PG490 (Triptolide) Cooperates with Tumor Necrosis Factor-α to Induce Apoptosis in Tumor Cells*

(Received for publication, December 2, 1998, and in revised form, February 6, 1999)

Kye Young Lee, Wen-teh Chang, Daoming Qiu, Peter N. Kao, and Glenn D. Rosen‡

From the Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, California 94305-5236

Progress in the treatment of solid tumors has been slow and sporadic. The efficacy of conventional chemotherapy in solid tumors is limited because tumors frequently have mutations in the p53 gene. Also, chemotherapy only kills rapidly dividing cells. Members of the tumor necrosis factor (TNF) family, however, induce apoptosis regardless of the p53 phenotype. Unfortunately, the cytotoxicity of TNF-α is limited by its activation of NF-κB and activation of NF-κB is proinflammatory. We have identified a compound called PG490, that is composed of purified triptolide, which induces apoptosis in tumor cells and sensitizes tumor cells to TNF-α-induced apoptosis. PG490 potently inhibited TNF-α-induced activation of NF-κB. PG490 also blocked TNF-α-mediated induction of c-IAP2 (hiap-1) and c-IAP1 (hiap-2), members of the inhibitor of apoptosis (IAP) family. Interestingly, PG490 did not block DNA binding of NF-κB, but it blocked transactivation of NF-κB. Our identification of a compound that blocks TNF-α-induced activation of NF-κB may enhance the cytotoxicity of TNF-α on tumors in vivo and limit its proinflammatory effects.

Despite the development of new chemotherapeutic agents and aggressive treatment of solid tumors there has been sporadic improvement in long term survival. We have focused our efforts on enhancing cytotoxicity in solid tumor cell lines with members of the tumor necrosis factor (TNF) family. Tumor necrosis factor-α (TNF-α), for example, shows broad cytotoxicity against many tumor cell lines but its clinical use is limited because it induces a profound inflammatory response through activation of NF-κB. Moreover, activation of NF-κB inhibits TNF-α-induced apoptosis. We, therefore, set out to identify compounds that would sensitize tumor cells to TNF-α through inhibition of NF-κB.

Extracts of traditional Chinese herbs have been used for many years in China to treat a variety of inflammatory conditions such as rheumatoid arthritis. An alcohol extract of the Chinese herb Tripterygium Wilfordii hook called T2 has potent antiinflammatory properties and has been suggested to be effective for the treatment of arthritis. The purified compound of T2, which possesses immunosuppressive activity is the diterpene triepoxide, triptolide. Interestingly, triptolide also possesses anticancer activity. For example, it shows potent antileukemic activity in animal models, it inhibits proliferation of tumor cell lines, and it shows antitumor activity in a murine breast tumor model.

NF-κB is the prototype of a family of dimeric transcription factors that have Rel regions, which bind to DNA, interact with each other, and bind the IκB inhibitors (reviewed in Ref. 4). NF-κB regulates the expression of many biologically important genes such as those encoding inflammatory cytokines, interferons, growth factors, cell adhesion molecules, and viruses. A recent role for NF-κB in the control of apoptosis has been demonstrated based on the observation that mice lacking RelA (p65) die during embryogenesis from massive liver cell apoptosis. Several studies have now demonstrated an essential role for NF-κB in preventing apoptosis induced by TNF-α and chemotherapy. In these studies, cells were made sensitive to TNF-α and chemotherapy-induced apoptosis through inhibition of NF-κB activity. A recent study demonstrated that the downstream effectors of NF-κB activation, which include TRAF-1, TRAF2, and c-IAP1, known as hiap-2, and c-IAP2, also known as hiap-1, were required to suppress TNF-α-induced apoptosis. These clearly demonstrate that activation of NF-κB suppresses apoptosis in tumor cells and tumor cells are sensitized to TNF-α-induced apoptosis through inhibition of NF-κB.

Here we show that purified triptolide sensitizes several solid tumor cell lines to TNF-α-induced apoptosis through inhibition of NF-κB. Additionally, triptolide alone induces apoptosis in tumor cells. Interestingly, triptolide inhibited transcriptional activation of NF-κB but not DNA binding of NF-κB. Also, triptolide blocked induction of c-IAP2 and c-IAP1 by TNF-α. We propose that the ability of triptolide to augment TNF-α-induced cytotoxicity while simultaneously inhibiting activation of NF-κB may enhance the cytotoxic potential of TNF-α and limit its proinflammatory effects in vivo.

MATERIALS AND METHODS

Source of Triptolide—PG490 (triptolide, molecular weight, 360) was obtained from Pharmagenesis (Palo Alto, CA). The material was composed of white to off-white crystals, had a melting point of 226–240 °C, and conformed to a standard triptolide preparation by proton nuclear magnetic resonance (10), and was 97% pure by reverse phase high pressure liquid chromatography evaluation using acetonitrile:methanol:water (18:9:73).²

Cells and Transfections—A549 (nonsmall cell lung cancer) and HT1080 (fibrosarcoma) cell lines were purchased from ATCC. An MCF-7 (breast cancer) cell subline was provided by Dr. Ron Weigel (Stanford University). Cells were cultured in the appropriate media with 10% fetal calf serum supplemented with l-glutamine, penicillin, and streptomycin. TNF-α was obtained from R&D Systems (Minneap-

* This work was supported by a University of California Berkeley Breast Cancer Grant 4JB-0057 and a gift from Pharmagenesis and Jan DiCarli. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Stanford University Medical Center, Div. of Pulmonary and Critical Care Medicine, 300 Pasteur Dr., Stanford, CA 94305-5236. Tel.: 650-725-9536; Fax: 650-725-5468; E-mail: grosen@leland.stanford.edu.

¹ The abbreviations used are: TNF, tumor necrosis factor; CBP, cAMP response element-binding protein; RT-PCR, reverse transcription-polymerase chain reaction.

² J. Fidler and R. L. Jin, private communication.
PG490 Cooperates with TNF-α to Induce Apoptosis

PG490 alone decreased cell viability by 40–70% of cells in several solid tumor cell lines at dosages between 5–20 ng/ml (Table I). Cell death was confirmed as apoptotic by Annexin staining followed by fluorescence-activated cell sorter analysis (data not shown). Cell death was maximal at 48 h after the addition of PG490. Several tumor cell lines such as HT1080 cells are resistant to TNF-α because of NF-κB activation following stimulation with TNF-α. We also found that A549 lung cancer cells are resistant to TNF-α-induced apoptosis (Table I). MCF-7 breast cancer cells show intermediate sensitivity to TNF-α with TNF-α inducing cell death in approximately 30% of cells (Table I). A recent study confirms differences in the sensitivity of MCF-7 sublines to TNF-α (20). PG490 alone (20 ng/ml) induced cell death in approximately 30% of cells in the A549 cell line but the combination of PG490 and TNF-α-induced cell death in over 80% of cells (Table I). In contrast to A549 cells, PG490 alone (20 ng/ml) induced cell death in 70–75% of cells in the MCF-7 cell line and 50–55% of cells in the HT1080 cell line (Table I). PG490 at a concentration of 5 ng/ml induced cell death in approximately 35% of MCF-7 cells and the combination of PG490 (5 ng/ml) plus TNF-α induced cell death in approximately 80% of MCF-7 cells (Table I). In HT1080 cells the combination of PG490 (20 ng/ml) plus TNF-α induced cell death in more than 99% of cells (Table I).

In view of recent studies, which show that inhibition of NF-κB following TNF-α stimulation augments TNF-α-induced apoptosis in tumor cells, we examined whether PG490 inhibits TNF-α-mediated activation of NF-κB in HT1080, A549, and MCF-7 cells. We found that PG490 inhibited TNF-α-induced activation of an IgG NF-κB luciferase reporter gene construct in all three cell lines but PG490 did not affect basal NF-κB activity (Fig. 1). PG490 alone did not affect basal NF-κB activity in A549 and HT1080 cells but it slightly induced NF-κB transcriptional activity in MCF-7 cells. We have observed that PG490 inhibits expression of the IκBα protein in MCF-7 cells leading to increased binding of NF-κB, which may explain the increase in NF-κB transcriptional activity (data not shown). We confirmed, through overexpression of an IκBα super-repressor construct, that inhibition of NF-κB sensitizes HT1080 cells to TNF-α as was described previously (7) (data not shown). We also found that inhibition of NF-κB with MG132 (3 μM), a proteasome inhibitor, or with a calpain inhibitor sensitizes A549 and MCF-7 cells to TNF-α-induced apoptosis (data not shown).

PG490 Does Not Inhibit DNA Binding of NF-κB—To determine whether PG490 inhibits activation of NF-κB through the inhibition of DNA binding of NF-κB, we examined the effect of PG490 on TNF-α-induced binding of NF-κB by electrophoretic mobility shift assay. TNF-α induced binding of NF-κB in A549 cells, and an antibody to p65 (Rel A) (Santa Cruz Biotechnology) supershifted the complex demonstrating that p65 is part of TNF-α-induced apoptosis.

### Table I

| Treatment            | Cell Line |
|----------------------|-----------|
| PG490 5 ng/ml        | A549      |
|                      | HT1080    |
|                      | MCF-7     |
| PG490 20 ng/ml       | 67.5 ± 5.4|
| TNF-α 10 ng/ml       | 94.7 ± 3.5|
| PG490 20 ng/ml +     | 79.6 ± 9.1|
| TNF-α 10 ng/ml       | 71.2 ± 2.9|
| PG490 20 ng/ml +     | 17.4 ± 10.4|
| TNF-α 10 ng/ml       | 0.2 ± 0.3 |
| PG490 20 ng/ml +     | 15.6 ± 4.1|

PG490 induces apoptosis in solid tumor cells and sensitizes tumor cells to TNF-α-induced apoptosis.

RESULTS

PG490 Induces Apoptosis in Solid Tumor Cells and Sensitizes Tumor Cells to TNF-α-induced Apoptosis. In an effort to sensitize tumor cells to TNF-α, we sought out compounds that would sensitize tumor cells to TNF-α through inhibition of NF-κB. PG490 (triptolide) is a diterpene epoxide derived from a traditional Chinese herb that possesses potent immunosuppressive and antitumor activity in vitro (1, 2, 17–19). Triptolide was shown to inhibit induction of cytokine expression in T cells (17). Cytokine expression in T cells depends at least in part, on activation of NF-κB. NF-κB is, therefore, a putative target for triptolide. We examined, therefore, whether PG490 inhibits TNF-α-mediated activation of NF-κB and sensitizes tumor cells.
Our results suggest, therefore, that PG490 blocks transactivation of NF-κB but not DNA binding activity. Because PG490 blocks transactivation of NF-κB but not DNA binding, we hypothesized that PG490 would inhibit transcriptional activity of p65. A plasmid encoding a fusion protein of the transactivating domains of p65, Gal4-p65521–551 (Gal4-p65 TA1) or Gal4-p65526–551 (Gal4-p65 TA2), with the DNA binding domain of the yeast transcription factor Gal4, was transfected into A549 cells along with a luciferase reporter containing upstream Gal4 binding sites. The TA1 domain is contained within the TA2 construct. We found that PG490 blocked transcriptional activity of the TA1 domain of p65 by 20–25% and of the TA2 domain by over 50% (Fig. 3). TNF-α did not stimulate transcriptional activity of the TA1 or TA2 domain even when the A549 cells were grown in medium containing 0.1% serum, which reduced basal NF-κB activity (data not shown). This lack of response to TNF-α may be explained by our observation that A549 cells contain significant basal NF-κB activity (see Fig. 4). Nonetheless, PG490 significantly blocked transcriptional activity of the transactivating domains of p65.

**PG490 Does Not Affect the Interaction of p65 with CBP—** Recent studies suggest that CBP/p300 are transcriptional co-activators of the p65 subunit of NF-κB (21, 23). The interaction of p65 with CBP/p300 requires phosphorylation of p65 at serine 276 (21). p65 then interacts with CBP/p300 in the nucleus, which enhances NF-κB-dependent transcription (21). We examined whether PG490 inhibits transactivation of NF-κB by blocking the interaction of p65 with CBP/p300. We found that p65 associated with CBP/p300 in unstimulated A549 cells, and this complex was induced by TNF-α (Fig. 4). PG490, however, did not affect the intensity or migration of the p65/CBP complex in TNF-α-treated A549 cells (Fig. 4). PG490 alone, in fact, increased the association of CBP with p65 in unstimulated A549 cells (Fig. 4).

**PG490 Suppresses the Induction of c-IAP2 and c-IAP1 by TNF-α—** Recent studies demonstrate that members of the inhibitor of apoptosis family such as c-IAP1 (hiap-1) and c-IAP2 (hiap-2) suppress TNF-α-mediated cell death (9, 24). We hypothesized that PG490 would block induction of c-IAP2 and c-IAP1 by TNF-α. TNF-α induced a 6-fold increase in c-IAP2 mRNA and a 4-fold increase in c-IAP1 mRNA in A549 cells, which was almost completely blocked by PG490 (Fig. 5, A and B). TNF-α also induced a 4-fold increase in c-IAP2 mRNA in
NGF-...full-length 32P-labeled c-IAP2 probe. The blot was then stripped and Northern blot analysis in A549 cells of c-IAP2 (A) with 10 mg of total RNA per lane probed with a full-length 32P-labeled c-IAP2 probe. The blot was then stripped and reprobed with a full-length 32P-labeled c-IAP1 probe (B). Then, the blot was restriped and probed with a 115-base pair 32P-labeled 28 S ribosomal cDNA probe to demonstrate equal loading of RNA. Panel C shows RT-PCR with RNA from MCF-7 cells using c-IAP2 oligonucleotide primers.

MCF-7 cells, which was blocked by PG490 (Fig. 5C). These results suggest that PG490 sensitizes tumor cells to TNF-α-induced apoptosis, at least in part, by suppressing the induction of c-IAP2 and c-IAP1.

DISCUSSION

Over 50–70% of solid tumors harbor mutations in p53, which confers relative chemoresistance. TNF family members such as TNF-α, Fas, and TNF-related apoptosis-inducing ligand, also known as Apo2L, induce apoptosis in tumor cells regardless of the p53 phenotype. Unfortunately, TNF-α-induced apoptosis is limited by activation of NF-κB. Additionally, activation of NF-κB induces the release of pro-inflammatory cytokines, which damage the host. We set out to identify a compound that sensitizes tumor cells to TNF-α-induced apoptosis through inhibition of NF-κB. PG490 (triptolide) is an oxygenated diterpene derived from a traditional Chinese herb that has been used as an immunosuppressant in China for the treatment of rheumatoid arthritis. There are also studies that show PG490 is cytotoxic in leukemia and breast cancer cell lines. We show here that PG490 cooperates with TNF-α to induce apoptosis in diverse solid tumor cell lines.

PG490 alone induces apoptosis in 30–70% of cells in the A549, HT1080, and MCF-7 cell lines. We have found that other tumor cell lines are even more sensitive to PG490-induced apoptosis (data not shown). We are presently investigating the mechanism of PG490-induced apoptosis. We also show that PG490 almost completely suppresses TNF-α-induced activation of NF-κB and sensitizes tumor cell lines to TNF-α-induced apoptosis. Recent studies suggest that c-IAP1 (hiap-2) and c-IAP2 (hiap-1) mediate, at least in part, the resistance of some tumor cells to TNF-α-induced apoptosis. We found that TNF-α induced c-IAP1 and c-IAP2 and that PG490 blocked TNF-α-mediated induction of c-IAP2 and c-IAP1 (Fig. 5).

PG490 did not affect the DNA binding of NF-κB but it blocked transactivation of NF-κB. Recent studies suggest that transactivation of NF-κB is enhanced through the phosphorylation of p65 at Ser-276 and Ser-529 (21, 22). Lipopolysaccharide induces phosphorylation of p65 at Ser-276, which promotes the interaction of p65 with CBP/p300 in the nucleus (21). This interaction enhances transactivation of NF-κB. A recent study showed that TNF-α induces phosphorylation of p65 at Ser-529 in the C-terminal region of p65 (22). This phosphorylation of p65 at Ser-529 also enhances transactivation of NF-κB but a transcriptional coactivator that interacts with p65 phosphorylated at Ser-529 has, as yet, not been identified. We show that PG490 inhibits transactivation of both the TA1 and TA2 regions of p65 (Fig. 3). PG490 did not, however, affect the interaction of p65 with CBP in TNF-α treated cells (Fig. 4). Our results suggest, therefore, that PG490 may block the phosphorylation of NF-κB at Ser-529 and/or the association of p65 with an, as yet, unidentified transcriptional cofactor in the nucleus. We are presently examining the effect of PG490 on the phosphorylation of p65. Our data, nonetheless, suggests that PG490 inhibits activation of NF-κB by a novel mechanism.

In A549 cells we observed some basal NF-κB activity reflected by the interaction of p65 with CBP/p300 in unstimulated cells (Fig. 4). Also, the IgG NF-κB luciferase reporter construct was active in unstimulated cells (data not shown). Basal NF-κB activity in A549 cells may explain why TNF-α did not induce Gal4-p65 TA1 transcriptional activity and it suggests that phosphorylation of p65 in A549 cells is partly constitutive. In support of this possibility, a recent study showed that TNF-α only weakly induces phosphorylation of p65 in COS cells, because phosphorylation of p65 is largely constitutive (22).

The identification of compounds that are both cytotoxic and block activation of NF-κB may enhance the cytotoxicity of TNF family members in vivo. We are presently examining the effect of PG490 in combination with TNF-α and in combination with chemotherapy in tumor xenograft models in vivo.

Acknowledgments—we thank Emily Anderson and Sonali Gotmare for technical assistance. We acknowledge Albert Baldwin for the kind gift of the Gal4 and Gal4-p65 constructs, Dean Ballard for providing the Iε3Ca super-repressor construct, and David Goeddel for the c-IAP1 and c-IAP2 DNAs. We also thank John Fidler and Ed Lennox from Pharamagenes for providing PG490 and for critical reading of the manuscript.

REFERENCES

1. Gu, W. Z., and Brandwein, S. R. (1998) Int. J. Immunopharmacol. 20, 389–400
2. Shamon, L. A., Pezzuto, J. M., Graves, J. M., Mehta, R. R., Wangcharoentrakul, S., Sangsuwan, R., Chaichana, S., Tuchinda, P., Cleason, P., and Reutrakul, V. (1997) Cancer Lett. 112, 113–117
3. Kupchan, S. M., and Schubert, R. M. (1976) Science 185, 791–793
4. Bauerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
PG490 Cooperates with TNF-α to Induce Apoptosis

5. Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995) *Nature* **376**, 167–170
6. Beg, A. A., and Baltimore, D. (1996) *Science* **274**, 782–784
7. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274**, 787–789
8. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274**, 787–789
9. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1996) *Science* **274**, 1680–1683
10. Kupchan, S. M., Court, W. A., Dailey, R. G., Jr., Gilmore, C. J., and Bryan, E. F. (1972) *J. Am. Chem. Soc.* **94**, 7194–7195
11. Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. W. (1995) *Mol. Cell. Biol.* **15**, 2809–2818
12. Schmitz, M. L., dos Santos Silva, M. A., and Bauehrle, P. A. (1995) *J. Biol. Chem.* **270**, 15576–15584
13. Wen, L. P., Fahrni, J. A., Troie, S., Guan, J. L., Orth, K., and Rosen, G. D. (1997) *J. Biol. Chem.* **272**, 26056–26061
14. Lee, K. A., Bindereif, A., and Green, M. R. (1988) *Gene Anal. Tech.* **5**, 22–31
15. Rosen, G. D., Barks, J. L., Iademarco, M. F., Fisher, R. J., and Dean, D. C. (1994) *J. Biol. Chem.* **269**, 15652–15660
16. Wen, L. P., Madani, K., Fahrni, J. A., Duncan, S. R., and Rosen, G. D. (1997) *Am. J. Physiol.* **273**, L1921–L1929
17. Yang, S. X., Xie, S. S., Gao, H. L., Ma, D. L., and Long, Z. Z. (1994) *Int. J. Immunopharmacol.* **16**, 895–904
18. Tao, X., Davis, L. S., Hashimoto, K., and Lipsky, P. E. (1996) *J. Pharmacol. Exp. Ther.* **276**, 316–325
19. Tao, X., Schulze-Koops, H., Ma, L., Cai, J., Mao, Y., and Lipsky, P. E. (1998) *Arthritis Rheum.* **41**, 130–138
20. Burrow, M. E., Weldon, C. B., Tang, Y., Navar, G. L., Krajewski, S., Reed, J. C., Hammond, T. G., Clejan, S., and Beckman, B. S. (1998) *Cancer Res.* **58**, 4940–4946
21. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1**, 661–671
22. Wang, D., and Baldwin, A. S., Jr. (1998) *J. Biol. Chem.* **273**, 29411–29416
23. Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2927–2932
24. Chu, Z. L., McKinsey, T. A., Liu, L., Gentry, J. J., Malim, M. H., and Ballard, D. W. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10057–10062