Pingyangmycin stimulates apoptosis in human hemangioma-derived endothelial cells through activation of the p53 pathway

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Abstract. Pingyangmycin (also known as Bleomycin A5) is produced by *Streptomyces verticillus var. pingyangensis* n.sp., and has anti-tumor activities against a variety of tumor cells. The aim of the present study was to determine the molecular mechanism(s) underlying the therapeutic effects of pingyangmycin against infantile hemangiomas. Human hemangioma-derived endothelial cells (HemECs) were treated with pingyangmycin at varying concentrations (100, 200 or 300 μg/ml), and the morphological changes and apoptosis levels were assessed. The gene expression changes were determined by cDNA microarray technology. Transmission electron microscopy examination revealed that the pingyangmycin-treated HemECs exhibited typical apoptotic characteristics, including chromatin condensation and the formation of apoptotic bodies. Annexin-V staining demonstrated that pingyangmycin caused a significant and dose-dependent induction of apoptosis in the HemECs. In the pingyangmycin-treated HemECs, 4,752 genes demonstrated at least 2-fold expression changes at the mRNA level. Quantitative polymerase chain reaction confirmed that pingyangmycin significantly upregulated the expression of p53, p53-induced protein with death domain, Bax, p53 upregulated modulator of apoptosis and p53 inducible gene 3, and downregulated the expression of murine double minute 2. The data demonstrated that the pro-apoptotic activity of pingyangmycin against infantile hemangiomas involves p53 pathway activation.

Introduction

Hemangioma is a common vascular tumor that develops during early life, with an incidence in newborns of 1-3%, which increases to ~10% by 1 year of age (1). Hemangiomas are regarded as benign neoplasms and are typically characterized by rapid postnatal growth followed by slow involution. The course of disease is often uneventful, culminating in a complete regression of the tumor. However, a number of cases with infantile hemangioma may suffer from complications, including ulceration, visual and airway impairment, and residual scarring and disfigurement (2). These complications, along with the potentially life-threatening consequences, are a definite indication for treatment. There are several pharmacological therapies available for patients with problematic hemangiomas, including oral corticosteroids, interferon α, vincristine and β-blockers (3,4).

X-chromosome inactivation pattern analysis has led to the hypothesis that infantile hemangioma arises from the clonal expansion of endothelial cells (ECs) (5). Drug-induced hemangioma regression can occur as a result of the induction of apoptosis in proliferating ECs (6,7). Apoptosis is characterized by numerous typical morphological features, including cell shrinkage, nuclear fragmentation, chromatin condensation and the formation of membrane-bound apoptotic bodies (8). Apoptosis is usually contrasted to necrosis, which is characterized by cell swelling, disruption of cellular organelles and plasma membrane rupture. The loss of cell membrane integrity results in the release of intracellular contents into the surrounding tissue, consequently leading to the induction of inflammatory responses. p53 is a pivotal transcription factor that activates numerous genes to restrict cell growth and induce apoptosis (9). Dai *et al* (10) reported that harmine (a β-carboline alkaloid) inhibits tumor angiogenesis through activation of p53 signaling in ECs. Ji *et al* (11) demonstrated that the pro-apoptotic effect of propranolol on hemangioma-derived ECs (HemECs) is linked to the stimulation of p53 and the modulation of the Bcl-2 family. These findings indicate that pharmacological activation of p53 signaling represents a promising strategy in the treatment of hemangioma.

Pingyangmycin (also known as Bleomycin A5) is produced by *Streptomyces verticillus var. pingyangensis* n.sp., and has cytotoxic activities against a variety of tumors (12,13). Several studies have also documented the therapeutic benefit of

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pingyangmycin in infantile hemangiomas (14,15). For example, Hou *et al* (14) demonstrated that pingyangmycin sclerotherapy for infantile hemangiomas in oral and maxillofacial regions lead to complete resolution or pronounced improvements in 66 consecutive patients. However, the molecular mechanism(s) underlying the therapeutic effects of pingyangmycin against hemangiomas is poorly understood. The present study profiled the gene expression in HemECs treated with pingyangmycin, using cDNA microarray technology, and aimed to identify the important signaling pathways involved in the antitumor activity of pingyangmycin.

Materials and methods

Isolation and culture of HemECs. The present study was approved by the Ethical Committee of Xi'an Jiaotong University (Xi'an, Shaanxi, China). HemECs were previously isolated from a proliferating infantile hemangioma (16) that was resected at the Department of Pediatric Surgery, Second Hospital of Xi'an Jiaotong University. Written informed consent was obatined from the patient. The cells were cultured in M199 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37˚C with 5% CO_2. The culture medium was changed every two days and the confluent cells were routinely subcultured using trypsin-EDTA solution (0.05%; Invitrogen Life Technologies). The cells at passages 6-8 (Invitrogen Life Technologies) were used in the present study.

Drug treatment. The HemECs were seeded at a density of $2x10^6$ cells/well into 6-well plates. Following incubation overnight at 37˚C, the cells were left untreated as the control, or treated with different concentrations (100 and 300 μg/ml) or 200 μg/ml (if not stated otherwise) of pingyangmycin (Tianjin Lisheng Pharmaceutical Co., Ltd., Tianjin, China) for 16 h. The cells were harvested and examined for apoptosis and gene expression.

Transmission electron microscopy (TEM). Following the treatments, the cells were prefixed 2.5% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide. The samples were dehydrated in an ascending series of ethanol to 100%, embedded and cut into ultrathin sections (50-70 nm). The sections were stained with 0.5% uranyl acetate and saturated lead citrate and examined on an electron microscope (H-600; Hitachi, Tokyo, Japan).

Apoptosis analysis. Following treatment, the cells were collected, washed and subjected to apoptosis analysis using an Annexin V-FITC kit (Trevigen, Gaithersburg, MD, USA), according to the manufacturer's instructions. The cells were analyzed on a FACScan flow cytometer with CellQuest software (BD Biosciences, San Jose, CA, USA).

cDNA microarray analysis. For microarray analysis, the Whole Human Genome Microarray kit (4x44K; Agilent Technologies, Santa Clara, CA, USA) was used. The sample preparation and microarray hybridization were performed according to the manufacturer's instructions. Briefly, the total RNA from the HemECs with or without pingyangmycin treatment was amplified and transcribed into fluorescent complementary RNA (cRNA) using the Quick Amp Labeling (one color) kit (Agilent Technologies). The Cy3-labeled cRNAs were hybridized onto the Agilent Whole Human Genome Microarray. Following washing, the arrays were scanned on a DNA microarray scanner (G2565BA; Agilent Technologies).

Feature Extraction software (version 10.7.3.1; Agilent Technologies) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Differentially-expressed genes were identified with at least 2-fold changes between the control and pingyangmycin-treated cells. Pathway and gene ontology analysis of differentially-expressed genes was conducted.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted with TRIzol according to the manufacturer's instructions (Invitrogen Life Technologies). Reverse transcription was performed using the AMV First Strand cDNA Synthesis kit (Bio Basic, Inc., Amhurst, NY, USA). qPCR amplification was conducted on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR primers used are listed in Table I. As an internal quantitative control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in a parallel reaction. All assays were performed in triplicate, and the threshold cycle (Ct) was calculated. The relative mRNA expression level normalized by that of GAPDH was determined using the $2^{-\Delta\Delta Ct}$ method (17).

Table I. Primers used for qPCR.

Gene	Primer sequence
p53	F: 5'-GGAAATCTCACCCCATCCCA-3' R: 5'-CAGTAAGCCAAGATCACGCC-3'
PIDD	F: 5'-CATCAAGCTGCCGAGACTTC-3' R: 5'-TGCTCATCCAGATCATCCCG-3'
PIG3	F: 5'-AGTGACCGAAATCCAGGAGG-3' R: 5'-GCTTTAAACGGCTCTGGAGG-3'
PUMA	F: 5'-CCCACCACCATCTCAGGAAA-3' R: 5'-GTGGTCACGTTTGGCTCATT-3'
MDM2	F: 5'-TCCCAGCCTAGGTTTCAGAC-3' R: 5'-AACACGGAGCTTGAGAGGAA-3'
Bax	F: 5'-AAGAAGCTGAGCGAGTGTCT-3' R: 5'-GTTCTGATCAGTTCCGGCAC-3'
GAPDH	F:5'-TGGGTGTGAACCATGAGAAGT-3' R: 5'-TGAGTCCTTCCACGATACCAA-3'

qPCR, quantitative polymerase chain reaction; F, forward; R, reverse; PIDD, p53-induced protein with death domain; PIG3, p53 inducible gene 3; PUMA, p53 upregulated modulator of apoptosis; MDM2, murine double minute 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

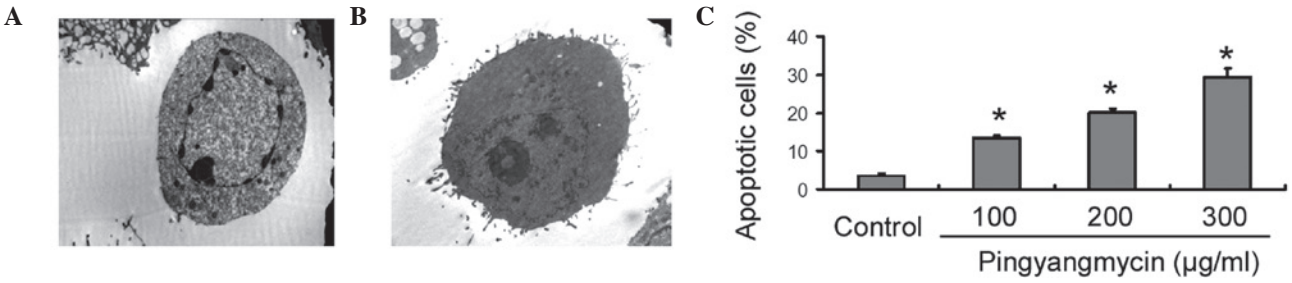

Figure 1. Pingyangmycin induces apoptosis in HemECs. TEM examination analysis of morphological changes of the HemECs treated with (A) 200 μg/ml pingyangmycin for 16 h and (B) untreated cells. Magnification, x4,000. (C) Flow cytometric analysis of the annexin-V-stained HemECs following treatment with different concentrations of pingyangmycin for 16 h. Error bars represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. untreated control. HemECs, hemangioma-derived endothelial cells; TEM, transmission electron microscope.

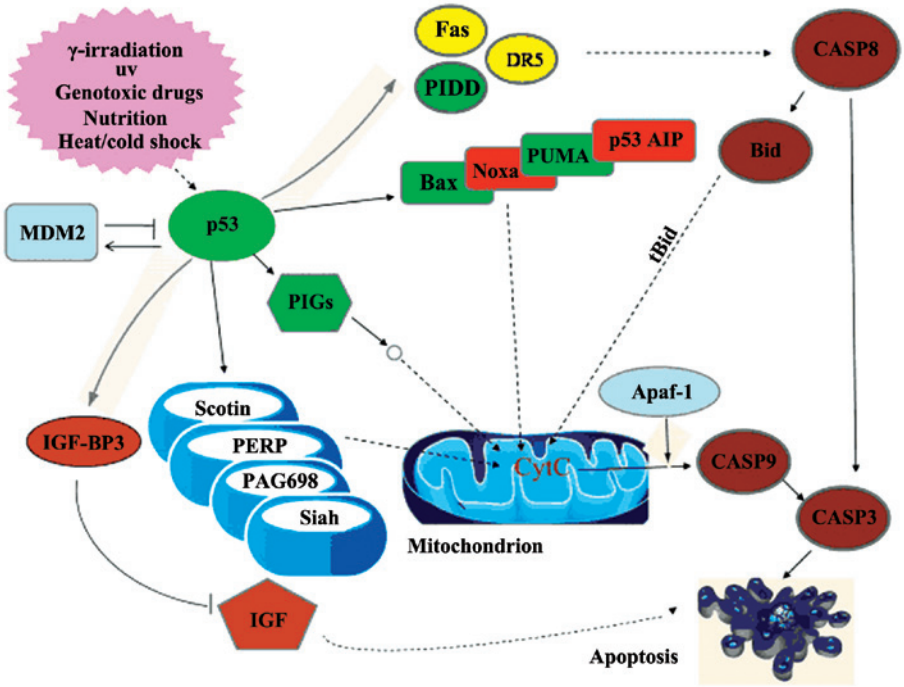

Figure 2. Pathway analysis of differentially-expressed genes upon pingyangmycin treatment using cDNA microarray technology. The results revealed that the p53 pathway has a central role in mediating the cytotoxicity of pingyangmycin in HemECs. HemECs, hemangioma-derived endothelial cells; PIDD, p53-induced protein with death domain; PUMA, p53 upregulated modulator of apoptosis; PIG3, p53 inducible gene 3; MDM2, murine double minute 2.

Statistical analysis. The statistical differences between the groups were calculated using Student's t-test. One-way analysis of variance, followed by Tukey's post-hoc test, was used to examine the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Pingyangmycin induces apoptosis in HemECs. TEM examination revealed that the pingyangmycin-treated HemECs exhibited typical apoptotic characteristics, i.e., intact cell membranes, chromatin condensation and the formation of apoptotic bodies (Fig. 1A). By contrast, the untreated control cells had a normal morphology (Fig. 1B). Flow cytometric analysis further demonstrated that the treatment with pingyangmycin for 16 h resulted in a dose-dependent induction of apoptosis in the HemECs (Fig. 1C). Pingyangmycin at 300 μg/ml caused an ~2-fold increase in the number of apoptotic cells compared with pingyangmycin at 100 μg/ml (29.2±2.4 vs. 13.5±0.5%, respectively).

Regulation of mRNA expression by pingyangmycin. Microarray expression analysis of the pingyangmycin-treated and untreated HemECs was performed to determine the differentially-expressed genes. It was identified that 4,752 genes demonstrated at least 2-fold expressional changes at the mRNA level. Among them, 2,544 were upregulated and 2,208 were downregulated by pingyangmycin (data not shown). Pathway analysis of the differentially-expressed genes revealed that the p53 pathway appeared to be a central pathway involved in the action of pingyangmycin in the HemECs (Fig. 2).

Verification of differentially-expressed genes using qPCR. To improve the quantitative verification of the gene expression alterations determined by the microarray, six genes of the p53 pathway were selected for qPCR analysis. It was identified

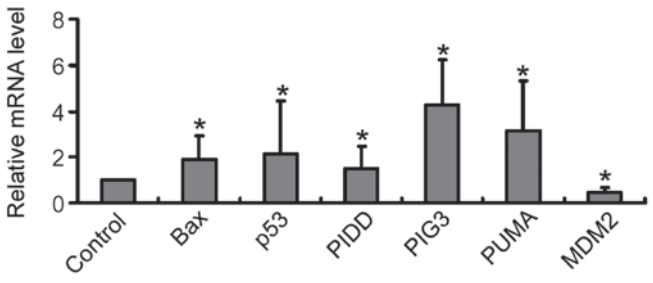

Figure 3. qPCR analysis of the mRNA levels of the indicated genes in HemECs treated with 200 μg/ml pingyangmycin for 16 h. Data are expressed as the fold change relative to the corresponding mRNA levels in the untreated control (set to 1). Error bars represent the mean ± standard deviation of three independent experiments. *P<0.05 vs. untreated control. qPCR, quantitative polymerase chain reaction; HemECs, hemangioma-derived endothelial cells; PIDD, p53-induced protein with death domain; PUMA, p53 upregulated modulator of apoptosis; PIG3, p53 inducible gene 3; MDM2, murine double minute 2.

that pingyangmycin treatment significantly (P<0.05) raised the mRNA abundance of p53, p53-induced protein with death domain (PIDD), Bax, p53 upregulated modulator of apoptosis (PUMA) and p53 inducible gene 3 (PIG3), and decreased the mRNA level of murine double minute 2 (MDM2) compared with the untreated control cells (Fig. 3). The results from the qPCR analysis are in agreement with the cDNA microarray data.

Discussion

Pingyangmycin has been shown to exhibit cytotoxicity against numerous types of tumor cells, including tongue carcinoma, sacrococcygeal chordoma and oral carcinoma cells (18,19). The induction of apoptosis constitutes an important mechanism for the eradication of tumor cells by therapeutic agents. Several drugs, including propranolol (11) and interferon α (20), have demonstrated pro-apoptotic activity in infantile hemangiomas. The retention of plasma membrane integrity during apoptosis prevents the onset of an inflammatory response that can favor tumor progression (21). Therefore, drug-induced apoptosis may represent a preferred and superior strategy for eradicating tumor cells. Notably, the present data demonstrated that pingyangmycin induced significant apoptosis in the HemECs. Furthermore, this pro-apoptotic effect was dose-dependent. These findings provide an explanation for the clinical benefits of pingyangmycin in infantile hemangiomas (14,15).

As an active suicidal response, apoptosis is characterized by nuclear condensation and fragmentation, cellular shrinkage without the loss of plasma membrane integrity and apoptotic body formation. The TEM examination in the present study revealed typical apoptotic characteristics in the pingyangmycin-treated HemECs, including shrinkage of the cytoplasm, chromatin condensation and formation of apoptotic bodies. Gene expression profiling studies have indicated that apoptotic morphological changes are a consequence of the coordinated regulation of a large number of genes (22). Consistent with this view, the present study demonstrated that pingyangmycin treatment upregulated 2,544 genes and downregulated 2,208 genes in the HemECs. The network pathway analysis indicated that the p53 pathway is involved in the pro-apoptotic effects of pingyangmycin in HemECs. To validate the microarray data, qPCR analysis was performed to examine the expressional changes of several key components of the p53 pathway. It was identified that pingyangmycin treatment significantly increased the mRNA expression levels of p53, PIDD, PIG3 and PUMA, and decreased the MDM2 mRNA level compared with the untreated control cells. p53 is a well-established tumor suppressor that is able to induce cell cycle arrest and apoptosis (23). Therefore, reactivation of p53 represents a promising therapeutic strategy against tumor growth. Indeed, upregulation of p53 is causally linked to the apoptosis-inductive effects of propranolol in HemECs (11).

The p53-dependent induction of apoptosis is largely associated with its transcriptional regulation of numerous target genes. PIDD is a downstream target gene of p53 and mediates p53-dependent apoptosis through interactions with components of the mitochondrial and death receptor signaling pathways (24). In addition to PIDD, the PUMA, PIG3 and Bax genes are another three targets of p53. Compelling evidence indicates that PUMA and PIG3 also function as critical mediators of p53-dependent apoptosis in a variety of cells (25,26). PUMA may bind to the anti-apoptotic protein Bcl-2 and induce cytochrome *c* release from the mitochondria, consequently leading to the activation of caspases 9 and 3 and the induction of caspase-dependent apoptosis. PIG3 has been indicated to be involved in the accumulation of reactive oxygen species and the induction of apoptosis (27). A conserved intronic p53-response element has been identified in the promoter of the Bax gene, which is sufficient to mediate p53-dependent transcriptional activation (28). In response to pro-apoptotic stimuli, Bax forms a homodimer and releases cytochrome *c* from the mitochondria, consequently resulting in caspase-9 activation (29). Yang *et al* (30) reported that the Bax level is significantly higher in the ECs of involutive hemangioma than in that of proliferating hemangioma and normal skin tissue, indicating that this gene is involved in the involution of hemangioma through the induction of apoptosis. By contrast, MDM2 is a major negative regulator of p53. This protein interferes with the p53 activity in two ways. Firstly, through the ubiquitination of p53, signaling for its degradation by the proteasome, and secondly, through directly binding to p53, masking its transactivation domain (23). Taken together, these data indicate that the pro-apoptotic effect of pingyangmycin on HemECs is largely mediated through the coordinated regulation of the p53 pathway components, particularly p53, PIDD, PUMA, PIG3, Bax and MDM2.

However, there are certain limitations to the present study that should be noted. Firstly, there is no direct evidence for the role of the p53 pathway in the induction of apoptosis by pingyangmycin. Genetic manipulation of the key p53 pathway components is valuable to determine to what extent the activation of the p53 pathway mediates the apoptosis-inductive effect of pingyangmycin in HemECs. Additionally, it remains unclear whether the findings of the present study may be translated into the *in vivo* setting.

In conclusion, the present study demonstrated that pingyangmycin is capable of inducing apoptosis in HemECs, coupled with the upregulation of p53, PIDD, PUMA, PIG3 and Bax, and the downregulation of MDM2. These data indicate that the therapeutic role of pingyangmycin against infantile hemangiomas is associated with the induction of p53-dependent apoptosis.

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