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Original Article

Pachymic acid modified carbon nanoparticles reduced angiogenesis via inhibition of MMP-3

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Abstract: Angiogenesis is a process of new blood vessel generation, which is consistently and robustly correlated with tumor formation, growth, and metastasis. The disruption of angiogenesis, and the imbalanced endothelial remodeling and regression, are the main pathogenesis of malignant tumor. Recently, multi-walled nanotubes (MWNTs) have been proposed as a new tool for drug delivery in cancer treatment, which also displayed anti-angiogenic property. In the present study, we modified MWNTs with pachymic acid (PA) extracted from *Heterosmilax chinensis*, a traditional Chinese medicine used for cancer treatment, and compared their effects on blood vessel development. MWNTs and PA/MWNTs were evaluated for their influences on chorioallantoic membrane (CAM) vessel morphology and extracellular matrix metalloproteinase-3 (MMP-3) expression, a crucial proteinase associated with tumor metastasis. MWNTs functioned as an inhibitor of forming branch while PA was not able to promote this inhibition. Subsequently, MWNTs suppressed the endothelial cell maturation, accounting for the ceased elongation of CAM blood vessel, while PA/MWNTs increased the suppressive effect, indicating the potential roles of PA in preventing angiogenesis. PA/MWNTs also showed greater anti-angiogenic property as MMP-3 expression in CAM tissue was significantly decreased by PA/MWNTs compared to MWNTs. These results emphasize the anti-angiogenic activities of PA, supporting a new promising therapy for cancer from the perspective of traditional Chinese medicine.

Keywords: Angiogenesis, metalloproteinase-3, multi-walled nanotubes, metastasis

Introduction

Angiogenesis is a process of generating new blood vessels, which occurs as a consequence of sprouting new capillaries from preexisting vessels by assembling of endothelial cells, and vasculogenesis [1, 2]. It is based on the activation, migration, maturation, remodeling and regression of endothelial cells in normal circumstances. Endothelial cells can be stimulated by angiogenic stimuli to re-enter the cell cycle, degrade the underlying basement membrane to migrate, form new capillary sprouts, and then exit from the cell cycle to differentiate into new vessels [3, 4].

Tumor growth has been well documented to be accompanied by new blood vessel formation in order to acquire nutrients by enhancing the expression of cell growth factors and receptors such as vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR-2) and fibroblast growth factor receptor (FGFR) [5-9]. Therefore, the disruption of angiogenesis, and the absence of endothelial remodeling and regression are the pivotal pathological features of malignant tumor. During sprouting angiogenesis, endothelial cells are able to sense the growth factors released from tumor cells in extracellular matrix (ECM), and then they produce proteases that degrade and remodel the ECM. This is in part achieved by the production of extracellular matrix metalloproteinase (MMPs), which cleaves the fibers of nearby ECM to alter the mechanical properties of ECM [10]. It was demonstrated that the overexpression of several types of MMPs was associated with the metastasis of tumor [11-13]. Potentially, the inhibition of MMPs activities can be a feasible and striking way to prevent tumor invasion by reducing angiogenesis hence blocking nutrient supply for tumor.

Over the last decade, VEGF inhibitors have emerged as a new anti-angiogenic cancer ther-

apy. However, anti-VEGF therapy is effective only in a limited number of cases and can lead to serious toxicity [14, 15]. In addition, inhibition of VEGF signaling pathway seems to be insufficient since angiogenesis involves a well-balanced crosstalk of hundreds of proteins. It has been documented that nanoparticles of diamond, graphite, graphene, nanotubes and fullerenes display low cytotoxicity and could serve as vehicle for drug delivery in cancer treatment [16, 17]. Interestingly these nanoparticles themselves also displayed anti-angiogenic properties [18]. In this study, we modified multi-walled nanotubes (MWNTs) with pachymic acid (PA) extracted from *Heterosmilax chinensis*, a traditional Chinese medicine used for cancer treatment, and observed improved effect on inhibition of angiogenesis.

Materials and methods

Pachymic acid separation

Extracts from heterosmilax chinensis were separated and analyzed by high performance liquid chromatography (HPLC) equipped with a Waters 1525 pump, a 2707 auto sampler and a 2998 PDA detector. The chromatographic separation was achieved at 30°C on Waters Sunfire™ C18 (250 mm × 4 mm i.d., 5 μm particle size) column. The run time was set at 35 min; the flow rate was 1.0 ml/min; and the sample injection volume was 10 μl. The mobile phase was 0.1% (v/v) phosphoric acid (A)-100% acetonitrile (B) filtered through a 0.45 μm filter and degassed prior to use. Separation was achieved with gradient elution using 0.1% phosphoric acid as a solvent. The gradient was reduced by 90% from 0 to 10 min, 75% from 10 to 20 min and 50% from 20 to 30 min, and was increased by 90% from 30 to 35 min to equilibrate the column. The flow rate was set at 1.0 ml/min and the samples were detected at 515 nm.

Modification of MWNTs

MWNTs were dispersed in distilled water and sonication was performed. Initially, MWNTs were incubated in a mixture of 20% (V/V) H_2SO_4 and 20% HNO_3 for 24 hours, followed by incubating in 10 mM PA and 20% H_2SO_4 for 24 hours at 37°C. After that, MWNTs were washed three times using distilled water and evaporated to dryness under a stream of warm air. Finally MWNTs/PA-MWNTs were re-dispersed in PBS with sonication. Sonication was required for dispersing MWNTs/PA-MWNTs before each experiment. The shape and sized of MWNTs and PA-MWNTs were visualized using a JEM-2000EX transmission electron microscope (JEOL Ltd., Japan) at 200 kV.

Chorioallantoic membrane (CAM) assay

Chorioallantoic membrane (CAM) implants were made from sterile Waterman filter paper with a diameter of 5 mm. PBS (control), MWNTs or PA-MWNTs (500 mg/L) were respectively added to the implants (final amount of MWNTs on the implant was 0.01 mg). The implants were pre-treated with 3 mg/ml of hydrocortisone sodium succinate (Sigma, USA) and air dried under sterile conditions. Fertilized eggs from Ross line 308 hens were obtained from a certified hatchery and kept for 4 days at 12°C. The eggs were sterilized using UVC light. Embryos were incubated at temperature 37°C, humidity 60% and turned once per hour. Embryonic day 0 (E0) is the first day when the eggs were placed into the incubator. At day E6, small holes (1 cm^2) were made on the shell above air space, the inner membrane was gently stripped off, and the implants were placed on CAM. The chicken embryos were incubated until day 7 of embryonic development, when implants with CAM were prefixed with 1.5 ml of 4% paraformaldehyde. After 30 min of incubation at 4°C, CAM with implants were cut out and fixed at 4°C in 4% paraformaldehyde for 60 min (total fixation time, 90 min). After fixation, the implants were gently stripped off.

CAM tissues from implants were investigated with a stereomicroscope under a 12.5-fold magnification (SZX10, Olympus Corporation, Japan). Photos were analyzed with ImageJ (NIH). The level of angiogenesis was determined by counting the branch (represents the vessel splitting process) and stop (represents the cell migration of endothelium) points in the area of direct contact of MWNTs or PA-MWNTs, usually with a diameter of 3 mm.

Western blot

The MMP-3 (~54 kDa) expressed in CAM tissue was determined by Western blot. 100 μl of each sample was harvested and added to 20 μl sample 2 × loading buffer (0.125 M of 5 M Tris-HCl, Amresco; 20% glycerol, USB; 4% of 10%

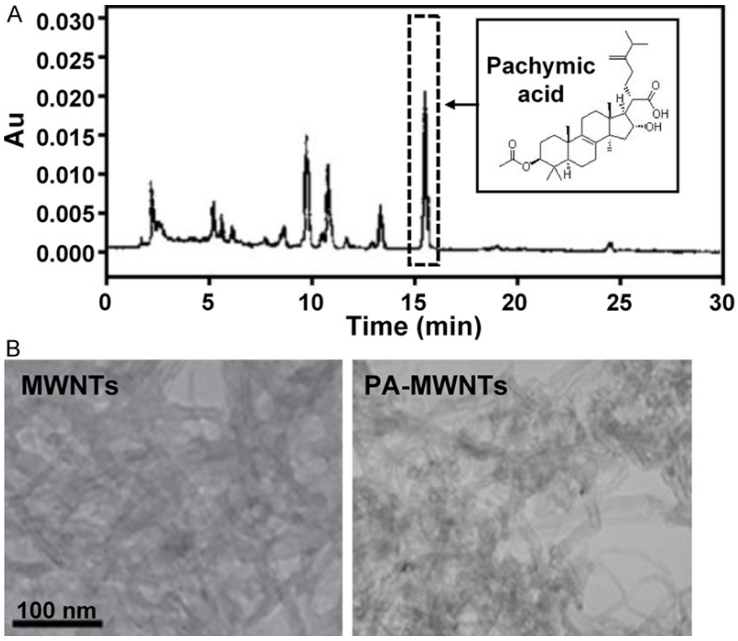

Figure 1. Modification of MWNTs with pachymic acid from heterosmilax chinensis. A. HPLC profile of heterosmilax chinensis and chemical structure of pachymic acid. B. Scanning electron microscopy of MWNTs and pachymic acid modified MWNTs (PA-MWNTs); scale bar, 100 nm.

sodium dodecyl sulfate, Amresco; 1% β-mercaptoethanol, Amresco; 0.2% of 0.05% (w/v) bromophenol blue, Sigma). Actin was used as a loading control. The samples were boiled for 5 min before loading. 10% running gel (25% of 40% acrylamide stock, Beyotime; 0.375 M of 1.5 M Tris-HCl, pH 8.8; 1% of 10% sodium dodecyl sulfate; 1% of 10% ammonium persulfate; 0.1% tetramethylethylenediamine) was utilized based on the range of molecular weights, which was from 15 to 100 kDa. After separation, proteins on the gel were transferred to a same size membrane (Nitrocellulose transfer membrane, Protian) within transfer buffer (25 mM Tris base, 192 mM Glycine, 0.037% sodium dodecyl sulfate, and 20% methanol) under 45 V for 40 min. The proteins that transferred onto the membrane were incubated with the primary antibody (ab52915 for MMP-3, Abcam; ab69512 for Actin as a loading control, Abcam) with a 1/1000 dilution in blocking buffer (50 mM Tris base; 100 mM NaCl; 0.02% Tween 20; and 3% BSA) overnight. The membrane was washed by TTBS (0.1% Tween 20, 10 mM Tris base, 100 mM NaCl, pH 7.5) for three times before adding secondary antibody (ab6721, Abcam) with 1/5000 dilution in blocking buffer for 2 hours. Background color was reduced by carefully washing with TTBS. The results were visualized by developer and fixing solution

Statistical analysis

Data are presented as mean ± SD. Comparisons between control, MWNT and PA-MWNT groups were made by a two-tailed Student's t test and $P<0.05$ was considered as statistical significant. All data were computed using Excel Microsoft® Version 14.0.7128.5000 (© 2010 Microsoft Corporation, Santa Rosa, USA).

Results

Preparation of PA modified MWNTs

Purification of PA (Molecular weight: 528.76) from extracts of *Heterosmilax chinensis* was achieved by HPLC at 515 nm (**Figure 1A**). The shape and size of MWNTs and PA modified MWNTs (PA-MWNTs) were observed by transmission electron microscopy (**Figure 1B**). Compared to MWNTs, PA-MWNTs are sparser and spreader, indicating the existence of PA between nanotubes.

PA-MWNT inhibited angiogenesis in CAM vessel essay

To investigate the impacts of PA on angiogenesis, we compared the development of blood vessels in embryo CAM between MWNTs and PA-MWNTs groups using CAM implantation method. Changes of CAM morphology by PA were observed (**Figure 2A**). Branch sprouting from the established blood vessels is an indicator of angiogenesis, and more branches indicate more dynamic activities of vessel development [19]. In control group (PBS-treated), the branch shown as red point was not affected, indicating angiogenesis was not affected. The branch and stop numbers in each group were all accounted (**Figure 2B**). Branch number was significantly decreased in MWNT group compare to PBS group ($P<0.001$). PA did not have statistically significant impact on the reduction of branches. More stops were formed in the presence of MWNT ($P<0.001$). Interestingly, PA

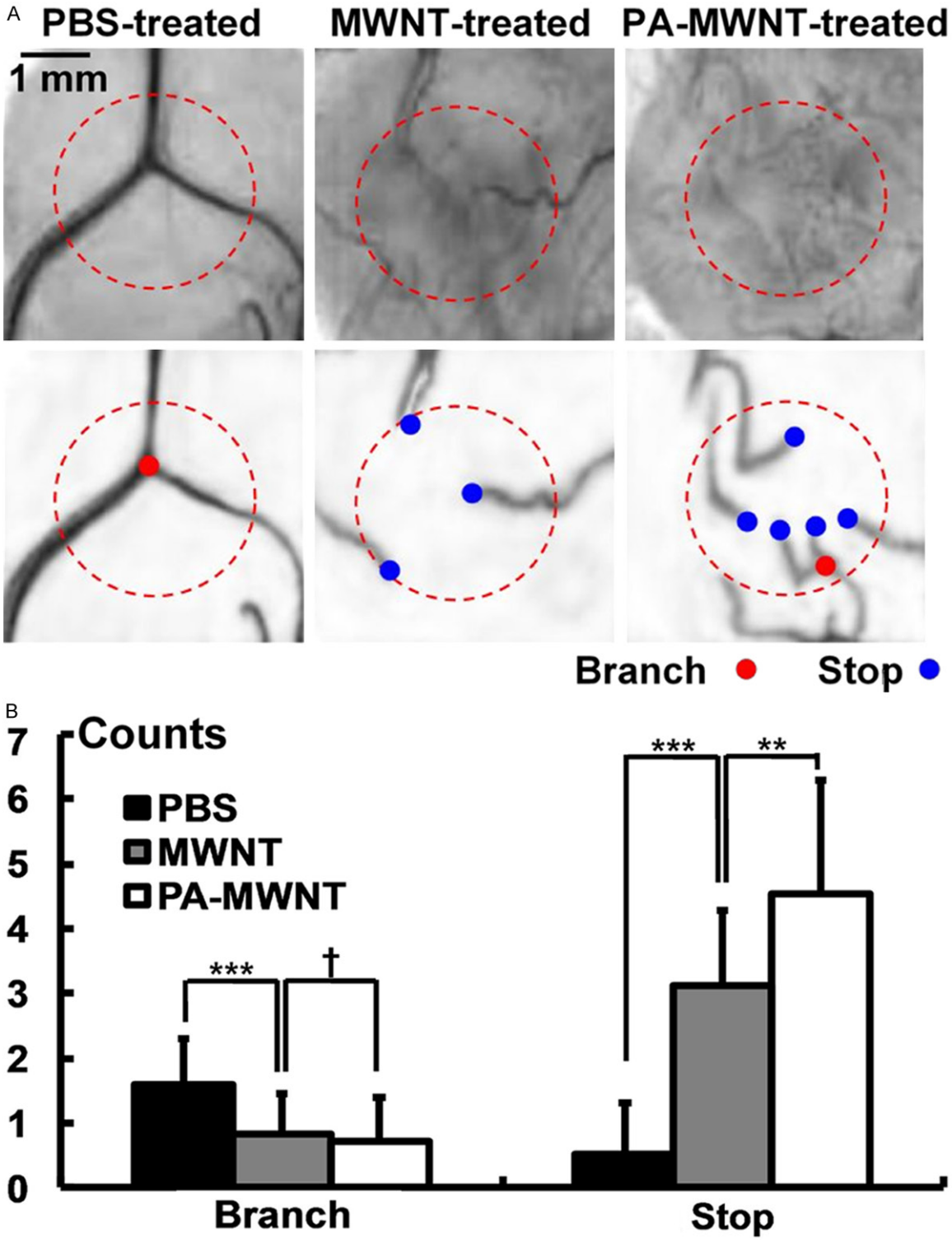

Figure 2. CAM vessel morphology in response to treatment with MWNTs and PA-MWNTs. A. Up, CAM vessel formation under microscope; down, schematic figures show the definition of "Branch" and "Stop" points. B. Summary of CAM essays; n=17 for each bar; ** and ***represents $P<0.01$ and 0.001, respectively; †represents not significantly different.

modification also led to a significant increasing of stops ($P<0.01$), suggesting PA may decrease vascular network via inhibiting the maturation but not migration of endothelial cells.

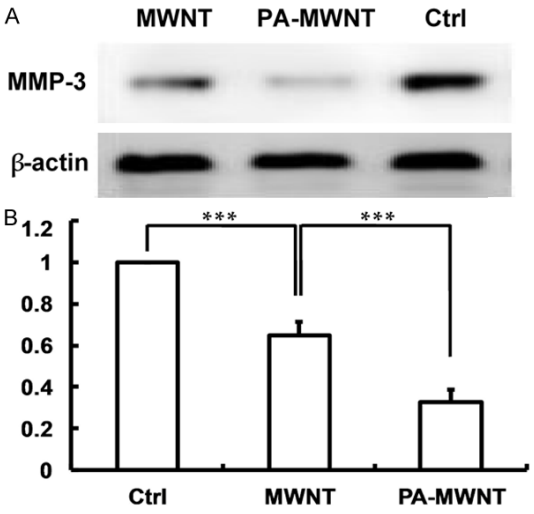

Figure 3. Western blot shows the inhibitory effect of MWNT and PA-MWNT on the expression of MMP-3. A. Representative gel graph of MMP-3 and β-actin under different treatment. B. Summary of Western blot; n=4 blots; ***represents P<0.001.

Stromelysin (MMP-3) expression affected by PA-MWNT

In order to know whether MWNT and PA-MWNT affect MMP-3 expression, western blot analysis was performed within control, MWNT and PA-MWNT groups respectively, with β-actin (~42 kDa) as loading control. As shown in **Figure 3A** and **3B**, MMP-3 expression was significantly down-regulated ($P<0.001$) by MWNT treatment, suggesting the inhibitory role of MWNT in MMP-3 expression. In comparison to MWNT group, PA-MWNT led to a substantially decreased ($P<0.001$) MMP-3 expression, supporting the potential roles of PA in inhibiting the MMP-3 expression.

Discussion

Previous studies showed striking evidence of the inhibition effects of MWNT on VEGF and fibroblast growth factor-2 (FGF2) induced angiogenesis [18]. In our study, MWNT slightly prevented angiogenesis, as indicated by the appearance of stops (blue point) in MWNT treated group (**Figure 2**). Whereas PA-MWNT exhibited significantly enhanced negative influences on angiogenesis, supported by more stops formed instead of branches. It suggests that PA plays a promising role in stop formation.

Endothelial cells can be stimulated by growth factors such as FGF-2 and VEGE. FGF-2 plays an essential role in inducing VEGE expression in forming blood vessels. VEGE, an angiogenic mitogen released from cells, is able to induce mitosis, differentiation, migration and survival of endothelial cells to form capillaries from primary vascular plexus and blood vessels during embryonic development or tumor growth [20-23]. The synergism between various growth factors and receptors maintains the balanced regulation of angiogenesis. MWNT and PA may also synergize as inhibitors of FGE-2 and VEGE. In order to obtain deeper insights of the influence of MWNT and PA on FGE-2 and VEGE, we measured the expression of MMP-3, a crucial protease in angiogenesis (**Figure 3**). MMP-3 is one of the most important metalloproteinases playing critical roles in degradation of extracellular matrix in tumor cell microenvironments, contributing in tumor invasion and angiogenesis [24-26]. As MMP-3 was predominantly associated with invasive carcinoma [27-29], the reduction of MMP-3 by PA-MWNT can be a remarkable step for treating cancer. The results in this study suggest the potential roles of PA in inhibiting the MMP-3 expression.

VEGE stimulates proteolytic activity of endothelial cells, and MMP-3 expression was shown to be regulated by VEGE [30, 31]. Stimulation of VEGF and MMP expressions dramatically elevated tumor angiogenesis and growth [32]. On the other hand, the decrease of MMP-3 expressions may be attributed to reduced VEGF activities. MWNT and PA-MWNT therefore can be a promising and effective way to inhibit VEGF/FGF-2 to prevent tumor growth and invasion.

MMP-3 indirectly controls endothelial cells migration via remodeling of nearby ECM. The decrease of MMP-3 may in part influence blood vessel sprouting. Nevertheless, the morphology of CAM showed no significant difference in branch numbers between MWNT and PA-MWNT treated groups. Furthermore, inhibition of MMP-3 and enhancement of stop formation imply the potential role of PA in anti-angiogenesis. Proteins involved in endothelial cells proliferation or maturation and the relevance between PA and angiogenesis inhibitors such as maspin [33] can be investigated in the future.

In this study, we have found the potential roles of MWNTs and particularly PA-MWNTs in con-

trolling angiogenesis by comparing the morphological changes of blood vessel in CAM from embryo treated with MWNT and PA-MWNT implantation. According to our results, MWNT alone showed anti-angiogenic properties as it decreased the branches as well as increased the stops of blood vessels in CAM. PA appeared to function by assisting the stop formation rather than reducing branches. Moreover PA-MWNT also suppresses MMP-3 expression. Taken together MWNT and PA-MWNT could be a novel and striking anti-angiogenic method to prevent tumor invasion. Considering the potential role of PA in inhibiting endothelial cells growth and proliferation, further study is warranted to investigate their correlation.

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Disclosure of conflict of interest

None.

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