Cell Cycle-independent Regulation of p21\(^{\text{Waf1/Cip1}}\) and Retinoblastoma Protein during Okadaic Acid-induced Apoptosis Is Coupled with Induction of Bax Protein in Human Breast Carcinoma Cells

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Abstract

Okadaic acid (OA) is a serine/threonine protein phosphatase inhibitor and has been shown to induce apoptosis in a number of different tumor cell lines, including human breast carcinoma (HBC) cells. The molecular basis of OA-induced apoptosis remains to be investigated. Here, we demonstrate that the OA concentration that inhibits only protein phosphatase 1 and 2A was sufficient to induce apoptosis in HBC cells. In MCF-7 cells, the OA-induced apoptosis was coupled with the overexpression of endogenous p53, p21\(^{\text{Waf1/Cip1}}\), and Bax proteins, whereas the Rb protein levels were decreased. OA also induced apoptosis and concomitantly enhanced the p21\(^{\text{Waf1/Cip1}}\) and Bax levels in human papilloma virus protein E6-transfected variants of MCF-7 cells, in which p53 function had been disrupted. OA, by contrast, had no effect on the levels or the subcellular localization of Gadd45 and Bcl2 proteins in either wild-type or E6-transfected MCF-7 cells. Bcl-x\(_L\), Bcl-x\(_S\), and Bak levels were also unchanged after OA treatment in both cell types. OA-induced apoptosis and its effect on the expression of the above molecular markers occurred in the absence of any detectable changes in the cell cycle phase distribution. On the basis of our findings, we conclude the following: (a) OA-induced apoptosis in HBC cells occurs independently of cell cycle arrest; (b) the wild-type p53 function is not an absolute prerequisite for OA-induced cell death; and (c) OA-induced apoptosis is associated with up-regulation of endogenous p21\(^{\text{Waf1/Cip1}}\) and Bax protein levels.

Introduction

Apoptosis is a genetically controlled program of cell death (1, 2). Apoptotic elimination of cells during physiological and pathological processes is important for normal tissue homeostasis (1, 2). Recent studies have demonstrated that the program of cell death is controlled via a number of signaling events (3). These signals, some of which are mediated via membrane receptors, are either protective or inductive of cell death (3). Fas and tumor necrosis factor receptor belong to a family of membrane receptors that are thought to mediate death signals (Ref. 4 and references therein), and the growth factor receptors are believed to transduce protective signals (3, 4).

Characterization of the signaling pathways involved in the receptor-mediated extracellular regulation of apoptosis is an area under intense investigation. Although these signaling pathways appear to involve protein-protein interactions as well as serine/threonine and tyrosine phosphorylation of effector proteins (3, 4), very little is known about the intracellular molecules that mediate these signals, and their ultimate targets remain elusive (3). It is possible that various apoptotic or antiapoptotic signals may feed into common intermediate phosphoproteins and, depending on the type of signal, may activate or inactivate common target(s).

A number of chemotherapeutic agents eliminate cancer cells by inducing apoptosis. A better understanding of the molecular events regulating the process of apoptosis is, therefore, required to develop novel, more efficacious, and less toxic cancer therapeutic agents. PP\(_I\) inhibitors are pharmacologically important agents that can be used to study cellular signal transduction and to develop better therapeutic strategies (5). OA is a polyether fatty acid that can directly pass through the plasma membrane to mediate its intracellular actions (5). OA increases protein phosphorylation by inhibiting serine/threonine-type intracellular PPs and was shown to induce apoptosis in a variety of mammalian tumor cell lines, including HBC cells (6, 7). The molecular events involved in OA-induced apoptosis remain unclear. Given that OA induces apoptosis in breast cancer cells, which is the most frequently occurring neoplasm in women worldwide, investigating the molecular mechanisms of OA-induced apoptosis in HBC cells is relevant. In an attempt to understand the molecular basis of OA-induced apoptosis and to identify the molecular mediators of OA-induced apoptosis, we monitored the OA effect on the apoptotic and antiapoptotic mo-

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2 The abbreviations used are: PP, protein phosphatase; OA, okadaic acid; Rb, retinoblastoma; DAPI, 4'-diamidino-2-phenylindole; HBC, human breast carcinoma; HRP, horseradish peroxidase.
Table 1  OA-induced apoptosis in MCF-7 HBC cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>Apoptotic nuclei (%)</th>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
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<tr>
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<td>OA</td>
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<td>9.1</td>
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<tr>
<td>Experiment 2</td>
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<tr>
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</tr>
<tr>
<td>OA</td>
<td>24</td>
<td>1.5</td>
</tr>
<tr>
<td>OA</td>
<td>48</td>
<td>11.9</td>
</tr>
</tbody>
</table>

Results

OA Inhibiting Only Phosphatases 1 and 2A Is Sufficient to Induce Apoptosis in MCF-7 Cells

Subconfluent cultures of logarithmically growing MCF-7 cells were treated with 20 nM OA, because at this concentration, OA only inhibits PP1 and PP2A and has no effect on the other types of phosphatases (5). Our results shown in Fig. 1 and Table 1 demonstrate that OA indeed increases the number of cells undergoing apoptosis and thus confirms the results of Kiguchi et al. (7). The OA-induced apoptosis was noted clearly within 24 h of treatment, and the effect was increased markedly by 48 h. Because the floating dead cells were not included in the quantitation, the percentage of apoptotic cells after each treatment as shown in Table 1 is an underestimate of the overall percentage of cells that underwent apoptosis.

Effects of OA on p53 and Other Apoptosis-related Proteins

We sought to investigate whether the OA-induced apoptosis is mediated via p53 signal transduction pathways. Therefore, we monitored the OA effects on the expression profiles of p53 and its downstream effectors, such as p21<sub>Waf1/Cip1</sub> (8-10), Rb (reviewed in Ref. 11), Gadd45 (12), as well as the Bcl-2 family of proteins (13-16). We chose to use an immunohistochemical staining method, because this method allows one to determine the alterations in levels, as well as in the subcellular localization of the proteins in question at a single cell level, in a heterogeneous cell population that contains nonapoptotic, apoptotic, and mitotic cells. Fig. 2 illustrates the representative photomicrographs showing the immunostaining profiles of these proteins before and after OA treatment.

OA Increases p53 Levels but Differentially Regulates p21<sub>Waf1/Cip1</sub> and Gadd45. We used p53-specific antibody 1801 to monitor the p53 immunostaining profile in untreated and OA-treated MCF-7 cells. As shown in Fig. 2, the untreated cells exhibited a heterogeneous pattern of nuclear p53 immunostaining with only a few p53-positive cells. These results are in agreement with the previously published p53-specific immunostaining profile of these cells (17, 18). Treatment with 20 nM OA for 48 h (data not shown) as well as 48 h clearly increased the number of p53-positive cells (Fig. 2).

The representative results in Fig. 2 show that the untreated MCF-7 cells also exhibited a heterogeneous pattern of p21<sub>Waf1/Cip1</sub> staining, and that OA treatment markedly increased the number of p21<sub>Waf1/Cip1</sub>-positive cells as well as the p21<sub>Waf1/Cip1</sub> staining intensity (Fig. 2). The OA-induced accumulation of p21<sub>Waf1/Cip1</sub> occurred as a function of time, and the OA effect, although less pronounced, was clearly obvious by 8 h (see below). The OA effect was, however, significantly more pronounced after 48 h of OA treatment.

We also noted that the mitotic cells in the untreated cell population were predominantly negative for p53 and p21<sub>Waf1/Cip1</sub> staining and that the OA treatment also increased the number of mitotic cells that stained positive for both proteins. Furthermore, all the apoptotic cells in the untreated as well as in the OA-treated cell population were always p21<sub>Waf1/Cip1</sub>-positive, whereas the same was not the case for p53, because a number of apoptotic cells were negative for p53 staining.

Gadd45 protein is the product of another p53-regulated gene and is expressed at lower levels in these cells (12, 19). We therefore used a more sensitive immunofluorescence...
Fig. 2. Representative photomicrographs depicting immunohistochemical and immunofluorescent staining of various proteins in untreated (C) or OA-treated MCF-7 cells. Cells grown on Lab-Tek slides were processed for immunostaining using different antibodies, as described in "Materials and Methods." Photographs were taken at a ×400 magnification using a Zeiss or Nikon microscope. p21\textsuperscript{Waf1}, arrowheads, mitotic cells. Bax, arrowheads, spontaneous and OA-induced apoptotic cells exhibiting strong Bax immunostaining.
method to investigate the OA effect on the levels and the subcellular localization of Gadd45. It is clear from the representative photomicrographs shown in Fig. 2 that the immunoreactive Gadd45 was detected predominantly in the nuclei of these cells, and the OA treatment altered neither the fluorescent intensity nor the subcellular distribution of Gadd45. Thus, despite the fact that OA enhanced the p53 and p21Waf1/Cip1 levels, it did not alter the levels of Gadd45 in these cells. It is also noteworthy that, although these cells exhibited a heterogeneous pattern of p53 and p21Waf1/Cip1 immunostaining, the Gadd45 protein was detected uniformly in all the cells, suggesting that some of the cells that do not express p53 and p21Waf1/Cip1 do in fact express Gadd45.

OA Regulation of the Bcl-2 Protein Family. Bax, Bcl-2, Bcl-xL, Bcl-xS, and Bak belong to the Bcl-2 family of proteins (13–16). The members of this family of proteins are believed to function as homodimers or heterodimers, and therefore, the relative stoichiometry of these proteins determines the cellular responses to apoptotic or antiparotic cues (13–16). We investigated whether the levels of Bcl-xL, Bcl-xS, and Bak are modulated as the cells undergo OA-induced apoptosis.

From the representative photomicrographs of Bax immunostaining shown in Fig. 2, Bax is clearly a cytoplasmic protein that localizes predominantly in the perinuclear region. The Bax immunostaining profile noted in our studies is consistent with the recently published Bax immunostaining pattern (20). We noted that OA treatment increased the overall intensity of Bax immunostaining, and that the cells undergoing either spontaneous or OA-induced cell death (particularly at the later stages, i.e., the rounded and refractile cells; see Fig. 2) always exhibited intense Bax immunostaining. The OA effect on Bax expression was that of a delayed type and was noted by 24 h (data not shown) and 48 h (Fig. 2), whereas 8 h of treatment did not exhibit any obvious increase in the Bax staining intensity (data not shown). The immunostaining profile described above was specific for Bax, given that the nonspecific rabbit polyclonal antibody did not exhibit similar immunostaining (data not shown).

The immunoreactive Bcl-2 protein was also detected in the cytoplasm, particularly in the perinuclear region. OA treatment for 24 h (data not shown) or 48 h did not alter the Bcl-2 levels nor its subcellular localization (Fig. 2). It is clear from the preceding results that OA increases Bax levels during apoptosis induction with no apparent effect on Bcl-2 levels. Bcl-xL, Bcl-xS, and Bak are the other proteins that belong to the Bcl-2 family of proteins. Because the antibodies for these proteins were not suitable for immunohistochemistry, we performed immunoblot analysis to monitor their levels. As shown in Fig. 3, the OA treatment for 48 h (or for 24 h; data not shown) did not alter the steady-state levels of Bcl-xL and Bak proteins. The antibody used for Bcl-xL detection cross-reacts with Bcl-xS; Bcl-xS migrates as a Mr, 21,000 protein (21). Prolonged exposure revealed a faint band in the same approximation most likely representing Bcl-xS, but its levels were not modulated by OA treatment (data not shown).

A recent study has demonstrated that OA-induced apoptosis in lymphoid cells was coupled with increased Bcl-2 phosphorylation (22). It was therefore concluded that OA-induced apoptosis might result from inactivating phosphorylation of Bcl-2 on serine/threonine (22). Because OA-induced phosphorylation was shown to significantly reduce the mobility of Bcl-2 during SDS-PAGE (22), we used a similar strategy to investigate the effects of OA on Bcl-2 phosphorylation. Immunoblot analysis as shown in Fig. 3 illustrates that OA treatment did not alter the mobility of the band representing Bcl-2, as was reported by Haldar et al. (22).

Down-Regulation of Rb during OA-induced Apoptosis.

It is now widely believed that some of the p53-mediated signals that are transmitted via p21Waf1/Cip1 ultimately modulate the phosphorylation status of Rb (reviewed in Ref. 11). From the above results, it is clear that OA enhances the p53 and p21Waf1/Cip1 levels. We next investigated whether the levels or phosphorylation status of Rb are modulated by OA. The OA effect on Rb is illustrated in Fig. 4 and, as is shown, treatment with OA, while increasing the p21Waf1/Cip1 levels, decreased the overall levels of Rb protein irrespective of the Rb phosphorylation status. Similar results were also obtained in p53-negative MDA-MB-231 HBC cells. The issue as to why Rb was not hypophosphorylated following OA treatment remains unclear. It is possible that OA may inhibit the PP responsible for Rb hypophosphorylation.

p53-independent Regulation of p21Waf1/Cip1, Bax, and Apoptosis.

From the preceding results, it is clear that OA-induced apoptosis is coupled with the induction of p53, p21Waf1/Cip1, and Bax, whereas the levels of Gadd45, Bcl-2, Bcl-xL, Bcl-xS, and Bak remain unaltered. The roles of Bax and p53 in regulation of cell death are known; however, although
OA induces Apoptosis without Modulating the Cell Cycle Progression

The OA effect on the cell cycle phase distribution in MCF-7 and MCF-7/E6 cells is illustrated in Table 3. It is clear that the OA treatment for 24 or 48 h did not alter the cell cycle progression of the asynchronously growing cells. It is of note that the OA concentration (20 nM) used to analyze cell cycle progression markedly induces p21\textsuperscript{Waf1/Cip1} and apoptosis in these cells within the same time frame. Nocodazole is a known mitotic inhibitor and was included to block the cells from leaking into G\textsubscript{1} phase of the cell cycle (25). Table 3 illustrates that the percentage of the cells in G\textsubscript{2}-M phase was increased considerably after overnight treatment with nocodazole, and the cell cycle profiles of the cells treated with nocodazole either alone or in combination with OA were essentially identical. Thus, the overexpression of endogenous p21\textsuperscript{Waf1/Cip1} (and p53 in wild-type MCF-7 cells) in response to OA treatment was not associated with alterations in the cell cycle phase distribution but was coupled with the induction of apoptosis.

Discussion

In this study, we demonstrate that the OA-induced apoptosis was coupled with the up-regulation of endogenous p53, p21\textsuperscript{Waf1/Cip1}, and Bax proteins, whereas the Rb protein levels were decreased in MCF-7 cells. OA by contrast had no effect on the expression profiles of Gadd45, Bcl-2, Bcl-x\textsubscript{L}, Bcl-x\textsubscript{S}, and Bak. At the concentration used in this study, OA effects are mediated predominantly via inhibition of serine/threonine PP2A (5). Thus, the OA-induced apoptosis should be coupled with the sustained phosphorylation of PP2A substrate(s). The role of p53 in OA-induced apoptosis is not excluded in the wild-type MCF-7 cells, and it is possible that OA may also affect p53 function by promoting p53 phosphorylation. Several lines of evidence, however, indicate that p53 is not absolutely required for OA-induced cell death. For example, the E6-transfected MCF-7 cells in which p53 function was abrogated retained their ability to respond to OA-induced apoptosis. Additionally, OA-induced apoptosis as well as up-regulation of p21\textsuperscript{Waf1/Cip1} and down-regulation of Rb (see Fig. 4) were also noted in MDA-MB-231 HBC cell line, which is another p53-negative cell line (7, 26).

p21\textsuperscript{Waf1/Cip1} and BAX genes are under direct p53 control (8, 27, 28), and thus, a part of the OA enhancement of p21\textsuperscript{Waf1/Cip1} and Bax levels might in fact be p53 dependent in wild-type MCF-7 cells. OA enhancement of Bax and p21\textsuperscript{Waf1/Cip1} in MCF-7/E6 cells, however, suggests that the regulation of both of these molecular markers does not absolutely require wild-type p53 function. The possibility that OA might prevent E6-mediated degradation of p53 is less likely, because untreated or OA-treated MCF-7/E6 cells were...
predominantly negative for p53 immunostaining (data not shown). p53-independent regulation of p21Waf1/Cip1 is well established (26, 29). Biggs et al. (30), using U937 leukemia cells, have recently identified a p53-independent OA-responsive region in the proximal promoter of the p21Waf1/Cip1 gene and thus demonstrate that OA regulation of p21Waf1/Cip1 is transcriptional and occurs in a p53-independent manner (30).

p53-independent regulation of Bax was also shown in recent studies (31, 32). Our results also demonstrate that Bax regulation can in fact occur in a p53-independent manner.

The role of Bax as a mediator of cell death is well known, although the BAX knockout mouse exhibited increased apoptosis in the testicular tissue (33). The results presented in this study, that the Bax levels were increased in response to OA-mediated cell death, whereas Bcl-2, Bcl-xL, and Bcl-xS levels were unaltered, suggest that the overexpression of Bax might be sufficient to favor Bax-Bax homodimers and to overcome the antideath effects of Bcl-2 and Bcl-xL. However, we cannot rule out the possibility that OA-induced posttranslational alterations of Bcl-2 and Bcl-xL, not grossly affecting their mobility on electrophoresis, may inactivate their function. Bak is a recently identified member in the Bcl-2 family of proteins, the enforced overexpression of which was shown to induce apoptosis (16, 34, 35). The exact mechanisms of Bak-initiated apoptosis are not clear. However, based on its physical interaction with Bcl-xS, Bak was proposed to elicit its actions by inactivating Bcl-xS (34, 35). Our results demonstrate that MCF-7 cells express easily detectable levels of Bak protein, and its expression was unaltered during the OA-induced apoptosis.

Unlike Bax, the role of p21Waf1/Cip1 or Gadd45 in the activation of cell death remains unclear. In a recent study (36), the interleukin 3 withdrawal-induced apoptosis was found to be associated with up-regulation of Gadd45 and down-regulation...
ulation of p21\textsuperscript{Waf1/Cip1} in a nonmalignant murine lymphoma cell line (36). From our results, it is clear that OA did not alter the Gadd45 expression profile, although it enhanced the levels of wild-type p53 and p21\textsuperscript{Waf1/Cip1} in MCF-7 cells. The expression of p21\textsuperscript{Waf1/Cip1} and Gadd45 is under p53 transcriptional controls (8, 12), and both proteins were shown recently to also interact with each other (37). Thus, the inability of OA to induce Gadd45 in these cells is very intriguing. The possibility that these cells might contain an inherently defective Gadd45 induction pathway is less likely, because ionizing radiation has been shown to induce p53, p21\textsuperscript{Waf1/Cip1}, and Gadd45 in these cells (19). It therefore appears that the regulation of p21\textsuperscript{Waf1/Cip1} and Gadd45 is not always coupled, even in the cells that are capable of mounting a p53 response and otherwise exhibit coordinate regulation of both Gadd45 and p21\textsuperscript{Waf1/Cip1} in response to certain signals. These results, therefore, highlight the importance of stress specificity and cellular context in investigating the regulation of these important molecules.

A large body of evidence supports the view that p21\textsuperscript{Waf1/Cip1} is one of the key players responsible for the G1-G2 cell cycle arrest (reviewed in Ref. 11). During the cell cycle progression, p21\textsuperscript{Waf1/Cip1} levels are high in G0-G1 phase and decline as the cells enter S phase; p21\textsuperscript{Waf1/Cip1} is essentially undetectable during G2-M phase (Ref. 38; reviewed in Ref. 11). Our results demonstrate that, although OA-induced apoptosis and up-regulation of p21\textsuperscript{Waf1/Cip1} were readily evident within 24 and 48 h of OA treatment, there was no evidence of G1 arrest during the same time. In the wild-type MCF-7 cells, OA also induced the expression of p21\textsuperscript{Waf1/Cip1} in mitotic cells that otherwise do not express p21\textsuperscript{Waf1/Cip1} (Fig. 2, p21\textsuperscript{Waf1}, arrowheads). Thus, the OA-induced "deregulated" expression of p21\textsuperscript{Waf1/Cip1} was associated with apoptosis and was not responsible for G1 arrest or for the consequences of it. Similar "deregulated" expression of p21\textsuperscript{Waf1/Cip1} was also evident in a significant proportion of mitotic cells in constitutively growing (i.e., not treated with OA) E6-transfected MCF-7 cells (Fig. 5, p21\textsuperscript{Waf1}, arrowheads). It is of note that the MCF7/E6 cells exhibited a much higher propensity to undergo spontaneous apoptosis than their wild-type counterparts. Our recent demonstration that enforced overexpression of exogenous p21\textsuperscript{Waf1/Cip1} was sufficient to sensitize MCF-7 and another HBC cell line, T47D, to apoptosis (39) would further strengthen an association between "deregulated" expression of p21\textsuperscript{Waf1/Cip1} and apoptosis. Cumulatively, these findings suggest that the "deregulated" expression of p21\textsuperscript{Waf1/Cip1}, either spontaneous, drug induced, or gene transfer mediated, might trigger the process of apoptosis at least in some cell types. Because MCF-7 cells exhibit a heterogeneous p21\textsuperscript{Waf1/Cip1} immunostaining pattern, we thoroughly monitor the p21\textsuperscript{Waf1/Cip1} immunostaining profile in the untreated and OA-treated cells. We noted repeatedly that apoptotic cells exhibiting nuclear fragmentation (see Figs. 2 and 3) were almost always p21\textsuperscript{Waf1/Cip1} positive. However, a number of nonapoptotic cells also exhibited very strong p21\textsuperscript{Waf1/Cip1} immunostaining (see Figs. 2 and 3). Considering the heterogeneity in the tumor cell population, it is likely that some cells might be able to tolerate very high levels of endogenous or exogenous p21\textsuperscript{Waf1/Cip1}.

Precisely how OA regulation of Bax, p21\textsuperscript{Waf1/Cip1}, and Rb can be linked to the induction of the apoptotic process remains unclear and requires additional investigation. It is possible that an OA-mediated decrease in the Rb levels would relieve E2F from the Rb controls; access-activated E2F would try to promote cellular entry into the S phase. The concomitant OA-mediated up-regulation of p21\textsuperscript{Waf1/Cip1} on the other hand, would tend to induce G1 arrest. As a result of these conflicting signals, cells may activate the inherent program of cell death by activating Bax and perhaps other as yet unidentified proteins, all of which may act in concert to induce cell death.

Materials and Methods

Cell Lines and Cell Culture. The origin of MCF-7 cells has been mentioned previously (24, 25, 39). MCF-7/E6 cells were established in our laboratory as described previously (24, 25). Cells were maintained regularly in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Interogen Co., Purchase, NY).

Antibodies and Reagents. p53-specific 1801 and antihuman p21\textsuperscript{Waf1/Cip1} monoclonal antibodies were purchased from Oncogene Science (Cambridge, MA). Anti-Bax, Bcl-\textsubscript{x}\textsubscript{a}, Bak, and Gadd45 rabbit polyclonal antibodies were obtained from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal anti-Bcl-2 antibodies were purchased from PharMingen (San Diego, CA). Fluorescein-conjugated goat antirabbit secondary antibodies were purchased from Oncogene Science (Cambridge, MA). HRP-conjugated goat antirabbit, goat antimouse antibodies, and mouse monoclonal oxidase peroxidase antiperoxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Chromom 3,3'-diaminobenzidine tetrahydrochloride tablets, normal goat serum, and Myer's hematoxylin solution were purchased from Sigma Chemical Co. DAPI was obtained from Boehringer Mannheim (Indianapolis, IN). OA was purchased from Life Technologies, Inc.

Immunohistochemistry. Cells grown on Lab-Tek tissue culture slides were washed twice with PBS [120 mm NaCl, 11.5 mm NaH\textsubscript{2}PO\textsubscript{4}, and 31.3 mm KH\textsubscript{2}PO\textsubscript{4} (pH 7.4–7.6)]. Special care was exercised to avoid the excessive loss of dead cells during washes. Cells were fixed in a 3.7% formaldehyde solution for approximately 12 min, followed sequentially by incubation for 5 min in ice-cold absolute methanol and 30 s in acetone. After two washes with PBS, the endogenous peroxidase activity was quenched with 1% hydrogen peroxide, and the slides were incubated in normal goat serum for 30 min. Cells were incubated with primary antibodies used at 1:100 dilution in PBS for 1–2 h at room temperature. For p53 and p21\textsuperscript{Waf1/Cip1} detection, goat antiserum IgG1 and peroxidase antiperoxidase were used, and HRP-conjugated goat antirabbit secondary antibody was used to detect Bax. The color was developed using 3,3'-diaminobenzidine tetrahydrochloride chromogen and 0.1% (v/v) H\textsubscript{2}O\textsubscript{2} in PBS; counterstaining was performed with hematoxylin. For Gadd45 and Bcl-2 detection, fluorescein-conjugated goat antirabbit secondary antibody was used. Photomicrographs were generated using a Zeiss Axiosplan or Nikon microscope.

Analysis for Apoptosis. Cells were analyzed for apoptotic features, as we have described recently (39). In brief, the logarithmically growing cells in six-well plates were either untreated or treated with OA for 24 or 48 h. Cells were washed carefully with PBS prior to fixation with ice-cold absolute methanol for 3–5 min. After rehydration in PBS, cells were incubated with DAPI solution for 30 min in the dark. Cells were washed with PBS, mounted with coverslips using 10% polyvinyl alcohol, and analyzed using a Zeiss fluorescent microscope at 420 nm. The apoptotic cells exhibiting condensed and fragmented nuclei were readily visible and scored positive. Using a 1 × 40 Neofluar objective lens, apoptotic nuclei were counted in five to seven randomly selected fields. A minimum of 500–1000 nuclei was examined for each case, and the results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

Western Immunoblot Analysis. OA-treated and untreated cells were harvested, and floating and adherent cells were pooled and lysed in
radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined using a Bio-Rad assay, and aliquots containing equal amounts of protein were size fractionated via 12 or 10% SDS-PAGE under denaturing conditions. The rainbow molecular mass markers were run in parallel. Protein transfer was accomplished via semidyey method. After transfer, the blots were washed once in PBS and then stained with Ponceau S to ascertain an equal amount of protein in each lane. Blots were blocked in 3–5% skim milk and 0.01% Tween 20 in PBS for 2–4 h and then incubated at room temperature in the same solution containing the primary antibodies for 1–2 h. Blots were washed three times in blocking solution 10 min each and then incubated either with HRP-conjugated goat antirabbit or goat antitmouse antibodies for 1 h. After washing three times with blocking solution and once for 10 min in cold PBS, bands were developed using the Amersham enhanced chemiluminescence system.

Flow Cytometry. Logarithmically growing cells were either not treated or treated with OA (20 mM) for 24 or 48 h. Nocodazole, a mitotic inhibitor, was added either alone for 16–18 h or simultaneously with OA for 24 h. Sample preparation for cell cycle analysis was as described by Fan et al. (25). Briefly, the adherent and nonadherent cells were pooled together and washed with ice-cold PBS and then incubated with ice-cold 70% ethanol for 1 h at 4°C. After fixation, the cells were washed with PBS and incubated with RNase A (500 units/ml) at 37°C for 15 min. DNA was stained with propidium iodide (50 μg/ml), and cell cycle analysis was performed on approximately 15,000 cells/determination using a fluorescence-activated cell analyzer.

Quantitation. Representative images of untreated or OA-treated cells were digitized using the image analysis program Adobe Photoshop (Version 6.0) for the Macintosh computer. This program allows the determination of the staining intensity at a single cell level. The brightness, contrast, and color balance of respective pairs of untreated and OA-treated images were adjusted to the same values prior to quantitation. The intensities of several areas in a particular image were determined, and the values were converted to a mean value. The mean values for the background intensity were also similarly obtained and were subtracted from the mean values of the image intensities. The percentage of p53- and p21WAF1/CIP1-positive cells before and after OA treatment were determined by direct microscopy; the positive nuclei were scored in several randomly selected microscopic fields, and the values were expressed as a percentage of positive nuclei over the total number of nuclei counted. At least 800–1000 nuclei were counted in each case.

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References


