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Methylene blue inhibits angiogenesis in chick chorioallontoic membrane through a nitric oxide-independent mechanism

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Abstract

Angiogenesis is the process of generating new blood vessels from preexisting vessels and is considered essential in many pathological conditions. The purpose of the present study was to evaluate the effect of methylene blue in chick chorioallantoic membrane angiogenesis model *in vivo*. In this well characterized model, methylene blue inhibited angiogenesis in a concentration-dependent manner. In addition, when methylene blue was combined with sodium nitroprusside, a spontaneous generator of nitric oxide, an inhibition of angiogenesis was evident which was comparable with that observed by the application of methylene blue alone. Sodium nitroprusside, alone, caused a significant inhibition in basal angiogenesis. These results provide evidence that methylene blue inhibits angiogenesis independently of nitric oxide pathway and suggest that methylene blue may be useful for treating angiogenesis-dependent human diseases.

Keywords: methylene blue - angiogenesis - chick chorioallantoic membrane -nitric oxide - guanylyl cyclase

Introduction

Angiogenesis, the growth of new capillaries, is accomplished by sprouting or bridging from existing vessels. The angiogenic process is matched exquisitely to changes in tissue mass and metabolic demands in order to maintain adequate oxygen delivery. However, pathological disruption of this process is a hallmark of both vascular insufficiency (myocardial or critical limb ischemia) and vascular overgrowth (malignant tumors, retinopathies, hemangiomas). Thus, numerous therapeutic benefits may be realized through the successful understanding and subsequent manipulation of angiogenesis.

Numerous molecules perform critical functions within the complex angiogenic cascade [1]. Production and activation of these growth factors, endothelial cell growth factor receptors, and intracellular signaling mediators and transcription factors trigger the phases that comprise the abluminal sprouting form of angiogenesis. In addition to its well-recognized vasodilatory properties, nitric oxide (NO), which is endogenously synthesized from L-arginine by nitric oxide synthases, is impli-

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cated as a mediator of angiogenesis, [2]. NO activates cytosolic guanylyl cyclase and triggers capillary endothelial cell growth and differentiation *via* cyclic GMP dependent gene transcription. The regulation of capillary growth by NO is complex because both angiogenic and angiostatic effects of NO have been demonstrated [3, 4].

Despite the fact that methylene blue (methylthioninium chloride, MB) has direct inhibitory effects on both constitutive (cNOS) and inducible (iNOS) nitric oxide synthase (NOS) [5], its effect on angiogenesis has not been reported. The additional effects on soluble guanylyl cyclase [6] add to the inhibition of the NO/c-GMP pathway by MB. MB inhibits guanylyl cyclase by binding to the heme group of the enzyme and blocks the catalytic functions of NOS by oxidation of enzyme-bound ferrous iron [7]. Here, we show for the first time that MB is capable of inhibiting *in vivo* angiogenesis in the chick embryo model through a mechanism independent to the inhibition of the NO pathway.

Materials and methods

Materials

Fresh fertilized chicken eggs were obtained locally (Ioannina, Greece) and were kept at 10°C until incubation at 37°C. Methylene blue, sodium nitroprusside (SNP), collagenase type VII from clostridium histolyticum were purchased from Sigma (Athens, Greece). [^{14}C]-proline was purchased by ICN Biomedicals, Inc (Irvine, CA).

Methylene blue was initially dissolved (stock solution) in dimethyl sulfoxide (DMSO) and subsequent dilutions (working solutions) were prepared in water. Sodium nitroprusside dissolved in water.

Chick chorioallantoic membrane assay

The *in vivo* chick chorioallantoic membrane (CAM) angiogenesis model was used as described previously [8]. Briefly, fertilized eggs were incubated at 37°C for 4 days, when a window was opened on the egg shell, exposing the CAM. The window was covered and the eggs were returned to the incubator until day 9. The test materials or vehicle (DMSO in the case of methylene blue) and 0.5 μCi [U-^{14}C]-labeled proline were placed on sterile plastic disks and allowed to dry under sterile conditions. The control disks (containing only vehicle and radiolabeled proline) were placed on the CAM one cm away from the disk containing the test material. The eggs were incubated until day 11, when assessment of angiogenesis took place.

Biochemical evaluation of newly formed vessels was performed by determining the extent of collagenous protein biosynthesis in the area of CAM lying directly under the disks as previously described. This method has been shown to provide a reliable index of angiogenesis [9]. Briefly, the tissue under the pellets was subjected to collagenase digestion. The resulting radiolabeled tripeptides, corresponding to basement membrane collagen and other collagenous materials synthesized by the CAM, were counted and expressed as dpm/mg protein.

In morphological evaluation of angiogenesis, eggs were treated as above in the absence of radiolabeled proline. At day 11, the eggs were flooded with 10% buffered Formalin, the plastic pellets were removed and the eggs were kept at 37°C until dissection. A large area around the pellet was removed and placed on a glass slide. Representative specimens were mounted on a stereoscope and photographed.

Statistical analysis

For each egg, collagenous protein biosynthesis under the test pellet was expressed as a percentage of that under the control pellet in the same egg. It was used at least 10 eggs for each group. Results are mean ± SD expressed as % of control. Statistical analysis was performed with Student's t-test.

Results

Effect of MB on angiogenesis in the CAM model

MB at concentrations ranging from 1 to 100μg/disk caused a dose-dependent inhibition in collagenous protein biosynthesis up to a level of 61% as compared to basic angiogenesis (Fig. 1A). This inhibitory effect was not toxic for the chick embryo, even at as high concentration as 300 μg/disk. The inhibition in collagenous protein biosynthesis is related to a reduction in vascular density as shown

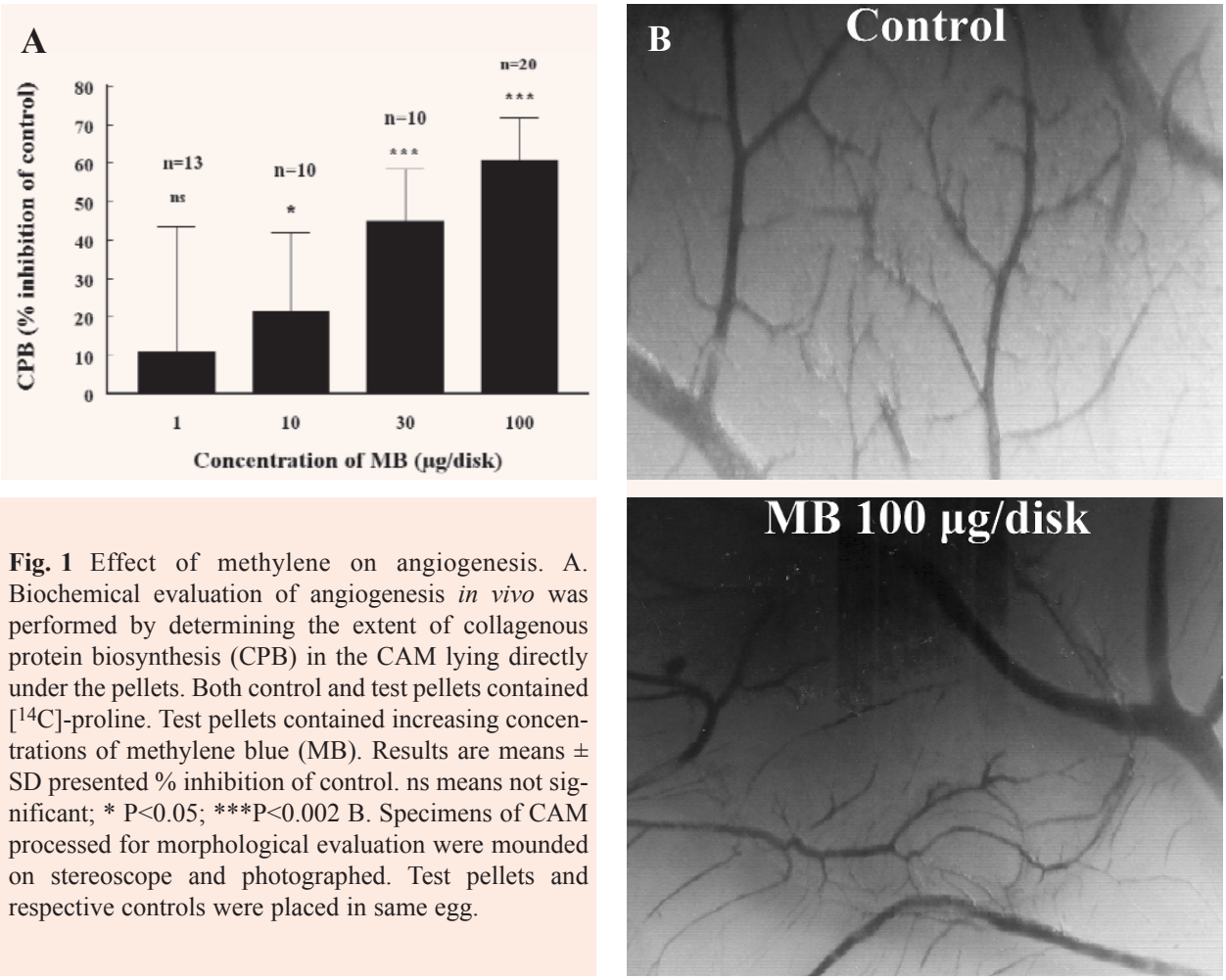


Fig. 1 Effect of methylene on angiogenesis. A. Biochemical evaluation of angiogenesis *in vivo* was performed by determining the extent of collagenous protein biosynthesis (CPB) in the CAM lying directly under the pellets. Both control and test pellets contained [^{14}C]-proline. Test pellets contained increasing concentrations of methylene blue (MB). Results are means ± SD presented % inhibition of control. ns means not significant; * P<0.05; ***P<0.002 B. Specimens of CAM processed for morphological evaluation were mounded on stereoscope and photographed. Test pellets and respective controls were placed in same egg.

in specimens presented in Fig. 1B. Photographs of the area of the CAM lying under the control and the test disk, in the same egg, provide visual confirmation of the anti-angiogenic effect of MB (Fig. 1B).

Effect of sodium nitroprusside, alone and in combination with MB, on angiogenesis in the CAM model

In order to explore the possibility that the action of MB was due to inhibition of nitric oxide synthase by MB, it was attempted to reverse the anti-angiogenic effect of MB by sodium nitroprusside (SNP), which is a spontaneous generator of NO [10]. SNP at concentration of 8,5 µg/disk and in agreement with previous observations [11, 12] caused a significant inhibition in basal angiogenesis as evidenced by a decrease in collagenous protein biosynthesis (Fig. 2A). This inhibition was 21% of control. When SNP (8,5 µg/disk) was combined with MB (100 µg/disk) an inhibition in angiogenesis was evident which was comparable (59%) with that observed by the application of MB alone (61%) (Fig 2A). The combination (MB plus SNP) had also significant inhibitory effect on angiogenesis in the CAM as shown in a representative specimen in Fig. 2B.

Discussion

Angiogenesis, the process of blood-vessel growth, is important during both normal development and tumor growth and metastasis. It is also a fundamental process essential in inflammation and wound repair. In addition, angiogenesis appears to play an important role in the development of intraabdomi-

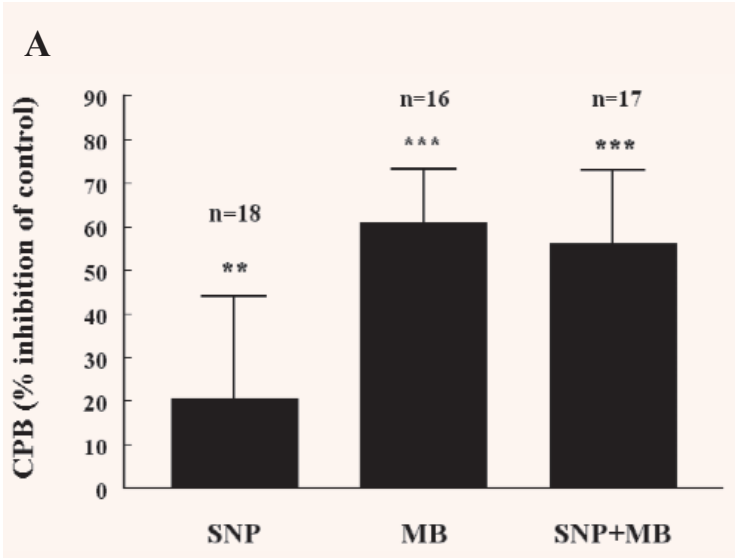
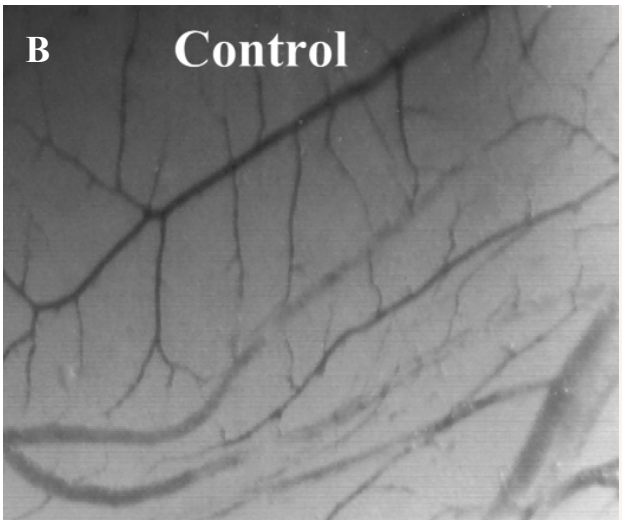
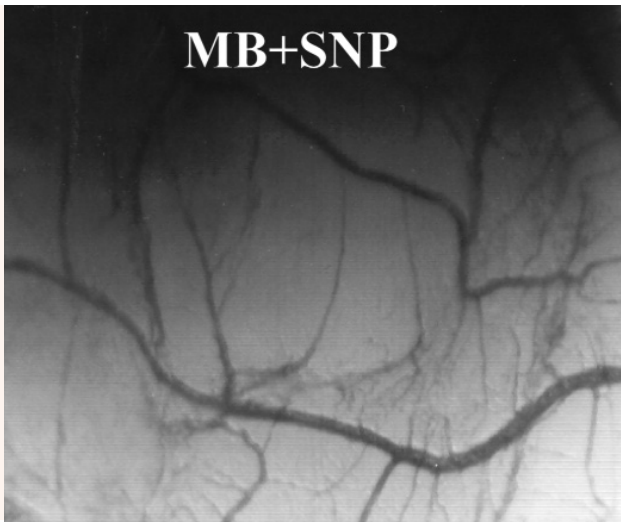


Fig. 2 Effect of sodium nitroprusside alone and in combination with methylene blue on angiogenesis. A. Biochemical evaluation of angiogenesis *in vivo* was performed by determining the extent of collagenous protein biosynthesis (CPB) in the CAM lying directly under the pellets. Both control and test pellets contained [^{14}C]-proline. Test pellets contained sodium nitroprusside (8,5 µg/disk, SNP) or methylene blue (100 µg/disk, MB) or the combinations as indicated. Results are means ± SD presented % inhibition of control. ** P<0.01; **P<0.002 B. Specimens of CAM processed for morphological evaluation were mounded on stereoscope and photographed. Test pellets and respective controls were placed in same egg.





nal adhesions after surgical procedures because the extent of early neovascularization correlates with adhesion formation. TNP-470, which is an inhibitor of angiogenesis, reduced neovascularization and adhesion formation in mice model of adhesions induced by Silastic [13]. Interestingly, methylene blue reduced the formation of peritoneal adhesions in rats [14]. Consistent with this finding, our results show that MB has antiangiogenic activity in CAM model, and most importantly may provide a basis for explaining the inhibitory effects of MB on the adhesion formation.

Previous studies demonstrated that MB inhibits the NO/cGMP signaling pathway and that most of the actions of MB are due to this effect [15]. However, our experiments demonstrate that SNP, an NO donor, did not reverse MB-induced inhibition of angiogenesis in CAM model. On the contrary, SNP caused a significant inhibition of angiogenesis in the CAM *in vivo* and the combination of MB with SNP did not alter the inhibition of angiogenesis by MB. These findings are in agreement with previous results showing that NO may be an endogenous antiangiogenic molecule of pathophysiological importance [4, 11, 12,].

In these studies, neither compound which modulate the intracellular NO levels had any effect on the growth of endothelial cells suggesting that the antiangiogenic effect by NO may not be due to inhibition of endothelial cell proliferation. A possible target for NO might be the blood platelet since platelet activation, aggregation and adhesion are inhibited by NO [16]. Deininger *et al.* provided evidence that the expression and release of endostatin, a natural

potent endogenous inhibitor of angiogenesis, by human brain endothelial cells was reduced by the NO synthase inhibitors and induced by NO donors, while SNP mediated endostatin induction was abrogated by a soluble guanylyl cyclase inhibitor [17]. In addition, YC-1, which activates soluble guanylyl cyclase through an NO-independent pathway, inhibited endothelial cell functions induced by angiogenic factors *in vitro* and angiogenesis *in vivo* [18]. These observations support the hypothesis that the inhibition of NO/cGMP parthway by MB is not the precise mechanism by which MB inhibit angiogenesis in CAM angiogenesis model.

MB might possess novel molecular properties that interfere with common angiogenic signaling pathways triggered by growth factor-mediated induction of angiogenesis in CAM system. Recently, Duenas *et al.* reported that light- and chemical-induced oxidation of methionine residues of recombinant human vascular endothelial growth factor (rhVEGF) can modulate binding to VEGF receptor [19]. Light-induced oxidation of all 6 methionine residues of rhVEGF *in vitro* can decrease its receptor binding capacity possibly due to the role of Met 18 in receptor binding [19]. The photo-oxidation of VEGF by MB, plus visible light, is unlikely because incubation of CAM with MB took place in the dark. However chemical-induced oxidation of methionine residues of VEGF by MB on CAM is possible. MB oxidized in some extent methionine residues of lysozyme in the dark and decreased enzyme activity by 21% [20].

Another possibility is that cationic MB can interact electrostatically with heparin sulfate proteoglycans (HSPG) on endothelial cells competing with VEGF binding to HSPG. Endothelial surface HSPGs act as co-receptors for a wide spectrum of angiogenic factors, being involved in the control of angiogenesis [21], and mediate the binding to their receptors on endothelial cells [22]. The VEGF-A isoforms 121, 165, 189 and 206 have heparin-binding domains, which help anchor them in extracellular matrix and are involved in binding to heparin sulfate and presentation to VEGF receptors. The capacity of VEGF to interact with heparin sulfate from HSPG raises the possibility that molecules able to interfere with this interaction may act as inhibitors of angiogenesis [23]. Noteworthy, is the general acceptance of the 1,9-dimethylmethylene blue (DMMB) (a derivative of MB) assay as a quick and simple method of measuring the sulphated glycosaminoglycan (sGAG) content of tissues and fluids. This assay is based on DMMB binding to negatively charged GAGs, and forming a complex that can be identified spectrophotometrically [24, 25].

Methylene blue has several notable uses in clinical medicine. Examples include use as a bacteriostatic genitourinary antiseptic, use as a topical agent (at a 0.1% solution) in conjunction with polychromatic light to photoinactivate viruses such as herpes simplex, use in combination with vitamin C for the management of chronic urolithiasis, and use as an indicator dye [26]. MB is also used investigationally to increase vascular tone and myocardial function in patients with septic or anaphylactic shock [27, 28]. It is as the antidote of choice in the treatment of symptomatic methemoglobinemia [29], whereas the therapeutic effects of MB in vasoplegic syndrome have been addressed in studies on hypotensive patients requiring high inotropic support [15]. Our present data reveal a novel action of MB, with the potential to become a useful agent for new clinical applications in the treatment of angiogenesis-related diseases.

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