Specific downregulation of death-associated protein kinase enhances Fas-mediated apoptosis in the human differentiated endometrial adenocarcinoma cell line, HHUA

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Summary

Purpose of investigation: Death-associated protein kinase (DAPK) is a serine/threonine kinase that is well-known as a positive mediator of Fas-mediated apoptosis. Previous reports have shown that DAPK and Fas are expressed in human endometrial adenocarcinoma cells. In this study, we examined the effects of specific downregulation of DAPK expression on Fas-mediated apoptosis in the human endometrial adenocarcinoma cell line, HHUA. *Methods and results:* Transfection of DAPK small-interfering RNAs (siRNAs) into the HHUA cells reduced DAPK protein expression, and enhanced Fas-mediated apoptosis, in a dose-dependent manner. *Conclusions:* These results indicate that, in contrast to cases with other malignant tumor cells, DAPK negatively regulates Fas-mediated apoptosis in these human differentiated endometrial adenocarcinoma cells.

Key words: Death-associated protein kinase; Fas; Endometrial adenocarcinoma; siRNA.

Introduction

Death-associated protein kinase (DAPK) is a 160-kDa Ca^{2+}/calmodulin-dependent serine/threonine kinase that functions as a positive mediator of apoptosis triggered by interferon-γ, tumor necrosis factor-α, anti-Fas antibodies, transforming growth factor-β, c-myc and E2F oncogenes, ceramide and by detachment from the extracellular matrix [1-8]. Moreover, loss of DAPK expression has been implicated in tumorigenesis and metastasis [9, 10] thus suggesting a crucial role for DAPK in the apoptotic process under pathological conditions. On the other hand, several lines of evidence have indicated that DAPK may have an anti-apoptotic function. Inhibition of DAPK expression in HeLa cells, 3T3 fibroblasts and primary human vascular smooth muscle cells using an antisense DAPK was found to increase apoptosis [11, 12]. In our previous studies, we detected higher DAPK protein expression levels in differentiated endometrial adenocarcinoma cells than in normal primary endometrial cells or in several ovarian and uterine carcinoma cells including HeLa cells [13]. These results suggest that DAPK regulates cell survival or apoptosis of human endometrial adenocarcinoma cells.

Constitutive expression of apoptosis-inducible cytokine receptors, such as type 1 receptor of tumor necrosis factor (TNFR1) and Fas antigen (CD95) have been reported in normal human endometrial tissues [14-16] and in differentiated endometrial adenocarcinoma cells [17]. Some of these endometrial adenocarcinoma cells express functional Fas antigens that mediate Fas-mediated apoptotic signals [17]. The DAPK protein is also expressed in endometrial adenocarcinoma cells [13]. DAPK was first reported to positively regulate tumor necrosis factor-α– and Fas-induced apoptosis via the death domain of the DAPK molecule [3]. Co-immunoprecipitation studies in brief seizure-induced neuronal death revealed binding of DAPK to the cytoplasmic domain of TNFR1 and to the Fas-associated death domain protein, suggesting that DAPK directly and positively regulates apoptosis mediated by the TNFR-family [18]. We recently reported that Fas-mediated apoptosis in chemically damaged ovarian granulosa cells is strongly suppressed in mutant murine ovaries in which the kinase domain of DAPK is deleted [19]. Epigenetic downregulation of DAPK gene expression by hypermethylation of its promoter region has been reported in many cancer cells including T-cell lymphomas [20], B-cell lymphomas [20], non-small cell lung carcinomas [21], head and neck cancers [22], gastric and colorectal carcinomas [23, 24], ovarian carcinomas [13] and uterine carcinomas [25]. However, Matsumoto *et al*. reported that the status of DAPK protein expression closely correlated with Fas expression but not with methylation of the promoter region [26]. Our previous studies also showed that DAPK protein expression levels correlated with Fas expression but not with methylation of the promoter region in differentiated endometrial adenocarcinomas [13], indicating that the relationship between DAPK and Fas may be organ- and tissue-dependent. In the present study, therefore, we directly investigated Fas-mediated apoptotic signals in the human differentiated endometrial adenocarcinoma cell line (HHUA) using specific DAPK small-interfering RNAs (siRNAs) to knock down endogenous DAPK expression in HHUA cells [27].

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Materials and Methods

Cell line and culture

The HHUA cell line [27] was obtained from the Riken Cell Bank (Tsukuba, Japan). The cells were cultured in OPTI-MEM (Invitrogen, Corp., Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Equitech Bio, Inc., Ingram, TX), penicillin (100 U/ml), streptomycin (100 U/ml) and Fungizone (0.25 μg/ml; Invitrogen, Corp.) under 5% CO_2 and 95% air at 37°C.

Transfection of DAPK siRNAs

Two DAPK siRNA duplexes were designed and synthesized by iGENE Therapeutics, Inc. (Tsukuba, Japan). The siRNA sequences are shown in Table I. A negative control siRNA was purchased from Ambion, Inc. (Austin, TX). Lipofectamine 2000 (Invitrogen, Corp.) was used as the transfection reagent according to the manufacturer's instructions. For experiments, cells were seeded in 6-well plates (2.5×10^5 cells/well) or 10-cm dishes (2×10^6 cells/dish), cultured for 24 h and then transfected with the DAPK siRNAs or control siRNA. Subsequently, the cells were cultured for 48-72 h for protein assays before being harvested as indicated.

Western blotting

For Western blotting analysis, the cells were collected 48-72 h after transfection with the DAPK siRNAs or control siRNA and lysed in phosphate-buffered saline containing 1% NP-40, 0.1% sodium dodecyl sulfate, complete protease inhibitor cocktail (Roche Diagnostics, Corp., Indianapolis, IN) and 1 mM phenylmethyl sulfonyl fluoride. The protein concentrations of the cell lysates were quantified by Coomassie Plus Protein assays (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of the total proteins were separated by SDS-PAGE using a 7.5% gel and then transferred to a polyvinylidene fluoride membrane (Atto, Corp., Tokyo, Japan). After sequential incubations with primary and secondary antibodies, the immunocomplexes on the membranes were detected using enhanced chemiluminescence (ECL) or ECL plus kits (Amersham Pharmacia Biotech, Uppsala, Sweden). The antibodies used were purchased from the following sources: mouse monoclonal anti-DAPK antibody (Sigma, St. Louis, MO); rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) and anti-cleaved PARP antibodies (Cell Signaling Technology, Beverly, MA). The membranes were stripped and reprobed with an anti-β-actin antibody (Sigma).

Cell viability assay

A Cell Counting kit (Dojindo Chemical Laboratory Co., Ltd., Tokyo, Japan) was used to evaluate the abilities of the DAPK siRNAs to enhance the cytotoxic effects of an agonistic anti-Fas IgM antibody (Medical & Biological Laboratory Co., Ltd., Nagoya, Japan). Cells were plated in quadruplicate in 96-well plates at 5×10^3 cells/well and cotransfected with Lipofectamine 2000 and 25-50 nM DAPK siRNAs or with 50 nM control siRNA. At 24 h after transfection, cells were incubated with various concentrations of anti-Fas IgM for a further 48-72 h. At the end of the treatments, viable cell numbers were determined using the Cell Counting kit according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader. Cell viability of 100% was defined as absorbance obtained for cells without anti-Fas IgM. The data are expressed as means ± SD. Comparisons between experimental groups were performed by analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

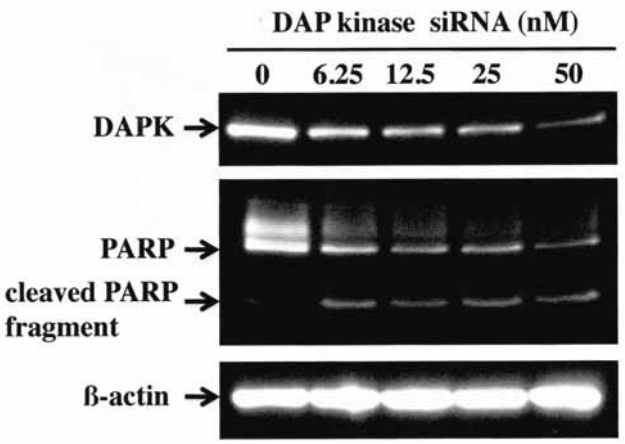

Figure 1. — Downregulation of endogenous DAPK expression in DAPK siRNA-transfected HHUA cells.
Western blot analyses of HHUA cell lysates shows that DAPK siRNA transfection dose-dependently reduces DAPK protein expression and increases the cleavage of PARP. The expression of β-actin was assayed as an internal control. The figure shows a representative of transfection experiments for both DAPK siRNA-1 or DAPK siRNA-2–transfected cells.

Results

Specific downregulation of endogenous DAPK expression by DAPK siRNA transfection induces cleavage of PARP in HHUA cells

To investigate the role of endogenous DAPK protein in HHUA cells, we first assayed the effect of specific downregulation of DAPK expression in HHUA cells following transfection with double-stranded siRNAs (DAPK siRNA-1 or siRNA-2) (Table 1) targeted against human DAPK mRNA. Western blot analyses revealed dose-dependent suppression of DAPK protein expression in DAPK siRNA-transfected HHUA cells at 48 h after transfection (Figure 1). Concurrently, specific downregulation of endogenous DAPK expression by DAPK siRNA transfection induced cleavage of PARP, an apoptosis marker, in the cells. No nonspecific inhibitory effects of DAPK siRNA were detected on β-actin protein expression, used as an internal control.

Table 1. — *DAPK siRNA sequences*.

DAPK siRNA	siRNA sequence
DAPK siRNA-1	5'-CAACAUCAUGCAAAGUGAAACAGUU-AG-3'
	3'-AU-GUUGUAGUACGUUUCACUUUGUCAA-5'
DAPK siRNA-2	5'-AGCCAAGAAUUAAGCUCAAGCUGUU-AG-3'
	3'-AU-UCGGUUCUUAAUUCGAGUUCGACAA-5'

Reduced endogenous DAPK expression increases the sensitivity of HHUA cells to anti-Fas IgM treatment

Since HHUA cells express functional Fas antigen [17], we directly examined the effects of DAPK knock down on Fas-mediated apoptosis in HHUA cells. In these experiments, the dose-response curve of the DAPK siRNA-transfected HHUA cells in response to anti-Fas IgM antibody treatment was significantly shifted to the

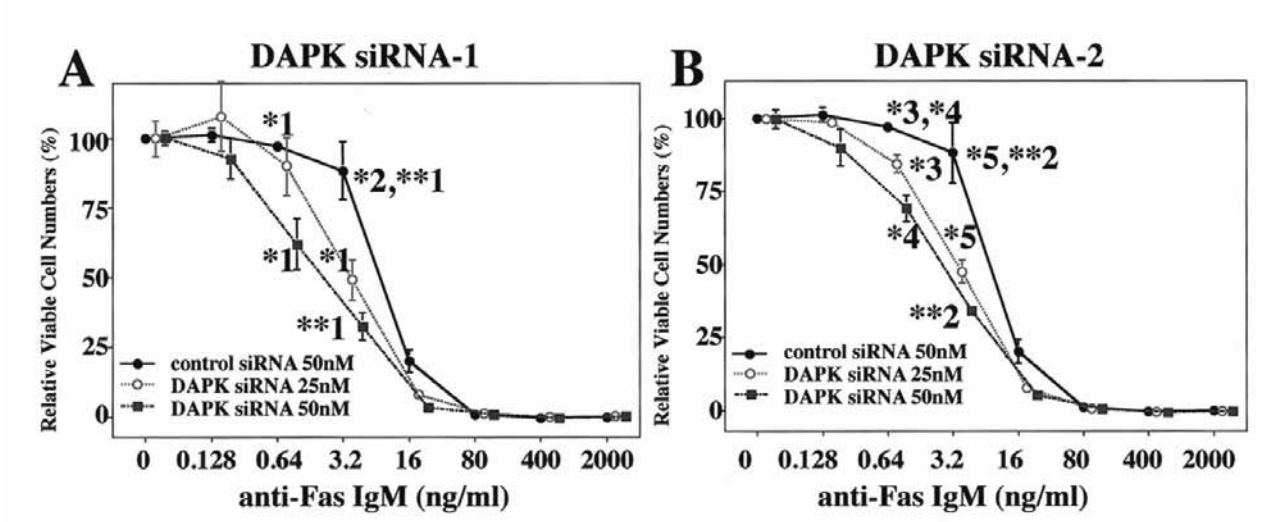

Figure 2. — Effect of DAPK siRNA transfection on cellular Fas-mediated apoptosis in HHUA cells.
The effect of transfection of 25 nM (open circles, dotted lines), or 50 nM (closed squares, dotted lines), of DAPK siRNA-1 (A) or siRNA-2 (B) on the anti-Fas IgM-dose-dependent decrease in cell viability was assayed. Control siRNA (closed circles, solid line) was assayed at a concentration of 50 nM). Transfection of DAPK siRNA dose-dependently enhances Fas-mediated cell death in HHUA cells. *1, *2, *3, *4, *5: $p < 0.05$. **1, **2: $p < 0.01$.

left compared with that of the control siRNA-transfected cells (Figure 2). This shift indicates that a reduction in DAPK expression enhances the susceptibility of HHUA cells to Fas-mediated apoptosis.

Discussion

This is the first report to show a relationship between Fas-mediated apoptosis and DAPK expression in human endometrial adenocarcinoma cells. Specific downregulation of DAPK significantly and dose-dependently enhanced Fas-mediated apoptosis in HHUA cells, indicating that endogenous DAPK expression negatively regulates Fas-mediated apoptosis. Moreover, suppression of DAPK expression may itself have induced apoptotic signals in the cells since DAPK siRNA transfection induced the cleavage of PARP, an apoptosis marker, in HHUA cells (Figure 1). It has been widely believed that DAPK positively and directly mediates apoptosis via the TNFR family members [3, 18, 28-30]. However, this study has shown that DAPK can negatively regulate Fas-mediated apoptosis in certain cancer cells. Our results also suggest that DAPK might have potential as a target of molecular targeting anticancer therapy.

HHUA cells express high levels of functional Fas antigen [17] as well as functional estrogen and progesterone receptors [27], similar to that in normal human endometrial epithelium. HHUA cells also form glandular luminal structures in collagen gel cultures, similar to the structures formed by normal glandular epithelial cells [31]. Analysis of 20 HHUA cells indicated that all cells had a normal 46XX karyotype [32]. Based on these characteristics, HHUA cells are considered to retain many of the intracellular signaling pathways found in normal endometrial epithelial cells. The present experimental data indicate that endogenous DAPK expression inhibits Fas-mediated apoptotic signaling in HHUA cells. There are several reports that DAPK plays cytoprotective roles in healthy cells under normal growth conditions [11, 12]. Therefore, our results may suggest the possibility that menstrual cycle-dependent changes in DAPK expression in the endometrium can also regulate normal endometrial epithelial cell survival or human endometrial remodeling. Since DAPK expression is often regulated by the methylation status of the DAPK promoter region in various cells [13, 20-25], some human endometrial remodeling may also be regulated epigenetically.

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