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Inhibition of angiogenesis by extracellular protein kinase A

Maria Szkudlarek [a,1], Rual M. Bosio [b,1], Qiong Wu [a], Khew-Voon Chin [a,c,d,*]

[a] Department of Medicine, The University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614, USA
[b] Department of Surgery, The University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614, USA
[c] Department of Biochemistry and Cancer Biology, The University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614, USA
[d] Center for Diabetes and Endocrine Research, The University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614, USA

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ABSTRACT

The cyclic-AMP dependent protein kinase (PKA) signaling pathway regulates cell growth, development, metabolism, and gene expression. Peripheral blood of cancer patients but not normal individuals, shows increased catalytic subunit levels of PKA (PKAc). We showed here that this extracellular form of PKAc (ECPKA) from conditioned media of cultured cancer cells as well as purified PKAc inhibit angiogenesis, using the *in utero* chicken embryo chorioallantoic membrane assay. Inhibition of angiogenesis is partially reversed by PKI, a peptide inhibitor of PKA, thus suggesting an anti-angiogenic role for ECPKA. The significance of ECPKA in cancer is discussed.

1. Introduction

The cAMP-dependent protein kinase (PKA) is a ubiquitous tetrameric holoenzyme composed of two regulatory (R) and two catalytic (C) subunits, activated by cAMP that dissociates into a dimer of R and two free C subunits [1]. The enzyme is intracellularly localized [2]. It was first reported in prostate and various other cancers that the C subunit kinase (PKAc) is released and found in the peripheral blood of cancer patients [3,4]. This anomalously secreted PKAc is constitutively active and does not require activation by cAMP, and its activity is inhibited specifically by the protein kinase A peptide inhibitor (PKI). It was further determined that PKAc is also found in the conditioned media of various cultured cancer cells [4,5]. This extracellular form of PKAc is termed ECPKA, which is distinct from the intracellular tetrameric holoenzyme that is activated by cAMP.

The release of ECPKA into the extracellular milieu is not due to cell lysis, because intracellular marker protein, such as the mitochondrial lactate dehydrogenase, was neither detectable in the serum nor in the conditioned media of cultured cancer cells [4]. Moreover, the release of ECPKA in peripheral blood has been further confirmed in other reports [6,7]. More recently, autoantibody against ECPKA has been detected in the serum of a large number of patients with various types of cancer by an autoantibody enzyme immunoassay [8], thus suggesting that ECPKA autoantibody may serve as a universal biomarker for cancer detection.

Activation of intracellular PKA by cAMP and the subsequent protein phosphorylation mediated by the kinase in the regulation of cell growth, development, metabolism, and gene expression [9,10] have been widely investigated for more than four decades. Hence, the discovery of free PKAc in peripheral blood in cancer is intriguing. Though the function of the secreted kinase is currently unknown, it is clear that there are marked differences between ECPKA

* Corresponding author. Address: Department of Medicine, The University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614, USA. Tel.: +1 419 383 3502; fax: +1 419 383 4473.
E-mail address: khew-voon.chin@utoledo.edu (K.-V. Chin).
[1] These authors contributed equally to this study.

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and intracellular PKA. Therefore, it is imperative to elucidate the physiological role of ECPKA, as well as identifying its extracellular targets of phosphorylation in cancer. In this report, we investigated the role of ECPKA in angiogenesis and characterized the PKAc isozymes released by cancer cells. The significance of ECPKA in cancer is also discussed.

2. Materials and methods

2.1. Chicken embryo chorioallantoic membrane (CAM) assay

The chicken embryo chorioallantoic membrane (CAM) assay is conducted as describe before [11]. Briefly, one day old fertilized eggs (Hertzfeld Poultry Farms, Waterville, OH, USA) were cleaned with Spor-Klenz solution (Steris, Mentor, OH, USA) and the chick embryos were incubated for 3 days at 37 °C, in 0.5% CO_2 with 85% humidity. CAM was separated from the egg's shell by cracking the egg and dropping the chick embryo with intact yolks into 100 mm tissue culture dish containing 5 ml of RPMI-1640 medium and incubated further for 3 days. Vascular endothelial growth factor (VEGF), thalidomide, purified PKAc, and concentrated serum-free conditioned media containing total secreted proteins including ECPKA from HCT-116 and LnCap cells were embedded in circular 0.45% methylcellulose (Sigma, St. Louis, MO, USA) discs formed on glass cover slips, and air-dried. The discs were lifted from the cover slips and placed directly onto CAM surface of the chicken embryos and incubated at 37 °C for approximately 72 h. New blood vessels growth or vascular zones on CAM tissue were imaged and the area of treatment under the methylcellulose disc was scored for new vessels and capillaries growth using the ImageJ software suite (National Institute of Health, Bethesda, MD, USA). Studies were conducted in triplicate (three chick embryos per treatment condition) and standard deviations were evaluated.

2.2. Immunoblotting

Serum-free conditioned media of HCT-116 and LnCap cells were collected and concentrated using Amicon column (Millipore, Billerica, MA, USA). Approximately 20 μg of total proteins were electrophoresed on 10% SDS–polyacrylamide gel and blotted onto nitrocellulose membranes. Immunoblot detection was performed with the corresponding rabbit antiserum or mouse monoclonal antibody specific against the various PKAc isozymes (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using an enhanced chemiluminescence detection kit and exposure to photographic film.

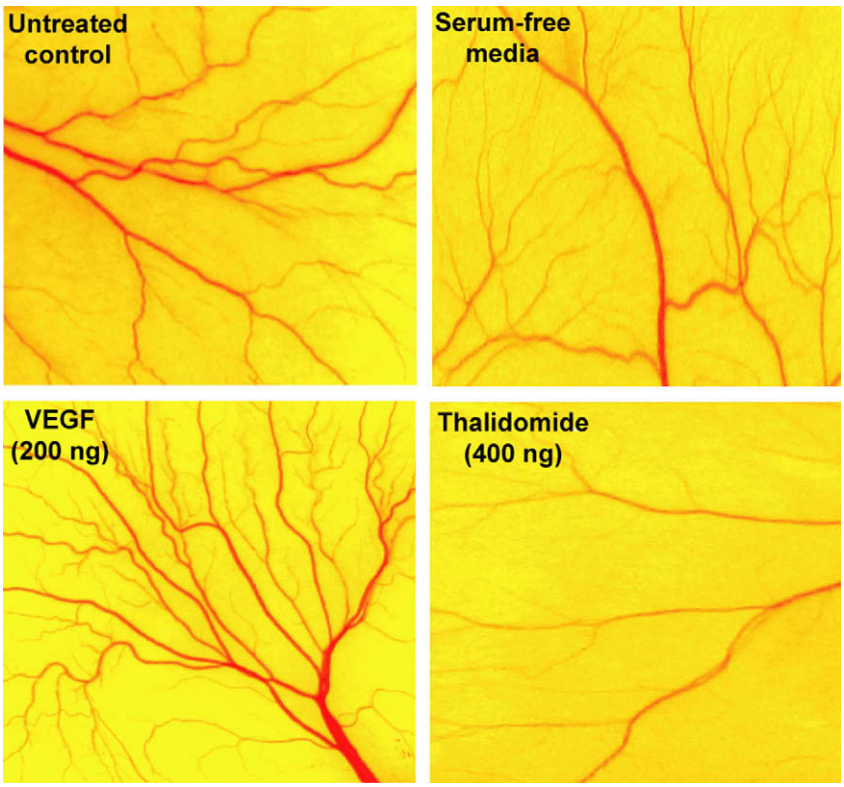

Fig. 1. Angiogenic activity assessed by the chick embryo CAM assay. Methylcellulose discs loaded with RPMI-1640 media (Control), VEGF (200 ng), or thalidomide (400 ng), were placed on CAM surface of chick embryo. Photomicrograph view of the CAM at the site of the disc approximately 72 h following drug treatment showing either induction or inhibition of allantoic blood vessels growth. Photomicrographs were obtained from representatives of triplicate experiments.

2.3. PKA and activity assay

Purified C subunit of PKA was purchased from Promega (Madison, WI, USA). PKA activity was measured using the Omnia Lysate PKA Assay Kit (Invitrogen/BioSource, Carlsbad, CA, USA) according to manufacturer's specifications.

3. Results

The identification of ECPKA in the serum of cancer patients and in conditioned media of cultured cancer cells raises questions about its function in the tumorigenic development of cancer. As cancer growth and metastasis are angiogenesis-dependent [12], we asked here whether ECPKA might be involved in tumor angiogenesis. We applied the chicken embryo chorioallantoic membrane (CAM) assay to determine the role of PKAc, and ECPKA from the conditioned media of cultured colon (HCT-116) and prostate (LnCAP) cancer cells, on new blood vessels formation.

We first examined the effects of the angiogenic vascular endothelial growth factor (VEGF) [13] and found distinct enhancement of new vessels and capillaries growth by VEGF compared to either untreated or serum-free RPMI-1640 media-treated control (Fig. 1). The anti-angiogenic drug thalidomide [14], in contrast, inhibited the formation of new blood vessels (Fig. 1), characterized by fewer major vessels and capillaries under the area of treatment. The amount of new blood vessels growth was also quantified using the ImageJ software suite, focusing on the area of treatment under the methylcellulose disc, which showed approximately 50% inhibition of angiogenesis by thalidomide (Fig. 2A). Exposure of the chicken embryo to various concentrations (10, 50, and 100 ng) of PKAc resulted in the inhibition of new blood vessels growth as evident by the lack of capillaries, compared to untreated, media alone, or VEGF-treated controls (Fig. 3). Inhibition of new blood vessels growth by PKAc was not dose-dependent. Furthermore, PKAc mediated inhibition of angiogenesis seemed to be comparable to that of thalidomide (Figs. 2A and 3).

Since activated intracellular PKA activity is normally terminated by either the hydrolysis of cAMP or the inhibition of the kinase by PKI [15], we asked whether co-treatment of chicken embryo with PKI might reverse the inhibition of new blood vessels growth by PKAc. Results in Fig. 2A showed that PKI alone did not have an effect on angiogenesis, but the inhibition by PKAc was partially reversed by PKI.

We next examined the effects of ECPKA obtained from the serum-free conditioned media of the cultured colon cancer HCT-116 and the prostate cancer LnCaP cell lines. The media were concentrated and constitutively active PKA activities were detected by a fluorescent peptide substrate, and the presence of ECPKA in the conditioned media of HCT-116 and LnCap cells was determined by Western blotting (Fig. 2B and C). Isozyme-specific antibodies against the C subunit of PKA showed that Cα and Cβ (Fig. 2C), but not Cγ (data not shown), were found in the conditioned media of HCT-116 and LnCAP cells. Addition of various concentrations (10, 50, or 100 μg) of total secreted proteins from the conditioned media of either HCT-116 or LnCap cells, which contained ECPKA, to the chicken embryo caused reduction of new blood vessels growth in a dose dependent-manner (Fig. 3). The extent of inhibition was comparable to the effects of thalidomide and PKAc. However, unlike PKAc, inhibition of new blood vessels growth by ECPKA from either HCT-116 or LnCap cells was not reversed by PKI (data not shown). These results showed that PKAc, and ECPKA of conditioned media of cultured cancer cells, inhibited new blood vessels growth in the CAM assay.

It is noteworthy that application of ECPKA-positive serum from cancer patients onto CAM induced rapid death to the chick embryo within 10–15 h (data not shown), possibly resulting from the cross-interaction of patients' antibodies with chicken embryonic antigens. These results hampered further direct examination of clinical serum sample using this assay.

4. Discussion

Release of the free C subunit of PKA into the extracellular milieu including the serum of cancer patients is an intriguing phenomenon [3,4,6,7]. We speculate that the aberrant and ubiquitous secretion of ECPKA by cancer cells into the serum of cancer patients serves to fuel the growth of the cancer either by promoting angiogenesis or tumor metastasis. However, our results in this study showed paradoxically that purified PKAc and ECPKA in the conditioned

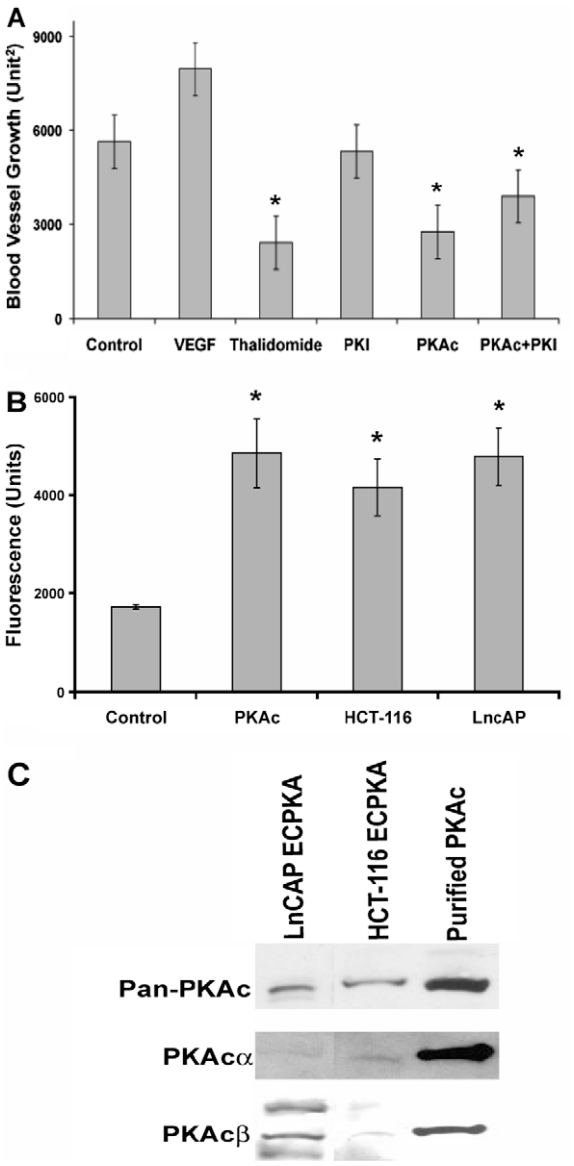

Fig. 2. (A) Effects of PKAc and PKI on allantoic vessels growth. Methylcellulose discs were embedded with either PKAc (10 ng), PKI (1 μM), or PKAc + PKI. Effects of PKAc and PKI on blood vessels growth were compared to serum-free RPMI-1640 media-treated control, VEGF (200 ng) and thalidomide (400 ng). (B) Kinase activity of ECPKA of HCT-116 and LnCAP. Conditioned media of HCT-116 and LnCAP cells were collected, concentrated, and 10 μg of total protein from the concentrates were used for the PKA activity assay according to manufacturer's specification, and compared to PKAc control (10 ng). (B and C, results are means ± SE of triplicate experiments and statistical significance was assessed using Student's *T*-test; asterisk, $P < 0.05$). (C) Western blot analysis of ECPKA. Conditioned media containing ECPKA from HCT-116 and LnCAP cells were probed with isozyme-specific antibodies against either the Cα or the Cβ subunit of PKA, or with a pan-PKAc antibody.

media of cultured cancer cell inhibited angiogenesis by the CAM assay (Figs. 1 and 3). Therefore, it is counterintuitive that cancer cells release a constitutively active protein kinase that inhibits angiogenesis, which might actually impede their growth and metastatic potential, thus compromising tumorigenesis.

While the role of the intracellular PKA holoenzyme in cell growth has been widely investigated [10], little is known, however, regarding the function of this recently identified secreted ECPKA. It has been shown that enhanced activity of the intracellular PKA promotes endothelial tube formation, thus facilitating angiogenesis [16]. In contrast, it has also been shown that activation of intracellular PKA causes the phosphorylation of the transcriptional repressor Id1 (a major contributor to angiogenesis) [17], and disrupts its nucleocytoplasmic shuttling, thus inhibit-

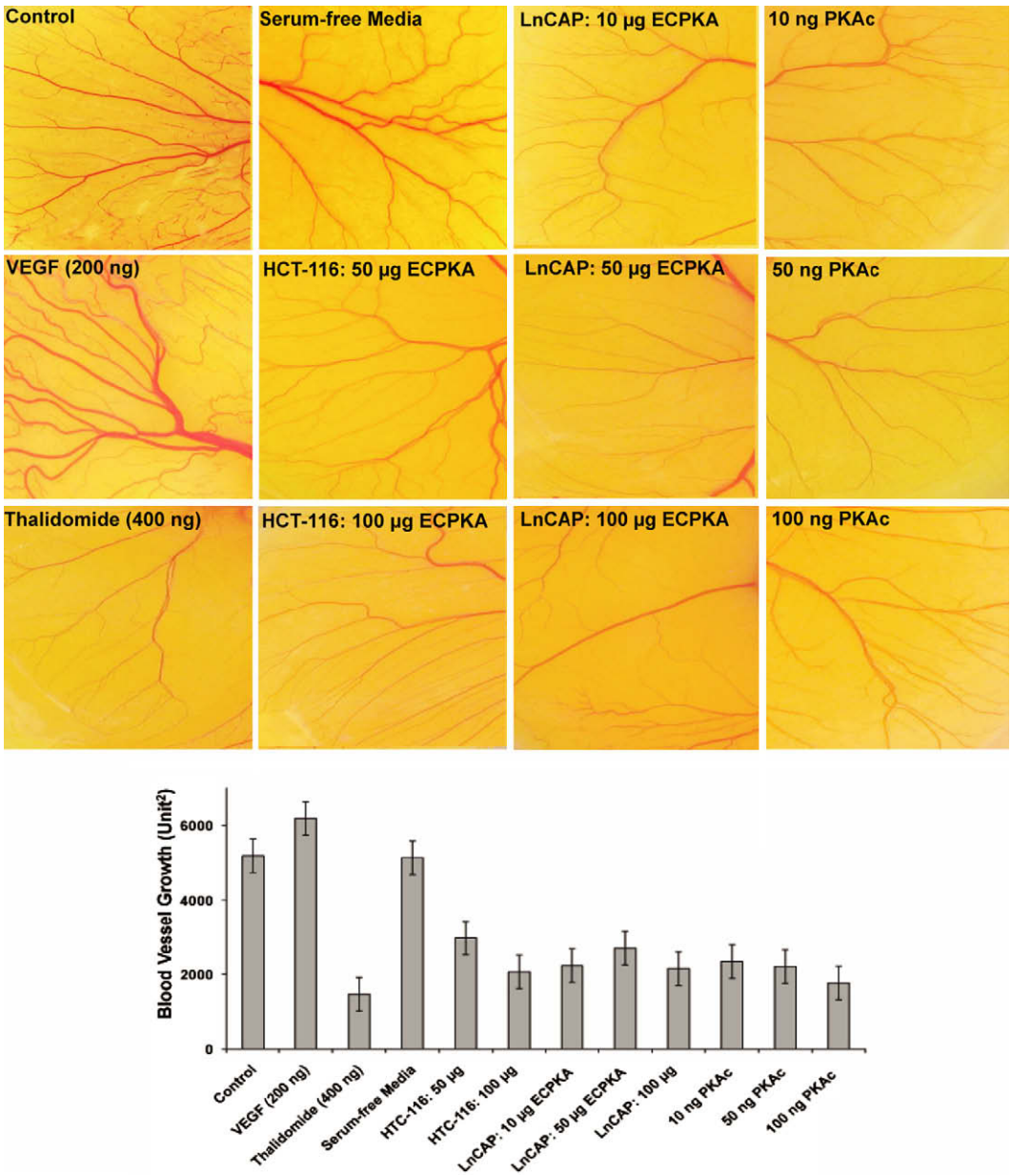

Fig. 3. Angiogenic response to PKAc and ECPKA by the CAM assay. Effects of various concentrations of either PKAc (10, 50, or 100 ng) or ECPKA obtained from conditioned media of HCT-116 (50 or 100 µg of total secreted protein) and LnCAP (10, 50, or 100 µg of total secreted protein) cultured cancer cells, on blood vessels growth in chick embryo CAM. Number of blood vessels at the methylcellulose disc and CAM boundary were compared with untreated, serum-free media, VEGF and thalidomide controls. Photomicrographs were obtained from representatives of triplicate experiments. Histogram showed quantitative data from the experiments in triplicate and results are means ± SE of triplicate experiments and statistical significance was assessed using Student's *T*-test; asterisk, $P < 0.05$.

ing angiogenesis [18]. In addition, either overexpression of the C subunit gene of PKA or pharmacological activation of PKA induces the death of endothelial cell by apoptosis [19], hence inhibiting angiogenesis. Though others and we have shown that in normal individuals, levels of ECPKA activity in the peripheral blood are negligible [3,4,6,7], but we have not explored whether ECPKA might be secreted when a system is perturbed in normal subject. Therefore, it is conceivable that intracellular PKA has angiogenic function during development that is distinct and independent of the action of ECPKA. While we did not measure endothelial cell death in this study, it is possible that ECPKA inhibits angiogenesis by inducing endothelial apoptosis. Moreover, overexpression of the Cα subunit gene of PKA has been shown to increase the production of ECPKA [3], which might account for the inhibition of angiogenesis noted in our study, and is consistent in part with the observed endothelial cell death.

We currently do not understand the underlying reasons for the release of the free C subunit of PKA in cancer. Once secreted, does ECPKA play the role of a driver gene in cancer development, or is it merely a passenger gene [20], which confers no growth advantage, amidst the myriad of genes targeted for alterations? For one, we speculate that the aberrant secretion of ECPKA might be an unintended consequence of tumor progression. Resultantly, the release of ECPKA into the serum of cancer patients elicits an autoimmune response, because PKA is normally localized inside the cell and the extracellular C subunit might be antigenic. This hypothesis is supported by the discovery of autoantibody against ECPKA in the serum of cancer patients [8]. We surmise that the production of autoantibody against ECPKA rapidly neutralizes its activity, hence preventing it from inhibiting angiogenesis and acting on its extracellular substrates or targets.

What is the pathogenic mechanism underlying ECPKA secretion in cancer? In human, there are three C subunit isoforms, Cα, Cβ, and Cγ, which have different biochemical and functional properties [21]. We showed here that Cα and Cβ are the predominant isozymes that constitute the ECPKA secreted by HCT-116 and LnCAP cells (Fig. 2C). The level of Cγ was not detectable in these conditioned media (data not shown). Therefore, Cα and Cβ are presumably the major isozymes released in cancer. Furthermore, the R and C subunits of PKA are normally present in a 1:1 stoichiometric ratio [21], hence there is little free PKAc intracellularly. It is conceivable that either the Cα or the Cβ subunit isozyme is overexpressed, or that the R subunits are downregulated in cancer, thereby resulting in excess free PKAc that are not associated with the R subunits, thus leading to their release out of the cells. Alternatively, mutations in the C subunit genes may result in the failure to retain these proteins in the cells, thus leading to their secretion into the extracellular milieu. Therefore, sequencing the C subunit genes in cancer might yield insights into their susceptibility to genetic alterations that predisposed them to secretion.

The ubiquitous nature of the release of ECPKA and its potential application as biomarker in cancer, and whether the aberrant release of ECPKA is just a bystander effect or that it might have important function in cancer development warrant further investigation.

Conflict of interest

There is no conflict of interest for any of the authors.

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