modifications. HeLa cells (1x10\(^5\)/2 ml) were treated with 5 µM of siRNA in Eagle’s minimum essential medium supplemented with 10% FCS in the presence of the RNAiFect™ transfection reagent. After a 24-h incubation at 37°C, the medium containing the mixture of RNAiFect™ and siRNA was replaced by RPMI 1640 medium that contained 10% FCS and the cells were incubated for an additional 24 h. Non-silencing control siRNA was purchased from QIAGEN, Inc.

Results

Induction of apoptosis in HeLa cells upon treatment with bufalin. Our previous study has shown that bufalin induced apoptosis in human leukemia cell lines, such as HL60 and U937 [7, 23, 24]. In the present study, the effect of bufalin on apoptosis was examined in human solid tumor HeLa cells and the involvement of the Tiam1 gene in the bufalin-induced apoptosis was clarified. The effect of bufalin on apoptosis in HeLa cells is shown in Figure 1. Apoptotic cell death in HeLa cells was investigated with a cell death detection ELISA PLUS kit, which includes mouse monoclonal antibodies directed against DNA and histones and allows for the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Assays using the ELISA PLUS kit confirmed that the extent of apoptosis in HeLa cells that had been treated with 10\(^{-8}\) M bufalin increased in a time-dependent manner. The induction of apoptosis continued to increase 24 h after the start of exposure of cells to bufalin. These results suggest that bufalin has the ability to induce apoptosis in HeLa cells.
Effect of bufalin on the expression of Tiam1. Tiam1 may be a mediator of bufalin-induced apoptosis through the activation of the Rac1, PAK, and JNK pathway in human leukemia U937 cells. The effect of bufalin on the expression of Tiam1 protein was examined by Western blotting analysis. The level of expression of Tiam1 protein was normalized to the level of actin expression. The results are typical of three experiments.

**Effect of bufalin on the expression of Tiam1.**

Figure 2. Effect of bufalin on the expression of Tiam1 protein. After treatment of HeLa cells with $10^{-8}$ M bufalin for the indicated times, the expression of Tiam1 protein was examined by Western blotting analysis. The level of expression of Tiam1 protein was normalized to the level of actin expression. The results are typical of three experiments.

![Figure 2](image2.png)

![Figure 3](image3.png)

Figure 3. Effect of bufalin on the expression of Tiam1 gene. After treatment of HeLa cells with $10^{-8}$ M bufalin for the indicated times, the expression of Tiam1 gene was examined by real-time PCR analysis as described in the Materials and Methods. The results are presented as mean ±SD of the results of three independent experiments.

In our previous study, bufalin has been found to activate mitogen-activated protein kinase via a small group of Ras superfamily G-proteins during the induction of apoptosis in human leukemia U937 cells. Moreover, by a

**Discussion**

In our previous study, bufalin has been found to activate mitogen-activated protein kinase via a small group of Ras superfamily G-proteins during the induction of apoptosis in human leukemia U937 cells. Moreover, by a
differential display technique, we recently analyzed genes involved in the bufalin-induced apoptosis in leukemia U937 cells and found that the Tiam1 gene activated the Rac1, PAK and JNK pathway (15). In the present study, we demonstrate that bufalin was able to induce apoptosis in a solid tumor HeLa cell line and that Tiam1 mRNA and its protein were transiently expressed during bufalin-induced apoptosis.

Small GTP-binding proteins of the Ras superfamily are molecular switches downstream of receptors controlling cell growth, differentiation, cytoskeletal rearrangements and vesicle transport (25, 26). One subfamily, the Rho-related GTPases, including RhoA, Rac1, and Cdc42, induces changes in the actin cytoskeleton in response to extracellular stimuli (26). Tiam1 was originally identified in T-lymphoma cells as a gene that confers invasive and metastatic properties (16). Tiam1 activates the Rho-like GTPase Rac1 and this activation leads to invasive behavior of T-lymphoma cells (22). Recent reports have implicated Tiam1 in the regulation of apoptotic process (27, 28). Tiam1 directly binds to c-Myc and interferes with c-Myc-mediated apoptosis in rat-1 fibroblasts (27). Tiam1 is cleaved by caspases during apoptosis, suggesting a novel mechanism for regulating guanine nucleotide exchange factor activity and GTPase-mediated signaling (28). The induction of the expression of Tiam1 protein and mRNA during bufalin-induced apoptosis in HeLa cells prompted us to study the possible role of Tiam1 in the regulation of apoptosis by bufalin and to examine the regulatory relationship between the expression of Tiam1 and apoptosis. To assess the role of Tiam1 in the regulation of bufalin-induced apoptosis in HeLa cells, the effect of siRNA silencing of the Tiam1 gene in apoptosis was investigated. In the present study it was demonstrated that the siRNA suppression of Tiam1 expression inhibited bufalin-induced apoptosis.

The biological effect of Tiam1 appears to be cell-type specific. For example, Tiam1 promoted the formation of E-cadherin-mediated cell-to-cell adhesion and, thus, the inhibition of epithelial cell migration (29). On the other hand, Tiam1 was also shown to promote migration of epithelial cells cultured on collagen (29, 30). Our present results and previous data (15) suggest that Tiam1 produces signals for inducing apoptosis in human leukemia HL60 and U937 cell lines, as well as in the solid tumor HeLa cell line. In contrast, a recent report described Tiam1 acting as an anti-apoptotic factor in rat-1 fibroblasts (27). The differences in biological effect of Tiam1 among cell types may be due to the differences in the regulation of the apoptotic process. Additional studies are necessary to clarify the role of Tiam1 in the regulation of the induction of apoptosis in tumor cells.

References


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