BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 270–275 (1997)
ARTICLE NO. RC976385

Cytotoxic Effect of Cytokines on Murine Colon Carcinoma Cells Involves TNF-Mediated Apoptosis

B. Sveinbjørnsson,[1] C. Rushfeldt, R. Olsen, B. Smedsrød, and R. Seljelid
Institute of Medical Biology, University of Tromsø, N-9037, Tromsø, Norway

Received March 3, 1997

We have studied the cytotoxic effect of TNF-α on C-26 murine colon carcinoma cells in vitro. Treatment with TNF-α alone did not result in any demonstrable cytotoxicity. However, when combined with IFN-γ, the cytotoxic effect of TNF-α was enhanced in a dose-dependent manner. An agonistic TNF-R1 specific antibody and recombinant human TNF-α both exerted a cytotoxic effect when combined with IFN-γ, suggesting that the cytotoxicity was mediated through the TNF-R1. The cytotoxicity was associated with production of nitric oxide without any direct involvement in the cytotoxic effect. At the ultrastructural level, treated cells displayed a typical apoptotic morphology which was not accompanied by internucleosomal cleavage of DNA as shown by conventional electrophoresis. © 1997 Academic Press

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic cytokine, originally identified on the basis of its ability to induce haemorrhagic necrosis in murine transplantable tumors (1) and of its direct cytotoxic effect against transformed cell lines in vitro (2,3).

At present, TNF is known to play a physiological role in ontogeny and in the control of major inflammatory and immune reactions affecting both myelogenic and non-myelogenic cells (4). It is primarily produced by activated macrophages and exerts its effect through two distinct cell surface receptors, denoted TNF-R1 and TNF-R2, which have been cloned and expressed in both murine and human systems. The two TNF-α receptors are present on virtually all nucleated cell types (5,6). TNF-α preferentially kills transformed cells in vitro (7,8), while it is not cytotoxic to normal diploid cells (3,7). The mechanisms underlying the cytotoxic effect of TNF-α are not completely understood. Inhibition of DNA topoisomerase (9), production of reactive oxygen radicals in the mitochondria (10) and induction of proteases (11) appear to be involved.

The cytotoxic effect of TNF-α on neoplastic cells is synergistically enhanced by IFN-γ both in vitro (3) and in vivo (12) although the molecular mechanism underlying this synergism is not known.

In the present study, we examined the cytotoxic effect of TNF-α and IFN-γ on a murine colon carcinoma cell line (C-26) in vitro. Our results demonstrate that the C-26 cells are resistant to TNF-α alone but undergo apoptosis in the presence of both TNF-α and IFN-γ, a cytotoxic effect demonstrated to be mediated through the TNF-R1 receptor.

MATERIALS AND METHODS

Reagents. Murine recombinant IFN-γ with approximate specific activity of 4.5×10^6 U/mg, murine recombinant TNF-α with approximate specific activity of 2×10^8 U/mg, murine recombinant IL-1β with specific activity of 1×10^7 U/mg, polyclonal anti-murine TNF-α antibody and agonistic monoclonal hamster anti-mouse TNF-R1 receptor (clone 55R-593) were purchased from Genzyme (Cambridge, MA, USA). Spermine NONOate was from Molecular Probes (USA). Human recombinant TNF-α with specific activity of 2×10^7U/mg, aminoguanidine (AGN), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum (MTT) sulphanilamide and naphthylendiamine dihydrochloride were obtained from Sigma Chemical Company (St. Louis, MO, USA). [*methyl*-^{3}H] thymidine was from ICN Radiochemicals (Irvine, CA, USA). Polyclonal anti-murine inducible nitric oxide synthase (iNOS) was purchased from Affiniti Research Products Ltd. (Nottingham, U.K.). Biotinylated goat anti-rabbit antibody was from Zymed (San Francisco, CA, USA) and phycoerythrin conjugated streptavidin was from Boehringer Mannheim (Mannheim, Germany).

Cells. The murine colon carcinoma cell line C-26 is derived from Balb/c mice after repeated injection of *N*-methyl-*N*-nitrosourethane (13) and was cultivated as monolayer culture in RPMI-1640 supplied with 10% fetal calf serum and antibiotics. Cultures were kept at 37°C in an atmosphere of 5% CO_2. Cells were passaged twice a week up to 10 passages.

Treatment. The C-26 cells (5×10^4 cells/well) were treated as indicated, through the addition of recombinant murine IFN-γ (100U/

[1] To whom correspondence should be adressed. Fax: +47-77-64 54 00. E-mail: baldur@fagmed.uit.no.

Abbreviations: mTNF-α, recombinant murine tumor necrosis factor-α; hTNF-α, recombinant human tumor necrosis factor-α; IFN-γ, interferon-gamma; TNF-R, TNF-receptor; AGN, aminoguanidine.

0006-291X/97 $25.00

ml), recombinant murine TNF-α (1000U/ml) and recombinant murine IL-1β (1000U/ml) either in combination or alone, to the cultures. After 24 hours of incubation experiments were carried out as explained below. The inhibitor of nitric oxide synthase, aminoguanidine (150μM) (14), neutralizing polyclonal anti-murine TNF-α antibody (10μl/ml), agonistic anti-TNF-R1 antibody (1μg/well) and recombinant human TNF-α (5000U/ml) were included in the cultures as indicated.

Cytotoxicity assay. Cytotoxicity against tumor cells was assayed by the MTT-assay as described (Mosman et al 1981).

Nitrite assay. Nitrite (NO_2^-), the stable endproduct of NO was measured by a novel colorimetric assay (15). Briefly, 50μl aliquots of medium were removed from individual wells and treated with equal volume of Griess reagent (1% sulphanilamide and 0.1% naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4) at room temperature for 10 minutes. The optical density of the samples was measured with a TiterTek-Multiskan at 540 nm. A standard curve obtained with $NaNO_2$ in culture medium was employed as a standard solution for calculating the NO_2^- concentration.

Tumor cell culture with spermine NONOate. C-26 cells (5 $\times 10^5$cells in a 200μl medium) were cultured in the presence of Spermine NONOate at a concentration of 500μM, 250μM, 125μM, 75μM and medium only for 18h. The concentration of NO_2^- in the medium and the cytotoxic effect on the tumor cells were determined as described above.

DNA electrophoresis. After incubation with TNF-α and IFN-γ for 24 hours, C-26 cells were washed twice in 1×PBS. All samples were digested in 0.5 ml lysis buffer (100mM NaCl, 10mM Tris-Cl, 25mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K, pH 8.0) overnight at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Equivalent amounts of DNA from a fixed number of cells were subjected to electrophoresis on 1% agarose gel and visualized by ethidium bromide staining. A 1 Kb ladder (Gibco, USA) was included as a molecular weight marker.

Immunohistochemistry. Identification of iNOS-positive cells was performed by using polyclonal anti-iNOS antibody. C-26 cells were grown on fibronectin coated chamber slides (Nunc, Denmark) for 12h in the presence of IFN-γ and TNF-α. Cultures were then washed and fixed for 12 h in 4% paraformaldehyde in phosphate buffer containing 0,2M sucrose. After washing with TBS buffer, antibody was diluted 1:100 and incubated with C-26 cultures overnight at 4°C. After rinsing in TBS, sections were incubated for 45 min. with secondary biotinylated antibody and thereafter with phycoerythrin-conjugated streptavidin. The cultures were examined in a Zeiss Axiophot photomicroscope equipped with phase contrast and incident-light fluorescence optics (Carl Zeiss, Oberkochen, Germany), and micrographs were taken with Kodak Ektachrome EP1600 film.

Transmission electron microscopy (TEM). After treatment with IFN-γ and TNF-α for 24 hours, C-26 cells were fixed in McDowellś fixative overnight, followed by postfixation in OsO_4, dehydration and embedding in Epon Araldite according to standard procedures. Ultrathin sections were cut on a Reichert Ultracut S microtome and then contrasted in 5% Uranyl acetate and Reynold's lead citrate. Sections were examined in a Jeol LEM-1010 transmission electron microscope.

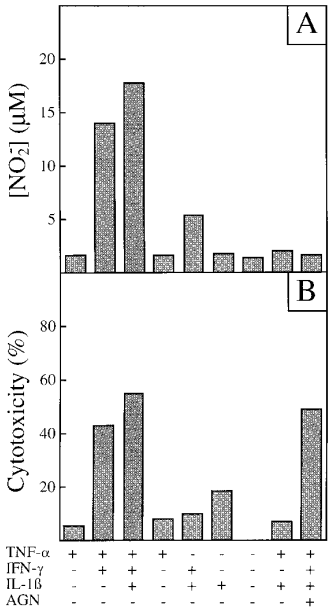

FIG. 2. (A) Nitrite formation in C-26 cells after treatment with different combinations of cytokines (24h). TNF-α = Tumor necrosis factor-alpha (1000U/ml), IFN-γ =interferon-gamma (100U/ml), IL-1β = interleukin-1-β (1000U/ml), AGN = aminoguanidine (150μM). (B) Cytotoxic effects (MTT assay) of different combinations of cytokines on C-26 cells (24h). Values are mean of three different experiments (S.E.M. < 10%).

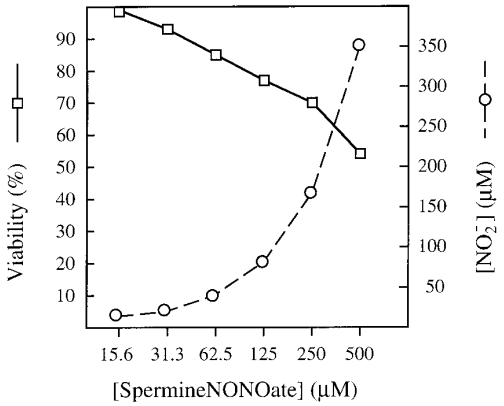

FIG. 1. Cytotoxic effect (MTT assay) of Spermine NONOate on C-26 cells.

RESULTS

We first examined how the presence of NO affected the viability of C-26 cells by cultivating the cells in different concentrations of Spermine NONOate, an exogenous NO-donor (figure 1). Increasing amounts of Spermine NONOate produced increasing amount of NO_2^- in the culture medium. There was a clear negative correlation between the concentration of NO_2^- and the viability of the C-26 cells as demonstrated by the MTT-assay. The concentration of Spermine NONOate required to kill approximately 50% of the C-26 cells came close 0.5mM.

C-26 cells were then examined for their ability to produce NO in response to different combinations of cytokines. Analysis of the culture medium revealed that IFN-γ, TNF-α and IL-1β added alone to C-26 cells

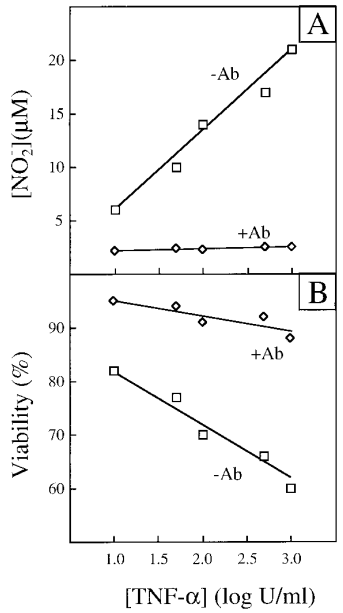

FIG. 3. (A) TNF dose-dependent response of NO_2^- formation by C-26 cells cultivated in the presence of IFN-γ (100U/ml) and different concentrations of TNF-α. The NO_2^- formation was totally blocked by anti-TNF-α neutralizing antibody. (B) TNF dose-dependent cytotoxicity towards C-26 cells cultivated in the presence of IFN-γ (100U/ml) and different concentrations of TNF-α. The cytotoxic effect was abrogated in the presence of anti-TNF-α neutralizing antibody. Values are mean of three different experiments (S.E.M. <10%)

did not result in a significant enhancement of nitrite production, compared to control cells (figure 2). The combination of TNF-α and IFN-γ was the optimal combination for enhanced nitrite production, while combinations of other cytokines were less effective.

Addition of aminoguanidine (150μM), an inhibitor of nitric oxide synthase, to the medium decreased the production of NO_2^- but did not have any significant effect on the sensitivity of the cells to the cytotoxic effect of TNF-α suggesting that the cytotoxic effect was not due to increased NO production.

The cells were subsequently cultivated in the presence of a constant amount of IFN-γ (100U/ml) and different concentrations of TNF-α for 24h and assessed for viability by the MTT-assay. NO_2^- accumulation in the medium was determined simultaneously. Under these conditions increasing concentrations of TNF-α (10-1000U/ml) resulted in increased NO_2^- production in a dose-dependent manner. Similarily, the cytotoxic effect increased with increasing concentrations of TNF-α and correlated well with the NO_2^- concentration. In both cases, the effect of TNF-α was abrogated by the presence of neutralizing antibody against TNF-α (figure 3).

An agonistic TNF-RI -specific antibody (clone 55R-593) exerted a cytotoxic effect and stimulated NO_2^- production to a degree similar to that of TNF-α (figure 4). Moreover, recombinant human TNF-α (hTNF-α) which has been shown to bind to and activate murine TNF-R1 but not murine TNF-R2 (16), also induced cytotoxic effect and NO_2^- production, suggesting that the cytotoxic effect was mediated through the TNF-R1. The cytotoxic effect of recombinant human TNF-α was not abolished in the presence of anti-murine TNF-α or anti-murine IL-1β, which rules out the possibility of endogenous induction of mTNF-α and mIL-1β by hTNF-α (data not shown)

Strong immunoreactivity for iNOS was seen in C-26 cells cultured in presence of TNF-α and IFN-γ (figure 5) for 12h, while stimulation with other combinations of cytokines and unstimulated control showed only week staining (data not shown). Twenty four hours after addition of TNF-α and IFN-γ to the medium, a prominent part of the cells, when examined by light microscopy, were detached and surrounded by small bodies which appeared to bud off from the cell membranes.

At the electron microscopical level, cells that had been cultivated in the presence of TNF-α and IFN-γ for 24 h, showed marked condensation of the chromatin, vacuolization and plasma membrane blebbing, all characteristic features of apoptotic cells (figure 6). When DNA was extracted from the cells and subjected to conventional gel electrophoresis, no distinct

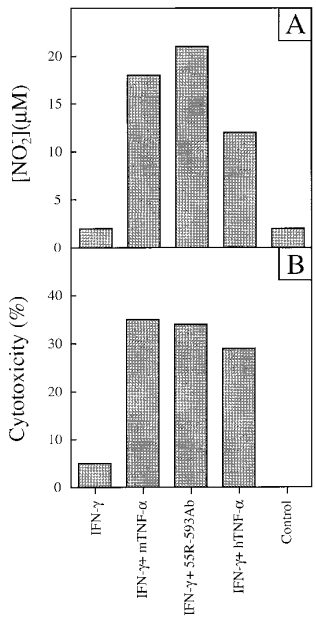

FIG. 4. Nitrite formation and cytotoxic effect of TNF-α on C-26 cells is mediated through the TNF-R1 receptor (24h). IFN-γ = interferon - gamma (100U/ml), mTNF-α = mouse tumor necrosis factor-alpha (1000U/ml), 55R-593 Ab = anti-tumor necrosis factor-receptor-1 (1μg/well), hTNF-α = recombinant human tumor necrosis factor-alpha (5000U/ml). Values are mean of three different experiments (S.E.M. <10%).

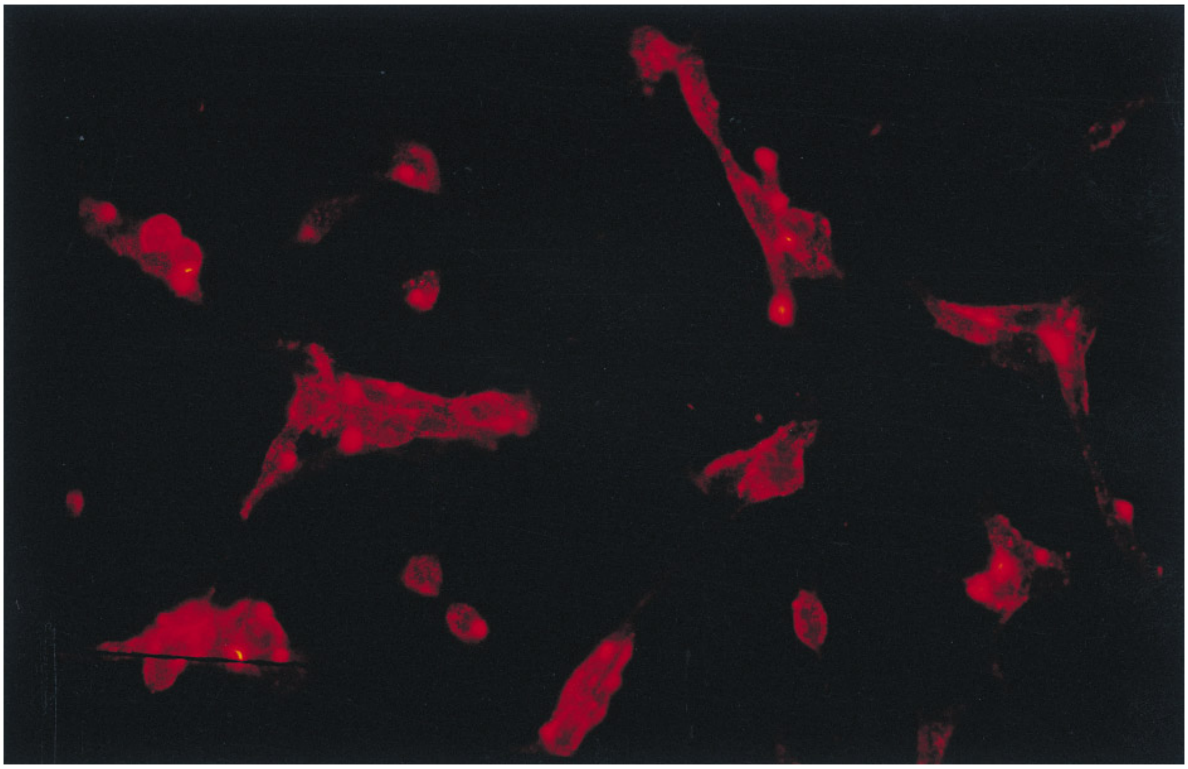

FIG. 5. Immunohistochemical localization of inducible nitric oxide synthase (iNOS) in C-26 cells after treatment with TNF-α (100U/ml) and IFN-γ 100U/ml for 12h.

inter-nucleosomal fragmentation was observed (figure 7).

DISCUSSION

Tumor necrosis factor- alpha is a pleiotropic cytokine with a wide variety of biological effects. Two distinct receptors for TNF-α have been characterized, the TNF-R1 ($\approx$55 kDa) and TNF-R2 ($\approx$75 kDa). Their extracellular domains share 28% homology, while their intracellular domains are apparently unrelated, suggesting that the two receptor types employ different signal transduction pathways.

The roles of the two receptors in mediating different biological effects of TNF-α have primarily been investigated by using agonistic and antagonistic receptor-specific antibodies and experiments with TNF-R deficient mice. The clustering of the TNF-R1 induces a large variety of responses in different cell lines including cytotoxicity of susceptible cells while the TNF effects on thymocytes have been attributed to the TNF-R2 (17).

Experiment involving deletion mutagenesis of the intracellular region of TNF-R1 has led to the identification of a so called "death domain" appearing to play a major role in the cytotoxic effect of TNF-α (18). It consists of 80 amino acids and its sequence shows weak homology to an intracellular region of the Fas antigen (28%). Agonist stimulation of Fas (CD95) leads to apoptosis in different cell types suggesting that the two receptors may share a common signal transduction pathway (19). The signal initiated from the death domain may also be necessary for NO synthase induction. The use of agonistic antibodies to murine TNF-R1 has demonstrated that NO synthesis in L929 cells is mediated by murine TNF-R1 (18). In addition, recent studies on hepatocytes from TNF-R1-null mice indicate that the TNF-R1 is essential for induction of nitric oxide production (20)

In this study the agonist anti-TNF-R1 antibody and recombinant murine TNF-α induced similar levels of NO_2^- production and cytotoxicity. Moreover, recombinant human TNF-α which only binds to the murine TNF-R1 had a similar effect (figure 4). Thus, our data indicate that the TNF-induced cytotoxicity against C-26 cells is mediated through the TNF-R1.

The cytotoxic effect of TNF is synergistically enhanced by IFN-γ (3,12). Therefore, IFN-γ and TNF-α can act synergistically in increasing NO production in macrophages (21) and are capable of inducing NO production in some tumor cell types in vitro (22, 23).

Furthermore, the study shows that C-26 cells treated with TNF-α and IFN-γ express the inducible isoform

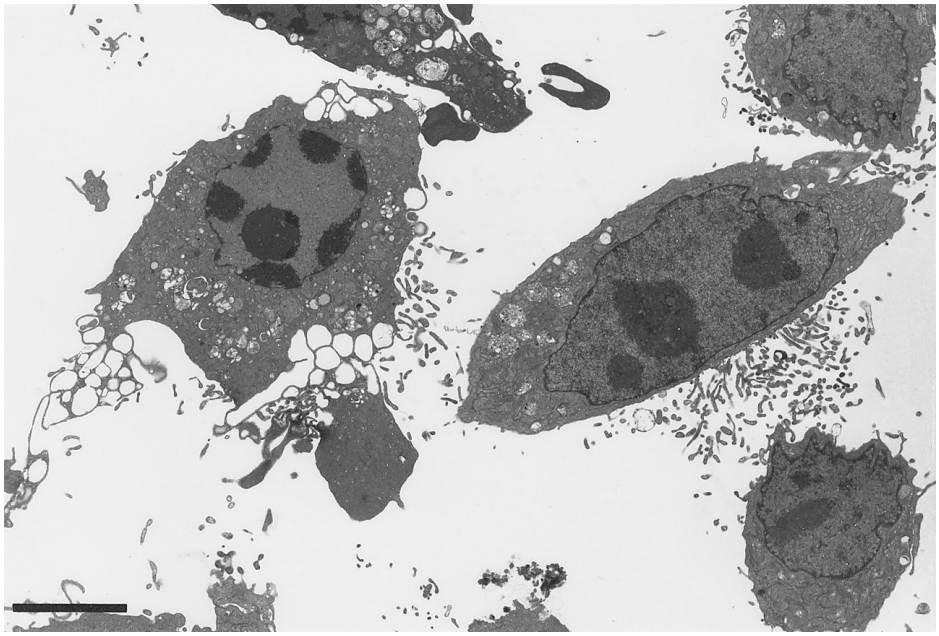

FIG. 6. Transmission electron micrographs of C-26 cells after treatment with TNF-α (1000U/ml) and IFN-γ (100U/ml) for 24 hours, showing typical apoptotic morphology as evidenced by condensation of the chromatin along the nuclear membrane, vacuolization and membrane blebbing. Scalebar = 5μm.

of nitric oxide synthase (figure 5) with a concomitant production of relatively small amounts of NO (figure 2). However, we were unable to demonstrate that NO plays a direct role in the cytotoxic effect mediated by TNF-α and IFN-γ towards C-26 cells. The C-26 cells were shown to be relatively resistant to killing by exogenous NO (figure 1) which is in agreement with a previous study showing that cytotoxicity exerted by Kupffer cells against C-26 cells in vitro is not mediated by NO (24). Thus, nitric oxide production may be an important indicator for the susceptibility of tumor cells to the induction of the "death domain" and TNF-mediated cytotoxicity, rather than being involved directly in the cytotoxic effect.

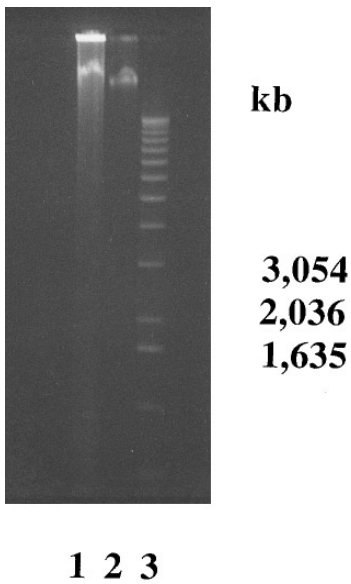

FIG. 7. DNA electrophoresis pattern. Lane 1; DNA from C-26 cell culture treated with TNF-α (1000U/ml) and IFN-γ (100U/ml) for 24h ; Lane 2; DNA from non-treated culture ; Lane 3, Kb-ladder.

Although the tumor cells displayed classical morphological features of apoptotic cell death after treatment with IFN-γ and TNF-α, the DNA electrophoretic fragmentation pattern of DNA did not result in demonstrable internucleosomal cleavage. Several investigators have proposed that cleavage of DNA into large fragments is an early apoptotic event, the larger DNA fragments being precursors for the smaller fragments (25). Larger fragments of this type would not produce an internucleosomal cleavage pattern under the conditions employed here.

Recent reports suggest that the appearance of a DNA "ladder" is not an obligatory event in an apoptotic cell death. (26). Thus, the absence of a typical ladder pattern does not contradict the involvement of apoptosis in the cytotoxic process triggered by TNF-α and IFN-γ.

The mechanism underlying the IFN-γ-mediated susceptibility of cells to the cytotoxic action of TNF-α is not known. IFN-γ has been shown to upregulate the number of TNF-α receptors on many cell types but there is no correlation between the degree of receptor

expression and susceptibility to the cytotoxic effect (27,28,29). Also, there is no correlation between susceptibility to TNF-α and histological origin of the tumor cell types, although many susceptible cell lines are derived from epithelia.

A recent study has shown that IFN-γ enhances the expression of the interleukin 1β-converting enzyme (ICE) gene (30) which is a protease required for TNF-induced apoptosis (31). Furthermore, IFN-γ has been shown to upregulate Fas on a number of different tumor cell types (32) suggesting that IFN-γ may influence a common apoptotic pathway.

In summary, we have shown that C-26 colon carcinoma cells, which are commonly used in a variety of murine tumor models, are susceptible to the cytotoxic effect of TNF-α and IFN-γ apparently through the TNF-R1 receptor, and without the direct involvement of NO as an effector molecule.

REFERENCES

1. Carswell, E., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3666–3670.
2. Helson, L., Helson, C., and Green, S. (1979) *Exp. Cell. Biol.* **47,** 53–60.
3. Fransen, L., Ruysschaert, M-R., Van Der Heyden, J., and Fiers, W. (1986) *Cell. Immunol.* **100,** 260–267.
4. Tracy, K. J., and Cerami, A. (1993) *Crit. Care Med.* **21,** S415–422.
5. Hohmann, H-P., Remy, R., Brockhaus, M., and van Loon, A. P. G. M. (1989) *J. Biol. Chem.* **264,** 14927–14934.
6. Vandenabeele, P., Declerq, W., Beyaert, R., and Fiers, W. (1995) *Trends in Biol.* **5,** 392–399.
7. Dollbaum, C., Creasy, A. A., Shahnaz, H. D., Rudolph, A. R., Lin, L., Vitt, C., and Smith, H. S. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4740–4744.
8. Haranaka, K., and Satomi, N. (1981) *Jpn. J. Exp. Med.* **51,** 191–194.
9. Baloch, Z., Cohen, S., and Coffman, F. D. (1990) *J. Immunol.* **145,** 2908–2913.
10. Gossens, V., Grooten, J., De Vos, K., and Fiers, W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8115–8119.
11. Suffys, P., Beyaert, R., Van Roy, F., and Fiers, W. (1988) *Eur. J. Biochem.* **178,** 257–265.
12. Brouckaert, P. G. G., Leroux-Roels, G. G., Guisez, Y., Tavernier, J., and Fiers, W. (1986) *Int. J. Cancer.* **38,** 763–769.
13. Corbett, T. H., Griswold, D. P., Roberts, B. J., Peckham, J. C., and Schabel, F. M. (1975) *Cancer Res.* **35,** 2434–2439.
14. Griffiths, M. J. D., Messent, M., MacAllister, R. J., and, Evans, T. W. (1993) *Br. J. Pharmacol.* **110,** 963–968.
15. Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988) *J. Immunol.* **141,** 2407–2412.
16. Lewis, M., Tartaglia, L. A., Lee, A., Bennet, G. L., Rise, G. C., Wong, G. H., Chen, E., and Goeddel, D. V. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2830–2834.
17. Tartaglia, L. A., and Goeddel, D. V. (1992) *Immunol. Today* **13,** 151–153.
18. Tartaglia, L. A., Ayres, T. M., Wong, G. H. W., and Goeddel, D. V. (1993) *Cell* **74,** 845–853.
19. Varfolomeev, E. E., Boldin, M. P., Goncharov, T. M., and Wallach, D. (1996) *J. Exp. Med.* **183,** 1271–1275.
20. Leist, M., Gantner, F., Jilg, S., and, Wendel, A. (1995) *J. Immunol.* **154,** 1307–1316.
21. Drapier, J-C., Wietzerbin, J., and, Hibbs, J. B. (1988) *Eur. J. Immunol.* **18,** 1587–1592.
22. Amber, I. J., Hibbs, J. B., Taintor, R. R., and, Vavrin, Z. (1988) *J. Leukocyte Biol.* **44,** 58–65.
23. Fast, D. J., Lynch, R. C., and Leu, R. W. (1992) *J. Leukocyte Biol.* **52,** 255–261.
24. Curley, S. A., Roh, M. S., Feig, B., Oyedeji, C., Kleinerman, E. S., and Klostergaard, J. (1993) *J. Leukoc. Biol.* **53,** 715–721.
25. Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R., and Sikorska, M. (1993) *EMBO J.* **12,** 3679–3684.
26. Bortner, C. D., Oldenburg, N. B. E., and Cidlowski, J. A. (1995) *Trends in Cell Biol.* **5,** 21–26.
27. Kull, F. C., Jacobs, S., and Cuatrecasas, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5756–5760.
28. Tsujimoto, M., Yip, Y. K., and Vilcek, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7626–7630.
29. Ruggiero, V., Latham, K., and Baglioni, C. (1987) *J. Immunol.* **138,** 2711–2717.
30. Tamura, T., Ueda, S., Yoshida, M., Matsuzaki, M., Mohri, H., and Okubo, T. (1996) *Biochem. Biophys. Res. Commun.* **229,** 21–26.
31. Tewari, M., and Dixit, V. M. (1995) *J. Biol. Chem.* **270,** 3255–3260.
32. Moller, P., Koretz, K., Leithauser, F., Bruderlein, S., Henne, C., Quentmeier, A., and Krammer, P. H. (1994) *Int. J. Cancer* **57,** 371–377.