Characterization of p53-mediated Up-regulation of CD95 Gene Expression upon Genotoxic Treatment in Human Breast Tumor Cells*

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Death receptor CD95 gene expression is frequently low in human breast tumors and is up-regulated by genotoxic treatments in a p53-dependent manner. We have evaluated the relative contribution of promoter and intronic p53 consensus sites to the regulation of the human CD95 gene in breast tumor cells following doxorubicin treatment. Deletion constructs of the promoter region and site-directed mutagenesis of p53 consensus sites in a fragment spanning 1448 bp of the 5 ′promoter demonstrate that these sites are not involved in the observed up-regulation of the CD95 gene upon doxorubicin treatment. In contrast, a p53 consensus site located within the first intron of CD95 gene is absolutely required for the inducible expression of CD95 upon genotoxic treatment in breast tumor cells. Analysis of the transcriptional activity of the two most common p53 mutants found in human breast tumors that are associated with resistance to doxorubicin reveals that these mutations completely eliminate the ability of p53 protein to transactivate CD95 gene expression. On the other hand, Bcl-2 overexpression albeit preventing doxorubicin-induced apoptosis, has no effect on p53-mediated CD95 up-regulation in breast tumor cells. Altogether, these results indicate the lack of involvement of p53 consensus sites of the CD95 promoter region and the pivotal role of intronic p53-responsive element in the regulation of human CD95 gene expression in breast tumor cells. Our results also suggest that in breast cancer patients with certain mutations in the p53 gene, expression of death receptor CD95 in response to genotoxic treatments could be severely compromised.

The CD95 (Fas/APO-1) receptor, a member of the TNF1/NGF receptor superfamily, is a potent inducer of apoptosis in cells of the immune system upon interaction with its natural ligand, CD95L (1). CD95 is also expressed in a broad panel of non-transformed cells outside the immune system (2). Although the CD95/CD95L system could play a role in tumor regression (3, 4), tumor cells seem to down-regulate CD95 expression as a mechanism of resistance to CD95L-induced killing by T lymphocytes and NK cells (5, 6). In this respect, many tumor cells, including breast carcinoma cells, have been shown to express low levels of CD95 mRNA and protein and are usually not sensitive to CD95L or CD95 antibodies (7–11). Therefore, treatments that either induce the expression of CD95 or sensitize tumor cells to CD95-mediated apoptosis could be of therapeutic importance.

Genotoxic drugs as well as radiation therapy have been widely used to induce apoptosis in breast tumor cells (12, 13), the most common neoplasia among women in the western world. We and others have reported recently that different antitumor agents, such as DNA-damaging drugs, ionizing radiation, and interferon-γ, up-regulate the expression of CD95, and other pro-apoptotic proteins in breast cancer cells, thereby sensitizing these tumor cells to CD95-mediated apoptosis (10, 14–16). The tumor suppressor protein p53 has been involved in the modulation of CD95 levels in response to chemotherapeutic drugs (17) and radiotherapy, not only in breast cancer cells but also in other tumor cell lines (14, 15, 17, 18).

Although several activities have been reported for p53, its tumor suppressor function is mainly due to its ability to block cell cycle progression and to induce apoptosis in response to different genotoxic stresses. Transcriptional activation by p53 contributes to both functions, growth arrest and apoptosis, even though some transcriptionally independent activities of p53 has also been implicated, particularly in the apoptotic response (19–21). The p53 gene is one of the most often mutated in human tumors and the majority of these mutations are missense in one allele, producing full-length mutant inactive proteins with increased stability, while the other allele is often lost (20, 22, 23). Major mutational “hot-spots” occur in four of five domains that are highly conserved in evolution and contain amino acids essential for p53 function. Therefore, these mutations usually create proteins that fail to bind to p53-specific DNA responsive sites and activate transcription (24, 25).

In breast cancer, p53 mutations have been found in about 20% of the cases (22, 26) and they seem to be associated with poor prognosis and resistance to chemotherapy (27, 28). On the other hand, expression of the anti-apoptotic protein Bcl-2 can modulate the sensitivity of breast tumor cells to apoptotic cell death (30). Bcl-2 and p53 seem to mutually modulate each other since p53 down-regulates the expression of...
Bcl-2 (27–29) while Bcl-2 inhibits p53-mediated apoptosis and suppress transactivation of p53 target genes in some cell systems (30–33). However, despite the importance of p53 mutations and deregulation of Bcl-2 expression in breast tumor development, the impact of these changes on CD95 expression has not been evaluated.

A consensus binding site for p53 (34) located in the first intron of the CD95 gene has been involved in the transcriptional regulation of this gene by p53 (18, 36). Furthermore, it has been suggested that other p53 consensus elements of the CD95 promoter region may collaborate with the intronic element in the regulation of CD95 expression (18). However, direct evidence supporting this proposition is still lacking. In this study, we analyzed the p53-mediated transcriptional regulation of the CD95 gene in human breast tumor cells. We have searched for p53 responsive elements in the upstream regulatory region (URR) of the CD95 gene that cooperate with the p53 intronic site in the transcriptional activation of this gene upon genotoxic stress. For this purpose we have generated constructs harboring different fragments of the URR of the CD95 gene fused to a p53 enhancer of the first intron. Despite the presence of several p53 consensus sequences, we have found no functional p53 responsive elements within a 1448 bp region of the URR of the CD95 gene. A minimal region of this promoter functional p53 responsive elements within a 1448 bp region of the URR of the CD95 gene fused to the p53 enhancer of the first intron. EMSA and ChIP studies further demonstrated the importance of the intronic site. In addition, we analyzed the capacity of the two most common p53 missense mutants found in breast tumors to transactivate the CD95 gene, and investigated the effect of the anti-apoptotic Bcl-2 protein on p53-mediated up-regulation of CD95 in breast tumor cells.

EXPERIMENTAL PROCEDURES

Cell Cultures and Treatments—The human breast tumor cell line MCF-7, expressing wild type p53, and the p53-null human lung carcinoma-derived cell line H1299 were kindly provided by Dr. M. Ruiz de Almodovar (University of Granada, Granada, Spain) and Dr. J. A. Pinto-Toro (Consejo Superior de Investigaciones Científicas, Sevilla, Spain), respectively. Cells were maintained in culture in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen), 1× streptomycin, and gentamycin, at 37 °C in a humidified 5% CO2/95% air incubator. MCF-7 cells stably expressing human papillomavirus type 16 E6 protein were generated as described previously (14). MCF-7 cells overexpressing human Bcl-2 protein were obtained as described previously (16). In some experiments, MCF-7 cells were treated with 500 ng/ml doxorubicin (Sigma Immunochemicals) for the indicated times.

Plasmids—Several putative p53 responsive elements are found within the promoter and first intron of the human CD95 gene. The nucleotide sequences and position relative to the ATG start codon of these sites are: p53RE1 (AGACAGAACCCT....ctACAAGaCT, numbering according to Ref. 37), was generated by PCR amplification of the human CD95 gene fused to the p53 enhancer of the first intron. The fragment was purified and subcloned into the polylinker, upstream from the promoter region of the previously described luciferase reporter plasmids, pCD95 1448Luc, pCD95 391Luc, and pCD95 170Luc, respectively. The 500-bp intronic fragment was also amplified by PCR from Jurkat genomic DNA using the primers sense 5′-CGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′ and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-CGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, respectively, with the antisense primer described above. Constructs plCD95 1448Luc, plCD95 391Luc, and pCD95 170Luc were generated by subcloning a 500-bp region from the first intron of the CD95 gene into the BamHI site of the pSP2 plasmid, upstream from the promoter region of the previously described luciferase reporter plasmids, pCD95 1448Luc, pCD95 391Luc, and pCD95 170Luc, respectively. The 500-bp intronic fragment was also amplified by PCR from Jurkat genomic DNA using the primers sense 5′-CGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, and antisense 5′-GGGGATCCGCT- GAGGCCCTCTGGCCGTTG-3′, respectively, with the antisense primer described above. Constructs plCD95 1448Luc, plCD95 391Luc, and pCD95 170Luc were generated by subcloning a 500-bp region from the first intron of the CD95 gene into the BamHI site of the pSP2 plasmid.
Regulatory Elements on p53-mediated CD95 Expression in Breast Tumor Cells

Characterization of Genotoxic Stress-induced p53-mediated Up-regulation of CD95 Gene Expression in Human Breast Tumor Cells—Induction of a p53 response upon DNA damage leads to the up-regulation of several apoptotic proteins (29, 42, 43). Among these, death receptor CD95 (Fas/APO-1) has been shown to be elevated in a number of tumor cells following genotoxic insults (15, 18). In breast tumor cells, we recently reported that different DNA damaging treatments up-regulated the expression of CD95 protein in a p53-dependent manner (14). In Fig. 1A, we show that p53-dependent up-regulation of CD95 is also observed at the mRNA level in breast tumor MCF-7 cells. Treatment of these cells with the DNA-damaging drug doxorubicin up-regulated CD95 mRNA expression (Fig. 1A). However, doxorubicin-induced CD95 expression was completely abrogated in MCF-7 cells expressing the human papillomavirus protein E6, that drives p53 to degradation (34, 42). The genotoxic drug doxorubicin elevates CD95 expression in breast tumor MCF-7 cells in a p53-dependent manner (Fig. 1A and Ref. 14). However, as shown in Fig. 1B, the 1448-bp promoter fragment was not sufficient to up-regulate transcription of luciferase gene in MCF-7 cells upon doxorubicin treatment. A 500-bp region of the first intron of CD95 gene, containing the reported binding element for p53 (p53-RE3) (18, 36), was required for doxorubicin-induced transcriptional activation (pI-CD95 1448Luc, Fig. 1B). Likewise, co-transfection of reporter plasmids together with a p53 expression vector demonstrated that wild-type p53 was sufficient to markedly stimulate the transcriptional activity of luciferase reporter when the intronic region was present (Fig. 1C). In these experiments, a mutant and inactive p53 (p53A41E) did not stimulate transcription from the pI-CD95 1448Luc construct.

These results prompted us to analyze the potential role of the −329 to −1302 bp p53 motif on the transcriptional regulation by p53 of the pI-CD95 1448Luc construct. To this end, the activity of a luciferase reporter construct including a shorter fragment (391 bp) of the CD95 promoter lacking this site (p53-RE1) was analyzed in the presence (pI-CD95 391Luc) or absence (pCD95 391Luc) of the intronic region. Interestingly, in MCF-7 cells treated with doxorubicin the activity of the pI-CD95 391Luc construct lacking the p53-RE1 site was even higher than that displayed by the pI-CD95 1448Luc construct (Fig. 1, B and D). These results were further substantiated in co-transfection experiments with p53-expressing plasmids. As shown in Fig. 1E, wild-type p53 but not mutant p53 (p53A41E), markedly stimulated the transcriptional activity of the pI-CD95 391Luc construct, which lacks the p53-RE1 site. Furthermore, we analyzed whether the doxorubicin-induced activation of the pI-CD95 391Luc construct required the presence of endogenous p53. As shown in Fig. 1F, the effect of the genotoxic drug was abrogated in MCF-7 cells constitutively expressing the human papillomavirus protein E6, that drives p53 to degradation by the proteasome pathway (44).

A computer-based program for the analysis of transcription factor binding sites (45) revealed the presence of two imperfect binding sites for p53 located at positions −222 to −200 (p53-RE2) and −110 to −91 (p53-RE3) of the promoter region. These sites match 14 (p53-RE2) and 15 (p53-RE3) bp of the 20 bp consensus sequence. A functional contribution of the p53-RE2 site to the p53-mediated transcriptional activity of the pI-CD95 391Luc plasmid was excluded in experiments using a construction containing a shorter promoter fragment together with the intrinsic element (pI-CD95 171Luc). These results (Fig. 2A) demonstrated that elimination of the −391 to −171 bp region containing the p53-RE2 site failed to affect doxorubicin-induced transcriptional activation when compared with pI-CD95 391Luc construct. Furthermore, site-directed mutagenesis of both p53-RE2 and p53-RE3 in pI-CD95 391Luc (pCD95 391Luc) did not result in loss of transcriptional activity (Fig. 2A) thus also excluding the relevance of the p53-RE3 site in...
Fig. 1. A p53 consensus element located in a region spanning from nucleotide −1329 to −1302 relative to the ATG start codon is not necessary for doxorubicin-induced transactivation of the human CD95 gene in MCF-7 cells. A, MCF-7<sup>Neo</sup> and MCF-7<sup>E6</sup> cells were incubated in the presence or in the absence of 500 ng/ml doxorubicin for 8 h and CD95 mRNA was detected by Northern blot analysis. B, D, F, cells were transfected with 0.5 µg of the indicated luciferase reporter plasmids. 10 h after transfection cells were treated with or without 500 ng/ml doxorubicin for an additional 15 h before luciferase activity was determined. C and E, MCF-7 cells were co-transfected with 0.5 µg of the indicated luciferase reporter plasmids and 0.5 µg of wild-type p53 (wt p53), mutant p53 (m p53), or empty vectors. Luciferase activity was measured 48 h after transfection. Results are representative of at least two independent experiments.
doxorubicin-induced activation of the intron-promoter construct. In contrast, evidence for the functional involvement of the p53 intronic site in doxorubicin-induced activation of the CD95 construct was provided by site-directed mutagenesis experiments. Mutation of this p53 site resulted in a complete abrogation of both basal and inducible transcriptional activity of the pI-CD95 391Luc construct (pmI-CD95 391Luc, Fig. 2A).

Further evidence for the role of the intronic p53 site in regulation of transcription was obtained in the p53-null human lung carcinoma-derived cell line H1299. In these cells, co-transfection of pI-CD95 391Luc with a wild-type p53-expressing vector markedly stimulated luciferase transcription (Fig. 2B). This p53-induced transcriptional activity was completely inhibited in experiments with mutant pmI-CD95 391Luc construct. In contrast, no loss of inducible activity was observed when co-transfecting wild-type p53 and the double mutant of the promoter region (pI-CD95 m391Luc) in H1299 cells (Fig. 2B).

It is worth mentioning that in co-transfection experiments in MCF-7 cells with p53 plasmids and the promoter constructs lacking the intrinsic element (pCD95 1448Luc and pCD95 391Luc), wild-type p53 plasmid always stimulated luciferase transcription when compared with mutant p53 or empty plasmids (Fig. 1, C and E). Although this may indicate the presence of p53 responsive elements in the promoter region, data obtained with doxorubicin (Figs. 1, B, D, and F and 2A) do not support this conclusion. It is possible that the high p53 levels probably achieved in co-transfection experiments (our data and Ref. 37) could result in the activation of the p53 promoter sites. Alternatively, these high levels of p53 could act indirectly by cooperation with other transcriptional activators (46).

To further analyze the role of the intronic p53-responsive enhancer in CD95 expression in breast tumor cells we performed EMSA and ChIP studies. EMSAs with nuclear extracts from MCF-7 cells and a probe including the p53 consensus site of the CD95 intronic enhancer showed that p53 binding activity in the presence of p53-activating antibody pAb421 (47) was clearly induced upon treatment with doxorubicin for 5 h (Fig. 3A, upper panel). Western blot analysis showed that p53 expression was maximal after 5 h treatment with doxorubicin (Fig. 3A, lower panel).

Chromatin immunoprecipitation was also performed to examine whether the p53-binding site in the first intron of the human CD95 genomic locus can be bound in vivo. MCF-7 cells treated or untreated with doxorubicin were then subjected to chromatin immunoprecipitation with an antibody against p53 (pAb421) or a nonspecific antibody of similar isotype. Results shown in Fig. 3B demonstrate that the region containing the intronic p53 consensus site was PCR-amplified only in the samples immunoprecipitated with pAb421. These results also indicate that doxorubicin treatment clearly increased the amount of PCR product amplified from genomic DNA derived from p53 immunocomplexes over control levels.

Two Mutant Forms of the p53 Protein Frequently Found in Human Breast Tumors Are Completely Devoid of CD95 Tran-
FIG. 3. Doxorubicin induces p53 protein accumulation and DNA binding activity in nuclei from MCF-7 cells. A, upper panel, nuclear extracts from MCF-7 cells treated with or without doxorubicin (500 ng/ml) for 5 h were analyzed by EMSA with a probe containing the p53 site of the human CD95 intronic enhancer. EMSAs were performed in the presence of the anti-p53 antiserum pAb421 or conditioned medium from X63 myeloma cell line as control antibody. The specific DNA-p53 complex is indicated by the arrow, and a representative result of four independent experiments is presented. Lower panel, 10 μg of nuclear extracts from MCF-7 treated without (--) or with 500 ng/ml doxorubicin for the indicated times was analyzed by Western blot with an anti-p53 antibody as described under “Experimental Procedures.” B, MCF-7 cells were incubated without or with 500 ng/ml doxorubicin for the indicated times and processed as described under “Experimental Procedures” for the ChIP assay. Extracts were immunoprecipitated either with λ-p53 antibody (α-p53) or with a nonspecific IgG2 monoclonal antibody as negative control. PCR of immunoprecipitated DNA (IP) was performed with primers specific for the intronic p53 consensus site of CD95 gene. To verify that at each time point an equivalent amount of chromatin was used in the immunoprecipitation, a sample of the total input chromatin (Input) was included in the PCR reactions. Results are representative of four independent experiments.

Discussion

Death receptor CD95 is involved in the lytic response of cytotoxic T lymphocytes and NK cells against tumor cells (6). In this context, down-regulation of CD95 receptor signaling appears to be a common strategy used by tumor cells to escape from the host immune system (5). Breast tumor cell lines are also less susceptible to CD95-induced apoptosis than non-malignant mammary epithelia in most cases because tumor cells lack expression of CD95 antigen at the cell surface (10). Furthermore, in breast tumors, CD95 mRNA expression inversely correlates with tumor malignancy, suggesting that the loss of CD95 receptor may be an important event associated with de-differentiation of breast tissue during cancer development (11). In different human malignancies somatic mutations have been found that result in CD95 deficiency and/or resistance to CD95-induced apoptosis (53, 54). In contrast, loss of CD95 expression or function during breast tumor development does not seem to result from somatic mutations in a fragment encompassing 5′-untranslated and coding regions of exon I and the p53 responsive intronic enhancer or a death domain-containing exon IX fragment (55).

In a previous report, it was suggested that p53 consensus sites located in the 5′ promoter region of the CD95 gene may collaborate with a p53 responsive element located at the first intron of the gene in the regulation of gene transcription by transfected p53 (18). Here we report that transcription of CD95 gene in breast tumor cells following genotoxic drug treatment mainly depend on the integrity of the p53 intronic site. Three additional p53REs found in the 5′-untranslated region of CD95, one of them (−1329 to −1302) showing high homology with the p53 consensus sequence (34), do not contribute to the doxorubicin-induced p53-dependent transcriptional activity of constructs containing the p53iRE. Interestingly, data obtained in the KILLER/DR5 gene, another p53-regulated death receptor (56) indicate that an intronic p53 consensus element is also

Transcriptional Activity—It has been reported that p53 mutants may differ in their ability to activate transcription of a number of gene promoters (48, 49). Furthermore, a particular p53 mutant may show different transactivation capabilities depending on the promoter analyzed (49–51). However, the effect of the two most frequent p53 mutations (248Trp and 273His, Fig. 4A) detected in human breast carcinomas (27) on p53-mediated up-regulation of CD95 expression has not been examined. To ascertain whether these two mutations of p53 gene, associated to doxorubicin resistance (28) could affect the p53-mediated transcriptional activation of CD95 gene in MCF-7 cells, we examined the activity of pI-CD95 391Luc construct in co-transfection experiments with plasmids coding for wild-type p53, mutant p53−248Trp or mutant p53−273His. Results depicted in Fig. 4B indicate that both mutations completely abrogated the transcriptional activity of human p53. Parallel control experi-
responsible for the up-regulation of KILLER/DR5 gene expression following a genotoxic insult. However, we cannot exclude the possibility that in the chromosomal context, p53REs of the promoter region may have a role in the transcriptional regulation of the CD95 gene by p53. In fact, at least in the case of KILLER/DR5 gene, other p53 binding elements have been found in the promoter of this gene (56). Although similar elements were not found in the promoter region of CD95 gene (39) when searched for high affinity binding sites, it is possible that under low stringency conditions the promoter p53 consensus sites described in our work could have been detected.

Genotoxic stress-induced CD95 expression may be also subjected to other control mechanisms. In this respect, high expression of the anti-apoptotic protein Bcl-2 has been reported to inhibit p53-mediated gene transcription in several cell models (31, 32). Although the exact mechanism of this effect was not determined, it was indicated that either Bcl-2 may interfere with p53 translocation to the nucleus (31) or reduce the capacity of p53 to transactivate target genes (32). Our results show that although Bcl-2 overexpression in breast tumor MCF-7 cells partially inhibited Dox-induced activation of the intronic p53 responsive element in an artificial construct, it did not affect genotoxic stress-induced CD95 protein up-regulation in these cells. These apparently contradictory data may indicate either that the intronic p53 responsive element in the endogenous CD95 gene is not susceptible to Bcl-2-mediated regulation or that in MCF-7 cells overexpressing Bcl-2 a possible inhibition of CD95 gene transcription is compensated by an increased stability of the CD95 mRNA or protein resulting from the suppression of apoptosis by Bcl-2. Therefore, our results also suggest that although genotoxic treatments may not directly eliminate tumor cells that express elevated levels of Bcl-2, they may sensitize these cells to a death receptor-operated mitochondria-independent apoptotic pathway through the up-regulation of death receptor expression (57, 58) and facilitate the action of the immune system. This could explain why patients with breast tumors expressing high Bcl-2 levels still have a favorable clinical outcome following therapy.

Although it has been suggested that up-regulation of CD95 expression upon genotoxic stress is required for p53-dependent apoptosis in certain tumor cells (18), our previous data in breast tumor cells indicate that CD95 up-regulation is not involved in genotoxic stress-induced apoptosis in these cells (14). However, the increased CD95 expression observed in breast tumor cells harboring a wild type p53 following genotoxic treatment (14) may facilitate immune surveillance through the activation of an apoptotic program by CD95L-expressing lymphocytes or NK cells in vivo (5). In this respect, our results also indicate that mutations in the p53 gene such as those used in our study that are frequently found in breast tumors (27, 28), may lead to a decreased transcriptional activity of p53 protein and thus markedly reduce the effectiveness of genotoxic therapies in inducing CD95 receptor expression in the tumor cells. Similarly, the p53 mutant 273 (Arg to His) has been reported to lose the capacity to transactivate the DR5/TRAIL receptor 2 gene (56). As TRAIL has been implicated in

![Fig. 4. Lack of transcriptional activation of the CD95 gene by two p53 mutants frequently found in human breast tumors. A, schematic diagram of the structure of human p53 protein. Dotted boxes with roman numerals represent the five evolutionary conserved regions. The main domains of the protein are indicated. Nucleotide substituted to generate the mutant codons 248\(^{Trp}\) and 273\(^{His}\), are also shown. B, MCF-7 cells were co-transfected with 0.5 \(\mu\)g of pl-CD95 391Luc plasmid and 0.5 \(\mu\)g of the indicated vectors. Luciferase activity was measured 48 h after transfection (B). In parallel, p33 protein expression was analyzed by Western blotting. Results are representative of at least two independent experiments.](image-url)
the control of tumor development by NK cells (59), inhibition of genotoxic treatment-induced DR5/TRAIL-R2 gene expression in tumors harboring this p53 mutant could lead to a decreased antitumor effect of the innate immune system or therapeutic combination strategies (60). Furthermore, the p53 mutants 248 (Arg to Trp) and 273 (Arg to His) used in our work have been previously shown not only to lack transcriptional activity on certain p53 target genes but also to have a dominant negative effect on pro-apoptotic Bax promoter transactivation by wild type p53 (61). Accordingly, these results suggest that breast tumor cells expressing these p53 mutants should be rather refractory to genotoxic treatment-induced death by at least two mechanisms, the suppression of the intrinsic apoptosis pathway and the prevention of death receptors DR5/TRAIL-R2 and CD95 up-regulation following DNA damage.

However, loss of CD95 expression during malignant progression might occur in the absence of p53 mutations through reversible transcriptional silencing of CD95 gene caused by histone acetylation (5). Restoration of CD95 expression either by transfection of CD95 or treatment with an inhibitor of histone deacetylase, suppresses tumor growth and restores chemosensitivity in vivo (5). In other tumor cells such as those transformed with oncogenic Ras, a farnesyltransferase inhibitor but not a histone deacetylase inhibitor, reversed CD95 down-regulation (62). In addition, other cis-regulatory elements present in the promoter region of the CD95 gene may have an important role in regulating CD95 expression in tumor cells and should therefore be examined (35, 63–64). Among these elements, adjacent GAS-AP1 sequences located in a 460-bp region of the CD95 promoter have been implicated in the down-regulation of CD95 expression in melanoma (64). In conclusion, these results indicate that CD95 expression in tumor cells can be regulated by multiple elements, some of them located in the promoter region of the gene. Therefore, a complete definition of these elements is of paramount importance to understand the regulation of CD95 expression during tumor development and to design strategies to elevate the levels of this death receptor in therapeutic approaches.

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Fig. 5. Overexpression of Bcl-2 in MCF-7 cells prevents doxorubicin-induced apoptosis but does not inhibit p53-mediated up-regulation of CD95 gene expression. MCF-7Neo and MCF-7Bcl-2 cells were treated with or without 500 ng/ml doxorubicin. After a 48-h incubation, PS externalization was analyzed by flow cytometry (A). Bcl-2, p53, and CD95 protein expression levels were determined by Western blot after 24-h treatment (B). C. MCF-7Neo and MCF-7Bcl-2 cells were transfected with 0.5 µg of pL-CD95 391Luc plasmid, and 10 h after transfection they were treated with 500 ng/ml doxorubicin. Luciferase activity was determined after 15-h treatment with doxorubicin. Results are representative of three independent experiments.

A

B

C
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