Ribozymes targeting serine/threonine kinase Akt1 sensitize cells to anticancer drugs

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(Received May 21, 2005/Revised June 22, 2005/Accepted June 27, 2005/Online publication September 5, 2005)

The serine/threonine kinase Akt is a key component of the cellular signaling pathway for survival and drug-resistance in cancer cells. In the present study we confirmed this view by expressing an antagonist of Akt, a dominant negative form of Akt, in HCT116 colon carcinoma cells and observing apoptosis induction in cells in which expression of the mutant protein had been induced. Three isoforms of Akt have been found: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ. However, the function of individual isoforms with respect to tumorigenicity and drug-resistance of cancer cells is largely unknown. We designed ribozymes targeting the Akt1 protein in mammalian cells. Our data indicate that Akt1 ribozymes downregulate Akt1 expression to less than half that of control cells. Downregulation of Akt1 expression appears to sensitize HEK293 and HeLa cells to typical chemotherapeutic agents. However, Akt1 ribozymes had little effect on the proliferative activity of the cells. Thus, Akt as a whole and even just the Akt1 isozyme is an excellent target for chemotherapy. We further suggest a synergistic effect for combination therapy targeting Akt and other vital molecules such as tubulins, topoisomerases and protein kinases. (*Cancer Sci* 2005; *96*: 620–626)

Akt (EC 2.7.1.37), the cellular homologue of the viral oncogene *v-akt*, encodes a serine/threonine kinase that is ubiquitously expressed in mammalian cells.[1,2] Activated Akt phosphorylates various substrates in the cytoplasm and in the nucleus, and elicits a variety of biological effects, including growth stimulation and suppression of apoptosis.[3] The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is frequently deregulated in cancer cells.[4,5] Akt is thought to play a critical role in preventing cancer cells from undergoing apoptosis.[6] Thus Akt plays an antiapoptotic role by phosphorylating caspase-9,[7] BAD[8,9] and the forkhead transcription factors involved in the expression of the Fas ligand,[10,11] suppressing their pro-apoptotic effects. Akt is constitutively activated in PTEN-negative cancer cells.[12] *PTEN* is a tumor suppressor gene that is frequently mutated in many advanced tumors.[13,14] PTEN is a phosphatase that dephosphorylates phosphatidylinositol 3,4,5-phosphates (PIP_3) and reverses the effect of PI3K.[14]

The Akt/PKB family is composed of three member isoforms: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, respectively. The isoforms all contain an N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal regulatory domain.[3–6] Akt1 and Akt2 are frequently amplified and overexpressed in many cancer cells.[15,16] It is known that Akt1 is ubiquitously expressed in human tissues[2] and amplified in gastric adenocarcinomas.[15] Based on these properties of Akt, we hypothesized that inhibition of Akt kinase activity would lead to suppression of growth and tumorigenic potential of cancer cells and/or to induction of apoptosis. In the present study we used an inducible expression system, 'Cre/loxP', for the expression of a dominant negative form of Akt1, which most likely inhibits all members of the family, and in fact has been observed to induce apoptosis. We further examined the role of the Akt1 isozyme in drug sensitivity of cancer cells: we attempted to inhibit the expression of Akt1 by using a ribozyme-expression vector and studied the effect of the downregulation of the isozyme on proliferation and sensitivity to chemotherapeutic agents. We report that downregulation of Akt1 expression sensitized 293 and HeLa cells to typical chemotherapeutic agents, but did not affect the proliferative activity of the cells.

Materials and Methods

Materials

Camptothecin, staurosporine, and paclitaxel were purchased from Sigma (St. Louis, MO, USA). Teniposide was obtained from Calbiochem (San Diego, CA, USA). Monoclonal antibody for hemagglutinin (HA), Y-11, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Plasmid pCALNLw DNA was kindly donated by Dr Izumu Saito, Institute of Medical Science, University of Tokyo, Japan. HA-tagged dominant-negative bovine Akt1, HA-Akt (DN), cDNA (K179A, T308A and S473A) was kindly donated by Dr James R. Woodgett of Ontario Cancer Institute, Canada.[17] The cosmid DNA harboring the β-galactosidase (*lacZ*) gene, pAXCAiLacZ, was purchased from Takara Shuzo, Tokyo, Japan. The recombinant adenovirus expressing β-galactosidase, AxCALacZ, was prepared by using the COS-TPC method as described elsewhere.[18] Another recombinant adenovirus expressing Cre recombinase, AxCANCre, was obtained from the Riken DNA Bank (Tsukuba, Japan).

Construction of the 'Cre/loxP' inducible expression system for the expression of HA-Akt (DN)

HA-Akt (DN) cDNA was excised from the plasmid by *Eco*RI/*Stu*I double digestion and the ends were blunted. The Akt cDNAs were ligated into a *Swa*I site in an inducible expression vector, pCALNLw. Orientation of these cDNAs relative to the promoter

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doi: 10.1111/j.1349-7006.2005.00088.x

in the vectors was determined by sequencing the constructs using a Texas-red-labeled cytomegalovirus promoter primer (5′-ggtaggcgtgtacggtg-3′), a Texas-red-polyA signal primer (5′-cagatggctggcaactagaaggcac-3′), a Thermo Sequenase Core Sequencing kit (Amersham Biosciences, Piscataway, NJ, USA) and an SQ-5500 Personal DNA Sequencer (Hitachi, Tokyo, Japan) according to the manufacturers' instructions.

Construction of the ribozyme expression vector

Because *trans*-acting hammerhead ribozymes preferentially recognize and cleave the GUC sequence, we searched for the GUC sequences in Akt1 mRNA as possible cleavage sites. Ignoring the complementarity between the upstream and downstream sequences at each GUC site, we chose two target sites: 445–447 and 561–563 nucleotides from the 5′ end of the Akt1 mRNA. These ribozymes were named ribozyme 1 and ribozyme 2, respectively. Then two single-stranded oligodeoxynucleotides were synthesized: ribozyme 1, 5′-ccggttcgaaaccgggcactacaaaaaccaacgttcgatctgatgaggccgaaaggccgaaacagtggaggtaccccggatatctttttttt-3′ and ribozyme 2, 5′-ccggttcgaaaccgggcactacaaaaaccaactgagcccctgatgaggccgaaaggccgaaaccggaaaggtaccccggatatctttttttt-3′. These single-stranded ribozymes were made double-stranded by using the polymerase chain reaction (PCR) with these oligonucleotides as templates and random hexamers as primers. The ribozymes were cloned into the *Kpn*I/*Bst*BI sites of the vector pUC19-KE/tRNA.[19]

Cell culture and vector transfection

Human colon carcinoma cell line HCT116, human embryonic kidney cell line 293 and human cervical carcinoma cell line HeLa were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Transfection of the HCT116 cells with the inducible expression vector of HA-Akt (DN) or an empty vector and transfection of the 293 cells with ribozyme expression vectors were carried out by using the calcium-phosphate precipitation method.[20] The cells were cotransfected with the neomycin resistance gene-expressing plasmid pcDNA3 for the selection of transfectants. Three days after transfection, G418 (Gibco BRL, Rockville, MD, USA) was added to the medium to a final concentration of 0.8 mg/mL and the cells were cultured for 3 weeks. Individual colonies were isolated and cloned for further analysis. Transient transfection of HeLa cells was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Western blot analysis

Clones harboring the inducible expression vector of HA-Akt (DN) were infected with AxCALacZ or AxCANCre at a multiplicity of infection (MOI) of 5 to 50, respectively. Two days after infection, cells were solubilized with RIPA buffer (25 mM Tris, pH 8.0/150 mM NaCl/0.1% SDS/0.5% sodium deoxycholate/1% NP-40/10% glycerol/2 mM EDTA).[21] The cell lysates were then subjected to 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis. The proteins were transblotted onto a nitrocellulose membrane. After blocking, the membrane was incubated with an anti-HA antibody, Y-11. The membrane was then incubated with an appropriate peroxidase-conjugated secondary antibody and developed with an enhanced chemiluminescence mixture (Amersham Bioscience).

Observation of the HA-Akt (DN) expressing clone

Two days after infection of AxCALacZ or AxCANCre at an MOI of 5, morphologies of the clones were observed with a phase-contrast microscope (IX70, Olympus, Tokyo, Japan). The nucleus of these clones was stained with Hoechst33258 (Wako, Osaka, Japan) and the clones were observed with a fluorescent microscope (BX50, Olympus).

DNA ladder formation analysis

Two days after infection of each virus at an MOI of 5, cells (3×10^6) were washed once with phosphate-buffered saline, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM ethylenediamine tetraacetic acid [EDTA], 0.5% Triton X-100) and centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant was then treated with 0.2 mg/mL RNase for 1 h at 37°C, followed by treatment with 0.4 mg/mL proteinase K for 30 min at 50°C. To this mixture was added 20 μL 5 M NaCl and 120 μL isopropanol, then the mixture was stood overnight at −20°C. After centrifugation at 12 000 *g* at 4°C for 5 min, the precipitate was dissolved in 20 μL of TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 4 μL of 6× loading dye (50% glycerol, 0.1% Bromo Phenol Blue) was added, then the mixture was electrophoresed on 2% agarose gels containing 0.1 μg/mL ethidium bromide.

Quantitative growth inhibition assay

Two thousand cells from each clone harboring inducible expression vectors were seeded in 200 μL growth medium in collagen-coated 96-well plates and incubated at 37°C for 24 h. Cells were then infected with AxCANCre at an MOI of 5. After infection, cells were further incubated at 37°C for 2–6 days, after which the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed.[22] Medium was removed and 200 μL of medium containing 250 μg/mL MTT solution (Sigma) was added to each well and incubated at 37°C for 4 h. Plates were centrifuged, the supernatant removed and the precipitates dissolved in 150 μL of dimethylsulfoxide (DMSO). The absorbance at 570 nM was measured for each well using a microplate reader (BioRAD, Hercules, CA, USA).

Reverse transcription-PCR

Total RNA was extracted from the transfectants and parental 293 cells by using the guanidine isothiocyanate method according to the standard laboratory manual.[23] One microgram of total RNA from each transfectant was reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase (MMLV-RT; Gibco BRL) and oligo-dT primer (Invitrogen) or pd $(N)_6$ random primer (Amersham Biosciences) to generate the first strand cDNA. To detect the expression of ribozymes, a set of primers, 5′-ccggttcgaaaccgggcac-3′ and 5′-aaaaaaagatatccggggtacct-3′, was designed based on the sequences surrounding the ribozymes in the pUC-KE/tRNA vector. The conditions used for amplification were as follows: 95°C for 30 s followed by 55°C for 30 s and 74°C for 30 s in a 50-μL reaction buffer containing first strand cDNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer and 2.5 U *Taq* polymerase (Promega, Madison, WI). As a control, a ribozyme 2-expressing vector was used as a template under the same conditions. PCR products after 25 cycles were quantified

by electrophoresis on 18% polyacrylamide gels. As a loading control, reaction mixtures of cDNA preparations were run on the same gels, and the 18S ribosomal RNA was shown.

RNase protection assay

The RNase protection assay was carried out as described elsewhere.[24] Total RNA was extracted as described earlier. ^{32}P-labeled antisense RNA probes targeting portions of Akt1 or Akt2 were prepared by *in vitro* transcription of the pBluescript II plasmid containing each Akt fragment in the presence of (α-^{32}P)UTP. The Akt fragments used were generated by RT-PCR using sets of primers, 5′-cccttctacaaccaggaccatgagaagctt-3′ and 5′-tctttccatctgggctcgagaggacagcgt-3′ for Akt1; 5′-cccaag-cttggcccctgatcagactcta-3′ and 5′-ccgctcgagtcctcagtcgtggaggagt-3′ for Akt2, and *Taq* DNA polymerase (Promega), and cloned into the *Hin*dIII/*Xho*I site of pBluescriptII SK+. The length of each probe was 501 bp for Akt1 and 575 bp for Akt2, respectively. Hybridization of cellular RNA with both probes was carried out at 42°C overnight. The protected fragments were precipitated, analyzed with 15% polyacrylamide-urea gel electrophoresis and quantified using the Bass-2000 Phosphorimager (Fuji, Tokyo, Japan).

Proliferation and drug sensitivity of 293 and HeLa cells

The 293 cells stably expressing ribozymes were plated in 96-well plates at an initial density of 2000 cells per well in culture medium. Viable cells were quantified by using the MTT method, with the following day set as day 1, for 4 days. In assessing drug sensitivity, cells were plated at 2000 cells per well onto 96-well plates. One day after plating, cells were treated with various concentrations of chemotherapeutic agents and cell survival was estimated by using the MTT method. The IC_{50} values of drugs were calculated from the survival of cells treated for 3 days. IC_{50} was defined as the concentration of drug causing 50% inhibition of cell growth, as compared with a solvent (DMSO) control. HeLa cells were transiently transfected with ribozyme expression vectors. Cells were harvested 2 days after transfection, and 2000 cells were plated per well onto 96-well plates. One day after plating, cells were treated with 10 nM of camptothecin or solvent (DMSO). Viable cells were quantified by using the MTT method, with the following day set day 1, for 5 days.

Results

Inducible expression of a dominant-negative Akt in HCT116 colon carcinoma cells leads to apoptosis

We used the 'Cre/loxP' system to inducibly express a dominant-negative form of Akt, Akt (DN).[25,26] We constructed a pCALNLw plasmid expressing Akt (DN) under the control of recombinase Cre, then transfected HCT116 colon carcinoma cells with the plasmid and established stable transformant clones, HCT/Akt (DN), harboring Akt (DN). From several clones we selected a clone named DN1-48 in which Akt (DN) was able to be induced by infection with a recombinant adenovirus, AxCANCre, which constitutively expresses Cre recombinase. Figure 1 shows the inducible expression of Akt (DN) 2 days after infection as a function of the MOI of AxCANCre (C), whereas AxCALacZ (L) constitutively expressing LacZ did not induce Akt (DN) at all. Next, we assessed the effect of Akt (DN)

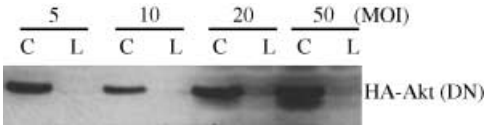

Fig. 1. Inducible expression of Akt (DN) in an Akt (DN)-transformed clone (DN1-48) by infection with AxCANCre. DN1-48 cells were infected with either AxCALacZ (L) or AxCANCre (C) at a multiplicity of infection of 5, 10, 20 or 50. Two days after infection, cells were harvested and subjected to western blotting using an anti-HA antibody.

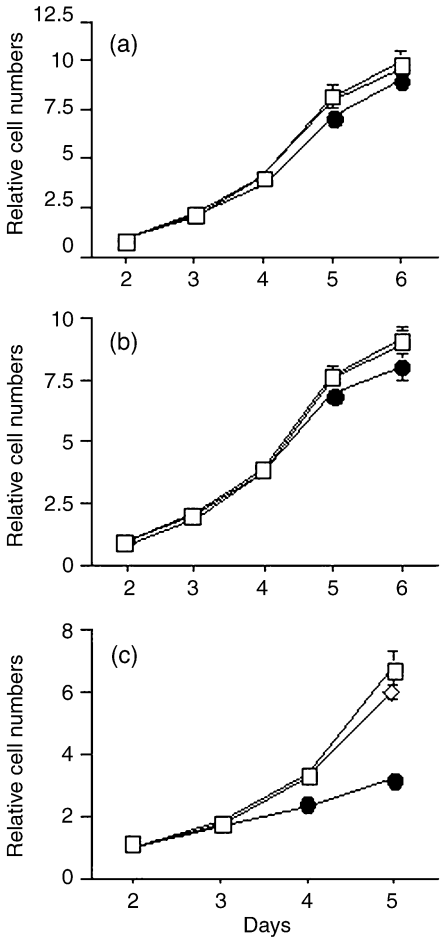

Fig. 2. Growth suppression of DN1-48 cells by inducible expression of Akt (DN). HCT116, the vector-transformed clone Vec-1, and DN1-48 cells were each infected with either AxCALacZ or AxCANCre. One day after infection, 2000 cells per well were plated onto 96-well microtiter plates and growth of the cells was monitored every day from the second day of infection by using an 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. (a) HCT116, (b) Vec-1 and (c) DN1-48 cells were (□) mock-infected or infected with either (◇) AxCALacZ or (●) AxCANCre.

expression on the growth of cells. We selected an MOI of 5 to minimize the cytotoxic effect of adenovirus infection. One day after infection of DN1-48 cells with AxCANCre, 2000 cells per well were plated onto 96-well microtiter plates and the growth of the cells was monitored by using the MTT method. As shown in Figure 2, growth of the parental HCT116 cells and the vector-transformed clone named Vec-1 were little

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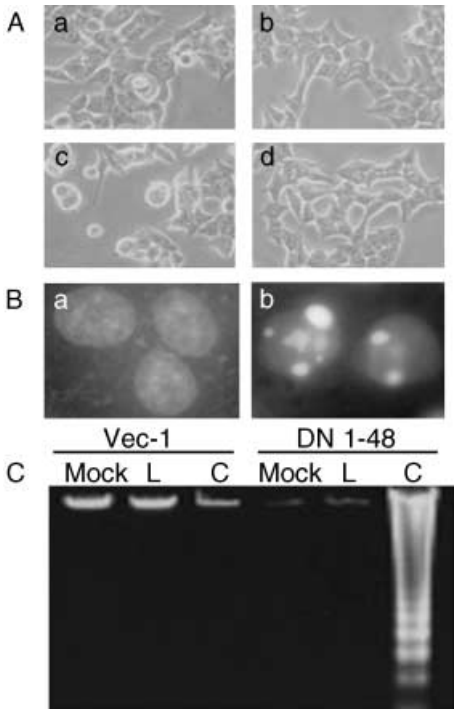

Fig. 3. Apoptosis of DN1-48 cells induced by expression of Akt (DN). HCT116, Vec-1 and DN1-48 cells were infected with either AxCALacZ or AxCANCre, harvested 2 days after infection, then monitored for apoptosis. (A) Phase contrast micrographs of (a) HCT116, (b) Vec-1 and (c and d) DN1-48 cells infected with (a, b and c) AxCANCre or (d) AxCALacZ. (B) Hoechst33258-stained (a) Vec-1 and (b) DN1-48 cells infected with AxCANCre. (C) Vec-1 and DN1-48 cells were infected with either AxCALacZ (L) or AxCANCre (C), and DNA was extracted from the cells and analyzed for DNA degradation.

affected by infection with either AxCALacZ or AxCANCre. However, growth of DN1-48 cells infected with AxCANCre was suppressed, whereas AxCALacZ and mock-infected DN1-48 cells grew normally. We next examined whether AxCANCre-infected DN1-48 cells undergo apoptosis. Two days after infection, cells appeared to undergo apoptosis by the morphological disintegration of cells (Fig. 3A), the induction of apoptotic bodies (Fig. 3B), and nucleosome-level DNA degradation (Fig. 3C), as opposed to vector-transformed cells infected with AxCANCre, or DN1-48 cells infected with AxCALacZ.

Ribozymes downregulate Akt1 expression in 293 cells but have little effect on their growth

We designed two kinds of ribozymes corresponding to appropriate sequences for Akt1, that is, GUC sequences at nucleotides 457–460 and 563–565, just downstream of the PH domain and upstream of the kinase domain, respectively, of Akt1 mRNA. The nucleotide sequences chosen are specific for Akt1 and are presumed to be conformationally open to ribozyme access. We constructed ribozyme expression vectors in the transfer RNA-based vector pUC-KE/tRNA.[19] 293 cells were cotransfected with the ribozyme plasmid and neomycin resistance gene-expressing plasmid pcDNA3. Stable transformant clones were isolated and analyzed for the expression of transduced ribozymes. As shown in Figure 4, the expression of ribozymes 1 and 2 was estimated by RT-PCR in one each of the

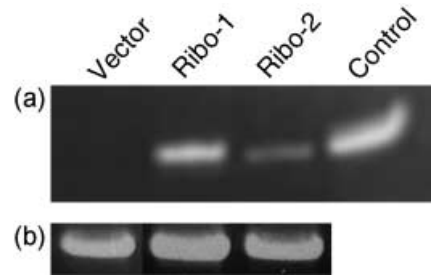

Fig. 4. Expression of ribozyme 1 and 2 in stable transformant clones (Ribo-1 and Ribo-2, respectively). (a) Reverse transcription–polymerase chain reaction analysis was performed on total RNA from vector-transformants and each ribozyme-transformant clone (Ribo-1 and Ribo-2) with primers specific for the ribozyme expression vector. Ribozyme 2 plasmid DNA was used as a control template. Products were analyzed by electrophoresis on 18% polyacrylamide gels. (b) Images of the 18S ribosomal RNA in each reverse transcription–polymerase chain reaction are shown as a loading control.

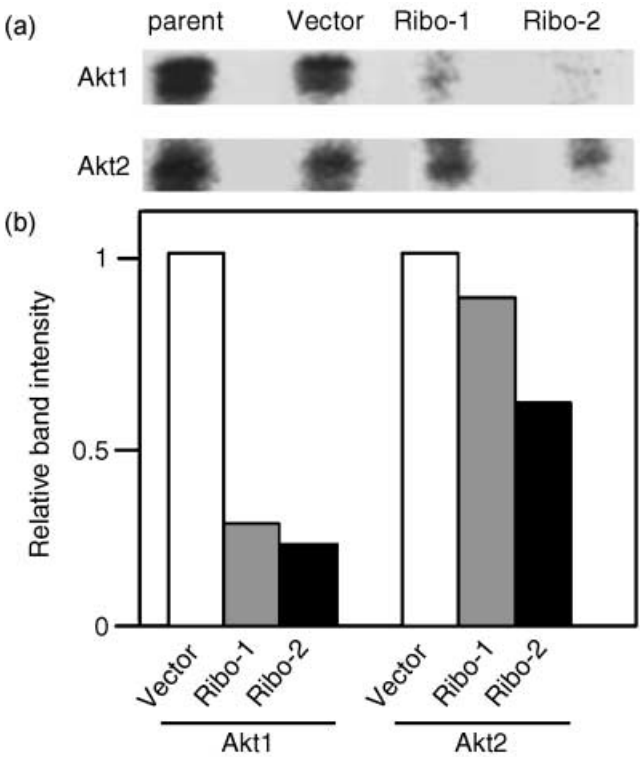

Fig. 5. Akt1 ribozymes efficiently and preferentially downregulate Akt1 expression. (a) Total RNA was prepared from each transformant and expression levels of Akt1 and Akt2 mRNA were estimated by the RNase protection method with Akt1 and Akt2 riboprobes. (b) Band intensities of Akt1 and Akt2 in the vector control clones; Ribo-1 and Ribo-2 were quantified by using a Phosphorimager analyzer. The values of Akt1 and Akt2 mRNA in Ribo-1 and Ribo-2 are expressed relative to those of the vector control, which was set at 1.

respective five clones of the ribozyme 1- and ribozyme 2-transfectants, Ribo-1 and Ribo-2. The expression level of ribozyme 1 appeared to be a few times greater than that of ribozyme 2. Akt1 and Akt2 mRNA levels in these clones were measured by using an RNase protection assay. A representative result of the two experiments is shown in

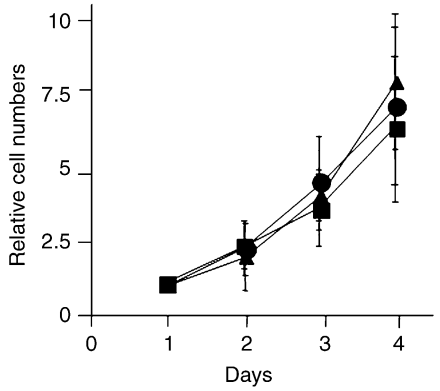

Fig. 6. Downregulation of Akt1 does not affect the proliferation of 293 cells. Two thousand cells of (■) the vector-transformant clones and the Akt1-ribozyme stable transformant clones of 293 cells, (●) Ribo-1 and (▲) Ribo-2, were plated per well onto 96-well plates. Viable cell numbers were estimated by using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method, with the following day set at day 1, for 4 days. Viable cell numbers are expressed relative to the values of day 1. Values are mean ± SD for four independent experiments.

Figure 5a, where both ribozymes reduced the Akt1 mRNA level dramatically compared with the vector control, whereas the effect of ribozymes on Akt2 was much less pronounced. Quantitation of the protected band, as illustrated in Figure 5b, showed that Akt1 mRNA levels were reduced to 29% of that of the vector control by ribozyme 1 (71% inhibition), and to 22% by ribozyme 2 (78% inhibition). In contrast, Akt2 mRNA levels were reduced to 88% of that of the vector control by ribozyme 1 (22% inhibition), and to 60% by ribozyme 2 (40% inhibition). These results demonstrate a preferential inhibition of Akt1 mRNA by the ribozymes, although a certain level of cross-reaction with Akt2 mRNA was observed. It is relevant to note here that the homology between the nucleotide sequences of Akt2 mRNA and Akt1 mRNA targeted by ribozyme 1 and ribozyme 2 are 80 and 53%, respectively, although this disparity between the two Akt isozymes was not precisely reflected in the results shown in Figure 5.

Then what effects does the downregulation of Akt1 have on cellular function? First, we examined the effect of ribozymes on the growth of 293 cells. Surprisingly, the proliferation rates of cells stably expressing ribozymes 1 and 2 were almost the same as those of the vector controls (Fig. 6), suggesting that the proliferative activity of 293 was not affected by the downregulation of Akt1.

Downregulation of Akt1 confers sensitivity to some chemotherapeutic drugs

Because Akt1 is thought to be an antiapoptotic factor, we examined the possibility that Akt1 downregulation affects the susceptibility of cells to antineoplastic drugs. The drugs used were camptothecin (CPT), a topoisomerase I inhibitor; teniposide (VM-26), a topoisomerase II inhibitor; staurosporine (STS), a protein kinase C inhibitor; and paclitaxel (PTX), a tubulin depolymerization inhibitor. Table 1 shows the IC_{50} values of various chemotherapeutic drugs in the vector control- and ribozyme-expressing cells, Ribo-1 and Ribo-2, and the 'sensitivity indices (SI)', that is, the ratios of the IC_{50} values of vector control cells divided by those of the Ribo-1 and Ribo-2 cells. Sensitivity index (SI) values for the drugs appeared to be higher in Ribo-2 than in Ribo-1, and the trend of higher sensitivity of ribozyme-expressing cells over vector control cells holds more or less for all drugs tested. These results indicate that downregulation of Akt1 makes 293 cells more sensitive to chemotherapeutic drugs.

Table 1. IC_{50} values and sensitivity indices of ribozyme-transformed clones to anti-tumor drugs

Drugs	Vector control	Ribo-1		Ribo-2	
	IC_{50}	IC_{50} (μM)	SI	IC_{50} (μM)	SI
CPT	0.20 ± 0.012	0.06 ± 0.004	3.57	0.06 ± 0.004	3.57
VM-26	7.84 ± 0.88	4.96 ± 0.55	1.59	2.21 ± 0.24	3.55
STS	0.12 ± 0.009	0.02 ± 0.002	6.00	0.02 ± 0.002	6.00
PTX	0.60 ± 0.052	0.60 ± 0.052	1.00	0.18 ± 0.015	3.33

IC_{50} values were calculated from the sensitivity curves for several antitumor drugs (data not shown). The sensitivity index (SI) is the IC_{50} of the vector control cells divided by that of each ribozyme transformant clone. CPT, camptothecin; VM-26, teniposide; STS, staurosporine; PTX, paclitaxel.

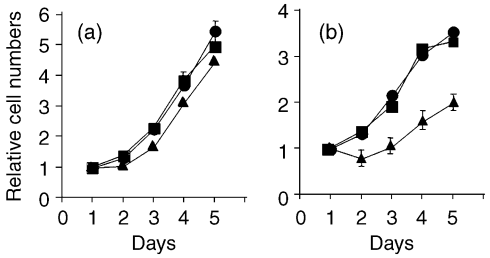

Fig. 7. Growth inhibition of HeLa cells by transient expression of ribozymes. (a) Cells were transfected with each ribozyme-expressing plasmid, (●) ribozyme 1, (▲) ribozyme 2, and (■) mock transfectants, as described in the Materials and Methods section. Cells were harvested 2 days after transfection, and 2000 cells were plated onto 96-well plates. Viable cell numbers were estimated by using the 3-(4,5-dimethyl-2 thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method, with the following day set at day 1, for 5 days, and the numbers are expressed relative to the values on day 1. Values are mean ± SD for four independent experiments. (b) One day after plating as in part a, cells were treated with 10 nM camptothecin. The rest of the experiment was carried out as in part a.

Additionally, we tested the effect of transient expression of ribozymes on the proliferative activity and drug sensitivity of HeLa cells (a cervical carcinoma cell line). No effect on proliferative activity was observed (Fig. 7a); however, ribozyme 2 but not ribozyme 1 appeared to sensitize cells to CPT (Fig. 7b).

Discussion

Apoptosis, a form of programmed cell death, plays a crucial role in the regulation of development and maintenance of tissues in metazoans.[3,27] In apoptosis, a genetic program is activated where a balance is shifted such that proapoptotic gene functions dominate those of antiapoptotic genes, resulting in cell death.[27] Deregulation of apoptosis has been implicated in the pathogenesis of many diseases, including cancer.[4,5] In recent years, it has been reported that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in the survival of many cell types.[28,29] Three isozymes of Akt, Akt1, Akt2 and Akt3,

 doi: 10.1111/j.1349-7006.2005.00088.x

have been shown to be expressed in various normal cells, as well as in tumor cells.[6,30] It seems that the three isozymes have the same substrate specificity, as the homology of these Akt isozymes is very high in mRNA and at the protein level, and both Akt1 and Akt2 have been shown to phosphorylate Bad *in vivo*.[31] Thus it seems difficult to differentiate between the functions of the individual isoforms, for example by overexpressing the protein in cells. However, recent mouse gene knockout studies have shed some light on this problem in that Akt1, Akt2 and Akt3 have been shown to be functionally distinct. Mice that are Akt1-deficient are reduced in size and have increased spontaneous apoptosis in the testes and thymus, whereas mice deficient in Akt2 are normal in size, but have impaired ability to maintain glucose homeostasis.[32,33,34] Akt3-deficient mice have no gross observable phenotype, but have uniformly reduced brain size.[35]

Given the fact that Akt plays an important role in tumorigenesis, downregulation of Akt activity should therefore suppress tumorigenic potential. In the present study we showed that apoptosis can be induced in tumor cells by the ectopic expression of a dominant-negative form of Akt (Figs. 2, 3). We further constructed two ribozyme expression vectors for Akt1 and transfected 293 and HeLa cells. It is known that 293 cells express all three types of Akt with the same level of mRNA, whereas Akt3 expression is barely detectable in HeLa cells.[30] We first tested the ability of these ribozymes to downregulate Akt1. Although the homology between Akt1 and Akt2 is 77.5% in mRNA and 81.6% in protein, the ribozymes we constructed appeared to preferentially downregulate Akt1 (Fig. 5), demonstrating that the preferential targeting Akt isozymes can be realized. We next examined the effect of ribozyme expression on some cellular functions. As shown in Figures 6 and 7a, transfection of ribozymes did not affect the proliferation rate of the cells, whether the cells were stably or transiently transfected. These results suggest that Akt1 alone is not critically involved in growth regulation or that its function is complemented by other Akt isozymes. Given the differential expression of Akt3 (i.e. it is expressed in 293 but not in HeLa cells), Akt3 may not play an important role in cell proliferation. Thus Akt2 alone or in conjunction with Akt1 could play an important role in the proliferation of cells.

Our data show that downregulation of Akt1 expression alone by either ribozyme 1 or 2 is sufficient to sensitize 293 cells to stresses from chemotherapeutic drugs (Table 1). In HeLa cells, however, only ribozyme 2 but not ribozyme 1 affected cell growth under CPT stress (Fig. 7b). This phenomenon may well be due to the fact that ribozyme 1 is weaker than ribozyme 2 in downregulating Akt1 expression, despite the lower expression level of ribozyme 2, as shown in Figure 4. Actually, ribozyme 2 appears to inhibit the mRNA levels of Akt1 and Akt2 more effectively than ribozyme 1 (Fig. 5), and the SI values of Ribo-2 to typical antitumor drugs were generally higher than those of Ribo-1 (Table 1). Thus, in HeLa cells, it might have been difficult to sensitize cells to CPT by transient transfection with ribozyme 1 but not ribozyme 2.

It is relevant here to refer to recent reports by others showing the importance of Akt isozymes in the proliferation and chemosensitivity of tumor cells. In accordance with our result, Krystal *et al*. report that expression of a dominant-negative allele of Akt (kinase dead) in human small cell lung cancer cells inhibits growth of the cells.[36] Liu *et al*. report that downregulation of Akt1 by an antisense oligonucleotide led several human cancer cell lines to apoptosis.[37] However, others have reported that downregulation of Akt1 and/or Akt2 does not affect the growth of tumor cells,[38,39] but does sensitize cells to some antitumor agents.[39] It is interesting that some tumor cells are sensitive to the downregulation of Akt isozymes with respect to growth inhibition, whereas others are not. This may well be due to differences in the cell type used and/or the extent to which downregulation of Akt is achieved. Our results and others have shown that Akt1 alone is a good target for anticancer therapy in combination with selected chemotherapeutic agents, and Akt2 could also be an excellent target. Thus, an anticancer strategy involving a combination of agents that target both the PI3K/Akt pathway and other vital molecules of tumor cells would be possible, as evidenced by recent reports,[36,40] where a synergistic enhancement of antitumor effect was obtained by just such a treatment; examples are: (i) a combination of LY294002 targeting PI3K, and etoposide targeting topoisomerase II in small cell lung cancer cells;[36] and (ii) rapamycin targeting mTOR downstream of Akt and adriamycin targeting topoisomerase II, or cyclophosphamide, an alkylating agent of DNA.[40]

In summary, we demonstrated that in cancer therapy, Akt is an excellent target for an ectopically expressed dominant-negative allele of Akt, which alone can induce the apoptosis of cells. This effect is most likely due to its suppression of all three isozymes. We also found that Akt1-targeting ribozymes were effective in downregulation of the isozyme and in sensitizing cells to anticancer chemotherapeutic agents.

Acknowledgments

We are grateful to Drs Izumu Saito of the Institute of Medical Science, University of Tokyo, and to James R. Woodgett of the Ontario Cancer Institute of Experimental Therapeutics for their kind gifts of pCALNLw and HA-tagged dominant negative-bovine Akt1 cDNA, respectively. This research was supported by the Screening Committee of New Anticancer Agents supported by a Grant-in-Aid for Scientific Research on the Priority Area 'Cancer' from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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 doi: 10.1111/j.1349-7006.2005.00088.x