Strategies for Manipulating Apoptosis for Cancer Therapy With Tumor Necrosis Factor and Lymphotoxin

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Abstract   Tumor necrosis factor (TNF) and lymphotoxin (LT), initially described as tumoricidal proteins, may be useful as adjuncts in cancer therapy. Treatment with TNF or LT was found to protect cells and animals against damage mediated by radiation or cytotoxic anticancer drugs. By contrast, tumor cells treated with TNF or LT were sensitized to these insults. We present a model in which TNF or LT induces both the synthesis of "protective" proteins such as manganous superoxide dismutase (MnSOD) and the activation of "killing" proteins, such as proteases, depending on the level of the inducing signal. Although the p55-TNF/LT receptor is structurally related to the Fas receptor, they can each signal apoptosis by distinct pathways. Furthermore, activation of both receptors acts synergistically in stimulating apoptosis.   © 1996 Wiley-Liss, Inc.

Key words: TNF, LT, MnSOD, Cancer, radioprotection, radiosensitization, Fas, apoptosis, ICE proteases, oxygen free radicals

Tumor necrosis factor (TNF) and lymphotoxin (LT) are cytokines that play a role in regulating immune responses and protecting cells against oxidative stress [Beutler, 1992]. Human TNF and LT exist as trimers and are encoded by linked genes located on chromosome 6. The production of these cytokines is tightly regulated and is induced by immunological stimuli or oxidative insults [Beutler, 1992]. TNF can be produced by activated macrophages, mast cells and lymphocytes, while LT is produced by activated lymphocytes [Beutler, 1992]. The two cytokines bind to common receptors (p55 and p75), which are expressed on most cells, although LT can also bind indirectly to another receptor (see chapter by Dr. C. Ware).

TNF or LT has been shown to induce apoptosis in vitro and to protect animals from reperfusion injury [Eddy et al., 1992; Nelson et al., 1995], radiation [Wong et al., 1992b], and other oxidative stresses [Wong et al., 1992a]. Here, we describe two anticancer strategies. One strategy relies on the ability of LT to selectively protect normal cells while sensitizing tumor cells to oxidative stress. The other involves the synergistic induction of apoptosis via the p55 TNF-R1 and Fas receptors.

EFFECT OF LT ON NORMAL AND TUMOR CELLS

TNF or LT has been shown to protect normal cells in vitro [Wong, 1995; Wong et al., 1992a,b] and in animals against oxidative insults [Eddy et al., 1992; Wong et al., 1992a,b]. Pretreatment with LT was found to protect mice against lethal doses of ionizing radiation. At day 12 after irradiation, 9 of 10 mice pretreated with LT were still alive, whereas all the control irradiated mice (10 of 10) were dead. Furthermore, pretreatment of neonatal rats with LT protected them against hair loss induced by cytosine arabinoside and lethal doses of doxorubicin (Wong et al., manuscript in preparation). Interestingly, pretreatment with LT actually sensitized tumors to killing by radiation or cytotoxic drugs in vitro and in vivo (manuscript in preparation). These preclinical studies suggest that LT has potential as a therapeutic agent by protecting normal cells while sensitizing tumor cells to radiation or chemotherapy.

PROTECTIVE ROLE OF MnSOD

The mechanisms by which TNF or LT pre-treatment protect cells against the toxic effects of radiation or chemotherapy are not clear but may involve the induction of protective proteins. Inhibition of protein synthesis by cycloheximide increases the sensitivity of cells to TNF killing, indicating that newly synthesized proteins are necessary for protection. One such protective protein has been identified as manganous superoxide dismutase (MnSOD), a mitochondrial enzyme involved in detoxification of superoxide ($O_2 . -$) radicals [Wong et al., 1989]. TNF and LT have been shown to induce the expression of MnSOD [Wong and Goeddel, 1988a]. The MnSOD induction is rapid and long lived and occurs in vitro and in vivo. However, TNF and LT do not induce MnSOD in some tumor cells [Wong and Goeddel, 1988a] or in certain human immunodeficiency virus (HIV)-infected cells [Wong et al., 1991]. Engineered overexpression of mitochondrial MnSOD (but neither of cytosolic nor of extracellular CuZn–SOD) protected cells against radiation damage [Wong, 1995]. Transfection of cells with MnSOD lacking its mitochondrial matrix signal did not provide protection against radiation [Wong, 1995]. How-ever, insertion of the MnSOD-derived mitochondrial targeting sequence into CuZn–SOD cDNA resulted in significant radioprotection [Wong, 1995]. These data indicate that the mitochondrial localization of MnSOD is critical for its unique protective property. Since MnSOD is necessary, but not sufficient, to give full protection from oxidative stress, additional proteins may be involved in TNF- or LT-induced protection [Wong, 1995].

POSSIBLE MECHANISMS OF TNF OR LT ACTIONS

The mechanisms of TNF or LT action is unclear despite years of intensive effort in many laboratories. We hypothesize that activation of the TNF-R1 by either TNF or LT triggers signals that can have opposite effects: induction of protective proteins on one hand and activation of killing proteins on the other. Radioprotection and radiosensitization are consequences of variations in the levels of the same signals (Fig. 1). One of these signals could be reactive oxygen radicals (ROR) produced in response to TNF and LT. Low concentrations of RORs in normal cells may cause radioprotection through induction of protective proteins such as MnSOD [Wong
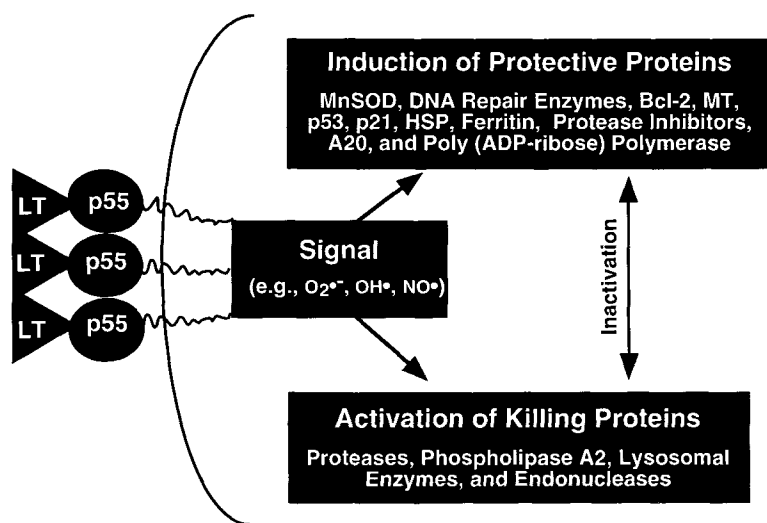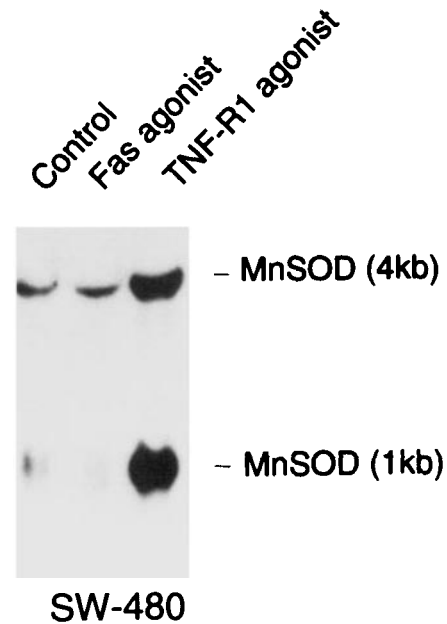

Fig. 1. Possible mechanisms of LT action. Trimers of LT complexed with three receptor molecules result in an increase in the concentration of the putative second messengers such as reactive oxygen radicals (RORs). The concentration of RORs induced by LT depends on the cell type, and the cellular response may vary accordingly. A high concentration of RORs may lead to the activation of killing proteins, which overcome protective proteins and result in either apoptosis or increased sensitivity to further insults. Low levels of RORs may not be sufficient to activate killing proteins, yet still induce the synthesis of protective proteins, resulting in protection against oxidative stress. Furthermore, an additional layer of regulation may occur in which existing protective proteins may inhibit the action of killing proteins and vice versa. These mechanisms may also apply for TNF.
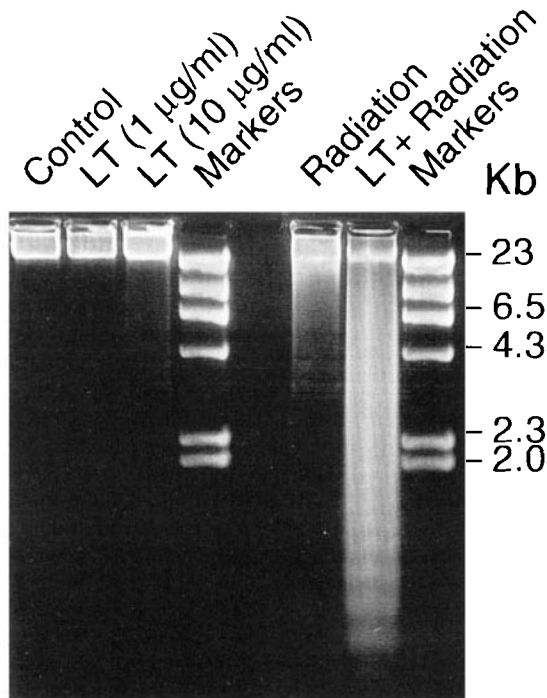
Fig. 2. LT and radiation synergistically induce fragmentation of cellular DNA. DNA isolated from human SK-N-SH neuroblastoma cells pretreated with 1 or 10 μg/ml of LT for 12 hours was not degraded. Pretreatment of cells with LT (1 μg/ml) followed by radiation (700 cGy) showed significant DNA fragmentation characteristic of apoptosis [Wong and Goeddel, 1994]. Radiation treatment alone had little effect on DNA fragmentation.

Fig. 3. TNF-R1 agonist (anti-R1), but not Fas agonist (anti-Fas), induces the expression of MnSOD mRNA. Human SW-480 colon carcinoma cells were treated with agonistic antibodies (1:50 dilution) to either Fas or TNF-R1 for 3 h. Northern blot analysis was performed with 3 μg of poly(A⁺) RNA/lane prepared from these cells. The resulting RNA blot was hybridized with MnSOD cDNA probe as previously described [Wong and Goeddel, 1988a].

et al., 1989, 1992a; Wong and Goeddel, 1988a]; DNA repair enzymes, Bcl-2 [Talley et al., 1995], p53, p21, A20 [Opipari et al., 1992]; metallothioneins [Sciavolino et al., 1992; Wong and Goeddel, 1988b]; heat-shock proteins [Simon et al., 1995]; ferritin [Torti et al., 1988]; poly(ADP-ribose)polymerase (PARP) [Lichtenstein et al., 1991]; and protease inhibitors [Kumar and Baglioni, 1991]. On the other hand, high levels of RORs in tumor cells may inactivate DNA repair enzymes, and/or activate killing proteins such as TRADD [Hsu et al., 1995]; proteases [Martin and Green, 1995; Tewari and Dixit, 1995; Tewari et al., 1995]; phospholipase A2 [Neal et al., 1988]; lysosomal enzymes [Liddil et al., 1989]; and endonucleases (Fig. 1). Thus, the susceptibility of tumor cells to killing by TNF or LT, radiation, or other oxidative insults may be determined by the relative balance of the synthesis of protective proteins and activation of killing proteins. Pretreatment with cycloheximide can inhibit p55-mediated apoptosis, indicating that protein synthesis is required [Wong and Goeddel, 1994]. One such killing protein could

be interleukin-1β converting enzyme (ICE) which is homologous to the cytotoxic protein ced-3 identified in the nematode *Caenorhabditis elegans* [Miura et al., 1993] (see paper by Dr. J. Yuan). Overexpression of ICE has been shown to induce apoptosis in some cells [Miura et al., 1993]. This "killing" activity can be inhibited by the cowpox CrmA gene product or Bcl-2 [Talley et al., 1995; Tewari and Dixit, 1995]. Interestingly, CrmA also blocks TNF-induced apoptosis [Tewari and Dixit, 1995; Tewari et al., 1995]. Recently, overexpression of other ICE-like proteases (e.g., YAMA/Cpp32) has been shown to induce apoptosis and are also inhibited by CrmA [Hsu et al., 1995; Tewari and Dixit, 1995; Tewari et al., 1995]. These results suggest that ICE and/or YAMA or other proteases are candidate killing proteins or are involved in activating killing proteins. We have examined whether TNF or LT regulates the level of ICE mRNA in TNF-sensitive and TNF-resistant cell lines. Our results indicate that ICE mRNA is detectable in TNF-sensitive cells but not TNF-resistant cells and that the levels of ICE mRNA do not change with treatment with either TNF or LT, even in the presence of interferon-γ (IFN-α), which of-

Fas (Apo-1)   TNF-R1 (p55)
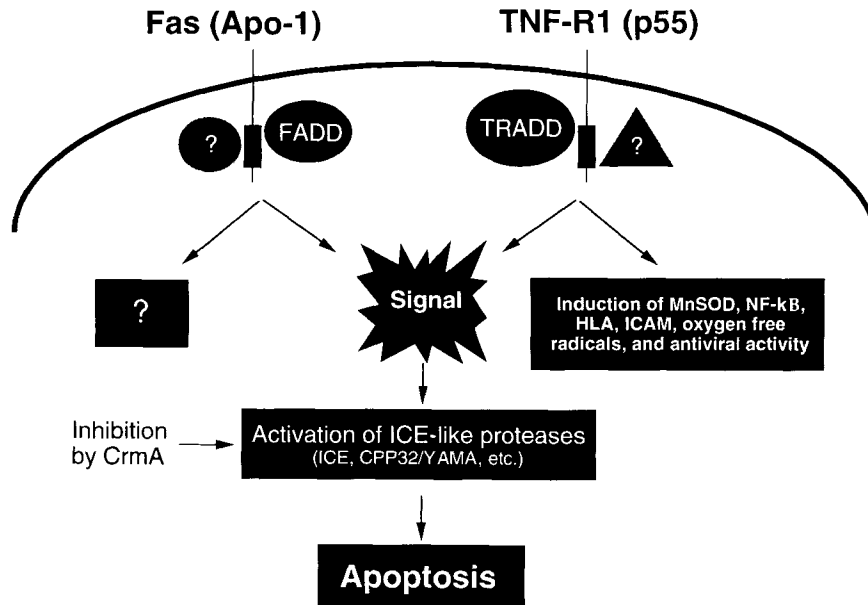
Fig. 4. Synergistic signaling mediated by Fas and TNF-R1 receptors. Both Fas and TNF-R1 receptors contain homologous cytoplasmic death domains which interact with the death proteins FADD (Fas) and TRADD (TNF-R1). We hypothesize that activation of FADD and TRADD may trigger a common signaling pathway, which includes activation of ICE-like proteases (e.g., ICE, Cpp32/YAMA) prior to cleavage of PARP and cell death. Our results support the hypothesis that activation of both receptors simultaneously can result in synergistic apoptosis [Wong and Goeddel, 1994]. Additional cellular responses signaled by TNF-R1, but not by Fas, are indicated.

ten enhances their cytotoxicity (R. Kaspar and G. Wong, in preparation). However, it is possible that these agents may activate ICE at a post-transcriptional level. One model is that LT treatment generates oxygen free radicals that directly or indirectly activate killing proteins, such as ICE or YAMA, and thereby sensitize tumor cells to radiation. For example, treatment of human neuroblastoma cells with LT alone or radiation alone did not result in DNA fragmentation. However, significant DNA fragmentation was detected when these cells were treated with LT followed by irradiation (Fig. 2).

ACTIVATION OF p55 AND Fas RECEPTORS SYNERGISTICALLY INDUCES APOPTOSIS

Agonistic antibodies to the Fas antigen, or the p55 TNF/LT receptor can induce apoptosis in some tumor cells. Homology has been found between regions of the intracellular domains of Fas antigen (also known as Apo-[1]) and the p55 receptor [Itoh et al., 1991]. The pathways induced by TNF and Fas ligand binding to their receptors are beginning to be understood. Using the yeast two-hybrid system, death proteins such as TRADD for p55 [Hsu et al., 1995] and FADD for Fas [Chinnaiyin et al., 1995], which specifically interact with the cytoplasmic domains of

these receptors, have been isolated and characterized. Overexpression of TRADD or FADD results in apoptosis, which is blocked by CrmA, suggesting that their killing activity is mediated by a similar mechanism which involves ICE or ICE-like proteases [Chinnaiyin et al., 1995; Enari et al., 1995; Hsu et al., 1995; Los et al., 1995]. Additional evidence demonstrates that the two receptors trigger apoptosis by at least two distinct mechanisms. The sensitivity of a particular cell type to Fas activation does not correlate with sensitivity to p55 activation, even though both receptors are expressed [Wong and Goeddel, 1994], and activation of both receptors can produce synergistic cytotoxicity [Wong and Goeddel, 1994]. Pretreatment with cycloheximide blocked apoptosis mediated by p55, but not by the Fas receptor [Wong and Goeddel, 1994]. Therefore, labile proteins are involved in p55, but not Fas killing. Inducible proteins that protect against p55 mediated apoptosis, such as MnSOD, do not protect against Fas-mediated apoptosis. Furthermore, activation of p55, but not of Fas, induces MnSOD expression (Fig. 3). While TNF and LT induce a multitude of cellular responses (Fig. 4), signaling through Fas seems to lead solely to apoptosis. An understanding of the distinct pathways of apoptosis medi-

ated by p55 and Fas may enable us to design new modes of anticancer therapy.

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