Role of the Raf Signal Transduction Cascade in the *in Vitro* Resistance to the Anticancer Drug Doxorubicin[1]

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ABSTRACT

The precise molecular events involved in the development of drug resistance (DR) remain largely unknown. Raf is an intermediate in the signal transduction cascades initiated by growth factors. The hypothesis behind the following studies is that deregulated Raf-1 expression plays a role in the development of drug resistance. A positive correlation was observed between increased Raf-1 activity and increased values for IC_{50} for doxorubicin in established cell lines. The National Cancer Institute/Adriamycin Resistant (NCI/ADR-RES) cell line exhibited both the highest Raf-1 activity and the highest IC_{50} values for doxorubicin (Adriamycin). In contrast, the MCF-7 cell line exhibited both lower Raf activity and lower IC_{50} values for doxorubicin. While MCF-7 cells transfected with either constitutively active ΔRaf-1 or conditionally active ΔRaf-1:AR demonstrated increased IC_{50} values for doxorubicin and a reduced capacity to undergo apoptosis after doxorubicin treatment as compared with parental cell lines. Moreover, growth curves performed show that both the constitutively and conditionally active forms of Raf-1 do not increase growth as compared with the parental MCF-7 cell line. This is important because it implies that higher cell counts between Raf transfectants and the parental MCF-7 cell line are attributable to differences in DR, not growth rates. These observations suggest a role for the Raf-1 protooncogene in the regulation of DR.

INTRODUCTION

DR[4] is one of the main obstacles to successful cancer treatment (1). There are several mechanisms that can contribute to the phenomenon of DR. These include, modification of the target, increased drug inactivation, extrusion of the drug by a variety of cell membrane pumps, and blockade of apoptosis (2–10). However, the precise molecular mechanisms involved in the regulation of these events remain largely unknown. Understanding the mechanisms of DR at the molecular level will provide a background for the rational design of adjuvant therapies that will reverse DR, thus giving new life to old drugs.

Most of the water-soluble growth factors, which regulate the growth, proliferation, and differentiation of normal and transformed cells, exert at least part of their effects by signaling through the highly conserved Ras/Raf/MEK/ERK pathway. A cascade of kinase activation occurs after the cognate receptor is ligated (11, 12). Some reports have suggested that this signal transduction pathway may be involved in the regulation of several aspects of DR. For instance, expression of the P-gp extrusion pump is regulated by the Ras/Raf/MEK/ERK pathway (13, 14). Moreover, a correlation between high Raf-1 activity and resistance to paclitaxel (Taxol™) has been reported in early passage human cervical tumors (15).

The purpose of this study was to examine the role of the *Raf-1* oncogene in the modulation of DR. A correlation between decreased Raf-1 activity and sensitivity to doxorubicin was observed in the cell lines examined. Drug sensitivity was studied in cell lines expressing constitutively active as well as conditionally active *Raf-1* oncogenes. The capacity of the Raf-infected cells to undergo doxorubicin-induced apoptosis was evaluated using annexin V/propidium iodide binding and DNA fragmentation assays. Cell proliferation assays were also performed to assess the growth rate of each cell line. All of the above techniques have suggested a role for the *Raf-1* oncogene

Received 7/12/00; revised 4/23/01; accepted 6/7/01.
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[1] This work was supported in part by Grants R01CA51025 from the National Cancer Institute and 9805-ARG-0006 and 2000-ARG-0003 from the North Carolina Biotechnology Center (to J. A. M.). R. A. F. was supported in part by Grants IRG-97-149 from the American Cancer Society and 9930099N from the American Heart Association. R. A. F. and J. A. M. were supported in part by the East Carolina University Interdisciplinary Program Supporting Breast Cancer Research.
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[4] The abbreviations used are: DR, drug resistance; FBS, fetal bovine serum; GFP, green fluorescent protein; GLB, Gold Lysis Buffer; PVDF, polyvinylidene difluoride; P-gp, P-glycoprotein; AR, androgen receptor; RT-PCR, reverse transcription-PCR; Ras/Raf/MEK/ERK, signaling pathway initiated by Ras resulting in ERK activation.

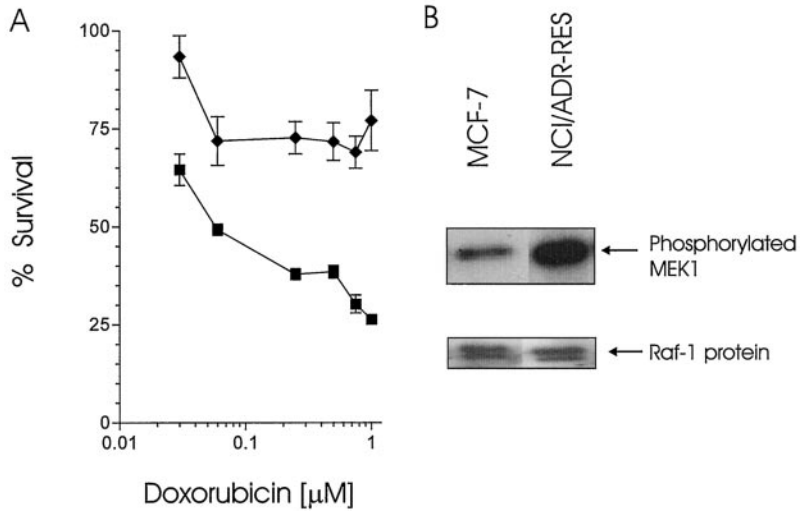

Fig. 1 Sensitivity of MCF-7 and NCI/ADR-RES cells to doxorubicin and Raf-1 kinase activity. *A*, survival curves after doxorubicin treatment for the MCF-7 (■) and the NCI/ADR-RES (◆) cell lines, previously known as MCF/ADR. Cells were plated in 96-well plates and treated with doxorubicin concentrations in the range of 0–1 μM for 48 h. After 1 day of growth in drug-free media, the cells were fixed by precipitation with TCA, and cell survival was estimated by staining with sulforhodamine. The absorbance of each well is proportional to cell survival, and the absorbance of untreated cells was set to 100% survival. The curve corresponds to a representative example of three independent experiments. Doxorubicin concentrations are plotted on a Log_{10} scale. *B*, Raf-1 immunocomplex assay for the MCF-7 and NCI/ADR-RES cell lines. Cells, which were starved overnight, were lysed, and Raf-1 was immunoprecipitated with anti-Raf-1 antibodies and protein A-Sepaharose beads. Then Raf-1 kinase activity was examined after incubation of the immunocomplexes with the inactive MEK1 substrate and [γ-^{32}P]ATP.

in the generation of breast cancer DR. Overexpression or inhibition of Raf-1 activity altered susceptibility of MCF-7 cells to effects of the chemotherapeutic drug doxorubicin.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells (American Type Culture Collection, Rockville, MD) and their retrovirally infected counterparts were grown in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), 2 mM glutamine, 100 units/liter penicillin, and 100 μg/ml streptomycin. The cells were cultured in a humidified incubator with a 5% CO_2 atmosphere. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Testosterone stock was 1 mM in ethanol, and doxorubicin (Adriamycin) stock was 2 mg/ml in sterile water.

Retroviral Infection of Cells. Plasmid DNAs containing recombinant retroviruses were transfected into the retroviral packaging cell line PA317, and φ2 with lipofectin (Life Technologies, Inc.) and retroviruses were passed sequentially from one cell line to the other to amplify their titers as described (16–18). MCF-7 cells were infected with viral stocks as described (19). The following G418-resistant (*neo*^{r}) retroviruses were used in this study: (*a*) pLNCRaf-1, which encodes the full-length human Raf-1 cDNA, inserted into the pLNCX retroviral vector; (*b*) pLNCΔRaf-1, which contains the ΔRaf-1 cDNA, an NH_2-terminal truncated and constitutively active form of the *Raf-1* gene, inserted into the pLNCX retroviral vector (20); (*c*) pLS v-Ha-Ras, which encodes v-Ha-Ras, inserted into the pLS retroviral vector (21); and (*d*) pLNTAR305ΔRC (RafAR), which contains the ΔRaf:AR cDNA, an NH_2-terminal truncated form of Raf-1 fused to the CAAX motif domain of K-Ras and the hormone binding domain of the human AR, cloned in-frame with the NH_2-terminal 9E10 Myc epitope (TAG) into the pLNSX3 retroviral vector (22). MCF-7 cells were also infected with a ΔRaf-1:AR retrovirus containing the AR hormone-binding domain linked to an activated ΔRaf-1, which was ligated to the GFP. The GFPΔRaf-1:AR insert is contained in the pBP3puro retroviral vector, which encodes resistance to puromycin. Some MCF-7 cells were infected with either the pZipneo or pBabepuro retroviruses, which are empty retroviral vectors encoding *neo*^{r} or

Table 1 IC_{50} values for doxorubicin in the MCF-7 cell line and the oncogene-overexpressing counterparts

To examine the cell survival in different doxorubicin concentrations, the cells were plated at 5000 cells/well in 96-well plates. Each doxorubicin concentration (0–1 μM) was tested in triplicate. Cell survival was tested using the sulforhodamine assay (22). The data were adjusted to a logistic model to calculate the doxorubicin IC_{50} and the confidence intervals, which appear within parentheses. The data presented correspond to one representative experiment of at least three independent replicates.

Cell line	IC_{50} (confidence interval)	Fold increase
MCF-7	0.1 (0.05; 0.2)	Reference
MCF/pZipNeo	0.09 (0.01; 0.2)	Reference
MCF/Raf-1	0.32 (0.16; 0.48)	3.2
MCF/ΔRaf-1	0.9 (0.2; 1.56)	9
MCF/GFPΔRaf-1:AR (ethanol)	0.16 (0.01; 0.6)	1.6
MCF/GFPΔRaf-1:AR (testosterone)	1.2 (0.6; 1.8)	12
MCF/Ha-v-Ras	0.5 (0.3; 0.7)	5

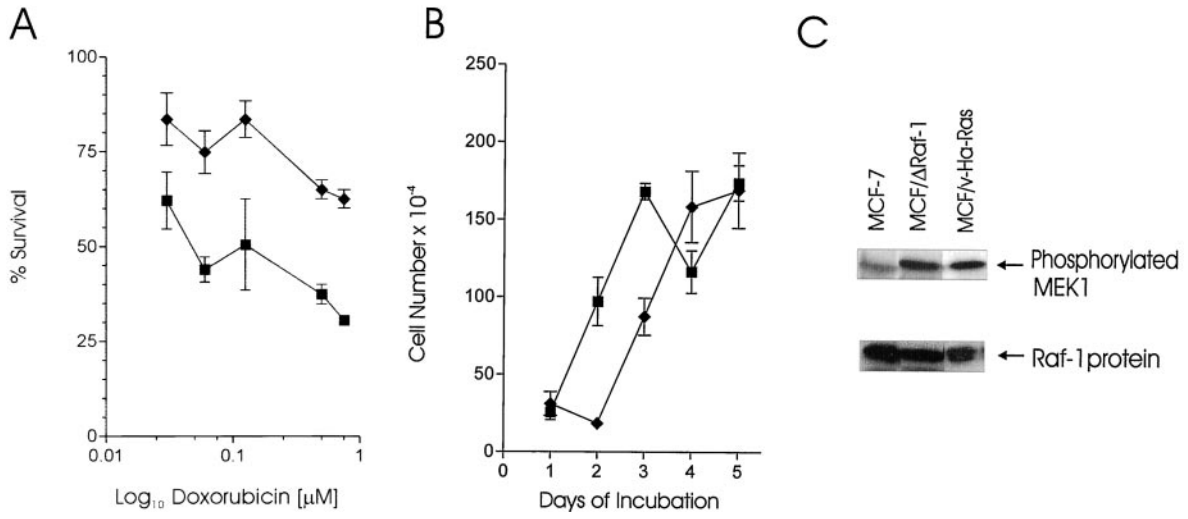

Fig. 2 Sensitivity of activated Ras- and Raf-transfected cells to doxorubicin and Raf-1 activity. *A*, survival curves after doxorubicin treatment for the MCF-7 cell line (■), the MCF/ΔRaf-1 cell line (♦), and the MCF/v-Ha-Ras cell line (●). The curve is a representative example of three independent experiments, and each concentration was tested in triplicate. Doxorubicin concentrations are plotted on a Log_{10} scale. *B*, cell proliferation of the MCF-7 cell line (■) and the MCF/ΔRaf-1 cell line (♦). The cells were incubated in a 24-well plate with RPMI 1640 containing 5% FBS. There were ~2.5×10^5 cells/well. The cells were collected and counted on a hemocytometer each day for 5 days. Both cell lines were tested in quadruplicate. *C*, Raf-1 immunocomplex assay for the MCF-7, MCF/ΔRaf-1, and MCF/v-Ha-Ras cell lines. The cells were starved overnight and then lysed. The Raf-1 protein was immunoprecipitated with anti-Raf-1 antibody and protein A-Sepharose beads. Then beads were incubated with inactive MEK1 in the presence of [γ-^{32}P]ATP. The products of the reactions were electrophoresed and electrotransferred to a PVDF membrane, which was exposed furthur to an X-ray film. The membranes were then Western blotted with anti-Raf-1 antibody for control of the amount of protein immunoprecipitated.

puro^r, respectively. *neo*^{r} or *puro*^{r} MCF-7 cells were isolated by selection in medium containing G418 (2 mg/ml; Life Technologies, Inc.) or puromycin (2 μg/ml; Sigma Chemical Co.), respectively. The nomenclature of the MCF-7 cells infected with the different oncogenes is MCF/Raf-1, MCF/ΔRaf-1, MCF/ΔRaf-1:AR, MCF/GFPΔRaf-1:AR, MCF/v-Ha-Ras, MCF/Zipneo, and MCF/pBP3puro for cells infected with the Raf-1, ΔRaf-1, ΔRaf-1:AR, GFPΔRaf-1:AR, v-Ha-Ras, pZipneo, and pBP3puro retroviruses, respectively.

Sulforhodamine Cell Viability Assay. Cells were trypsinized, and 5000 cells/well were seeded in a 96-well plate. Then the different dilutions of doxorubicin or media were added for 48 h. Each condition was performed in triplicate. After this time period, the drug-containing media was removed, and drug-free media was added, to allow the remaining viable cells to proliferate. After 24 h, the cells were fixed by precipitating with 60% trichloroacetic acid for 1 h. After extensive washing with H_2O, the cells were stained with 0.4% sulforhodamine for 10 min (23). After washing with 1% acetic acid, the plates were air-dried, and the dye was solubilized in unbuffered 10 mM Tris. The absorbance was read in an Anthos (Anthos Labtec Instruments, Salzburg, Austria) plate reader at 540 nm. Whenever the effects of testosterone were examined, charcoal-stripped FBS and phenol red-free RPMI 1640 media were used to minimize possible androgenic effects of either the phenol red in the media or the steroids in the FBS.

IC_{50} Estimation. A logistic model, modified from Van Ewijk and Hoekstra (24), was adjusted to the data. The model is given by:

$$Y = \frac{k}{1 + \left(\frac{d}{x_0}\right)^b}$$

The variables in this model are: *Y*, absorbance, and *d*, doxorubicin dose in μM. The parameters in this model are: *k*, the absorbance when the dose is 0; x_0 is the IC_{50}, and *b* represents the monotonicity of the function (*i.e.*, if *b* is >0, the response is monotonically decreasing).

The nonlinear model was adjusted using the procedure PROC NLIN from SAS (Statistical Analysis Systems, Cary, NC), version 7.1. The IC_{50} and their 95% confidence intervals were calculated for each cell line.

Immunocomplex Kinase Assay. Preconfluent cells were either starved overnight or stimulated with 2 μM testosterone for different time intervals. The cells were harvested and lysed using GLB [20 mM Tris (pH 7.4), 137 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 15% glycerol] containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 100 μM β-glycerophosphate, 1 mM EGTA, 10 mM sodium fluoride, 1 mM $Na_4P_2O_7$, and 1 mM phenylmethylsulfonyl fluoride. Lysate equivalent to 1.25×10^6 cells was immunoprecipitated by incubation with 0.4 μg of anti-Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and then another hour of incubation with 25 μl of 50% v/v protein A-Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ). The beads were washed extensively with GLB buffer, GLB + 0.5 mM NaCl, and kinase wash buffer [25 mM HEPES (pH 7.4) and 10 mM $MgCl_2$]. The kinase reaction was

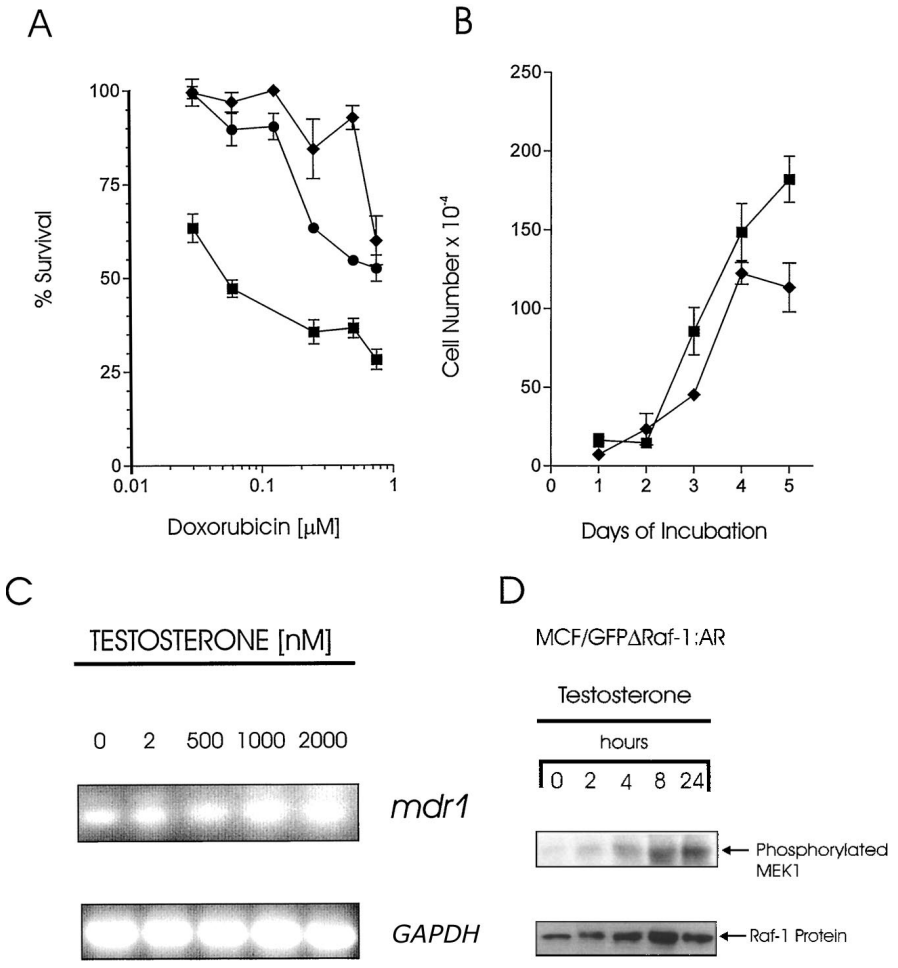

Fig. 3 Sensitivity of activated Raf-transfected cells to doxorubicin and increased levels of *mdr*-1 mRNA caused by Raf activation. *A*, survival curves to doxorubicin in the MCF/GFPΔRaf-1: AR cells cultured in the presence of 1 μM testosterone (♦) and the vehicle, ethanol (■). The curve corresponds to a representative example of three independent experiments, and each concentration was tested in triplicate. Doxorubicin concentrations are plotted on a Log_{10} scale. *B*, growth of the MCF/ΔRaf-1: AR cell line (■) and the MCF/ΔRaf-1: AR cell line cultured in the presence of 1 μM testosterone (♦). The cells were incubated in a 24-well plate with RPMI 1640 containing 5% FBS. There were $\sim 2.5 \times 10^5$ cells/well. The cells were collected and counted on a hemocytometer each day for 5 days. Both cell lines were tested in quadruplicate. *C*, MCF/GFPΔRaf-1: AR cells grown in the testosterone concentrations indicated for 16 h. Then cells were harvested by scraping, and the RNA was isolated by the Trizol method. RT-PCR was performed using 1 μg of RNA and 35 cycles of PCR after the reverse transcription reaction for the *mdr-1* gene. For the *glyceraldehyde-3-phosphate dehydrogenase* gene, 0.5 μg of RNA was used, and 28 cycles of PCR were done after the reverse transcription. This experiment was performed twice with similar results. *D*, Raf-1 immunocomplex assay for the MCF/GFPΔRaf-1: AR cell line. The cells were starved overnight and then treated with 1 μM testosterone for the indicated time periods. The immunoprecipitated Raf-1 protein was incubated with the substrate-inactive MEK1 in the presence of [γ-^{32}P]ATP. The products of the reactions were electrophoresed and electrotransferred to a PVDF membrane, which was then exposed to film. Membranes were then blotted with an anti-Raf-1 antibody to determine the amount of Raf-1 immunoprecipitated.

performed by incubating the immunocomplexes in a reaction mixture containing kinase wash buffer, 1 mM DTT, 50 mM ATP, 1 mM $MnCl_2$, 10 μCi [γ-^{32}P]ATP, and 12.5 μg of inactive MEK1 (Upstate Technologies, Lake Placid, NY) for 30 min. The product of the reaction was electrophoresed on an 8% SDS-polyacrylamide gel and electrotransferred to a PVDF membrane (Immobilon-P, Millipore Corporation, Bedford, MA), which was later exposed to film to visualize phosphorylated MEK1. Band intensity levels were determined by scanning films with a Pharmacia LKB densitometer.

Determination of the Levels of the Proteins Immunoprecipitated in the Immunocomplex Kinase Assays (Back-Blot). The same PVDF membrane that was exposed to the X-ray film was probed further with anti-Raf-1 antibody (Santa Cruz Biotechnology) in a 1:2000 dilution and then incubated with a donkey antigoat antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) in a 1:2000 dilution. The blot was developed using a chemiluminescence system (NEN, Boston, MA) and exposed for 10 s to Hyperfilm (Amersham Pharmacia Biotech). Band intensity levels were determined by scanning films with a Pharmacia LKB densitometer. Intensity levels of phosphorylated MEK1 were normalized to Raf-1 Western blot intensity levels to enable comparison of the Raf-1

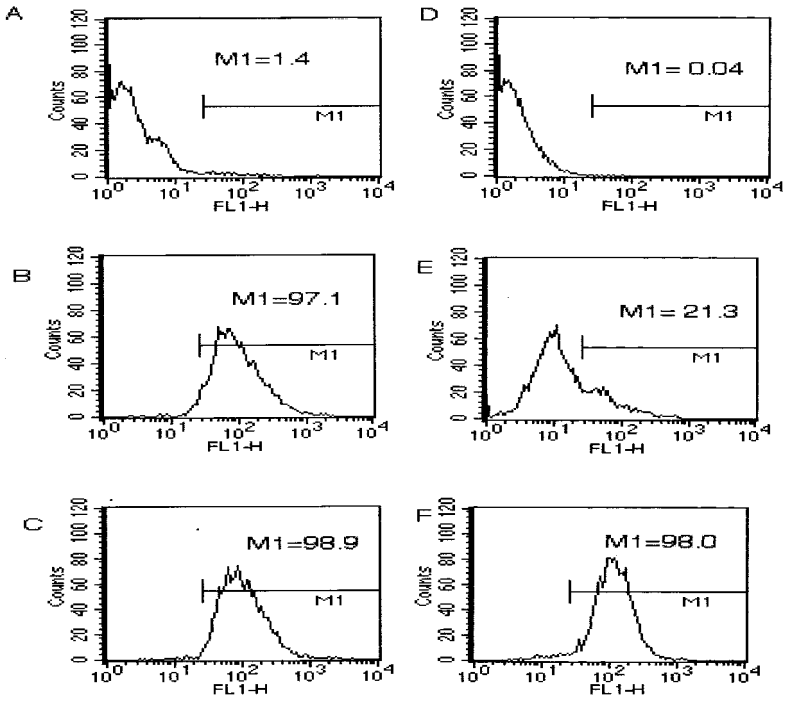

Fig. 4 Expression of activated Raf-1 induces P-gp activity. The activity of the P-gp extrusion pump was measured by the capacity of cells to extrude the fluorescent dye rhodamine 123 in the presence and absence of the P-gp inhibitor verapamil. Histograms of FL1 fluorescence are presented. Autofluorescence is represented in *A* and *D* in MCF-7 cells and MCF/ΔRaf-1 cells, respectively. *B* and *E*, fluorescence of MCF-7 and MCF/ΔRaf-1 cells stained with rhodamine 123, respectively; *C* and *F*, fluorescence of MCF-7 and MCF/ΔRaf-1 cells stained with rhodamine 123 in the presence of verapamil. *M1*, mean fluorescence of the cells under the marker bar. The experiment was performed twice with similar results.

activity of NCI/ADR, MCF/ΔRaf-1, and MCF/v-Ha-Ras cells to that of MCF-7 cells.

Cell Proliferation Assay. MCF/ΔRaf-1:AR cells containing the AR were incubated in the presence and absence of 1 μM testosterone. Cells were recovered with trypsin and EDTA (Life Technologies, Inc.) and washed with 5 ml of PBS. Cells were stained with trypan blue and counted with a hemocytometer. Cells were counted for 5 days in quadruplicate.

Rhodamine 123 Extrusion. Exponentially growing cells were harvested and washed with warm PBS. The cells were incubated in flow cytometry tubes with a warm solution of PBS alone, 10 μM rhodamine 123, or 10 μM rhodamine plus 20 μM verapamil (a P-gp pump inhibitor) for 1 h. Then cells were washed with warm PBS and the fluorescence of rhodamine 123 in each cell line was detected with a FACScan on the FL1 channel.

Confocal Microscopy. The MCF-7 cells as well as the MCF/GFPΔRaf-1:AR cells cultured in the absence or presence of testosterone (1 μM) were grown on cover slides overnight.

The cover slides were then observed under a Leica TCS SP Spectral confocal microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) with 490 nm for excitation and 520 nm for emission.

Flow Cytometric Analysis. The MCF-7 cells as well as the MCF/GFPΔRaf-1:AR cells grown in the presence or absence of testosterone (1 μM) were harvested after trypsinization. The fluorescence of the cells was detected using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. For each cell line, 10,000 events were collected, and channel 1 fluorescence was registered.

DNA Fragmentation Assays. Preconfluent cells were recovered after trypsinization. The cells were counted on an hemocytometer using the viability dye trypan blue. The volume of lysis buffer [20 mM Tris (pH 7.4), 10 mM EDTA, and 0.2% Triton X-100] was adjusted to yield a concentration of 1×10^7 cells/ml. The lysates were shaken at 4°C for 15 min. Afterward, the lysates were centrifuged at 19,700 × *g* for 10 min. Equal volumes of the supernatants containing low molecular weight DNA, were treated overnight with proteinase K (6 mg/ml; Life Technologies, Inc.) and RNase (6 mg/ml) and then subjected to DNA purification by phenol extraction and then choloroform:isoamyl alcohol extraction. DNA was precipitated overnight with 1 M sodium acetate and ethanol, at −20°C. The precipitate was resuspended in sterile water, and the absorbance was determined. After vacuum-drying, the DNAs were resuspended in DNA loading buffer (0.025% bromphenol blue, 0.025% xylene cyanol, and 0.25% Ficoll) and subjected to electrophoresis in 2% agarose gels containing ethidium bromide to visualize the DNA. Tris-borate EDTA [90 mM Tris (pH 8.0), 80 mM boric acid, and 0.2 mM EDTA (pH 8.0)] buffer was used as running buffer.

Annexin V/Propidium Iodide Binding. The cells were recovered after trypsinization using EDTA-free trypsin (Life Technologies, Inc.). The cells were washed once with EDTA-free PBS and then incubated for 15 min with a mixture containing annexin V and propidium iodide (Roche Diagnostics, Indianapolis, IN) in binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 5 mM $CaCl_2$]. After the incubation period, the supernatants were removed, and 500 μl of binding buffer was added to each sample. The fluorescence was measured using a Becton Dickinson FACScan. Compensation on FL1 and FL2 channels was performed as required, as well as the necessary adjustments on the FL2 detectors, using a nonstained doxorubicin-treated sample to account for doxorubicin autofluorescence.

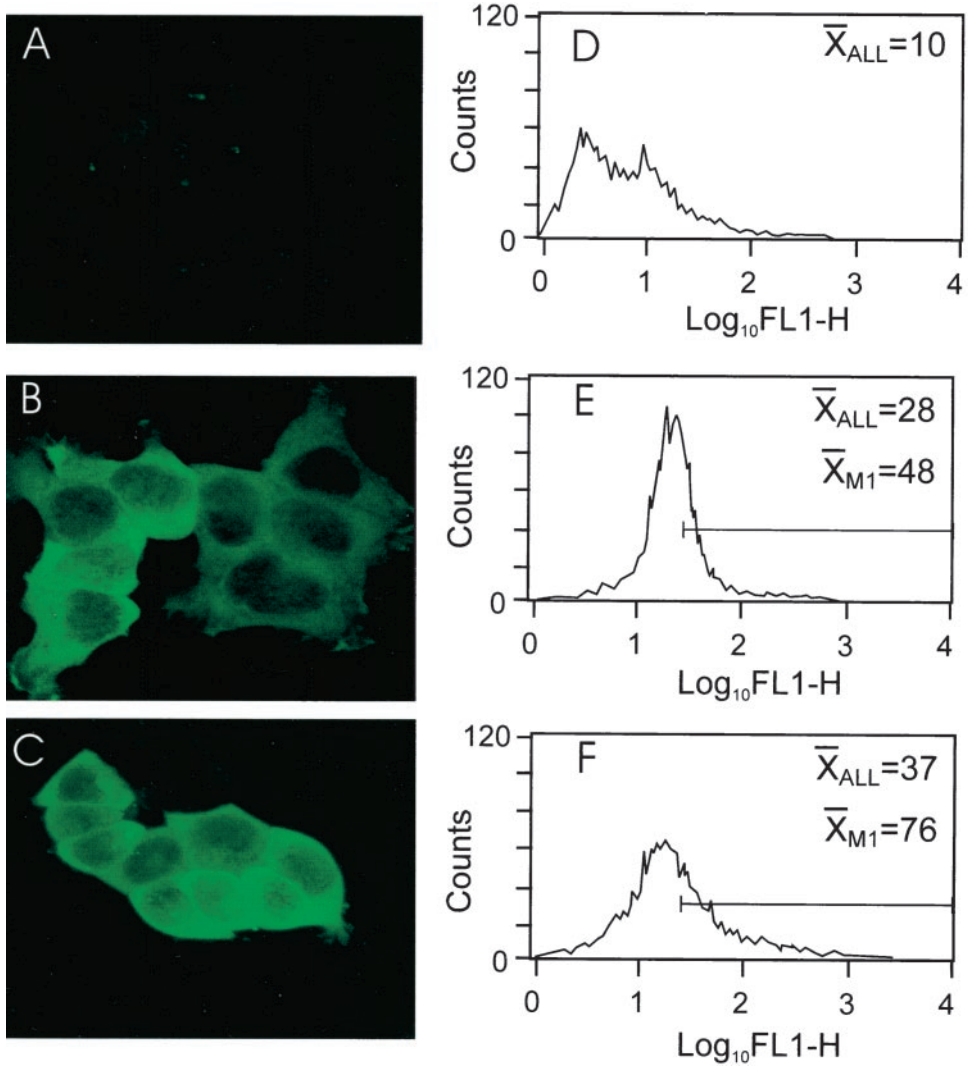

Fig. 5 GFPΔRaf-1: AR expression in MCF-7/GFPΔRaf-1: AR cells. *A*, *B*, and *C*, MCF-7 (*A*) and MCF/GFPΔRaf: AR (*B* and *C*) grown in the absence or presence of testosterone (1 μM), respectively, and then observed by confocal microscopy. The cells were grown for 24 h on coverslips. *D*, *E*, and *F* show the flow cytometric analysis performed to determine the levels of fluorescence displayed under the same conditions. The MCF/ΔRaf-1: AR cells were grown for 24 h in nonactivating (*E*) or activating conditions (1 μM testosterone; *F*). Fluorescence was determined by flow cytometry with the FL1 detector and plotted in *D*, *E*, and *F* on a Log_{10} scale. The MCF-7 parental cell line was used as a negative control (*D*).

RESULTS

To determine the role of the Ras/Raf/MEK/ERK pathway in DR, we examined the levels of Raf-1 activity and the IC_{50} for the chemotherapeutic drug doxorubicin in two established cell lines. We observed a positive correlation between IC_{50} values for doxorubicin and the levels of Raf-1 activity in the established cell lines MCF-7 and NCI/ADR-RES (Fig. 1). Although the levels of the Raf-1 protein were detected at similar levels in both cell lines, the activity of the Raf-1 kinase as measured by the capacity to phosphorylate the physiological substrate, MEK1, was increased in the NCI/ADR-RES cell line. The NCI/ADR-RES cell line represents an accepted model for the study of resistance to the drug doxorubicin caused by increased expression of the extrusion pump P-gp (25). In comparing the MCF-7 and NCI/ADR-RES cells and their response to doxorubicin, MCF-7 cells were found to be more sensitive. A 4.5-fold increase in Raf-1 activity was observed in NCI/ADR-RES cells relative to MCF-7 cells, which is consistent with the hypothesis that Raf-1 kinase plays a role in DR.

To test this hypothesis further, MCF-7 breast cancer cells were infected with retroviral constructs containing constitutively active Raf-1 (ΔRaf-1). In addition, cells were infected with a construct encoding the activated v-Ha-Ras protein, an upstream activator of Raf-1. To determine the effects of Ras and Raf-1 on the IC_{50} values for doxorubicin, dose-response curves were performed. Constitutively active Raf-1 and v-Ha-Ras increased the survival and the IC_{50} values for doxorubicin as compared with the parental MCF-7 (Table 1). There was a moderate increase in the IC_{50} for doxorubicin in cells overexpressing full-length Raf-1 (Table 1).

Cell proliferation assays were also performed on the parental MCF-7 and the constitutively and conditionally activated Raf constructs infected cell lines, both in the presence and absence of 1 μM testosterone (Fig. 2). These assays were performed to determine whether increased survival of the Raf transfectants was attributable to the ability of the protooncogene to increase growth rates. Cell proliferation was observed over a 5-day period. In comparing MCF-7 proliferation with the con-

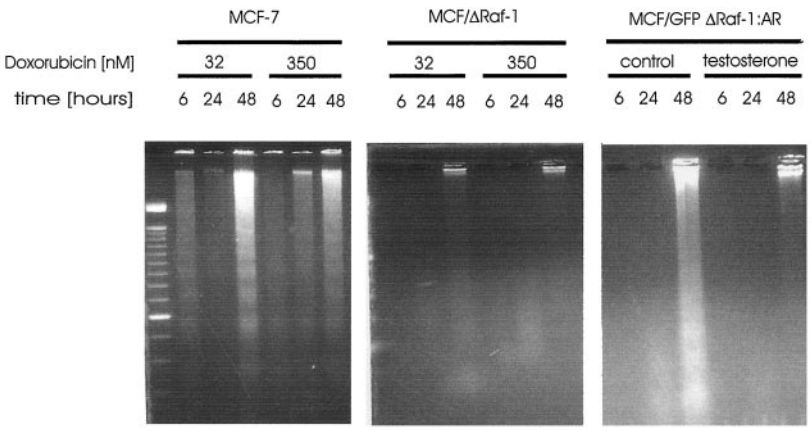

Fig. 6 Effects of activated Raf-1 on DNA fragmentation in response to doxorubicin. Cells were treated for the indicated periods of time with either 32 or 350 nM doxorubicin. The supernatants of the cell cultures containing detached cells as well as attached cells were recovered after trypsinization and lysed, and low molecular weight DNAs were recovered by organic solvent extraction and ethanol/sodium acetate precipitation. The purified DNAs were electrophoresed in agarose gels containing ethidium bromide. This experiment was repeated twice with similar results.

stitutively active Raf transfectant, it can be observed that they are roughly equivalent. These results support the hypothesis that Raf-1 activation does not increase growth as compared with the MCF-7 parental cell line.

To determine whether the introduced Δ*Raf-1* and *v-Ha-Ras* genes resulted in increased Raf-1 activity, *in vitro* Raf kinase assays were performed. *Panel C* in Fig. 2 shows increased Raf-1 activity in the MCF/ΔRaf-1 and MCF/v-Ha-Ras cell lines, as measured by the phosphorylation of the physiological substrate MEK1. These levels of Raf-1 activity are 5.2- and 3.6-fold higher than in the MCF-7 parental cell line, respectively. At the *bottom* of each *panel* are Western blots with an anti-Raf-1 antibody showing roughly equivalent Raf-1 immunoprecipitation.

To test further the hypothesis that activated Raf-1 is contributing to the DR of breast cancer cells, MCF-7 cells were also infected with a conditionally active GFPΔRaf-1:AR construct consisting of the NH_2-terminal truncated Raf-1 protein fused to the hormone binding domain of the AR and GFP. Binding of testosterone to GFPΔRaf-1:AR fusion proteins induces Raf-1 kinase activity. The presence or absence of testosterone in the culture media allows control of GFPΔRaf-1:AR activity. The MCF/GFPΔRaf-1:AR cell line exhibited enhanced survival to doxorubicin under activating conditions (testosterone) as compared with control conditions (cells treated with the vehicle for testosterone, ethanol). The parental cell line did not display enhanced survival to doxorubicin when cultured in testosterone-containing media (data not presented).

Cell proliferation assays were also performed on MCF-7 cells transfected with the conditionally activated form of Raf-1, ΔRaf-1:AR, to show that the presence of a fused AR does not increase cell proliferation. The cells were grown in both the presence and absence of testosterone. Even when MCF/ΔRaf-1:AR cells were activated by testosterone, they appeared to have essentially the same growth rate as those cells which were not activated.

Panel C links Raf-1 activation to increased levels of *mdr*-1 mRNA. As MCF/GFPΔRaf-1:AR cells were exposed to increased concentrations of testoserone, *mdr*-1 levels increased, as determined by RT-PCR. This illustrates a directly proportional relationship between Raf-1 activation and *mdr*-1 expression. *Panel D* represents a time course of the activation of Raf-1 after the addition of testosterone. Activation of Raf-1 started at 4 h and increased at 8 and 24 h (Fig. 3). These results also support the hypothesis that Raf-1 activation plays a role in DR.

To examine further the effect of activated Raf-1 on P-gp activity, MCF-7 and MCF/Δ Raf-1 cells were stained with the fluorescent dye, rhodamine 123 (Fig. 4). As can be seen in *Panel B*, MCF-7 cells were unable to extrude the dye. MCF/ΔRaf-1 cells, however, were able to extrude almost all of the rhodamine 123 (*Panel E*). Both cell lines were also treated with verapamil, an inhibitor of P-gp. *Panels C* and *F* show that neither MCF-7 cells or MCF-7 cells transfected with the constitutively activated form of Raf-1 (ΔRaf-1), were able to extrude the dye. Autofluorescence of both MCF-7 and MCF/ΔRaf-1 cells are shown in *Panels A* and *D* respectively.

To examine whether the GFP/ΔRaf-1:AR construct was expressed in the MCF/GFPΔRaf-1:AR cell lines, the cells were analyzed by confocal microscopy and flow cytometry (Fig. 5). The parental MCF-7 cells only displayed background fluorescence (*Panels A* and *D*). The cells grown in the absence of testosterone displayed some fluorescence, as expected because of the transcription of the construct (*Panels B* and *E*). The MCF/GFPΔRaf-1:AR cells grown in the presence of testosterone (1 μM) exhibited brighter fluorescence (*Panels C* and *F*), probably caused by stabilization of the protein from the binding of testosterone to GFPΔRaf-1:AR.

The capacity of the Raf-1-overexpressing cells to undergo doxorubicin-induced apoptosis was investigated. DNA fragmentation assays were used to investigate whether apoptosis induced by treatment with doxorubicin was hindered in the cells overexpressing Raf-1. Fig. 6 shows the DNA fragmentation assay for the MCF-7 cells as well as for the MCF/ΔRaf-1 cells. Cells which expressed either constitutively active Raf-1 or conditionally active Raf-1 in the presence of testosterone were protected from doxorubicin-induced apoptosis.

To demonstrate further that drug-induced apoptosis was blocked in the Raf-1-overexpressing cell lines, annexin V-FITC/propidium iodide binding experiments were performed (Fig. 7). The ΔRaf-1:AR construct not fused to the GFP was used for flow cytometric detection of apoptosis, because it lacks the GFP moiety that would complicate this type of analysis. The annexin

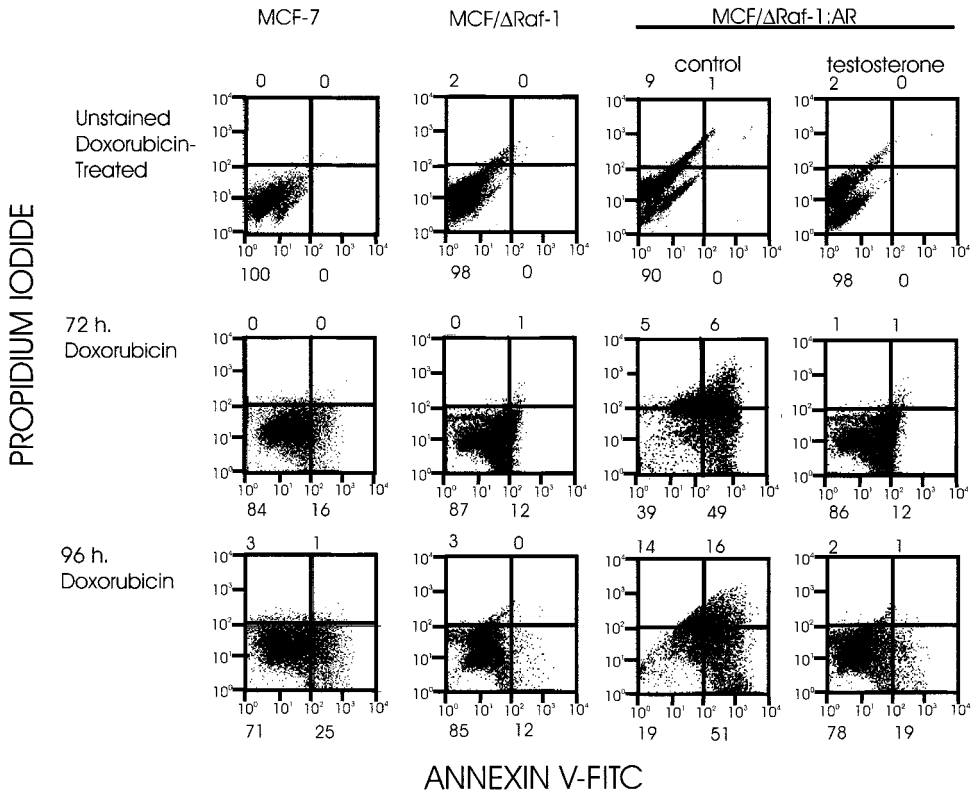

Fig. 7 Effects of activated Raf-1 on the induction of apoptosis in response to doxorubicin. Annexin V/propidium iodide binding analyses of the MCF-7, MCF/ΔRaf-1: AR (cultured in the presence or absence of 1 μM testosterone) and MCF/ΔRaf-1 cells treated with doxorubicin (1 μM) for the indicated periods of time are presented. The cells were harvested after trypsinization with EDTA-free trypsin and incubated for 20 min in a mixture containing annexin V-FITC and propidium iodide. After removing the staining solution, the cells were resuspended in the binding buffer, and the fluorescence was detected with a Becton Dickinson FACScan flow cytometer. The *X* axis shows annexin V-FITC fluorescence and the *Y* axis shows the propidium iodide fluorescence. The percentages of cells in each quadrant are presented. The *first row* of graphs shows the controls for autofluorescence for the doxorubicin-treated, nonstained cells. The first column shows the time course for annexin V/propidium iodide binding for the parental MCF-7 cells after 72 and 96 h of doxorubcin (1 μM) treatment. The next two columns present the time course for the annexin V/propidium iodide binding after 72 and 96 h of doxorubicin-treatment for the MCF/ΔRaf: AR cells cultured in the absence or presence of testosterone (1 μM). The last column presents the annexin V/propidium iodide binding graphs after 72 and 96 h of doxorubicin treatment for the MCF/ΔRaf-1 cells. *Lower left quadrant*, viable cells; *lower right quadrant*, early apoptotic cells; *upper left quadrant*, necrotic cells; *upper right quadrant*, late apoptotic and necrotic cells.

V/propidium iodide assay further confirmed that both conditionally active Raf-1 in the presence of testosterone and constitutively active Raf-1 protected the cells from doxorubicin-induced apoptosis. Testosterone did not protect the parental MCF-7 cells from doxorubicin-induced apoptosis (data not presented). A dose of 1 μM doxorubicin was used to ensure that the antiapoptotic effects of Raf-1 activation were observed at a dose that is 10 times the IC_{50} for the parental MCF-7 cell line. The experiment was also performed at 32 nM doxorubicin, and similar results were observed (data not presented).

DISCUSSION

In this study, we report that a higher Raf-1 activity correlates with a higher IC_{50} value toward the anticancer drug doxorubicin. This initial observation encouraged us to investigate the role of Raf-1 in the development of DR to doxorubicin. To obtain a better model in which cells of the same origin could be compared, we infected the MCF-7 breast cancer cell line with either a constitutively active form of Raf-1 (ΔRaf-1) or with the Raf-1 upstream activator, v-Ha-Ras. In both transfectants, a higher IC_{50} value of doxorubicin was observed in comparison with MCF-7. Moreover, in both transfected cell lines, increased MEK1 phosphorylation was observed, indicating increased Raf-1 activation.

The parental MCF-7 cell line was also transfected with a conditionally active form of Raf-1 that becomes activated in the presence of testosterone (ΔRaf-1:AR). Like its constitutively active counterpart, when activated, it had increased IC_{50} values in comparison with treatment of ethanol (testosterone vehicle).

Cell proliferation assays done on the MCF-7 cell line, MCF-7 transfected with the constitutively active form of Raf-1, and MCF-7 transfected with the conditionally active form of Raf-1, both in the presence and absence of testosterone, showed that simple Raf-1 transfection does not increase survival. Therefore, failure of Raf-1 transfectants to undergo doxorubicin-induced apoptosis was not attributable to an increase in growth,

and it is concluded that Raf-1 protein activation contributed to DR.

To examine the pathway by which this DR is conferred, *mdr*-1 expression was studied in cells transfected with conditionally active Raf-1 in response to treatment with different amounts of testosterone. *mdr*-1 expression increased with increased amounts of testosterone added, as illustrated by RT-PCR. A Raf-1 immunocomplex assay also showed that testosterone increased Raf-1 activation in cells transfected with conditionally active Raf-1.

These observations suggest that Raf-1 is regulating the expression of *mdr*-1, presumably accounting for the DR phenotype some cells display. Increased activation of Raf-1 up-regulates *mdr*-1 (13, 14). To substantiate this hypothesis further, P-gp extrusion pump function was assessed in MCF-7 cells transfected with constitutively active Raf-1.

Extrusion of rhodamine 123 was 4-fold higher in MCF/ΔRaf-1 than in MCF-7 cells. Moreover, when MCF/ΔRaf-1 cells were treated with verapamil, the membrane pump was inhibited, and the dye was retained inside the cells. This evidence suggests that the increase in expression of *mdr*-1 downstream of Raf-1 activation is accompanied by increased ability of P-gp to pump toxins out of the cell, and it supports the hypothesis that up-regulation of *mdr*-1 is one mechanism by which Raf-1 mediates DR (14).

The current model for the mechanism of action of most anticancer drugs and radiation hypothesizes that treated cells die by apoptosis. Those cells that fail to undergo doxorubicin-induced apoptosis consequently are believed to develop DR. For this reason, the capacity of the cells to undergo apoptosis after doxorubicin treatment was also examined. The cells overexpressing Raf-1 displayed an impaired capacity to undergo apoptosis, whereas MCF-7 cells displayed a higher degree of apoptosing cells after doxorubicin treatment. This validates further the hypothesis that Raf-1 overexpression mediates DR. Raf-1, as well as its downstream substrate MEK, have previously been implicated in the inhibition of apoptosis (26–29). It has been suggested that the MCF-7 cells would not undergo DNA fragmentation when undergoing apoptosis caused by the lack of functional caspase 3 (30–31). We have tested the capacity of the MCF-7 cells to activate caspase 3 in response to both starvation and doxorubicin. Both conditions resulted in caspase 3 activation that was inhibited by a specific caspase 3 inhibitor.[5]

Thus, our studies conclude that Raf-1 activation mediates the DR of human breast cancer cells. It is reasonable to assume that increased activation of Raf-1 may up-regulate transcription of P-gp or other cell-membrane extrusion pumps (14). The distal events triggered by Raf-1 activation are the ultimate effectors of the DR phenotype and are currently being examined in MCF-7 cells overexpressing Raf-1 and the parental counterparts.

[5] Unpublished observations.

ACKNOWLEDGMENTS

We thank Catherine Spruill for the artwork. We acknowledge Dr. Hartmut Land, who generously provided us with the pLNTAR305ΔRC vector.

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Clin Cancer Res 2001;7:2898-2907.