Involvement of NF- κ B in the Protection of Cell Death by Tumor Necrosis Factor in L929 Derived TNF Resistant C12 Cells

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The Tumor necrosis factor (TNF)-resistant C12 cell line was established by continuous exposure of a toxic concentration of TNF to parental murine fibrosarcoma L929 cells. Introduction of the cytosolic phospholipase A2 (cPLA₂) gene to C12 cells resulted in restoration of the TNF sensitive phenotype (CPL4 cells). DNA ladder formation and nuclear condensation by TNF exposure suggested that TNF induced apoptotic cell death in L929, C12 and CPL4 cells. TNF-induced activation of transcription factor nuclear factor- κ B (NF- κ B) was observed in all 3 cell lines. The activation reached the maximum level at 30 min after the exposure to TNF and thereafter declined slowly. The amount of activated NF- κ B in C12 cells was about twice as high as that of L929 cells with either dose of TNF tested in this study. It was found that C12 cells expressed latent NF- κ B twice that of L929 cells. This abundance of latent NF- κ B would provide a higher response of NF- κ B in C12 cells. Pretreatment with the known potent NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC) profoundly suppressed the activation of NF- κ B induced by TNF and potentiated TNF cytotoxicity in all 3 cell lines. These results are consistent with the recently found anti-apoptotic action of NF- κ B and suggest that NF- κ B acts as an acquired TNF resistant factor in C12 cells.

Key words TNF; NF- κ B; apoptosis

Tumor necrosis factor (TNF) is a pleiotropic cytokine mainly produced by activated macrophage. TNF possesses a wide variety of biological effects such as the induction of apoptosis, activating transcription factors and production of cytokines (for review, see ref. 1–3). These signals are transduced mainly by the specific interaction of the TNF receptor's inner domain and other cytosolic proteins. When TNF binds to the outer domain of its 55 kDa receptor (TNF-R1), trimerization of receptors occurs. Then TNF receptor-associated death domain-containing protein (TRADD)⁴ is recruited to TNF-R1 and acts as an adapter molecule for Fas-associated death domain-containing protein (FADD)⁵ or TNF receptor-associated factor 2 (TRAF2).^{4,6} If FADD is recruited to the complex, apoptosis will occur through activation of caspase-8.^{5,6} When TRAF2 interacts with the complex, liberation of nuclear factor κ B (NF- κ B) from its inhibitory subunit I- κ B will occur by activation of recently found NF- κ B inducing kinase (NIK)⁷ and I- κ B kinase (IKK).⁸ Activated NF- κ B then translocates to the nucleus where it stimulates expression of a number of genes involved in immune and inflammatory responses. Recently, the apoptosis-protecting activities of NF- κ B were reported.^{9–11} Although the precise mechanism of anti-apoptosis still remains unclear, NF- κ B is thought to enhance expression of a yet unidentified anti-apoptotic protein(s). Despite the fact that these two opposite signals, death and survival, originate from same receptor, we do not yet know the determinant factor(s) for survival or death.

Hayakawa *et al.* have established a TNF resistant cell line (C12 cells) from TNF sensitive murine fibrosarcoma L929 cells by continuous exposure to a toxic concentration of TNF.^{12–14} Employing these cell lines, it was shown that cytosolic phospholipase A₂ (cPLA₂) was crucial in the cytotoxic action of TNF. In TNF resistant C12 cells, expression of cPLA₂ is maintained at a very low level and the fact that C12 cell-derived CPL4 cells which express exogenously in-

troducted cPLA₂ confirms the involvement of cPLA₂ in TNF-induced cell death.^{12,13} However, the mechanism(s) of TNF resistance still remains largely unknown.

By comparing TNF induced NF- κ B activation in both cell lines, we found that the TNF resistant cell line C12 cells had a constitutively high level of latent NF- κ B (NF- κ B and I- κ B complex). Consequently, TNF caused the production of active NF- κ B in C12 cell twice as high as that in L929 cells. Moreover, TNF-induced cytotoxicity was significantly enhanced by pretreatment with NF- κ B inhibitor in L929, C12 and CPL4. These results suggest that higher expression of NF- κ B might act as an acquired TNF resistant factor in C12 cell line.

MATERIALS AND METHODS

Cell Lines Murine fibrosarcoma L929 cells, its TNF resistant subline C12 cells and C12 derived cPLA₂ expression clone CPL4 cells were used through out the experiments.^{13,14} These cells were cultured in Dulbecco's Modified Eagles MEM (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented 5% fetal calf serum (Intergen, Purchase, NY, U.S.A.), 0.29 mg/ml L-glutamin, 100 μ g/ml streptomycin and 100 U/ml penicillin (Life Technologies, Inc., Rockville, MD, U.S.A.).

Reagents Recombinant human TNF was purchased from R&D systems (Minneapolis, MN, U.S.A.). Poly(dI-dC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). All other chemicals not listed were obtained from Wako Pure Chemical Industries (Osaka).

Nuclear Extract Preparation and Electrophoretical Mobility Shift Assay (EMSA) Cells (1×10^5) were seeded in a 3.5 cm dish 24 h before the experiments. Cells were fed fresh medium and then incubated with the indicated concentrations of TNF or IL-1 α for 30 min. After removal of

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medium, cells were washed once with ice-cold PBS(-), then harvested in PBS(-) with a rubber policeman. After brief centrifugation (400×*g*, 2 min at 4 °C), pelleted cells were frozen in a dry ice-ethanol bath. Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM dithiothreitol (DTT)) was then added and cells were suspended by gentle pipetting. Nuclei were obtained by centrifugation (800×*g*, 10 min at 4 °C). Nuclear extracts were obtained by gentle agitation of nuclei for 30 min at 4 °C in buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM DTT, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), and 25% glycerol) followed by centrifugation (14000×*g*, 20 min at 4 °C). Nuclear extracts (20 μl) were diluted with 30 μl of buffer D (20 mM HEPES pH7.9, 50 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 25% glycerol). Protein contents were determined using commercially available Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.). For the binding reaction, 10 μg of nuclear extract were incubated with radiolabelled 22-mer double stranded NF-κB nucleotide (5'-AGTTGAGGGGACTTCCCCAGGC-3', 2×10⁴ cpm) in HDKE buffer (100 mM HEPES pH 7.9, 25 mM DTT, 250 mM KCl, 5 mM EDTA, and 2 μg/ml bovine serum albumin) containing 1 μg poly(dI-dC) (total 20 μl) on ice for 30 min. After incubation, 4 μl of 6× glycerol dye mix was added. DNA-protein complexes were resolved on 4% native poly acrylamide-Tris Borate EDTA (TBE) gel run in 1× TBE. The gels were dried and autoradiography was performed.

Determination of NF-κB Contents Whole cell extracts were prepared as follows: Cells (1×10⁷) were collected and frozen in a dry ice-ethanol bath. Cells were thawed and suspended in 50 μl of whole cell extract buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). Whole cell extracts were obtained by 10000×*g* for 5 min centrifugation at 4 °C. Whole cell extracts (10 μg of protein) were incubated with 0.5% deoxycholic acid on ice for 30 min. Then NF-κB was determined by EMSA as described above.

Cytotoxicity Assay Cells were seeded 1×10⁴ per well of 96 well microplate the day before experiment. Various amounts of TNF were added and incubated for 3 d. Survival of cells was determined by the MTT method.¹⁵⁾

Detection of DNA Ladder Each cell line was seeded at 1×10⁶ cells in a 3.5 cm dish. On the next day, the cells were treated with the indicated concentrations of TNF or other reagents. After a 3 d incubation, cells were collected with incubation medium and then pelleted by centrifugation. Pellets were washed once with ice-cold PBS(-) then lysed by addition of 1% SDS containing PBS(-). RNase (100 μg) was then added and incubated at 37 °C for 1 h. Proteins were degraded by incubating with proteinase K (100 μg) at 37 °C overnight. After incubation, equal volumes of 6 M NaI solution were added and further incubated at 60 °C for 15 min. DNA was pelleted by adding an equal volume of isopropanol followed by centrifugation. DNA pellets were dissolved in TE buffer after 3 times wash with isopropanol. After additional RNase treatment, DNA was resolved on 2% agarose gel electrophoresis then stained by ethidium bromide and photographed under UV illumination.

Measurement of Nuclear Condensation Cells (2×10⁵) were incubated with TNF for 18 h and harvested in PBS(-) with a rubber policeman. Cells were pelleted by centrifuga-

Table 1. Sensitivity to TNF in L929, C12 and CPL4 Cells

Cell line	LD ₅₀ (ng/ml)	Fold increase (vs. L929)
L929	0.15	1
C12	20	133
CPL4	0.81	5.4

Cells were treated with various concentrations of TNF for 3 d. Survival ratio was determined by MTT assay.

Fig. 1. TNF Induced DNA Ladder Formation

Each cell line was treated with the indicated concentrations of TNF for 3 d. Then DNA was extracted and electrophoresed in 2% agarose gel.

tion and fixed with 1% glutaldehyde for 30 min at room temperature. Cells were washed twice with PBS(-) and then stained by 200 μM Hoechst 33258. Photographs were taken under fluorescence microscope.

RESULTS

Resistance of C12 Cells to the Apoptosis Caused by TNF Table 1 shows the LD₅₀ values for the L929, C12 and CPL4 cell lines to TNF. The LD₅₀ of the C12 cells was 133-fold higher than that of the L929 cells. The CPL4 cells showed moderate sensitivity to TNF. DNA ladder, an indicator of apoptosis, was observed in L929 cells by the treatment with 1 ng/ml TNF (Fig. 1). In C12 cells, a relatively high dose of TNF (10 ng/ml) was required to induce the same extent of DNA ladder as compared to L929 cells. Moderate DNA ladder formation by TNF treatment was again observed in CPL4 cells as was seen in TNF-induced cytotoxicity. Figure 2B displays a typical picture of TNF treated cells stained with Hoechst 33258. As compared to nontreated cells (Fig. 2A), cells carrying condensed nuclei were observed. The population of cells carrying condensed nuclei increased in a dose dependent-fashion (Fig. 2C). These data suggested that these cells apoptotically died by TNF treatment.

TNF Induced Activation of NF-κB Activation of NF-κB by TNF was determined by EMSA. As shown in Fig. 3B, activation of NF-κB reached a maximum at 30 min in L929, C12 and CPL4 cells after exposure to 5 ng/ml TNF and thereafter decreased until 60 min. Activation of NF-κB was detected by 0.5 ng/ml of TNF in L929 cells and the amounts of activated NF-κB increased in a dose-dependent manner (Fig. 3A). Activation of NF-κB in C12 and CPL4 cells was also observed at 0.5 ng/ml TNF, and activated NF-κB in both cell lines was approximately twice as high as that of L929 cells. Slightly higher activation of NF-κB was observed in C12 cells as compared to CPL4 cells. IL-1α, a well known activator of NF-κB, also activated NF-κB in L929, C12 and

CPL4 cells (Fig. 3C) in nearly the same fashion as TNF.

Determination of the Amount of Latent NF- κ B in Non-Stimulated Cells To compare the amount of the latent form of NF- κ B in these cell lines, whole cell lysate was treated with deoxycholate to remove I- κ B from NF- κ B complex and the amount of NF- κ B was determined by EMSA (Fig. 4). The latent form of NF- κ B in non-stimulated C12 and CPL4 cells was about twice as high as that of L929 cells. These results suggested that an abundance of latent NF- κ B

in C12 and CPL4 cells would provide the high amounts of active NF- κ B when these cells were stimulated with TNF.

Effect of PDTC on TNF Induced NF- κ B Activation and Cytotoxicity Activation of NF- κ B by TNF was markedly suppressed in all 3 cell lines by pretreatment with pyridine dithiocarbamate (PDTC) which is a potent antioxidant and metal chelator (Fig. 5A). Under the condition of inhibition of NF- κ B activation, the cytotoxicities of TNF to these cell lines were significantly potentiated at the several doses of TNF used in the experiment (Fig. 5B). For example, the survival ratio of C12 cells by 10 ng/ml TNF decreased 77% to 56% by pretreatment with PDTC. These results suggest that NF- κ B activation would suppress the apoptotic signaling of TNF in L929, C12 and CPL4 cells.

DISCUSSION

C12 cells were resistant to TNF cytotoxicity compared to L929 cells due to its low expression of cPLA₂¹³ which is required for the release of arachidonic acid from the *sn*2 position of phospholipids. Several reports have shown that there is a close relation between TNF induced arachidonic acid release and TNF cytotoxicity.^{15,16} However, we still do not know how cPLA₂ acts as a determinant factor to TNF sensitivity. It has been reported that cPLA₂ activity was required for the production of ceramide,^{12,17} which is a lipid second messenger that is believed to induce apoptosis. Ceramide did not increase in response to TNF in C12 cells, suggesting the non-involvement of this lipid metabolite in TNF cytotoxicity.^{12,17}

As shown in Fig. 1, C12 cells were resistant to TNF-in-

Fig. 2. TNF Induced Nuclear Condensation

(A) No treatment. (B) L929 cells were treated with 1 ng/ml TNF for 18 h. Arrow heads show typical examples of condensed nuclei. (C) Dose dependent increase of nuclear condensation in L929 cells. The percentage of cells which carry condensed nuclei was plotted. The data were expressed as mean of 3 independent counting among at least hundred cells per count.

Fig. 3. TNF Induced NF- κ B Activation

(A) Dose dependent NF- κ B activation. Cells were treated with the indicated amount of TNF for 30 min. (B) Time course experiment of TNF induced NF- κ B activation. Cells were treated with 5 ng/ml TNF for the indicated time. (C) IL-1 α induced NF- κ B activation. NF- κ B were measured after 30 min exposure to 0.1 ng/ml IL-1 α . EMSA was performed as described in Materials and Methods.

Fig. 4. Latent NF- κ B Contents in Non-Stimulated Cells

Whole cell extracts (10 μ g) of each cell line were incubated with 0.5% deoxycholic acid for 30 min on ice. Then EMSA was carried out as previously described.

Fig. 5. PDTC Pretreatment Suppresses TNF Induced NF- κ B Activation and Enhances TNF Cytotoxicity

(A) Cells were treated with 100 μ M PDTC for 30 min then further incubated with TNF for 30 min. (B) Cells were treated with 100 μ M PDTC for 30 min then medium was replaced with TNF containing medium. Open column; no pretreatment, shaded column; PDTC pretreatment. Survival ratio of the cells was determined by MTT assay 3 d after addition of TNF.

duced apoptosis as compared with L929 cells; however, the resistance of C12 cells is likely to be limited to TNF-induced apoptosis since C12 cells did not show any resistance to apoptosis induced by typical apoptotic treatment such as UV-B irradiation, etoposide and *cis*-platinum (data not shown). Enari *et al.*, also demonstrated that C12 did not resist the apoptosis caused by anti-Fas.¹⁸⁾ These experimental results suggested that C12 should have anti-apoptotic factor(s) which acts specifically to TNF-induced apoptosis since both anti-Fas and TNF utilize FADD as a signaling molecule to induce apoptosis.

TNF transduces several different signals *i.e.*, JNK (c-Jun N-terminal kinase) activation which contributes to activate

activator protein 1 (AP-1),¹⁹⁾ caspase activation which leads cell to apoptosis,⁵⁾ and NF- κ B nuclear translocation.⁴⁾ Recently, it was revealed that NF- κ B participates in the regulation of the apoptosis signal of TNF as an anti-apoptotic factor.^{9–11)} Liu *et al.* demonstrated that exogenously introduced NF- κ B component prevented TNF induced apoptosis in HeLa cells.⁹⁾ Human T cell line HuT-78 which expresses constitutively active NF- κ B was resistant to TNF.²⁰⁾ NF- κ B is thought to enhance transcription of a yet unidentified anti-apoptotic protein(s). These reports lead us to determine TNF-induced NF- κ B activation in L929, C12 and CPL4 cells. NF- κ B was activated in all 3 cell lines by exposure to TNF, however, the amount of active NF- κ B was two fold higher in C12 and CPL4 cells than that of L929 cells. This could be explained by the intracellular available NF- κ B contents since C12 and CPL4 have about two fold more latent NF- κ B. We were not able to determine why more NF- κ B exists in TNF resistant cell lines. This might not be explained by cPLA₂ activity, since CPL4 have almost same amount of latent NF- κ B compared to C12. The long term exposure to TNF might alter expression of NF- κ B so it became an acquired phenotype. Jayadev *et al.* reported that no difference was observed in activation of NF- κ B by TNF between L929 and C12.¹⁷⁾ At the present time, we do not have any answer to clarify this incompatibility. It may be explained by different experimental or cellular conditions.

PDTC has both antioxidative and metal chelating activities and is known as a potent NF- κ B inhibitor.²¹⁾ PDTC pretreatment suppressed TNF induced NF- κ B activation in L929, C12 and CPL4 cell lines almost completely and enhanced TNF cytotoxicity to all 3 cell lines. These results are consistent with the report of Giri *et al.* which demonstrated that PDTC pretreatment potentiated TNF-induced apoptosis.²⁰⁾ Although PDTC potentiates TNF-induced apoptosis, the incomplete restoration of TNF cytotoxicity to the same level as L929 cells in C12 cells suggested that NF- κ B is not the single determinant factor of TNF resistance. There might be other factor(s), such as ceramide and arachidonate pathway products, contributing to the resistance to TNF in TNF resistance in C12 cells. CPL4 cells showed moderate sensitivity to TNF so that almost the same amount of NF- κ B was activated as C12 cells. The abundance of NF- κ B may contribute to the moderate resistance (5.4-fold resistant) of this cell line to TNF. The production of ceramide and cPLA₂ metabolites were observed at a relatively late phase as compared to NF- κ B activation.^{12,13,17)}

These experimental results suggest a complex mode of action for these anti-apoptotic and apoptotic factors. It also indicates a certain contribution of NF- κ B to the inhibition of TNF cytotoxicity as an early phase anti-apoptotic factor. In conclusion, we propose that the abundance of NF- κ B is one of the acquired TNF resistant factors in addition to loss of cPLA₂ expression in TNF resistant C12 cells.

Acknowledgment The authors thank M. Okazaki, R. Fukasawa, R. Aizawa, and T. Takayama for their technical assistance, Dr. S. Hara for providing a NF- κ B probe and his helpful advise. This work was supported in part by a grant for Somatic Cell Transformation project from RIKEN and by a grant from Takeda Science Foundation.

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