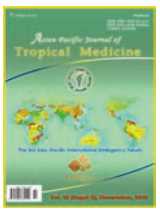

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Anti-tumor activity of a recombinant endoglin-MIP3α Fc-fusion protein in mice with hepatocellular carcinoma

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ABSTRACT

Objective: To investigate the effects of a recombinant endoglin-macrophage inflammatory protein 3α Fc-fusion protein (EM) vaccine on tumor angiogenesis and growth in mice with H22 hepatocellular carcinoma.

Methods: An *in vivo* hepatoma mouse model was established. Seven days after subcutaneous inoculation of H22 tumor cells, mice were randomly divided into four groups: EM, endoglin Fc-fusion protein, macrophage inflammatory protein 3α Fc-fusion protein, and normal saline groups. Tumor volume and survival rate of mice were studied at 3-day intervals. Microvessel density of the tumors and tumor cell proliferation were detected by immunohistochemistry, and tumor cell apoptosis was detected by TdT-mediated biotinylated-dUTP nick-end label staining. The number of CD11c and CD86 positive dendritic cells were detected by flow cytometry.

Results: Compared with the other groups, the tumor volume became smaller, and the survival time was longer in the EM-treated group. Besides, microvessel density and cell proliferation index were significantly lower, while the tumor cell apoptosis index was significantly higher in the EM-treated group. Besides the number of CD11c and CD86 positive dendritic cells in EM-treated mice was larger than that in other groups.

Conclusions: EM Fc-fusion protein could effectively inhibit tumor growth through inhibiting endoglin-related tumor angiogenesis and cell proliferation, promoting tumor cell apoptosis, and could induce a certain degree of antitumor immune responses.

1. Introduction

Hepatocellular carcinoma is the most common type of liver cancer, causing approximately 1 million deaths annually[1]. In 2012, approximately 50% of new cancer cases occurred in Asia, mostly in China[2]. The primary aetiological factors in these regions are chronic infections by hepatitis viruses and dietary exposure to aflatoxins, a group of mycotoxins that are natural contaminants of the staple diet[3]. Thus, it is essential to seek multidisciplinary approaches for the treatment of hepatocellular carcinoma.

A series of preclinical and clinical studies have indicated that endoglin is one of the most important angiogenic growth factors for tumor angiogenesis, and it is overexpressed and upregulated in tumor-associated angiogenic vasculature relative to normal tissue vasculature[4-7]. Therefore, it is conceivable that using endoglin as a target of tumor immunotherapy strategy could inhibit endoglin-related angiogenesis and further suppress tumor growth. Our previous work also found that targeted endoglin immunotherapy of tumors showed an effective and synergistic anti-tumor activity[8-11]. Macrophage inflammatory protein 3α (MIP3α) is the most

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important specific chemokine of dendritic cells (DCs)[12]. By attracting immature DCs which express the DC-unique receptor CCR6, MIP3α chemoattracts pathogenic infected tissues, enabling DCs to contact and present antigens[12-15]. Our previous study found that recombinant MIP3α adenovirus used as a vaccine effectively attracted DCs and induced antitumor immunities[16]. Therefore, if we try to increase the level of MIP3α in tumor tissues, we can promote the chemotaxis of DCs to tumor tissues and improve anti-tumor immunity. The purpose of our study was to evaluate the anti-tumor efficacy of the endoglin-MIP3α (EM) Fc-fusion protein in mice with H22 liver cancer.

2. Materials and methods

2.1. Proteins preparation

In this study, the lyophilized recombinant Fc-fusion proteins of endoglin-MIP3α, endoglin, and MIP3α were prepared as described previously[9].

2.2. Animals model and treatment

To established the H22 hepatoma model, forty six to eight-week-old female and specific pathogen-free (SPF) BALA/c mice were randomly divided into four groups of ten mice each and injected subcutaneously with 2×10^6 corresponding tumor cells in the right flank. The temperature in the animal facility was maintained at 20-26 ℃, with a relative humidity between 40% and 70%. Environmental conditions in the laboratory animal center were in line with the national standard for SPF-grade facilities. The EM, endoglin and MIP3α Fc-fusion protein were injected intratumorally once a week for 4 wk with a dose of 10 μg (100 μL) per mouse, respectively. Additional untreated control animals were injected with 100 μL normal saline. Tumor growth was evaluated every 3 days, and tumor volume was estimated using the formula for an ellipsoid (0.5×length×width×height, cm). This study followed the animal protocols of the College's Animal Care and Use Committee and was ethically approved by the First Affiliated Hospital of Hainan Medical University Committee (code of ethics: ZDYF2017165).

2.3. Immunohistochemistry

For microvessel density and cell proliferation analyses, frozen sections were fixed in acetone, incubated, and stained with antibodies reactive to either CD31 or proliferating cell nuclear antigen (BD Pharmingen, USA) respectively. Moreover, sections of tissue were fixed with 1% paraformaldehyde in phosphate buffer solution (PBS) and stained for apoptosis analysis using TdT-mediated biotinylated-dUTP nick-end label (TUNEL) assay (In Situ Cell Death Detection Kit; Roche, UK) as previously described[9].

2.4. Flow cytometry

Lymphocytes were isolated from the tumor using a lymphocyte separation medium. The cells were collected by centrifugation and then were washed twice using PBS. The suspension was removed by centrifugation. The cells were resuspended in 90 μL PBS, then mixed with antibodies (CD11c: 0.25 μg/test; CD86: 0.125 μg/test) and incubated with antibodies at 4 ℃ for 30 min. After the addition of 400 μL PBS, FACS data collection was performed on a FACScan (Becton Dickinson) using CellQuest Pro software.

2.5. Statistical analysis

To compare data at individual time points, ANOVA and an unpaired Student's *t*-test were used. Survival curves were constructed according to the Kaplan-Meier method. $P<0.05$ was considered statistically significant.

3. Results

3.1. Inhibition of tumor growth and extended survival time

The tumor volume was smaller and the survival time was significantly longer in the EM-treated group than that in other groups ($P<0.001$, Figure 1).

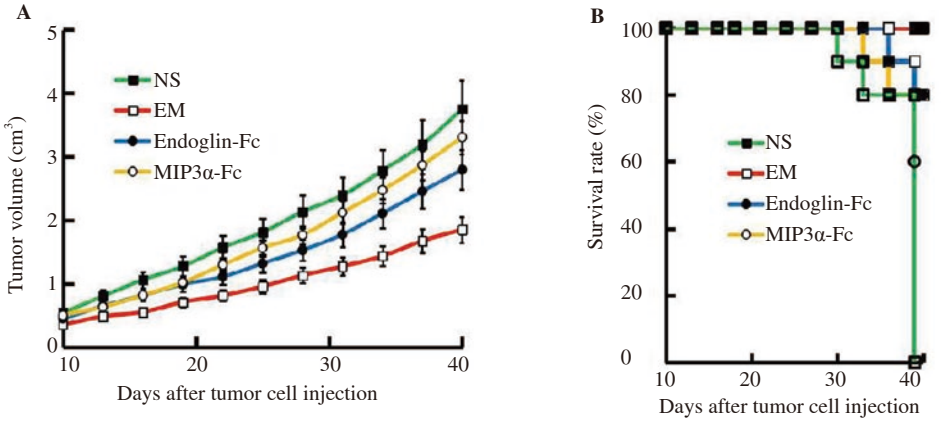

Figure 1. Induction of anti-tumor activities in the H22 hepatoma model. A: Tumor volume, B: Survival rate. NS: normal saline group, EM: endoglin-MIP3 Fc-fusion protein group, Endoglin-Fc: endoglin Fc-fusion protein group, MIP3 -Fc: MIP3 Fc-fusion protein group.

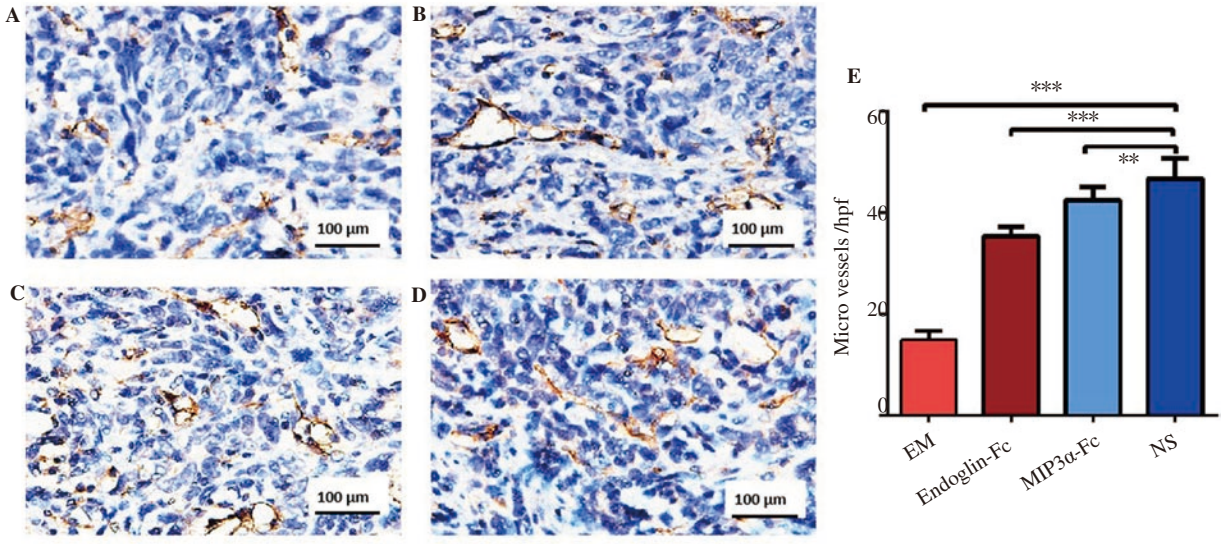

Figure 2. Inhibition of tumor angiogenesis by immunohistochemical analysis. Endoglin-MIP3α Fc-fusion protein group (A), endoglin Fc-fusion protein group (B), MIP3α Fc-fusion protein group (C), and normal saline group (D). Vessel density (E) was determined in H22 tumor sections stained with an antibody reactive to CD31 (***P*<0.01,****P*<0.001).

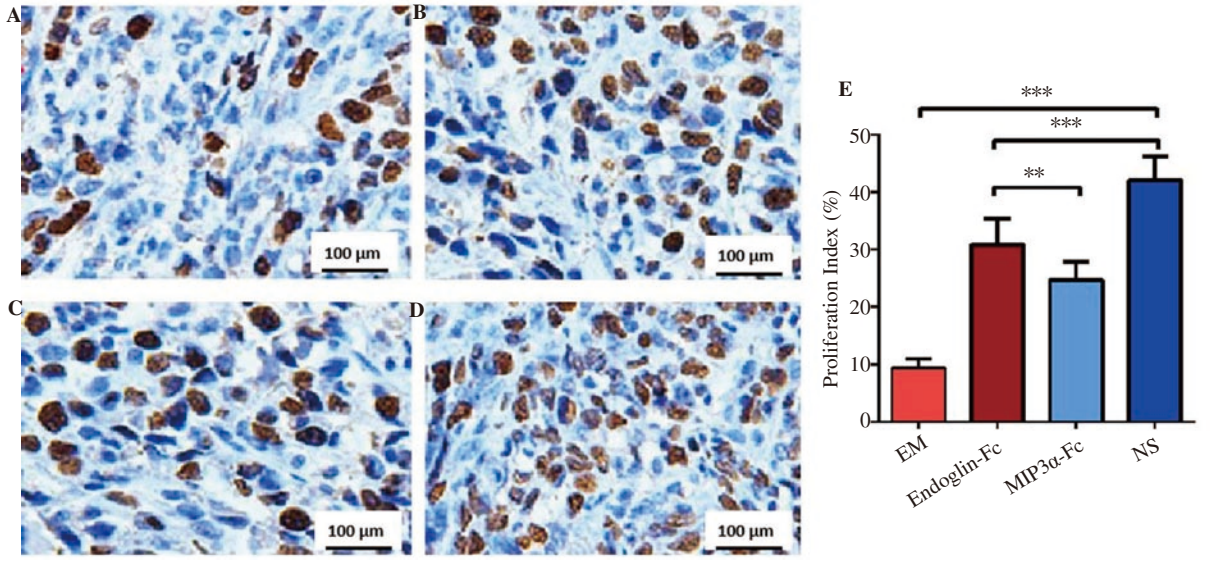

Figure 3. Inhibition of tumor cell proliferation by immunohistochemical analysis. Endoglin-MIP3α Fc-fusion protein group (A), endoglin Fc-fusion protein group (B), MIP3α Fc-fusion protein group (C), and normal saline group (D). Tumor cell proliferation (E) was determined in H22 tumor sections stained with an antibody reactive to proliferating cell nuclear antigen (***P*<0.01,****P*<0.001).

3.2. Suppression of angiogenesis

The microvessel density staining with an antibody reactive to CD31 was lower in the EM-treated mice than in controls [14.9±1.9 *vs.* 35.4±1.8 (endoglin) *vs.* 42.4±2.8 (MIP3α) and 46.8±3.7 (NS)] (*P*<0.001, Figure 2).

3.3. Suppression of cell proliferation

Tumor cell proliferation index detected by immunohistochemistry (Figure 3A-D) was significantly higher in the EM-treated group than that in endoglin, MIP3α, and NS group (9.4 ± 1.6 *vs.* 30.8 ± 4.6, 24.7 ± 3.1, 42.1 ± 3.9, *P*<0.001) (Figure 3E).

3.4. Induction of tumor cell apoptosis

Tumor cell apoptosis index (Figure 4) was significantly higher in the EM-treated group [(15.2 ± 3.2)%] than that in endoglin group [(6.8±1.3)%], MIP3α group [(2.7±0.9)%], and NS group [(1.4 ± 0.7)%] (*P*<0.001 for all).

3.5. Induction of anti-tumor immunity

Flow cytometry result showed that the number of CD11c and CD86 positive dendritic cells was significantly larger in the EM-treated group [(37.9 ± 3.5)%] than that in endoglin group [(7.3 ± 1.2)%], MIP3α group [(34.2 ± 1.5)%] , and NS group [(1.2 ± 0.3)%] (*P*<0.001).

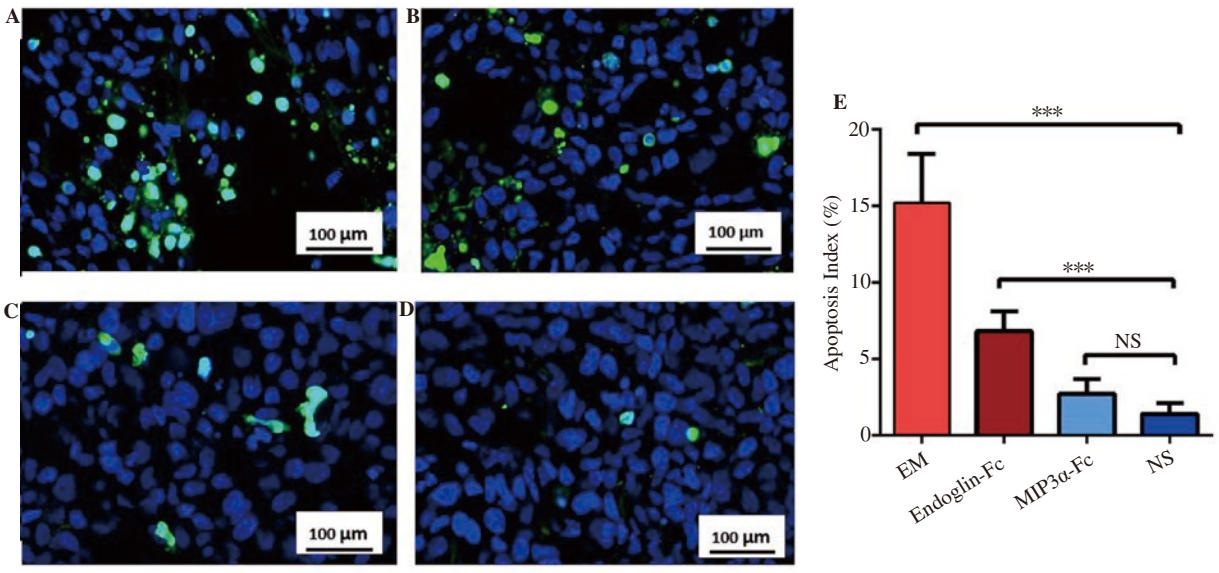

Figure 4. Induction of tumor cell apoptosis. Section of frozen H22 model tumor tissues was stained for apoptosis by TUNEL. Endoglin-MIP3α Fc-fusion protein group (A), endoglin Fc-fusion protein group (B), MIP3α Fc-fusion protein group (C), and normal saline group (D)(***P<0.001, NS P>0.05).

4. Discussion

Angiogenesis is critical to the growth and metastasis of tumors, and endoglin is one of the most critical angiogenic growth factors for tumor angiogenesis[4-7]. Since endoglin is a crucial regulatory molecule of angiogenesis in the solid tumor as described previously[7-10], it is viable to use anti-cancer angiogenesis therapy to block the TGF-β/endoglin signal pathway. However, due to inadequate DC targeting, the inadequate anti-tumor activity of endoglin alone, and inhibition of tumor microenvironment, it is necessary to seek effective treatment strategies. MIP3α is the most important specific chemokine of DCs and could effectively attract DCs and induce antitumor immunities[12-16]. Therefore, this research is to explore the anti-cancer activities of a recombinant EM Fc-fusion protein in mice with H22 hepatocellular carcinoma.

The results of this study suggest that the treatment of the EM Fc-fusion protein could effectively inhibit tumor growth, not only by inducing more effective anti-angiogenesis, but also by promoting apoptosis and suppression of cell proliferation in tumor tissues. Moreover, the number of CD11c and CD86 positive DCs was more significantly increased in the EM-treated group as compared with other groups, suggesting that the Fc fusion protein brings about better chemotactic recruitment of DCs.

Our findings demonstrate that the endoglin-MIP3α Fc-fusion protein treatment may be used as a useful and new approach to treat solid tumors. In addition, we also find that endoglin-MIP3α Fc-fusion protein induces a certain degree of the anti-tumor immune response, the specific mechanism remains to be further explored.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Funding project

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Authors' contribution

Y.D.L. and Z.H.H. conceived and coordinated the study. S.R.L., X.L.W., Y.H.H., Y.C. and P.P.X. carried out the experiments. X.L.W. and Y.D. collected and analysed the data. Both Z.H.H. and Y.D.L. authors contributed to the final version of the manuscript.

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