Cell Death and Differentiation (2005) 12, 98–100

www.nature.com/cdd

Letter to the Editor

TNFα resistance in MCF-7 breast cancer cells is associated with altered subcellular localization of $p21^{CIP1}$ and $p27^{KIP1}$

Cell Death and Differentiation (2005) **12,** 98–100. doi:10.1038/sj.cdd.4401515

Dear Editor,

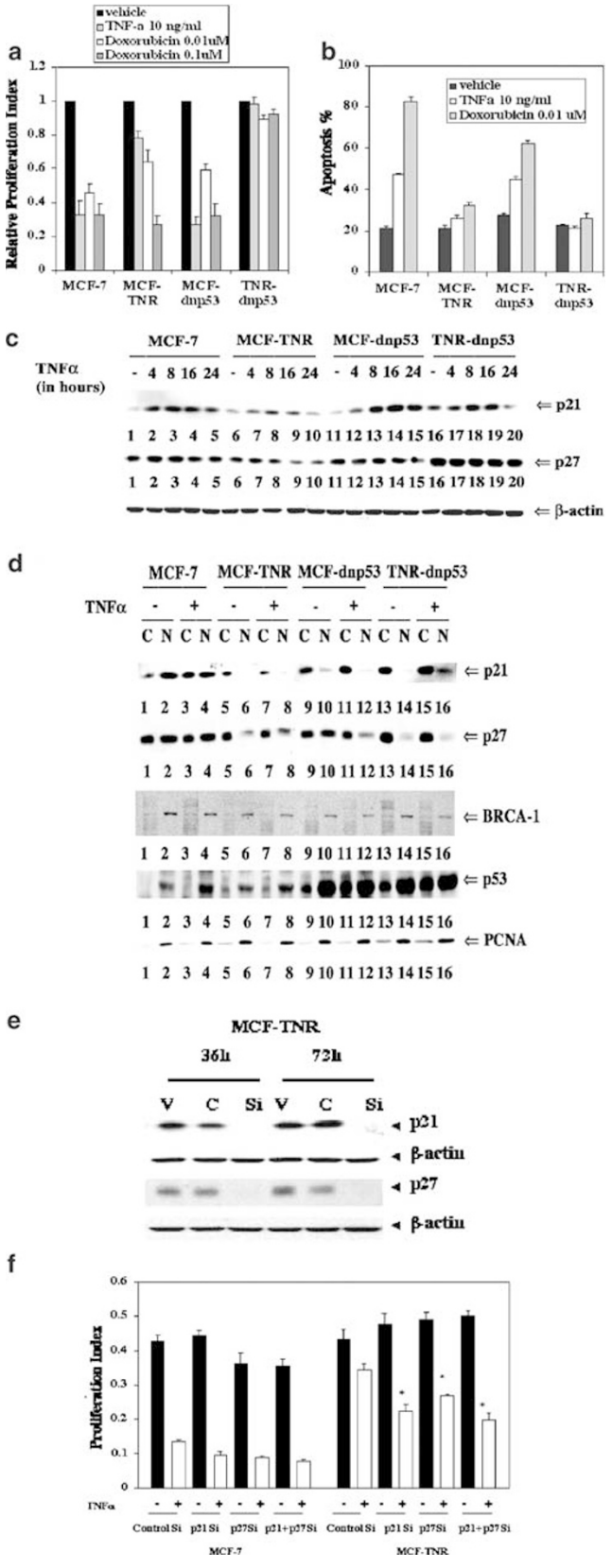
Tumor necrosis factor alpha (TNFα) is a pleiotropic cytokine that plays an important role in immunity and inflammation as well as in the control of cell proliferation, differentiation, and apoptosis. TNFα is produced mainly by macrophages and enhances tumor regression mediated by cytotoxic T cells.[1,2] Although systemic toxicity dashed early hopes of clinical use of this cytokine as an antitumor agent, the development of new strategies such as site-directed delivery of TNFα in combination with chemotherapy drugs significantly reduced side effects and enhanced antitumor activity.[3,4] Unfortunately, a subset of tumor cells responds poorly or become resistant to TNFα, thus evading the host's immune defense. Breast cancer cells isolated from ~70% of patients are sensitive to TNF-induced apoptosis. Resistance to TNF is observed in the remaining patient samples, which are also cross-resistant to the chemotherapeutic drug, doxorubicin.[5] Although we and others have demonstrated that NF-κB plays a role in TNF-resistance of breast cancers,[6,7] an NF-κB-independent TNF resistance mechanism in breast cancer has not been described.

Here, we used TNF-sensitive and TNF-resistant MCF-7 and MCF-TNR breast cancer cells, respectively; and their derivatives expressing a p53 dominant-negative mutant MCF-dnp53 and TNR-dnp53, to study the mechanisms of TNF resistance. We observed for the first time an association between cytoplasmic mislocalization of cell cycle regulators $p21^{CIP1}$ and $p27^{KIP1}$ and TNF resistance of MCF-7 breast cancer cells.

As shown in Figure 1a, TNFα inhibited the proliferation of parental MCF-7 and MCF-dnp53, despite reduced p53 activity in MCF-dnp53 cells. Conversely, MCF-TNR cells with wt p53 were resistant to TNF. Interestingly, TNR-dnp53 cells were completely resistant to TNF and simultaneously crossresistant to the chemotherapeutic drug doxorubicin, suggesting that loss of p53 function in part contributes but not sufficient for TNF resistance. To further determine whether defects in the apoptosis pathway is responsible for TNF-resistance and crossresistance of TNF-resistant cells to doxorubicin, we performed the carboxyfluorescein FLICA assay[8] to measure TNFα and doxorubicin-induced cell death (Figure 1b). Both MCF7 and MCF-dnp53 cells showed significantly increased apoptosis as well as decreased cell survival when treated with TNF or doxorubicin compared to MCF-TNR and TNR-dnp53 cells, demonstrating that the resistance to TNF and doxorubicin is due to a compromised apoptotic pathway in TNF-resistant cells. In addition, flow cytometry assay revealed a modest decline in G0/G1 arrest by TNF in MCF-TNR and TNR-dnp53 cells compared to MCF-7 and MCF-dnp53 cells (data not shown).

To understand the mechanism of TNF-resistance and associated crossresistance, we then examined whether TNF-resistance is associated with altered expression and/or localization of cell cycle regulatory proteins $p21^{CIP1}$ and $p27^{KIP1}$ since recent studies suggest that subcellular localization affects the function of p21 or p27.[9,10] It is proposed that nuclear p21 and p27 are cell cycle inhibitors whereas cytoplasmic p21 and p27 function as antiapoptotic proteins.[11,12] Overall, basal and TNF-inducible expression levels of p21 and p27 did not show any correlation with the resistant phenotype at both the protein level (Figure 1c) and mRNA level (data not shown). In fact, the highest level of p27 protein was detected in TNR-dnp53 cells, suggesting that TNF and doxorubicin resistance is less likely due to the altered expression of p21 and p27. Western blot analysis of cell fractions revealed that p21 was predominantly localized in the nucleus of MCF-7 cells and in the cytoplasm of MCF-TNR cells. Most of the cellular p21 was in the cytoplasm of MCF-dnp53 and TNR-dnp53 cells. In addition, we observed equal cytoplasmic and nuclear distribution of p27 in TNF-sensitive MCF-7 and MCF-dnp53 cells. In contrast, p27 was localized exclusively in the cytoplasmic extracts of MCF-TNR and TNR-dnp53 cells (Figure 1d). There was no difference in the subcellular localizations of BRCA-1, p53, PCNA, and GRB2 among the four cell types, suggesting that TNF resistance is not associated with a global change in the nuclear and cytoplasmic localization of proteins.

To further determine the functional significance of cytoplasmic p21 and p27 in TNF resistance, we used siRNA against p21 and p27 to reduce their expression levels. The p21- and p27-specific siRNA but not control siRNA reduced the level of respective proteins (Figure 1e). MCF-TNR cells treated with siRNA against p21 or p27 became sensitive to TNF, suggesting that cytoplasmic p21 or p27 is actively involved in suppressing TNF-induced cell death (Figure 1f). The fact that MCF-dnp53 cells with cytoplasmic p21 were sensitive to TNF suggests that cytoplasmic p21 on its own cannot confer TNF resistance but cooperates with additional factors in reducing TNF sensitivity. The carboxyfluorescein FLICA assay revealed that MCF-TNR cells treated with siRNA



against p21 and p27 undergo apoptotic cell death in response to TNF treatment (data not shown).

Cytoplasmic accumulation of p21 and p27 has been observed in primary breast cancer and is associated with poor prognosis.[9,10,13] The results in the present study provide an *in vitro* correlation to the observation *in vivo*. In addition, we show for the first time that altered subcellular distribution of the cell cycle inhibitors p21 and p27 contribute to TNF resistance and associated crossresistance to doxorubicin, demonstrating that mislocalization of proteins itself is sufficient for drug resistance. Cooperation between cytoplasmic p21 and p27 is essential for drug resistance given that MCF-dnp53 cells with cytoplasmic p21 but not p27 were sensitive to TNF and doxorubicin. In addition, we propose that altered cytoplasmic localization of the cell cycle controllers p21 and p27 may lead not only to loss of their growth inhibitory functions in the nucleus, but also to gain of antiapoptotic functions in the cytoplasm. Based on studies with siRNA against p21 and p27, it appears that the gain of antiapoptotic function is responsible for TNF resistance. Further identification of the antiapoptotic function of cytoplasmic p21 and p27 responsible for TNF resistance is being rigorously pursued. In conclusion, cytoplasmic localization of the cell cycle and apoptosis regulators p21 and p27 may be the molecular mechanism whereby breast cancer cells circumvent TNFα-induced growth inhibition and apoptosis, thus evading the host's immune defense. The TNFα resistance mechanisms along with p53 mutation may be responsible for the intrinsic resistance of breast cancer cells to the chemotherapeutic drug doxorubicin. Thus, the development of novel small molecules that enhance nuclear import or block nuclear export of p21 and p27 may be promising strategies for more effective cancer therapy.

Figure 1 Cytoplasmic mislocalization of $p21^{CIP1}$ and $p27^{KIP1}$ cell cycle regulators is associated with TNF resistance of MCF-7 breast cancer cells. (**a**) Cell survival was determined in the absence or presence of TNFα or doxorubicin by the MTT assay (Promega, Madison, WI, USA) after 6 days of treatment. In each experiment, cells (2×10^3) in eight wells of 96-well plate were treated with TNF or doxorubicin. The results are presented as means $\pm$ S.E. and represent three independent experiments. (**b**) Apoptosis assay in the absence or presence of 10 ng/ml TNFα or 0.01 μM doxorubicin using the carboxyfluorescein FLICA detection kit (Immunohistochemistry Technologies, LLC, Bloomington, MN, USA) was performed as described.[8] Cell death was measured 5 days after treatment. The results are presented as means $\pm$ S.E. and represent three independent experiments. (**c**) Total p21 and p27 protein expression levels were examined in various cell types with or without 10 ng/ml TNFα treatment by Western blot. (**d**) Subcellular distribution was determined by cellular fractionation and Western blot analysis as described.[14] Extracts from cells treated with or without TNFα 10 ng/ml for 24 h were examined for the distribution of the indicated proteins. PCNA was used as a marker for nuclear fraction. C, cytoplasmic extracts; N, nuclear extracts (**e**) MCF-TNR cells were transfected with siRNA that specifically targets p21 or p27 (5 nM) and with nonspecific control siRNA (5 nM) using the TransIT-TKO transfection reagent (Mirus Corporation, Madison, WI, USA) according to instructions of the manufacturer. The siRNA that specifically targets p21 and p27 as well as control siRNA were designed according to the manufacturer's protocol (Dharmacon Research, Lafayette, CO, USA). Cellular expression of p21 or p27 was analyzed by Western blot at the indicated time points after transfection. V, transfection reagent alone without siRNA; C, cells transfected with nonspecific control siRNA; Si, anti-p21 or anti-p27 siRNA. (**f**) Sensitivity of MCF-7 and MCF-TNR cells to TNFα 10 ng/ml in the presence of nonspecific control, p21 or p27 siRNA (5 nM) was measured by MTT assay 4 days after siRNA transfection. $^*P < 0.001$

Acknowledgements

We thank Drs. P Chambon, P Leder, R Roth, M Smith, and B Vogelstein for various reagents. We also thank Dr. Hui Lin Chua for critical reading of the manuscript. This work is supported by American Cancer Society Grant RPG-00-122-01-TBE and Public Services Award CA-89153 from the National Cancer Institute (to HN).

Z Wang[1], H Kishimoto[1], P Bhat-Nakshatri[3], C Crean[1] and H Nakshatri[*1,2,3,4]*

[1] Department of Surgery, Indianapolis, IN, USA;
[2] Department of Biochemistry and Molecular Biology, Indianapolis, IN, USA;
[3] Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN, USA;
[4] Walther Cancer Institute, Indianapolis, IN, USA

* Corresponding Author: Harikrishna Nakshatri, R4-202 Indiana Cancer Research Institute, 1044 West Walnut Street, Indianapolis, IN 46202, USA; Tel.: 317 278 2238; Fax: 317 274 0396; E-mail: hnakshat@iupui.edu

1. Baud V and Karin M (2001) Trends Cell Biol. 11: 372–377
2. Locksley RM, Killeen N and Lenardo MJ (2001) Cell 104: 487–501
3. Eggermont AM (2000) J. Immunother. 23: 505–506
4. Hambek M *et al.* (2001) Cancer Res. 61: 1045–1049
5. Dollbaum C *et al.* (1988) Proc. Natl. Acad. Sci. USA 85: 4740–4744
6. Bhat-Nakshatri P *et al.* (2002) Oncogene 21: 2066–2078
7. Tang F *et al.* (2002) Mol. Cell Biol. 22: 8571–8579
8. Pozarowski P *et al.* (2003) Cytometry 54A: 118–124
9. Zhou BP *et al.* (2001) Nat. Cell Biol. 3: 245–252
10. Viglietto G *et al.* (2002) Nat. Med. 8: 1136–1144
11. Blagosklonny MV (2002) Cell Cycle 1: 391–393
12. Coqueret O (2003) Trends Cell Biol. 13: 65–70
13. Winters ZE *et al.* (2001) Eur. J. Cancer 37: 2405–2412
14. Asada M *et al.* (1999) EMBO J. 18: 1223–1234