Original Article

Effect of DAPT, a gamma secretase inhibitor, on tumor angiogenesis in control mice

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Abstract

Background: Notch signaling is a key factor for angiogenesis in physiological and pathological condition and γ-secretase is the regulator of Notch signaling. The main goal of this study was to assess the effect of (N-[N-(3,5-Difluorophenaacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) DAPT, a γ-secretase inhibitor, on serum angiogenic biomarkers, and tumor angiogenesis in control mice.

Materials and Methods: Tumor was induced by inoculation of colon adenocarcinoma cells (CT26) in 12 male Balb/C mice. When tumors size is reached to a 350 ± 50 mm^3, the animals were randomly divided into two groups: control and DAPT (n = 6/group). DAPT was injected subcutaneously 10 mg/kg/day. After 14 days, blood samples were taken and the tumors were harvested for immunohistochemical staining.

Results: Administration of DAPT significantly increased serum nitric oxide concentration and reduced vascular endothelial growth factor receptors-1 (VEGFR1) concentration without changes on serum VEGF concentration. DAPT reduced tumor vascular density in control mice (280.6 ± 81 vs. 386 ± 59.9 CD31 positive cells/mm^2), although, it was not statistically significant.

Conclusion: It seems that γ-secretase inhibitors can be considered for treatment of disorders with abnormal angiogenesis such as tumor angiogenesis.

Key Words: Angiogenesis, obesity, tumor, γ-secretase

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INTRODUCTION

Angiogenesis is a regulated process, which requires several steps including degradation of basement membrane by protease, migration, and proliferation of endothelial cells and maturation of blood vessels.[1] Several angiogenic and anti-angiogenic factors are involved during angiogenesis. Vascular endothelial growth factor (VEGF) is the known angiogenic factor. The role of VEGF on angiogenesis has been documented in several *in vivo* and *in vitro* studies.[2] VEGF has two receptors: Vascular endothelial growth factor receptors-1 (VEGFR1) and VEGFR2.[3] VEGFR1 has higher affinity to VEGF than VEGFR2. VEGFR1 is a potent negative regulator of VEGFR2 action.[4] VEGFR1 is a negative regulator of angiogenesis.[5] VEGFR1 is the dominant receptor in tumor vasculature.[6] Nitric

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oxide (NO) is the main endothelium-relaxing factor, which has several effects on cardiovascular system. It also considered as an angiogenic factor, which is documented in different studies.[7]

Notch signaling is a key factor for angiogenesis in physiological and pathological condition including carcinoma.[8,9] First time, the role of Notch signaling was shown in lymphoblastic leukemia.[10] γ-secretase is the regulator of Notch signaling, and is a key molecule in postnatal angiogenesis. γ-secretase is required for processing of several proteins involve in angiogenesis including Notch and CD44 and the drugs oppose angiogenesis by altering the processing of those proteins.[11] In this study, we used a γ-secretase inhibitor, (N-[N-(3,5-Diflurophenaacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) DAPT (N-[N-(3,5-Dif lurophenaacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) to evaluate its effect on serum NO, VEGF, soluble form of VEGFR1 (sVEGFR1), and tumor angiogenesis in control mice.

MATERIALS AND METHODS

Animals and diets

Male Balb/C mice (n = 12) were purchased from Pasteur Institute (Tehran, Iran) and housed two per cage in standard animal house conditions, room temperature between 20 and 25°C, constant humidity and 12 h light/dark cycle. The experimental procedures were reviewed and approved by the ethical committee of the Isfahan University of Medical Sciences. The mice (at age of 20 weeks) were randomly divided into two groups: Control and DAPT.

Induction of tumor and drug administration

CT26 colon adenocarcinoma cells (5×10^5 cells) in 500 μl of Phosphate buffer solution (PBS) were inoculated subcutaneously into the dorsum of all mice using a syringe fitted with a 21 gauge needle. Tumor growth and development was then followed-up and monitored after inoculation. Once tumors became palpable and their sizes reached to approximately to 350 ± 50 mm^3, the animals were treated by DAPT.[12] DAPT was prepared by dissolving in Dimethyl sulfoxide (DMSO) and subcutaneously injected (10 mg/kg/day).[11] Control group received the same amount of DMSO. After 14 days, the mice were sacrificed. Blood samples were collected by cardiac puncture and the serums were separated and kept at –30°C for further analysis.

Histological analysis

By the end of experiment, the induced tumors were collected and processed for histological analysis. The tumors were put in formalin solution. Paraffin-embedded tissues were sectioned at a thickness of 5 μm and stained with standard immunohistochemical protocol with a monoclonal rat anti-mouse CD31 antibody (Abcam Co., USA, Cat# Ab28364). CD31 positive cells were counted in 20 fields of 10 sections at ×40 magnification and reported per mm^2.

Serum biochemical and angiogenic measurements

Blood glucose was measured by a glucometer. ELISA kits were used for determination of serum nitrite (Promega Corp, USA, Cat#G2930), the main metabolite of NO, VEGF, and sVEGFR1 (R and D systems, Mineapolis, USA) concentrations.

Statistics

Data are presented as mean ± SEM. The significant differences between groups were tested by Students t-test (SPSS version 16). $P < 0.05$ was considered statistically significant.

RESULTS

Serum NO concentrations

Figure 1 illustrates the changes of serum concentration in experimental groups. As shown, administration of DAPT significantly increased serum NO concentration in control mice ($P < 0.05$).

Serum VEGF and sVEGFR1 concentrations

The changes of serum VEGF and sVEGFR1 concentrations in experimental groups are shown in Figure 2. Administration of DAPT significantly reduced serum sVEGFR1 while, could not change serum VEGF concentration in control mice.

Angiogenesis assay

Immunohistochemical study of the tumors showed that CD31 positive cells were reduced after DAPT administration (280.6 ± 81 vs. 386 ± 59.9 CD31 positive cells/mm^2), although it was not statistically significant [Figure 3a]. Samples of immunohistochemical staining are shown in Figure 3b.

DISCUSSION

This study investigated the impact of DAPT, a γ-secretase inhibitor on tumor angiogenesis and serum angiogenic factors in control mice. Results showed that DAPT increased serum NO, reduced serum sVEGFR1 and non-significantly reduced tumor vascular density.

Notch signaling has been implicated in for vascular development and angiogenic process.[9] γ-secretase is required for processing of several proteins involve in angiogenesis including Notch and CD44. Thus, the drugs oppose the γ-secretase, inhibits angiogenesis

by altering the processing of those proteins.[11] In the present study, we used DAPT, a γ-secretase inhibitor and we expected that it reduced vascular density and angiogenesis in tumor cells. Our results showed that administration of DAPT reduced tumor vascular density, although it was not statistically significant.

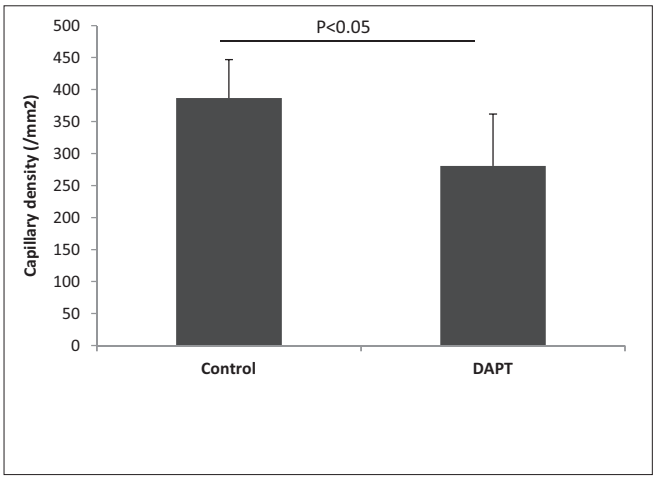

Figure 1: Serum nitric oxide concentration in experimental groups. *: *P* < 0.05 compare to other group

Previous studies suggested that DAPT may be affect tumor growth by disturbance of angiogenesis.[13] Inhibition Notch signaling enzyme complex (γ-secretase) disrupt vascular structure and function, however it increases vascular density.[14] Paris *et al*. revealed that γ-secretase inhibitors inhibit angiogenesis and tumor growth in rat aortic ring model of angiogenesis and glioblastoma and human lung adenocarcinoma and they showed that DAPT dose dependently inhibited the sprouting of new capillaries in rat aortic ring model.[11] The mice lacking γ-secretase activity exhibits cerebral hemorrhage due to abnormal vessel formation.[15]

In this study, we found that administration of DAPT increased serum NO and reduced sVEGFR1 concentration and it seems that it may affect tumor angiogenesis through altering the angiogenic factors. An *in vitro* study in the mouse micro vascular endothelial cell line showed that DAPT increased endothelial nitric oxide synthase (eNOS) and VEGFR2 protein and expression and decreased VEGFR1 at both the expression and protein and they showed that DAPT up regulates NOS in a concentration-dependent

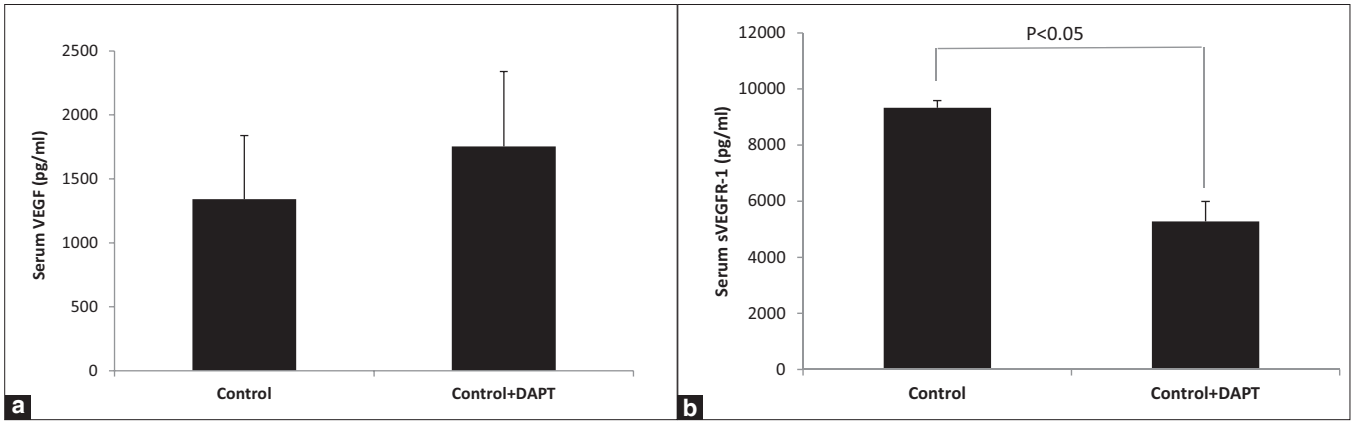

Figure 2: Changes of serum vascular endothelial growth factor (a) and s vascular endothelial growth factor receptors1 (b) in experimental groups *: *P* <0.05 compare to other group

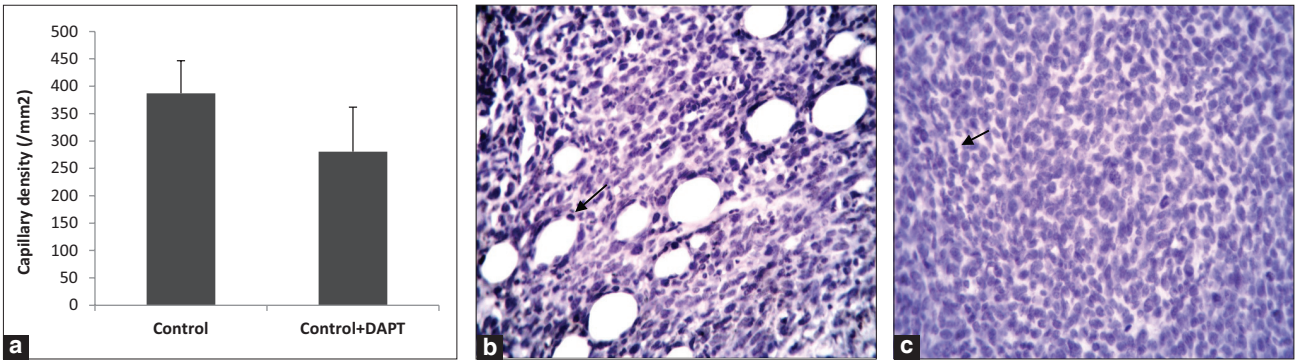

Figure 3: capillary density in adenocarcinoma tumors expressed as CD31 positive cells per mm^2 (a) Samples of immunohistochemical staining (×400) from tumor tissue in control (b) and control + DAPT (c) Arrows indicate CD31 positive cells

manner.[16] They also indicated that DAPT regulates VEGF signaling via alteration of VEGF receptors expression. VEGF has two receptors: VEGFR-1 and VEGFR2. VEGFR1 is a negative regulator of angiogenesis and VEGFR1 is the dominant receptor in tumor vasculature.[6] DAPT reverse VEGFR1:VEGFR2 ratio and DAPT treatment down-regulate VEGFR1 but up-regulates VEGFR2.[17]

We conclude that γ-secretase inhibitors are associated with disruption of eNOS and VEGF signaling and may be considered for treatment of excessive angiogenesis disorders such as colon adenocarcinoma.

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