Communication

DOI: 10.5582/bst.2018.01105

The proangiogenic role of polymorphonuclear myeloid-derived suppressor cells in mice infected with *Echinococcus granulosus*

Jianhai Yin, Yujuan Shen, Aiping Yu, Congshan Liu, Jiaqing Yao, Wenci Gong, Jianping Cao*

National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Chinese Center for Tropical Diseases Research, WHO Collaborating Centre for Tropical Diseases, National Center for International Research on Tropical Diseases, Ministry of Science and Technology, Key Laboratory of Parasite and Vector Biology, Ministry of Health, Shanghai, China.

Summary **The aim of this study was to first evaluate the proangiogenic activity of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC) in mice infected with *Echinococcus granulosus*. PMN-MDSCs derived from experimentally infected mice were collected and cultured *in vitro*, and their effect on angiogenesis was investigated using a human umbilical vein endothelial cell (HUVEC) tube-formation assay stimulated with the supernatant by microscope and the Angiogenesis module of the software NIH Image J. In addition, the expression levels of several functional factors related to proangiogenic activity were analyzed. The results showed that vascular endothelial growth factor (VEGF) was increased in the serum from infected mice, and the PMN-MDSCs expressed VEGF directly. The culture supernatant from PMN-MDSCs significantly promoted HUVECs to form tubes. VEGF mRNA was higher and soluble fms-like tyrosine kinase-1 levels were lower, in PMN-MDSCs from infected mice than in those from control mice. In conclusion, host angiogenesis in mice infected with *E. granulosus* appeared to be promoted by PMN-MDSCs. Other specific angiogenic factors derived from PMN-MDSCs and parasites in the microenvironment of infection foci should be clarified in further studies, in order to provide more information for the prophylaxis and treatment of echinococcosis.**

Keywords: Polymorphonuclear myeloid-derived suppressor cells, proangiogenic, *Echinococcus granulosus*, vascular endothelial growth factor, soluble fms-like tyrosine kinase-1

1. Introduction

The circulatory system, consisting of the cardiovascular and lymphatic systems, transports circulating nutrients, oxygen, hormones, growth factors, and their metabolites to tissues and cells. Angiogenesis, the growth of new capillary blood vessels in the body, is an important physiological process (*1*). It is highly regulated by a precise balance of growth and inhibitory factors produced in healthy tissues (*2,3*). However, abnormal blood vessel growth due to the imbalance of these factors is related to many deadly and debilitating pathological conditions, including cancer, diabetic ulcers, and rheumatoid arthritis (*1,4*). More and more diseases are being reported to have angiogenesis as an underlying mechanism.

Vascular endothelial growth factor (VEGF) is considered to be the strongest stimulator of angiogenesis (*5*). It promotes the proliferation and migration of endothelial cells, antagonizes cell apoptosis and promotes differentiation through activating intracellular tyrosine kinase, and finally directly forms blood vessels. Moreover, VEGF can also initiate the proteolytic enzyme system to participate in the degradation of extracellular matrix and promote the formation of new blood vessels (*6*). In addition, VEGF may increase vascular permeability resulting in plasma protein extravasation, participate in the formation of vascular basement membrane and extracellular matrix, and provide support for endothelial cell migration and blood vessel growth (*7*).

**Address correspondence to:*
Dr. Jianping Cao, National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Chinese Center for Tropical Diseases Research, WHO Collaborating Centre for Tropical Diseases, National Center for International Research on Tropical Diseases, Ministry of Science and Technology, Key Laboratory of Parasite and Vector Biology, Ministry of Health, Shanghai 200025, China.
E-mail: caojp@yahoo.com

In contrast, soluble fms-like tyrosine kinase-1 (sFlt-1) is a tyrosine kinase protein that disables proteins that cause blood vessel growth (*8,9*).

The growth, maturation, and reproduction of parasites in a host are inseparable from nutrient supply and metabolite excretion, both of which depend on the circulatory system (*10*). Angiogenesis in a host during a parasitic infection can be activated by various factors produced by the parasites themselves. For example, echinococcosis caused by *Echinococcus multilocularis* produces tumor-like infiltrative growth in the host liver, and causes the development of hydatid cysts wrapped by granulation tissues, with abundant new vessels visible (*11*). This is partially attributed to the glucose phosphate isomerase expressed by *E. multilocularis*, which is not only essential for the growth, development, infiltration, and metastasis of *E. multilocularis*, but also plays a potential role in host angiogenesis (*12*).

In addition to parasite-derived factors, parasite-infected hosts can also produce factors that promote angiogenesis in the host. Myeloid-derived suppressor cells (MDSC) including polymorphonuclear MDSCs (PMN-MDSC) and monocytic MDSCs (M-MDSC) are activated and enriched during parasitic infections, and play an important role in immunosuppression (*13*). These cells have a proangiogenic role in the processes of tumor growth, invasion and metastasis (*14-16*), but whether they function similarly during parasitic infection is unclear. It has been reported that MDSCs secrete angiogenic factors such as VEGF and/or directly differentiate into vascular endothelial cells to promote host angiogenesis, which is beneficial to tumor development. In our previous studies, we found that hydatid cysts of *Echinococcus granulosus* promoted the formation of tubes by human umbilical vein endothelial cells (HUVEC) *in vitro* (*17*). We also found that MDSCs were significantly enriched in the spleen and peripheral blood of BALB/c mice infected with *E. granulosus*, and played an immunosuppressive role (*18*). Moreover, M-MDSCs from the spleen of BALB/c mice infected with *E. granulosus* could significantly promote HUVECs into forming tubes.

We therefore investigated the potential proangiogenic role of PMN-MDSCs from the spleen of BALB/c mice infected with *E. granulosus* using the tube formation assay and analysis of key angiogenic factors. To our knowledge, this is the first time this has been investigated, and the results have important implications for the prophylaxis and treatment of echinococcosis.

2. Angiogenesis caused by *E. granulosus* infection

Host angiogenesis caused by parasite infection is an extremely complicated process that is not only affected by parasitic factors; host response to infection is also important (*10*). In general, the exchange of angiogenic factors between parasite and host plays an important role. In this study, around 2,000 live protoscoleces from sheep liver hydatid cysts were injected intraperitoneally into each BALB/c mouse (aged 4 weeks), and it was clear that new blood vessels were wrapped around the outside of cysts developed 8 months post infection through visual observation (Figure 1A). Meanwhile, CD31 was highly expressed in these tissues using immunohistochemistry (Figure 1B), suggesting that the host cells stimulated by this parasite have a tendency to differentiate into endothelial cells, thereby possessing the potential for the tube formation that is necessary for angiogenesis.

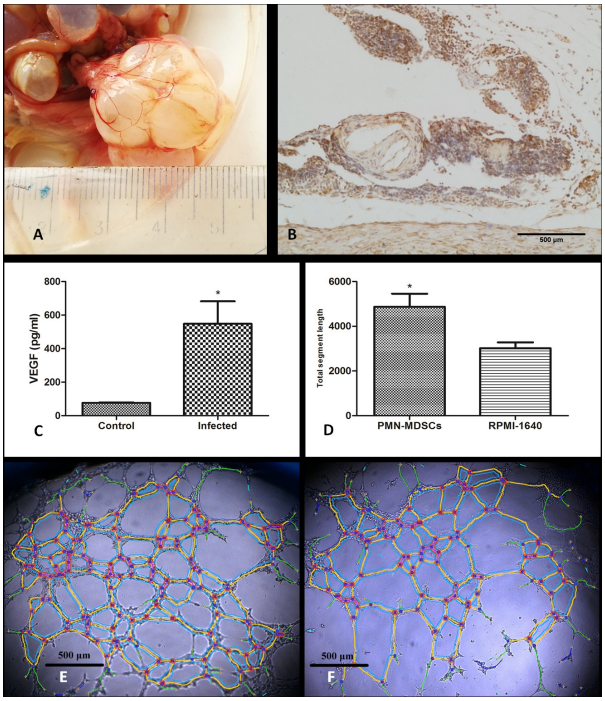

Figure 1. Angiogenesis casused by *E. granulosus*. (A) New blood vessels wrapped around the hydatid cysts isolated from mice infected with *Echinococcus granulosus*. (scale: cm) **(B)** CD31 was highly expressed in the tissues surrounding the hydatid cysts using immunohistochemical staining. **(C)** Higher level of VEGF in serum from mice infected with *E. granulosus* compared with controls (n = 7, each) detected by ELISA. **(D)** Total tube segment length induced by supernatant of PMN-MDSCs from infected mice was higher than that from controls. **(E)** HUVEC tube formation induced by the supernatant from PMN-MDSCs from infected mice. **(F)** HUVEC tube formation induced by RPMI-1640. *$p < 0.05$.

It has been reported that the supernatant from hydatid cysts isolated from mice infected with *E. granulosus* significantly induced HUVEC tube formation *in vitro* (*17*), suggesting that parasite-derived angiogenic factors are involved in the regulation of host angiogenesis. In the current study, a significantly higher level of VEGF was found in serum from mice infected with *E. granulosus* (548.100 ± 134.200 pg/mL) than in serum from control mice (76.950 ± 2.760 pg/mL; t = 4.109, p = 0.001) by Enzyme-linked immunosorbent assay according to the instructions of Mouse VEGF ELISA Kit (RayBiotech, USA) (Figure 1C). It is therefore likely that this increased level of VEGF could boost host angiogenesis.

3. PMN-MDSCs promotes angiogenesis

MDSCs are enriched in peripheral blood and spleen during infection with *E. granulosus*, especially in the period of chronic infection (*18*). We showed that M-MDSCs may have a proangiogenic role using the HUVEC tube formation assay and transcriptome analyses. In the present study, PMN-MDSCs were isolated from spleens of BALB/c mice infected with *E. granulosus* after 8 months post-infection and normal control mice (n = 7, each) using a mouse Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec, Germany) and flow cytometry (purity higher than 90%) (*18*), VEGF was detected by Mouse VEGF ELISA Kit (RayBiotech, USA) in the supernatant of PMN-MDSCs from mice infected with *E. granulosus* (5.444 ± 1.191 pg/mL) at a density of 10^6 cells/well in 24-well plates with Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) containing 100 U/mL penicillin and 100 U/mL streptomycin incubating at 37°C in a 5% CO_2/95% air atmosphere for 24 h, but it couldn't be detected in the culture supernatant of PMN-MDSCs from control mice.

An *in vitro* tube formation assay was used to evaluate the effect of PMN-MDSCs on HUVECs differentiation on a Matrigel matrix (BD Biosciences, USA). In brief, 100 μL of HUVECs (3-5 passages) at a density of 3 × 10^5 cells/mL per well of 96-well plates were stimulated with 100 μL PMN-MDSC culture supernatant from infected mice, or RPMI-1640 medium with 100 U/mL penicillin and 100 U/mL streptomycin as a control. After 3 h of incubation at 37°C in 5% CO_2/95% air, the total segment length of tube-like structures in three random fields per well was quantified using the angiogenesis module of the NIH ImageJ software. As a result, HUVEC tube formation was much greater with the supernatant (4873 ± 586.1) than control group (3015 ± 269.3) (t = 2.685, p = 0.025) (Figure 1D-1F). Moreover, analysis of the transcriptional expression of VEGF (forward: 5′-GAGTACCCCGACGAGATAGA-3′, reverse: 5′-GGCTTTGGTGAGGTTTGAT-3′) and sFlt-1 (forward: 5′-GGGAAGACATCCTTCGGAAGA-3′, reverse: 5′-TCCGAGAGAAAATGGCCTTTT-3′) genes by quantitative real-time PCR showed that the expression of VEGF mRNA was higher in the PMN-MDSCs of *E. granulosus*-infected mice than in those of normal control mice (t = 13.81, p < 0.001) (Figure 2A), and that the expression of sFlt-1 mRNA (t = 7.550, p = 0.026) was downregulated (Figure 2B).

In conclusion, host angiogenesis in mice infected with *E. granulosus* appeared to be promoted by PMN-MDSCs, as shown by their high expression of VEGF, promotion of tube formation in the *in vitro* HUVEC assay, and significantly higher expression of VEGF mRNA and lower expression of sFlt-1 mRNA. Taken together, it suggests that MDSCs, including M-MDSCs and PMN-MDSCs, from mice infected with *E. granulosus* play a role in host angiogenesis. Furthermore,

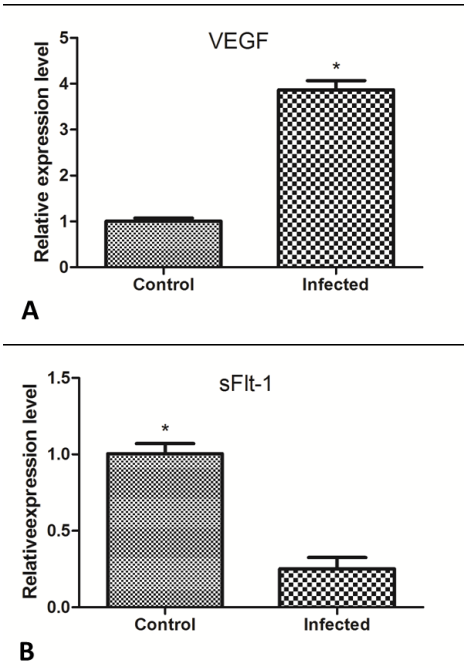

Figure 2. The relative mRNA expression levels of target genes. (A) VEGF mRNA was expressed more highly in PMN-MDSCs from mice infected with *E. granulosus* than in normal control mice. **(B)** sFlt-1 mRNA was expressed at a lower level in PMN-MDSCs from mice infected with *E. granulosus* than in controls. *p < 0.05.

other angiogenic factors in addition to VEGF derived from host cells (such as MDSCs) and pathogens (such as *E. granulosus*) in the microenvironment of local lesions should be clarified in future studies. This would provide ideas for the further study of host angiogenesis induced by *E. granulosus* infection.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant numbers 81702030, 81772224) and the Fourth Round of Three-Year Public Health Action Plan of Shanghai, China (grant number 15GWZK0101).

References

1. Carmeliet P. Angiogenesis in life, disease and medicine. Nature. 2005; 438:932-936.
2. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: Mechanisms of blood vessel formation and remodeling. J Cell Biochem. 2007; 102:840-847.
3. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000; 6:389-395.
4. Polverini PJ. Angiogenesis in health and disease: Insights into basic mechanisms and therapeutic opportunities. J Dent Educ. 2002; 66:962-975.
5. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology. 2005; 69 Suppl 3:4-10.
6. McColl BK, Stacker SA, Achen MG. Molecular regulation of the VEGF family -- inducers of angiogenesis

and lymphangiogenesis. APMIS. 2004; 112:463-480.
7. Melgar-Lesmes P, Tugues S, Ros J, Fernandez-Varo G, Morales-Ruiz M, Rodes J, Jimenez W. Vascular endothelial growth factor and angiopoietin-2 play a major role in the pathogenesis of vascular leakage in cirrhotic rats. Gut. 2009; 58:285-292.
8. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003; 111:649-658.
9. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A. 1993; 90:10705-10709.
10. Dennis RD, Schubert U, Bauer C. Angiogenesis and parasitic helminth-associated neovascularization. Parasitology. 2011; 138:426-439.
11. Guerret S, Vuitton DA, Liance M, Pater C, Carbillet JP. *Echinococcus multilocularis*: Relationship between susceptibility/resistance and liver fibrogenesis in experimental mice. Parasitol Res. 1998; 84:657-667.
12. Stadelmann B, Spiliotis M, Muller J, Scholl S, Muller N, Gottstein B, Hemphill A. *Echinococcus multilocularis* phosphoglucose isomerase (EmPGI): A glycolytic enzyme involved in metacestode growth and parasite-host cell interactions. Int J Parasitol. 2010; 40:1563-1574.
13. Van Ginderachter JA, Beschin A, De Baetselier P, Raes G. Myeloid-derived suppressor cells in parasitic infections. Euro J Immunol. 2010; 40:2976-2985.
14. Gabrilovich DI. Myeloid-derived suppressor cells. Cancer Immunol Res. 2017; 5:3-8.
15. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends Immunol. 2016; 37:208-220.
16. Binsfeld M, Muller J, Lamour V, De Veirman K, De Raeve H, Bellahcene A, Van Valckenborgh E, Baron F, Beguin Y, Caers J, Heusschen R. Granulocytic myeloid-derived suppressor cells promote angiogenesis in the context of multiple myeloma. Oncotarget. 2016; 7:37931-37943.
17. Yin JH, Shen YJ, Yu AP, Cao JP. *In vitro* pro-angiogenic activity of *Echinococcus granulosus* hydatid cysts from experimentally infected mice. Chin J Schisto Contrl. 2017; 29:320-323. (in Chinese)
18. Pan W, Zhou HJ, Shen YJ, Wang Y, Xu YX, Hu Y, Jiang YY, Yuan ZY, Ugwu CE, Cao JP. Surveillance on the status of immune cells after Echinnococcus granulosus protoscoleces infection in Balb/c mice. PloS One. 2013; 8:e59746.

(Received May 17, 2018; Revised June 23, 2018; Accepted June 26, 2018