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PTEN Expression Elicited by *EGR-1* Transcription Factor in Calyculin A-Induced Apoptotic Cells

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Abstract *PTEN* is a tumor suppressor gene encoding a phosphatase that negatively regulates cell survival mediated by the PI3-kinase-Akt pathway. The gene for transcription factor *EGR-1* is an early response gene essential for cellular growth, proliferation, and differentiation. Protein phosphatase inhibitors including calyculin A and okadaic acid are potent inducers of apoptosis in several cell lines; however, the molecular mechanisms underlying their action are unknown. The purpose of this study was to examine the expression of PTEN and EGR-1 and the phosphorylation status of EGR-1 and Akt in calyculin A-treated human squamous carcinoma cells (SCCTF). Phosphorylation of EGR-1 and upregulation of PTEN expression were observed to occur in SCCTF cells treated with calyculin A in time- and dose-dependent fashions. The level of phosphorylated Akt decreased as the expression of PTEN protein increased in the calyculin A-treated SCCTF cells. Calyculin A-stimulated expression of EGR-1 and PTEN might be p53 independent, because the expression of them was also detected in p53-null Saos-2 cells. RNA interference using double-stranded RNA specific for the *EGR-1* gene inhibited not only EGR-1 expression but also PTEN expression in SCCTF cells treated or not with calyculin A. Calyculin A induced nuclear fragmentation and chromatin condensation in SCCTF cells. The present results suggest that the level of PTEN expression and the phosphorylation status of Akt were associated with apoptosis induced by calyculin A. These observations also support the view that EGR-1 regulates PTEN expression in the initial steps of the apoptotic pathway. J. Cell. Biochem. 94: 117–125, 2005.

Key words: Akt; apoptosis; calyculin A; EGR-1; PTEN

Apoptosis is a morphologically and biochemically distinct mode of cell death that plays a major role during embryogenesis, carcinogenesis, cancer treatment, and immune and toxic cell killing, as well as one in cell proliferation [Arends and Wyllie, 1991; Steller, 1995]. Apoptosis can be regulated by extrinsic factors, including hormones, growth factors, cell-surface receptors, and various forms of cellular stress. The action of apoptosis-related factors is often affected by the modulation of the phosphorylation status of key elements active in the apoptotic processes [Cross et al., 2000].

Calyculin A and okadaic acid are potent inhibitors of protein phosphatases type 1 (PP1) and type 2A (PP2A), which dephosphorylate serine and threonine residues in eukaryotic cells [Ishihara et al., 1989; Cohen et al., 1990]. We previously reported that calyculin A or okadaic acid induced apoptosis in osteoblastic cells [Morimoto et al., 1997; Morimoto et al., 1999, 2004; Kito et al., 2003; Yoshida et al., 2003], oral squamous carcinoma cells [Fujita et al., 1999, 2004; Okamura et al., 2001, 2002, 2004; Goto et al., 2002], and submandibular

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gland cells [Morimoto et al., 1999, 2001]. However, the mechanisms whereby the phosphatase inhibitors act in cell killing are still unclear.

The early growth response-1 (*EGR-1*) gene product is a nuclear transcription factor and is implicated in the regulation of a number of genes that are involved in immune response, growth, development, and differentiation [Milbrandt, 1987; Lemaire et al., 1988; Sukhatme et al., 1988]. The *EGR-1* gene belongs to a group of early response genes, as stimulation with many environmental signals including growth factors, hormones, and neurotransmitters dramatically and rapidly induces *EGR-1* gene expression [Liu et al., 1998]. Despite the discovery of EGR-1 as a growth-promoting protein, there have appeared several reports published in recent years describing EGR-1 as a pro-apoptotic protein [Thiel and Cibelli, 2002]. Interestingly, it was reported that calyculin A or okadaic acid induced a sustained expression of the *EGR-1* gene in human and mouse fibroblasts [Cao et al., 1992].

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), also referred to as mutated in multiple advanced cancers (MMAC), was discovered as a tumor suppressor gene [Li et al., 1997; Steck et al., 1997]. It was later found to be a phosphatase that cleaves the 3′ phosphate from phosphatidylinositol 3, 4, 5-trisphosphate to generate phosphatidylinositol 4, 5-bisphosphate [Maehama and Dixon, 1999]. PTEN opposes the action of phosphoinositide 3-kinase (PI3-kinase), which phosphorylates the 3'OH group of the inositol ring in inositol phospholipids. PI3-kinase regulates cell survival via phosphorylation and activation of Akt/protein kinase B, which is important for cell growth and survival [Tsugawa et al., 2002]. PTEN negatively regulates Akt activation by preventing its phosphorylation. Loss of PTEN activity has been suggested to cause enhanced cell proliferation and decreased apoptosis [Stambolic et al., 1998; Davies et al., 1999]. Although the importance of PTEN in suppressing tumor growth has been well studied, little is known about the expression and regulation of PTEN in the cells undergoing apoptosis induced by phosphatase inhibitors. The principal aim of this study was to investigate the expression and regulation of PTEN and the phosphorylation status of EGR-1 and Akt in calyculin A-induced apoptotic cells.

MATERIALS AND METHODS

Materials

Dulbecco's and α modified Eagle's Minimal Essential Medium (D-MEM and α-MEM, respectively) were purchased from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS). Calyculin A and okadaic acid were purchased from Wako Chemical (Osaka, Japan). Both stock solutions (100 μM), prepared in dimethylsulfoxide (DMSO) and protected from the light, were diluted to the appropriate concentrations with medium. Anti-EGR-1 and anti-PTEN polyclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA); and anti-Akt monoclonal antibody and anti-phospho-Akt (p-Ser472/473/474) polyclonal antibody, from BD Biosciences (San Jose, CA). An anti-nucleolin monoclonal antibody (Clone 4E2) was obtained from MBL (Nagoya, Japan). Plastic dishes were from Iwaki (Chiba, Japan). Other materials used were of the highest grade commercially available.

Cells and Culture Conditions

Human squamous carcinoma cell lines, SCCTF and SCCKN cells were purchased from Riken Cell Bank (Tsukuba, Japan). Human squamous carcinoma cell line SCC25 cells and human osteoblastic cell lines Saos-2 and MG63 cells were obtained from the American Type Culture Collection (Rockville, MD). SCCTF and SCCKN cells, and Saos-2 and MG63 cells were cultured in plastic dishes containing D-MEM and α-MEM, respectively. Each medium contained 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For cytochemistry, the cells were plated on 18 mm round coverslips placed in 60 mm plastic dishes and cultured. An Olympus IMT-2 phase-contrast microscope equipped for photomicroscopy was used to monitor cell modification. Phase-contrast microphotographs were taken on Fuji Presto 100 films.

SDS–PAGE and Immunoblotting

After appropriate periods of cultivation, cells were washed twice with phosphate-buffered saline (PBS) and scraped into lysate buffer containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 mM EGTA

in PBS. The cells were sonicated for 10 s with a sonifier cell disrupter, and the sonicates were then centrifuged for 10 min at 10,000*g*. The supernatants were denatured in sample buffer and heated in boiling water for 5 min. Equal amounts of protein estimated with the Bio-Rad protein assay kit (Richmond, CA) and prestained molecular weight markers (Gibco BRL) were separated by 10% SDS–PAGE and transferred electrophoretically from the gels to polyvinylidene difluoride (PVDF) transfer membranes (Immobilon, Millipore, Bedford, MA). The membranes were incubated in a blocking solution containing 5% skim milk and 0.05% Tween-20 in PBS (PBS-Tween) and incubated for 2 h at ambient temperature. The membranes were washed briefly in PBS-Tween and incubated at 4°C overnight with each antibody diluted (1:100–1:500). The membranes were next washed four times within 30 min in PBS-Tween by using a rotary shaker at ambient temperature. The washed membranes were incubated for 1 h at ambient temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit or -mouse IgG (diluted 1:5,000) in PBS-Tween. The membranes were washed as described above and the proteins recognized by the antibodies were visualized by using an ECL detection kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's directions. The intensities of staining were measured by using an image-analyzing program (NIH image) and standardized with the control.

RNA Synthesis

We prepared the following DNA oligos specific for human *EGR-1* sequences (1206-1226), which were designed with 3′ T7 promoter sequences.

EGR-1 sense: 5′-aagaaagtttgccaggagcgacctgtc tc-3′
EGR-1 antisense: 5′-aatcgctcctggcaaactttccctg tctc-3′

Target sequences were aligned to the human genome database in a BLAST search to eliminate sequences with significant homology to other genes. Transcription was performed in 100 μl of transcription mix: 5× T7 transcription buffer, 100 mM rNTP, 5 μg template DNA, and T7 RNA polymerase (T7 RiboMAX Express Large Scale RNA Production System, Promega, Madison, WI). After incubation at 37°C for 4 h, RNase-free DNase (Promega) was added; and incubation was continued for 15 min at 37°C. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions and heating them at 95°C for 5 min followed by 1 h at 37°C to obtain the synthesized small interfering dsRNAs. The RNAs were extracted with phenol/chloroform, ethanol precipitated, and dissolved in water. Formation of the dsRNA was monitored by electrophoresis on a 1.5% agarose gel.

Transfection

SCCTF cells were plated in 90 mm plates and maintained in 10% FBS D-MEM until they had reached 70–80% confluence. Before transfection, the cells were washed once with serum-free medium. Transfections were performed with LipofectAMINE reagents (Invitrogen, Carlsbad, CA). Synthesized RNA (1.5 μg) or non-specific control dsRNA (1.5 μg, DHARMACON, Lafayette, Colorado) was mixed with Plus reagents (Invitrogen) for 15 min in OPTI-MEM (Gibco BRL), and then they were mixed with LipofectAMINE reagent for another 45 min at room temperature. Serum-free medium was added to the mixture, and the complexes were overlaid onto the cells. After incubation at 37°C, the medium was replaced with fresh medium containing 10% FBS and incubation was continued for another 48 h. The sequences of non-specific control dsRNA are as follows:

Sense: 5′-auucuaucacuagcgugacuu-3′
Antisense: 5′-gucacgcuagugauagaauuu-3′

Nuclear Fragmentation Assay With Hoechst Staining

Morphological assessment of apoptotic cells was performed by using the Hoechst 33342 staining. After appropriate cultivation, the coverslips were removed from the dishes and placed directly into 10% formalin in PBS for 10 min at ambient temperature. The fixed cells were washed three times with PBS and permeabilized with methanol for 10 min at −20°C. After having been washed three times with PBS, the coverslips were incubated with Hoechst 33342 (10 μg/ml) for 10 min at ambient temperature. After a final rinse with PBS, coverslips were mounted while wet in Gel/Mount aqueous mounting medium (Biomeda, Foster City, CA). The coverslips were examined under an Olympus microscope (BX50) equipped for epifluorescent illumination (BX-FLA) and for

photomicroscopy (PM-30). Photomicrographs were taken on Fuji Presto 400 films.

RESULTS

Expression of EGR-1 in SCCTF Cells

Figure 1 shows the reaction between the anti-EGR-1 antibody and the proteins extracted from various human cell lines at the confluent stage including SCCKN, SCCTF, SCC25, Saos-2, and MG63 cells. In the samples prepared from SCCKN and SCCTF cells, the anti-EGR-1 antibody interacted with a major band of protein with an estimated molecular weight of 82,000. The staining intensity of EGR-1 protein was higher in SCCTF cells than that in SCCKN cells. However, a weak or no reaction was detected in the extracts prepared from SCC25, Saos-2, and MG63 cells at the confluent stage (Fig. 1).

Phosphorylation of EGR-1 and Upregulation of PTEN Expression in Calyculin A- Treated SCCTF Cells

Cell extracts were prepared from SCCTF cells that had been treated with various concentrations of calyculin A for 6 h. Figure 2 shows the reaction of the EGR-1 (upper panel) and the PTEN (lower panel) antibodies with the proteins extracted from the calyculin A-treated SCCTF cells. When the cells were treated with 5 nM calyculin A, the EGR-1 antibody interacted with an additional band that migrated slower than the control band. The intensity of this band increased in a dose-dependent manner up to 10 nM calyculin A (Fig. 2A). This finding indicates that the EGR-1 protein was phosphorylated in the SCCTF cells treated with calyculin A. The level of phosphorylated EGR-1 in the cells treated with calyculin A increased in a time-dependent manner (Fig. 2B). The level of PTEN protein expression also increased in dose- and time-dependent manners in the calyculin A-treated cells (Fig. 2). No band corresponding to EGR-1 or PTEN was detected in the blot incubated with the same dilution of normal rabbit or normal goat serum (data not shown). Okadaic acid, another protein phosphatase inhibitor, induced phosphorylation of EGR-1 protein in SCCTF cells, and also stimulated the expression of PTEN in these cells in a dose-dependent manner (Fig. 3). To examine whether p53 is involved in PTEN expression, we prepared proteins from Saos-2 cells, which lack

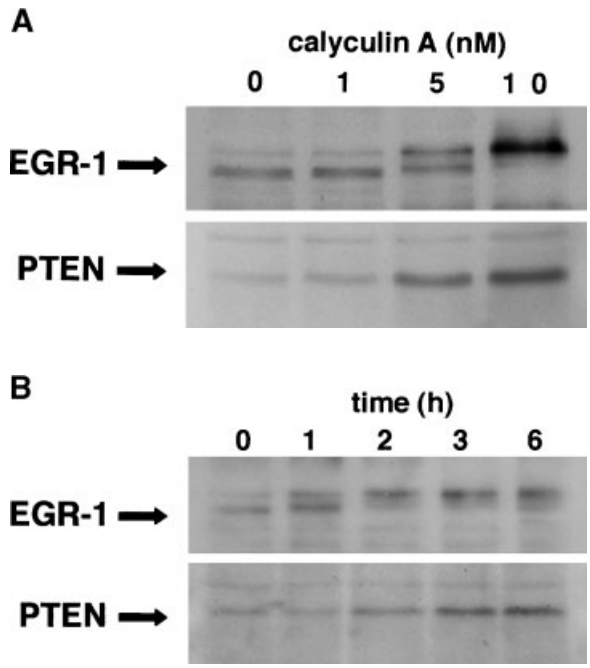

Fig. 2. Identification of EGR-1 and PTEN in SCCTF cells treated with calyculin A. Proteins prepared from the cells cultured with calyculin A were analyzed by Western blotting. **A**: Dose-response of the expression of both proteins in the cells treated with calyculin A. **Upper panel**, EGR-1; **lower panel**, PTEN. **B**: Expression of both EGR-1 and PTEN proteins in SCCTF cells treated with 10 nM calyculin A for various time periods. **Upper panel**, EGR-1; **lower panel**, PTEN.

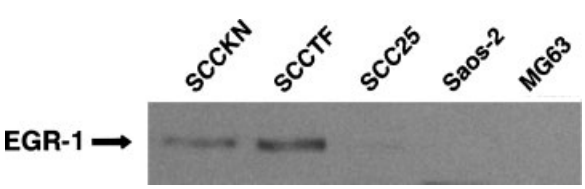

Fig. 1. Examination of SCCKN, SCCTF, SCC25, Saos-2, and MG63 cells at the confluent stage for expression of EGR-1 protein. Proteins prepared from each cell line were analyzed by Western blotting using anti-EGR-1 antibody.

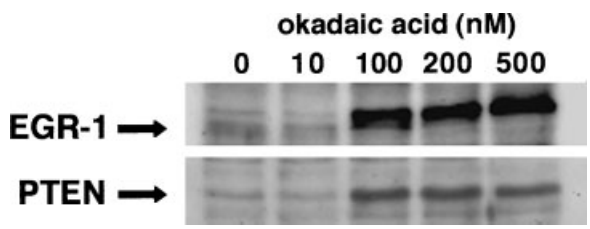

Fig. 3. Identification of EGR-1 and PTEN in SCCTF cells treated with okadaic acid. Proteins prepared from cells cultured for 6 h with various concentrations of okadaic acid were analyzed by Western blotting. **Upper panel**, EGR-1; **lower panel**, PTEN.

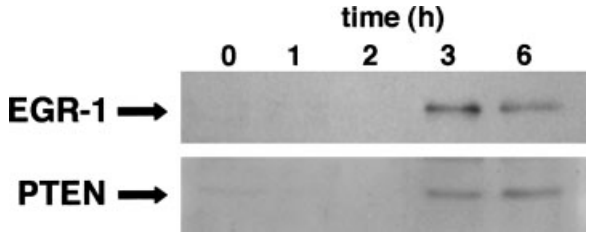

Fig. 4. Identification of EGR-1 and PTEN in Saos-2 cells treated with calyculin A. Proteins prepared from cells cultured with 10 nM calyculin A for various time periods as indicated were analyzed by Western blotting. **Upper panel**, EGR-1; **lower panel**, PTEN.

the *p53* gene, after having treated them for various time periods with 10 nM calyculin A and then performed Western blotting with the anti-EGR-1 and the anti-PTEN antibodies (Fig. 4). Although the expression was minimal in the Saos-2 cells at the confluent stage, calyculin A-induced the expression of EGR-1 in these cells. Calyculin A also stimulated the PTEN expression in Saos-2 cells in a dose-dependent fashion.

Downregulation of Phospho-Akt in the Calyculin A-Induced Apoptotic Cells

To investigate whether the level of active Akt in SCCTF cells could be changed in response to calyculin A treatment, we examined the amount of the activated form of Akt, i.e., its phosphorylated form, by Western blotting using an anti-phospho-Akt-specific antibody. Figure 5 shows that the treatment with 10 nM calyculin A decreased the phosphorylation status of Akt (Fig. 5, upper panel) in a time-dependent manner in SCCTF cells. This antibody was then stripped off from the PVDF membrane, and the membrane was re-probed with an anti-pan Akt antibody, which recognized non-phosphorylated form of Akt. The amount of non-phosphorylated Akt did not change notably in the calyculin A-treated cells (Fig. 5, lower panel).

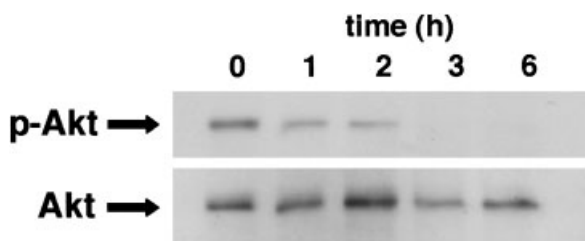

Fig. 5. Expression of Akt and its phosphorylation by calyculin A treatment. The expression of Akt and its phosphorylation form was analyzed in calyculin A-treated SCCTF cells by Western blotting. The cells were treated with 10 nM calyculin A for various time periods as indicated. Twelve micrograms of protein of each sample was separated on a 10% of SDS–PAGE, transferred to a PVDF membrane, and incubated with anti-phospho-Akt antibody (**upper panel**). The antibody was then stripped off the membrane, which was subsequently re-incubated with the whole Akt-recognizing antibody (**lower panel**).

Involvement of EGR-1 in the Expression of PTEN by Calyculin A

To further assess the role of EGR-1 in PTEN expression, we transfected SCCTF cells with synthesized dsRNA specific for the *EGR-1* gene. To analyze the change in *EGR-1* gene expression at the protein level in the transfected cells, we performed Western analysis by using the anti-EGR-1 and anti-nucleolin antibodies. Expressions of EGR-1 and nucleolin were not affected in the SCCTF cells transfected with mock or non-specific dsRNA as controls. Compared with that in the control cultures, the staining intensity of EGR-1 decreased in the SCCTF cells that had been transfected with the *EGR-1*-specific dsRNA (Fig. 6). However, this interference had no effect on the nucleolin expression, suggesting that this interference was specific for *EGR-1* (Fig. 6). We next examined whether EGR-1 is involved in PTEN expression in SCCTF cells treated with calyculin A. Figure 7 shows the reaction of the EGR-1

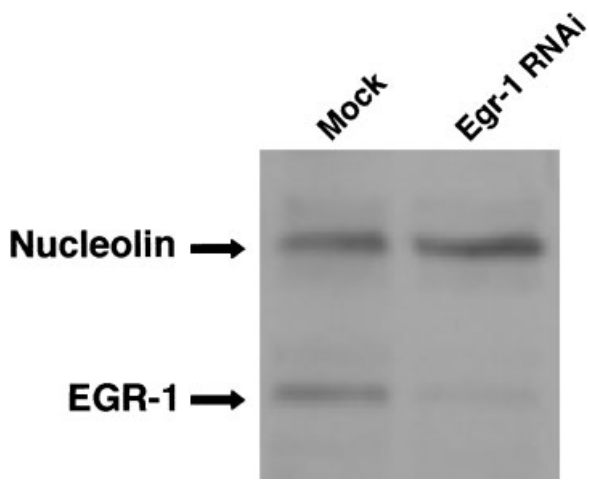

Fig. 6. Western analysis of EGR-1 and nucleolin in SCCTF cells transfected with dsRNA for *EGR-1*. The cells were transfected with mock and dsRNA specific for *EGR-1* gene. After 48 h cell extracts were prepared, separated on 12.5% gel, and transferred to Immobilon membrane. The membrane was probed with anti-EGR-1 and anti-nucleolin antibodies.

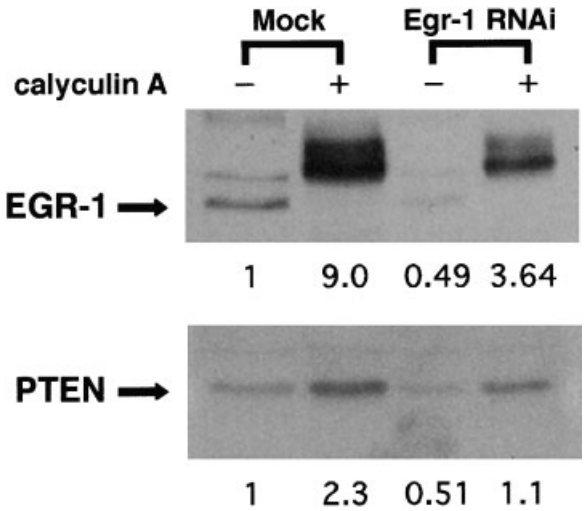

Fig. 7. Western analysis of EGR-1 and PTEN in SCCTF cells treated with calyculin A. The cells were transfected with mock or *EGR-1*-specific dsRNA. After 48 h, the cells were incubated with or without 10 nM calyculin A for 6 h. Cell extracts were prepared, separated on 12.5% SDS–PAGE gel, and transferred to Immobilon membrane. The membrane was probed with anti-EGR-1 and anti-PTEN antibodies. The values under the columns were the relative-fold values of staining intensity standardized by the controls.

(upper panel) and the PTEN (lower panel) antibodies with the proteins extracted from SCCTF cells treated with calyculin A. Higher level of the EGR-1 expression was observed in the control SCCTF cells. The band migrated to the origin of the gel was stained intensively in the cells treated with calyculin A, indicating the phosphorylated form of EGR-1. In the cells in which *EGR-1*-specific dsRNA was transfected, the staining intensity of EGR-1 decreased to the half levels of the control. The intensity of the slower migrating band of EGR-1 decreased in the cells treated with calyculin A compared with that of the mock control cells treated with calyculin A. The amount of PTEN was half of the control level in the cells transfected with the *EGR-1*-specific dsRNA. Also the increased staining level of PTEN in the cells treated with calyculin A was diminished in the cells subjected to *EGR-1* interference compared with that in the cells without the interference. Same results were obtained in four experiments and the most representative result was shown. The values of the signals of EGR-1 or PTEN were presented below the columns as the fold intensity. Transfection of non-specific control dsRNA had the same effects on the expression of EGR-1 or PTEN in the mock-transfected cells (data not shown).

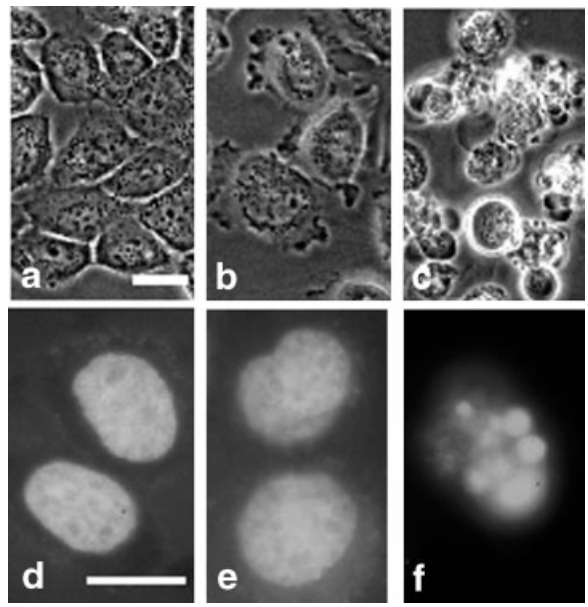

Fig. 8. Phase-contrast appearance (**a**–**c**) and nuclear morphology (**d**–**f**) of SCCTF cells treated with calyculin A. After they had reached confluence, SCCTF cells were left untreated (a and d) or treated with calyculin A at a concentration of 10 nM for 3 h (b and e), or 6 h (c and f). Cells were stained with Hoechst 33342 and observed under a fluorescence microscope (d–f). Bar represents 10 μm.

Calyculin A-Induced Apoptosis in SCCTF Cells

The confluent SCCTF cells were treated with 10 nM calyculin A for various time periods (Fig. 8). The control cultures of SCCTF cells (Fig. 8a) did not show any evidence of cytotoxicity. The cells treated with calyculin A for 3 h (Fig. 8b) showed a low level of cytotoxicity; however, the cells still remained attached to the culture dish. Cell rounding and shrinking was obvious in SCCTF cells treated with calyculin A for 6 h (Fig. 8c). To determine whether calyculin A-induced cell death was due to apoptosis, we evaluated nuclear fragmentation and condensation of chromatin in SCCTF cells. After having been fixed and permeabilized, the cells were incubated with Hoechst 33342 for 10 min. The control cultures of SCCTF cells (Fig. 8d) and the cells incubated with 10 nM calyculin A for 3 h (Fig. 8e) did not show any apoptotic features. However, the treatment with 10 nM calyculin A for 6 h induced nuclear fragmentation and condensation of chromatin in SCCTF cells (Fig. 8f). Calyculin A also induced DNA ladder formation in SCCTF cells in time- and dose-dependent manners (data not shown).

DISCUSSION

In the present study, we used SCCTF cells to examine the changes in the phosphorylation status of EGR-1 effected by calyculin A or okadaic acid because EGR-1 protein was constitutively expressed at a higher level in SCCTF cells than in other cell lines including SCCKN cells. SCCTF cells exhibited minimal sensitivity to antitumor drugs, whereas SCCKN cells were highly sensitive to these drugs [Urade et al., 1992; Okamura et al., 2001]. We showed that calyculin A or okadaic acid stimulated phosphorylation of EGR-1 protein in SCCTF cells. This phosphorylation was not observed in SCCKN cells and okadaic acid suppressed the expression of EGR-1 in these cells [Okamura et al., 2002]. This discrepancy might depend on the sensitivity of the cells to the inhibitors. EGR-1 was strongly and rapidly induced in human and mouse fibroblasts by calyculin A or okadaic acid treatment [Cao et al., 1992], and multiple species of the phosphorylated forms of the EGR-1 protein were detected in the cells treated with either of these phosphatase inhibitors. It was also reported that binding of hyper-phosphorylated EGR-1 to its consensus sequence was strongly stimulated by okadaic acid in HeLa cells and fibroblasts [Cao et al., 1993; Huang et al., 1998]. These reports and our observations support the notion that modification of EGR-1 by phosphorylation may have profound effect on its biological functions.

PTEN is known to dephosphorylate the PI3-kinase product at the 3′ position of the inositol ring, resulting in the reduced Akt activation [Maehama and Dixon, 1999; Choi et al., 2002]. In the present study, the level of the phosphorylation of Akt decreased in accord with the induction of PTEN expression in SCCTF cells. Considering that the PTEN substrate is PI (3, 4, 5) P_3, a lipid second messenger whose levels are limiting for activation of the PI3-kinase-dependent survival cascade, we confirmed that the changes in PTEN expression are sufficient to produce profound effects on signaling downstream of PI (3, 4, 5) P_3. In the present study, we also demonstrated that treatment with 10 nM calyculin A for 6 h induced apoptosis and PTEN expression in SCCTF cells. However, apoptosis and PTEN expression could not detected in the SCCTF cells treated for 6 h with 1 nM calyculin A. In other situations calyculin A or okadaic acid could protect against apoptosis induced by various stimuli [N'cho and Brahmi, 1999]. This discrepancy might be due to the concentrations and the terms of treatment with these reagents in each experiment. In addition to calyculin A, okadaic acid also strongly stimulated PTEN expression in SCCTF cells, indicating that this expression was not restricted to the case of calyculin A. These results are consistent with reports that the expression of PTEN resulted in growth inhibition and increased apoptosis in a variety of normal and transformed, human and murine cells [Paramio et al., 1999; Choi et al., 2002]. It was shown that embryonic fibroblasts from PTEN knockout mouse exhibited reduced sensitivity to apoptosis and that extrinsic PTEN could restore the sensitivity to agonist-induced apoptosis [Stambolic et al., 1998]. It was also pointed out that the expression of PTEN seems to regulate certain apoptotic signals affecting PI3-kinase, although okadaic acid only slightly affected the expression of PTEN in Neuro-2a cells [Kyrylenko et al., 1999].

In view of the transcriptional regulation in PTEN expression, a 2 kb fragment upstream of the translation start site of PTEN is highly GC rich and contains several potential binding sites for the EGR-1 protein [Virolle et al., 2001]. The hypothesis that EGR-1 might regulate the transcription of the *PTEN* gene is attractive, because the two genes have growth-suppressing activity toward and some similar other effects on cells, with variation depending on specific cell types. We showed that the stimulation of PTEN expression by calyculin A treatment was observed in accordance with the expression of EGR-1 in Saos-2 cells, which are p53 defective [Chandar et al., 1992]. These observations strengthen our hypothesis that EGR-1, not p53, plays a positive role in PTEN expression induced by calyculin A. Furthermore, the RNA interference using dsRNA specific for the *EGR-1* gene inhibited the expression of EGR-1 and PTEN in SCCTF cells untreated or treated with calyculin A, indicating EGR-1 involvement in PTEN expression in either case. The increase of EGR-1 and PTEN expressions by calyculin A treatment was detected in the cells transfected with *EGR-1*-specific dsRNA. This might be explained by the fact that EGR-1 expression was not completely blocked by RNAi. Calyculin A might stimulate the expression of EGR-1 and PTEN and phosphorylation of EGR-1 even in the cells treated with dsRNA. It was

reported that EGR-1 could directly regulate PTEN, triggering the initial step in the apoptotic pathway after irradiation [Virolle et al., 2001]. These researchers suggested that the loss of EGR-1 expression, which often occurs in human cancers, could down-regulate the *PTEN* gene expression and thus contribute to the radiation resistance of some cancer cells [Virolle et al., 2001]. However, these results do not eliminate the possibility that other transcription factors activated or inactivated in response to another cellular stress might also contribute to the regulation of PTEN expression in SCCTF cells. For example, it was demonstrated that the selective PPARγ ligand rosiglitazone increased PTEN expression in AsPC-1 human pancreatic cancer cells. Concurrent treatment with GW9662, which inhibits PPARγ activation, prevented the rosiglitazone-induced increase in PTEN protein levels [Farrow and Evers, 2003]. Another group reported that induction of p53 in primary and tumor cell lines with wild-type p53 increased *PTEN* mRNA levels [Stambolic et al., 2001].

In summary, our data show that either calyculin A or okadaic acid induced the expression of PTEN through the activation of EGR-1 during apoptosis in SCCTF cells. Our results suggest that EGR-1 can regulate PTEN expression triggered by calyculin A in the initial step in apoptotic pathway. However, further studies are needed to clarify whether up-regulation of PTEN is the most essential event for apoptosis induced by protein phosphatase inhibitors.

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