Vasoactive intestinal peptide represses activation of tumor-associated macrophages in gastric cancer via regulation of TNFα, IL-6, IL-12 and iNOS

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Abstract. Vasoactive intestinal peptide (VIP) has been regarded as deactivator for macrophages. However, the depressive effect of VIP on tumor-associated macrophages (TAM) has not been recognized. In the present study, we investigated the effect of VIP on gastric cancer via TAM by suppressing expression levels of TNFα, IL-6, IL-12 and iNOS. Real-time PCR was carried out to examine the expression of CD68 to determine the levels of TAM. The effect of VIP on cell activities was assayed by proliferation assay, colony formation and flow cytometry analysis. The co-culture of TAM and human gastric cancer cell line MKN-45 were performed to understand whether the VIP affects the gastric cancer cells via TAM. Further, the tumor formation in a nude mouse model and VIP injection were performed to illustrate the effect on tumor progression *in vivo*. CD68 was high expressed in gastric cancer indicating high level of TAM in gastric cancer. Treatment with VIP significantly depressed TAM activation. Moreover, the expression of TNFα, IL-6, IL-12 and iNOS in TAM were depressed by VIP treatment, and the VIP treated TAM depressed gastric cancer cells. The experiment in the nude mouse model also suggested that by injection with TAM+VIP, the tumor volume and tumor weight were both decreased significantly. These data suggest that treatment with VIP inhibits gastric cancer.

Introduction

Gastric cancer is one of the most common cancers in the world (1). Up to date, the castration-resistant gastric cancer has only limited curative effect and has shown poor prognosis in clinical practice (2). New treatment strategy of targeting driver pathways provides optional treatment for cancers. For example, induced robust $CD8^+$ T cell response against tumor-associated macrophages (TAM) suggested a novel strategy against breast cancer (3). In tumor progression, TAM is increased and thereby remodels the tumor microenvironment which promoted carcinogenesis (4). By secreting growth and proangiogenic factors, TAM participates in tumor cell proliferation and metastasis via regulating the function of fibroblasts in the tumor stroma (5). Recent studies suggested that anti-TAM effects by small molecule inhibitors could depress tumor suppression, such as cytotoxic (6), biphosphonate compound (7) and zoledronic acid (7). However, these inhibitors showed low infiltrate and limited effects on tumor growth. Thus, the therapeutic targeting of TAM needs to be elucidated and more inhibitors should be developed.

Vasoactive intestinal peptide (VIP) belongs to a superfamily of peptides which also includes pituitary adenylate cyclase-activating polypeptide (PACAP), secretin, and glucagon (8). In recent studies, VIP was shown to regulate the production of TNFα, IL-6, IL-12 and iNOS (8-10). Moreover, VIP also inhibits expression levels of cyclooxygenase-2 (COX2) and high mobility group box-1 (HMGB1) in activated macrophages (11,12). In peritonitis, VIP reduced recruitment of neutrophils, macrophages and lymphocytes via controlling the expression of transcription factors including AP-1, CREB and IRF-1 (12-14). Although inhibiting the expression levels of signaling pathways, VIP also induces the expression of toll-like receptors (TLRs) (15,16), indicating the multiple-effects on the immune system. In macrophages, similar to PACAP, VIP was able to bind to specific membrane receptors, including PAC1 and VPAC (17). The receptors of VIP interact with G proteins, and mediate cAMP-dependent pathway as well as calcium mobilization, protein kinase C, phosphoinositide 3-kinase (PI3-K) and mitogen-activated protein kinase MEK1/2 pathways (18,19). Thus, the expression of VIP in organisms plays a crucial role in multiple biological actions including immunomodulation, muscle relaxation, cell proliferation and differentiation. Several studies have shown that VIP has potential effects on increasing vessel formation in a xenograft model providing insight into VIP treatment in clinical practice (10,20). Similarly, Vacas and colleagues indicated that VIP suppresses clear cell renal cell carcinoma by inducing oxidative stress (9). Therefore, VIP as deactivator of macrophages may also contribute to the suppression of TAM. However, the molecular mechanism underlying this suppression effect of VIP remains poorly understood.

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The effects of VIP on expression of TNFα, IL-6, IL-12 and iNOS in macrophages were reported previously (12). TNFα, IL-6, IL-12 and iNOS are important regulators and indicators in the process of physiological and pathological immune system (21-23). Herein, we hypothesized that VIP may directly interact with TAM in gastric cancer and modulate the activation of TAM by regulating TNFα, IL-6, IL-12 and iNOS. The aim of the present study was to understand the effects of VIP on TAM in gastric cancer and illustrate the mechanism by which VIP represses the activation of TAM. For this purpose, the increasing TAM profile in patients and the depressive effects of VIP on TAM in gastric cancer were studied. Furthermore, by *in vivo* and *in vitro* experiments, the TNFα, IL-6, IL-12 and iNOS expression levels after VIP treatment was also assayed.

Materials and methods

Patients. All the samples from gastric cancer were obtained from Xiangya School of Medicine, Central South University (Changsha, China). The experiments in the present study were according to the ethical guidelines of Xiangya School of Medicine Research Ethics Committee. All the patients signed informed consent forms and the study was approved by the Hospital Ethics Committee. In total, 38 patients with gastric cancer were involved in this study. Tissues were collected during the operation. Tissue adjacent to the tumors were determined under a microscope as normal control tissues. The characteristics of the patients were shown in Table I.

Table I. The patient characteristics.

Variables	Data
Sample size	38
Age (years)	
Median	53.69
Range	37-63
Histology	
Distal	30
Proximal	8
Size (mm)	
<11	13
11-20	11
21-30	7
30-40	6
>40	1
Histological grade	
I	21
II	11
III	6
Stage	
I	14
II	12
III	7
IV	5

Cell culture and treatment. Human gastric cancer cell line MKN-45 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. TAM was induced from human monocytes THP1 as previously reported (24). Briefly, with 48-h treatment by 320 nmol/l phorbol myristate acetate (Merck Chemical Division, Rahway, NJ, USA), the suspended cells were transferred into adherent cells. Then the cells were treated with 20 ng/ml IL-4 and IL-13 for 72 h. The induced TAM was demonstrated using flow cytometry by the biomarkers CD14, CD68, CD206 and CD204.

The MKN-45 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) [supplemented with 10% fetal bovine serum (Gibco)] at 37˚C in an incubator with atmosphere of 5% CO_2. For TAM, the medium was supplemented with 500 U/ml IFN-γ and 100 ng/ml LPS (Sigma-Aldrich, St. Louis, MO, USA).

To determine the effect of VIP on TAM, cells were planted at $3x10^4$ cells/ml in 6-well plates (Gibco). The 20 wells were randomly divided into five groups (n=4), including 0, 0.5, 1, 2 and 10 μM VIP supplemented groups. After 48-h culture, the TAM responses to the VIP were detected by flow cytometry, proliferation assay and colony formation. The maximal responses of the VIP concentration was confirmed as 1.0 μM, and used to treat the TAM in following analysis. Subsequently, to understand the effects of TAM and VIP in the treated TAM on the gastric cancer cells, the MKN-45 cells at $3x10^4$ cells/ml were plated in 6-well plates (Gibco). The plated cells were divided into five groups (n=4), including control, TAM+MKN-45, TAM+VIP (1 μM), MKN-45+VIP (1 μM) and TAM+MKN-45+VIP (1 μM). The groups which contained TAM and MKN-45 were co-cultured at concentration of $3x10^4$ cells/ml for each cell type. For the groups with VIP supplementation, the VIP was added within 48 h after plating. Subsequently, the cells were analyzed within 96 h. Three independent experiments were performed.

Real-time PCR. Total RNAs from tissues and cells were isolated using RNA TRIzol (Invitrogen, Carlsbad, CA, USA). By agarose gel electrophoresis and BioPhotometer Plus (Eppendorf AG, Hamburg, Germany), the integrity and amount were assayed. Then 2 μg of total RNA was reverse transcribed into first cDNA using reverse transcriptase (Invitrogen) according to the manufacturer's protocols. The primers of the present study were designed as shown in Table II. The PCR was performed on ABI 7500 Real-Time PCR system (Applied Biosystems, Austin, TX, USA). The conditions were: 95˚C for 3 min, 40 cycles at 95˚C for 12 sec and 55˚C for 40 sec. In this experiment, GAPDH mRNA is the internal control gene for normalization. Tests without DNA template were performed as negative control and melt curves were performed to remove the DNA contamination. The relative expressions of mRNAs were calculated using $2^{-\Delta\Delta Ct}$ method.

Western blotting. The protein expression levels were assayed using western blotting. After homogenized in RIPA buffer

Table II. Primers used for real-time PCR.

Gene names	Forward primers (5'→3')	Reverse primers (5'→3')
CD68	CGGAATTCTGCTGGGGCTACTGGCAG	TGATCTAGAGTCCCCTGGGCTTTTGGCAG
TNF-α	GGAGAAGGGTGACCGACTCA	CTGCCCAGACTCGGCAA
IL-6	AGCACATTAAGTACATCCTCGGC	CCAGATTGGAAGCATCCGTC
IL-12	TGGAGTGCCAGGAGGACAGT	TCTTGGGTGGGTCAGGTTTG
iNOS	GGATGACTTTCGAGGACATGC	GGGCCCTCTGGTCATACTTTT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

(50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM DTT, protease and phosphatase inhibitors), the protein were isolated and then the quantity was determined by BCA protein assay kit (Beyotime, Wuhan, China). For each sample, 20 μg total protein was separated by 10% dodecyl sulfate polyacrylamide gel. The protein on gel was then transferred into polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 4% skim milk for 1 h at room temperature, primary polyclonal antibodies of CD68 (IS613, Dako, Denmark, produced in rabbit, 1:1,000), TNFα (T8300, Sigma-Aldrich, produced in rabbit, 1:2,000), IL-6 (MFCD00162579, Sigma-Aldrich, produced in rabbit, 1:1,000), IL-12 (I4153, Sigma-Aldrich, produced in goat, 1:1,000), iNOS (SAB4502011, Sigma-Aldrich, produced in rabbit, 1:1,000) and GAPDH (SAB2100894, Sigma-Aldrich, produced in rabbit, 1:500) were incubated with membranes at 4˚C overnight. All the antibodies were purchased from Sigma-Aldrich. After 3 times washing in TBST buffer (pH 7.6, 20 mM Tris-HCl, 137 mM NaCl, 0.01% Tween-20), the second antibodies (HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-goat IgG, Sigma-Aldrich, 1:2,000) and enhanced chemiluminescence (ECL, Millipore) were used to visualized the protein signals.

Immunohistochemical staining (IHC). Tissues were cut into 6-μm thick sections in paraffin wax. After de-waxing, the sections were blocked with 4% skim milk and incubated with primary antibodies (CD68, 1:200; TNFα, 1:200; IL-6, 1:500; IL-12, 1:200; iNOS, 1:200) at 4˚C overnight. Subsequently, the proteins were visualized after the second antibody incubation at room temperature for 1 h and visualized using streptavidin-biotinylated horseradish peroxidase complex kit (Beyotime). The TAM and MKN-45 cells were labeled by antibodies of FITC-CD68 (1:200) and cy5-MSI1 (1:200) (Bioss Co., China). Each staining was repeated 3 times.

Colony formation assay. After treatment, 2,000 cells from each group were plated into 6-well plates and incubated for 7 days at 37˚C in an incubator with atmosphere of 5% CO_2. After washing with phosphate buffer solution (PBS) three times, the cells were fixed with methanol for 15 min and stained using 0.2% crystal violet for 15 min. The colonies were counted and mean values were calculated from three independent experiments.

Flow cytometry. To analyze the apoptosis of the cells, we used flow cytometry (Coulter, Luton, UK) and Annexin V-FITC and propidium iodide (PI) (BioVision, Milpitas, CA, USA) staining according to the manufacturer's instructions. At least 30,000 cells for each sample were treated. The detection of biomarkers of TAM was performed using antibodies of CD14, CD68, CD206 and CD204. The cells were fixed using Cytofix/Cytoperm™ Fixation/Permeabilization solution (BD Biosciences, Franklin Lakes, NJ, USA). The FITC-CD68, FITC-CD14, PE-CD206, and PE-CD204 antibodies (Sigma-Aldrich) were incubated with the cells and labeled with PE-conjugated goat anti-mouse secondary antibody. Triplicate biological repeats were measured for this experiment.

Tumor formation in a nude mouse model. We used 5-week-old nude mice to generate the tumor model. The animal experiments were approved of the ethics committees of Xiangya School of Medicine, Central South University (Changsha, China). The tested mice were randomly divided into three groups (n=10 for each group), including control, VIP and VIP+TAM group. The mice were first injected with $3x10^4$ MKN-45 cells in 1 ml DMEM. The injections were performed every 2 days for 20 days until the tumor size was 100 mm^3. The tumor size was calculated as V=LxWxDx3.14/6. Subsequently, TAM and TAM+VIP (1 μM) were injected into the tumor tissues. Also, a blank group was performed by injection with saline. The tumor volumes were measured every 2 days until 20 days. After 20 days, the tumor tissues were excised and assayed using real-time PCR, western blotting and IHC.

Statistical analysis. The data are indicated as mean ± standard deviation (SD). The difference among the groups were confirmed using one-way-ANOVA analysis. The significant difference was determined at $P<0.05$. All the statistical analysis were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Increasing of TAM in gastric cancer. To assess the TAM level in gastric cancer, the indicator of TAM, CD68 was detected using real-time PCR. Compared to the adjacent normal tissues, the expression of CD68 was much higher in cancer samples ($P<0.001$, Fig. 1A). High level of CD68 was observed in the high histological grade (Fig. 1B) and advanced tumor stage (Fig. 1C). The results indicated high levels of TAM in gastric cancer.

VIP depresses TAM activation. The TAM were induced by THP1 human monocytes. The flow cytometry analysis of biomarkers including CD14 (marker for monocyte differentia-

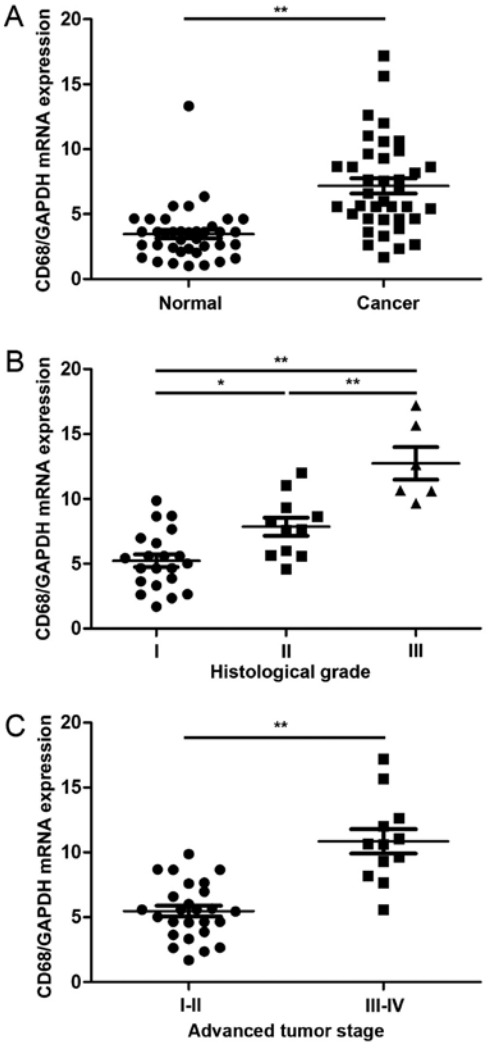

Figure 1. Expression of CD68 is upregulated in gastric cancer. (A) Compared to the normal tissues, the expression of CD68 was much higher in the gastric cancer tissues. (B) Higher expression of CD68 was observed based on the histological grade. (C) The expression of CD68 was much higher in III-IV advanced tumor stages compared to I-II advanced tumor stages. $^{*}p<0.05$; $^{**}p<0.001$.

tion), CD68 (marker for macrophages differentiation), CD206, and CD204 (both markers for M2 macrophages) proved that the TAM were successfully induced (Fig. 2).

After VIP treatment, the CD68 mRNA levels in TAM were significantly depressed ($P<0.05$). The 0.5 and 1.0 μM VIP treatment showed the most significant effects on expression of CD68 mRNA indicating the inhibition of TAM by VIP (Fig. 3A). The protein expression levels decreased significantly and was similar to the expression of mRNA. The 1.0 μM VIP treatment showed the most efficient depressive effects (Fig. 3B). The colony formation assay suggested that treatment with 1.0 μM VIP inhibited growth of TAM (Fig. 3C). By flow cytometry, we also showed that VIP treatment stimulated the apoptosis of TAM and 1.0 μM VIP induced apoptosis with the optimal dosage (Fig. 3D).

VIP inhibits TNFα, IL-6, IL-12 and iNOS in TAM. VIP has been shown to depress expression of TNFα, IL-6, IL-12 and iNOS in macrophages. In the present study, the effect of VIP on expression of TNFα, IL-6, IL-12 and iNOS in TAM was determined. The result showed that VIP depressed the expression of TNFα, IL-6 and IL-12 in all the treatment groups, including 0.5, 1.0, 2.0 and 10.0 μM VIP treatment (Fig. 4A-C). For iNOS, except the 0.5 μM VIP treatment, the other concentrations of VIP treatment, including 1.0, 2.0 and 10.0 μM VIP, inhibited the expression in TAM significantly (Fig. 4D). Western blotting showed similar changes (Fig. 4E). The protein expression of TNFα, IL-6, IL-12 and iNOS was dcreased after VIP treatment. The 1.0 μM VIP treatment group showed inhibition of these genes among all the groups, thus, the following experiment was performed using 1.0 μM VIP in treatment of TAM.

The VIP-treated TAM depressed gastric cancer cells. To examine the possible effects of VIP on gastric cancer cells via TAM, TAM were co-cultured with human gastric cancer cell line MKN-45 and treated with VIP. The co-culture of TAM and MKN-45 is shown in Fig. 5A. The CD68 and MSI1 as specific biomarkers for TAM and MKN-45 were used to identify the cells. The result showed co-existence of TAM and MKN-45 (Fig. 5A). Colony formation assay showed that the VIP-treated TAM remarkably reduced colony formation of gastric cancer cells (Fig. 5B). Moreover, without TAM, the MKN-45+VIP group had no significant decrease compared to control, which indicated the depressive effects of VIP on gastric cancer cells is indirect and meditated by TAM (Fig. 5B). The proliferation assay showed that the lowest cell viability of MKN-45 cells was observed in TAM+VIP and TAM+MKN-45+VIP group (Fig. 5C). The TAM+MKN-45 group had the highest cell viability, while, other groups showed medial cell viability. Apoptosis demonstrated by flow cytometry showed similar results. TAM+MKN-45+VIP group

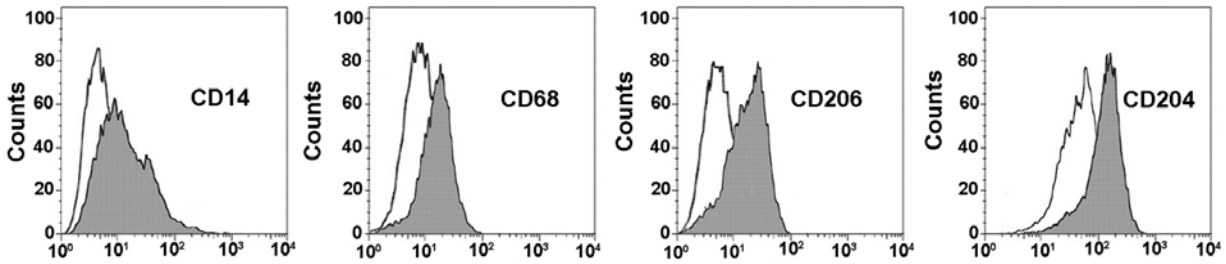

Figure 2. Confirmation of induced TAM by CD14 (marker for monocyte differentiation), CD68 (marker for macrophage differentiation), CD206, and CD204 (both markers for M2 macrophages).

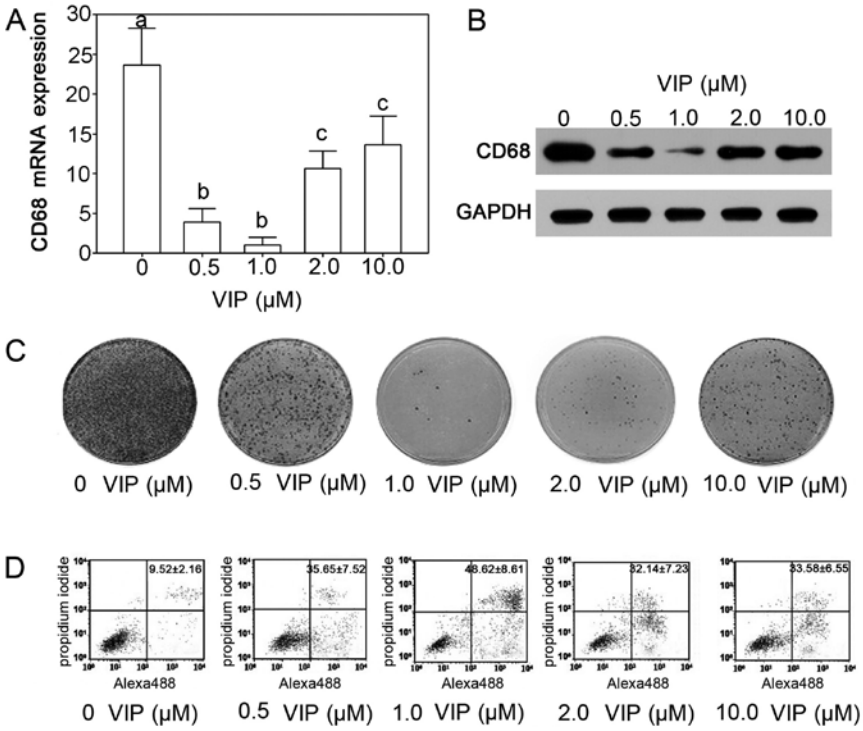

Figure 3. VIP depresses activation of TAM. (A) Real-time PCR shows inhibition of CD68 mRNA levels of TAM by VIP (n=4 for each group, data are expressed as the means ± SD; different lower case characters represent significant differences, P<0.05). (B) Western blotting indicates the depressive effects of VIP on protein levels of CD68. (C) Colony formation test of TAM after VIP treatment. (D) Flow cytometry assay indicates that VIP treatment induces apoptosis of TAM. The same letter indicates no significant difference while different letter superscripts represent significant difference.

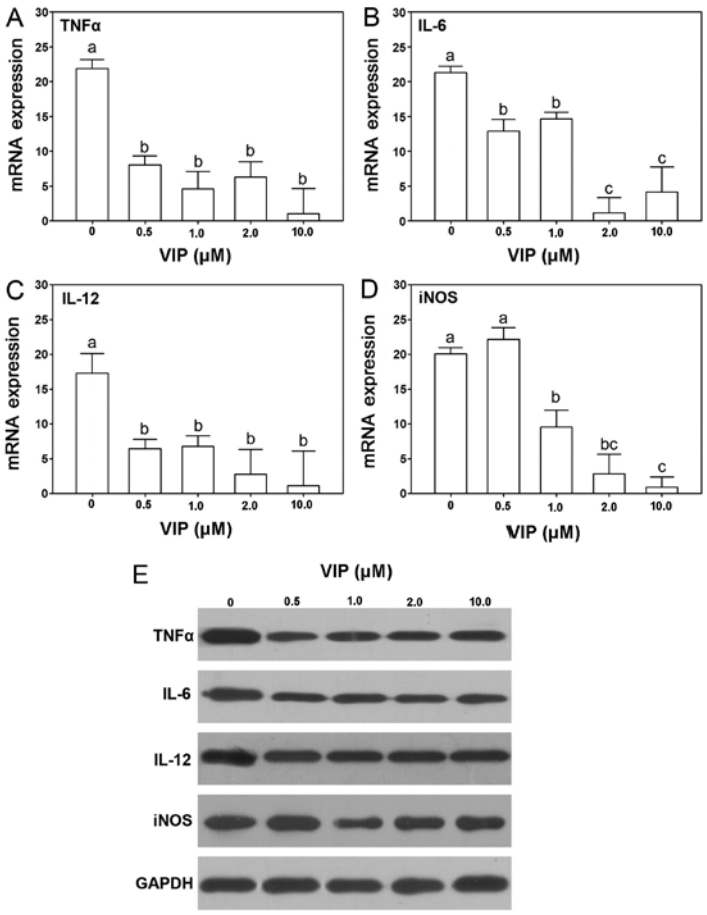

Figure 4. VIP inhibits expression of TNFα, IL-6, IL-12 and iNOS in TAM. Real-time PCR indicates that mRNA expression of TNFα (A), IL-6 (B), IL-12 (C) and iNOS (D) of TAM (n=4 for each group, data are expressed as the means ± SD; different lower case characters represent significant differences, P<0.05). (E) Western blotting also shows depressive effects of VIP on protein expression of TNFα, IL-6, IL-12 and iNOS in TAM.

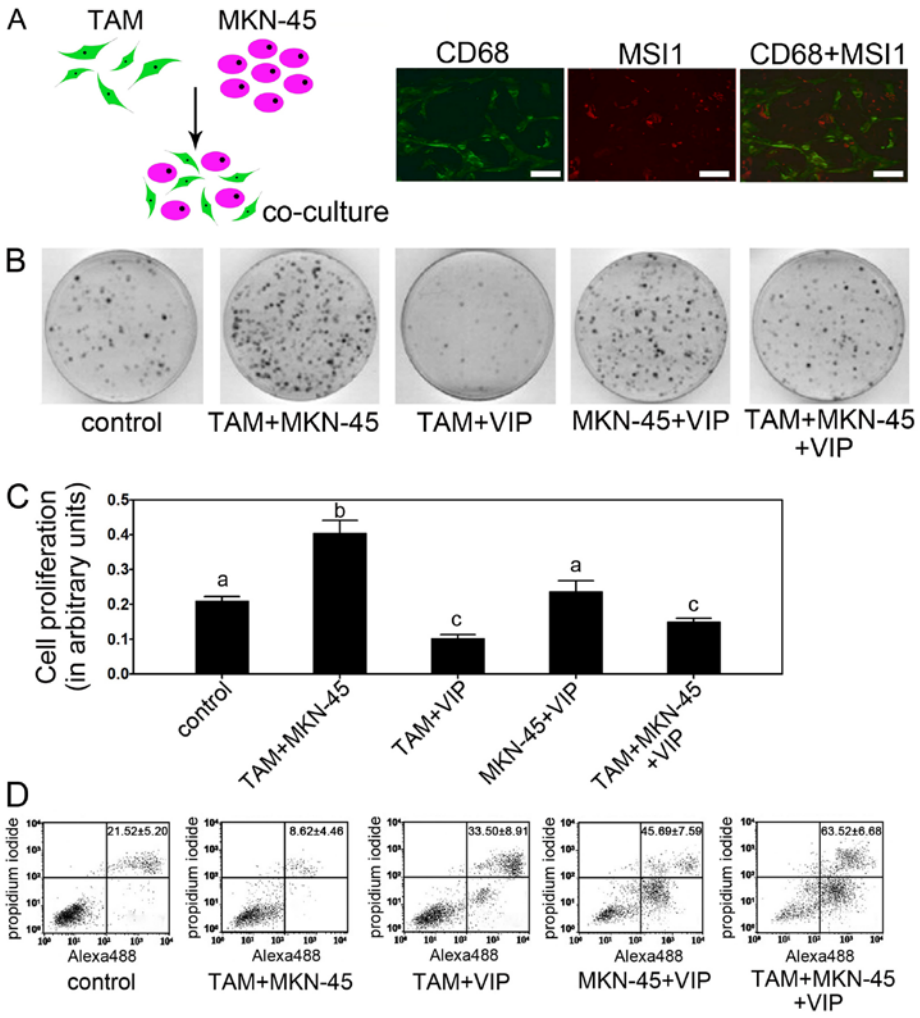

Figure 5. VIP inhibits activation of gastric cancer cells via depressing TAM. (A) Co-culture of TAM and MKN-45. CD68 and MSI1 are labeled TAM and MKN-45, respectively. (B) Colony formation test of gastric cancer cells (MKN-45) and TAM after VIP treatment. (C) Cell proliferation of gastric cancer cells (MKN-45) and TAM after VIP treatment (n=4 for each group, data are expressed as the means ± SD; different lower case characters represent significant differences, P<0.05). (D) Flow cytometry assay indicates apoptosis of gastric cancer cells (MKN-45) and TAM after VIP treatment. The same letter indicates no significant difference while different letter superscripts represent significant difference.

showed the highest apoptosis rate while TAM+MKN-45 group had the lowest apoptosis rate (Fig. 5D).

As the VIP depressed activation of gastric cancer cells via TAM, we next determine if VIP affects gene expression levels in cultured gastric cancer cells directly or indirectly. VIP downregulated TNFα, IL-6, IL-12 and iNOS were found in TAM+VIP and TAM+MKN-45+VIP groups while MKN-45+VIP group showed no significant difference of expression compared to control, which suggested that the depressive effects of TNFα, IL-6, IL-12 and iNOS was mediated by TAM (Fig. 6).

VIP depresses TAM and tumor formation in the nude mouse model. To illustrate the effect of VIP on gastric cancer *in vivo*, the tumor formation in the nude mouse model was constructed by $3x10^4$ MKN-45 cell injections. After tumor formation, the TAM and TAM+VIP were injected into the tumors. The tumor volume and tumor weight of TAM+VIP group were significantly lower compared with the TAM group (P<0.05 after 20 days) (Fig. 7A and B). The CD68 was increased accordingly in TAM groups while depressed CD68 was found in TAM+VIP group indicating the depressive effect of TAM *in vivo* (Fig. 7C-E).

Consistent with the result *in vitro*, the expression levels of TNFα, IL-6, IL-12 and iNOS in xenograft tumor tissues were downregulated by VIP in TAM+VIP group compared with the TAM group (Fig. 8). Thus, the results of the xenograft model suggested that VIP inhibits the tumor progression of gastric cancer mediated by TAM *in vivo* via downregulating expression of TNFα, IL-6, IL-12 and iNOS.

Discussion

The present study showed the primary findings on the VIP depressive effect on TAM. Moreover, the treatment with VIP inhibits the expression of TNFα, IL-6, IL-12 and iNOS in TAM, which results in deactivation of TAM. For cancer cells, TAM acts as mediators for interacting growth factors, cytokines and chemokines and change the tumor microenvironment to stimulate tumor progression (25-27). We demonstrated that the VIP inhibited gastric cancer via TAM both in cultured cells and in the nude mouse model.

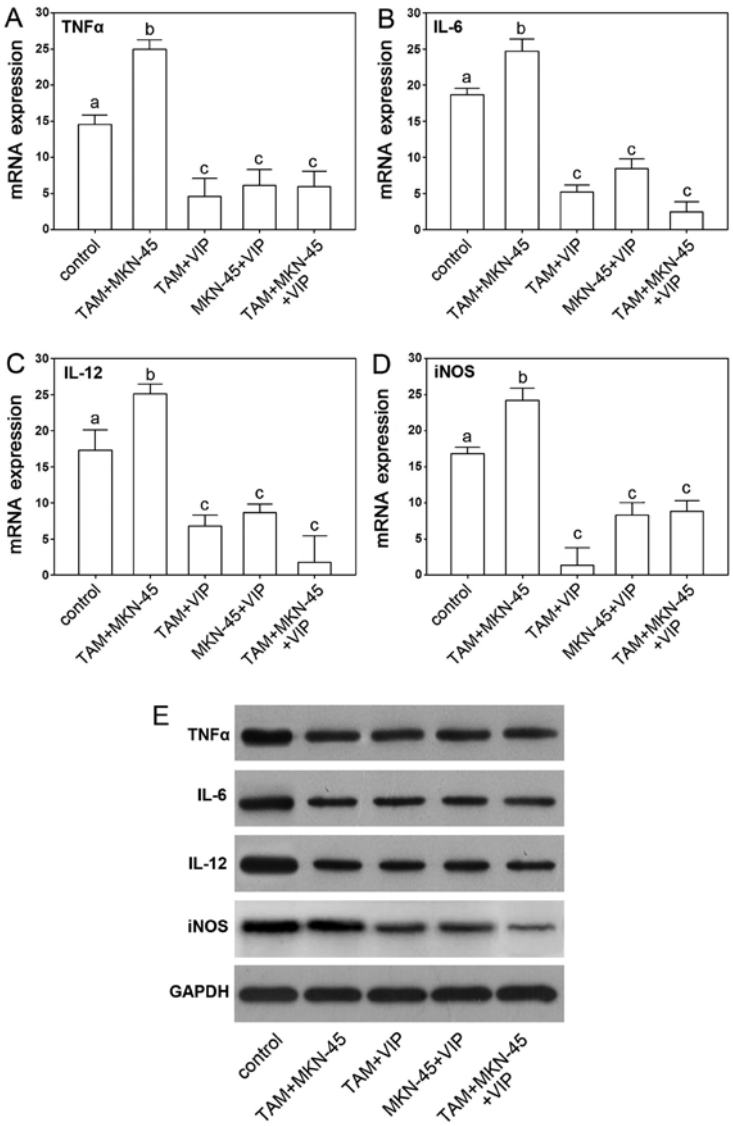

Figure 6. VIP depression of TNFα, IL-6, IL-12 and iNOS after VIP treatment in TAM co-cultured with gastric cancer cells. Real-time PCR indicates that mRNA expression of TNFα (A), IL-6 (B), IL-12 (C) and iNOS (D) of TAM co-cultured with gastric cancer cells (n=4 for each group, data are expressed as the means ± SD; different lower case characters represent significant differences, $P<0.05$). (E) Western blotting also shows depressive effects of VIP on protein expression of TNFα, IL-6, IL-12 and iNOS in TAM co-cultured with gastric cancer cells.

Macrophages originating from blood monocytes are divided into M1 (classically activated) and M2 types (alternatively activated) (28). TAM has been regarded as M2 phenotype and play mostly pro-tumoral functions such as promoting tumor cell survival, proliferation and invasion (29). High levels of TAM are correlated with poor prognosis (30). Simultaneously, CD68 as indicator of TAM has been used as molecular signature to determine the prognosis of cancer (31,32). With no surprise, as we found in the present study, CD68 was highly expressed in gastric cancer tissues compared to normal tissues showing similar results with previous reports with higher level of TAM in tumor than normal tissues. Accordingly, depression of TAM may be a potential therapy for gastric cancer treatment. Further, we used VIP, a pleiotropic peptides to intervene in TAM to demonstrate the possibility of VIP as new therapeutic strategy.

Tumorigenesis as a complex process, results from molecular and cellular variation with a variety of compounds including oncoproteins and tumor proteins. During this process, TAM are the promotional factor for tumorigenesis (33). VIP is a neuropeptide that exerts multiple actions in different types of cells (8). Several studies showed that the de-activated function of VIP in macrophages (11,12,34). As we found in the present study, VIP depressed TAM as well. It is known that VIP affects the expression of both pro- and anti-inflammatory factors after LPS and IFNγ induced macrophages (35,36). In the present study, the treatment of VIP depressed activities of TAM by suppressing expression of CD68 and colony formation as well as inducing apoptosis. Based on these findings, it seems likely that the depressing effect of VIP on the proliferation of macrophages also exists in TAM. However, there is still a lack of straight forward answer as to how VIP suppressed the TAM and whether VIP could inhibit tumorigenesis by depressing TAM *in vivo*.

The molecular mechanism by which VIP exerts its deactivated effects in macrophage is well studied. VIP has been

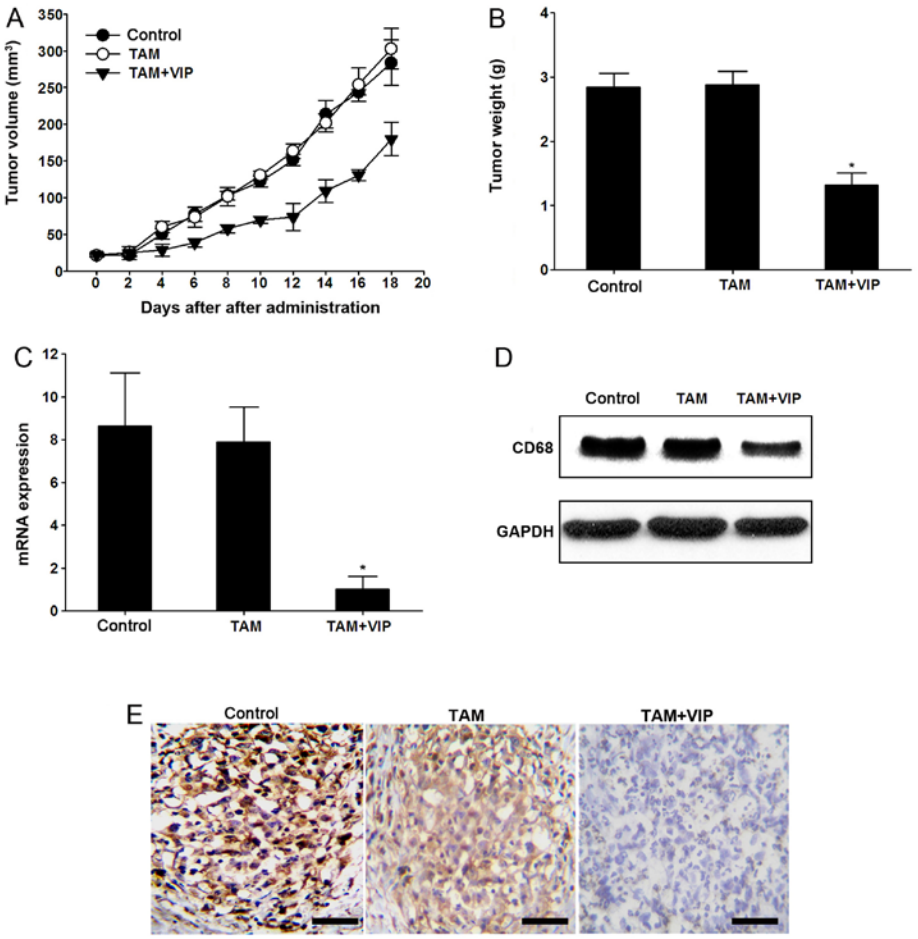

Figure 7. VIP treatment of TAM depresses tumor formation. (A) Tumor volume changes after injection with TAM and VIP treated TAM (n=10 mice in each group). (B) Tumor weight after injection with TAM and VIP treated TAM (n=10 mice in each group, data are expressed as the means ± SD; asterisks showed significant difference between the groups, P<0.05). (C) Expression of CD68 mRNA in xenograft tumors after injection with TAM and VIP treated TAM (n=5 mice in each group, data are expressed as the means ± SD; asterisks showed significant difference between the groups, P<0.05). (D) Expression of CD68 protein in xenograft tumors after injection with TAM and VIP treated TAM assayed by western blotting (data are expressed as the means ± SD; asterisks show significant difference between the groups, P<0.05). (E) Distribution and expression of CD68 detected by IHC. Bar, 50 μm.

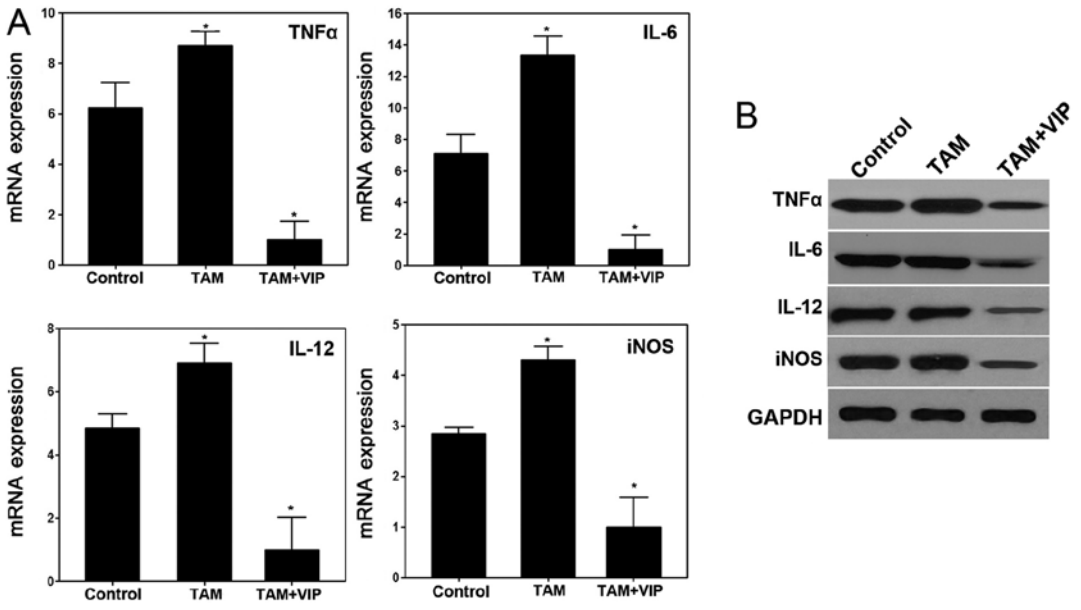

Figure 8. Expression of TNFα, IL-6, IL-12 and iNOS in xenograft tumors after injection with TAM and VIP treated TAM. (A) Real-time PCR result indicates depressive effect of VIP treatment on mRNA expression of TNFα, IL-6, IL-12 and iNOS in xenograft tumors after TAM injection (n=5 mice in each group, data are expressed as the means ± SD; asterisks show significant difference between the groups, P<0.05). (B) Western blotting shows depressive effect of VIP treatment on protein expression of TNFα, IL-6, IL-12 and iNOS in xenograft tumors after TAM injection.

shown to regulate the expression and/or transactivating of transcription factors such as AP-1, NFκB, CREB and IRF-1 (13,14,34,36) and mediate the expression of chemokines, tumor necrosis factors, COX2, interleukin and toll-like receptors (8,15). TNFα showed tumor-promoting roles in previous studies and is regarded as a target in malignant cancer (21,37). Inhibition of TNFα reduces metastatic activity in tumors (37). The present study showed that VIP inhibited production of TNFα and the incubation of VIP suppressed the effects of TAM. These data are consistent with previous studies showing that VIP reduced the growth of macrophage via regulating the growth factor, and in macrophages, IL-6, IL-12 and iNOS could be inhibited by VIP which showed deactivation effects of VIP on macrophages (8,11,12). IL-6 and IL-12 are synthesized by macrophages and participate in inducing antibody secretion, acute phase reaction, hematopoiesis and regulating production of IFN-γ and TNFα (8). iNOS, also participates in the immune response by binding to calmodulin and produces NO as an immune defense mechanism which also indicates the activities of macrophages (23). The inhibited expression levels of IL-6, IL-12 and iNOS by VIP showed decreased activity of TAM. These results indicate that treatment with VIP inhibits TNFα and IL-6, IL-12 and iNOS of TAM which then results in depressed cell proliferation.

Our data showed that VIP inhibits progression of TAM, and the role of TAM as cancer promoter has been demonstrated previously. This evidence suggests TAM as potential therapeutic target in human cancer and VIP could be an efficient inhibitor for TAM. In the present study, VIP is found to inhibit gastric cancer cells as well as tumor formation in the nude mouse model. We found the depressive effects of VIP are indirect via first suppressing TAM. The decreased expression of TNFα and IL-6, IL-12 and iNOS after VIP treated required co-culture of TAM and MKN-45. In a previous study, VIP suppressed metastatic human clear cell renal cell carcinoma by inducing oxidative stress (9). Vacas *et al* demonstrated that VIP inhibited invasion and metastasis of human clear cell renal cell carcinoma via decreasing β-catenin (9). On the contrary, high expression of VIP in pancreas could induce VIPoma which is a very rare type of cancer that usually derived from pancreatic cells (38). Thus, the roles of VIP in cancer progression seem contradictory in different types of cancer which needs to be elucidated in further study. The results of our present study showed that the VIP treatment with a proper dosage could inhibit progression of gastric cancer by deactivating TAM which is similar to the macrophages by decreasing expression levels of TNFα and IL-6, IL-12 and iNOS.

In conclusion, we demonstrated that VIP inhibits progression of gastric cancer mediated by TAM in the present study. The antitumor action of VIP appears to be initiated by interaction with TAM via depression of the expression levels of TNFα and IL-6, IL-12 and iNOS. The presented data provide new insight into the therapeutic application of VIP to inhibit gastric cancer both *in vivo* and *in vitro* via TAM.

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