Development and pharmacological characterization of a novel plasma-based *in vitro* angiogenesis assay

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Abstract

Background: *In vitro* angiogenesis assays were first described in the early 1980s, when it was found that endothelial cells form capillary-like structures (CLS) on matrices of collagen, laminin or fibrin. These assays have been useful in unravelling some of the molecular steps involved in angiogenesis, as well as in screening potential angiogenesis inhibitors.

Aim: The aim of our current study was to develop a novel *in vitro* angiogenesis assay.

Material and methods: A heat-coagulated plasma matrix was used as substrate for CLS formation by cultured human umbilical vein endothelial cells (HUVECs). Angiogenesis was quantified by counting CLS branch points and by the release of matrix metalloproteinase-2 (MMP-2) from HUVEC as measured by gelatin zymography.

Results: In our current study, we demonstrate the formation of CLS, by HUVECs stimulated with phorbol-12 myristate-13 acetate (PMA), on a heat-coagulated plasma matrix. CLS formation was inhibited by the angiogenesis inhibitor angiostatin and by the matrix metalloproteinase inhibitor o-phenanthroline.

Conclusions: Hence, we propose that this novel assay may be a useful tool to study angiogenesis in vitro and to screen possible pharmacological inhibitors of angiogenesis.

Key words: angiogenesis assay, coagulated-plasma matrix, angiostatin, matrix metalloproteinases.

Background

Angiogenesis *in vitro* was first described by Folkman and Haudenschild [1] in 1980 when long-term culture of endothelial cells in tumour-conditioned medium resulted in the spontaneous formation of capillary-like structures (CLS) on a matrix of gelatin. Subsequent models of *in vitro* angiogenesis showed that endothelial cells form CLS in 2 dimensions on matrices of collagen and basement membrane constituents [2], or in 3 dimensions when the endothelial cells are sandwiched in a collagen gel [3]. In addition to collagen, other basement membrane components such as laminin have been shown to play a crucial role in the differentiation of endothelial cells into capillary-like structures *in vitro*. Kubota and colleagues demonstrated the importance of laminin in CLS formation on matrigel, a basement membrane-like extract composed of laminin, collagen, heparan sulphate proteoglycan and nidogen/entactin [4]. In addition to basement membrane components, matrices derived from the coagulation system have shown to provide scaffolding for CLS formation. Nicosia et al. made use of a matrix formed by clotted chick plasma [5] and showed the importance of fibrin in a 3-dimensional model of *in vitro* angiogenesis [6]. In our present study, we report capillary-like structure formation by endothelial cells on a matrix derived from human plasma. Moreover, we pharmacologically characterize this novel 2D *in vitro* angiogenesis model.

Materials and methods

Reagents

If not otherwise specified all other reagents were purchased from Sigma (St. Louis, MO, USA). Human angiostatin protein containing kringles 1-4 (k1-4) was obtained from Oncogene (San Diego, CA, USA).

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Platelet poor plasma

Approval for the current study was obtained from the University of Texas institutional review board. Informed consent was obtained according to the Declaration of Helsinki. Blood was collected from healthy volunteers who had not taken any drugs for 14 days prior to the study. Platelet poor plasma (PPP) was prepared [7] and stored at –20°C until used for angiogenesis assays.

Cell culture

Human umbilical vein endothelial cells (HUV-EC-C) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The HUV-EC-C cell line was cultured in 75 cm^2 culture flasks at 37°C in a humidified atmosphere with 5% CO_2 as described previously [8, 9]. Cells were cultured in 90% Kaighn's F12K medium with gentamycin (0.05 mg/ml), penicillin (0.06 mg/ml), streptomycin (0.01 mg/ml), and with 10% fetal bovine serum (FBS). Furthermore, the medium was supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement. Cells were supplied with fresh medium every two days and subcultured once a week. Cells were detached from flasks using a Trypsin-EDTA solution. Following harvesting, HUV-EC-C cells were resuspended in serum-free Kaighn's F12K medium and counted. Cell concentration was then adjusted to 10^6 cells/ml.

Angiogenesis assays

In vitro angiogenesis assays in which endothelial cells align and form capillary-like structures on heat-coagulated plasma were performed as follows. Platelet poor plasma (200 µl per well) was spread to evenly cover each well (100 µl per cm^2). Plasma covered plates were incubated at 80°C for 20 minutes for the PPP to polymerize. After polymerization, plates were allowed to cool to room temperature. Thrombin (0.1 units/ml) was added to each well and incubated at 37°C for 1 hour. After 1 hour the thrombin was aspirated and its remaining activity was inhibited by adding F12K medium with 10% FBS. Subsequently, the wells were washed 3x with serum-free F12K. HUV-EC-C cells (25,000 per well) were seeded in 24 well plates in serum-free F12K medium with phorbol-12 myristate-13 actetate (PMA) (50 ng/ml). In some experiments, cells were seeded in the presence of o-phenanthroline or angiostatin, or their respective vehicles. Angiostatin experiments were carried out in the presence of F12K medium formulated to give a pH of 6.7 under conditions of 5% CO_2 as described previously [9]. HUV-EC-C cells were incubated at 37°C on the polymerized plasma for 24-48 hours. After incubation each well was documented using an Olympus CKX41 microscope (Olympus America Inc., Melville, NY, USA) equipped with a digital camera, and images were captured using PictureFrame™ software. HUV-EC-C releasates were collected for gelatin zymography and stored at –80°C until analysis. Each individual experiment was performed in duplicate. Angiogenesis was quantified by counting the total number of branch points in each well.

Gelatin zymography

To determine MMP-2 activity, gelatin zymography was performed as described previously [9]. Briefly, zymography was performed using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with co-polymerized gelatin (2 mg/ml). After electrophoresis, gels were washed 3x for 20 minutes in 2% Triton X-100. Next, gels were washed 2x for 20 minutes in developing buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3) and then incubated in developing buffer at 37°C overnight. Gels were stained with 0.05% Coomassie Brilliant Blue and then destained in a 4% ethanol and 8% acetic acid solution. Gelatinolytic activity was detected as transparent bands against a blue background of Coomassie-stained gelatin. 72 KDa pro-MMP-2 and 64 KDa MMP-2 activity was identified by comparison to standards from the conditioned medium of HT-1080 human fibrosarcoma cells [9]. MMP-2 gelatinolytic activity was quantified using a Bio-Rad (Hercules, CA, USA) VersaDoc gel documentation system with Quantity One software and expressed as Trace quantity per mg protein.

Statistics

Statistics were performed using Graph Pad Software Prism 3.0. (GraphPad Prism, San Diego, CA, USA). All means were reported with standard error. Paired t-tests were performed where appropriate. A p-value of less than 0.05 was considered as significant.

Results

Coagulated plasma acts as a matrix for capillary-like structure formation

HUV-EC-C cells incubated on a heat-coagulated plasma matrix formed capillary-like structures in response to phorbol-12 myristate-13 actetate (PMA) in 24-48 hours (fig. 1a). Compared to control, angiostatin, an endogenous angiogenesis inhibitor derived from plasminogen, inhibited capillary-like structure formation (fig. 1b). HUV-EC-C cells incubated in the presence of angiostatin k1-4 (5 µg/ml) formed capillary-like structures with an average of 4.8±0.5 branch points per well compared to control cells, which formed structures with an average of 27.0±0.5 branch points per well (fig. 1c). In the absence of the PMA stimulus, HUV-EC-C cells failed to form capillary-like structures on the plasma-derived matrix (fig. 1d).

Formation of capillary-like structures depends on matrix metalloproteinases

The formation of capillary-like structures by HUV-EC-C cells on heat-coagulated plasma was dependent on MMP activity. HUV-EC-C cells stimulated with 50 ng/ml PMA released MMP-2 in its active 64 KDa form (0.632±0.038 Trace quantity per mg protein), as measured by gelatin zymography (fig. 2c). Inhibition of endothelial cell MMP-2 activity by o-phenanthroline resulted in complete inhibition of capillary-like structure formation by HUV-EC-C cells on heat-

-coagulated plasma (fig. 2a-c). Not only did phenanthroline inhibit CLS formation, but also it completely inhibited the attachment of HUV-EC-C cells to the coagulated plasma (fig. 2b).

Discussion

Since the first reports of capillary-like structure formation by endothelial cells in culture, a number of *in vitro* angiogenesis models have been described [10]. In some models CLS formation occurs on basement membrane components such as collagen and/or laminin, while in other models CLS formation occurs on transitional matrices such as fibrin. Fibrin, which re-enforces the platelet plug at sites of vascular damage and around leaky tumour blood vessels, is thought to act as a provisional matrix for sprouting endothelial cells [11]. While fibrin angiogenesis models have been utilized to investigate the roles of MMPs and nitric oxide in angiogenesis [11, 12], these models often require a high thrombin concentration (0.5-2.5 U/ml) to convert fibrinogen to fibrin and often do not yield an even fibrin matrix. In our present study, we show that human platelet-poor plasma heated to 80°C forms an even matrix on which PMA stimulated endothelial cells align and form CLS. We used PMA to stimulate angiogenesis in our model, since tumour promoting phorbol esters have been shown to induce angiogenesis *in vitro* [13]. Furthermore, recently it has been shown that PMA alone is sufficient to induce formation of vessels that are indistinguishable from those formed by a cocktail of PMA, vascular endothelial growth factor (VEGF), and basic fibroblastic growth factor (bFGF) in an *in vitro* collagen model of angiogenesis [14].

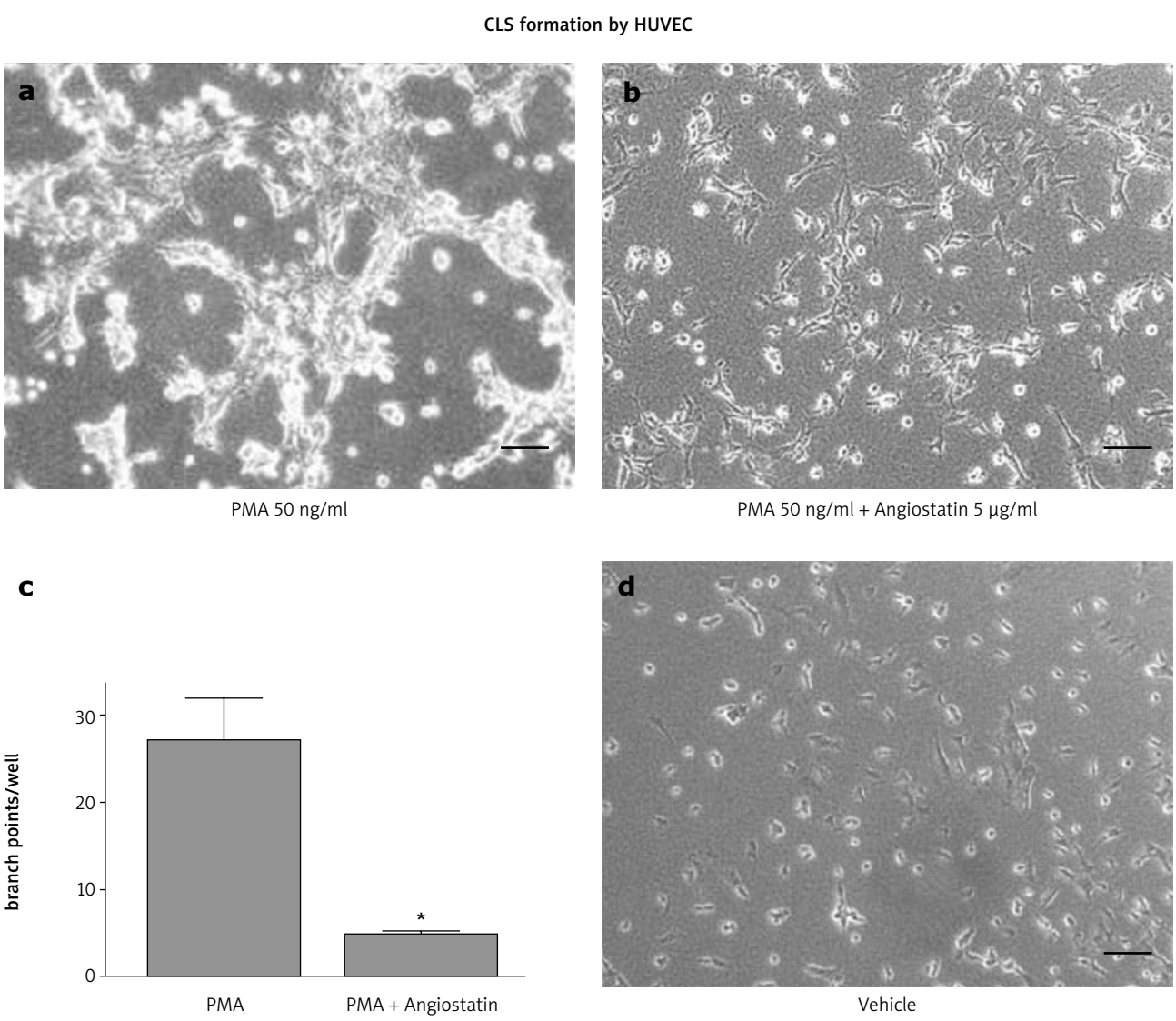

Fig. 1. (a-b) Representative micrographs showing formation of CLS by HUV-EC-C cells stimulated with 50 ng/ml PMA on a plasma matrix, and inhibition of CLS formation by 5 µg/ml angiostatin. (c) Quantification of *in vitro* angiogenesis by counting branch points in networks of capillary-like structures. Bars are means ± SE from four separate experiments. *****P<0.05. (d) Representative micrograph showing a lack of capillary-like structures on a plasma matrix in absence of PMA. Bar represents 100 µm

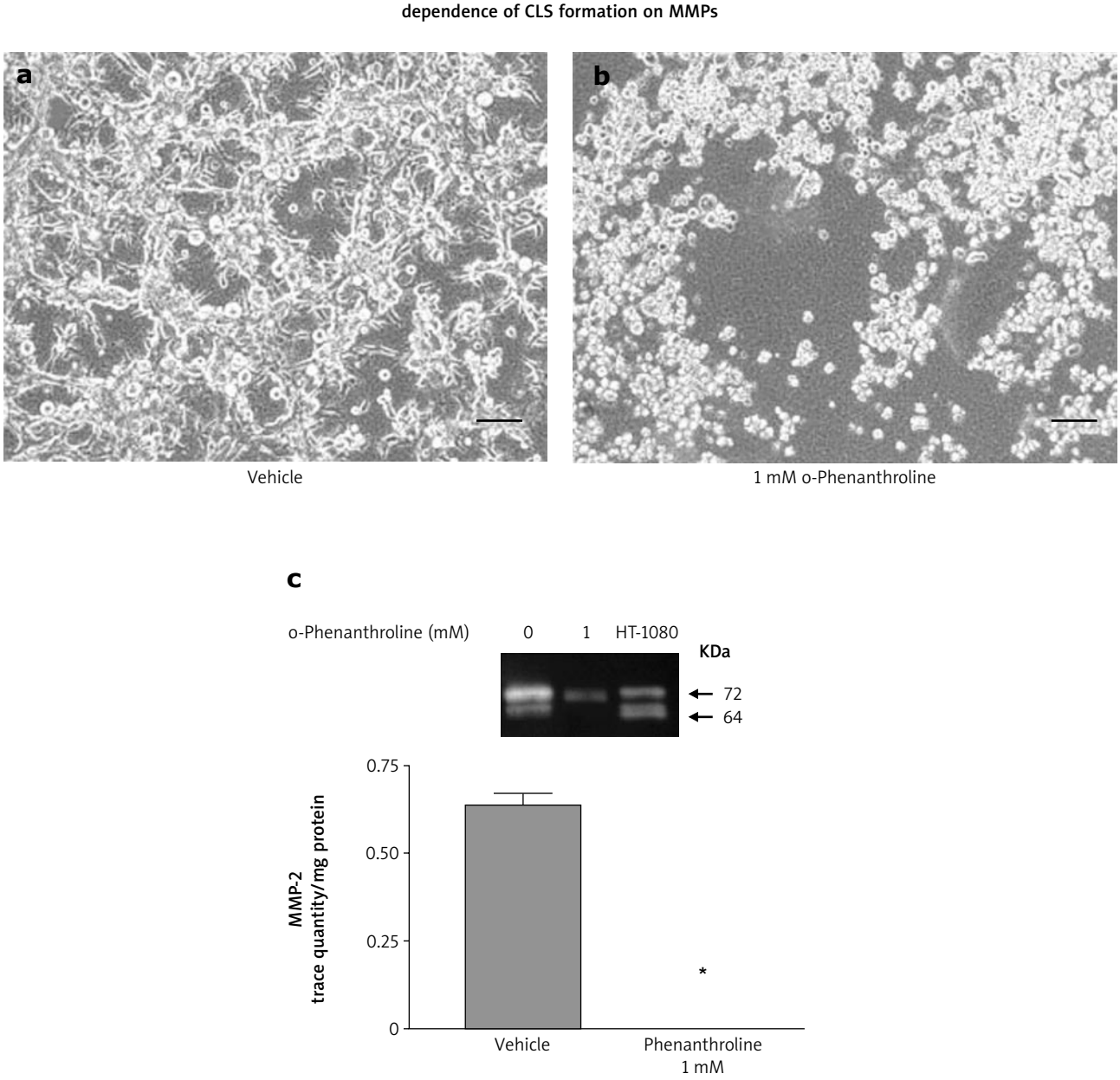

Fig. 2. (a-b) Representative micrographs showing formation of CLS by HUV-EC-C cells stimulated with 50 ng/ml PMA on a plasma matrix, and inhibition of CLS formation by 1 mM ortho-phenanthroline. Bar represents 100 μm. (c) Quantification of MMP-2 released by HUV-EC-C cells during CLS formation in the absence and presence of o-phenanthroline. Inset shows representative gelatin zymogram in presence of HT-1080 fibrosarcoma-derived MMP-2 standards. Bars are means ± SE from four separate experiments. *****P<0.05

We have previously shown that both recombinant and platelet-generated angiostatin can inhibit the formation of CLS on matrigel [8, 9]. Similar to matrigel, in our present model angiostatin inhibited CLS formation on the plasma-based matrix. Moreover, we have shown that CLS formation on the plasma-based matrix is dependent on MMPs since ortho-phenanthroline, a synthetic MMP inhibitor, inhibited both endothelial cell MMP-2 activity and CLS formation. The dependence of angiogenesis on MMPs has been demonstrated in both matrigel and fibrin models [15, 11]. Matrix metalloproteinases remodel the extracellular matrix during CLS formation, and MMP-2 has been used as a molecular marker of angiogenesis [9].

We have shown that our plasma-based model recapitulates molecular steps of other *in vitro* angiogenesis assays. Our model makes use of small amounts of human plasma from healthy volunteers (4.8 ml per 24 well plate); hence, a small donation of blood allows for a large number of experiments to be performed. Therefore, we feel our model is an inexpensive and rapid tool for studying angiogenesis *in vitro* and for screening of angiogenesis inhibitors.

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