Enhanced Sensitivity to Cytochrome c–Induced Apoptosis Mediated by PHAPI in Breast Cancer Cells

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Abstract

Apoptotic signaling defects both promote tumorigenesis and confound chemotherapy. Typically, chemotherapeutics stimulate cytochrome c release to the cytoplasm, thereby activating the apoptosome. Although cancer cells can be refractory to cytochrome c release, many malignant cells also exhibit defects in cytochrome c–induced apoptosome activation, further promoting chemotherapeutic resistance. We have found that breast cancer cells display an unusual sensitivity to cytochrome c–induced apoptosis when compared with their normal counterparts. This sensitivity, not observed in other cancers, resulted from enhanced recruitment of caspase-9 to the Apaf-1 caspase recruitment domain. Augmented caspase activation was mediated by PHAPI, which is overexpressed in breast cancers. Furthermore, cytochrome c microinjection into mammary epithelial cells preferentially killed malignant cells, suggesting that this phenomenon might be exploited for chemotherapeutic purposes. (Cancer Res 2006; 66(4): 2210-8)

Introduction

Apoptosis is critical for proper embryonic development and tissue homeostasis (1). Moreover, disruptions in apoptotic signaling pathways frequently contribute to disease progression. In particular, evasion of apoptosis is believed to be universally essential for cancer development (1). Mutations in or modifications to components of the apoptotic pathway can confer a survival advantage on malignant cells, allowing them to proliferate under conditions in which cell death would be beneficial to the organism.

The goal of many chemotherapy regimens is to activate the intrinsic cell death program in malignant cells to promote tumor reduction and/or elimination. Unfortunately, these approaches are frequently unsuccessful due either to preexisting defects in the apoptotic machinery or to the continued accrual of mutations in apoptotic components as malignant cells proliferate. This chemoresistance commonly leads to treatment failure. In addition, chemotherapeutics that target apoptotic pathways often lack specificity for malignant cells and thus may also promote the death of normal cells, causing adverse side effects and a loss of chemotherapeutic efficacy (2). Some new and effective chemotherapeutics, including Gleevec (imatinib mesylate), Herceptin (trastuzumab), and Iressa (gefitinib), have used knowledge of specific molecular differences between normal and malignant cells to selectively induce apoptosis in cancer cells (3–5). Thus, an understanding of apoptotic mechanisms in malignant cells may provide a powerful tool for the development of more effective chemotherapeutic strategies.

In general, death by apoptosis results from the activation of a group of cysteine proteases known as caspases, which are responsible for the orderly dismantling of the cell (6, 7). Activation of caspases is tightly regulated by factors that either promote or inhibit their activation; a variety of these regulatory mechanisms can be disrupted in cancerous cells, allowing them to evade death. Caspase activation often proceeds through an intrinsic pathway involving the mitochondria (8, 9). Receipt of a proapoptotic stimulus can cause permeabilization of the outer mitochondrial membrane, resulting in release of cytochrome c into the cytosol. Once cytosolic, cytochrome c can bind the adaptor protein Apaf-1, inducing its oligomerization (10). This oligomerization promotes the recruitment of caspase-9 (via its NH2-terminal prodomain) to the caspase recruitment domain (CARD) at the NH2 terminus of Apaf-1, resulting in formation of the caspase-9-activating “apoptosome” (11). Activated caspase-9 then cleaves and activates caspase-3 and caspase-7, which cleave many cellular substrates to cause the hallmark features of apoptosis (i.e., plasma membrane blebbing, nuclear condensation, and DNA fragmentation; ref. 12). Mitochondrial cytochrome c release is regulated by the Bcl-2 family of proteins which either promote (e.g., Bax and Bak) or impede (e.g., Bcl-2 and Bcl-XL) the release of cytochrome c (13). Caspase activation downstream of cytochrome c release is regulated as well. For example, the inhibitor of apoptosis family of proteins restrain cell death by direct binding and inhibition of active caspases (14).

Cancer cells typically evolve the capacity to hijack the regulatory pathways described above to evade apoptosis. Prevention of cytochrome c release from the mitochondria in malignant cells has been the subject of intense scrutiny. However, cancer cells frequently develop additional mechanisms to evade apoptosis by inhibiting caspases even after mitochondrial cytochrome c release. Malignant melanomas, ovarian cancers, non–small-cell lung carcinomas, renal cell carcinomas, and certain non–chronic myelogenous leukemia (CML) types of leukemia have all been shown to have decreased apoptosome activity leading to a decrease in apoptosis (15–22). Furthermore, we have previously shown that the oncogenic tyrosine kinase Bcr-Abl, directly involved in the etiology of CML, promotes a defect in apoptosomal assembly, contributing to apoptotic evasion (23).

Remarkably, little is known about apoptotic control downstream of cytochrome c release in some of the most prevalent and frequently studied cancers, including breast cancer. A few reports have shown decreased caspase-3 expression in a subset of breast cancer cells but the consequence and frequency of these alterations remains undetermined (24, 25). Whereas treatment of

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hormone-responsive breast cancer with the antiestrogen tamoxifen can induce growth arrest and apoptosis in malignant mammary epithelial cells (26), chemoresistance remains a daunting problem as many breast cancers become hormone independent and subsequently more metastatic (27). An in-depth understanding of apoptotic regulation (specifically downstream of cytochrome c release) could potentially allow for the development of more effective chemotherapeutics. We report here that, in contrast to the post-cytochrome c apoptotic inhibition described in other cancers, malignant mammary epithelial cells show increased caspase activation in response to cytochrome c compared with their normal counterparts. This sensitivity is unique to breast cancer cells as we found that malignant cells from several other epithelium-based tumors, some of which have been described previously, are resistant to caspase activation induced by cytochrome c. The sensitivity of breast cancer cells to cytochrome c seems to stem from an enhanced capacity of caspase-9 to bind to the Apaf-1 CARD. We have found this altered affinity to be mediated by the protein PHAPI, which was overexpressed in breast cancer cells. Furthermore, we showed that the introduction of cytochrome c into the cytosol of malignant epithelial cells preferentially kills malignant cells, establishing direct apoptosis activation as a promising strategy for breast cancer chemotherapy.

Materials and Methods

Cell culture and extract preparation. Cells were maintained according to the specifications of either American Type Culture Collection (Manassas, VA) or Clonetics (East Rutherford, NJ; for primary cells). Cell-free extracts for caspase activation were prepared and caspase activation was measured as previously described (23).

Western blot analysis. The following primary antibodies were used for immunoblotting: caspase-9 (Neomarkers, Fremont, CA), Apaf-1 (Alexis, San Diego, CA), caspase-3 (Upstate, Charlottesville, VA), β-actin (Sigma, St. Louis, MO), cytochrome c (BD Pharmingen, San Diego, CA), cleaved caspase-3 (Cell Signaling, Danvers, MA), and PHAPI (Prosci, Poway, CA). Alexa Fluor secondary antibodies were used to visualize primary antibodies using the Li-Cor Odyssey IR Imaging System.

Caspase-9 activity assays. Caspase-9 was immunoprecipitated as previously described (28). To isolate active caspase-9, 20 mmol/L b-VAD-fmk (Alexis), 3 ng/μL cytochrome c, and 1 mmol/L dATP were added to 500 μg cell-free extract (at 10 μg/μL) and incubated at 37°C for 30 minutes. Reactions were then brought to 300 μL with cell lysis buffer and 30 μL streptavidin-Sepharose beads (Pierce, Rockford, IL) were added and incubated overnight at 4°C. Beads were washed thrice in cell lysis buffer and resolved by SDS-PAGE.

Production of recombinant proteins. The Apaf-1 CARD was induced as previously described (23) and purified with glutathione beads by a standard protocol (29).

Apoptosome formation assays. To assess Apaf-1 binding to cytochrome c, cell extracts (500 μg total protein at 10 μg/μL) were incubated with mammalian or yeast cytochrome c (CN-Br coupled to Sepharose) for 30 minutes at 37°C, washed thrice with radiouimmunoprecipitation assay (RIPA) buffer, and resolved by SDS-PAGE. To assess caspase-9 binding to the Apaf-1 CARD, 1 μL of the glutathione S-transferase (GST)-Apaf-1 CARD fusion protein was incubated with 500 μg cell extracts (at 10 μg/μL) for 15 minutes at 37°C. The GST-CARD was recaptured on 25 μL glutathione-Sepharose beads, washed thrice with RIPA buffer, and associated proteins resolved by SDS-PAGE for immunoblotting. To immunoprecipitate the apoptosome, 10 μg of caspase-9 antibody (Upstate) were pre-coupled to 25 μL of protein G-Sepharose. Fifty microliters of lysate (at 10 μg/μL) were activated with 1 mmol/L dATP and 1 ng/μL cytochrome c for 10 minutes at 37°C. Activated lysate was precleared on 25 μL protein G-Sepharose and incubated with precoupled antibody/beads overnight at 4°C. Beads were washed thrice with RIPA buffer and bound proteins were immunoblotted.

PHAPI assays. Recombinant PHAPI (packaged as I1PP3A; Calbiochem, San Diego, CA) was added at 75 ng to 500 μg of cell-free extract (at 10 μg/μL) and incubated at 37°C for 30 minutes. Cytochrome c was then added at 3 ng/μL and caspase-3 activation measured. For antibody blocking, 15 μg of PHAPI antibody (Abcam, Cambridge, MA) were added to 500 μg of cell-free extract (at 10 μg/μL) and incubated at 4°C for 1 hour. For add-back experiments, 10 μL PHAPI protein was incubated with 15 μg antibody before antibody addition to the extract. PHAPI knockdown was achieved using small interfering RNA (siRNA) duplexes generated by Eurogentec. T47D cells were transfected using the Transit-TKO reagent (Mirus, Madison, WI) with either the siRNA negative control duplex or the following duplex targeting PHAPI (30 at 100 nmol/L: 5'-GGAGCCCCUCUGAUGGAAT-3'; 5'-UUCAACUAGGGCCUGUCCT-3').

Protein extraction from tumors. The integrity of frozen specimens was tested via H&E staining. Specimens were added to a bead beater tube with 300 μL of lysis buffer [50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/μL aprotinin, 5 μg/μL leupeptin, 50 mmol/L NaF]. Tissue was homogenized for 40 seconds by bead beating. Lysate was collected and spun at 14,000 rpm (Eppendorf 5415C) for 30 minutes at 4°C. Lysate concentration was tested via Bradford assay.

Microinjections. Cells were microinjected with cytochrome c as described previously using 10 μg/μL bovine heart cytochrome c (31). To mark microinjected cells, the microinjection solution [100 mmol/L KCl, 10 mmol/L KPi, (pH 7.4)] contained 4 mg rhodamine dextran/mL. Cell viability was determined by counting rhodamine-positive cells that had intact, phase-bright cell bodies immediately after injection and at various times postinjection.

Results

Cytosolic extracts from breast cancer cell lines show enhanced caspase activation in response to cytochrome c. The addition of exogenous cytochrome c to cytosolic mammalian cell lysates allows for the faithful reconstitution of caspase-activating pathways in a cell-free setting, providing a powerful tool for the study of apoptosome formation, caspase activation, and cleavage of downstream cellular substrates (32). We used this approach to examine cytochrome c–induced caspase activation in a variety of normal and malignant mammary epithelial cell lines. Addition of cytochrome c to extracts prepared from the breast cancer cell lines T47D, MDA-MB-231, MDA-MB-361, BT-474, and MDA-MB-453 elicited markedly enhanced cleavage of the synthetic caspase-3 substrate Ac-DEVD-pNA when compared with extracts prepared from immortalized normal mammary epithelial cell lines (MCF-10a, HMEC kTERT) or from primary human mammary epithelial cells (HMEC, Fig. 1A). These data suggest that malignant mammary epithelial cells are inherently more sensitive to cytochrome c–induced apoptosis than their normal counterparts. Furthermore, although the breast cancer cells were uniformly hypersensitive to cytochrome c, they were highly varied with respect to their estrogen receptor and HER2 status, aggressiveness, and tumorigenicity in mice (33).

Cytochrome c–hypersensitive breast cancer cells are resistant to mitochondrial cytochrome c release and are unique among cancer cells in their cytochrome c hypersensitivity. Because malignant mammary epithelial cells seemed to have an increased propensity to undergo apoptosis in response to cytochrome c, we sought to understand why tumor cells would be so sensitive to a proapoptotic signal. As described above, the evasion of apoptosis is a hallmark of malignant cells and, hence, the hypersensitivity of breast cancer cells to cytochrome c was perplexing (1). We hypothesized that breast cancer cells might be resistant to apoptosis due to inhibition of mitochondrial cytochrome c release.
and would therefore never "see" cytoplasmic cytochrome c. To test this, we treated our cells with the apoptosis-inducing chemotherapeutics Adriamycin and cisplatin. Treatment of the cytochrome c–hypersensitive breast cancer cell line MDA-MB-231 with these agents triggered very little cell death whereas HMEC cells were efficiently killed by treatment with these drugs (Fig. 1B). Consistent with these observations, we found that both Adriamycin and cisplatin induced mitochondrial cytochrome c release in normal HMEC cells whereas the malignant mammary epithelial cells released considerably less cytochrome c. We also wished to determine whether cytochrome c sensitivity was unique to malignant mammary epithelial cells. Accordingly, we prepared cytosolic extracts from malignant prostate, ovarian, lung, and colon cells and tested their ability to cleave the caspase-3 substrate Ac-DEVD-pNA in response to cytochrome c addition. Surprisingly, we discovered that breast cancer cells were the only cell type to show any notable hypersensitivity to cytochrome c–induced caspase activation (Fig. 2). As previously reported, malignant ovarian and lung cells were both resistant to cytochrome c–induced apoptosis (15, 17, 18, 21). Malignant prostate and colon cells also shared this resistance to cytochrome c–induced caspase activation (Fig. 2). These data distinctively separate malignant mammary cells from other malignant cells of epithelial origin with regard to their apoptotic regulation downstream of cytochrome c release.

**Hypersensitivity of breast cancer cells to cytochrome c is due to increased caspase-9 activation and is independent of changes in expression of core apoptosomal components.** As many of the previous reports of apoptotic regulation downstream of cytochrome c release have identified the expression of core apoptosomal components as a point of control (22, 24), we examined the protein expression of caspase-9, Apaf-1, and caspase-3 in breast cancer cells. All of the cytochrome c–hypersensitive breast cancer cell lines, as well as the nonmalignant HMEC and MCF-10a cells, expressed equivalent levels of caspase-9, Apaf-1, and caspase-3 (Fig. 3A–C).

Although cytochrome c hypersensitivity in breast cancer cells was not due to alterations in the expression of apoptosomal proteins, the observed range of hypersensitivity did correlate well with the degree of cleavage of both procaspase-3 and procaspase-9 in the cytochrome c–supplemented lysates (Fig. 4A and B). Whereas caspase-9 cleavage is not required for its enzymatic activity (34), its processing does provide evidence of caspase-3 and caspase-9 activity as both contribute to the cleavage of caspase-9. Additionally, caspase activation can be monitored by assaying caspase binding to a biotinylated caspase substrate (b-VAD-fmk) that interacts only with active caspases (32). As shown in Fig. 4C, substantially more caspase-9 bound to b-VAD-fmk in extracts from malignant mammary epithelial cells than from the normal HMECs. It should be noted that b-VAD-fmk can also serve as a caspase

![Figure 1](image_url)
inhibitor, which explains why uncleaved procaspase-9 is observed in the tumor cell extracts in Fig. 4C but not in the tumor cell extracts in Fig. 4B (which do not contain b-VAD-fmk and have very little detectable procaspase-9). These data are again consistent with an increase in cytochrome c–induced caspase-9 activation in the breast cancer cells.

Caspase-9 has an enhanced affinity for the Apaf-1 CARD in breast cancer cells. Because our data indicated that caspase-9 activation by cytochrome c was enhanced in breast cancer cells, we wished to explore the possibility that altered apoptosome assembly was responsible for this enhanced activity. In our previous studies, we have used a cytochrome c–Sepharose resin to examine the interaction between cytochrome c and Apaf-1 (23). Hence, we incubated cell-free extracts prepared from nonmalignant HMECs and from T47D, MDA-MB-231, and MDA-MB-453 cells with either a mammalian cytochrome c resin or a yeast cytochrome c resin, which does not bind Apaf-1 (35). We then immunoblotted the resin-bound material for the presence of associated Apaf-1. As shown in Fig. 4D, equivalent levels of Apaf-1 were found associated with the mammalian cytochrome c resin dipped into either the normal or malignant mammary epithelial cell extracts, suggesting that an alteration in the affinity of Apaf-1 for cytochrome c was unlikely to underlie the enhanced sensitivity of breast cancer cells to cytochrome c–induced apoptosis.

In addition, we investigated the ability of Apaf-1 to recruit procaspase-9 in breast cancer cells. Although there are a number of ways to evaluate this, we have found that a simple method involves measuring the ability of the isolated CARD of Apaf-1 to retrieve procaspase-9 from cell-free lysates. Normally, cytochrome c promotes the oligomerization of full-length Apaf-1 so as to expose the CARD, making it accessible for caspase-9 binding. By using the isolated CARD, we circumvented this step, allowing us to observe recruitment of caspase-9 to Apaf-1 in the absence of cytochrome c. Therefore, we added GST-Apaf-1 CARD to cytosolic extracts prepared from both normal and malignant mammary epithelial cells and then isolated the Apaf-1 CARD and any associated proteins on glutathione-Sepharose. Resin-bound proteins were resolved by SDS-PAGE and immunoblotted to detect associated caspase-9. Strikingly, we found that caspase-9 association with the Apaf-1 CARD was enhanced in the T47D, MDA-MB-231, and MDA-MB-453 extracts as compared with extracts from normal HMECs (Fig. 4E). Additionally, we examined the interaction between endogenous Apaf-1 and caspase-9 by immunoprecipitating the apoptosome using anti-caspase-9 sera. Similar to our results using the Apaf-1 CARD, we observed an increase in the association of endogenous Apaf-1 with endogenous caspase-9 in malignant mammary epithelial cell extracts when compared with normal mammary
epithelial cell extracts (Fig. 4F). These data strongly suggest that an enhanced capacity of caspase-9 to associate with Apaf-1 could render malignant mammary epithelial cells more susceptible to cytochrome c–induced apoptosis.

**PHAPI overexpression in breast cancer cells drives their sensitivity to cytochrome c–induced apoptosis.** The increased caspase-9 association with Apaf-1 in malignant mammary epithelial cells led us to question the molecular mechanism governing this alteration. Intriguingly, a recent report indicated that PHAPI, a putative tumor suppressor protein, could enhance the association of caspase-9 with Apaf-1 (36). Very little is known about the function of PHAPI, also known as pp32, mapmodulin, and I1PP2A. It has been shown that PHAPI can combat Ras and Myc-induced oncogenic transformation and that its highly acidic COOH terminus is necessary for this function (37, 38). In addition, PHAPI has been implicated as an inhibitor of protein phosphatase 2A, a modulator of interactions between microtubules and associated proteins, and a regulator of histone acetylation (39–41). Furthermore, the role of PHAPI in apoptosomal formation does not seem to be due to its activity in the cytosol (37, 38, 42). This addition was sufficient to increase cytochrome c–induced cleavage of the caspase-3 substrate (Ac-DEVD-pNA) in HMEC lysates to levels observed in malignant cell lysates, suggesting that the overexpression of PHAPI is sufficient to enhance cytochrome c sensitivity in breast cancer cells (Fig. 5B). As these cytosolic extracts do not support transcription/translation, our results suggest that PHAPI enhances cytochrome c–induced caspase activation posttranslationally.

We also did experiments to examine the effects of inhibiting PHAPI function on cytochrome c–induced caspase activation in breast cancer cells. Reasoning that the PHAPI antibody might be able to interfere with its function, we preincubated breast cancer cell lysates with PHAPI antibody and then activated these extracts with cytochrome c. We discovered that the PHAPI antibody could act as a neutralizing antibody, diminishing caspase-3 activity in the cytochrome c–hypersensitive T47D, MDA-MB-231, and MDA-MB-453 cell lines (Fig. 5C), arguing that the elevated PHAPI levels seen in the tumor lines contributed significantly to the increased cytochrome c–induced caspase activation seen in these cells. Note that these experiments had to rely on PHAPI inactivation (which might not be complete) because available antibodies could not immunodeplete PHAPI. To further validate our findings, we preincubated the PHAPI antibody with recombinant PHAPI and added this antibody to extracts of one of our tested lines, MDA-MB-231. This preincubation was sufficient to restore sensitivity to cytochrome c, confirming the specificity of the PHAPI antibody to target endogenous PHAPI (Fig. 5D). Additionally, we wished to determine if decreasing PHAPI expression in breast cancer cells was sufficient to restore a normal apoptotic response to cytochrome c. It has been reported that PHAPI is remarkably difficult to knock down by RNA interference (36). Indeed, we tried a number of different cell lines and found that most were refractory to both siRNA oligonucleotides and short hairpin RNA constructs. However, using T47D cells, we were able to partially knock down PHAPI expression using a particular siRNA duplex. Importantly, we found that even a partial reduction in expression of PHAPI in T47D cells induced a correlative reduction in responsiveness to cytochrome c–induced caspase activation (Fig. 5E). Taken together, these data indicate

![Figure 4](Image)

**Figure 4.** Cytochrome c–hypersensitive breast cancer cells show an increase in caspase-3/caspase-9 activation and an increased affinity of caspase-9 for Apaf-1. A, extracts from the indicated cell lines were supplemented with either 0 or 5 ng/μL of cytochrome c and subsequently immunoblotted for cleaved caspase-3. B, caspase-9 antibody or immunoglobulin G (IgG) was added at equal concentrations to the indicated cell lysates after the addition of 1 ng/μL cytochrome c. Proteins were then isolated on protein G-Sepharose and immunoblotted for caspase-9. C, cytochrome c (3 ng/μL) and b-VAD-fmk (20 μM/L) were added to lysates from the indicated cell lines. Streptavidin beads were then used to isolate the b-VAD-fmk and associated proteins. Beads were then immunoblotted for caspase-9. D, yeast or mammalian cytochrome c-Sepharose beads were added to the indicated cell lysates. Beads were then immunoblotted for Apaf-1. E, GST or GST-Apaf-1 (CARD) was added to the indicated cell extracts and beads were subsequently immunoblotted for associated caspase-9. F, caspase-9 was immunoprecipitated from the indicated extracts in the presence of 1 ng/μL cytochrome c and precipitates were blotted for both caspase-9 and Apaf-1.
that the PHAPI overexpression is important for hypersensitivity to cytochrome c–induced apoptosis in breast cancer cells.

We were also interested in determining the relative expression of PHAPI in cell types that did not display high sensitivity to cytochrome c. Consequently, we prepared cytosolic extracts from normal (nonmalignant) prostate epithelial cells and normal human bronchial epithelial cells and compared the expression of PHAPI in these cells to expression levels in their tumorigenic counterparts. In contrast to mammary epithelial cells, there were no significant differences in PHAPI expression when normal and malignant epithelial cells of either prostate or lung origin were compared (Fig. 5F). As these other cell types did not exhibit elevated caspase activation in response to cytochrome c, these data further suggest that PHAPI overexpression is of fundamental importance to the sensitivity of breast cancer cells to cytochrome c.

To address the potential clinical relevance of this mechanism, we examined the expression of PHAPI in patient samples of breast tumor tissue. We observed that PHAPI was overexpressed in all of our breast tumor samples when compared with either normal breast tissue (which showed no detectable expression) or primary mammary epithelial cells (Fig. 5G). Furthermore, the degree of PHAPI overexpression in these patient samples, based on immunoblotting equal amounts of total protein, was quite similar to that observed in the malignant tissue culture cells (MDA-MB-453).

Introduction of cytochrome c into breast cancer cells results in dramatically enhanced apoptosis when compared with nonmalignant mammary epithelial cells. The hypersensitivity of breast cancer cells to cytochrome c suggested that cytochrome c, or an agent acting in a similar manner (i.e., a cytochrome c mimetic), might potentially be co-opted as a novel chemotherapeutic to selectively eliminate breast cancer cells. To evaluate this idea, we microinjected cytochrome c into the cytosol of normal and malignant mammary epithelial cells, which promotes caspase-dependent death in other cell types (42, 43). We found that a significantly higher percentage of malignant cells underwent apoptosis following cytochrome c microinjection when
compared with nonmalignant cells (Fig. 6A and representative micrographs in Fig. 6B). These data raise the exciting possibility that cytochrome c hypersensitivity in breast cancer cells might be exploited for chemotherapeutic purposes to specifically kill cancerous (and not noncancerous) mammary epithelial cells.

Discussion

Apoptotic signaling is altered at many loci in cancer cells. Although many tumors develop resistance to cytochrome c–induced apoptosis, we have discovered that breast cancer cells exhibit a unique hypersensitivity to cytochrome c–induced apoptosis. Interestingly, this sensitivity is not due to changes in core apoptosomal proteins (44) but is due to a PHAPI-mediated post translational event that enhances the recruitment of caspase-9 to the Apaf-1 CARD. These findings have the potential to affect breast cancer chemotherapy through the development of apoptosome activators or cytochrome c mimetics as shown, in principle, by the fact that malignant mammary epithelial cells could be more easily killed by cytosolic cytochrome c than their normal counterparts.

PHAPI-mediated increase in caspase activation in breast cancer. Whereas many inhibitory signaling pathways converge on the apoptosome, there are very few physiologic/pathologic examples of enhanced apoptosome activation. Our data suggest that recruitment of caspase-9 to the Apaf-1 CARD can be regulated in cancer cells to promote caspase activation. Although these observations suggest that apoptosome assembly is enhanced in breast cancer cells, future studies directly probing apoptosome assembly (e.g., using gel filtration) will be required to firmly establish this point. Presumably, increased association of caspase-9 with the Apaf-1 CARD leads to enhanced dimerization-induced caspase-9 activation. Our data indicate that a posttranslational mechanism dependent on PHAPI is responsible for the increased caspase-9 association with Apaf-1. Because supplementation of normal cell lysates with PHAPI rendered them similarly sensitive to cytochrome c and both anti-PHAPI antibody addition and RNAi-based PHAPI knockdown reduced the cytochrome c sensitivity of malignant cells to normal levels, it seems likely that the observed differences in PHAPI expression in cultured cells and tissue samples are of importance. Additionally, PHAPI overexpression was observed only in breast cancer cells and not in other tumor cell types (Fig. 5F). Interestingly, nonmalignant mammary epithelial cells seem to express less PHAPI than normal prostate or bronchial epithelial cells, suggesting that the baseline from which PHAPI can dictate sensitivity to cytochrome c is lower in breast cells than in the other cell types.

Although PHAPI seems to regulate apoptosome formation, it does not interact physically with the apoptosome (28). There are a variety of ways in which PHAPI might affect the post translational regulation of the apoptosome. Given that phosphorylation of apoptosomal components has been shown to regulate its function, kinase signaling pathways could potentially contribute to the enhanced binding of caspase-9 to Apaf-1, although we saw no evidence of altered phosphorylation of Apaf-1 or caspase-9 in breast cancer cells (data not shown). We have also shown that the ability of PHAPI to inhibit PP2A does not contribute to cytochrome c sensitivity in that other PP2A inhibitors do not modulate cytochrome c–induced caspase activation in mammary cell lysates (data not shown). It has been shown that the acidic COOH terminus of PHAPI is critical for its activation of the apoptosome and for its ability to oppose oncogene-induced transformation (28, 37). Interestingly, this region contains a nuclear localization signal, suggesting that import of PHAPI into nuclei may be important for its ability to enhance caspase activation.

Apoptotic sensitivity in cancer. Given that cancer cells typically evade apoptosis, it seemed paradoxical that breast cancer cells would be hypersensitive to cytochrome c. However, we also observed that cytochrome c–hypersensitive breast cancer cells were markedly resistant to chemotherapeutic release of cytochrome c from mitochondria. Thus, it seems likely that the apoptotic evasion in breast cancer cells is due to an inhibitory mechanism (mitochondrial cytochrome c release) that is dominant to (and upstream of) the observed point of apoptotic sensitivity (cytochrome c–induced caspase activation). Although we hypothesize that the inhibition of cytochrome c release in these cells is likely due to alterations in the activity of various Bcl-2 family members, it is also possible that the resistance of these tumor cells to chemotherapeutics stems in part from up-regulation of P-glycoprotein, a multidrug resistance gene product, allowing the cells to export anticancer drugs into the extracellular environment (45).

Interestingly, increased apoptosis in tumors has been observed under a variety of circumstances, particularly because tumor cells are chronically stressed and deprived of growth factors. One
intensively studied mechanism for this enhanced apoptosis involves the c-Myc oncogene, of which proapoptotic functions have been well characterized (46, 47). c-Myc also induces cellular proliferation, which is balanced by its proapoptotic signaling. However, when this proapoptotic signaling is later disrupted, c-Myc becomes extremely oncogenic. Whereas c-Myc per se has never been shown to regulate the apoptosis, it seems possible that oncogenes activated in breast cancers are proapoptotic and post-cytochrome c release and that subsequent evolution of the tumor cells enables the acquisition of antiapoptotic mechanisms upstream of the mitochondria. In accordance with its tumor-suppressive functions, PHAPI up-regulation may initiate a pro-apoptotic mechanism that is later overcome by mutations that inhibit mitochondrial cytochrome c release.

**Targeting apoptosis using chemotherapeutics.** The goal of chemotherapy is to selectively induce apoptosis in malignant cells (2). Whereas commonly used chemotherapeutics cause apoptosis by triggering cell cycle checkpoint-dependent release of cytochrome c (48), the specific targeting of caspase activation is also potentially promising. In particular, recent work has determined that Smac peptides or mimetics can serve as chemotherapeutics by targeting and inhibiting X-linked inhibitor of apoptosis in cancer cells where this protein plays a critical role in their evasion of cell death (21, 49–52). In addition, apoptosis activation by small molecules has shown some promise for selective targeting of cancer cells (53). It is attractive to speculate that cytochrome c sensitivity in breast cancers might be targeted in a similar manner. The design of peptides or small molecules that mimic cytochrome c-mediated activation of Apaf-1 could be of great utility in the treatment of breast cancers as our results show that malignant cells would be induced to undergo apoptosis preferentially. If cytochrome c sensitivity were maintained throughout the metastatic process, it might be possible to target malignant mammary epithelial cells even after distant metastasis.

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