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Biochemical Pharmacology 68 (2004) 729–738
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Participation of cyclin D1 deregulation in TNP-470-mediated cytostatic effect: involvement of senescence

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Received 20 November 2003; accepted 10 May 2004

Abstract

Inhibition of angiogenesis is becoming one promising, alternative approach to stop tumor from growth and spreading to distant organs. TNP-470, an analog of fumagillin, possesses potent anti-angiogenic effects with minimal toxicity in animal tumor models and is now in the phase III of human cancer trial. Although TNP-470 induced endothelial cell cycle arrest at G1 phase via p53 and p21(Cip1), the underlying mechanism of the cytostatic effect of TNP-470 on endothelial cells remains limited. We have found that TNP-470 did not only induce p53 and p21(Cip1) but also cyclin D1 in the basic fibroblast growth factors (bFGF)-treated endothelial cells. The TNP-470-mediated increase of cyclin D1 protein was due to the enhanced expression of mRNA. The induced cyclin D1 formed a complex with cyclin-dependent kinase4 (CDK4) and p21(Cip1). The ability of cyclin D1-associated CDK4 to phosphorylate retinoblastoma (Rb) protein was, however, reduced in the same cells. TNP-470 also significantly increased senescence-associated-β-galactosidase activity (SA-gal), hallmark of cells undergoing senescence. Interestingly, the effect of increased cyclin D1 protein mimicked by overexpression of cyclin D1 increased the sensitivity of human umbilical vein endothelial cells (HUVECs) to TNP-470. In summary, the cytostatic effect of TNP-470 on endothelial cells is in part mediated by induction of senescence and cyclin D1 is a key molecule participating in this event.

Keywords: Angiogenesis; Angiogenic inhibitor; Cell cycle; Endothelial cells; bFGF

1. Introduction

Angiogenesis, neovascularization from the pre-existing endothelium, is a complex, multi-step process including degradation of extracellular matrix, endothelial cell migration, proliferation and differentiation, and tube formation [1]. This process is indeed controlled by a finely tuned balance of angiogenesis inducers and inhibitors. The imbalance of this process has been associated with many pathological conditions including cancer [2]. Newly formed blood vessels not only do provide oxygen and nutrients for cancer cells but also a gateway for cancer cells to enter circulation then metastasize to distant organs. Recognition of the therapeutic potential for controlling the neovascularization has led to the search for safe and potent angiogenic inhibitors. A number of angiogenic inhibitors have been identified with the hope to treat vascular diseases like cancer.

Fumagillin, a metabolite of *Aspergillus fumigatus*, was discovered based on its potent endothelial cytostatic activity in vitro [3]. Both fumagillin and TNP-470 (AGM-1470, o-chloracetylcarbamoyl-fumagillol), a semi-synthetic derivative of fumagillin, possess potent anti-angiogenic activity to block solid tumor growth and metastasis except that TNP-470 manifested lower toxicity in animal studies. TNP-470 is currently under intensive investigation for the

Abbreviations: bFGF, basic fibroblast growth factors; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; Rb, retinoblastoma; PCNA, proliferation cell nuclear antigen; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SA-Gal, senescence-associated β-Gal

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doi:10.1016/j.bcp.2004.05.020

phase III anti-tumor trials and is considered to be one of the many leading anti-angiogenic compounds.

The inhibitory effect of TNP-470 on endothelial cell proliferation is relatively specific to endothelial cells and behaves in a biphasic manner, being cytostatic at dose lower than 3 μg/ml and cytotoxic at dose greater than 30 μg/ml [4]. TNP-470 does not inhibit early mitogenic events but inhibits retinoblastoma (Rb) phosphorylation, the activation of cell division cycle 2(CDC2) and cyclin-dependent kinase2 (CDK2). Little or no effect on the mRNA level of cyclin D1 was observed in same endothelial cells [5]. In contrast to this study, a 4 h-treatment with TNP-470 suppressed cyclin D1 mRNA expression in mid G1 phase [6]. The discrepancy with respect to the effect of TNP-470 on cyclin D1 mRNA remains to be clarified. Although methionine aminopeptidase-2 (MetAP2) is a target for TNP-470 and its covalent modification by TNP-470 leads to the loss of enzymatic activity, its role in regulating angiogenesis remains controversial [7–9]. Recently, the anti-angiogenic effect of TNP-470 requires p53 and p21(Cip1) [10,11]. Despite of many preclinical studies, the current knowledge about the molecular action of TNP-470 has not been fully elucidated.

D-type cyclin consisting of D1, D2 and D3 is one essential component of cell cycle machinery. Among the D-type cyclin, amplification of cyclin D1 gene has been found in various cancer types [12]. Activation of either Ras or Myc induces cyclin D1 expression [13,14], suggesting that cyclin D1 is a mediator of oncogenic transformation. Overexpression of cyclin D1, thus, promotes cell cycle progression via shortened G1-to-S transition [15,16]. However, in some cases, an increased level of cyclin D1 is a characteristic of arrested cells [17,18]. An increased level of cyclin D1 can, therefore, accelerate S1 entry or cause growth arrest depending on its cellular context. The expression of cyclin D1 is subjected to the stimulation by growth factors and tumor suppressor proteins like p53 and Rb [19–21]. Moreover, a proper growth arrest mediated by p53 often requires both p21(Cip1) and cyclin D1 [20,22].

With the importance of cyclin D1 being an key regulator for cell cycle progression and the requirement of p53 for TNP-470-mediated anti-angiogenesis, we investigated the role of cyclin D1 and other cell cycle mediators in exerting the cytostatic effect of TNP-470 on proliferating endothelial cells. Western blotting analysis in conjunction of immunoprecipitation and transient transfection were adopted to address possible involvement of cyclin D1 deregulation in TNP-470-mediated anti-angiogenic effect.

2. Materials and methods

2.1. Materials

TNP-470 was a generous gift from Takeda Chemical Industries Ltd., Japan. Human recombinant basic fibroblast growth factors (bFGF) was purchased from Pepro Tech. The plasmid pFLEX-D1 encoding a full-length cyclin D1 cDNA was a generous gift of Dr. C. Sherr at St. Jude Children's Research Hospital [23]. EGM2 for human umbilical vein endothelial cell (HUVECs) was from Bio-Wittaker. BSA, gelatin and all other chemicals were ordered from Sigma. Reagents and enzymes for PCR reaction and molecular biology, and CellTiter $96^{R}AQ_{ueous}$ One Solution Cell Proliferation Assay Kits were from Promega. Oligonucleotide primers were from MDbio Inc. Bio-Rad protein assay reagent and horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad. The antibodies to cyclin D1, p57(Kip2), CDK2, CDK4 and CDK6 were from Santa Cruz Biotechnology. The antibodies to p21(Cip1), p27(Kip1) and proliferation cell nuclear antigen (PCNA) were from BD Transduction Laboratories. The anti-α-tubulin antibody was from Neo Markers. The antibodies to p53, cyclin D1, and cyclin E were from Oncogene Research Products. Rb-C fusion protein corresponding to amino acids 706-928 of Rb was ordered from Cell Signaling Technology, Inc. Renaissance Chemiluminescence Reagent Plus was obtained from NEN Life Science Products.

2.2. Isolation and maintenance of HUVECs

Primary HUVECs were obtained from fresh human umbilical veins with a slight modification as previously described [24]. Isolated HUVECs, seeded in dishes precoated with 0.1% gelatin for 1 h, were routinely maintained in EGM2. To avoid genetic variation resulting from different individuals, HUVEC from five or more different donors were pooled together. HUVECs at no more than six passages were used for the following experiments.

2.3. Drug treatment

Subconfluent HUVECs at early passages were used for drug treatment. Following overnight incubation in the starvation medium [1% fetal bovine serum (FBS) and 0.1% BSA in M199], serum-starved cells were replenished with fresh starvation medium containing the indicated concentrations of TNP-470 or vehicle (0.1% dimethyl sulfoxide (DMSO)) for 1 h prior to incubation with 1 ng/ml of bFGF for the indicated time. Cells were then harvested for total RNA or protein isolation.

2.4. Cell proliferation assay

HUVECs at 5,000 cells/well were seeded in gelatin-coated 96-well tissue culture plate. Eight hours following seeding, HUVECs were serum-deprived in starvation medium for 12 h prior to the treatment. In the last hour of starvation, serum-starved cells were pretreated with various doses of TNP-470 (0.1–25 ng/ml) in starvation medium. DMSO (0.1%) was used as a vehicle control.

Following the pretreatment, 1 ng/ml of bFGF was used to stimulate HUVEC proliferation for 48 h prior to using Cell titer 96 one aqueous cell proliferation kit. Each treatment was seeded in quadruplicates. Each experiment was repeated three times.

2.5. RT-PCR followed by Southern hybridization

Serum-starved HUVECs were pretreated with 100 ng/ml of TNP-470 or 0.1% DMSO (vehicle control) followed by bFGF stimulation for the indicated times. Total RNA was isolated using TRIzol Reagents (Gibco/Invitrogen) based upon a modification of single-step RNA isolation [25]. Following treatment and total RNA isolation, 1 μg of DNA-free total RNA from sample was used as a template for reverse transcription using oligo $dT_{12\text{-}18}$ primers. The cDNA mix was then used a template for gene-specific PCR. The cDNA fragments encoding cyclin D1 and GADPH (housekeeping gene) were co-amplified in the same PCR reaction. The amplification cycle for each gene fragment was in the linear range. Following electrophoresis and membrane blotting, the membrane was hybridized with biotin-labeled probes using North2South™ Biotin Random Prime Kit as described by manufacture. Finally, the blot was detected using North2South™ Chemiluminescent Nucleic Acid Hybridization and Detection Kit followed by autoradiography. The gene-specific primers were as follows: cyclin D1 primers (5′-GAGCTCGGCCCCATAAATCAT-3′, 5′-CACTCAAGGTCCATCCCTCTG-3′); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′).

2.6. Western blotting analysis

Treated HUVECs were lysed in the boiled lysis buffer containing 1% sodium dodecyl sulfate (SDS) and 10 mM Tris-HCl (pH 7.4). Following brief sonication and centrifugation at 13,000 rpm for 10 min, the protein concentration in each lysate was measured by Bradford protein assay. Equal amounts of sample proteins were fractionated by SDS-PAGE then blotted onto PVDF membrane. The protein blot was hybridized with the primary antibody of interest then secondary antibody. The hybridized signals were then detected by Renaissance Chemiluminescence Reagent Plus followed by quantified with Fuji imaging system FLA3000 and its analysis software Image Gauge.

2.7. Preparation of nuclear and cytosolic proteins

Nuclear and cytosolic proteins were isolated from treated or control HUVECs as described [26]. HUVECs grown on 10 cm culture dishes were lysed in a lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% IGEPAL CA-630, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 100 μM phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 10 μg/ml aprotinin and 1 nM okadaic acid). The lysate was partitioned into supernatant and pellet by centrifugation at 5,400 rpm for 10 min at 4 °C. The supernatant containing cytosol was further centrifuged at 15,000 rpm for 15 min at 4 °C to obtain cytosolic fraction. The pellet containing nuclei was gently washed three times then resuspended in a washing buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 25 mM NaCl, 1 mM sodium orthovanadate, 50 mM NaF, 100 μM PMSF, 1 μg/ml leupeptin and 10 μg/ml aprotinin). The pellet resuspension was layered over a cushion of sucrose buffer (1 M sucrose, 1 mM sodium orthovanadate, 50 mM NaF, 100 μM PMSF, 1 μg/ml leupeptin and 10 μg/ml aprotinin) followed by centrifugation to remove non-sedimented cellular debris. The final pellet containing nuclei was extracted with a nuclear extraction buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 50 mM NaF, 100 μM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin and 1 nM okadaic acid). After extraction on ice for 30 min, the samples were centrifuged to collect supernatants containing nuclear proteins. Equal amounts of nuclear and cytosolic proteins were fractionated by SDS-PAGE for western blotting analysis.

2.8. Immunoprecipitation and in vitro kinase assay of cyclin D1-associated CDK activity

Following treatment for the indicated time, cellular extracts were prepared in a lysis buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 5 mM EDTA, pH 8.0, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM NaF, 100 μM PMSF, 1 μg/ml leupeptin and 10 ug/ml aprotinin). Cyclin D1-associated protein complex was immunoprecipitated with an anti-cyclin D1 antibody and protein A agarose. The cyclin D1 immunocomplex was then incubated in a kinase buffer containing 50 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, 10 μCi [γ-^{32}P] ATP and 5 μg of Rb-C fusion protein. Reactions were then incubated at room temperature for 30 min. Finally, the reaction mixtures were electrophoresed on a 10% SDS-PAGE followed by autoradiography.

2.9. Transient transfection by electroporation

Electroporation was used to transiently transfect plasmid DNA into HUVECs as previously described [27]. Briefly, HUVECs at a cell density of 2×10^6 were resuspended in M199 medium containing 10% FBS followed by electroporation at 270 V and 500 μF using GENE PULSER II (BioRad) and 20 μg of cyclin D1 plasmid DNA dissolved in electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, pH 7.0). Following transient transfection for

indicated time, cells were replated onto gelatin-coated 96 well plates for cell proliferation assay or lysed for isolation of protein lysates.

2.10. Senescence assay

Following the treatment with bFGF or bFGF and TNP-470 for 12 h, cells were fixed with 0.05 M glutaraldehyde at room temperature for 60 min. Then, the samples were washed three times with phosphate buffered saline and incubated for 24 h at 37 °C with senescence-associated β-Gal (SA-β-Gal) staining solution (1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, phosphate buffer, pH 6.0) [28]. The images following SA-β-Gal staining were recorded with a light phase contrast microscopy (OLYMPUS, IMT-2-21). Five random high power fields (200X) were chosen to count the percentage of blue cells in a total of 100 cells.

3. Results

3.1. TNP-470 at nanogram ranges dose-dependently inhibits HUVEC proliferation

Basic FGF is a potent mitogen for HUVEC. As low as 1 ng/ml, bFGF stimulated HUVEC proliferation by 2–3 folds [29]. To determine the optimal inhibitory dose of TNP-470 for proliferating endothelial cells, serum-deprived HUVECs were treated with 0–25 ng/ml of TNP-470 in the presence of bFGF at 1 ng/ml (Fig. 1A). TNP-470 significantly inhibited HUVEC proliferation. The inhibition was in a dose-dependent manner. Consistent with the reported cytostatic range (10 pg–3 μg/ml) for TNP-470 to inhibit endothelial cells [30], the IC_{50} of TNP-470 on HUVECs was approximately at 25 ng/ml in this study. To achieve a complete inhibition, we therefore used 100 ng/ml of TNP-470 to block bFGF-promoted HUVEC proliferation in the following experiments.

3.2. TNP-470 induces the expression of p53 and p21

Previous studies have shown that the anti-angiogenic effect of TNP-470 requires p53 and p21(Cip1) [9,31]. We used Western blotting analysis to investigate whether a cytostatic concentration of TNP-470 at 100 ng/ml also induced the expression of p53 and p21(Cip1) in the presence of bFGF (Fig. 1B). Basic FGF at 1 ng/ml slightly promoted the expression of p53 at 4 h post-treatment and the induced level of p53 was steadily increased thereafter. Treatment of the same cells with TNP-470 further increased the expression of p53. The increase of p53 was dramatic as early as 4 h. There was no further induction thereafter during the 24-h incubation (Fig. 1B, top panel). P21(Cip1) is one downstream target of p53. Like p53, TNP-470 increased the level of p21(Cip1) in the same

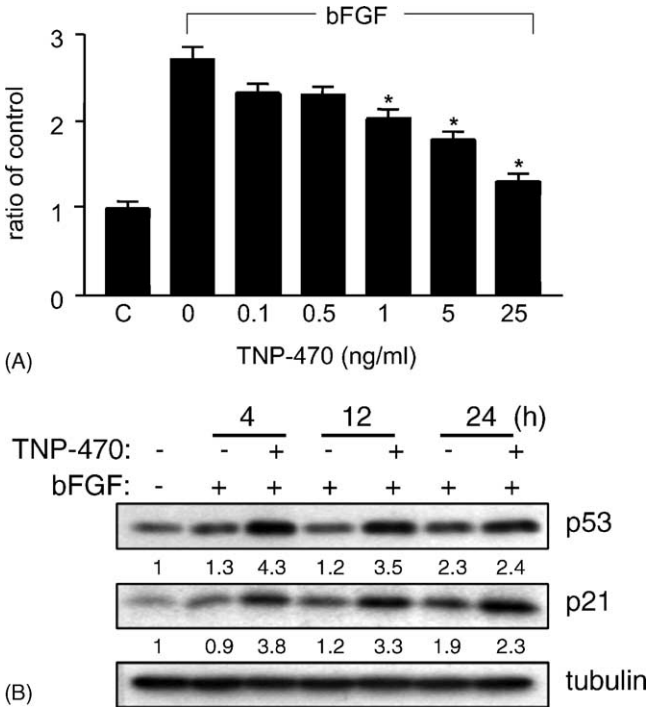

Fig. 1. Effect of TNP-470 on bFGF-induced cell proliferation, p53 and p21(Cip1). (A) Dose-dependent inhibition of HUVEC proliferation by TNP-470. Serum-starved HUVECs were pretreated with 0.1% DMSO (C) or various concentrations of TNP-470 for 1 h prior to their incubation with 1 ng/ml of bFGF for 48 h. Each bar was the average of quadruplicate values (mean ± S.D.). The same experiment was repeated two times with similar result. $^*P < 0.05$ for bFGF treatment alone vs. combined treatment with bFGF and TNP-470. (B) Time-dependent increase of p53 and p21 by TNP-470. Following treatment, cell lysates were harvested for immunoblotting using antibodies to p53, p21(Cip1) or α-tubulin. The α-tubulin serves as a loading control. The numbers below each blot represent the induction ratio of each protein of interest in samples following normalization to its loading controls.

cells. The increased profile of p21(Cip1) was similar to that of p53 in HUVECs (Fig. 1B, middle panel).

3.3. TNP-470 increases the expression of cyclin D1 but not cyclin E

Consistent with the cytostatic effect of TNP-470, TNP-470 indeed increased the percentage of HUVECs arrested at G1 while decreasing the number of S-phase cells (data not shown). Cyclins are one essential components of the cell cycle machinery. D-type cyclin is active in mid-G1 phase whereas E-type is active in late-G1 phase prior to the transition of cell cycle from G1 to S phase [32]. Deregulation of these cyclins may either arrest cycling cells in G1 or promote their transition into S phase. Western blotting analysis of serum-deprived HUVECs treated with bFGF for 4–24 h revealed that bFGF-promoted the expression of cyclin D1 with time. The level of cyclin D1 protein was further increased by TNP-470 and peaked at 12 h post-treatment (2.8 folds, Fig. 2A). Conversely, the expression of cyclin E decreased with time even in the presence of

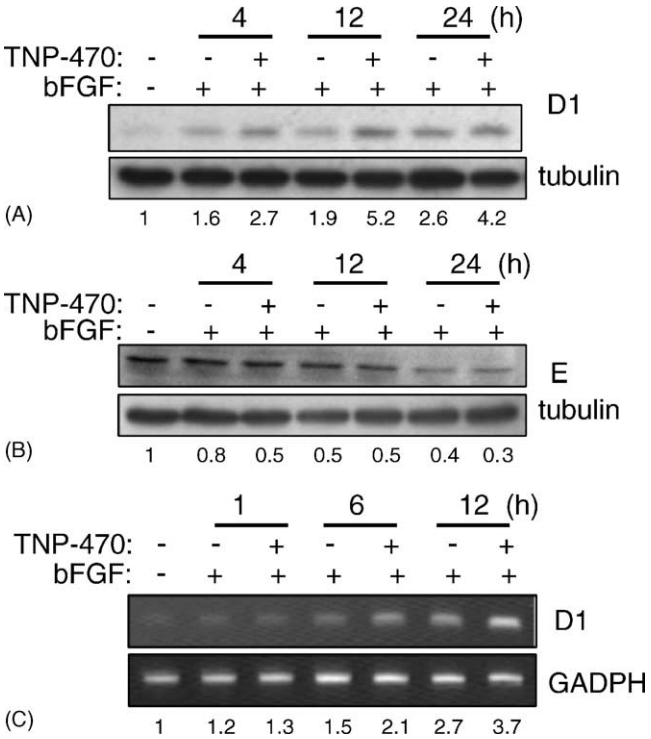

Fig. 2. Effects of TNP-470 on cyclin D1 and E in HUVECs. Following treatment with bFGF and TNP-470 for the indicated times, cell lysates were fractionated by SDS-PAGE followed by immunoblotting with antibodies to cyclin D1 (A) and cyclin E (B). The same blots were stripped and rehybridized to α-tubulin, which served as a loading control. (C) Total RNA from treated or not treated HUVECs for 1-12 h were isolated followed by RT-PCR and Southern blotting analysis, respectively, with primers to cyclin D1 (top panel) and GADPH (bottom panel). The numbers below each blot represent the induction ratio of each protein or mRNA in samples following normalization to its loading controls.

bFGF, and TNP-470 marginally attenuated its expression in endothelial cells (Fig. 2B). The increased expression of cyclin D1 but not that of cyclin E by TNP-470 suggests a possible involvement of cyclin D1 in TNP-470-mediated inhibition.

The observed change of cyclin D1 protein in TNP-470-treated HUVECs could result from up-regulation of cyclin D1 mRNA. To examine whether there was a change of cyclin D1 mRNA in the presence of TNP-470, we used RT-PCR followed by Southern blotting analysis. There was indeed a 1.4-fold increase of cyclin D1 mRNA after 12 h treatment with TNP-470 (Fig. 2C), suggesting that TNP-470-mediated increase of cyclin D1 protein is in part mediated by the enhanced synthesis of mRNA.

3.4. TNP-470 has differential effect on other cell cycle mediators

Since TNP-470 increased the percentage of G1-phase cells, we examined by Western blotting analyses whether G1 or G1-to-S mediators like CDK, CDK inhibitor (CKI), and PCNA were also deregulated. The expression of all three CDK members, CDK4, 6, and 2, attenuated with time

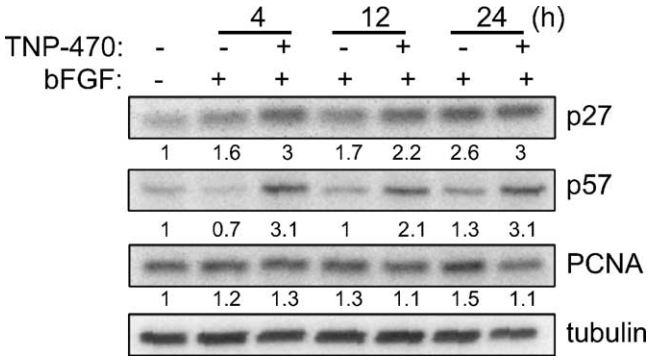

Fig. 3. Differential effects of TNP-470 on members of CDK, CKI and PCNA proteins. Serum-deprived HUVECs were treated with TNP-470 (100 ng/ml) or vehicle (0.1% DMSO) for 1 h, then incubated with bFGF (1 ng/ml) for the indicated times. Protein lysates were harvested then resolved by SDS-PAGE for immunoblotting. Shown are the results of immunoblots probed with antibodies against p27(Kip1) and p57(Kip2), and PCNA. Equal loading of proteins from each time point was confirmed by reprobing the membrane for anti-α-tubulin antibodies. The numbers below each blot represent the induction ratio of each protein in samples following normalization to the intracellular level of α-tubulin.

in bFGF-treated HUVECs. The presence of TNP-470 marginally reduced their expression in endothelial cells (data not shown). There are two classes of CKI, one being specific for CDK4/6-like INK4a family and the other being general for all CDK-like Cip/Kip family [33]. Little or no expression of p16 (INK4a) was detected in HUVEC (data not shown). By contrast, the expression of Cip/Kip family was readily detected in HUVEC. TNP-470 had stimulatory effects on the expression of both p27(Kip1) and p57(Kip2) (Fig. 3). Like p21(Cip1), the expression of p27(Kip1) and p57(Kip2) was evident within 4 h of treatment and the increase was maintained up to 24 h post-treatment. Unlike Cip/Kip family, the expression of PCNA was decreased in bFGF and TNP-470-treated cells compared to bFGF-treated cells (Fig. 3, third panel). These results demonstrated that TNP-470 increased the expression of p27(Kip1) and p57(Kip2), while marginally attenuating that of CDK members and PCNA.

3.5. Subcellular localization of cyclin D, p21 and p53 in TNP-470-treated HUVEC

Cyclin D1 accumulates in the nuclei of G1-phase cells then disappears from the nucleus as DNA replication begins [34]. We sought to analyze the subcellular location of the increased cyclin D1 in TNP-470-treated cells. Western blotting analysis of cytosolic and nuclear fractions revealed that there was no preferred subcellular localization for the TNP-470-induced cyclin D1 protein (Fig. 4, top panel). However, more p21(Cip1) protein was accumulated in the cytosolic than nuclear fractions. Consistent with p53 being a transcription regulator, p53 was predominantly accumulated in nuclei. No significant cross-contamination between cytosol and nuclei was observed as indicated by stripping and reprobed with

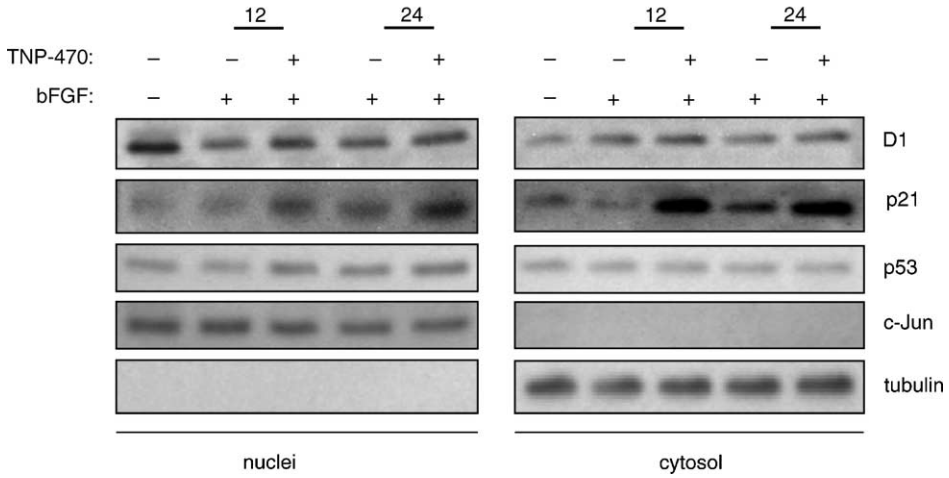

Fig. 4. Subcellular distribution of cyclin D1, p21(Cip1) and p53 in TNP-treated HUVECs. Serum-deprived HUVECs were treated with TNP-470 (100 ng/ml) or vehicle (0.1% DMSO) for 1 h, then incubated with bFGF (1 ng/ml) for the indicated times. Nuclear and cytosolic extracts were isolated as described in the Section 2. Equal amount of protein lysates, respectively, from nuclei and cytosol were resolved by SDS-PAGE followed by immunoblotting with anti-cyclin D1, p21(Cip1), and p53 antibodies. The same blot was, respectively, reprobed with antibodies against c-Jun (a nuclear protein) and α-tubulin (a cytoskeletal protein).

antibodies, respectively, to c-Jun and α-tubulin (Fig. 4, bottom two panels).

3.6. Increased association of cyclin D1 with CDK4 and p21(Cip1) but reduced cyclin D1-dependent CDK activity by TNP-470

Cyclin D1 assembles with CDK4/6 to form a holoenzyme that facilitates exit from G1 by phosphorylating Rb [16]. Activation of the holoenzyme is antagonized by CKI like p21(Cip1), which is induced by anti-proliferative signals [35,36]. To analyze the effect of TNP-470 on the association of cyclin D1 and its partner proteins including CDK4/6, CKI and PCNA, immunoprecipitation followed by western blotting analysis of HUVECs treated or not treated with TNP-470 in the presence of bFGF was performed. TNP-470 increased the association of cyclin D1 with CDK4 (Fig. 5A). Consistent with low level of CDK6 in HUVEC [37], no association of cyclin D1 with CDK6 could be detected in the immunocomplex of cyclin D1 (data not shown). The increased association of cyclin D1 with CDK4 was further confirmed by the co-immunoprecipitation using an antibody against CDK4. There was an evident increase of p21(Cip1) by TNP-470 in the CDK4 immunocomplex (Fig. 5B, top panel). Although TNP-470 also increased the protein levels of p27(Kip1) and p57(Kip2), there was no increased presence of p27(Kip1) or p57(Kip2) in the same CDK4 protein complex (Fig. 5B, middle and bottom panels). PCNA is another cyclin D1-associated protein, no PCNA was, however, found in this complex (data not shown).

To examine if the increased association of cyclin D1, CDK4 and p21(Cip1) had any effect on the kinase activity of cyclin D1-associated CDK complex, we used an in vitro

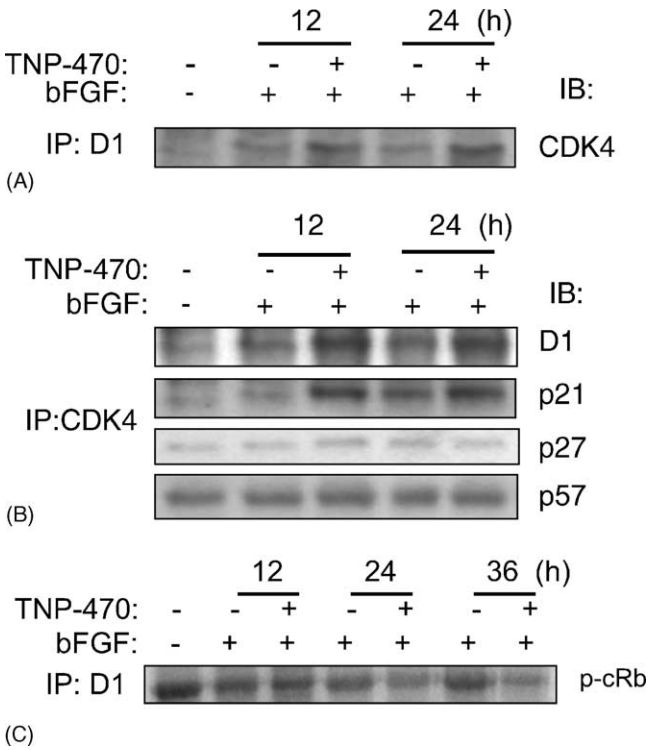

Fig. 5. Increased association of cyclin D1 with CDK4 and p21 (Cip1) but reduced cyclin D1-dependent CDK activity by TNP-470. Serum-deprived HUVECs were treated with TNP-470 (100 ng/ml) or vehicle (0.1% DMSO) for 1 h, then incubated with bFGF (1 ng/ml) for the indicated times. (A) Following the harvest of protein lysates, equal amounts of cellular proteins were immunoprecipitated with anti-cyclin D1 antibodies followed by immunoblotting with anti-CDK4 and anti-CDK2 antibodies. (B) Equal amounts of total proteins were immunoprecipitated with CDK4 followed by immunoblotting with antibodies to cyclin D1, p21(Cip1), p27(Kip1) and p57(Kip2). (C) Equal amounts of total protein lysates were immunoprecipitated with anti-cyclin D1 antibodies followed by in vitro kinase assay using γ-^{32}P-ATP.

kinase assay of cyclin D1-associated immunocomplexes collected from vehicle- or TNP-470-treated cells under the influence of bFGF. The ability of cyclin D1-dependent CDK to phosphorylate the C-terminal portion of Rb was reduced by TNP-470 at 24 h post-treatment and thereafter (Fig. 5C), suggesting that co-precipitated p21 might behave as a universal inhibitor for the CDK4 activity.

3.7. Overexpression of cyclin D increases the sensitivity of HUVECs to TNP-470

TNP-470 induced the expression of cyclin D1 in HUVECs. To examine whether the overexpression of cyclin D1 had any role in mediating the inhibitory effect of TNP-470 on endothelial cells, a cDNA clone encoding the full-length cyclin D1 (pFLEX-D1) was introduced into HUVECs by electroporation. Under this condition, we routinely achieved 50–70% of transfection efficiency. The overexpressed cyclin D1 in the transfected cells was confirmed by Western blotting analysis (Fig. 6A). Mock-transfected cells and cells overexpressing cyclin D1 were, respectively, subjected to cell proliferation assay. Basic FGF-promoted HUVEC proliferation. The bFGF-promoted proliferation of mock-transfected HUVECs was completely attenuated by TNP-470 at 1 ng/ml. However, the presence of exogenous cyclin D1 significantly increased the sensitivity of HUVECs to TNP-470 when compared to the bFGF-treated cells. The complete inhibition in the presence of exogenous cyclin D1 was achieved at a dose lower than 0.5 ng/ml (Fig. 6B). Taken together, the induced cyclin D1 appears to be required for the angiogenic inhibition mediated by TNP-470.

3.8. The increase of the senescence-like phenotype by TNP-470 possibly via association of CDK2 with cyclin D1

Overexpression of p21(Cip1) results in senescence-like growth arrest [38]. Since TNP-470-mediated cytostatic effect requires p21(Cip1), we next examined by the use of SA-gal staining if TNP-470 was able to promote senescence-like phenotype in bFGF-treated HUVECs. Following overnight starvation and the 12 h incubation, bFGF at 1 ng/ml induced 50% of HUVECs to become SA-gal-positive cells. Addition of TNP-470 to the treatment medium further increased the percentage of SA-gal-positive cells by 20% (Fig. 7A, $P < 0.05$). Although a mitogenic

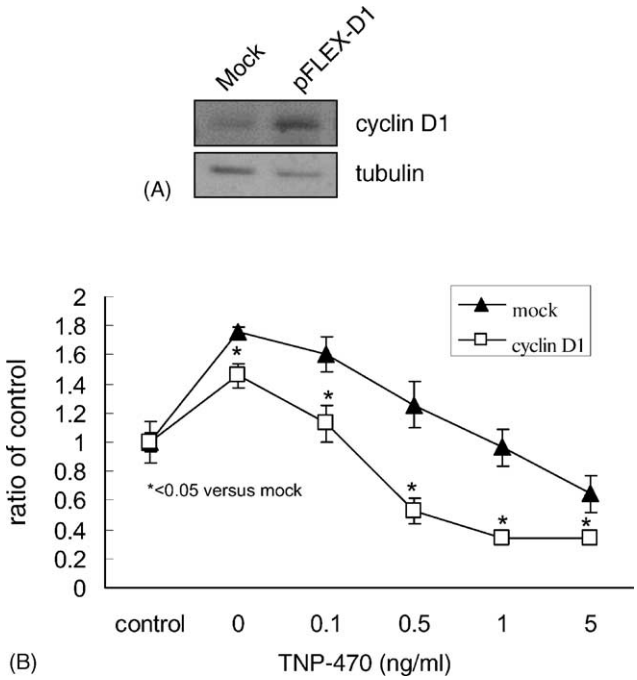

Fig. 6. Overexpression of cyclin D1 increases the susceptibility of HUVECs to TNP-470. Subconfluent HUVECs were transiently transfected with or without pFLEX-D1 cDNA by electroporation. (A) Forty-eight hours following transient transfection, the amount of cyclin D1 in the mock-transfected or pFLEX-D1 transfected HUVECs were analyzed by SDS-PAGE and immunoblotting analysis. (B) Twelve hours following then being replated onto gelatin-coated 96-well plates, transfected HUVECs were pretreated with the indicated concentrations of TNP-470 for 1 h followed by incubation with bFGF at 1 ng/ml for 2 days prior to cell proliferation assay. Each bar represents an average of two experiments, in each of which each treatment condition was performed in quadruplicate (mean ± S.D.). $^{*}P < 0.05$ for mock-transfection vs. cyclin D1-transfection in presence of increasing dose of TNP-470.

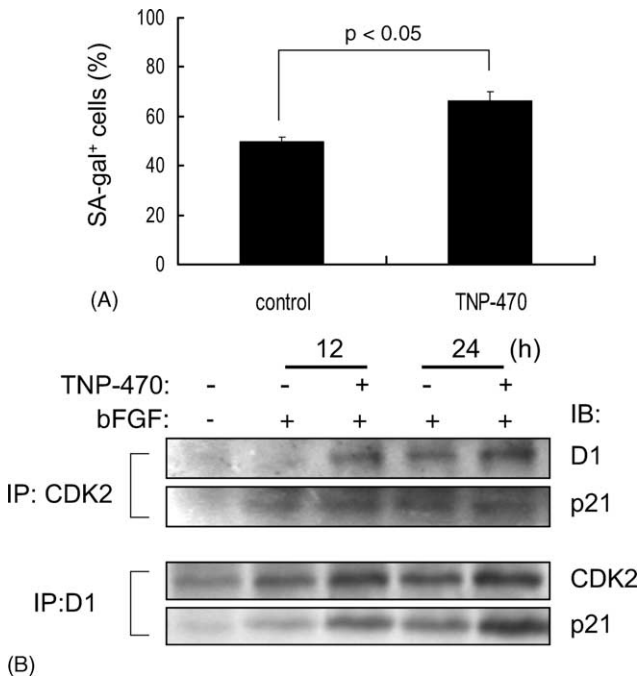

Fig. 7. Senescent effect o TNP-470 on proliferating HUVECs. (A) Twelve hours after the treatment of HUVECs with bFGF or TNP-470/bFGF, cells were fixed then stained with β-galactosidase in acidic pH. Five high power fields (200X) were randomly chosen to count the percentage of blue cells among the total cell numbers calculated. $^{*}P < 0.05$ for bFGF-treated vs. bFGF and TNP-treated cells. (B) Serum-deprived HUVECs were treated with TNP-470 (100 ng/ml) or vehicle (0.1% DMSO) for 1 h, then incubated with bFGF (1 ng/ml) for the indicated times. Following treatment, equal amounts of total proteins were immunoprecipitated with anti-CDK2 antibodies followed by immunoblotted with antibodies against cyclin D1 then p21(Cip1), or immunoprecipitated with anti-cyclin D1 then blotted with antisera against CDK2 and p21(Cip1).

dose of bFGF alone was not sufficient to provide all the survival signals required for HUVEC, TNP-470 significantly increased the percentage of cells with senescence-associated β-galactosidase, a hallmark of senescence. In senescent cells, not only cyclin D1 is overexpressed but also forms an inactive complex with CDK2 [39]. To study if similar complex was also formed in TNP-treated HUVECs, we used anti-CDK2 antibodies to precipitate the CDK2 protein complex followed by immunoblotting with anti-cyclin D1, p21(Cip1), and PCNA. There was an evident increase of cyclin D1 and p21(Cip1) in the CDK2 immunoprecipitates in TNP-treated cells (top two panels, Fig. 7B). No PCNA was found in the same CDK2 immunocomplex (data not shown). The same immunocomplex was further confirmed by an antiserum against cyclin D1 (bottom two panels, Fig. 7B). TNP-470 promotes HUVEC to undergo senescence possibly via increased formation of cyclin D1 with CDK2, reminiscent of an inactive CDK2 complex found in senescent fibroblasts.

4. Discussion

TNP-470 is a potent angio-inhibitory drug that inhibits angiogenesis both in vitro and in vivo. TNP-470 at doses ranging between 10 pg and 3 μg has a cytostatic effect on proliferating endothelial cells. This effect was previously shown via induction of p53 and p21(Cip1) [10,11]. In addition to these two proteins, we have also identified the up-regulation of other cell cycle mediators including cyclin D1, p27(Kip1) and p57(Kip2) in the TNP-470-treated cells. P21(Cip1), p27(Kip1) and p57(Kip2) all belong to the Cip/Kip family of CKI [40,41]. While Cip/Kip family inhibits all CDK kinases involved in the G_1 to S transition, another family, INK4a, including p15, p16, p18, p19, specifically inhibits the activity of CDK4 and CDK6 [42]. Since we were not able to detect the expression of p16 (INK4a) in HUVEC using western blotting analysis (data not shown), the effect of TNP-470 on the INK4a family, however, could not be completely ruled out. By contrast, there was only marginally attenuated effect on that of CDK family members and PCNA. The increased cyclin D1 also formed a complex with CDK2, reminiscent of similar complexes identified in aging fibroblasts. Treatment of HUVECs with TNP-470 indeed significantly increased the senescent phenotypes. Over-expression of cyclin D1 promoted the susceptibility of HUVECs to TNP-470. These studies establish that TNP-470-mediated angio-inhibitory effect was in part via induction of senescence and cyclin D1 is one important molecule involving this inhibition.

TNP-470-induced cell cycle arrest requires p53 and p21(Cip1). A proper G1 arrest mediated by p53 often associates with up-regulation of both p21(Cip1) and cyclin D1 proteins. In addition to the presence of a weak p53 binding in the promoter region of cyclin D1 gene, the mediation of cyclin D1 synthesis by p53 is in part via p21(Cip1) [20]. Cyclin D1 is, thus, believed to be a mediator of p53 growth suppression. Consistent with this notion, we observed a concurrent increase of cyclin D1 protein with the increase of p53 and p21(Cip1) protein in the TNP-470-treated endothelial cells. TNP-470-induced expression of cyclin D1 protein was in part via increased expression of its mRNA. This finding was different from two earlier reports in which that of cyclin D1 mRNA was either unchanged or suppressed in TNP-470-treated endothelial cells [6,43]. The differences might stem from different time points used to harvest mRNA (1, 6 and 12 h versus 20 h) or from the sequence of drug and growth factor treatment (pretreatment versus drug treatment at 2 h post-stimulation). Using a cyclin D1 promoter-driven reporter assay in conjunction with transient transfection should be able to address whether TNP-470 has any effect on the promoter activity of cyclin D1. We are now undertaking experiments to address this issue.

TNP-470 increased the level of cyclin D1 transcript by 1.4 folds in the endothelial cells. However, the increase of cyclin D1 protein by TNP-470 might be attributed to regulation at levels other than transcription. Pulse-chase labeling of HUVECs using [^{35}S]methionine/cysteine demonstrated that the amount of cyclin D1 protein was higher in TNP-470 cells than that in vehicle-treated cells during both pulse and chase periods (data not shown), suggesting that transcription is a primary mechanism involved in TNP-470-mediated upregulation of cyclin D1. In addition to transcriptional and translational regulation, proteasomal degradation of D1 via threonine phosphorylation by glycogen synthase kinase 3-beta (GSK-3 beta) and nuclear export has been previously reported to be involved in regulating the intracellular level of cyclin D1 [44,45]. We have found that the level of cyclin D1 protein was neither predominantly accumulated in the nuclei of TNP-treated HUVEC (Fig. 4) nor the activity of GSK 3-beta was affected by TNP-470 (data not shown). Taken together, the increase of cyclin D1 protein observed in TNP-470-treated HUVECs is predominantly mediated by the enhanced synthesis of mRNA.

The expression level of cyclin D1 plays a determinant role in the functional status of this protein in the cells. Depending on the cell types, cyclin D1 possesses two contrasting roles, promoter or suppressor, for the cell cycle progression [20]. In most cells, a moderate increase of cyclin D1 promotes cell proliferation by accelerating the G1 transition, while its attenuated expression prevents S entry. However, in fibroblasts not only high level of cyclin D1 expression does antagonize cell proliferation by preventing S phase entry, but also the amount of cyclin D1 protein is greatly increased in senescent cells [17,46]. Consistent with cyclin D1 being a suppressor for the cell cycle, overexpression of cyclin D1 increased the susceptibility of HUVECs to TNP-470.

Angiogenic activity is controlled by a finely tuned balance of angiogenic inhibitors and inducers. To elucidate the mechanism of antagonistic actions of inhibitory TNP-470 and promoting bFGF, only these two molecules are included in this study. Although a mitogenic dose of bFGF was not able to completely prevent HUVECs from undergoing senescence, treatment of the same cells with TNP-470 for as short as 12 h significantly increased the percentage of senescent cells by 20% ($P < 0.05$). Induction of p21(Cip1) expression from an inducible promoter results in senescence-like growth arrest in a human fibrosarcoma cell line [38]. The increase of senescent cells was consistent with the induction profile of p21(Cip1) in TNP-470-treated cells. Moreover, like in senescent fibroblasts, an inactive complex of CDK2 with cyclin D1 was identified in TNP-470-treated endothelial cells. Over-expression of p57(Kip2) imposes a cell proliferation block and induces senescence in human astrocytoma cells [47]. The expression of p57(Kip2) was also increased in TNP-470-treated cells. Taken together, these data indicate that angiogenic inhibition mediated by TNP-470 may be in part through induction of senescent-like phenotype in endothelial cells.

In summary, we have identified that cyclin D1 is another key molecule participating the angiogenic inhibition mediated by TNP-470. The increase of cyclin D1 protein in TNP-470-treated cells predominantly results from its increased expression at transcriptional level. Although more studies may be needed, induction of senescence accompanied with the formation of a complex including cyclin D1, CDK2 and p21(Cip1) appears to be one mechanism whereby cytostatic TNP-470 mediates its anti-angiogenic effect in human endothelial cells.

Acknowledgments

First, we would like to express our great gratitude to Dr. Ming-Der Lai for his helpful discussion while preparing the manuscript. Second, we would like thank to Takeda Pharmaceuticals for TNP-470. This work was mainly supported by a 3-year Career Development Grant (NHRI-EX9016BC) from National Health Research Institute in Taiwan and in part by the MOE program promoting academic excellence of University under the grant number 91-B-FA09-2-4.

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