Doxycycline Induces Apoptosis in PANC-1 Pancreatic Cancer Cells

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Abstract. Background: Tetracyclines such as doxycycline are reported to possess cytotoxic activity against mammalian tumor cells, but the mechanism of their effects on cell proliferation remains unclear. Materials and Methods: The antitumor effect of doxycycline was investigated in human pancreatic cancer cell line, PANC-1. We also investigated the effect of doxycycline on expression of a potent proangiogenic factor, interleukin (IL)-8. Results: In excess of 20 μg/ml, cytotoxic effects of doxycycline were accompanied by G1-S cell cycle arrest and DNA fragmentation in PANC-1 cells. Doxycycline consistently activated transcription of p53, p21 and Fas/FasL-cascade-related genes, while reducing the expression of Bcl-xL and Mcl-1. Doxycycline (5 μg/ml) below the cytotoxic level suppressed endogenous and paclitaxel-induced IL-8 expression. In the mouse xenograft model, doxycycline treatment was shown to suppress tumor growth by 80%. Conclusion: These data suggest that doxycycline exerts its antitumor effect by activating proapoptotic genes, inhibiting IL-8 expression, and suppressing antiapoptotic genes.

Tetracyclines possess cytotoxic activity against several tumor cells in addition to their antimicrobial activities, for which the possibility of their use as anticancer agents is currently investigated (1, 2). The non-antibiotic property of tetracyclines was first discovered by their ability to inhibit various zinc-dependent enzymes of the matrix metalloproteinase (MMP) family, including MMP-1, -2, -3, -9, -13 and gelatinases, via multiple mechanisms (3, 4). Doxycycline, minocycline and newly developed chemically modified derivatives that do not have antimicrobial but retain anti-MMP activities are included in this family (5, 6). Recently, the ability of tetracyclines to induce apoptosis was reported in osteosarcoma, prostatic cancer cells, and Jurkat T lymphocytes (1, 7, 8).

Doxycycline, a semi-synthetic tetracycline, characterized by the presence of a dimethylamino group in C4 and a hydroxy group in C5, can inhibit MMP expression and cell proliferation in a number of cultured cell lines and animal models (1, 7, 11). Doxycycline-mediated antiproliferative activity may be associated with the regulation of cell proliferation as well as its ability to inhibit MMP activity (7, 9). Furthermore, doxycycline induces apoptosis, decreases the invasion of tumor cells, and suppresses the metastatic potential in breast cancer and melanoma cell lines at concentrations of 5-10 μg/ml (9, 12). Thus, doxycycline has been evaluated in preclinical cancer models and entered in early clinical trials in patients with malignant diseases (13).

Pancreatic adenocarcinoma is the fifth leading cause of cancer death in North America (14), for which surgery remains the sole curative option. Unfortunately, fewer than 15% of patients with pancreatic cancer are amenable to complete surgical resection, and the recurrence rate even after a successful pancreatectomy remains high (15). Therefore, the development of more effective treatment is urgently needed to improve outcome. To investigate the therapeutic efficacy of doxycycline in pancreatic cancer, we have studied the mechanism by which doxycycline induces apoptosis. Moreover, a tumor-bearing mouse xenograft model was used to determine whether doxycycline inhibits tumor growth in vivo.

Materials and Methods

Reagents. Oligodeoxyribonucleotides, doxycycline, tumor necrosis factor α (TNF-α) and paclitaxel were purchased from SIGMA (The Woodlands, TX, USA) and final concentration of 10 ng/ml for TNF-α or 30 μM for paclitaxel were used for the cell treatment. Stock solution of doxycycline was prepared freshly at 8 mg/ml in phosphate-buffered saline (PBS), pH 7.4 (pH was adjusted with 1 mol/l NaOH).
Stock solutions were diluted in PBS to allow a 1% volume addition of doxycycline to experimental wells. Control cells were treated with 1% volume of PBS. Antibody for p53 immunoblotting and electromobility supershift assay was purchased from Calbiochem (San Diego, CA, USA). Radiolabeled proteins were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA).

Cell line. The human pancreatic tumor cell line PANC-1 was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Seiryo-machi 4-1, Sendai 980-8575, Japan). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, nonessential amino acids, and L-glutamine. The culture was incubated at 37°C in a 5% CO2 humidified atmosphere.

Cell proliferation. PANC-1 cells were grown at a density of 10⁵ cells/ml in six-well Coster plates in DMEM at 37°C for 6 days in a 95% O2/5% CO2 incubator. Aliquots of cells and media were removed at 2-day intervals. Cells were treated with PBS in the absence or presence of 10-40 μg/ml doxycycline. Following treatment, cells were trypsinized and counted at each time-point.

DNA fragmentation. DNA fragmentation in apoptotic cells was determined by gel electrophoresis. PANC-1 cells (50×10⁶) were treated with doxycycline for 48 h, collected and washed with PBS, followed by lysing 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. Proteinase K (4 μM) was added, followed by incubation at 50°C for 3 h. DNA was extracted using phenol:chloroform and chloroform. The aqueous phase was precipitated with 2 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate on ice for 30 min, and then the DNA pellet was washed with 70% ethanol and resuspended in 50 μl Tris-EDTA buffer. Forty micrograms of DNA were subjected to electrophoresis on 0.8% agarose gel. The gel was stained with 50 μg/ml ethidium bromide for 30 min, and then photographed.

Cell cycle analysis. Subconfluent cells were treated with 0-40 μg/ml doses of doxycycline for 8, 12 and 24 h. Following treatment, cells were harvested and fixed with ice-cold 80% (v/v) ethanol for 24 h. After centrifugation at 200 × g for 5 min, the cell pellet was washed with PBS (pH 7.4) and resuspended in PBS containing propidium iodide (50 μg/ml), Triton® X-100 (0.1%, v/v), 0.1% sodium citrate and DNase-free RNase (1 μg/ml), followed by incubation at room temperature for 1 h. The DNA content was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Northern blot analysis. For Northern blot analysis, total RNA was extracted using TRIZOL Reagent (Life Technologies, Inc. Gaithersburg, MD, USA) according to the manufacturer’s protocol. Fifteen μg 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. The blots were hybridized either with random primed radiolabeled human p53 or with p21 cDNA (16). The cDNA probes were labeled with [α-32P]deoxycytidine triphosphate using a random labeling kit (Roche, Indianapolis, IN, USA) 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) as described by Hunt et al. (17). 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). RPA was used to detect and quantify 16 different mRNA apoptotic species expression with 2 human template probe sets. hApo-2 and hApo-3c. (The RiboQuant Multi-probe protection assay system: Pharmingen, San Diego, CA, USA). The probe set hApo-2 includes 8 Bcl-2-related gene templates including Bcl-xL, Bcl-xS, Bfl-1, Bik, Bak, Bax, Bcl-2 and Mcl-1. The hApo-3 probe set includes 8 proapoptotic and tumor necrosis factor family-related gene templates, namely, caspase-8, FasL, Fas, FADD, DR3, FAP, TRAIL. The 32P-labeled riboprobes were hybridized for 18 h with 15 μg of total RNA. The hybridized RNA was digested with RNase, and remaining RNase-protected probes were purified and resolved on denaturing polyacrylamide gels. Equal loading of mRNA samples was monitored by the two housekeeping genes L32 and GAPDH.

Animal study. Male nu/nu mice, 6-8 weeks old, were maintained in a specific-pathogen-free animal care facility according to institutional guidelines. Xenografts of tumors were established by subcutaneous (s.c.) injection of 1×10⁶ cells (suspended in PBS) into the flank. After the tumors reached an approximate volume of 100 mm³ (six weeks after injection), doxycycline-containing pellets (35 mg/pellet) with a timed-release of 60 days (Innovative Research of America, Sarasota, FL, USA) (n=7) or placebo pellets (n=7) were implanted s.c. on the other side of the xenograft. The two longest tumor diameters were measured every week, and tumor volumes were calculated according to the formula V=ab²/2 (18). Seven weeks after treatment, all mice were sacrificed and xenografts were resected and measured. Differences in tumor growth were compared by the two-way repeated measures ANOVA, and comparisons of tumor weight between the groups were made using the two-tailed Student’s t-test. A p-value of <0.05 is considered significant. All of the statistical analyses were performed using StatView 5.0 (Abacus Concepts Inc., Berkeley, CA, USA).

Results

Doxycycline effectively inhibits cell growth in PANC-1 cells. The effects of doxycycline at increasing concentrations on cell proliferation in human pancreatic cancer cell line PANC-1 are shown in Figure 1A. The treatment with 10 μg/ml doxycycline failed to show an inhibitory effect on cell proliferation, whereas the treatment with 20 μg/ml doxycycline resulted in up to 15% cell death on day 3, followed by complete cell death on day 6. Moreover, 40 μg/ml doxycycline exerted more enhanced cytotoxicity, leading to complete cell death 4 days after treatment. Thus, in excess of 20 μg/ml, doxycycline exerted a cell-killing effect in PANC-1 cells in a dose-dependent manner.

The effect of doxycycline on induction of apoptosis. To test the hypothesis that antitumor effect of doxycycline is attributable to its ability to induce apoptosis, we treated PANC-1 cells with different doses of doxycycline for 48 h. Following incubation, cells were harvested and genomic DNA was prepared to determine DNA fragmentation. DNA gel electrophoresis revealed a typical pattern of DNA

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fragment ladders among the cells treated in excess of 20 μg/ml doxycycline (Figure 1B). These results suggest that the cytotoxic effect of doxycycline may be a consequence of apoptosis induction in PANC-1 cells.

Doxycycline arrests cells in G1-S phase of the cell cycle. We investigated whether doxycycline interferes with the cell cycle as a leading phenomenon of the observed apoptosis. PANC-1 cells were treated with increasing doses of doxycycline for 8, 12, and 24 h, followed by cell cycle analysis using flow cytometry. As shown in Figure 2, cells treated with 20 μg/ml doxycycline started to arrest in G1-S phase at 24 h. For example, at 0 h, 28% of the cell population was in the G1-phase, and by 8, 12, and 24 h after treatment, the G1 population accounted for approximately 28%, 30%, and 48%, respectively. In contrast, treatment with PBS alone (indicated as 0 μg/ml) or 10 μg/ml doxycycline treatment did not change the size of the G1 population. The cells treated with 40 μg/ml doxycycline exhibited G1-S cell cycle arrest as early as 12 h after treatment, and these effects continued throughout the treatment periods. An increment in the sub-G1 population with apoptosis induction was not observed in any of the treatment groups within 24 h. These results suggest that the doxycycline induces cell cycle arrest at the G1-S phase.

Doxycycline induces the expression of p53 and its downstream target p21 in PANC-1 cells. The findings of the previous set of experiments suggested that doxycycline could induce cell cycle arrest and apoptosis in PANC-1 cells. One possible mechanism by which doxycycline participates in cell cycle arrest and apoptosis is the involvement of the tumor suppressor gene p53. The activation of p53 probably causes cell cycle arrest through the transcriptional up-regulation of its downstream targets including p21, and/or a transcription-independent apoptosis (16, 19). Therefore, we investigated the effect of doxycycline on the levels of p53 and p21. PANC-1 cells were treated with doxycycline for 48 h. Following treatment, cells were harvested and total RNA was prepared for Northern blot analysis. As shown in Figure 3A, p53 mRNA level was induced after exposure of PANC-1 cells to doxycycline. This induction was observed at as low as 15 μg/ml of doxycycline and increased at higher doses in a dose-dependent manner. Like p53 mRNA up-regulation, p21 mRNA was increased in excess of 15 μg/ml doxycycline (Figure 3A). Up-regulation of p53 mRNA appeared at as early as 24 h after exposure to 20 μg/ml doxycycline (Figure 3B), in agreement with G1-S arrest determined by FACS analysis. Furthermore, 40 μg/ml doxycycline treatment resulted in earlier p53 induction at 12 h (Figure 3B), followed by detectable p21 mRNA induction at 24 h. These transcriptional activations of p53 and p21 genes support the assumption of doxycycline-mediated G1-S cell cycle arrest and subsequent induction of apoptosis.

Doxycycline down-regulates antiapoptotic genes and induces proapoptotic genes. Uncontrolled growth of cancer cells results from the inability of these cells to undergo cell cycle arrest and apoptosis. The phenomenon of apoptosis is regulated by
integration of both proapoptotic and apoptotic signals. Doxycycline has been reported to induce apoptosis in several cancer cell lines, but its mechanism remains largely unknown (1, 7, 20). Therefore, we investigated the effect of doxycycline on pro- and antiapoptotic gene regulation. As demonstrated in Figure 4A, doxycycline down-regulated the antiapoptotic genes including Bcl-xL and Mcl-1 in a time- and dose-dependent manner. In contrast, 20 μg/ml doxycycline treatment significantly induced proapoptotic gene expression, including of caspase-8, FasL, Fas, FADD, DR3, FAP, FAF and TRAIL (Figure 4B). These results may raise the possibility that doxycycline enables cells to become highly susceptible to apoptosis by activation of proapoptotic genes and suppression of antiapoptotic genes.

Doxycycline blocks TNF-α- and paclitaxel-mediated IL-8 induction. In addition to its stimulatory effects on neutrophil migration, IL-8 has been shown to have a proangiogenic property in tumor metastasis (21, 22). It is supposed that IL-8-mediated angiogenesis is enhanced by TNF-α (23, 24). Recent reports have shown that paclitaxel-resistant ovarian cancer cells overexpress IL-8, suggesting the role of this angiogenic factor in paclitaxel-resistance (25, 26). For these reasons, we determined endogenous and TNF-α- and paclitaxel-mediated IL-8 expression in the presence of doxycycline. Endogenous IL-8 mRNA expression was reduced by doxycycline treatment in a dose-dependent manner (Figure 5A). TNF-α-induced IL-8 mRNA expression peaked at 3 h (Figure 5B, lane 3), which was effectively blocked by 48 h of doxycycline-pretreatment (20 μg/ml, lane 7), resulting in delayed induction of IL-8 mRNA at 6 h (lane 8) in accordance with the elimination of doxycycline (27). The inhibition of TNF-α-induced IL-8 expression by doxycycline-pretreatment was observed at as low as 5 μg/ml concentration (Figure 5C, lane 3), with a dose-dependent augmentation of this effect (lanes 4 to 7). We also confirmed the

Figure 2. Doxycycline arrests cells at the G1-S phase of the cell cycle. PANC-1 cells treated with different doses of doxycycline for 8, 12, and 24 h were evaluated by FACS analysis.
doxycycline effect on paclitaxel-mediated IL-8 regulation. Paclitaxel induced IL-8 expression in a time-dependent manner (Figure 5D, lanes 1-4), which was significantly blocked by doxycycline-pretreatment (lanes 6 to 8). Interestingly, 5 μg/ml doxycycline-pretreatment was sufficient to block paclitaxel-induced IL-8 expression (Figure 5E, lane 3). These results suggest that doxycycline blocks TNF-α- and paclitaxel-induced IL-8 expression.

Antitumor effect of doxycycline in a mouse xenograft model. Tumor growth was significantly suppressed in mice treated with doxycycline as compared to those treated with placebo pellet (p<0.05, Figure 6C). Doxycycline treatment led to an overall reduction by up to 80% in tumor weight at the end of the experiment (Figure 6A, B and D).

Discussion

Doxycycline has recently been shown to possess non-antibiotic properties including cytotoxic activity against several tumor types (7, 9, 12). Although the mechanism by which doxycycline exerts its apoptotic effect remains unclear (1, 7), it is proposed that doxycycline induces apoptosis through a Fas/Fas-ligand dependent pathway in Jurkat T lymphocytes (8). We demonstrated cytotoxic activity of doxycycline in excess of 20 μg/ml in PANC-1 cells. It is assumed that this activity of doxycycline is mediated by the induction of apoptosis, which was evidenced by DNA fragmentation. In the process of apoptotic cell death, two central pathways are known: activation of the caspase proteases and the mitochondrial pathway (28). Doxycycline induced both
caspase activators such as FADD and members of caspase cascade including FasL, Fas, DR3 and caspase-8. Regarding the mitochondrial pathway, doxycycline did not influence the level of proapoptotic factors Bax and Bak, but down-regulated antiapoptotic factors such as Bcl-xL and Mcl-1. These results suggest that activation of caspase proteases may be a major proapoptotic mechanism of doxycycline. It was previously shown that caspase-8 and -10 activity induced by doxycycline were responsible for induction of apoptosis in two other pancreatic cancer cell lines, T3M4 and GER (29). Our observations are consistent with these results. In the present study, we further investigated whether doxycycline inhibits growth of pancreatic cancer cells in vivo. Doxycycline successfully reduced tumor growth in this mouse xenograft model. This observation is the first report to show that apoptosis induction by doxycycline inhibited pancreatic cancer cell growth in vivo.

A variety of chemotherapeutic agents can cause some degree of DNA damage, resulting in a rapid up-regulation of p53 protein levels by a posttranscriptional mechanism (30, 31). The outcome of the activation of p53 is either apoptosis or cell cycle arrest, depending on the level of p53 expression: p53 at high levels promotes cell death by a transcriptionally independent mechanism, whereas lower levels initiate a transcriptionally controlled inhibition of cell cycle progress (32). We have previously reported that doxycycline-induced apoptosis is a nuclear factor (NF)-κB- and p53-dependent in colon cancer cell line HCT116 (33). Similarly, simultaneous activation of p53 and p21 as well as cell cycle arrest were observed in PANC-1 cells, suggesting that a p53-dependent mechanism is involved in doxycycline-mediated apoptosis.

In viable cells, doxycycline is transferred into the cytoplasm and localizes in mitochondria, where it is metabolized to stable photoproducts, such as lumidoxycycline, by photodegradation, which induce mitochondrial fragmentation and alter mitochondrial membrane integrity, leading to continuous inhibition of mitochondrial protein synthesis (34). These effects of doxycycline on mitochondria are likely to depend on generation of excited-state singlet molecular oxygen (O₂(1Δg)) (35). Reactive oxygen species (ROS) and mitochondria play an important role in the induction of apoptosis under both physiological and pathological conditions. ROS induce many types of DNA damage, including single- and double-stranded DNA breaks, base and sugar modifications, DNA-protein crosslinks, and depurination and depyrimidination. This DNA damage is followed by activation of the tumor-suppressor gene p53, which transcriptionally regulates the expression of Fas on various cell types (35). Thus, ROS produced by doxycycline photoproducts could potentially be a mediator of doxycycline-mediated apoptosis.
Overexpression of chemokine/cytokine genes, including IL-6, IL-8, monocytochemotactic protein 1 (MCP-1) and macrophage inflammatory protein 2α (MIP-2α), has previously been observed in a paclitaxel-resistant ovarian cancer cell line (25). Among them, IL-6, IL-8 and MCP-1 are induced by paclitaxel treatment in a paclitaxel-resistant phenotype, but not in parental cells, implying that constitutive and inducible expression of these molecules plays a crucial role in paclitaxel-resistance. In the present study, IL-8 mRNA was inducible in PANC-1 cells after paclitaxel treatment. In contrast, both basal and paclitaxel-inducible levels of IL-8 mRNA in doxycycline-pretreated cells were effectively inhibited. Interestingly, inhibitory effects of doxycycline on IL-8 synthesis for paclitaxel treatment could be achieved at a low dose (5 μg/ml). Importantly, a daily oral dose of 800 mg, which is tolerated by most patients, can maintain serum levels of doxycycline between 5 and 10 μg/ml. Although further investigation is required to determine whether doxycycline has synergetic properties with anticancer agents, our findings suggest that doxycycline could be used as an adjunct for the treatment of chemoresistant cancer cells.

In conclusion, doxycycline exhibited cytotoxic effects in pancreatic cancer PANC-1 cells and a mouse xenograft model. Doxycycline-mediated apoptosis seems to be involved in the inhibitory effects of doxycycline on IL-8 synthesis.
in several apoptotic signal pathways, including caspase activation and the p53 signal pathways. Additionally, low-dose doxycycline blocked paclitaxel-induced IL-8 expression. Further studies are warranted to determine the potential efficacy of doxycycline as an anticancer agent as well as an enhancer of conventional chemotherapeutic agents.

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References


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