CC-PLA2-1 and CC-PLA2-2, two *Cerastes cerastes* venom-derived phospholipases A₂, inhibit angiogenesis both *in vitro* and *in vivo*

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Integrins are essential in the complex multistep process of angiogenesis and are thus attractive targets for the development of antiangiogenic therapies. Integrins are antagonized by disintegrins and C-type lectin-like proteins, two protein families from snake venom. Here, we report that CC-PLA2-1 and CC-PLA2-2, two novel secreted phospholipases A₂ (PLA₂) isolated from *Cerastes cerastes* venom, also showed anti-integrin activity. Indeed, both PLA₂s efficiently inhibited human brain microvascular endothelial cell adhesion and migration to fibrinogen and fibronectin in a dose-dependent manner. Interestingly, we show that this anti-adhesive effect was mediated by $\alpha 5\beta 1$ and αv -containing integrins. CC-PLA2s also impaired *in vitro* human brain microvascular endothelial cell tubulogenesis on Matrigel and showed antiangiogenic activity *in vivo* in chicken chorioallantoic membrane assay. The complete PLA₂ cDNAs were cloned from a venom gland cDNA library. Mature CC-PLA2-1 and CC-PLA2-2 contain 121 and 120 amino acids, respectively, including 14 cysteines each and showed 83% identity. Tertiary model structures of CC-PLA2-1 and CC-PLA2-2 were generated by homology modeling. This is thus the first study describing an antiangiogenic effect for snake venom PLA₂s and reporting first clues to their mechanism of action on endothelial cells.

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Angiogenesis is fundamental to normal healing, reproduction and embryonic development. However, this process is also important in the pathogenesis of a broad range of disorders such as arthritis and cancer.¹ Angiogenesis is thus required to sustain malignant cells with nutrients and oxygen for tumors to grow beyond a microscopic size. The development of new blood vessels in the tumor also facilitates metastasis formation by providing a mechanism to increase the likelihood of tumor cells entering the blood circulation. Thus, the microvascular endothelial cell recruited by a tumor is an important target in cancer therapy and has the advantage of being genetically stable. Therefore, treating both the cancer cell and the endothelial cell in a tumor may be more effective than treating the cancer cell alone.²

Angiogenesis is a complex process, typically consisting of enzymatic degradation of the basement membrane, vascular endothelial cell migration into perivascular space, proliferation

and alignment to form capillary-like structures, and new vessel formation. All these events are supported by the action of the integrin adhesion receptors.³ Integrins are heterodimeric, transmembrane glycoproteins that are involved in cell–extracellular matrix (ECM) and sometimes in cell–cell adhesion. Integrins are composed of noncovalently associated α - and β -subunits that can assemble into at least 24 known distinct integrins, and heterodimer composition generally confers ligand specificity.⁴ In addition to their structural role as bridges between the ECM proteins and the actin cytoskeleton, integrins also have a critical role in signal transduction events that regulate cell shape, survival, proliferation and migration.⁵

Snake venom contains components able to disrupt cell–ECM interactions. Among these molecules, disintegrins are the most widely characterized and they are now studied for potential use in medicine (for a review, see McLane *et al*⁶).

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Most disintegrins contain an Arg-Gly-Asp (RGD) or RGD-like sequence in the C-terminal half of the molecule, which is essential to their ability to block integrin interaction with ligands.⁷ Another family of venom peptides, the C-type lectin proteins (CLPs),⁸ has also been reported to impair cell–ECM interactions. They inhibit cell adhesion, migration, proliferation and angiogenesis by interacting with integrins.^{9–12}

Phospholipases A₂ (PLA₂, EC 3.1.1.4) are generally Ca²⁺-dependent enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of glycerophospholipids. Secreted PLA₂s are small proteins (14–18 kDa) usually containing 5–8 disulfide bonds and possessing a His/Asp dyad required for catalysis.¹³ Snake venoms contain high amounts of secreted PLA₂s that belong to either group I (elapid and hydrophid venoms) or group II (vipereid and crotalid venoms). In addition to their possible role in the digestion of preys, snake venom PLA₂s exhibit a wide spectrum of pharmacological effects by interfering in normal physiological processes. They include neurotoxicity, myotoxicity, hemolytic activity, anticoagulant and antiplatelet effects (see Kini¹⁴ for a review). Furthermore, they can show antiviral and bactericidal properties.^{15–17} Several lines of evidence indicate that, in many instances, these effects are independent of the catalytic phospholipid hydrolysis. Moreover, to explain the susceptibility of a tissue to a particular PLA₂, it has been proposed that venom PLA₂s have selective membrane-binding sites that direct them to specific cellular targets, and that such binding is crucial in their mechanism of action.¹⁴

In this work we reported *in vitro* and *in vivo* anti-angiogenic activities of two secreted PLA₂s (CC-PLA2-1 and CC-PLA2-2) isolated from *Cerastes cerastes* venom, the Tunisian horned viper. We showed that their effect is likely due to the inhibition of $\alpha 5\beta 1$ and αv -containing integrins.

MATERIALS AND METHODS

Reagents

Venom was collected from *C. cerastes* snake in the Pasteur Institute's serpentarium (Tunis, Tunisia) and stored at –20°C. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were purchased from Lonza (Levallois-Perret, France), fetal calf serum (FCS) and horse serum were from Invitrogen (Cergy-Pontoise, France), and endothelial cell growth supplement (ECGS) was from BD Biosciences (Le Pont de Claix, France). Poly-L-lysine, human fibrinogen and mouse laminin-1 were from Sigma (St Quentin Fallavier, France). Rat type I collagen was from Upstate (Lake Placid, NY, USA) and human fibronectin from Millipore (St Quentin en Yvelines, France). Human vitronectin was purified according to Yatogho *et al.*¹⁸

Rat monoclonal antibody (mAb) 69.6.5 against αv integrin was produced as previously described.¹⁹ Mouse mAbs FB12 (anti- $\alpha 1$), P1B5 (anti- $\alpha 3$), P1D6 (anti- $\alpha 5$), LM609 (anti- $\alpha v\beta 3$) and P1F6 (anti- $\alpha v\beta 5$) were purchased from Millipore. Mouse mAbs Gi9 (anti- $\alpha 2\beta 1$), Lia1/2 (anti- $\beta 1$)

and rat mAb GoH3 (anti- $\alpha 6$) were from Beckman Coulter (Marseille, France).

Cell Culture

Human colonic adenocarcinoma (HT29-D4)²⁰ and fibrosarcoma (HT1080) (ATCC) cells were routinely cultured in DMEM containing 10% FCS. Human leukemia (K562; ATCC) and ovarian adenocarcinoma (IGROV1) cells, provided by Dr J Benard (Institut Gustave Roussy, Villejuif, France) were cultured in RPMI 1640 medium containing 10% FCS. Rat pheochromocytoma (PC12; ATCC) cells were cultured in DMEM supplemented with 5% FCS and 10% heat-inactivated horse serum. Immortalized human brain microvascular endothelial cells (HBMECs), kindly provided by Dr K Kim (JHU, Baltimore, MA, USA), were maintained in RPMI 1640, 20% heat-inactivated FCS, ECGS (50 μ g/ml), heparin (50 U/ml) and L-glutamine (2 mM).

Cell Adhesion and Migration Assays

Adhesion assays were performed as previously described.²¹ Briefly, cells in single-cell suspension were added to wells coated with purified ECM proteins and allowed to adhere to the substrata for 1 h (HBMEC, HT1080, IGROV1 and PC12 cells) or 2 h (HT29-D4 and K562 cells) at 37°C. Incubation of K562 cells was performed in the presence of 1 mM MnCl₂ and 100 nM phorbol 12-myristate 13-acetate (PMA) to activate $\alpha 5\beta 1$ integrin. After washing, attached cells were fixed, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 600 nm.

In vitro cell migration was evaluated using haptotaxis assays in modified Boyden chambers (NeuroProbe Inc., Bethesda, MD, USA) as previously described²¹ in the presence of DMEM/10% FCS. After staining cells with 0.1% crystal violet, cell migration was quantified by measure of absorbance at 600 nm.

Solid-Phase Receptor Assay

Microtiter plates (96-well) were precoated with 0.5 μ g CC-PLA2 for 2 h at 37°C, then blocked with PBS/3% BSA, overnight at 4°C. HBMECs were solubilized with 20 mM Tris-HCl (pH 7.6), 200 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% BSA and a mixture of protease inhibitors (1 mM PMSF, 500 U/ml aprotinin, 1 μ g/ml leupeptin, 1 μ M pepstatin, 1 mM iodoacetamide and 1 mM o-phenanthroline) for 30 min at 4°C. The extract was clarified by centrifugation at 10 000 g for 10 min at 4°C and then added (200 μ g of total proteins) to CC-PLA2-coated wells for 4 h at 4°C. Unbound proteins were removed by three washes with PBS/0.1% Triton X-100. Integrins binding was detected by sequential incubations with 100 μ l of 10 μ g/ml mouse mAbs AMF-7 (anti- αv), Sam-1 (anti- $\alpha 5\beta 1$) or Gi9 (anti- $\alpha 2\beta 1$) and with sheep anti-mouse IgG HRP-conjugated antibodies for 1 h at room temperature. Finally, 100 μ l of peroxidase substrate (3,3',5,5'-tetramethylbenzidine) were added for 10 min at room

temperature, the reaction was stopped by adding 50 μ l of 1 mol/l H₂SO₄ and absorbance was measured at 450 nm.

Chemical Inhibition of PLA₂ Activity

Chemical modification of CC-PLA2-1 and CC-PLA2-2 enzymes by p-bromophenacyl bromide (BPB) and enzymatic activity measurements were performed as described.²²

Tubulogenesis on Matrigel™ and Chicken Chorioallantoic Membrane Assay

Tubulogenesis assays were performed using HBMEC cells on Matrigel-coated wells as already described.¹¹ The capillary-like structures formed in the gel were quantified using ImageJ software.

Chick embryos from 3-day-old eggs were opened and placed in double Petri dishes with water for humidity. After 5 days at 37°C, filter paper disks (diameter 6 mm) soaked in buffer (0.9% NaCl) or CC-PLA2-1 or CC-PLA2-2 (0.5 or 1 μ M) were applied on the chicken chorioallantoic membrane (CAM). After 48 h, spontaneous angiogenesis was observed and photographed with a digital camera at $\times 10$ magnification.

To check the effect of CC-PLA2-1 and CC-PLA2-2 on growth factor-induced angiogenesis, bFGF or vascular endothelial growth factor (VEGF) (200 ng per embryo)-impregnated filter disks were administered topically on the CAM of 8-day-old embryos. After 24 h, CC-PLA2-1 and CC-PLA2-2 (1 μ M) were applied to the CAM and blood vessels were photographed after 72 h of incubation.

cDNA Library Construction and Clones Screening

The first strand of cDNA was reverse-transcribed by a standard method according to the manufacturer's instructions (Promega) using the universal oligo(dT)-containing adapter primer 5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTC AAGCTT₁₆-3' (Proligo). 12 μ l of 0.1% diethylpyrocarbonate-treated water containing 0.1 μ g of primer and 1 μ g of total venom gland RNA were heated to 70°C for 10 min to denature any possible secondary structure, cooled in ice and mixed with 4 μ l of 5 $\times$ First Strand Buffer, 2 μ l of 0.1 M dithiothreitol, 1 μ l of RNasin (ribonuclease inhibitor at 40 U/ μ l), 1 μ l of dNTP (10 mM each) and 200 U RNase H-free Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h, followed by 10 min at 95°C to inactivate the enzyme.

CC-PLA2 encoding sequences were amplified by PCR using venom gland cDNAs as template and the following primers: 5'-TCTGGATTCTGGGAGGATGAGG-3' (forward primer, corresponding to highly conserved 5' untranslated region of reported cDNA sequences encoding group II PLA₂s) and 5'-GAGGACTCGAGCTCAAGC-3' (reverse primer, which is contained in the oligo(dT) primer). The PCR protocol included an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C) and extension (1 min at 72°C), and

a final extension step of 7 min at 72°C. The amplified fragments were separated on 1% agarose gel electrophoresis, purified using QIAquick gel extraction kit (Qiagen) and cloned into pGEM-T vector (Promega) before transforming *Escherichia coli* MOSBlue cells.

Positive clones, selected by growing the transformed cells in Luria Broth medium containing 10 μ g/ml ampicillin, were confirmed by PCR amplification using the above primers and sequencing of the inserts on an Applied Biosystems model 377 DNA sequencer.

Molecular Modeling

Tertiary model structures of CC-PLA2-1 and CC-PLA2-2 were generated by homology modeling with the program Modeller 9v2.²³ Homologous polypeptides with known structures were identified by a Blast2 search of the PDB database (RCSB organization) using the sequence of CC-PLA2-1 and CC-PLA2-2 as entry. The solution structures of phospholipase A₂ inhibitor from Vipoxin (PDB code 1VPI) and Rv4/Rv7 complexes (PDB code 1OQ5) were used as templates. A total of 20 models were generated for each protein. All their pdb files were analyzed for their energetic and geometric characteristics. For each protein, only one model combining the best Ramachandran plot (<http://swift.cmbi.ru.nl/servers/html/ramaplot.html>), and good scores for the objective function values,²³ and the VICTOR/FRST energy function proposed by Tosatto²⁴ (<http://protein.cribi.unipd.it/frst/>) was considered. The best models were then visualized with the ViewerLite50 program (<http://www.accelrys.com/products/dstudio/>).

RESULTS

Molecular Cloning of CC-PLA2-1 and CC-PLA2-2 and Sequences Analysis

We recently purified to homogeneity and partially sequenced two nontoxic PLA₂s with a relatively high enzymatic activity from *C. cerastes* snake venom. These enzymes, named CC-PLA2-1 and CC-PLA2-2, inhibited blood coagulation, platelet aggregation, and tumor cell adhesion and migration.²² In this study, we cloned the cDNAs encoding PLA₂ isoforms from a *C. cerastes* venom gland cDNA library, amplified by PCR and sequenced.

Among the clones obtained, we retained two cDNAs that showed a complete identity with the N-terminal amino-acid sequences of purified CC-PLA₂s as determined by Edman degradation (Figure 1a, underlined). Both nucleotide sequences were deposited in GenBank under the accession numbers FJ754186 (CC-PLA2-1) and FJ754187 (CC-PLA2-2). The cDNAs contain open reading frames of 411 and 408 bp that encode two polypeptides of 137 and 136 amino-acid residues, respectively, including a 16 amino-acid signal peptide (Figure 1a). Mature CC-PLA2-1 and CC-PLA2-2 thus contain, respectively, 121 and 120 amino acids, including 14 cysteines each. The peptide sequences of CC-PLA2-1 and CC-PLA2-2 are very similar (83% identity). Indeed, they

BLAST search and amino-acid sequence alignment show that the two CC-PLA₂s have a high sequence identity with group IIA snake venom PLA₂s (Figure 1b). The highest amino-acid identity with CC-PLA₂-1 was observed with EC1 PLA₂ from *Echis coloratus* snake venom (75%), ammodytin I2 (D)

isoform from *Vipera ursinii* (75%), PLA2-CERCE from Pakistani *C. cerastes* (64%) and RV7 from *Daboia russelii siamensis* (62%). CC-PLA2-1 sequence also showed 45% identity with KDR-bp, a KDR binding PLA₂ from *Agkistrodon piscivorus* (Figure 1b).

CC-PLA2-1 and CC-PLA2-2 Affect Adhesion and Migration of Endothelial Cells

Although it has been reported that snake venom PLA₂s exert a cytotoxic activity on tumor cells,^{17,25} we showed that the two CC-PLA2s are not cytotoxic on human fibrosarcoma (HT1080) and melanoma (IGR39) cell lines.²² In this study, we used HBMECs to check the effect of CC-PLA2s on angiogenesis. We observed that none of the CC-PLA2s, at 1 μ M, neither induced cell death (data not shown), nor inhibited cell growth after 4 days in culture (Figure 2a). However, both CC-PLA2s affected cell adhesion depending on the purified ECM protein used as substratum (Figure 2b). Both CC-PLA2-1 and CC-PLA2-2 at 1 μ M abolished the attachment of HBMEC to fibrinogen and dramatically reduced their adhesion to fibronectin, whereas no effect was observed on type I collagen, vitronectin or laminin 1. The inhibition likely involved the integrin family of adhesion receptors, as adhesion of HBMEC was not affected when using the integrin-independent substratum poly-L-lysine. Both CC-PLA2-1 and

CC-PLA2-2 inhibited cell attachment in a dose-dependent manner (Figure 2c). The IC₅₀ values were, respectively, 0.12 and 0.20 μ M for fibrinogen and 0.12 and 0.30 μ M for fibronectin.

Moreover, as already observed with tumor cells,²² alkylated CC-PLA2s were still able to inhibit HBMEC adhesion as efficiently as the active enzymes, indicating a clear dissociation of the anti-adhesive effect of CC-PLA2s and their catalytic activity (Figure 2d).

Cell migration can be considered as a finely regulated process including successive steps of cell adhesion and de-adhesion. Because CC-PLA2s block HBMEC cell adhesion, we also looked at their effect on cell migration using haptotaxis assays in modified Boyden chambers. As illustrated in Figure 3a, both CC-PLA2s at 1 μ M completely blocked HBMEC cell migration toward fibronectin. The inhibitory effect of CC-PLA2s on cell migration to fibronectin and to fibrinogen was dose dependent, with an inhibition of 50–65% at a concentration as low as 0.1 μ M (Figure 3b).

CC-PLA2-1 and CC-PLA2-2 Effects are Mediated by $\alpha 5\beta 1$ and αv Integrins

Because HBMEC adhere on fibrinogen and fibronectin through $\alpha 5\beta 1$ and αv -containing integrins (see Supplementary Figure S1), these integrins are good candidate targets for

Figure 2 CC-PLA2-1 and CC-PLA2-2 inhibit endothelial cells adhesion. (a) HBMEC were seeded at 5000 cells per well and allowed to spread for 2 h at 37°C. They were then cultured without (■) or with 1 μ M of CC-PLA2-1 (◻) or CC-PLA2-2 (◼) for the indicated time. At daily intervals, cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet, solubilized by SDS and absorbance was measured at 600 nm. (b) HBMEC were preincubated without (■) or with 0.5 μ M of CC-PLA2-1 (◻) or CC-PLA2-2 (◼) for 30 min at room temperature. Cells were then added to 96-well microtiter plates coated with 10 μ g/ml type I collagen (Coll I), fibronectin (Fn), vitronectin (Vn) or laminin-1 (Ln 1), or with 50 μ g/ml fibrinogen (Fg) or poly-L-lysine (PLL) and allowed to adhere for 1 h at 37°C. After washing, adherent cells were fixed, stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. (c) HBMECs were preincubated with the indicated concentrations of CC-PLA2-1 (—○—) or CC-PLA2-2 (—■—) and allowed to adhere to plates coated with fibrinogen or fibronectin as described above. (d) HBMECs were preincubated without (control) or with 1 μ M of purified CC-PLA2-1 or CC-PLA2-2 (native) or with enzymes previously chemically treated in the absence (–BPB) or in the presence (+BPB) of p-bromophenacyl bromide. Cells were then allowed to adhere to plates coated with fibrinogen as described above. All data shown are means ($\pm$ s.d.) from at least three experiments performed in triplicate.

Figure 3 CC-PLA2-1 and CC-PLA2-2 inhibit endothelial cell migration. (a) HBMECs were treated for 30 min at room temperature with 1 μM CC-PLA2s and then added into the upper reservoir of a modified Boyden chamber with porous membrane precoated with 10 μg/ml fibronectin. After incubation at 37°C for 5 h, cells that migrated to the underside of the filter were stained with 0.1% crystal violet. Scale bar = 100 μm. (b) HBMECs were treated without (■) or with 0.1, 0.5 or 1 μM of CC-PLA2-1 (▨) or CC-PLA2-2 (▩) and migration to fibrinogen or to fibronectin was quantified as described above. Data shown (± s.d.) are from one experiment representative of three performed in triplicate. They are expressed as a percentage of adhesion in the absence of CC-PLA2s.

the inhibitory effects of CC-PLA2s. To test this supposition, we conducted several assays. We first performed adhesion assays to immobilized CC-PLA2s in the presence of blocking antibodies directed against various integrin subunits. As illustrated in Figure 4a, both CC-PLA2s were efficient in allowing HBMEC attachment. Antibodies against αv integrin subunit completely abolished cell adhesion to CC-PLA2s, whereas antibodies against αvβ3 reduced cell attachment by about 75–80%, suggesting that αv integrins may be involved.

We next checked the anti-adhesive effect of CC-PLA2s on various cell/ECM protein pairs involving each one a sole integrin: α1β1 (PC12/type IV collagen), α2β1 (HT1080/type I collagen), α5β1 (K562/fibronectin), α6β4 (HT29-D4/laminin-1), αvβ3 (IGROV1/fibrinogen), αvβ5 (HT29-D4/vitronectin) and αvβ6 (HT29-D4/fibronectin). As shown in Figure 4b, none of the CC-PLA2s modified cell adhesion through α1β1, α2β1, α6β4 or αvβ5 integrins. However, both PLA₂s efficiently blocked the adhesive function of α5β1, αvβ3 and αvβ6 integrins.

The integrin adhesive function can be regulated by subcellular relocalization or by intracellular signals, a phenomenon known as inside-out signaling. We therefore used a solid-phase binding assay to test the interaction of the integrins with immobilized CC-PLA2 in a cell-free environment. In these conditions, we observed that solubilized αv and α5β1 integrins still bound to both CC-PLA2s, whereas binding of α2β1 integrin was very low (Figure 4c). Altogether these results suggest that CC-PLA2s likely inhibit cell

adhesion and migration by interacting directly with αv and α5β1 integrins.

CC-PLA2-1 and CC-PLA2-2 Display *in vitro* and *in vivo* Antiangiogenic Activity

Cell adhesion and migration are integral parts of the angiogenic process and affect tubulogenesis. Because HBMEC tubulogenesis can be inhibited by specific anti-αv antibodies and by C-type lectins that target αv-containing integrins,¹¹ we hypothesized that PLA₂-mediated impairment of HBMEC adhesion and migration could therefore affect tubulogenesis. HBMECs were thus treated with 1 μM of CC-PLA2 and *in vitro* tubulogenesis assays were performed on Matrigel. As shown in Figure 5A, both CC-PLA2s completely blocked *in vitro* tubulogenesis. Besides, this effect was dose dependent (Figure 5B).

To further characterize the antiangiogenic properties of CC-PLA2s, we used the *in vivo* CAM assay. Both CC-PLA2-1 and CC-PLA2-2 (1 μM) strongly reduced vasculature development. A marked reduction in the number of new capillaries and branching, without affecting the mature blood vessels, was also observed (Figure 5C, b and c). Quantification shows that the total vessel length was reduced by 76 and 78% for CC-PLA2-1 and CC-PLA2-2, respectively, compared with the untreated conditions (Figure 5D). Chicken CAM assays were also performed under the induction of angiogenesis by bFGF or VEGF (200 ng per embryo) with or without the addition of CC-PLA2. Either bFGF or VEGF induced a pronounced angiogenic response in this model (Figure 5C, d and g, respectively). Both CC-PLA2-1 and CC-PLA2-2 reduced by 54% the bFGF-induced angiogenesis (Figure 5C, e and f; Figure 5D). CC-PLA2-1 and CC-PLA2-2 also reduced by 60 and 66%, respectively, VEGF-induced angiogenesis (Figure 5C, h and j; Figure 5D). Consequently, we can conclude that both CC-PLA2s are able to block normal as well as induced angiogenesis *in vitro* and *in vivo*.

Molecular Modeling of CC-PLA2-1 and CC-PLA2-2

The three-dimensional models of CC-PLA2-1 and CC-PLA2-2, obtained using solution structures of PLA₂ inhibitor from Vipoxin (PDB code 1VPI)²⁶ and Rv4/Rv7 complex (PDB code 1OQ5)²⁷ as templates, were refined with suitable stereochemical parameters. Statistics for the Ramachandran plot from an analysis gave 94.2% for CC-PLA2-1 and 96.6% for CC-PLA2-2 of nonglycine residues in the most favored regions. The overall structures of CC-PLA2-1 and CC-PLA2-2 resemble that of classic group II PLA₂ enzymes (Figure 6), consisting of an N-terminal α-helix (residues Leu² to Thr¹³), a loop equivalent to Ca²⁺-binding loop (Tyr²⁷, Gly²⁹, Gly³¹ and Asp⁴⁹), two long antiparallel α helices (α2, residues Ala³⁹ to Gly⁵²; α3, residues Pro⁷⁹ to Glu⁹⁷), the short two-stranded antiparallel β-sheet (β-wing) (Leu⁶⁵ to Phe⁶⁷ and Ile⁷² to Cys⁷⁴), and the C-terminal loop region. In this model, the 14 cysteine residues of CC-PLA2-1 and CC-PLA2-2 form seven disulfide bonds for each protein. The patterns of the disulfide

Figure 4 Anti- $\alpha 5$ and anti- αv antibodies inhibit cell adhesion to CC-PLA2s. **(a)** HBMECs were preincubated without (–) or with function-blocking antibodies against integrin subunits for 30 min at room temperature. Antibodies were used as follow: FB12 (anti- $\alpha 1$), Gi9 (anti- $\alpha 2\beta 1$), GoH3 (anti- $\alpha 6$), 69.6.5 (anti- αv), Lia1/2 (anti- $\beta 1$) and LM609 (anti- $\alpha v\beta 3$) at 10 μ g/ml and P1F6 (anti- $\alpha v\beta 5$) at 1:100. Cells were then added to 96-well microtiter plates coated with 1 μ M CC-PLA2-1 or CC-PLA2-2 for 1 h at 37°C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Cell adhesion to type I collagen (Coll I) is shown as a control. Data are means ($\pm$ s.d.) from 3–5 experiments performed in triplicate. **(b)** Adhesion assays were performed with various cell/ECM protein pairs involving each one a sole integrin: $\alpha 1\beta 1$ (PC12/type IV collagen), $\alpha 2\beta 1$ (HT1080/type I collagen), $\alpha 5\beta 1$ (K562/fibronectin), $\alpha 6\beta 4$ (HT29D4/laminin-1), $\alpha v\beta 3$ (IGROV1/fibrinogen), $\alpha v\beta 5$ (HT29D4/vitronectin) and $\alpha v\beta 6$ (HT29D4/fibronectin). Cells were preincubated in the absence (■) or in the presence of 1 μ M CC-PLA2-1 (□) or CC-PLA2-2 (▨) and plated on ECM protein-coated wells at 37°C for 1 or 2 h depending on the cell line. Adhesion was then quantified as described above. Data shown ($\pm$ s.d.) are from one experiment representative of 2 or 3 performed in triplicate. **(c)** Solubilized proteins (200 μ g) from HBMEC were added to 96-well plates precoated with 0.5 μ g of CC-PLA2-1 (□) or CC-PLA2-2 (▨). After incubation for 4 h at 4°C and extensive washing, bound integrins were detected by sequential incubations with 10 μ g/ml mouse mAbs AMF-7 (anti- αv), Gi9 (anti- $\alpha 2\beta 1$) or Sam-1 (anti- $\alpha 5\beta 1$) and with sheep anti-mouse IgG HRP-conjugated antibodies for 1 h at room temperature. Finally, peroxidase substrate was added for 10 min at room temperature, the reaction was stopped by H₂SO₄ and absorbance was measured at 450 nm.

bridges are identical to that of classic group II PLA₂ (Cys²⁶–Cys¹¹³, Cys²⁸–Cys⁴⁴; Cys⁴³–Cys⁸¹; Cys⁴⁹–Cys¹²¹, Cys⁵⁰–Cys⁸⁶; Cys⁵⁷–Cys⁷⁹ and Cys⁷⁴–Cys⁸⁴). Other main structural features include highly conserved catalytic residues (His⁴⁷, Tyr⁵¹ and Asp⁸⁹).

DISCUSSION

PLA₂s are among the best-characterized components of snake venom. In addition to their possible role in the digestion of the prey, many snake venom PLA₂s are toxic and exhibit a large variety of pharmacological effects, including tissues toxicity, modulation of platelet aggregation and hypotension.¹⁴ In this work, we demonstrated for the first time that CC-PLA2-1 and CC-PLA2-2, two snake venom PLA₂ enzymes from *C. cerastes* venom, can also exert antiangiogenic activity *in vitro* and *in vivo*. In some cases, *in vivo* tissue damages caused by PLA₂s have been correlated with *in vitro* cytotoxicity.^{28,29} However, the antiangiogenic activity of CC-PLA2s is likely not due to any cytotoxic effect on HBMEC cells. Indeed, endothelial cells proliferation was not affected

by CC-PLA2s over a 4-day period (see Figure 2a), cells did not detach from the substratum during the test and no cell debris could be observed. Moreover, their proliferative potential was neither altered by treating cells in suspension, as performed in most of the experiments, excluding the possibility that endothelial cells undergo apoptosis secondary to anoikis (data not shown). Rather, the CC-PLA2s, used at concentrations lower than 1 μ M, efficiently inhibit several key steps of the angiogenic process *in vitro*, such as endothelial cell adhesion, migration and tubulogenesis.

To the best of our knowledge, such inhibitory activities of secreted PLA₂ on endothelial cells have not been reported to date. Some studies have suggested instead an increased motility of epithelial cells and an increased invasion of fibroblasts in response to *Naja naja* PLA₂.^{30,31} In the case of endothelial cells, Rizzo *et al*³² also showed evidences for a chemotactic effect of secreted PLA₂. This discrepancy with our own observations may be due to the differences in the techniques, as we used haptotaxis assays to analyze cell migration. Moreover, we analyzed the effect of CC-PLA2s on

Figure 5 CC-PLA2s block *in vitro* and *in vivo* angiogenesis. **(A)** Representative tubulogenesis assay after pretreatment of HBMEC with 1 μ M CC-PLA2-1 or CC-PLA2-2 for 30 min at room temperature. Cells (2×10^4) were then added to the Matrigel-coated wells in culture medium and allowed to form capillary-like structures for 18 h at 37°C. Scale bar = 100 μ m. **(B)** Quantification of the capillary-like structures formed in the gel after treatment with the indicated concentrations of CC-PLA2-1 or CC-PLA2-2. Presented is a mean ($\pm$ s.d.) from at least three independent experiments. **(C)** The CAM models were prepared using 8-day-old chick embryos treated as described in Materials and methods. Filter disks were soaked in (a) 0.9% NaCl alone; (b) 1 μ M CC-PLA2-1; (c) 1 μ M CC-PLA2-2; (d) 200 ng bFGF; (e) 200 ng bFGF and 1 μ M CC-PLA2-1; (f) 200 ng bFGF and 1 μ M CC-PLA2-2; (g) 200 ng VEGF; (h) 200 ng VEGF and 1 μ M CC-PLA2-1; (i) 200 ng VEGF and 1 μ M CC-PLA2-2. After incubation for 72 h, CAMs were photographed with a digital camera. Each group contained four CAMs and the experiment was repeated three times. **(D)** The quantitative measurement of total vessel length was performed on 50% of the total CAM surface treated in the absence (■) or in the presence of CC-PLA2-1 (▨) or CC-PLA2-2 (▩).

Figure 6 Surface electrostatic potential models for CC-PLA2-1 and CC-PLA2-2. Tertiary model structures of CC-PLA2-1 and CC-PLA2-2 were generated by homology modeling with the program Modeller 9v2 and visualized with the ViewerLite50 program. The dotted circles indicate N-terminal α -helix and active site regions. The β -wing and C-terminal loop regions are emphasized.

cell migration after 5 h, a period of time giving no more chemotactic response in the case of endothelial cells.³² Alternatively, secreted PLA₂s may show tissue-specific anti-angiogenic activities. This is supported by the fact that PLA₂ are unable to induce migration of human vascular smooth muscle cells.³² This is also the case of sphingosine-1-phosphate, a generally proangiogenic molecule, that inhibits brain-derived endothelial cells migration and *in vitro* tubulogenesis.³³ The physiological relevance of our *in vitro* findings on endothelial cells is confirmed by the blockage of *in vivo* angiogenesis induced by CC-PLA₂s.

The inhibitory effect of CC-PLA₂s on cell migration and angiogenesis is likely due to the reduced attachment to ECM proteins observed in the presence of the enzyme. As primary receptors for ECM proteins, integrins are required for cell motility. Integrins have also a major role in angiogenesis along with others promoters, such as cadherins, matrix metalloproteinases or growth factors.¹ Initially, the anti-integrin activity among snake venom proteins was considered to be characteristic for disintegrins.³⁴ However, in the last years increasing evidences show that some CLP can also inhibit integrin-dependent function.^{11,12,35} Here, we extend this observation to another class of snake venom proteins, the group IIA PLA₂s.

That CC-PLA₂-1 and CC-PLA₂-2 impaired angiogenesis by interfering with integrin function is suggested by the fact that both proteins affected adhesion to ECM proteins, but not to the integrin-independent substratum poly-L-lysine. However, all integrins are likely not affected because inhibition was only observed with fibrinogen and fibronectin, but not with other integrins ligands such as type I collagen, vitronectin or laminin 1. In HBMEC, fibrinogen and fibronectin are ligands of $\alpha 5 \beta 1$ and αv -containing integrins (Supplementary Figure S1), suggesting that these receptors may be targeted by CC-PLA₂s. Indeed, using blocking antibodies and various cell/ECM protein pairs, we observed that both CC-PLA₂-1 and CC-PLA₂-2 inhibited $\alpha 5 \beta 1$ and αv -containing integrins, while having no effect on $\alpha 1 \beta 1$, $\alpha 2 \beta 1$ and $\alpha 6 \beta 4$. Furthermore, CC-PLA₂s are able to discriminate between αv -containing integrins, as $\alpha v \beta 3$ and $\alpha v \beta 6$ were inhibited, whereas $\alpha v \beta 5$ was not. HBMEC cells express at their cell surface similar high levels of $\alpha v \beta 3$ and $\alpha v \beta 5$, two vitronectin receptors. The fact that $\alpha v \beta 5$ is not inhibited by CC-PLA₂s may therefore explains the quasi-absence of effect on HBMEC adhesion to vitronectin. Because each cell type shows its own repertoire of integrins, the functional redundancy of these receptors could also explain the absence of PLA₂ effect reported for some cell types.^{31,32}

The inhibitory effect of CC-PLA₂s on integrin function could be either direct (eg, physical association with integrins) or indirect (through a third partner). We report in this study that integrins $\alpha v \beta 3$ and $\alpha 5 \beta 1$ are still able to interact with CC-PLA₂s in a cell-free assay, excluding any effect of PLA₂s on integrin subcellular relocalization or activation by intracellular signals. This is in agreement with a recent report

showing that secretory group IIA PLA₂s bind to $\alpha v \beta 3$ and $\alpha 4 \beta 1$ integrins and that integrin-PLA₂ interactions may mediate proinflammatory activity.³⁶

The role of $\alpha v \beta 3$ in the angiogenic process is well documented,³⁷ with $\alpha v \beta 3$ expression being the most significant alteration in angiogenic endothelial cells relative to quiescent endothelium.³ In the last decade, several clinical trials evaluating the efficacy of $\alpha v \beta 3$ blockers have led to encouraging results in cancer therapy and diagnosis. However, integrins $\alpha 5 \beta 1$ are also involved, as $\alpha 5$ -null mouse embryos showed a marked decrease in the complexity of the vasculature when compared with wild-type embryos.³⁸ The heterodimer $\alpha 5 \beta 1$ has also been shown to be expressed in growing vessels but its expression disappears in mature vessels.³⁹ Consistent with this, our results showed that CC-PLA₂s do not affect stable, established blood vessels on CAMs, but strongly reduce the number of new capillaries and vascular branching. Moreover, CC-PLA₂s do not affect the viability of stable confluent endothelial cells *in vitro*.

Thus, the antiangiogenic effect of CC-PLA₂s can, at least partly, be due to the blockage of the $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrins function. However, inhibition of angiogenesis can also result from blockage of VEGF or its receptor. Thus, it has been recently reported that inactive PLA₂ homologues, such as KDR-bp isolated from Eastern cottonmouth venom, are common antagonists of KDR, a VEGF receptor.⁴⁰ Moreover, interaction with KDR involved clusters of positively charged amino acids present in the C-terminal loop (residues 115–120) and β -wing (Lys³⁸ and Lys⁵³) regions of KDR-bp.⁴¹ However, as shown in surface electrostatic potential models (Figure 6), both CC-PLA₂-1 and CC-PLA₂-2 contain negatively charged clusters within their C-terminal loop (residues Glu¹¹⁵, Glu¹¹⁶ and Glu¹¹⁷ instead of Lys in KDR-pb) and β -wing (Asp³⁸ and Glu⁵³ instead of Lys in KDR-pb) regions. Taken altogether, this confirms that CC-PLA₂-1 and CC-PLA₂-2 likely impair angiogenesis by inhibiting integrins, in particular $\alpha v \beta 3$, rather than the VEGF receptor.

In conclusion, we show for the first time that two PLA₂s from snake venom are potent integrin inhibitors. These enzymes are able to block endothelial cell adhesion and migration by interfering with $\alpha 5 \beta 1$ and αv integrins function, resulting in a blockage of angiogenesis. It could thus be interesting to confirm whether this inhibition requires physical interaction with integrins and which are the amino-acid residues involved.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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