Desensitization of NFκB for Overcoming Chemoresistance of Pancreatic Cancer Cells to TNF-α or Paclitaxel

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Abstract. *Background: Chemotherapy-induced nuclear factor kappaB (NFκB) activation is thought to play a key role in acquisition of chemoresistance by cancer cells. We focused on blockade of this activation by using the observation so-called 'desensitization' of NFκB using known NFκB activator, doxycycline. Materials and Methods: The human pancreatic cancer cell line PANC-1 was incubated with doxycycline, followed by treatment with tumor necrosis factor (TNF)-α or paclitaxel. NFκB activity and the regulation of NFκB-related genes was analyzed. Results: Doxycycline induced sustained NFκB activation, followed by desensitization to further NFκB activation by TNF-α -or paclitaxel, which was accompanied by decreased expression of TNF receptor p55, p75, and epidermal growth factor receptor. Consistent with these observations, doxycycline-pre-treatment resulted in an augmentation of TNF-α- and paclitaxel-mediated cytotoxicity and apoptosis. Conclusion: These data indicate the possible clinical application of desensitization of NFκB to overcome chemoresistance by conventional chemotherapy for pancreatic cancer.*

Pancreatic cancer is a common malignant cancer worldwide, with a poor median survival rate of 12 months after surgery, due to advanced stage at the time of diagnosis, rapid tumor growth and high potential for distant metastasis (1, 2). Moreover, pancreatic cancer is one of the most intrinsically drug-resistant tumors, and resistance to chemotherapeutic agents is a major cause of treatment failure. Therefore, there is a dire need for designing new and targeted therapeutic strategies that can overcome such drug-resistance and improve the clinical outcome for patients diagnosed with pancreatic cancer.

Nuclear factor kappa B (NFκB) is one of the major transcription factors associated with cancer development and progression, and is involved in cell proliferation, inhibition of apoptosis, tissue invasion and metastasis (3). NFκB is typically a heterodimer consisting of p65 (RelA) and p50 proteins, and most inactive NFκB molecules are sequestered in the cytoplasm by inhibitory κBα (IκBα) protein. NFκB activating signaling leads to IκBα phosphorylation, followed by NFκB protein release from IκBα and its translocation into the nucleus. After translocation, NFκB in the nucleus activates transcription target genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation and suppression of apoptosis (4). Constitutive activation of NFκB has been described in a great number of solid tumors and this activation appears to support cancer cell survival and to reduce the sensitivity to chemotherapeutic drugs (5, 6). Additionally, some of these drugs induce NFκB themselves and through this mechanism they lower their cytotoxic potential. Therefore, inhibition of NFκB by various means has been shown to enhance the sensitivity to antineoplastic-induced apoptosis and thus, NFκB inhibitors are more likely to be of use in cancer therapy (7). On the other hand, sustained NFκB activation and subsequent decrease in sensitivity of NFκB further stimulation by stress responses, so-called 'desensitization' or 'toleration' have been reported (8, 9). We reported that doxycycline, a classic antimicrobial tetracycline, activates NFκB by generating superoxide (10). Here, we investigated the efficacy of doxycycline as a desensitizer of NFκB for further cytotoxic stimulation in a pancreatic cancer cell line.

Materials and Methods

Reagents. Oligodeoxyribonucleotides and doxycycline were synthesized by SIGMA (The Woodlands, TX, USA). Primary antibodies for immunoblotting [IκBα, IκBβ, β-actin, epidermal growth factor receptor (EGFR), and activated EGFR] and antibodies for the electromobility supershift assay (EMSA) (RelA, p50, p52,

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c-Rel) were purchased from Santa Cruz biotechnology, Inc. (Santa Cruz, CA, USA). Phospho-IκBα antibodies for immunoblotting were purchased from Cell Signaling Technology (Beverly, MA, USA). Radioisotopes were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA).

Cell culture. The human pancreatic adenocarcinoma cell line PANC-1 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-incubated fetal bovine serum, sodium pyruvate, nonessential amino acids, and L-glutamine. The culture was incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Cell proliferation. PANC-1 cells were grown at a density of 10^6 cells/ml in six-well Coster plates in DMEM at 37˚C for six days in an incubator with 95% O_2/5% CO_2. Aliquots of cells and medium were removed at two-day intervals. Following examination, attached cells were trypsinized and counted.

DNA fragmentation. DNA fragmentation in apoptotic cells was determined by DNA gel electrophoresis. The cells were lysed in extraction buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, 0.5% SDS and 20 μg/ml RNase) at 37˚C for 1 h. Then 25 mg/ml of proteinase K was added and the sample was incubated at 50˚C for 3 h. DNA was extracted with phenol/chloroform and chloroform. The aqueous phase was precipitated with two volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate on ice for 30 min. The DNA pellet was then washed with 70% ethanol and re-suspended in 50 μl Tris-EDTA buffer. The absorbance of the DNA solution at 260 nm and 280 nm was determined by spectrophotometry. The extracted DNA (40 μg/lane) was electrophoresed in 2.0% agarose gel. The gel was stained with 50 μg/ml ethidium bromide for 30 mim, then photographed.

EMSA. EMSA was performed using nuclear extracts prepared from control and treated PANC-1 cells, as described previously (11). End-labeled DNA probes (wild-type κB: Upper strand; 5'-AGTTGAGGGGACTTTCCCAGGC-3', mutant κB: Upper strand; 5'-AGTTGAGGCGACTTTCCCAGGC-3', and organic cation transporters 1 (OCT1): Upper strand; 5'-TGTCGAATGCAAATCA CTAGAA-3') were mixed with 10 μg of nuclear extract in a 10 μl reaction volume containing 75 mM NaCl, 15 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 1.5 mM DTT, 25% glycerol and 20 μg/ml bovine serum albumin (BSA) and 1 μg of poly-deoxyinosinic-deoxycytidylic (dI-dC). The reaction mixture was incubated on ice for 40 min and applied to a 4% non-denatured polyacrylamide gels containing 0.25×TBE (22.5 mM Tris, 22.5 mM borate, 0.5 mM EDTA, pH 8.0) buffer. Equal loading of nuclear extracts was monitored by OCT1 binding. For competition assays, a 100-fold molar excess of unlabeled oligonucleotides was added to the binding reaction. For antibody supershifts, 2 μl of the polyclonal antibodies against p65, p50, p52 and c-Rel were pre-incubated for 30 min at room temperature prior to adding the probe. After electrophoresis, the gel was dried for 1 h at 80˚C and exposed to Kodak film (Eastman Kodak Co., Rochester, NY, USA) at −70˚C.

Northern blot analysis. For northern blot analysis, total RNA was extracted using TRIZOL Reagent (Life Technologies, Inc. Gaithersberg, MD, USA) according to the manufacturer's protocol. Fifteen micrograms of RNA were electrophoresed on a 1% denaturing formaldehyde agarose gel, transferred to a nylon membrane in the presence of 20×SSC, and UV cross-linked. To obtain a cDNA probe for northern blot, reverse transcription (RT) and polymerase chain reaction (PCR) were performed as follows: 1 μg of total RNA made from PANC-1 cells was incubated at 42˚C for 1 h with 100 ng of Oligo (dT) 12-18 primer (Life Technologies, Inc.), 0.25 mM of dNTPs (Promega, Madison, WI, USA), 1× incubation buffer of AMV reverse transcriptase (Roche, Indianapolis, IN, USA), 20 U of RNase inhibitor (Roche) and 25 U of AMV reverse transcriptase (Roche) in a final volume of 20 μl. Subsequently, the samples were heated to 90˚C for 5 min to terminate the reaction. One microliter of the cDNA reaction was then subjected to 30 PCR cycles (denaturing at 94˚C for 1 min, annealing at 56˚C for 1 min, and polymerization at 72˚C for 1 min.), in the presence of 0.25 U of Taq DNA Polymerase (Roche), 1× PCR reaction buffer (Roche), 0.25 mM of dNTPs (Promega), and 0.5 μM of specific primers (TNFR1p75: 5'-primer; GTGTCCACACGAT CCCAACACAC, 3'-primer; GAAAGCCCCTCTGCAGAAAA GGA-5') in a final reaction volume of 50 μl. The PCR products were extracted and subsequently cloned in a pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequence analysis. The sequences of these cDNA fragments agreed with the sequence obtained from the Gene Bank (National Center for Biotechnology Information). The cDNA probes (*Eco*RI-*Eco*RI) were labeled with [a-^{32}P] deoxycytidine triphosphate using a random labeling kit (Roche) and used for hybridization. Equal loading of mRNA samples was monitored by hybridizing the same membrane filter with the cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Ribonuclease protection assay. PANC-1 cells were treated in the presence of doxycycline. Cells were scraped, and total RNA was harvested using Trizol reagent. A custom made Riboquant™ Multiprobe RNase Protection Assay System (Pharmingen, San Diego, CA, USA) and a multiprobe template set (hAPO-3) were used for RNase protection assay. The ^{32}P-labeled riboprobes were hybridized for 18 h with 15 μg of total RNA. The hybridized RNA was digested with RNase, and the remaining RNase-protected probes were purified, resolved on denaturing polyacrylamide gels. Equal loading of mRNA samples was monitored by the two housekeeping genes *L32* and *GAPDH*.

Reporter gene analysis. One microgram of HIV-κB reporter gene construct containing firefly luciferase was co-transfected into tumor cells with an internal control, p-TK renilla luciferase, using lipotransfection method (FuGENE 6; Roche) in triplicate. The HIV-κB reporter gene construct contains two HIV-κB enhancers. The activity of both firefly and renilla luciferase were determined 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

Western blot analysis. Cytoplasmic extracts were prepared as described previously (12). Soluble protein were separated on 10% sodium dodecyl sulfate-polyaclylamidegel electrophoresis (SDS-PAGE) by electrophoresis and electrophoretically-transferred onto polyvinylidene difluoride (PVDF) membranes (Osmonics, Westborough, MA, USA). Membranes were blocked with 5% nonfat milk in PBS containing 0.2% Tween-20 and incubated with affinity-purified mouse antibody against β-actin and rabbit antibody against

phospho-IκBα, IκBα, IκBβ, and regular and activated-EGFR. Membranes were washed in PBS containing 0.2% Tween-20 and probed with horseradish peroxidase-coupled secondary goat anti-rabbit or mouse IgG antibodies (Amersham, Arlington Heights, IL, USA). The probe proteins were detected using the Lumi-Light Western Blotting Substrate (Roche), according to the manufacturer's instructions.

Results

Sustained NFκB activation by doxycycline is followed by desensitization to further stimuli by TNF-α or paclitaxel. The NFκB pathway is a known survival pathway, and its activation may confer resistance to cancer cells from TNF-α- or chemotherapeutic-induced apoptosis (13). Indeed, most agents that induce apoptosis also activate NFκB and several reports have shown that NFκB inhibition sensitizes the cancer cells to apoptosis induced by TNF and cancer therapy (14). In our previous study, we found that doxycycline generates superoxide, which in turn activates NFκB (10). Therefore, we determined the effect of doxycycline on further NFκB activation by cytokine or a chemotherapeutic agent. Consistent with our previous report, 20 μg/ml of doxycycline increased DNA-binding activation of NFκB (Figure 1A, lane 2). The specificity of the observed band-shift, checked by competition and supershift experiments, indicated that doxycycline-activated NFκB complexes contained both p50 and RelA components (lanes 6-9). Interestingly, TNF-α-induced NFκB activation which peaked at 1 h, was significantly inhibited by preliminary incubation with doxycycline (Figure 1A, lanes 3 and 4; Figure 1B), and this effect was dose-dependent (Figure 1C). Doxycycline-mediated NFκB activation was observed at 2 h after dosage and was sustained during the experimental period (Figure 1D, lanes 2-7). On the other hand, the inhibitory effect of TNF-α-mediated NFκB activation, followed by doxycycline pre-treatment occurred at 4 h and was augmented in a time-dependent manner (Figure 1D, lanes 11-15).

Cytokine-mediated activation of NFκB is controlled by sequential phosphorylation, ubiquitination, and degradation of its inhibitory subunit IκB, triggered by the activation of protein kinase complex, IκB kinase (IKK) (15). We next determined the effect of doxycycline on TNF-α-mediated expression of IκBα protein. In agreement with NFκB binding activation, doxycycline increased the protein expression of phospho-IκBα (Figure 1E, lane 2). On the other hand, TNF-α strongly induced IκBα phosphorylation, which was prevented by pre-treatment with doxycycline (Figure 1E, lanes 3 and 4). Consistent with these data, although doxycycline led to protein degradation of IκBα, it inhibited subsequent TNF-α-mediated IκBα phosphorylation and degradation (Figure 1E).

Paclitaxel, an antimitotic agent which stabilizes the assembly of microtubules and arrests cell progression through mitosis, is claimed to be active as a single-agent for advanced pancreatic cancer (16, 17), although the objective effect of this agent seems to be marginal (18). Our previous report indicates that paclitaxel-mediated activation of NFκB and expression of downstream *BCL-XL* gene confer resistance to paclitaxel-induced apoptosis in pancreatic cancer cells (12). Recently, we reported that doxycycline itself reduces gene expression of BCL-XL (19). In the present study, we next determined the effect of doxycycline on paclitaxel-mediated NFκB activity. As shown in Figure 2A, paclitaxel induced NFκB binding activation, accompanied by specific IκBα degradation in a time-dependent manner, which was effectively inhibited by pre-treatment with doxycycline (Figure 2B).

In order to determine whether the effects of doxycycline were accompanied by the transcriptional regulation of NFκB-inducible genes, we carried out transfection experiments with a reporter gene construct, containing two NFκB enhancers. PANC-1 cells were transiently transfected with the reporter in the presence or absence of doxycycline, or its combination either with TNF-α or paclitaxel. Consistent with previous NFκB EMSA analysis, TNF-α, and paclitaxel increased the NFκB-driven transcriptional activity, while these activations were blocked in the presence of doxycycline (Figure 3).

Doxycycline reduces tumor necrosis factor receptor (TNFR) and EGFR expression. A recent report showed that cross-communication between TNFR and EGFR signals allows for the synergistic augmentation of the NFκB activation pathway (20). We determined whether doxycycline-mediated desensitization to NFκB for further stimuli was accompanied by the regulation of TNFR or EGFR. As shown in Figure 4A and B, doxycycline reduced mRNA expression of *TNFRp75* and *TNFRp55* in a time- and dose-dependent manner. The protein levels of activated EGFR at a molecular weight of 170 kDa were decreased in a dose- and time-dependent manner (Figure 4C). These results suggest that doxycycline reduces TNFR and EGFR expression, which may account for the desensitization to TNF-α- or paclitaxel-induced NFκB activation by doxycycline.

Doxycycline sensitizes cells to TNF and paclitaxel-induced apoptosis. In the present study, we have shown that doxycycline could have inhibited TNF-α- or paclitaxel-mediated NFκB activation. Our data and previously reported evidence (21, 22) suggests that doxycycline may sensitize cancer cells to paclitaxel. To test this hypothesis, we treated PANC-1 cells with either PBS or 10 μg/ml doxycycline for 24 h, followed by either 20 ng/ml TNF-α or 30 μM paclitaxel. Surviving cells were counted at each time point after TNF-α or paclitaxel addition. As shown in Figure 5A, compared with control cells, cells pre-treated with

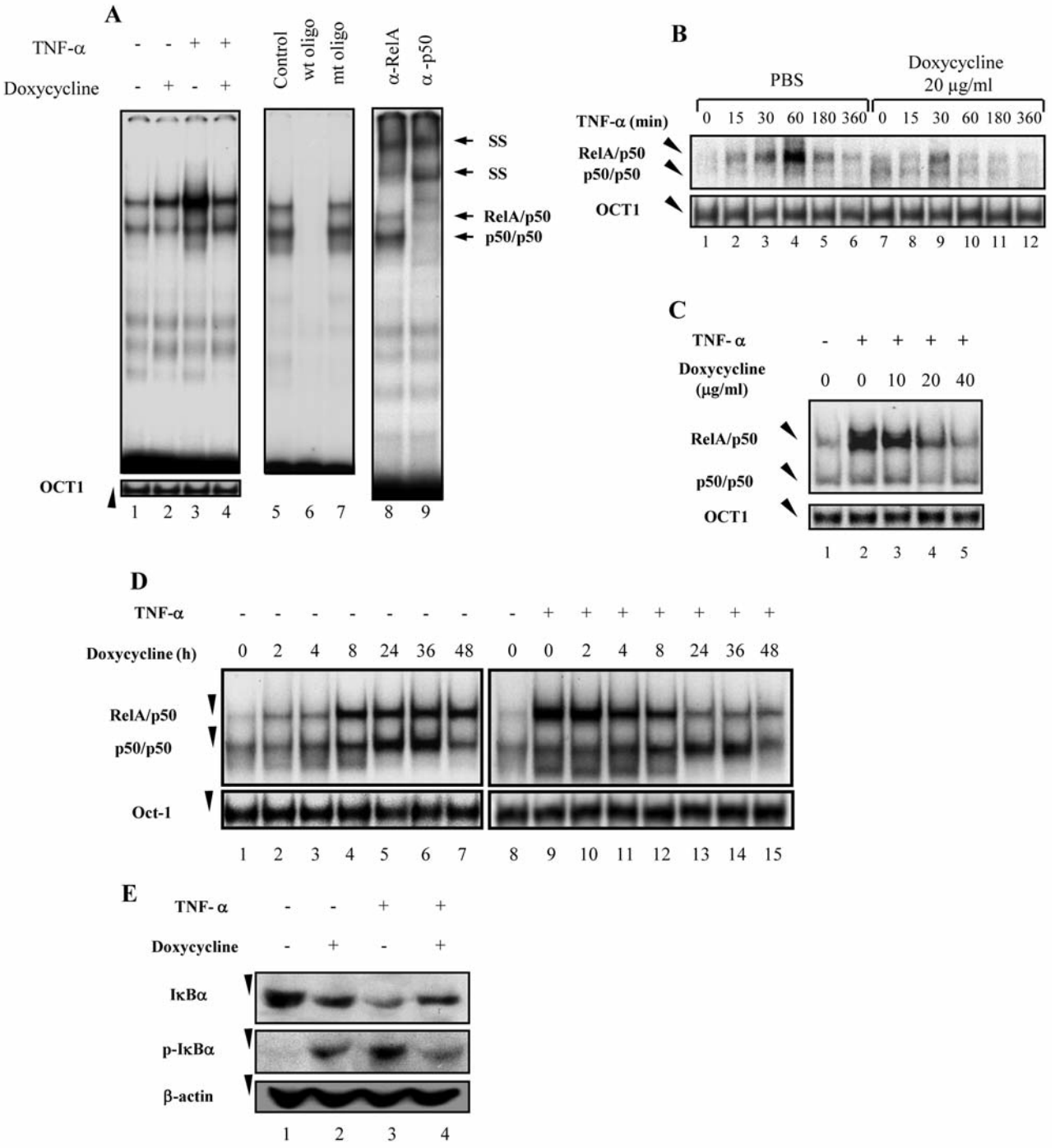

Figure 1. *Doxycycline induced nuclear factor κB (NFκB) activation and subsequent inhibition of further NFκB activation by tumor necrosis factor (TNF)-α or paclitaxel in PANC-1 cells. A-D, Representative electrophoretic mobility shift assays (EMSA) analysis for NFκB are shown. A. Cells were treated either with PBS (lane 1) or 20 μg/ml doxycycline (lane 2) for 48 h, followed by the treatment with 20 ng/ml TNF-α for 1 h (lanes 3 and 4). Competition assay was carried out using 100× excess of unlabeled wild-type (lane 6) or mutant (lane 7) κB probe, and supershift analysis was performed with anti-RelA (lane 8) and anti-p50 (lane 9). Arrows indicate the NFκB-DNA binding complexes; SS, supershifted band. OCT1 probe was used as a control for quality and quantity of cell extract. B. Cells were pre-treated either with PBS (lanes 1-6) or doxycycline (lanes 7-12) for 48 h, followed by the addition of 20 ng/ml TNF-α for indicated periods. C. Cells were pre-treated with increasing-dose doxycycline for 48 h, followed by the addition of 20 ng/ml TNF-α for 1 h. D. Cells were treated with 20 μg/ml doxycycline for the indicated periods (lanes 1-7), followed by the addition of 20 ng/ml TNF-α for 1 h (lanes 8-15). E. Western blots for phospho-IκBα, IκBα, and IκBβ were carried out using the cytoplasmic extract prepared from A. The membrane used for IκBα blots was stripped and re-probed for the expression of β-actin to control for loading.*

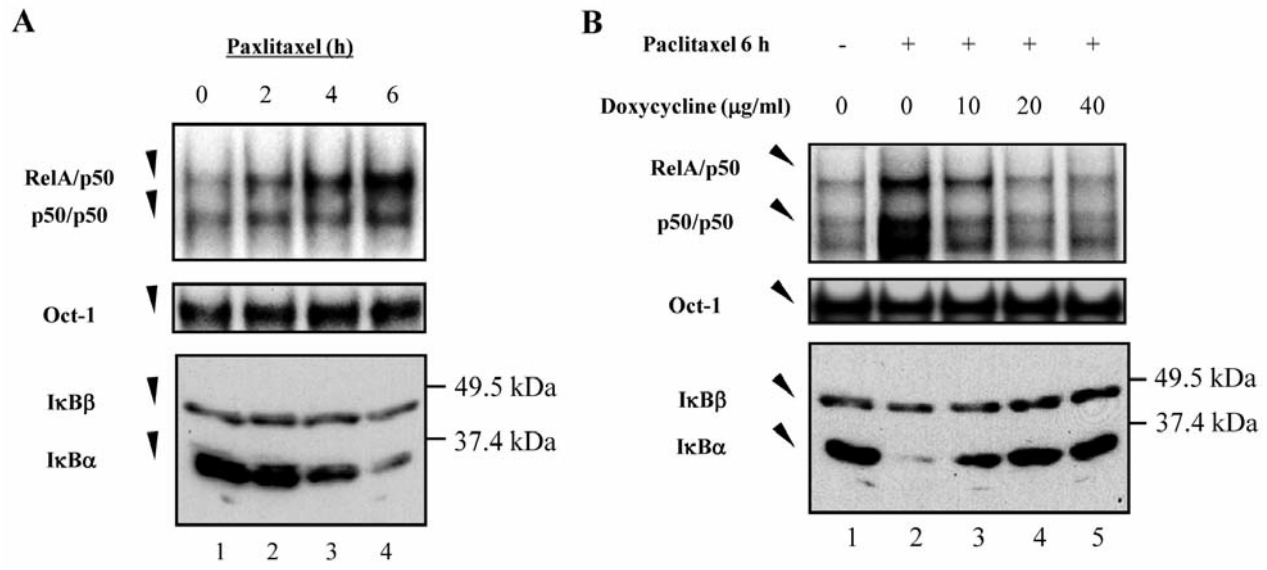

Figure 2. *Effect of doxycycline on paclitaxel-induced nuclear factor κB (NFκB) activation and inhibitory κB (IκB)α degradation. Electrophoretic mobility shift assay (EMSA) analysis for NFκB and western blot for IκBα and IκBβ are shown. A. Time-course treatment with 30 μM paclitaxel was carried out. B. Doxycycline inhibits paclitaxel-induced NFκB binding activation and IκBα degradation. Cells were treated with an increasing-dose of doxycycline for 48 h, followed by the medium exchange and the stimulation with 30 μM paclitaxel for 6 h.*

doxycycline were more sensitive to TNF-α and to paclitaxel treatment. Doxycycline pre-treatment efficiently induced apoptosis of TNF-α- and of paclitaxel-treated cells (Figure 5B), suggesting that doxycycline sensitized cells to TNF-α- and paclitaxel-induced apoptosis.

Discussion

In the present study, we focused on the role of doxycycline in inducing NFκB activation by cytokine and chemotherapeutic agents, because 'induced' NFκB is a key mediator of chemoresistance (4, 13, 23). Initially, doxycycline activated nuclear binding of NFκB, accompanied by specific IκBα phosphorylation and degradation. Subsequently, doxycycline prevented further activation of NFκB by TNF-α or paclitaxel stimulation. Previous reports suggest that persistent NFκB activation, induced by a variety of stimuli could be followed by decreased response to further stimulation (24-29). These physiological responses are thought to be an important means of self-defense against systemic toxicity because persistent NFκB activation can lead to devastating conditions, such as septic shock or acute inflammation (24). Indeed, stress responses, such as 'heat shock' stabilize IκBα and induce expression of IκBα, which in turn reduce TNF-α-mediated NFκB nuclear translocation (8). Thus, an autoregulatory loop whereby IκBα regulates the activity of

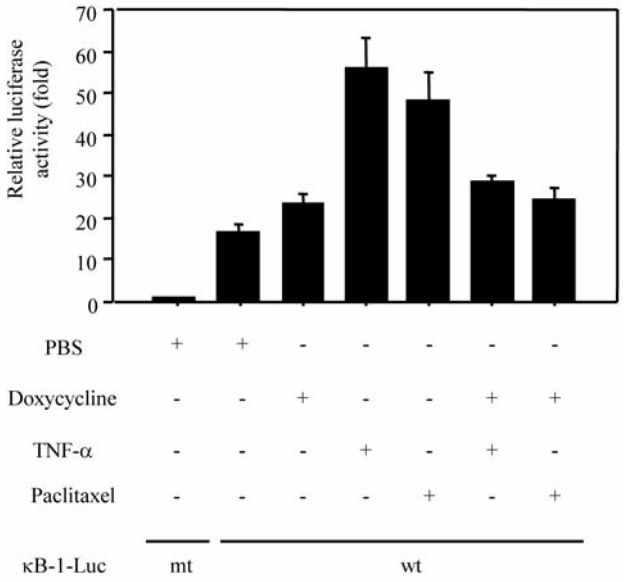

Figure 3. *Nuclear factor κB (NFκB)-dependent reporter gene activity. PANC-1 cells were transiently transfected with a wild-type or a mutant κB-Luciferase and a pTK-Renilla Luciferase reporter genes. Three hours after transfection cells were treated either with 20 ng/ml tumor necrosis factor (TNF)-α, 30 μM paclitaxel in the absence or presence of 20 μg/ml doxycycline for following 45 h. Fold-increases in Luciferase activity were calculated by normalizing to the activity of an internal control. Data represent the mean±standard error from three different experiments performed in triplicate.*

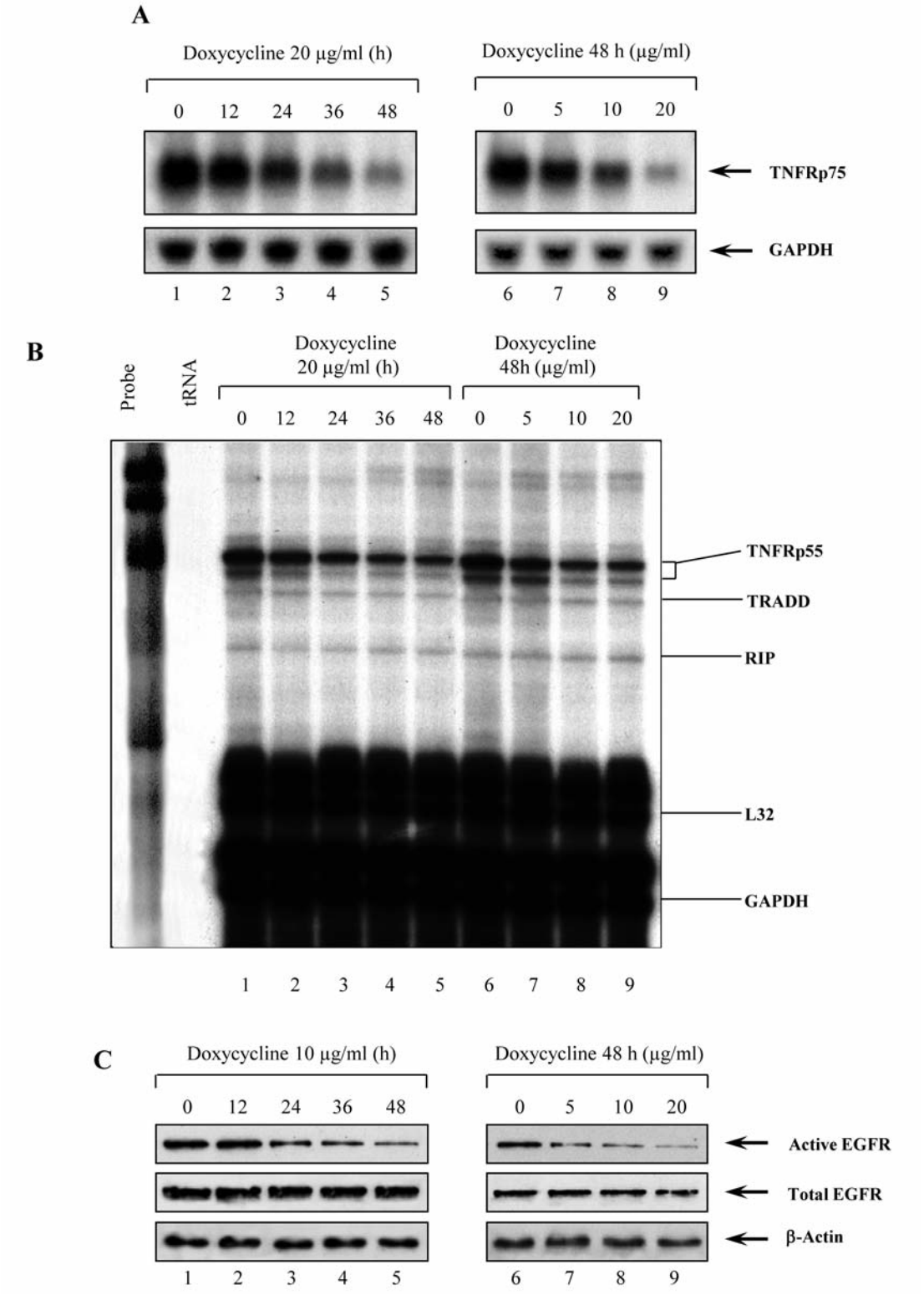

Figure 4. *Time- and dose-dependent down-regulation of tumor necrosis factor receptor (TNFR) mRNA expression by doxycycline. Northern blots for TNFRp75 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A) and Ribonuclease protection assay (B) are shown. Total RNA samples were prepared from PANC-1 cells treated with 20 μg/ml doxycycline for indicated times (lanes 1-5) and with increasing-dose doxycycline for 48 h (lanes 6-9). C. Western blots for total and activated epidermal growth factor receptor (EGFR) are shown. Cytoplasmic protein samples were prepared and subjected to western analysis following treatment as indicated in A and B.*

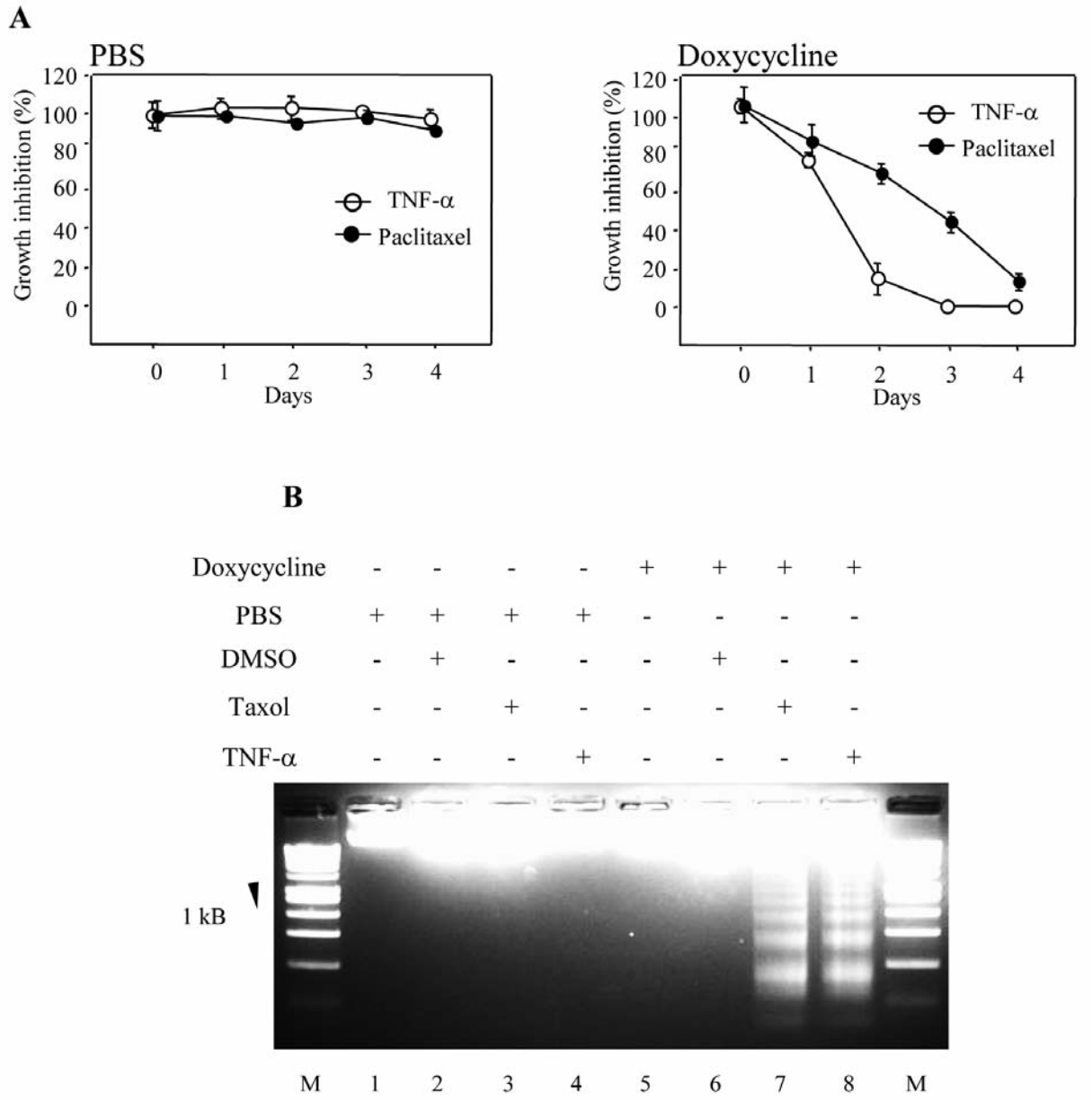

Figure 5. *Doxycycline enhances tumor necrosis factor (TNF)-α- and paclitaxel-mediated cytotoxicity and apoptosis. A. Cell proliferation. PANC-1 cells were pre-treated either with PBS or 10 μg/ml doxycycline for 24 h, followed by medium exchange and addition either of 20 ng/ml TNF-α or 30 μM paclitaxel in the culture dishes and incubated for the times indicated. Then, cells were collected at each indicated time-point and determined for the survival. B. Doxycycline sensitized PANC-1 cells to apoptosis mediated by TNF-α or paclitaxel. DNA fragmentation assay is shown. PANC-1 cells were pretreated either with PBS (lanes1-4) or 10 μg/ml doxycycline (lanes 5-8) for 24 h, followed by the medium exchange and the addition either of 20 ng/ml TNF-α or 30 μM paclitaxel in the culture dishes and incubated for 24 h. M; DNA markers.*

transcription factor NFκB, which in turn regulates IκBα activity may be involved in stress response-related desensitization of NFκB by further stimuli (11). In the present study, development of desensitization of NFκB was observed coincidentally with sustained NFκB binding activation, as well as decreased IκBα expression, by doxycycline treatment. These data suggest that autoregulation of IκBα protein itself is unlikely to be involved in this desensitization. On the other hand, in agreement with our data, down-regulation of TNFR is implicated as a mechanism of tolerance of macrophages to lipopolysaccharide or endotoxin (24, 26, 27).

EGFR is constitutively activated in many types of cancer cell lines and may play a role in chemoresistance in patients

with pancreatic cancer (30, 31). EGFR associates with NFκB-inducing kinase (NIK) and a TNFR-interacting protein (RIP), forming a multiprotein complex termed a signalosome, which has been implicated in EGFR-induced NFκB activation (32). Doxycycline reduced both TNFR and activated forms of EGFR, which may account for doxycycline-mediated desensitization.

NFκB subunit p50 has been characterized in LPS tolerance by a diminished production of TNF during prolonged exposure to LPS, in which the binding of p50 homodimers to the κB element on TNF promoter played an important role in the down-regulation of TNFR (33). Moreover, p50 blocks cytoplasmic localization activity of the RelA subunit, leading to the nuclear accumulation of both RelA and p50, which potentially permit sustained NFκB activation (34). Doxycycline-activated DNA-binding of p50/p50 homodimer, observed at 8 h and sustained up to 36 h, was synchronized with the development of TNF-α tolerance to further NFκB activation. These data suggest that increased nuclear binding of p50 homodimer may be involved in doxycycline-mediated desensitization of NFκB to further TNF-α stimulation.

The paclitaxel-resistant ovarian cancer cell line (SKOV-3TR) constitutively overexpressed chemokine/cytokine genes, including interleukin(IL)-6, IL-8, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-2α (22). Among them, IL-6, IL-8 and MCP-1 were induced by paclitaxel treatment in the paclitaxel-resistant phenotype, but not in parental cells, suggesting that inducible expression of these molecules plays a crucial role in paclitaxel resistance. We previously reported that paclitaxel-induced IL-8 expression was effectively blocked by as low as 5 μg/ml of doxycycline in PANC-1 cells (19). Indeed, PANC-1 cells resist to paclitaxel in terms of cell death and apoptosis, although they are made sensitive by pretreatment with doxycycline. Moreover, doxycycline-mediated augmentation of paclitaxel- or TNF-α-inducible apoptosis could be achieved by relatively low-dose (10 μg/ml) doxycycline. It should be noted that serum levels of 5-10 μg/ml doxycycline can be achieved with oral doses of 200-400 mg doxycycline administered once a day which is tolerated by most patients. Thus, our evidence may provide the basis for a new strategy for cancer treatment, using doxycycline as an adjuvant chemotherapeutic agent for reducing the chemoresistance of cancer cells to conventional chemotherapeutic agents.

In summary, we demonstrated desensitization of NFκB in PANC-1 cells and their subsequent susceptibility to apoptosis by further death stimuli such as TNF-α or paclitaxel. Given the critical role of NFκB as a promoter of cell survival through induction of target genes, our findings provides further evidence for clinical application of NFκB activators such as doxycycline for combination chemotherapy for patients with pancreatic cancer.

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