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Doxycycline Enhances the Ras-MAPK Signaling and Proliferation of Mouse Thymic Epithelial Cells

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ABSTRACT

Depletion of T-cell-dependent immunity is a major consideration for patients suffering from infections of human immunodeficiency virus (HIV), those undergoing organ transplantation, and those receiving anti-cancer chemotherapy and/or radiotherapy. In general, T-cell regeneration occurs in the thymus through thymopoiesis. We have found that doxycycline (Dox), a tetracycline derivative, enhances the proliferation of mouse thymic epithelial cells, which are unique in their capacity to support positive selection and are essential throughout the development of thymocytes. Cell cycle analysis indicates that the increased cell proliferation is due to a shortened G_0/G_1 phase. To reveal the underlying mechanisms, we examined the expression of an array of molecules that regulate the cell cycle. The results show that in mouse thymic medullary-type epithelial cell line 1 (MTEC1) Dox leads to elevated levels of H-Ras, phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), cyclin E, cyclin dependent kinase 4/2 (CDK4/CDK2), E2F3, and c-myc. These data, and the observation that the proliferation-enhancing effect is largely abolished following treatment with an ERK inhibitor support an active role of the Ras-ERK/mitogen-activated protein kinase (MAPK) signaling pathway. In conclusion, the present study reveals a new activity of an old family of antibiotics. The in vivo effect of Dox on immune reconstitution warrants further exploration. J. Cell. Biochem. 107: 494–503, 2009.

KEY WORDS: DOXCYCLINE; MOUSE THYMIC EPITHELIAL CELLS; CELL PROLIFERATION; MAPK

The acquired immunodeficiency syndrome (AIDS) is a set of symptoms and infections resulting from the damage to the human immune system caused by the human immunodeficiency virus (HIV) [Weiss, 1993]. While progressive depletion of $CD4^+$ T-cells is characteristic of the impact of HIV on the immune system, HIV-infected patients often lose their lymphoproliferative responses to antigens, alloantigens, and mitogens as they progress from the early to advanced stages of the disease. Furthermore, thymocytes are infected with HIV and destroyed during the infection [Al-Harthi and Landay, 2002]. Thus, immune reconstitution is necessary to reverse the effects of HIV on the immune system, so that normal levels of functional T-cells can be produced. As in HIV-infected patients, those who are subjected to transplantation or to anti-cancer chemotherapy may develop an immune deficiency or immune dysfunction. A characteristic of immune deficiency following transplantation or dose-intensive chemotherapy or radiotherapy is the depletion of $CD4^+$ T-cells. Chemotherapy, radiotherapy, and transplant regimens often result in a severe and protracted lymphopenia. The recovery of T-cell populations is delayed, relative to that for myeloid, natural killer cells (NK) or B cells [Hakim et al., 1997].

The thymus is primarily a lymphoid organ dedicated to the development and maturation of T lymphocytes. Within the thymus,

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bone marrow-derived precursors undergo a complex process of cell proliferation, T-cell receptor (TCR) gene rearrangement, TCR repertoire selection and functional maturation, eventually leading to positively selected thymocytes [Fischer and Malissen, 1998; Sant'Angelo et al., 1998]. Most $CD4^+CD8^+$ double-positive (DP) immature thymocytes are cortically located, whereas $CD4^+$ and $CD8^+$ single-positive cells are found in the medulla. The medullary thymocytes undergo further maturation before being exported to the periphery [Anderson et al., 1996; Li et al., 2007; Jin et al., 2008]. This developmental program depends upon interactions with stromal cells such as thymic epithelial cells (TECs), which provide signals for T-cell proliferation, survival, and differentiation. Lymphostromal interactions within the thymus not only result in the normal development of T-cells, but also control the development of the thymic microenvironment [van Ewijk et al., 1994]. Thus, TECs have an important function in the maintenance of T-cell number and balance of immune function.

Doxycycline (Dox), a tetracycline antibiotic, is a potent, nonselective inhibitor of matrix metalloproteinases (MMPs). Due to their anti-tumor, anti-metastatic, anti-inflammatory, and anti-angiogeneic properties, tetracyclines are widely used in clinical research. By inhibiting MMPs, tetracyclines suppress tumor invasion and metastasis [Lokeshwar et al., 2002]. They also induce apoptosis of tumor cells via activation of caspases and up-regulation of Fas/FasL expression [Liu et al., 1999; Iwasaki et al., 2002; D'Agostino et al., 2003]. Tetracyclines demonstrate regulatory effects on inflammatory responses. In addition to suppressing MMP activity associated with inflammatory conditions [Nordstrom et al., 1998; Grenier et al., 2002], they inhibit the expression and enzymatic activity of nitric oxide synthase, down-regulate the interleukin-1β-converting enzyme expression [Cillari et al., 1998; D'Agostino et al., 2001; Suk, 2004; Lai and Todd, 2006], and block the generation of oxygen radicals [Gabler and Creamer, 1991; Soory, 2007]. Dox is therefore being evaluated for its therapeutic potential in diseases such as metastatic cancer, rheumatoid arthritis, periodontal disease, cardiac hypertrophy, and aortic aneurysm [Nordstrom et al., 1998; Liu et al., 1999; Grenier et al., 2002; Iwasaki et al., 2002; Lokeshwar et al., 2002; D'Agostino et al., 2003; Sho et al., 2004; Soory, 2007; Errami et al., 2008].

Although Dox has many clinical applications, its influence on thymic stromal cells and immune function is not clear. Answers to such questions should allow a better understanding of its potential as an anti-cancer and anti-inflammatory agent. The aim of the present study was to explore the effect of Dox on mouse thymic epithelial cell line 1 (MTEC1). The results show that Dox enhances the proliferation of MTEC1 cells by shortening the G_0/G_1 phase of the cell cycle. The activation of the Ras-ERK/mitogen-activated protein kinase (MAPK) signaling pathway was at least partly responsible for this effect.

MATERIALS AND METHODS

CELL CULTURE

MTEC1, a mouse thymic medullary-type epithelial cell line established in our laboratory [Liu et al., 1996], supports the functional maturation of CD4 single-positive thymocytes in vitro [Ge and Chen, 2000]. These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Shanghai, China) containing 10% fetal calf serum (FCS; Invitrogen).

REAGENTS

Dox, NP-40, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were obtained from Sigma–Aldrich Corporation (Shanghai, China). Horseradish peroxidase-conjugated goat anti-rabbit IgG, Monoclonal rabbit anti-Ras, anti-ERK1/2, anti-phospho-ERK1/2, anti-cyclin D1, anti-c-myc, and anti-GAPDH antibodies were purchased from Cell Signaling Technology (Shanghai, China). Polyclonal rabbit anti-cyclin E antibody was obtained from Santa Cruz Biotechnology (Shanghai, China). [^{3}H]-Thymidine ([^{3}H]-TdR; specific activity, 60 Ci/mM) was purchased from the China Institute of Atomic Energy. Stock solutions of Dox (10 mg/ml) were prepared in sterile, phosphate-buffered saline (PBS, pH 7.2). The ERK inhibitor U0126 was purchased from Cell Signaling Technology, and dissolved in DMSO (10 mM). The final concentration of DMSO in the culture medium was 0.1%.

CELL PROLIFERATION ASSAY

The effect of Dox on MTEC1 cells was measured by the [^{3}H]-TdR incorporation assay, performed as previously described [Fife et al., 1998]. Briefly, MTEC1 cells were seeded into 96-well plates at a density of 5×10^4/well. Dox in serial dilutions (0.1, 1, 10, and 100 μg/ml) was added to the cultures, with triplicate wells for each concentration. Cells were cultured for 24, 48, or 72 h, and [^{3}H]-TdR (0.5 μCi/well) was added to cells for the final 12 h. Cells were collected on filter paper, and the mean counts per minute of [^{3}H]-TdR incorporated was calculated by liquid scintillation β counter (Beckman-Coulter). All experiments were repeated three times.

ANALYSIS OF THE KINETICS OF CELL GROWTH

MTEC1 cells were cultured in 10% FCS–DMEM in 50 ml flasks and harvested when their growth approached 80% confluence. The cells were seeded into 24-well plates at a density of 1×10^3/well and were exposed to Dox (0, 0.1, 1, or 10 μg/ml) for 8 days. Triplicate cultures were set up for each experimental group. The cell number was determined daily and plotted against time of culture.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

To observe Dox-induced changes in cell cycle progression, flow cytometry was used to determine the percentage of cells in each individual phase as previously described [Robison et al., 2007]. Briefly, 3×10^5 cells were seeded in 50 ml flasks and cultured in 10% FCS–DMEM. After overnight incubation, the cells were serum-starved in 0.5% FCS–DMEM for 36 h for synchronization in the G1 phase. The culture was shifted back into 10% FCS–DMEM with or without Dox (10 μg/ml) for 24 h. Subsequently, cells were detached with 0.1% EDTA–trypsin, washed with 5% FCS–PBS, fixed with cold 70% ethanol, and stored at 4°C until assay. The cells were then incubated for 30 min at room temperature in the dark with a staining a mixture containing PI (50 μg/ml) and RNase (100 μg/ml). DNA

contents of the stained nuclei were determined with a flow cytometer (BD FACS Calibur System). Debris and aggregates were gated out during data acquisition, and 30,000 gated events were collected for each sample. ModFit LT software was used for cell cycle analysis of the DNA histograms. Experiments were repeated three times.

TOTAL RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Cultures of MTEC1 cells in 10% FBS in the presence or absence of Dox (10 μg/ml) were used as a source of RNA. Total RNA (1 μg), isolated with the TRIZOL reagent (Invitrogen), was used for reverse transcriptase-PCR (TOYOBO) utilizing an oligo(dT) primer for cDNA synthesis. The amplification reactions were performed in a final volume of 20 μl containing SYBR Green (0.5 μl, diluted at 1:1,000, Invitrogen), rTaq polymerase (0.2 μl, 5 μM, Takara), $MgCl_2$ (0.8 μl, 25 μM), sense and anti-sense primers (0.3 μl, 10 mM), and cDNA (1 μl). Primer sequences are listed in Table I. Each value was normalized to the expression of β-actin. Values presented are the means ± SD. of triplicate measurements.

SDS–POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

MTEC1 cells (4×10^6) were exposed to Dox, harvested at the indicated time points, washed twice with ice-cold PBS and lysed with RIPA lysis buffer [Tris–HCl (50 mM, pH 7.4); NaCl (150 mM); Triton X-100 (1%); sodium deoxycholate (1%); SDS (0.1%); and EDTA (2 mM)] supplemented with a mixture of phosphatase and protease inhibitors (sodium orthovanadate, sodium fluoride, leupeptin, and phenylmethylsulphonyl fluoride). Equal amounts (50 μg) of proteins were loaded and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto a polyvinylidene difluoride membrane (Millipore, Shanghai, China). The membrane, blocked with 5% skim-milk, was incubated overnight at 4°C with primary antibodies to Ras, ERK1/2, phospho-ERK1/2, cyclin D1, cyclin E, c-myc, and GAPDH. After washing in 1× Tris–buffered saline with Tween-20 (0.1%), the bound primary antibodies were detected with 1:1,000 diluted horseradish peroxidase-conjugated secondary antibodies and visualized with the enhanced chemiluminescence detection system. Bands were detected with the Typhoon 9000 scan system (GE Healthcare, Shanghai, China). Experiments were repeated at least three times.

STATISTICAL ANALYSIS

The data are presented as group mean ± SD. Differences between groups were assessed by one-way analysis of variance followed by Dunnett's test. A P-value of 0.05 was considered significant.

RESULTS

DOX-INDUCED ACCELERATED PROLIFERATION OF MTEC1 CELLS

MTEC1, a mouse thymic epithelial cell line of medullary origin, has the unique property of supporting the maturation of CD4 single-positive thymocytes in vitro [Liu et al., 1996; Li et al., 2007]. In our studies of the molecular mechanisms underlying the functional maturation of CD4 single-positive thymocytes, we attempted to achieve inducible gene expression in MTEC1 cells using the Tet-on system with Dox as an inducer. Surprisingly, Dox was found to stimulate the growth of MTEC1 on its own. This observation prompted us to further explore the dose–response, the magnitude, and the time course of Dox-promoted MTEC1 cell proliferation. In the range of 0.1–100 μg/ml, Dox enhanced MTEC1 cell proliferation in comparison to the control; the maximum effect was at 10 μg/ml (Fig. 1A). At this concentration, cells proliferation increased by 2.27-, 5.04-, and 3.58-fold after 24, 48, and 72 h treatment, respectively. The proliferation-enhancing effect tended to decline when the concentration of Dox was further increased to 100 μg/ml, possibly due to drug-associated cytotoxicity. Thus, a concentration of 10 μg/ml was used in the following experiments. Subsequently, we analyzed the kinetics of cell growth under culture with Dox. With an initial cell density of 1×10^3/well in a 24-well plate, the growth of MTEC1 cells reached the highest level on day 6 of culture in the absence of Dox or at the low concentration of Dox (0.1 μg/ml); afterward, the cell numbers declined. In contrast, when cultured with higher concentrations of Dox (1 or 10 μg/ml), cells increased in number with an approximate doubling time of 24 h in the first 5 days of culture; from days 5 to 8, exponential growth was observed (Fig. 1B).

DOX-INDUCED CHANGES IN THE G_0/G_1 PHASE OF THE CELL CYCLE

The effect of Dox on the cell cycle progression of MTEC1 cells was determined by flow cytometry. In the presence of Dox (10 μg/ml), cells in G_0/G_1 phase decreased from 45% to 37%, and those in the S phase increased from 39% to 48% (Fig. 2). The representative data from three independent experiments demonstrated that both the decrease in G_1 phase and the increase in S phase were statistically significant ($P < 0.05$). This suggested that exposure to Dox led to a

TABLE I. Primer Sequences

Primer	Sequence (5′–3′)	Cycles	Annealing (°C)
β-Actin (Genebank#NM_007393)	Sense: TGGAATCCTGTGGCATCCATGAAAC; antisense: TAAAACGCAGCTCAGTAACAGTCCG	25	58
H-Ras (Genebank#NM_008284)	Sense: GACTCCTACCGGAAACAGGT; anti-sense: TGATGGATGTCCTCGAAGGA	35	56
Cyclin D1 (Genebank#NM_007631)	Sense: TGGAGCCCCTGAAGAAGAG; anti-sense: AAGTGCGTTGTGCGGTAGC	30	56
CDK4 (Genebank#NM_009870)	Sense: CTGCCACTCGATATGAACCCG; antisense: TAGATCCTTAATGGTCTCAACCG	30	58
Cyclin E (Genebank#NM_007633)	Sense: AGTGGCCTATGTCAACGACACG; anti-sense: CCACTGTCTTTGGAGGCAATG	35	56
CDK2 (Genebank#NM_016756)	Sense: TGAATTTCTGCACCAGGACCTC; anti-sense: AATCTTGCCGAGCCCACTTG	35	60
C-myc (Genebank#NM_010849)	Sense: CTGTATGTGGAGCGGTTTCT; anti-sense: TGCTGTCGTTGAGCGGGTA	35	59
E2F3 (Genebank#NM_010093)	Sense: CCAAGACCACAATGGGAATA; antisense: GGACAAACTGCGATACACG	35	59

The β-actin gene was used as an internal standard to normalize the amount of total RNA present in each reaction.

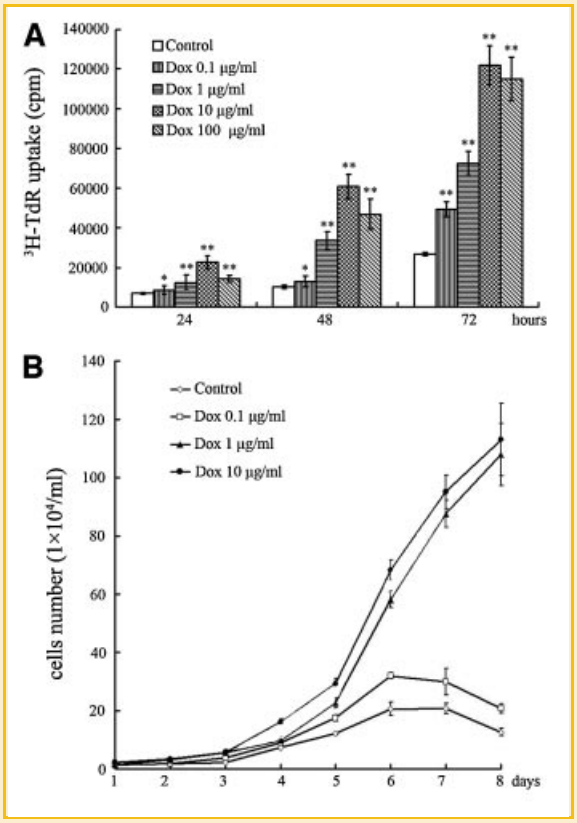

Fig. 1. The accelerated proliferation of MTEC1 cells by Dox. A: MTEC1 cells were seeded in 200 μl of medium at a density of 5 × 104/well in 96-well plates and cultured with Dox (0, 0.1, 1, 10, or 100 μg/ml) for 24, 48, or 72 h. The cells were pulsed with [^{3}H]-TdR (0.5 μCi/well) for 12 h, then harvested and assayed by liquid scintillation counting. The value of counts per min (cpm) was used to represent the extent of cell proliferation. Data are presented as mean ± SD as indicated by the error bar. $^*P < 0.05$, $^{**}P < 0.01$ compared with cultures in medium alone. B: MTEC1 cells were seeded at an initial density of 1 × 103/ml/well and cultured in the presence of Dox (0, 0.1, 1 or 10 μg/ml) for 8 days. Cell numbers were recorded daily and plotted against days of culture.

shortened G_0/G_1 phase and to an increased percentage of cells in S phase.

EFFECT OF DOX ON EXPRESSION OF CYCLINS AND CYCLIN-DEPENDENT KINASES

Since the transition from the G_1 to the S phase is regulated by the checkpoint kinases CDK2/CDK4 in association with cyclin E/cyclin D1 [Blagosklonny, 1999; Shapiro, 2006], the effects of Dox on the expression of cyclin D1, cyclin E, CDK2, and CDK4 in MTEC1 cells were examined. The mRNA expression of cyclin E, CDK2, and CDK4 was found to be significantly up-regulated, peaking at 30 min after exposure to Dox. The level of cyclin D1 mRNA, on the other hand, was not significantly altered (Fig. 3A). Western blot analysis revealed a similar pattern of protein expression for cyclin E and cyclin D1, with elevated levels of cyclin E but virtually unaffected cyclin D1 expression (Fig. 3B,C). It is interesting to note the time lag between the up-regulation of cyclin E protein versus that of cyclin E mRNA, suggesting the regulation is likely to be at the level of transcription.

INVOLVEMENT OF THE RAS-MAPK SIGNALING PATHWAY IN DOX-INDUCED PROLIFERATION OF MTEC1 CELLS

The Ras-Raf-MAP kinase signaling pathway has been implicated in the proliferative response of cells to a variety of stimuli [Malumbres and Pellicer, 1998; Perez-Sala and Rebollo, 1999], we next investigated whether exposure to Dox regulated the expression levels or the activation status of some critical components of this pathway. Real-time PCR revealed significant up-regulation of H-Ras mRNA at 30 min or 8 h (Fig. 4A). A similar increase was also seen at the protein level (Fig. 4B). Furthermore, Western blot analysis demonstrated that treatment with Dox resulted in the progressive accumulation of phosphorylated ERK1/2 (p-ERK1/2), which reached the highest levels between 1 and 2 h (Fig. 4C).

In addition to the upstream events, we analyzed the downstream genes activated by G_0/G_1 cyclins and cyclin-dependent kinases. Quantitative PCR indicated that both E2F3 and c-myc transcripts were elevated in MTEC1 cells treated with Dox (Fig. 4A). Western blotting further showed an abrupt and dramatic increase of the c-myc protein between 2 and 4 h after Dox treatment (Fig. 4B).

To evaluate the contribution of the Ras-MAPK signaling pathway to the accelerated cell proliferation induced by Dox, cells were pretreated with the ERK inhibitor U0126 (0.1, 1, or 10 μM) prior to the exposure to Dox. In comparison to the virtually normal proliferative response of vehicle-treated cells, Dox-induced proliferation was progressively suppressed with increasing concentration of U0126. However, the inhibition is not complete as the proliferation was still higher than the basal level even in the presence of 10 μM U0126 (Fig. 5). Therefore, activation of ERK seems to be partly responsible for the proliferation-enhancing effect of Dox.

We also examined the potential effects of ERK inhibition on the molecular changes induced by Dox. As shown in Figure 6A, the Dox-induced increase in mRNA expression of cyclin E, CDK2, CDK4, Ras, E2F3, and c-myc was all reverted following pretreatment with U0126. Western blot analysis indicated a similar inhibitory effect of U0126 on the expression of cylin E, Ras, and c-myc proteins (Fig. 6B). Notably, the phosphorylation of ERK was also seen to be suppressed in Dox-treated MTEC1 cells (Fig. 6C). Again, these data support an important role of ERK signaling in Dox-mediated effects.

DISCUSSION

Tetracycline and its derivatives exert effects that are distinct from their anti-microbial activities. In tumor cell lines, Dox causes arrest of cell growth in colorectal cancer, breast carcinoma, osteosarcoma, melanoma, and prostate cancer cells [Fife and Sledge, 1995; Fife et al., 1997; Fife et al., 1998; Onoda et al., 2004; Sun et al., 2007]. Furthermore, semi-synthetic tetracyclines, such as Dox and minocycline, induce cell proliferation and differentiation of human bone marrow osteoblastic cells [Gomes and Fernandes, 2007; Gomes et al., 2008]. In chemotherapy and radiotherapy, loss of TECs is the

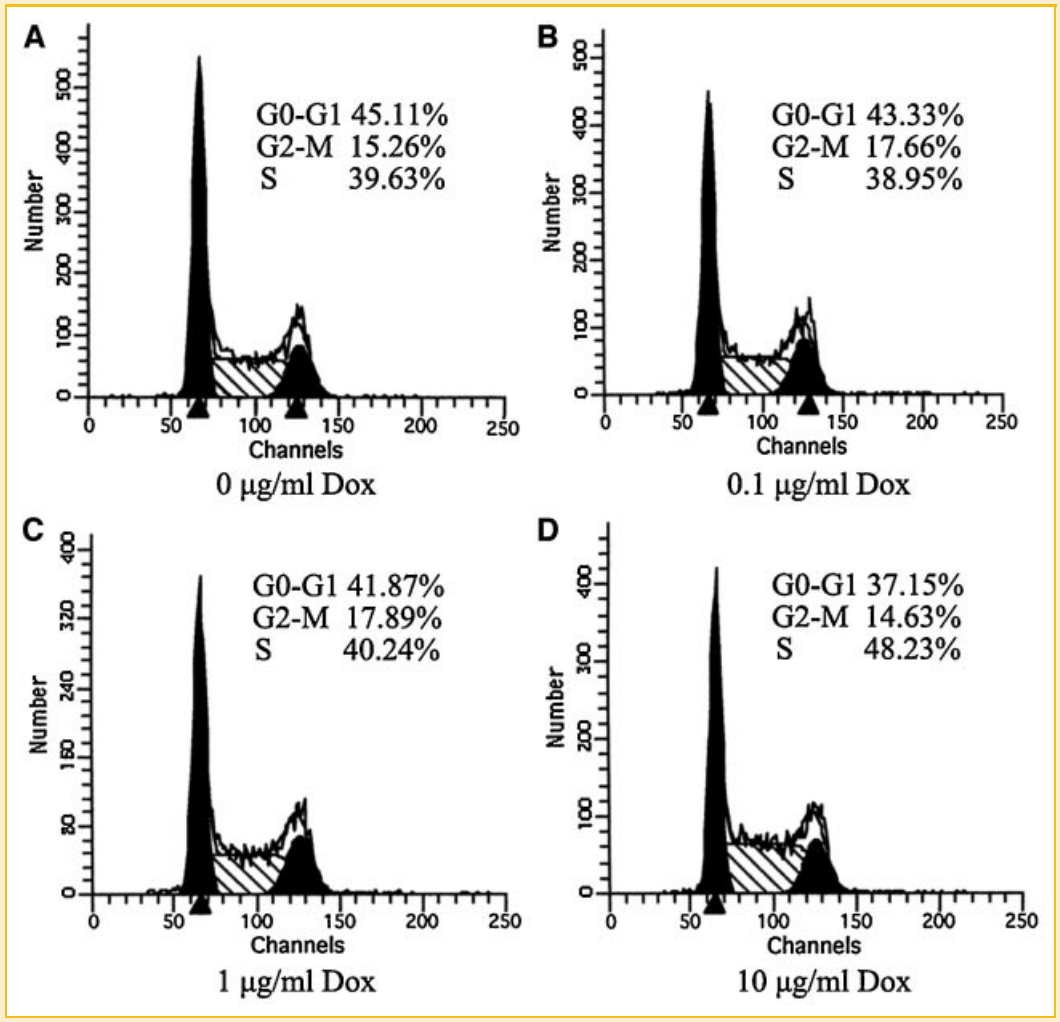

Fig. 2. Cell cycle analysis by flow cytometry of MTEC1 cells with or without exposure to Dox. MTEC1 cells were serum-starved for 36 h, switched back to medium containing 10% FCS, and cultured for additional 24 h with or without different concentrations of Dox. Cellular DNA content was determined with PI staining by flow cytometry, and the data were analyzed using ModFit LT program. The representative results of one out of three independent experiments are shown. A,B,C,D: Dox was added to 0, 0.1, 1, and 10 μg/ml, respectively. The percentage of cells at the G_0–G_1, G_2–M and S phases is indicated at the top-right corner.

major reason for the deficiency of T lymphocytes. In SCID mice, the abnormal organization of thymic medullary epithelial cells, which can be corrected by the introduction of wild-type bone marrow, causes a lack of production of functional T- and B-cells with antigenic receptors [Bosma et al., 1983; Shores et al., 1991]. Moreover, in mouse acute graft-versus-host disease caused by bone marrow transplataion, major histocompatability complex-mismatched donor T-cells home into the thymus of unconditioned recipients. There, activated donor T-cells secrete IFN-γ, which in turn stimulates the programmed cell death of TECs. For these reasons, reconstitution of T lymphocytes should protect the TECs [Hauri-Hohl et al., 2007].

Our findings of the proliferation-enhancing effect of Dox on MTEC1 cells revealed a new activity of the tetracycline family. The mechanisms behind the actions of Dox on MTEC1 remain to be fully defined. Cell cycle analysis indicated that the enhanced proliferation of MTEC1 cells in the presence of Dox was essentially due to the accelerated transition from the G_1 to the S phase, as suggested by a shortened G1 phase, a reduced number of G1 cells, and a concomitant increase of cells in the S phase (Fig. 2).

Cell cycle progression requires a coordinated expression of and interactions among different cyclins and CDKs. Cyclin E/D1, which bind to CDK2/4, are involved in cell cycle progression from the G_1 to the S phase [Shapiro, 2006]. Cyclin E/D1 binds and activates CDK2/4, and the complexes of cyclin D1/CDK4 and cyclin E/CDK2 phosphorylate the retinoblastoma (Rb) protein at different serine and threonine residues. Hyperphosphorylated Rb, no longer binding to the transcription factors of E2F family, releases the heterodimeric transcription factor complex E2F-DP [Botz et al., 1996; Geng et al., 1996]. E2F-DP complexes contribute to the activation of an array of genes that are necessary for the G1–S transition, including genes encoding DNA synthesis enzymes and protooncogenes, such as

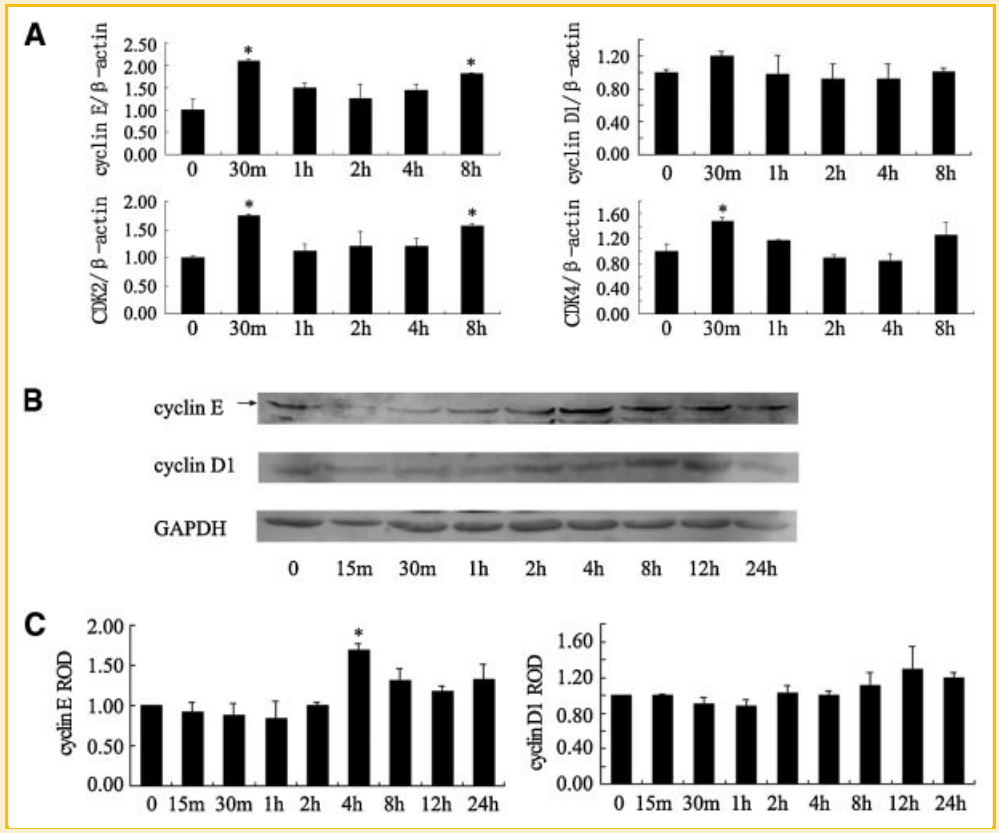

Fig. 3. Cyclin and cyclin-dependent kinase expression in Dox-treated MTEC1 cells. MTEC1 cells were treated with Dox (10 μg/ml) for time indicated. Total RNA and protein were extracted and examined for cylin and CDK expression. A: Real-time PCR analysis for cyclin E, CDK2, cyclin D1, and CDK4 versus β-actin mRNA expression. The ratio of the target gene versus β-actin at time 0 was set as 1. Data from three independent experiments are presented as means ± SD. B: Western blot analysis for cyclin E and D1. A representative gel out of three is shown. GAPDH was included as a loading control. C: Densitometry analysis of the Western blots for cylin E and D1. The ratio of the target protein versus GAPDH at time 0 was set as 1. Data from three independent experiments are presented as means ± SD. $^{*}P < 0.05$ versus time 0. ROD: relative optical density.

c-myc. The cell cycle thereby progresses to the DNA synthesis phase [Joyce et al., 2001]. In Dox-treated MTEC1 cells, cyclin E, CDK2, and CDK4, and some downstream molecules (E2F3 and c-myc) were found to be significantly up-regulated (Figs. 3, 4A,B, and 6A,B).

Mitogen-activated protein (MAP) kinases, especially ERK1/2, are involved in cell survival and proliferation, and drive the expression of cyclin D1/E. In MTEC1 cells, Dox up-regulates Ras expression and increases phosphorylation of ERK1/2 (Fig. 4). On the contrary, prior exposure to U0126, an inhibitor of ERK1/2, causes the significant decreases in cell proliferation, Ras expression, and ERK phosphorylation (Figs. 5 and 6A–C). Ras proteins are essential components of signaling pathways that link the activation of cell surface receptors with transcriptional events leading to the control of proliferation, differentiation, and apoptosis [Perez-Sala and Rebollo, 1999]. GTP-bound Ras binds cytoplasmic Raf-1 and translocates it to the plasma membrane. Activated Raf phosphorylates MAP kinase-extracellular signal-regulated kinase (MEK), which in turn phosphorylates and activates the ERK kinases. This Ras-Raf-MEK-ERK signaling pathway is involved in cell proliferation driven by various mitogens [Karnoub and Weinberg, 2008].

On the basis of our findings, we propose a model for the enhanced proliferation of MTEC1 cells by Dox. In cells exposed to Dox, induced Ras expression initiates a cascade of signaling steps: the phosphorylation of ERK/MAP kinase, induction and formation of cyclin D1/CDK4 and cyclin E/CDK2 complexes, release of the transcription factor E2F-DP, and the activation of genes directly involved in G_1–S progression. These events lead to an accelerated transition from the G_1 to the S phase, thereby shortening the cell cycle time and enhancing cell proliferation. The highly conserved MAP kinase cascades, which regulate growth, apoptosis, and differentiation of peripheral T-cells, are also involved in thymocyte selection, both negative [Bommhardt et al., 2000] and positive [McNeil et al., 2005]. Thus, we speculate that Dox, used as an immune regulator, may regulate the positive selection and negative selection progression of T-cells by inducing the proliferation of TECs via the Ras-MAPK signaling pathway. It may also contribute to immune regulation and immune reconstitution.

A subgroup of highly active anti-retroviral therapy (HAART)-treated, HIV-infected patients; immunosuppressant-treated patients; or chemotherapy-treated patients exhibit paradoxical deterioration in their clinical status. This clinical deterioration, known as the immune restoration syndrome or immune reconstitution inflammatory syndrome [Robertson et al., 2006; Singh, 2008], is a result of an enhanced inflammatory response towards previously diagnosed

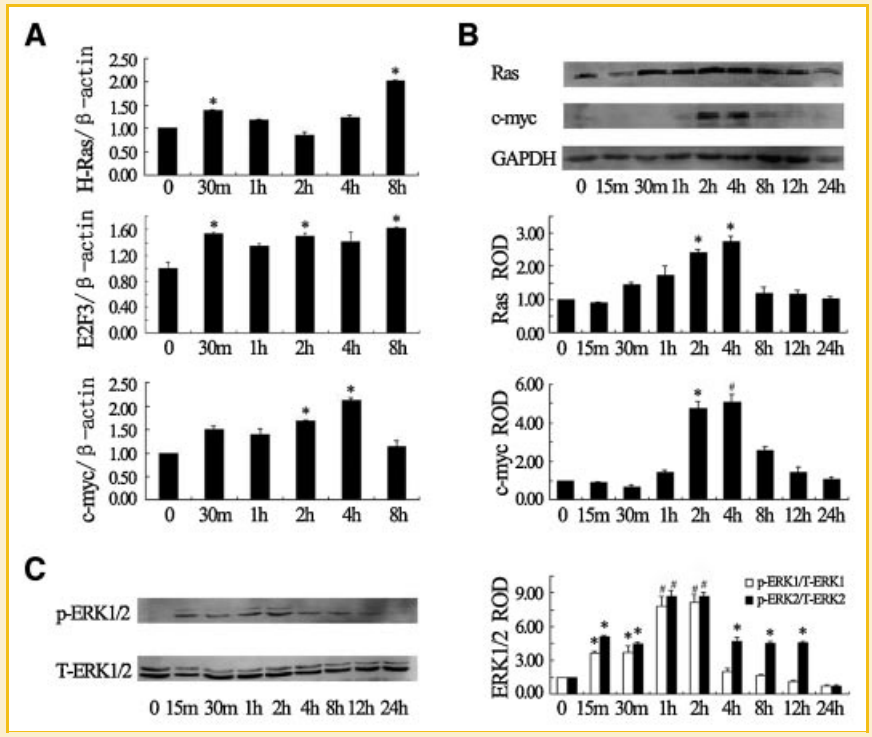

Fig. 4. H-Ras, E2F3, and c-myc expression and ERK phosphorylation in Dox-treated MTEC1 cells. MTEC1 cells were treated with Dox (10 μg/ml) for time indicated. Total RNA and protein were prepared for further analysis. A: Real-time PCR analysis for H-Ras, E2F3, and c-myc versus β-actin mRNA expression. The ratio of the target gene versus β-actin at time 0 was set as 1. Data from three independent experiments are presented as means ± SD. B: Western blot analysis and densitometry analysis for Ras and c-myc. A representative gel out of three is shown. GAPDH was included as a loading control. The ratio of the target protein versus GAPDH at time 0 was set as 1. C: Western blot analysis and densitometry analysis for total and phosphorylated ERK1/2. A representative gel out of three is shown. The ratio of the p-ERK1/2 versus T-ERK1/2 at time 0 was set as 1. Data from three independent experiments are presented as means ± SD. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus time 0. ROD: relative optical density.

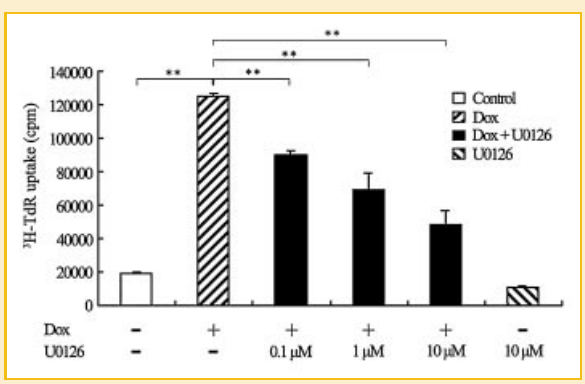

Fig. 5. Inhibition of the proliferation-enhancing effect of Dox by the ERK inhibitor U0126. MTEC1 cells were pretreated with different concentration of U0126 (0, 0.1, 1, or 10 μM) for 2 h prior to the addition of Dox (10 μg/ml). The cultures were carried on for another 72 h, and the cell proliferation was assayed by [^{3}H]-TdR incorporation. To control the vehicle effect, the concentration of DMSO (solvent for U0126) was adjusted to 0.1% in all cultures. Data from three independent experiments are presented as means ± SD. $^{**}P < 0.01$ versus Dox alone.

or incubating opportunistic pathogens, including fungus, mycoplasma, chlamydia, *Mycobacterium tuberculosis*, and non-tuberculous mycobacteria. Dox is relatively effective for these opportunistic pathogens, even for naturally multi-drug resistant species [Kang-Birken and Prichard, 2006; Rallis and Koumantaki-Mathioudaki, 2007].

T-cell reconstitution following lymphopenia caused by chemotherapy or transplantation is often slow and incompetent, contributing to the development of infectious diseases, relapse, and graft-versus-host disease. This is due to the fact that de novo T-cell production is impaired following cytoreductive regimens [Hakim et al., 1997]. Innate immunity (e.g., epithelial barriers, phagocytes, NK cells) typically recovers within weeks post-transplant, but the recovery of adaptive immunity takes longer, months for B-cells and years for T-cells [Storek, 2008], leading to persistently abnormal CD4:CD8 ratios [Novitzky et al., 2002]. Given the important role of TECs in the production of T lymphocytes, we speculate that Dox may find its applications in T-cell immune reconstitution through stimulating the proliferation of TECs.

In summary, our studies demonstrate that Dox reduces transition time from the G1 to the S phase and leads to accelerated proliferation of MTEC1 cells, possibly through the enhanced Ras-MAPK. Further exploration of its effect in vivo and potential influence on immune

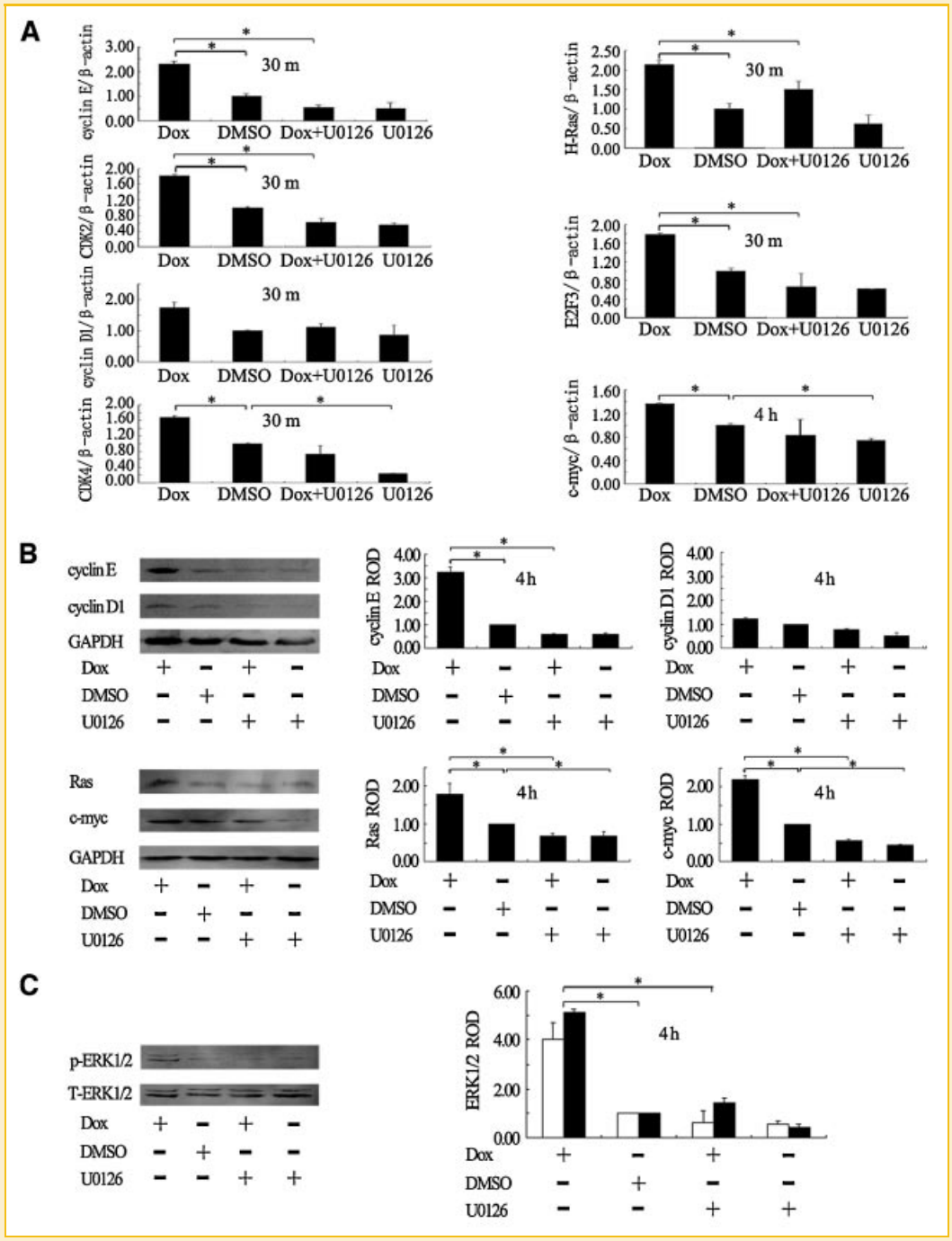

Fig. 6. Inhibition of the Dox-induced molecular events by the ERK inhibitor U0126. MTEC1 cells were pretreated with U0126 (10 μM) for 2 h. After the addition of Dox (10 μg/ml), cells were cultured for an additional period as indicated. Total RNA and protein were prepared for further analysis. Controls include untreated cells (DMSO) and cells treated with U0126 or Dox alone. All cultures contained 0.1% DMSO. A: Real-time PCR analysis for cyclin E, CDK2, cyclin D1, CDK4, H-Ras, E2F3, and c-myc versus β-actin mRNA expression. The ratio of the target gene versus β-actin in DMSO was set as 1. Data from three independent experiments are presented as means ± SD. B: Western blot analysis and densitometry analysis for cyclin E and D1, Ras, and c-myc. A representative gel out of three is shown. GAPDH was included as a loading control. The ratio of the target protein versus GAPDH in DMSO was set as 1. C: Western blot analysis and densitometry analysis for total and phosphorylated ERK1/2. A representative gel out of three is shown. The ratio of the p-ERK1/2 versus T-ERK1/2 in DMSO was set as 1. Data from three independent experiments are presented as means ± SD. $^{*}P < 0\ 05$. ROD: relative optical density.

reconstitution may provide a strategy for dealing with immunosuppression and immunodeficiency.

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