The forkhead transcription factor FOXO4 sensitizes cancer cells to doxorubicin-mediated cytotoxicity

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The forkhead superfamily of transcription factors, which play major roles in control of cellular proliferation, oxidative stress and apoptosis, are becoming more and more considered as crucial therapeutic targets in cancer. In this study, we addressed the contribution of class O of forkhead box transcription factor (FOXO) 4 transcription factor, a forkhead superfamily member, to cytotoxicity mediated by the anthracyclic drug doxorubicin. FOXO4 can be phosphorylated by phosphatidylinositol-3-kinase/AKT signaling resulting in its inactivation and nuclear exclusion. Under stress conditions, FOXO4 can be phosphorylated via jun N-terminal kinase (JNK) leading to increased transcriptional activation of the transcription factor. Our results show that doxorubicin incubation led to phosphorylation of AKT and concomitantly to AKT-dependent inactivation and nuclear exclusion of the tumor suppressor FOXO4 in Hct-116 cells. We found that inhibition of FOXO4 nuclear exclusion by blockage of AKT phosphorylation following overexpression of dominant-negative AKT enhanced doxorubicin-mediated cytotoxicity. Overexpression of wild-type FOXO4 led to an increase in doxorubicin-mediated cytotoxicity, which was further exacerbated by overexpression of a solely nuclear-localized FOXO4 mutant. In contrast, though doxorubicin resulted in JNK activation, modulation of JNK-dependent regulation of FOXO4 was of no effect to doxorubicin cytotoxicity. These results show for the first time that in Hct-116 cells sustained nuclear localization of FOXO4 seems to be one crucial point enhancing doxorubicin-induced cytotoxicity and apoptosis. Targeting FOXO4 or AKT may lead to new chances in sensitizing cancer cells to cytostatic drugs thereby allowing use of lower drug concentrations and minimizing drug-induced adverse effects in patients.

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Introduction

The cytostatic drug doxorubicin has been widely used for treatment of a broad spectrum of cancers (1). The mechanisms by which doxorubicin drives cancer cells into apoptosis are related to its ability to disturb DNA function and to induce DNA damage. Doxorubicin can intercalate into the DNA double helix, inhibit topoisomerase II and cross-link DNA strands (2). Furthermore, it is known that doxorubicin produces reactive oxygen species (ROS) via one-electron reduction to the corresponding semiquinone free radicals that then react rapidly with oxygen to generate superoxide radical anions (3). At moderate concentrations, doxorubicin-induced ROS can act as important effectors modulating diverse signaling pathways like the redox-sensitive nuclear factor-xB pathway (4), the p53 tumor suppressor pathway (5) and the phosphatidylinositol-3-kinase (PI3K)/AKT pathway (6).

The PI3K/AKT pathway is a well-known oncogenic signaling pathway linked to cellular survival and proliferation (7). Upon activating stimuli, the PI3K is recruited to the plasma membrane and phosphor-ylates phosphoinositol lipids that in turn recruit and activate phosphatidylinositol-dependent protein kinase and AKT. Activated AKT (PKB) then facilitates phosphorylation of multiple downstream targets on serine and threonine residues (8). PKB/AKT signaling is antagonized by phosphatase and tensin homologue (PTEN) that transforms phosphoinositol lipids back to their inactivated form (9,10). AKT has numerous downstream targets that are phosphorylated and thereby either activated or inactivated (8).

One group of identified AKT downstream targets is class O of forkhead box transcription factors (FOXOs), mammalian homologues of DAF-16, which is known to regulate life span and stress response of the nematode Caenorhabditis elegans (11) after caloric restriction as reviewed by Morris (12) and plant-derived polyphenol treatment (13). In mammals, the FOXOs include four members: FOXO1, FOXO3, FOXO4 and FOXO6 that regulate a variety of physiological and pathological processes and are currently becoming more and more important as a major target in preventing tumorigenesis (14). Three of four in human identified FOXO genes were initially found at chromosomal translocations associated with cancer supporting the view that FOXO factors may act as tumor suppressor genes (15). Furthermore, Paik et al. (16) showed that somatic deletion of FOXO transcription factors in mice indeed resulted in conditions of cancer progression, showing their role as tumor suppressors in mammals.

FOXO4 is one member of the subfamily of mammalian FOXO forkhead transcription factors (17) that are implicated to play major roles in control of cellular proliferation, cell cycle arrest, DNA repair, oxidative stress and apoptosis (18). In the absence of PI3K/AKT activation, FOXO4 is located in the nucleus, where it functions as a transcription factor (8). Upon PKB/AKT activation, FOXO4 becomes phosphorylated on three highly conserved serine and threonine residues (Thr-28, Ser-193 and Ser-258) followed by inactivation and nuclear exclusion (19). Besides the three AKT-dependent phosphorylation sites, FOXO4 is also known to carry two additional jun N-terminal kinase (JNK)-dependent phosphorylation sites (Thr447 and Thr451), which become phosphorylated following induction of ROS inside the cell. Phosphorylation via JNK results in transcriptional activation and subsequent induction of a negative feedback mechanism to counteract ROS (20).

Several studies show that doxorubicin treatment results in AKT phosphorylation and therefore proliferation and survival of breast cancer cells (21), gastric cancer cells (6) and hepatoma cells (22). We hypothesized that activation of PI3K/AKT pathway by doxorubicin may subsequently inhibit the AKT downstream target FOXO4 and by counteracting this pathway, cancer cells may be sensitized to the chemotherapeutic drug doxorubicin. The objective of this study was to determine the role of FOXO4 transcription factor in doxorubicin-induced cytotoxicity using human colon carcinoma cells (Hct-116) and human hepatoma cells (HepG2).

Here, we show that doxorubicin-mediated AKT phosphorylation leads to subsequent phosphorylation of FOXO4 transcription factor. Inactivation and nuclear exclusion of FOXO4 by AKT-mediated phosphorylation following doxorubicin treatment to our knowledge have not been shown before. In addition, we demonstrate for the first time that sustained nuclear localization of FOXO4 is associated with enhanced doxorubicin-induced cytotoxicity and sensitizes cancer cells to doxorubicin-mediated apoptosis. Our data indicate that targeting AKT activity or downstream AKT effectors (like FOXO transcription factors) may sensitize cancer cells to cytostatic drugs and increase efficacy in treatment of tumor cells.

Abbreviations: DN-AKT, dominant-negative AKT; ECS0, the half maximal effective concentration values; FOXO, class O of forkhead box transcription factor; HA, hemaglutinin; JNK, jun N-terminal kinase; MTT, 3-(4,5- dimethylthiazole-2-yl)-2,5-bisphenyl tetrazolium bromide; PCR, polymerase chain reaction; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homologue; ROS, reactive oxygen species; SEAP, secreted embryonic alkaline phosphatase.

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Materials and methods

Cell culture and transfection

Hct-116 human colon carcinoma cells were grown in Dulbecco’s modified Eagle’s medium with l-glutamine, 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories, Austria), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 5% CO2 and 37°C. HepG2 human hepatoma cells were grown in RPMI 1640 medium containing 2 mM l-glutamine, 10% heat-inactivated FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 5% CO2 and 37°C. Hct-116 and HepG2 cells were transiently transfected by using JetPEI™ (Polyplus Transfection, France) or Genejuice Transfection Reagent (Novagen, Germany) following the batch transfection protocol recommended by the manufacturer.

Plasmid vectors

pMT2-HA-FoxO4 [referred to as FOXO4wt(1)] and pMT2-HA-FoxO4-T447/451A [referred to as FOXO4-JNK-mut]) were used for overexpression of wild-type FOXO4 and FOXO4 in which both JNK-dependent phosphorylation sites (T447 and T451) are blocked by base exchange to alanine. Both vectors were kindly provided by Prof. Burgering (University Medical Center Utrecht, The Netherlands) (20). pT7B01-Flag-FoxO4-wt [referred to as FOXO4wt(2)] and pT7B01-Flag-FoxO4-triple mutant (referred to as FOXO4neu) were used for overexpression of wild-type FOXO4 and FOXO4 triple mutant in which all three AKT-dependent phosphorylation sites (T28, S193 and S258) are blocked by base exchange to alanine. This FOXO4 mutant protein persistently stays in the nucleus. Both vectors were kindly provided by Prof. Kikkawa (Biosignal Research Center, Kobe University, Japan) (19). pHA-AKT-DN entailed overexpression of dominant-negative AKT (DN-AKT) and was a kind gift from T. Franke (Columbia University, New York, NY).

Cytotoxicity assays

For assessing cell viability, 1-2 × 10^4 cells/well were plated in 96-multwell plates. Twenty-four or 72 h after plating, cells were incubated with doxorubicin for 3 h followed by growth medium for 24 h. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-biphenyl-tetrazolium bromide (MTT) and neutral red assay as published before (23,24). Both assays were quantitated using a Wallac Victor™ multilabel counter at 540 nm. The cells incubated with control medium were considered 100% viable.

Growth curve and cell cycle analysis

Growth curves of wild-type and transfected cells were generated by plating cells in 96-well plates at 5 × 10^3 cells/well and viable cells were measured at 12-96 h post-plating by using the MTT assay. For cell cycle analysis, 1 × 10^6 cells were transiently transfected and different cell cycle phases were determined as described in detail according to Dorn et al. (25).

SEAP reporter gene assay

For pSEAP-3xIRS vector construction, three copies of the insulin-responsive sequence (3xIRS) were subcloned from p3IRS-luc [kindly provided by A. Fukamizu, University of Tsukuba, Tsukuba, Ibaraki (26)] with KpnI and HindIII into pTAL-SEAP Vector (Clontech, France). Twenty-four hours after transfection of the secreted embryonic alkaline phosphatase (SEAP) reporter vector, cells were stimulated with doxorubicin for 3 h. SEAP activity in the medium was detected 24 h after the end of doxorubicin treatment by using Tropix CSPD substrate according to the manufacturer’s instructions. Results were calculated as relative intensity to untreated control and normalized for protein concentration.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was isolated from cells using Trizol® Reagent (Invitrogen, Germany). One microgram of total RNA was transcribed into complementary DNA using the M-MLV reverse transcriptase from Promega (Germany). Polymerase chain reactions (PCRs) were performed essentially as described elsewhere (24). The following primer pairs were used for amplification: human glyceraldehyde-3-phosphate dehydrogenase (sense, 5’-ACCACAGATCTTGATCCATAC-3’; antisense, 5’-TCCACACCTGTGTTGCATA-3’), human FoxO4 (sense, 5’-GCTCTGATCTGCGGAGG-3’; antisense, 5’-GGTGGCTGTACAGGGTG-3’). PCR products were analyzed by agarose gel electrophoresis at cycles within the linear range of complementary DNA amplification using the Quantity One system from Bio-Rad (Germany) for visualization and software Tina 2.09 for densitometric analysis.

Isolation of proteins and immunoblotting

Protein lysates were prepared essentially as described previously (4). Samples containing equal amounts of protein were separated by 10 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride membranes (Roche Diagnostics, Germany). Blots were probed by using antibodies against hemaglutinin (HA)-Tag, PTEN, PKB (Sigma, Germany), phospho-AKT, total-FoxO4, unphosphorylated-FoxO4, phospho-FoxO4, phospho-JNK (Cell Signaling, Germany) and JNK (Upstate, Germany). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, bound antibodies were visualized using enhanced chemiluminescence reagent (Roche Diagnostics). For reprobing with other antibodies, blots were stripped using Restore™ Western Blot Stripping Buffer (Pierce, Germany) according to the manufacturer’s instructions.

Analysis of DNA fragmentation

After incubation with doxorubicin for 3 h and adjacent incubation with growth medium for 24 h, 1 × 10^6 cells were lysed and DNAs were extracted using phenol–chloroform (2:1) and precipitated with isopropanol. The pellets were resuspended in tris-EDTA buffer and electrophoresed in a 1.75% agarose gel at 100 V for 2-3 h. The bands were visualized under ultraviolet light using the Quantity One system from Bio-Rad.

Caspase assays

Determination of caspase activity was carried out in 96-well plates using 75 µg protein. Colorimetric labeled substrates for caspase-3/7 (Ac-DEVD-pNA), caspase-8 (Ac-IETD-pNA) and caspase-9 (Ac-LEHD-pNA) were used following the protocol of the manufacturer (Calbiochem, Germany). Caspase activity was detected by measuring proteolytic cleavage of 200 µM caspase substrates using the absorbance of released p-nitroaniline at 405 nm. Caspase-3/7 activity was also measured using the APO-ONE Homogeneous Caspase 3/7 Assay (Promega) according to the manufacturer’s protocol by using 2 × 10^5 cells/ well. Increase in fluorescence was measured at 37°C (excitation: 485 nm and emission: 535 nm). Results were calculated as relative fluorescence units to untreated control and normalized to viability using MTT assay.

Measurement of intracellular ROS

The effect of doxorubicin on the production of ROS was determined by fluorimetric assay using 2’-7’-dichlorodihydrofluorescein diacetate from Invitrogen. In brief, cells were cultured with 50 µM 2’-7’-dichlorodihydrofluorescein diacetate and various concentrations of doxorubicin for 3 h, followed by incubation with fresh Dulbecco’s modified Eagle’s medium for 24 h containing 2’-7’-dichlorodihydrofluorescein diacetate. Cells were washed once with phosphate-buffered saline, pH 7.4, and cellular fluorescence was measured in a Wallac Victor™ multilabel counter (excitation: 485 nm and emission: 525 nm). Results were calculated as relative intensity to untreated control and normalized for protein concentration.

Statistical analysis

All data were analyzed using one-way analysis of variance, followed by least significant difference post hoc analysis to determine statistical significance. *P* values <0.05 were considered statistically significant.

Results

Effect of doxorubicin on Hct-116 cells

Doxorubicin induces cytotoxicity and oxidative stress. To investigate the effect of doxorubicin on cell viability, Hct-116 human colon carcinoma cells were incubated in the presence of increasing concentrations of doxorubicin for 3 h followed by incubation with complete growth medium for 24 h. Doxorubicin treatment resulted in concentration-dependent cytotoxicity, as confirmed by neutral red and MTT assay (Figure 1A). To assess whether the cytotoxicity was associated with apoptosis, proteolytic cleavage of caspase substrates was measured. A significant activation of effector caspase-3 (Figure 1B) and initiator caspase-8 (Figure 1C) was observed. On the other hand, mitochondria-activated caspase-9 (Figure 1D) showed only a slight but significant increase in activation. The doxorubicin-mediated apoptotic cell death was further confirmed by an oligonucleosomal DNA fragmentation assay detecting DNA cleavage by endonucleases during apoptosis (Figure 1E).

Since it is known that one major factor in doxorubicin-mediated apoptosis is the induction of ROS (2), we investigated the oxidant status of the cells. Using the oxidant-sensitive probe 2’-7’-dichlorofluorescein diacetate, we observed a significant accumulation of ROS in Hct-116 cells after incubation with 5 and 10 µM doxorubicin (Figure 1F).

FOXO4 is known to be involved in regulation of apoptosis and cell cycle progression, and therefore basal growth and apoptosis rates for Hct-116 cells overexpressing different FOXO4 constructs were evaluated. Under our experimental conditions, no significant changes in basal apoptosis and proliferation levels were observed after transient overexpression of various FOXO4 constructs in Hct-116 cells (supplementary Tables and Figures are available at Carcinogenesis Online).
JNK-mediated activation of FOXO4 is not required for FOXO4-induced sensitization against doxorubicin. Because incubation of Hct-116 with doxorubicin was accompanied by accumulation of ROS, we evaluated the contribution of the redox-sensitive transcription factor FOXO4 to doxorubicin-mediated cytotoxicity. Hct-116 parental cells and Hct-116 cells overexpressing wild-type FOXO4 were treated with various concentrations of doxorubicin. Overexpression of wild-type FOXO4 resulted in significantly enhanced doxorubicin cytotoxicity compared with parental Hct-116 cells (Figure 2B). Furthermore, the stress-responsive kinase JNK is known to be activated by ROS burst. To determine phosphorylation of JNK following doxorubicin administration, Hct-116 cells were treated with 5 μM doxorubicin for 3 h. This treatment induced a time-dependent phosphorylation of JNK with a maximum after 4 h. Thereafter, phosphorylation declined (Figure 2A). One of the downstream targets of JNK is the FOXO forkhead box O transcription factor FOXO4. FOXO transcription factors are known to be involved in doxorubicin-mediated apoptotic signaling (6). FOXO4 carries two phosphorylation sites (T447, T451) that are phosphorylated in a JNK-dependent manner upon ROS burst, leading to FOXO4 with enhanced transcriptional activity (20). We next examined the influence of JNK-dependent modulation of FOXO transcriptional activity on the cytotoxicity of doxorubicin. For this, Hct-116 cells were transfected using FOXO4-JNK-mut, in which JNK-dependent phosphorylation is blocked by

**Fig. 1.** Doxorubicin-dependent cytotoxicity and apoptosis and induction of ROS formation. Cells were treated with various concentrations of doxorubicin for 3 h followed by incubation with growth medium for 24 h. Viability was assessed using neutral red and MTT assay (A). Data are means ± SDs (n = 4), *P < 0.05 versus corresponding Hct-116 control. The activities of caspase-3/7 (B), caspase-8 (C) and caspase-9 (D) were measured as described under Materials and Methods. Data are means ± SDs (n = 3) *P < 0.05 versus corresponding Hct-116 control. For DNA fragmentation (E), DNA was extracted and separated on a 1.75% agarose gel. A representative image of three independent experiments is shown. Accumulation of ROS in Hct-116 cells was measured using dichlorofluorescein assay (F). Cells were cultured in the presence of 50 μM 2',7'-dichlorodihydrofluorescein diacetate and various concentrations of doxorubicin. After 3 h, doxorubicin-containing medium was replaced by medium comprising 50 μM 2',7'-dichlorodihydrofluorescein diacetate for 24 h. Results are expressed as fluorescence intensity (FI) normalized for protein concentration. Data are means ± SEMs (n = 4) *P < 0.05 versus corresponding Hct-116 control.
exchange of threonine 447 (T447) and threonine 451 (T451) to alanine (20). To confirm successful overexpression of wild-type FOXO4 and FOXO4-JNK-mut, their expression was assessed by PCR and immunoblotting as shown in Figure 2C. As expected, expression of FOXO4-JNK-mut resulted in significantly repressed basal FOXO4 transcriptional activity (Figure 2D). We hypothesized that blockage of JNK-dependent phosphorylation should render Hct-116 cells more resistant to doxorubicin. However, overexpression of FOXO4-JNK-mut did not alter Hct-116 cytotoxicity compared with FOXO4 wild-type overexpressing cells (Figure 2B), indicating that JNK-inducible transcriptional activity of FOXO4 is not required for its sensitizing effect toward doxorubicin.

Doxorubicin induces phosphorylation of AKT and FOXO4. It is known that doxorubicin activates the PI3K/AKT signaling pathway and that blocking the PI3K/AKT signaling pathway results in stronger doxorubicin-mediated apoptosis (22,27). Therefore, we investigated PI3K/AKT-dependent phosphorylation of FOXO4 at Ser-193 and its possible contribution to doxorubicin cytotoxicity and apoptosis. To analyze the expression and activation of proteins involved in PI3K/AKT signaling, Hct-116 cells were grown for 72 h, incubated with doxorubicin for 3 h and expression of proteins was assessed by immunoblot 24 h after doxorubicin treatment. Phosphorylation of AKT was induced in a dose-dependent manner after treatment with 1 and 5 μM doxorubicin (Figure 3A), whereas PTEN, a major negative regulator of AKT, was not affected by doxorubicin (Figure 3A). Phosphorylation of FOXO4, a known AKT downstream target, was determined by using an antibody that detects FOXO4 only when phosphorylated at Ser-193. We found a concentration-dependent phosphorylation of FOXO4 after treatment with doxorubicin (Figure 3A). Concurrently, levels of unphosphorylated FOXO4 declined as assessed by using an antibody that detects only the non-phosphorylated FOXO4 (Figure 3A). To show that phosphorylation of FOXO4 is tantamount to nuclear exclusion, cellular distribution of FOXO4 was assessed in nuclear and cytosolic fractions (Figure 3A). Our results confirmed that doxorubicin-induced FOXO4 phosphorylation led to an accumulation of cytosolic-located FOXO4, whereas nuclear-located FOXO4 declined. Therefore, doxorubicin incubation was accompanied by nuclear exclusion of FOXO4 in a dose-dependent manner.

Dominant-negative AKT aggravates doxorubicin-induced cytotoxicity. Next, we analyzed whether inhibiting this pathway had an effect on doxorubicin-mediated cytotoxicity. Hct-116 cells were transiently transfected using a dominant-negative AKT expression construct. Seventy-two hours after transfection, cells were treated with doxorubicin and cell viability was assessed. Overexpression of dominant-negative AKT significantly increased doxorubicin-mediated cytotoxicity (Figure 3B). The half maximal effective concentration values (EC50) values shifted from 3.92 μM for Hct-116 wild-type cells to 2.77 μM for Hct-116 cells overexpressing DN-AKT. As shown in Figure 3C, successful overexpression could be detected using HA antibody against HA-tagged DN-AKT. Our data suggest that doxorubicin sensitivity is linked to activation of AKT. We next treated wild-type and DN-AKT-overexpressing cells with doxorubicin and
assessed phosphorylation of FOXO4 at Ser-193. Figure 3D demonstrates that doxorubicin led to phosphorylation and nuclear exclusion of FOXO4, whereas inhibition of AKT kinase abolished doxorubicin-mediated FOXO4 phosphorylation and therefore nuclear levels of FOXO4 remained unchanged following doxorubicin incubation.

FOXO4 overexpression increases doxorubicin cytotoxicity and apoptosis. Our results show that inhibition of AKT led to an increase in doxorubicin-mediated cytotoxicity that was accompanied by hypophosphorylation of FOXO4. Therefore, we hypothesized that FOXO4 is a key factor in modulating doxorubicin sensitivity in Hct-116 cells. To test this hypothesis, overexpression of wild-type FOXO4 or FOXO4nuclear was used in which all three AKT phosphorylation sites are replaced by alanine through site-directed mutagenesis (19). Phosphorylation by AKT therefore is impossible and FOXO4 cannot be excluded from the nucleus.

Seventy-two hours after transfection, Hct-116 cells were incubated with doxorubicin and viability was assessed by MTT assay. As already shown in Figure 2B, overexpression of wild-type FOXO4 increased doxorubicin-mediated cytotoxicity (Figure 4A). Overexpression of FOXO4nuclear resulted in an additional significant increase of cytotoxicity compared with wild-type FOXO4 overexpression (Figure 4A), with EC50 values shifting from 4.32 μM for Hct-116 wild-type cells to 3.32 μM for wild-type FOXO4 and 2.61 μM for FOXO4nuclear, respectively (Table I). To assess if increased doxorubicin cytotoxicity was due to increased apoptosis, APO-ONE assay was used to measure proteolytic cleavage of caspase-3/7. The data suggest that increased cytotoxicity was the result of increased caspase-3/7 activity (Figure 4B).

We confirmed successful overexpression of FOXO4 by using PCR and immunoblotting as shown in Figure 4C. To test functionality of FOXO4 constructs, a reporter gene assay was performed by cotransfecting Hct-116 cells with 3xIRS-SEAP reporter together with wild-type FOXO4 or FOXO4nuclear constructs. As expected, FOXO4nuclear showed twice as high SEAP reporter activities than wild-type FOXO4 (Figure 4D). Our results indicated that doxorubicin incubation led to phosphorylation and nuclear exclusion of FOXO4 in Hct-116 cells. Figure 4E shows that doxorubicin treatment failed to reduce nuclear amounts of FOXO4 in Hct-116 cells overexpressing wild-type FOXO4 sufficiently. Therefore, cellular overload following transient transfection with wild-type FOXO4 resulted in increased doxorubicin sensitivity.

We show that Hct-116 cells are sensitized to doxorubicin-mediated apoptosis by inactivation of AKT and therefore sustained nuclear localization of its downstream target FOXO4. To determine whether the sensitizing effect of FOXO4 toward doxorubicin is limited to Hct-116 cells, we investigated the effect of FOXO4 on HepG2 human...
hepatoma cells. Overexpression of wild-type FOXO4 resulted in increased doxorubicin-mediated cytotoxicity in human hepatoma cells (Figure 5), indicating that the observed effect is a more general one and not restricted to the Hct-116 colon carcinoma cells.

Discussion

One major obstacle in chemotherapy is resistance of tumor cells against cytostatic drugs. Doxorubicin is one of the most important anticancer drugs for the treatment of solid tumors and it has been shown to induce apoptosis (28). Unfortunately, repetitive doxorubicin treatment induces resistance of cancer cells, resulting in therapeutic failure (29,30). Therefore, a better understanding of doxorubicin-induced signaling pathways in cancer cells is needed to overcome resistance and by this way to enhance the efficacy of anthracycline treatment. Our findings present for the first time that the AKT down-stream target FOXO4 plays a central role in doxorubicin-mediated cell death in Hct-116 colon carcinoma cells. The transcription factor FOXO4 is directly phosphorylated by AKT following doxorubicin treatment and thereby inactivated leading to relocation from the nucleus to the cytosol. We show that by overexpression of FOXO4, doxorubicin-induced cytotoxicity in colon carcinoma and hepatoma cells is exacerbated.

Table I. EC50 values obtained following treatment with doxorubicin in wild-type and FOXO4 overexpression Hct-116 cells

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<td>EC50 (µM)</td>
<td>4.32</td>
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overexpression of wild-type FOXO4 resulted in the same levels of doxorubicin cytotoxicity as overexpression of a FOXO4 mutant lacking the threonine residues responsible for induced JNK activation and concomitant FOXO4 phosphorylation at threonines 447 and 451. Phosphorylation at these sites provides FOXO4 with transcriptional activity (20). FOXO4 transcription factor can then act as a tumor suppressor by inducing cell cycle arrest and apoptosis (14,36). Doxorubicin induced a time-dependent JNK phosphorylation in Hct-116 cells and this was also shown by Zhao et al. (37) in human lung adenocarcinoma cells. However, our hypothesis that blocking JNK-dependent activation of FOXO4 transcriptional activity would protect Hct-116 cells against doxorubicin cytotoxicity could not be supported experimentally. FOXO4 overexpression did indeed result in increased doxorubicin-mediated cytotoxicity not only in Hct-116 cells but also in another tumor cell line, human HepG2 hepatoma cells. But in contrast to our expectations, this sensitization toward doxorubicin was completely independent of JNK-mediated transcriptional activity of FOXO4. Overexpression of a FOXO4 mutant lacking the threonine residues to be phosphorylated by JNK and thus lacking transcriptional activity resulted in the same levels of doxorubicin cytotoxicity as overexpression of wild-type FOXO4.

Therefore, contribution of FOXO4 to doxorubicin cytotoxicity must be dependent on a different way of regulation. Besides the two JNK-dependent phosphorylation sites, FOXO4 carries three AKT-dependent phosphorylation sites by which FOXO4 is negatively regulated, denoting its nuclear export and inactivation (38). To test the importance of PI3K/AKT signaling in doxorubicin cytotoxicity in our system, we used overexpression of a dominant-negative AKT that this FOXO4 phosphorylation is indeed AKT dependent. Blocking of FOXO4 phosphorylation by inhibition of AKT signaling leads to enhanced doxorubicin cytotoxicity. FOXO4 can only function as a tumor suppressor and control apoptosis if it is located in the nucleus. Following overexpression of wild-type FOXO4, cells are flooded with FOXO4 proteins by which AKT induced phosphorylation and nuclear export is counteracted. Accumulation of hypophosphorylated FOXO4 localized in the nucleus is sufficient to induce consequential increase of doxorubicin cytotoxicity. These results suggest that FOXO4 holds a crucial role for doxorubicin-mediated cytotoxicity in Hct-116 and HepG2 cells.

Our findings support the essential role of FOXO4 transcription factor to induce apoptosis and by this to sensitize cells to doxorubicin treatment as a tumor suppressor (14,15). These results implicate that in Hct-116 cells sustained nuclear localization of FOXO4 seems to be the most crucial point for enhanced doxorubicin-induced cytotoxicity and apoptosis. However, the exact mechanism by which nuclear- localized FOXO4 mediates enhanced doxorubicin cytotoxicity remains unknown. Further studies are needed to investigate in more detail the downstream effects of nuclear-localized FOXO4 by which cancer cells are sensitized to the cytostatic drug doxorubicin.

In conclusion, we have shown for the first time that FOXO4 holds a crucial role in doxorubicin-induced apoptosis. Doxorubicin incubation leads to activation of PI3K/AKT signaling and concomitantly to inactivation and nuclear exclusion of the tumor suppressor FOXO4. Inhibition of FOXO4 nuclear exclusion by blockage of AKT phosphorylation enhanced doxorubicin-mediated cytotoxicity. Consequently, overexpression of wild-type FOXO4 led to an increase in doxorubicin-mediated cytotoxicity, which was further exacerbated by overexpression of an exclusively nuclear-localized FOXO4 mutant. Our results implicate that targeting FOXO or AKT may provide new options in sensitizing cancer cells to cytostatic drugs thereby improving chemotherapeutic efficiency and minimizing drug-induced adverse effects.

Supplementary material

Supplementary Tables and Figures can be found at http://carcin.oxfordjournals.org/

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