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Copper promotion of angiogenesis in isolated rat aortic ring: role of vascular endothelial growth factor☆

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Abstract

Copper stimulation of angiogenesis at the organ system level is vascular endothelial growth factor (VEGF) dependent, but copper stimulation of vascular endothelial cell proliferation in cultures is VEGF independent. The present study was undertaken to use isolated rat aortic rings to understand the seemly controversial observations between *in vivo* and *in vitro* studies. The thoracic aorta was isolated from Sprague Dawley rats (8–10 weeks) and sectioned into 1.0-mm thick vascular rings for culturing. Copper sulfide at a final concentration of 5, 25, 50 or 100 μM was added to the cultures and maintained for 8 days. A copper chelator, tetraethylenepentamine (TEPA) at a final concentration of 25 μM, was added to some cultures to block the effect of copper. An anti-VEGF antibody was used to determine the role of VEGF in copper promotion of angiogenesis. The data obtained showed that copper at 5 μM in cultures stimulated the vascular formation; an effect was blocked by TEPA. Copper at concentrations above 50 μM lost the proangiogenesis effect. However, copper at 5 μM did not enhance the production of VEGF, and concentrations above 50 μM significantly increased VEGF production. On the other hand, the treatment with anti-VEGF antibody completely blocked the proangiogenesis effect of 5-μM copper. This study thus demonstrates that VEGF is essential for angiogenesis but the proangiogenesis effect of copper does not act through enhanced production of VEGF.

Keywords: Angiogenesis; Aortic ring; Copper; VEGF

1. Introduction

Copper promotion of angiogenesis in avascular corneas of rabbits was reported more than 30 years ago [1], and the mechanism of action of copper has been a focus for investigation recently. Enhanced vascular endothelial growth factor (VEGF) production was observed in copper promotion of wound healing [2]; therefore, the role of VEGF in copper promotion of angiogenesis and the mechanism by which copper regulates VEGF production have been extensively studied [3–5]. However, in the process of angiogenesis, there are multiple factors involved in the stimulation and propagation of angiogenesis [6], although VEGF plays a pivotal role in the initiation of angiogenesis process [7].

Previous studies using human umbilical vein endothelial cells have shown that copper stimulation of cell proliferation is independent of VEGF but dependent on endothelial nitric oxide synthase (eNOS) signaling pathways [8]. However, we also observed that in the pressure overload-induced cardiac hypertrophy in mice, copper supplementation-induced regression of cardiac hypertrophy requires myocardial angiogenesis, which is VEGF dependent [3]. In the mouse study, anti-VEGF antibody was used to block the angiogenic effect of copper; myocardial angiogenesis was suppressed.

In the angiogenesis, the proliferation of vascular endothelial cells is only one aspect of the process. Therefore, the *in vivo* observation that VEGF is required for the angiogenic effect of copper is more related to the overall action of VEGF, which involves multiple types of cells at the organ level and an integrated function of a diversity of cytokines in addition to VEGF [3]. On the other hand, the *in vitro* observation that the proliferation of vascular endothelial cells is independent of VEGF focuses on only the growth stimulating effect of VEGF on one cell type [8]. It remains unknown what is the exact role of VEGF in copper promotion of angiogenesis.

To address this question, the present study was undertaken to use isolated rat aortic ring to directly observe the effect of copper on angiogenesis of the cultured vascular tissue. This model system excludes the confounding effects of nonvascular tissues in the whole organ system, but it remains multiple biochemical and metabolic activities besides endothelial cell proliferation for the vascular formation.

2. Methods

2.1. Isolated rat aortic ring and treatment conditions

Thoracic aortas were removed from 8- to 10-week-old male Sprague Dawley (SD) rats (292–307 g) and immediately transferred to a culture dish containing ice-cold serum-free endothelial basal medium-2 (EBM-2, Lonza Cologne AG). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and

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iridectomy scissors; paying special attention not to damage the aortic wall. One-millimeter long aortic rings were sectioned and extensively rinsed in five consecutive washes with EBM-2.

2.2. Assay for angiogenesis

Forty-eight well tissue culture grade plates were covered with 100 μl of matrigel (GFR, BD) and allowed to gel for 45 min at 37°C, 5% CO_2. Aortic rings were placed on the matrigel-coated wells, covered with an additional 100-μl matrigel and allowed to gel again for 45 min at 37°C, 5% CO_2. The culture was then added 250-μl EBM-2 containing 1% fetal bovine serum (FBS). Copper sulfide solution was added to the culture at a final concentration of 5-, 25-, 50- or 100-μM copper element. The growth media were removed and replaced every 2 days. Aortic rings were photographed on Day 8.

2.3. Image analysis

The area of angiogenic sprouting was calculated using the Image J software program (NIH, Bethesda, MD). Microvessel densities are reported in square pixels.

2.4. Western blotting analysis of VEGF

Protein extracts were obtained after lysing rat aortic rings with matrigel in the radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Jiangsu, CN) containing 1% complete ethylene diamine tetraacetic acid (EDTA)-free protease inhibitor cocktail (Roche, Mannhein, DE) for 30 min on ice. Equal loading of protein was assured by prior quantitation using a bicinchoninic acid (BCA) protein assay kit (Thermo, Rockford, USA). An appropriate amount of protein in total lysates was resolved in an sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel and transferred onto a polyvinylidene difluoride membrane (Bio-rad, USA). Membranes were blocked for 1 h

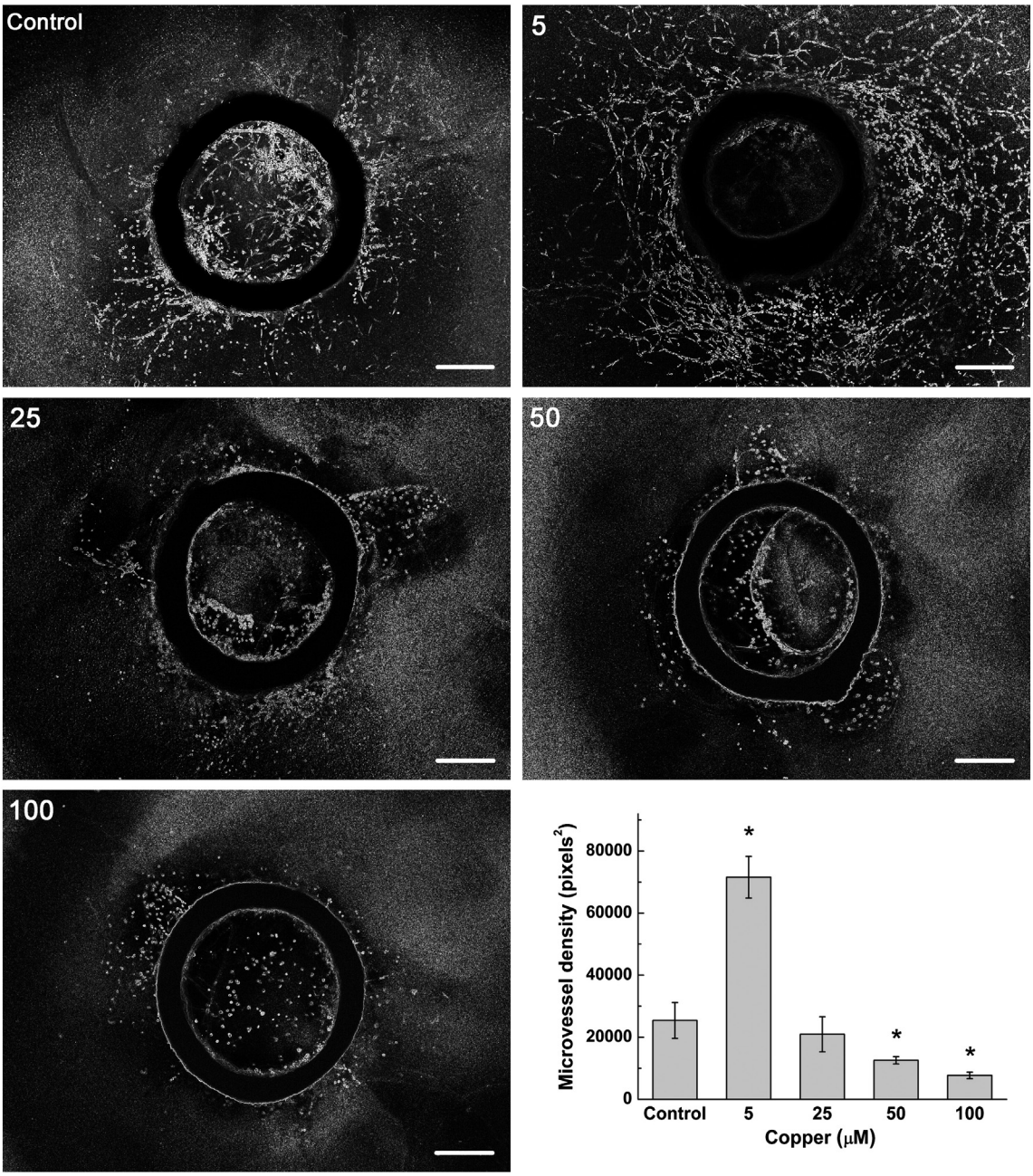

Fig. 1. Effects of varying concentrations of copper sulfide on angiogenesis of the isolated rat aortic rings cultured in EBM-2 with 1% FBS. Copper sulfide was added directly to the cultures at the final concentration of copper element of 0 (control), 5, 25, 50 or 100 μM and maintained for 8 days. The quantitative data were obtained from three separate experiments; each containing three samples for each treatment, and the data presented as mean±S.E.M.; *significantly different from the control group ($P<.05$) (bar=500 μm).