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Walker256 Sarcoma Stimulates Myocardial Angiogenesis after Acute Myocardial Infarction

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Abstract

Objective: Regulating angiogenesis is an attractive target for treating tumors or ischemic diseases. The present study was to establish the Wister rat model with acute myocardial infarction (AMI) and Walker256 Sarcoma, and observe angiogenesis and expression of vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGFβ1).

Method and results: Wistar rats were randomly assigned to AMI group (subjected to coronary artery ligation), AMI-tumor group (right regioaxillaris tumor induced by inoculation of tumor cell after AMI), sham group (the operation procedure was same to AMI rats except for the ligation of coronary artery or tumor cell inoculation) and sham-tumor group (inoculation of tumor cell added to rats in sham group). The capillary density in myocardium was investigated with optical microscope, and protein expression was examined with immunohistochemical staining. The left ventricular mass index (LVMI) of rats in AMI-tumor group decreased significantly compared with rats in AMI group, in parallel with increased expression of TGFβ1 and VEGF as well as capillary density.

Conclusions: Inoculation with walker256 sarcoma in AMI rats showed an increase of capillary density in the ischemic myocardium, which may be associated with elevated protein expression of VEGF and TGFβ1 in the serum and ischemic myocardium.

Keywords: Walker-256 sarcoma; Ischemic myocardium; Angiogenesis; Vascular endothelial growth factor; Transforming growth factor β1

Introduction

Angiogenesis is an attractive focus in the treatment for ischemic heart disease and malignant tumors. Although malignant tumors and myocardial infarction (MI) show different pathological changes, the treatment targeted to angiogenesis is the valuable strategy for them in recent years [1,2]. There is an increasing demand in oxygen and nutrient supplied from capillary during the tumor growing, as an important regulator of tumor angiogenesis, vascular endothelial growth factor (VEGF) is released by most solid tumors and various hematologic tumors to induce tumor angiogenesis [3]. Intervention of the VEGF pathway has emerged as a therapeutic strategy in oncology. Another cytokine associated with angiogenesis is transforming growth factor β1 (TGF-β1)which plays two conflicting roles of a tumor suppressor and a tumor promoter. In the early stage of cancer development TGF-β1 acts as a tumor suppressor, whereas in late stage it can take on role of tumor promoter, favoring of angiogenesis and invasion [4]. The convincing evidence has been provided by experimental studies that targeting the TGF-β pathway can inhibit tumor growth and metastasis *in vivo* [5]. Taken together, VEGF and TGF-β1 are two important factors involved in tumor angiogenesis and have been attached a great attention in the treatment for cancers [6,7].

Similarly, the release of these angiogenic factors might be an underlying mechanism of angiogenesis in ischemic myocardium after AMI [8]. Recent evidence showed that VEGF and TGF- β1 were associated with an increase in the angiogenesis of ischemic myocardium and improvement of myocardial perfusion in patients with refractory angina [9-11].

This study is to address two questions: (1) what are the differences in angiogenesis in ischemic myocardium between the rats with both MI and tumor originated from inoculation of walker256 sarcoma cells and those with MI alone; (2) whether the rats with both tumor and MI have an increase in angiogenesis of ischemic myocardium compared with rats only with MI. Therefore, we developed a new experimental model by inoculating walker256 sarcoma cells into rat with MI, and explored the possible mechanism of angiogenesis in ischemic myocardium by detecting the micro vessel density and the expression of VEGF and TGFβ1.

Materials and Methods

Animals

Wistar rats (180-200 g) were obtained from the Experimental Animal Center, China Academy of Chinese Medicine Sciences (SCK [Jing] 20005-0013). All experimental procedures performed in this study were approved by the Institutional Animal Care and Use Committee at China Academy of Chinese Medical Sciences. The

investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

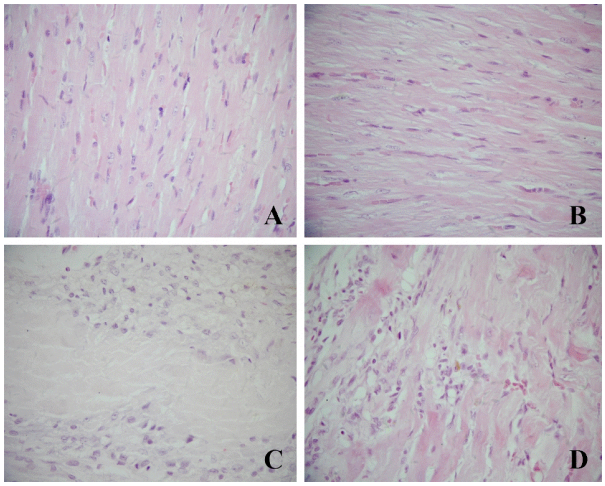

Figure 1: The HE stained sections of myocardium in different groups. Myocardial cells in both sham rats (A) and sham-tumor rats (B) were lined in order with clear nuclear staining. Myocardial cells in both AMI-tumor rats (D) and AMI rats (C) were atrophied with necrosis and surrounded by the large number of new granulation tissue (400X).

Myocardial infarction model

Animals were anaesthetized with a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg) via intraperitoneal injection. The operation procedures of MI were performed as described previously [12], briefly as follows: the left thoracotomy of rat was performed at third intercostal space, and muscles and pericardium were carefully dissected. The heart was squeezed out. The ligation site of left anterior descending artery (LAD) was between the pulmonary conus and left atrial appendage. The MI rat model was supposed to be succeeding, when J point on the II lead of synchronized electrocardiogram markedly elevated and left ventricular anterior wall became paler shade as reported previously [13]. The heart was then quickly put back into the chest and the incision was sutured. Meanwhile, the rats in sham group underwent the same procedures except for arterial occlusion. The adequacy of anaesthesia was monitored during the operation without withdrawal response to foot pinch. All rats were given 40000 units penicillin (intramuscular injection) for 3 d in order to prevent the wound infection. The mortality rate for AMI rat model in this study was 39%.

Inoculation of tumor cells into rats with AMI

The Walker 256 sarcoma is widely used in experimental tumor research, mainly based on easy transplantation, successful inoculation rate, lack of strain specificity, and rarity of spontaneous metastases [14]. The Walker 256 sarcoma strain was provided by the Institute of Pharmacology Affiliated to Chinese Academy of Medical Sciences. Seven days after intraperitoneal injection of Walker 256 sarcoma cells to the rats, its ascites was taken under sterile conditions. Cell viability was assessed by trypan blue exclusion, and the number of tumor cells was diluted with saline to 2×10^6/ml cells suspension. The tumor cells suspension of 0.2 ml was injected subcutaneously into right regio axillaris of rat with MI and the tumor was formed locally.

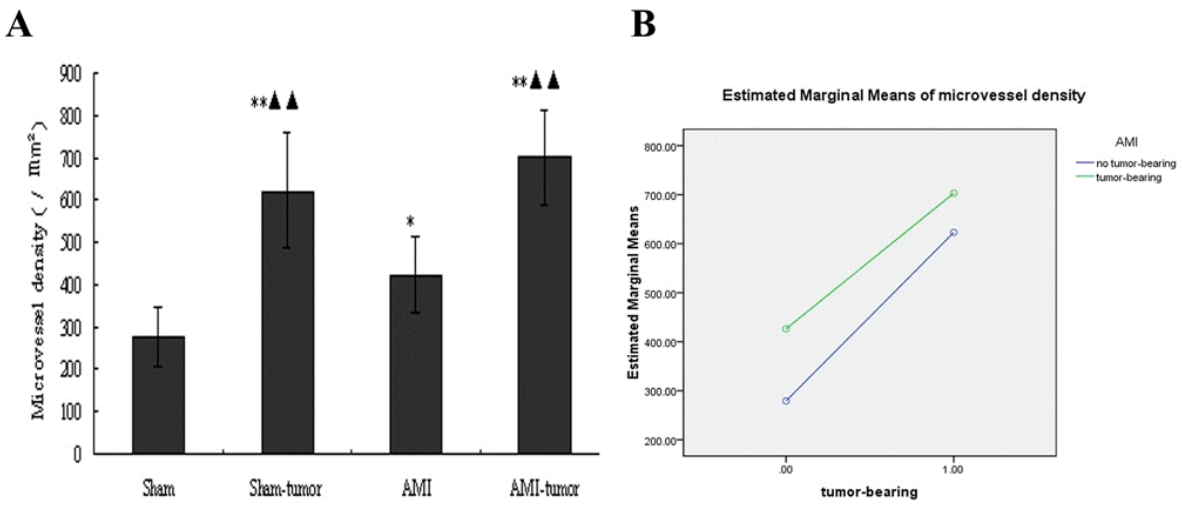

Figure 2: (A) The capillary density in myocardium of sham-tumor rats and AMI-tumor rats showed a significant increase compared with AMI rats. The results are expressed as mean ± SEM, n=5. $^{*}P<0.01$ and $^{**}P<0.01$, *vs.* sham group; ▲▲$P<0.01$ *vs.* AMI group. (B) The variance analysis of factorial experimental design showed an independent effect of tumor-bearing from AMI on promoting capillary density (F=0.510, P=0.485).

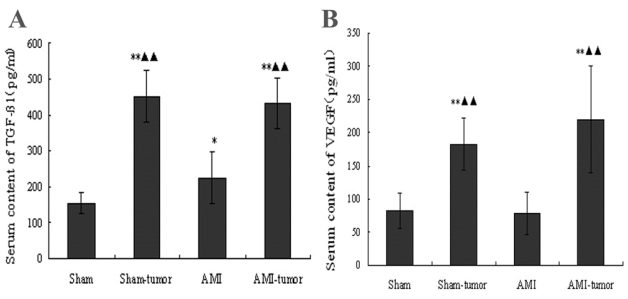

Figure 3: A and B showed quantitative results of TGFβ1 and VEGF in serum measured by ELISA, respectively. (n=7 for AMI group; n=6 for AMI-tumor group; n=8 for all other groups). *P<0.01 and **P<0.01 *vs.* sham group; ▲▲P<0.01 *vs.* AMI group.

Experimental protocol

Sixteen survival AMI rats were randomly allocated to either AMI group (rats with AMI) or AMI-tumor group (rats with AMI inoculated with Walker 256 sarcoma cells), 8 rats in each group. Sixteen rats with sham operation were randomly divided into sham group (no inoculation with Walker 256 sarcoma cells) and sham-tumor group (inoculation with Walker 256 sarcoma cells), 8 rats in each group.

Left ventricular mass index (LVMI)

At 15 d after inoculation with tumor cells, the rats were anesthetized with 1.5% isoflurane; the blood was taken out from abdominal artery for serological testing. And then the rats were sacrificed by cervical dislocation. The hearts were harvested quickly, and the left ventricle was separated and weighed. LVMI was measured as the ratio of left ventricle weight to body weight, and the left ventricle was fixed in the 10% neutral formaldehyde solution for histopathological and immunohistochemical examination.

Enzyme-linked immunosorbent assay

The content of VEGF and TGFβ1 in serum was measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction. The VEGF and TGFβ1 ELISA kits for murine were purchased from Rapid Biolab Co., US.

Histological staining

The myocardium in ventricle above transverse line, 2 mm below ligation site, was removed, and the rest ventricle was embedded in the paraffin and was cut transversely into sections (4 μm/section) for staining with haematoxylin-eosin (HE). To test the protein expression of VEGF and TGFβ1, the immunohistochemical staining with polyclonal rabbit anti-human-VEGF (Wuhan Boster Biological Engineering Co., China) and polyclonal rabbit anti-human-TGFβ1 (Wuhan Boster Biological Engineering Co., China) were performed respectively according to the manufacturer's instructions.

Five serial slices of the ischemic myocardium samples in each group were observed under light microscope (400X) and the positive reactions of VEGF and TGFβ1 with color of brownish yellow in cytoplasm of myocardial cells in three visual fields of each slice were analysed quantitatively. The expression rate of VEGF or TGFβ1 was presented as the percentage of positive cells to all cells in whole visual field.

Myocardial capillary density

The capillary density in ischemic myocardium 2 mm below ligation site was calculated in light of the method reported by Weidner [15]. Three visual fields of the highest vascular density in each slice were selected for calculating the capillary numbers (diameter<20 μm) under light microscope (400X) and the average capillary density (MVD) was expressed as capillary number/mm^2.

Statistical analysis

All data were analysed with Statistical Package for the Social Sciences (version 13.0) and presented as mean ± standard error of the mean (SEM)$\left(\overline{X}\pm S\right)$. The significant difference among groups was evaluated by a one-way analysis of variance (ANOVA). Correlation analysis between groups was evaluated by Pearson co rrelation coefficient, and the non-normal distribution data were evaluated by spearman correlation coefficient. The interaction between the factors was analysed by variance analysis of factorial experimental design.

Results

Histopathological observation

Three survivals AMI rats were died during the experiment, 1 in AMI group, died of cardiac rupture; 2 in AMI-tumor group, died of cachexia. The observation with HE stain showed that the cardiac structures in sham rats and sham-tumor rats were normal: myocardial cells were lined in order, with clear nuclear staining, and without degeneration or interstitial edema. Cardiac structures in AMI-tumor rats and AMI rats were disorganized: the myocardial cells were atrophy and necrosis surrounded by a large number of new granulation tissue; the necrotic myocardial tissue was wrapped like an island shape. However, comparing with AMI-tumor rats, the new granulation tissue was less and inflammatory cells infiltration was more severe in AMI rats (Figure 1).

Groups	N	Body weight (g)	LV weight (g)	LVMI (mg/g)
Sham	8	295.75 ± 29.58	0.68 ± 0.06	2.25 ± 0.37
AMI	7	306.29 ± 41.43	1.02 ± 0.11**	3.38 ± 0.60**
Sham-tumor	8	216.75 ± 28.32**▲▲	0.49 ± 0.04**▲▲	2.28 ± 0.20▲▲
AMI-tumor	6	215.67 ± 34.35**▲▲	0.57 ± 0.09*▲▲	2.70 ± 0.78▲

Table 1: The changes of LVMI. LV: Left Ventricle; LVMI: Left Ventricular Mass Index; AMI: Acute Myocardial Infarction. *P<0.05 and **P<0.01 *vs.* sham group. ▲P<0.05 and ▲▲P<0.01 *vs.* AMI group.

Left ventricular mass index (LVMI)

AMI rats revealed a significant increase in LVMI compared with sham rats (3.38 ± 0.60 mg/g versus 2.25 ± 0.37 mg/g; P=0.000), but there was no significant difference in LVMI between the sham-tumor rats and AMI-tumor rats (sham-tumor versus AMI-tumor: 2.28 ± 0.20

mg/g versus 2.70 ± 0.78 mg/g; P=0.129). In addition, LVMI in AMI rats inoculated with tumor cells was significantly reduced by 32.5% compared with AMI rats (P=0.026) (Table 1).

Given the cachexia after the rats inoculated with tumor cells, which might have an impact on LVMI, we analysed the correlation between rat's body weight and LVMI and the result showed that the body weight had not impacts on the LVMI (the correlation coefficient, -0.066, P=0.732).

Capillary density in ischemic myocardium

Capillary density was an important index for evaluating angiogenesis in ischemic myocardium. AMI rats showed a significant increase in capillary density of ischemic myocardium compared with sham rats (426.20 ± 90.31/mm^2 versus 279.20 ± 68.05/mm^2; P=0.042). In addition, compared with AMI rats, the AMI rats inoculated with tumor cells showed a further increase in capillary density of ischemic myocardium to 703.00 ± 112.53/mm^2(P=0.001) (Figure 2A).

To evaluate whether the inoculation with tumor cells have an additional effect on capillary density in ischemic myocardium of AMI rats, we analysed the interaction between tumor-bearing and myocardial infarction using variance analysis of factorial experimental design, and the results showed that the effect of tumor-bearing on the increase of capillary density was independent of AMI (F=0.510, P=0.485) (Figure 2B).

Serum contents of VEGF and TGFβ1

The sham-tumor rats showed a significant increase in the serum contents of VEGF and TGFβ1 compared with sham rats (P<0.01). Both AMI-tumor and sham-tumor rats also showed a significant increase in serum contents of VEGF and TGFβ1 compared with the AMI rats (P<0.01) in parallel with capillary density changes in ischemic myocardium of different rats (Figure 3).

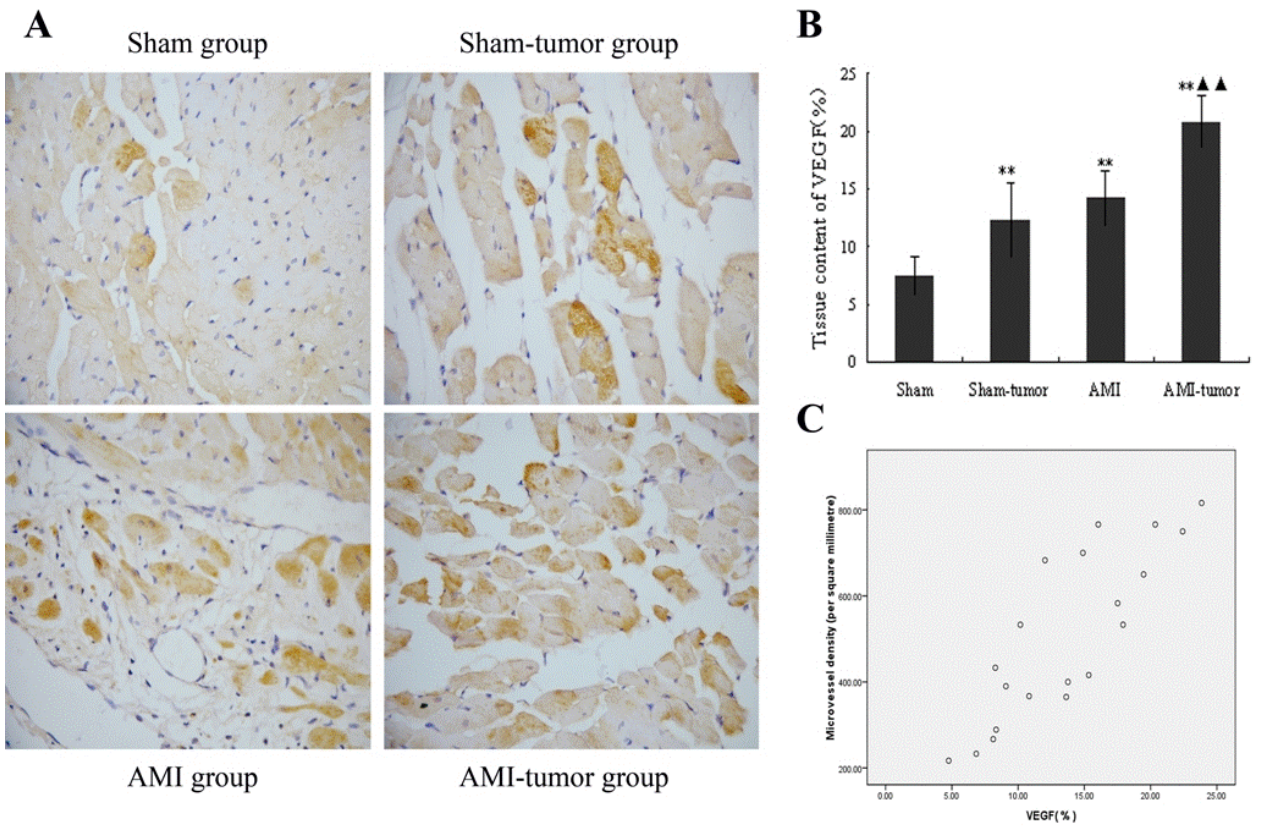

Figure 4: The changes of VEGF in the myocardium of each group rats. (A) Immunohistochemical sections of the ischemic myocardium 15 days after AMI. Brownish yellow color was the positive reaction of VEGF located in the cytoplasm of myocardial cells. (B) The protein expression of the VEGF in the ischemic myocardium was determined by immunohistochemical staining (n=5). **P<0.01, vs. sham group; ▲▲P<0.01 *vs.* AMI group. (C) The correlation analysis of VEGF expression and capillary density in the myocardium showed that the correlation coefficient between VEGF levels and capillary density was 0.834, P<0.01.

Protein expression of VEGF and TGFβ1 in the ischemic myocardium

VEGF and TGFβ1 protein expressions in the ischemic myocardium were measured by immunohistochemistry staining. AMI-tumor rats showed a significant increase in TGFβ1 and VEGF compared with AMI rats (P<0.01) (Figures 4B and 5B).

The correlation analysis of VEGF expression and capillary density in the ischemic myocardium showed a positive relationship between

VEGF levels and capillary density (coefficient=0.834, P<0.01) (Figure 4C). Similarly, the correlation analysis of TGFβ1 expression and capillary density in the myocardium also showed a positive relationship between TGFβ1 levels and capillary density (coefficient=0.837, P<0.01(Figure 5C).

Discussion

According to our knowledge, this study, for the first, investigated the changes of myocardial capillary density in AMI animal model coexisting with Walker 256 sarcoma. The inoculation of Walker 256 tumor cells into the Wistar rats with AMI was proved feasible and reproducible. The successful inoculation rate was near to 99%, which is consistent to previous report [16]. Because the rats with Walker 256 tumor cells have a higher mortality rate due to the cachexia compared with rats without inoculation, we performed the investigation at the 15th day after inoculation in this experiment.

The LVMI is an objective index of cardiac remodelling after AMI [17]. The present study showed a significant decrease in LVMI and body weight of AMI-tumor group compared with AMI group (P<0.01). However, due to the consumption caused by rapid tumor growth, a significant lowered body weight of tumor-bearing rat might be an influence factor on evaluation of LVMI. Therefore, we analysed the correlation of body weight and LVMI to determine if the body weight has an impact on evaluation of LVMI. The result showed that the body weight has no impact on the evaluation of LVMI in tumor-bear rats (P>0.05). Taken together, the inoculation of Walker 256 tumor cells into rats with AMI reduced LVMI.

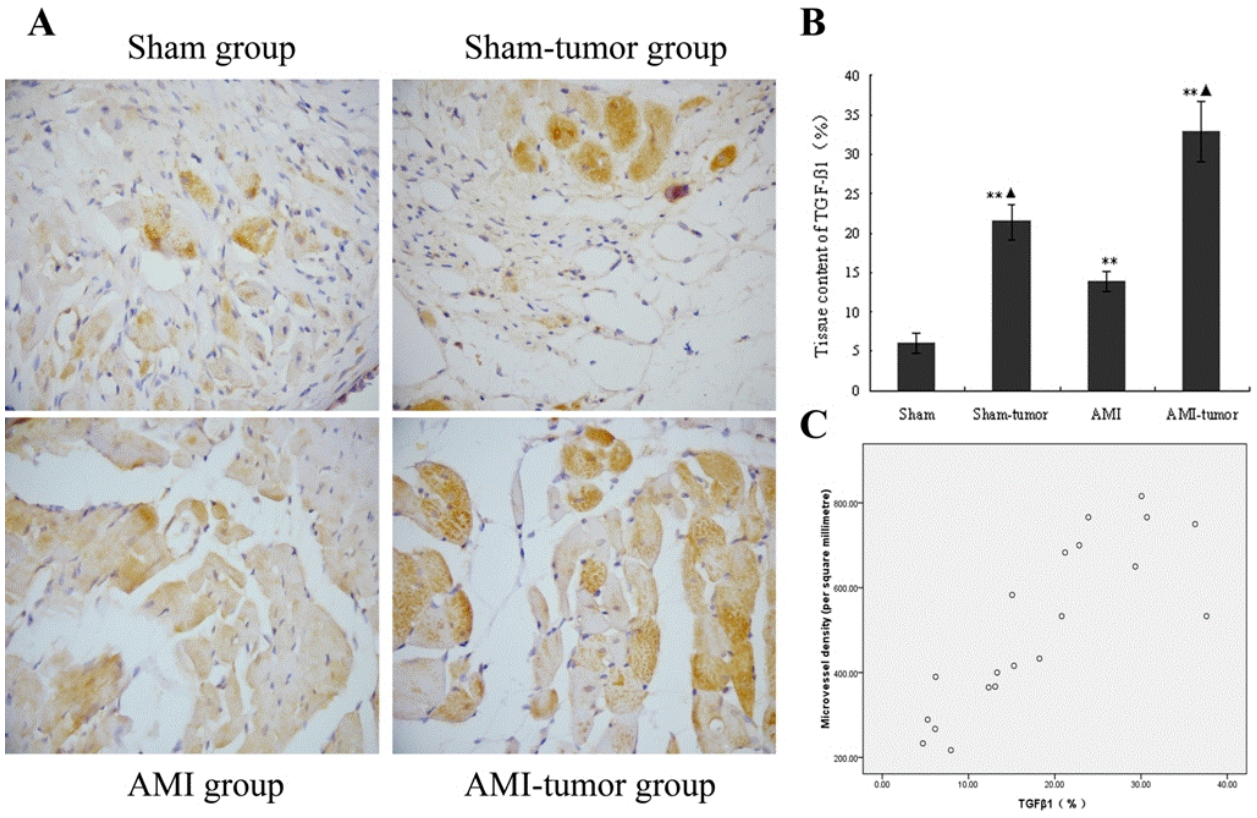

Figure 5: The changes of TGFβ1 in the myocardium of each group rats. (A) Immunohistochemical sections of the ischemic myocardium 15 days after AMI. Brownish yellow color was the positive reaction of TGFβ1 located in the cytoplasm of myocardial cells. (B) The protein expression of the VEGF in the ischemic myocardium was determined by immunohistochemical staining(n=5). **P<0.01 *vs.* Sham group; ▲▲P<0.01 *vs.* AMI group. (C) The correlation analysis of TGFβ1 expression and capillary density in the myocardium showed that the correlation coefficient between TGFβ1 levels and capillary density was 0.837, P<0.01.

Because angiogenesis is a preliminary condition for proliferation, invasion and metastasis of tumor cells, the capillary density in tumor tissue is an important indicator for its metastasis, recurrence and prognosis [18]. Angiogenesis also plays an essential role on the improvement of heart function after AMI. In this study, capillary density of ischemic myocardium was increased significantly in the AMI-tumor group and sham-tumor group, compared with AMI group and sham group respectively (P<0.01), which indicated that the inoculation of tumor cells into the rats with AMI further improved the angiogenesis in ischemic myocardium.

VEGF and TGFβ1 are the critical factors in regulation of cell migration, proliferation and survival, as well as mediators of tumor angiogenesis [18-23]. Both of them not only initiated the signal pathways of angiogenesis, but also were the downstream pathways of angiogenesis, which closely associated with microvessel regeneration [7,24]. Previous studies reported that low levels of TGFβ1 increased

VEGF and stimulated angiogenesis [25]. VEGF secreted from tumor cells linked to its receptor in the surface of vascular endothelial cell and bone marrow-derived cell, induced regional angiogenesis in ischemic myocardium and improved cardiac function [1,2]. VEGF could stimulate angiogenesis in different animal models [26,27]; low dose of recombinant human VEGF protein injected into the rabbit myocardium within the infarct region enhanced angiogenesis of infarcted myocardium [28]. In the present study, compared with rats in both sham group and AMI group, the rats in both tumor-bearing sham group and tumor-bearing AMI group showed a higher level of VEGF or TGFβ1 in both serum and ischemic myocardium ($P<0.01$). The correlation analysis showed that the concentration of VEGF or TGFβ1 in ischemic myocardium of each group was positively correlated with myocardial capillary density($P<0.01$), which indicated that the proangiogenic effect of walker 256 sarcoma on ischemic myocardium in AMI rats may be associated with elevated protein expression of VEGF and TGFβ1 in the serum and ischemic myocardium.

In this study, we established a new murine model with both myocardial infarction and tumor for the first time, and found that the tumor induced by walker 256 sarcoma in AMI rats increased capillary density in the ischemic myocardium, which associated with the elevated protein expression of VEGF and TGFβ1 in serum and ischemic myocardium. Our results suggest that exploring new target from angiogenesis stimulated by tumor might be a promising strategy for treatment of ischemic heart disease.

Acknowledgements

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