INHIBITION OF ANGIOGENESIS BY BLEOMYCIN AND ITS COPPER COMPLEX

Tsutomu OIKAWA,*,[a] Kenji HIROTANI,[a,b] Hiroyuki OGASAWARA,[a,b] Takashi KATAYAMA,[b] Hiromi ASHINO-FUSE,[a] Mariko SHIMAMURA,[a] Takao IWAGUCHI[a] and Osamu NAKAMURA[c]

Division of Cancer Therapeutics, The Tokyo Metropolitan Institute of Medical Science,[a] Honkomagome 3-18-22, Bunkyo-ku, Tokyo 113, Japan, Faculty of Pharmaceutical Sciences, Science University of Tokyo,[b] Ichigaya Funagawara-cho, Shinjuku-ku, Tokyo 162, Japan and Division of Neurosurgery, The Tokyo Metropolitan Komagome Hospital,[c] Bunkyo-ku, Tokyo 113, Japan

The effects of bleomycin (BLM) and its copper complex on embryonic angiogenesis were studied in the chorioallantoic membranes of 4.5-day-old chick embryos. Copper-free BLM inhibited embryonic angiogenesis in a dose-dependent manner, with activity detectable at 1 ng/egg and maximal at 1000 ng/egg. Also, treatment with copper-BLM complex dose-dependently caused inhibition of embryonic angiogenesis in a lower dose range. Since tumor growth is believed to depend on angiogenesis, the present results may indicate that the antiangiogenic activity of BLM is, at least in part, implicated in the antitumor activity of the drug.

KEYWORDS bleomycin; copper-bleomycin complex; embryonic angiogenesis; chorioallantoic membrane; angiogenesis inhibition

The bleomycins (BLMs), a family of glycopeptide antibiotics, were isolated by Umezawa et al. from fermentation broths of Streptomyces virticillus.[1] Subsequently, these antibiotics have been shown to exhibit antitumor activity against human as well as experimental animal tumors. At present the BLM is used clinically in chemotherapy for squamous cell carcinomas and malignant lymphomas. Also, combination therapy of BLM with other chemotherapeutic drugs, such as ifosfamide and cisplatin, has resulted in an improvement in therapeutic effectiveness in advanced and recurrent cervical cancer.[2] The BLMs, which were first isolated as a blue powder (i.e., copper-BLM complex), can form metal-complexes with different metal ions, including copper, iron, zinc and cobalt ions.[1]

Copper has been reported to affect angiogenesis, and, in particular, migration of angiogenic endothelial cells.[3] Our previous observation showed that the antiangiogenic activity of medroxyprogesterone acetate, which is used clinically as an endocrine-therapeutic drug for breast cancer, probably helped the drug to inhibit the growth of rat mammary tumors.[4] In addition, we reported that retinoids and vitamin D_3 analogues strongly inhibited embryonic angiogenesis, and proposed that these angiostatic vitamins were promising angiogenesis inhibitors for managing aberrant angiogenesis during several pathological processes such as during tumor growth.[5] Here we describe the antiangiogenic action of BLM and its complex with copper in a chick embryo chorioallantoic membrane (CAM).

BLM (which was mostly BLM A_2) was kindly provided by Nippon Kayaku Co., Ltd., Tokyo. The copper-BLM complex was prepared as follows: 10 μmol BLM, calculated on the basis of a molecular weight of 1487, was mixed with an equal dose of $CuSO_4$ in 0.9% NaCl and the mixture was adjusted with dilute NaOH to about pH 7.

Antiangiogenic activity was assessed in a CAM assay as described previously,[5] except that 1% methylcellulose (20-30 cps; Tokyo Kasei Co., Ltd., Tokyo) was used instead of ethylene-vinyl acetate copolymer. In brief, fertilized eggs (Ohmiya Kakin Lab., Ohmiya) were incubated in a humidified egg incubator at 37 ℃ for 4.5 days. A test sample (10 μl) in 1% methylcellulose (in 0.9% NaCl), was placed within a sterilized silicon ring (5-mm outer diameter,

3-mm inner diameter) put on the 4.5-day CAM. Following 2-day incubation at 37℃, about 0.5 ml of a 10% fat emulsion was injected into the chorioallantois to show the vascular network better. When the test sample caused an avascular zone of 3-mm diameter or over in the test CAM, the antiangiogenic response was scored as positive. At least 7 eggs were used for each experimental group. Data as to antiangiogenic activity were analyzed by Fisher's exact probability test, with $P < 0.05$ as the level of significance.

To determine whether copper-free BLM could modulate embryonic angiogenesis, CAMs of chick embryos were treated for 2 days with various doses of this agent (Fig 1). BLM inhibited the embryonic angiogenesis dose-dependently; the minimum effective dose for inhibition was 1 ng/CAM, and at 1 μg/CAM the inhibition reached a maximum of about 90%. A further increase in the dose of BLM rather depressed the antiangiogenic activity. Such a biphasic inhibitory effect is not limited to this agent. Similar dose-response curves were obtained with cotreatment of hydrocortisone and heparin,[6] and with an angiogenesis inhibitor in the conditioned medium of hamster cells.[7]

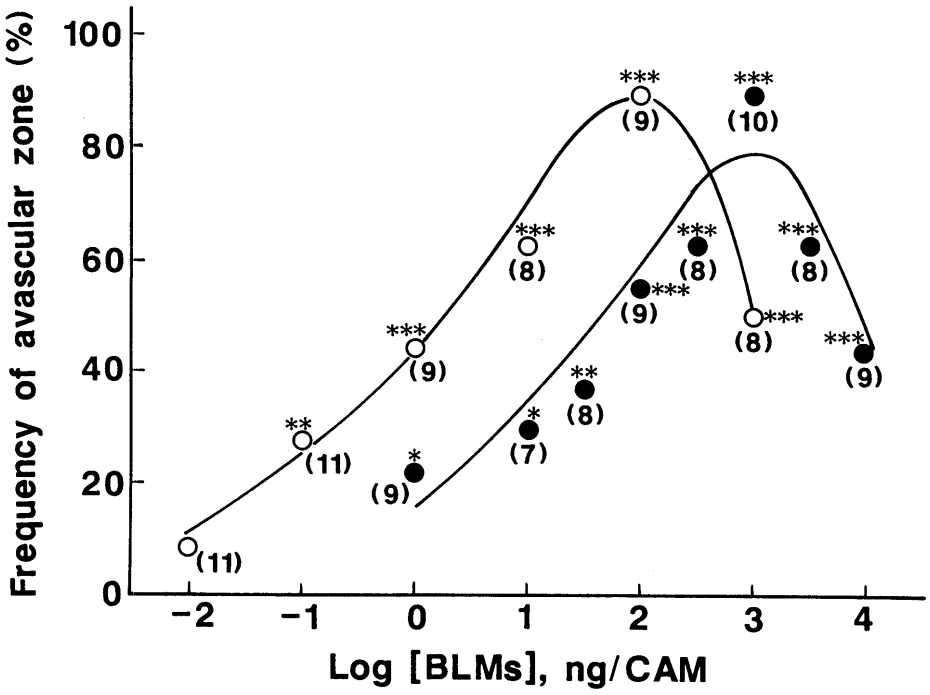

Fig. 1. Inhibitory Effects of BLMs on Embryonic Angiogenesis

The 4.5-day CAMs were treated with different doses of BLMs for 2 days, at which time their antiangiogenic activities were assayed by measuring the avascular zone. The number of eggs used are shown in parentheses. (●), Cu-free BLM; (○), Cu-BLM complex. The indicated BLM doses were expressed as the equivalent of Cu-free BLM doses. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

We also studied the effect of copper-BLM complex on embryonic angiogenesis (Fig. 1). Like Cu-free BLM, its complex with copper strongly inhibited angiogenesis, with an optimal dose of 100 ng/CAM. In comparison with methylcellulose alone, of 39 control CAMs examined, the minimum effective dose was 0.1 ng/CAM, a value one order of magnitude lower than that for copper-free BLM (the minimum effective dose of 1 ng/CAM). The dose for 50% inhibition of angiogenic activity was 1.5 ng/CAM for Cu-BLM complex, and 75 ng/CAM for Cu-free BLM, as estimated from their respective dose-response curves.

Figure 2 shows examples of these experiments. Both copper free-BLM (Fig. 2B) and BLM chelated with copper (Fig. 2C) caused significant avascular zones, whereas methylcellulose alone (Fig. 2A) had no effect on embryonic angiogenesis.

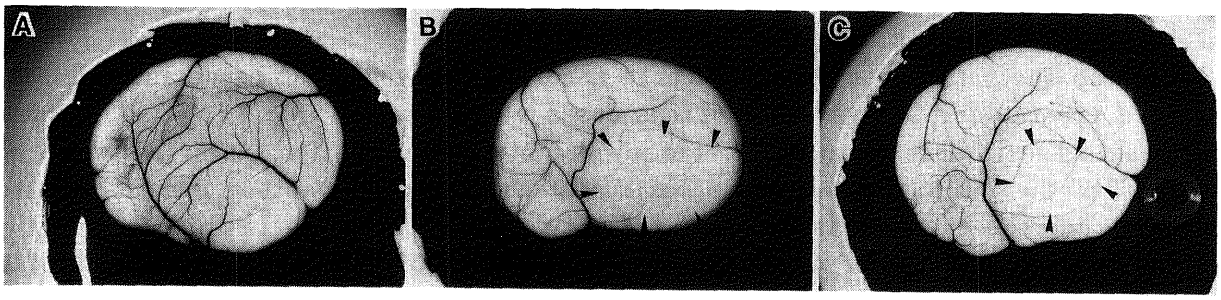

Fig. 2. Effects of BLMs on Angiogenesis in CAMs

Note the presence of an avascular zone (indicated by arrows) in CAM treated with BLM. A), methylcellulose alone; B),Cu-free BLM (100 ng/CAM); C), Cu-BLM complex (1 ng/CAM). magnification, x 1.6.

BLM is thought to exhibit its therapeutic potency through affecting deoxyribonucleic acid (DNA). On the other hand, Tanaka et al. reported that chemotherapeutic agents, such as mitomycin C and 5-fluorouracil, which exert their effects by acting on DNA, had no effect on embryonic angiogenesis.[8] Taken together, the present results demonstrate that BLM chelated with and without copper inhibits embryonic angiogenesis, although the mechanism of action remains to be elucidated. In addition, the antitumor activity of BLM might be, in part, due to the angiogenesis-inhibitory activity of the agent, since many of the hitherto reported angiogenesis inhibitors, including cortisone[9] and medroxyprogesterone acetate,[4,10] have the capacity to inhibit not only embryonic angiogenesis but also tumor neovascularization, and since it is believed that tumor growth depends on angiogenesis and that angiogenesis inhibition probably inhibits tumor growth.[4,11]

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