FEBS Letters 581 (2007) 962–966

Anti-angiogenic properties of *myo*-inositol trispyrophosphate *in ovo* and growth reduction of implanted glioma

Gabin Sihn[a,*,1], Thomas Walter[b], Jean-Claude Klein[b], Isabelle Queguiner[a], Hiroshi Iwao[c], Claude Nicolau[d], Jean-Marie Lehn[e], Pierre Corvol[a], Jean-Marie Gasc[a]

[a] *Laboratoire de Pathologie Vasculaire et Endocrinologie Rénale, Inserm U36, Collège de France, 11, place Marcelin Berthelot, 75005 Paris, France*
[b] *Ecole des Mines de Paris, Centre de Morphologie Mathématique, 35, rue St-Honoré, 77305 Fontainebleau, France*
[c] *Osaka City University Medical School, Osaka, Japan*
[d] *Oxyplus, Inc., 200 Boston Avenue, Medford, MA 02155, USA*
[e] *Laboratoire de Chimie Supramoléculaire, ISIS-Université Louis Pasteur, 8, allée Gaspard Monge, BP 70028, 67083 Strasbourg Cedex, France*

Received 21 November 2006; revised 29 January 2007; accepted 30 January 2007

Available online 14 February 2007

Edited by Vladimir Skulachev

Abstract **We investigate here the anti-angiogenic properties of the synthetic compound *myo*-inositol trispyrophosphate (ITPP). By increasing oxy-haemoglobin dissociation, ITPP has the potential to counteract the effects of hypoxia, a critical regulator of angiogenesis and cancer progression. ITPP inhibited angiogenesis of the chorioallantoic membrane (CAM), as analyzed with an original program dedicated to automated quantification of angiogenesis in this model. ITPP also markedly reduced tumor progression and angiogenesis in an experimental model of U87 glioma cell nodules grafted onto the CAM. These results point out the potential of ITPP for the development of a new class of anti-angiogenic and anti-cancer compounds.**

Keywords: Angiogenesis; Allosteric effector; Chorioallantoic membrane; Haemoglobin; Inositol hexakisphosphate; Inositol trispyrophosphate; Partial oxygen pressure; Tumor; Vascular quantification

1. Introduction

Angiogenesis, the main process of blood vessel formation, is a hallmark of tumor progression [1], and anti-angiogenic compounds are extensively studied for anti-cancer therapy [2]. However, angiogenesis involves complex regulatory pathways, and compounds that are currently used in clinical trials, notably blockers of vascular growth factors and inhibitors of their tyrosine kinase receptors, have limited efficacy and exert some secondary effects [3]. Therefore, new strategies are required to sharpen and increase the efficiency of anti-angiogenic therapy.

Myo-inositol trispyrophosphate (ITPP) is a synthetic compound derived from *myo*-inositol hexakisphosphate (IP_6), a natural molecule ubiquitously produced in mammalian cells and previously reported for antioxidant [4] and anticancer [5] properties. Unlike IP_6, ITPP, which bears lower negative charge, is soluble in both aqueous and lower polarity media [6]. It is thus able to cross the red blood cell (RBC) membrane and act as an allosteric effector, increasing the oxygen release from free haemoglobin as well as from whole blood [7]. In contrast, IP_6 must be introduced into the RBC by physical methods in order to increase their oxygen release [8].

It was hypothesized that ITPP, by increasing the partial pressure of oxygen (pO_2) in hypoxic tissues, may act as an anti-angiogenic compound. Indeed, pO_2 is a crucial regulator of angiogenesis: hypoxia is a major activator of blood vessel formation [9,10], while hyperoxia has opposite effects [10]. Human microvascular endothelial cells submitted to hypoxia in the presence of human red blood cells loaded with ITPP do not form capillary tube-like structures and do not express HIF-1 [11]. In this report, we show that ITPP triggers defects in the vascular network of the chick chorioallantoic membrane (CAM) *in ovo*, consistent with an anti-angiogenic effect during physiological angiogenesis. To describe quantitatively the effects observed, we developed a new program that allows automated quantification of several vascular network parameters on angiographic images of the CAM. This original program should prove useful for analysis of pro- or anti-angiogenesis in the model of the CAM as it allows accurate, quick and easy quantification on large series of images. Finally, we report the ability of ITPP to inhibit tumor growth and angiogenesis in an experimental model of human glioma grafted onto the CAM.

2. Materials and methods

Myo-inositol trispyrophosphate (ITPP) was prepared from *myo*-inositol hexakisphosphate (IP_6) as previously described [7,11].

2.1. Chick embryos and cells

Fertilized eggs from White Leghorn chickens were obtained from a commercial breeder (Haas, Kalten House, France). U87 human glioma cells (American Type Culture Collection) were maintained in modified E-MEM (LGC Promochem, France) with 10% FBS.

*Corresponding author. Present address: Max-Delbrück-Center for Molecular Medicine, Robert-Rössle Strasse 10, 13125 Berlin-Buch, Germany. Fax: +49 30 9406 2110.
E-mail address: g.sihn@mdc-berlin.de (G. Sihn).

[1]Gabin Sihn holds a fellowship from the Fondation pour la Recherche Médicale, Paris, France.

Abbreviations: CAM, chorioallantoic membrane; FOV, first order vessel; IP_6, inositol hexakisphosphate; ITPP, inositol trispyrophosphate; pO_2, partial oxygen pressure; SOV, second order vessel; VBR, vessel/background ratio

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.079

2.2. Chorioallantoic membrane angiogenic assay

The chorioallantoic membrane (CAM) assay was adapted from Celerier et al. [12]. At embryonic day 8 (E8), each CAM received two silicon rings (10 mm-ID) laid onto two areas looking alike for their vascularization. Twenty-five microliters of ITPP hexasodium salt 0.1 M [7] were applied on the first ring, and 25 μl of vehicle (0.15 M NaCl + 2.5 mM $CaCl_2$) on the second one. After 24 h treatment, CAM were either analyzed on angiographic pictures after i.v. injection of FITC-dextran (see details in [13]), or collected for molecular histology.

2.3. Quantification of vascular parameters of the CAM

Quantification was carried out on 1.8×-magnified angiographic images. Automated quantification was performed as follows:

(A) *Vessel segmentation*. After normalizing the dynamic range of the images, a prefiltering step was applied to remove the capillary bed (morphological closing by reconstruction, dynamic filtering [14] and volume leveling [15]). Then, the following threshold scheme was applied: (1) A first (high) threshold was applied on the prefiltered image in order to segment the bright (thick) vessels. (2) The small vessels were extracted from the prefiltered image by means of the top-hat transformation [14], smoothed in their main direction with a Gaussian profile filter [16], and segmented with a second (low) threshold. These results were combined resulting in a binary image representing the vessels.

(B) *Finding the extremities*. Extremities of a binary set coincide with the local maxima of its geodesic distance map [17,18]. However, parasite maxima may exist due to (1) border irregularities and (2) loops in the segmentation result. These two problems have been overcome by (1) removing all local maxima with low dynamic [14] and (2) carrying along a label image while constructing the geodesic distance map, allowing the identification of bifurcations, and therewith the identification of the parasite maxima [18]. This method has been implemented efficiently using FIFO-structures (queues). By the end of this procedure, all endpoints of the vascular tree were obtained, which coincided with the first order vessels [19].

(C) *Calculation of a clean skeleton*. The classical skeleton (set of one pixel wide lines) of the vascular tree was calculated. From this skeleton, we removed all branches with endpoints not coinciding with the ones calculated in section 2.

(D) *Calculation of parameters*. From the segmentation result (A) the ratio of the number of vascular pixels to the number of non vascular pixels (VBR, vessel background ratio) was calculated, reflecting the vascular density. Alternatively, this measure can be calculated for small vessels only (SVBR), in order to avoid the bias induced by large vessels. The extremities (B) gave the number of first order vessels (FOV). With the skeleton (C) the number of bifurcations (three outgoing branches), the number of crossings (four or more outgoing branches), the number of second order vessels (SOV) [19], and the length distribution and total length of the vascular tree were calculated.

2.4. Reliability of the automated quantification

Results obtained by manual observation by three different observers were compared to those of automated quantification. For this comparison, which was made on nine CAMs of a single experiment, the FOV parameter was investigated. Plotting the computed data against those of each observer revealed linear regression curves with scores (R^2) of 0.74 ± 0.02 (Fig. 1B). As well, inter-observer comparisons also led to linear curves with R^2 of 0.82 ± 0.03 (Fig. 1B). Equations of the regression curves are in Fig. 1B.

2.5. Experimental glioma assay

Experimental human glioma assays were performed on the CAM as described by Hagedorn et al. [20]: at E10, a silicon ring was laid onto the CAM, and 3–5 million U87 human glioma cells in 20 µl of medium were deposited after gentle laceration of surface. At E12, embryos bearing size-matching tumors were treated until E14 with 25 µl per day of either ITPP 0.1 M or the vehicle. The effect of ITPP was analyzed on the tumoral growth and vascularization, and appearance of hemorrhages within the tumor.

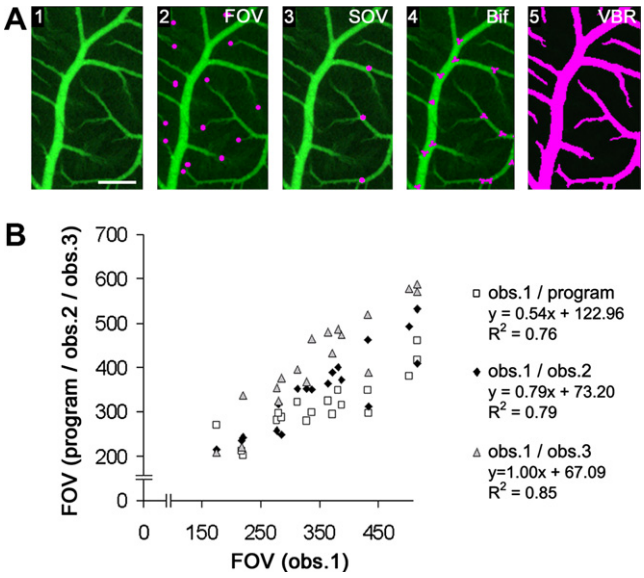

Fig. 1. Automated quantification. (A) Computed analysis of CAM vasculature, allowing, from an angiographic image (inset 1), the quantification of first order vessels (FOV, inset 2), second order vessels (SOV, inset 3) Bifurcations (Bif, inset 4) and vessel/background ratio (VBR, inset 5) parameters, revealed in purple (for details, see Section 2). (B) Plots of computed measurements for the FOV parameter against manual measurements of a first observer (obs. 1). As a comparison, plots of manual measurements of two other observers (obs. 2 and obs. 3) against obs. 1 are shown. The equations of the regression curves obtained for the different plots as well as their correlation scores (R^2) are indicated. Scale bar: 400 µm.

2.6. Histological procedures

Tissues were fixed overnight in 4% paraformaldehyde at 4 °C, dehydrated in graded alcohol, cleared in xylene, embedded in paraffin, and cut into 7 µm-thick sections. Chick blood vessels were labeled with biotinylated *Sambucus Nigra* lectin (SNA-lectin) (1:1000; Vector, Burlingame, CA), with routine signal amplification by ABC Elite (Vector, Burlingame, CA) and diaminobenzidine as chromogen. For quantification of vascular density within the U87 cell nodules, three fields per section of three different sections were analysed for each nodule as follows: images of SNA-lectin-labeled nodules were taken with a Coolsnap digital camera (Roper Scientific, Trenton, NJ), binarized with a threshold determined manually for each image, and quantified for the number of colored pixels with the IPLab software (IPLab, Scanalytics).

2.7. Statistical analysis

For manual and automatic quantification of vascular parameters of the CAM, a non parametric test of Mann and Whitney was used.

3. Results

3.1. Effects of ITPP on the CAM

ITPP at 0.1 M provoked obvious alterations of the CAM vascular tree as soon as after 24 h of treatment. Compared to the controls (Fig. 2A, C), the vascular network appeared disorganized (Fig. 2B, D) as observed on angiographic pictures, notably at the level of the microvascular bed (Fig. 2D vs C).

We evaluated these defects by measuring several parameters of the vascular network with the program described in Section 2, which allows an automated quantification. Thirteen CAM from two series of experiments were analyzed. A treatment with ITPP led to a significant decrease in parameters such as