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Lebectin, a *Macrovipera lebetina* Venom-Derived C-type Lectin, Inhibits Angiogenesis Both In Vitro and In Vivo

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Integrins play an essential role in endothelial cell motility processes during angiogenesis and thus present interesting targets for the development of new anti-angiogenic agents. Snake venoms naturally contain a variety of proteins that can affect integrin–ligand interactions. Recently, the C-type lectin proteins (CLPs) have been characterized as efficient modulators of integrin functions. In this study, we investigated the anti-angiogenic activity of lebectin, a newly discovered CLP from *Macrovipera lebetina* venom. Human brain microvascular endothelial cells (HBMEC), used as an in vitro model, express $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrins, as well as the $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\beta 4$ subunits. Our data show that lebectin acts as a very potent inhibitor ($IC_{50} \approx 0.5$ nM) of HBMEC adhesion and migration on fibronectin by blocking the adhesive functions of both the $\alpha 5\beta 1$ and αV integrins. In addition, lebectin strongly inhibits both HBMEC in vitro tubulogenesis on Matrigel™ ($IC_{50} = 0.4$ nM) and proliferation. Finally, using both a chicken CAM assay and a Matrigel™ Plug assay in nude mice, our results show that lebectin displays potent anti-angiogenic activity in vivo. Lebectin thus represents a new C-type lectin with anti-angiogenic properties with great potential for the treatment of angiogenesis-related diseases.
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Angiogenesis is the formation of new capillaries from preexisting blood vessels (Folkman, 2004). The angiogenic process is fundamental to normal healing, reproduction and embryonic development (Zadeh and Guha, 2003). It has also been implicated in the pathogenesis of a wide variety of disorders, including primary and metastatic tumors, aneurysms, arteriovenous malformations, and cavernous malformations (Harrigan, 2003). The process of angiogenesis is complex, 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 (Folkman, 2001). Although growth factors stimulate new blood vessel growth and survival (Folkman, 1995b, 2004), adhesion to the extracellular matrix (ECM) also regulates endothelial cell survival, proliferation, and motility during new blood vessel formation (Li et al., 2003). At the molecular level, interactions between vascular cells and ECM are mainly mediated by integrins, a family of transmembrane proteins that link the ECM to the actin cytoskeleton within the cell (Hwang and Varner, 2004).

Integrins are heterodimeric glycoproteins consisting of non-covalently associating α and β subunits. To date, 24 $\alpha\beta$ heterodimers, formed by combinations of 8 β and 18 α subunits, are known (Brakebusch et al., 2002). They are involved in various processes such as development, immune response, hemostasis, and maintenance of tissue integrity. Integrins also participate in various pathological processes such as chronic inflammation, tumor invasion, and metastasis (Hynes, 1992; Hemler, 1998). In addition to their role in cell adhesion, integrins relay molecular signals from the cellular environment which influence cell shape, survival, proliferation,

Abbreviations: CLP, C-type lectin protein; HBMEC, human brain microvascular endothelial cell; ECM, extracellular matrix; CAM, chorioallantoic membrane.

These two authors (Magali CONESA and Sameh SARRAY) equally contributed to this work.

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and gene transcription (Guo and Giancotti, 2004). More recently, integrins have also been shown to be pivotal molecules in the angiogenic process (Hodivala-Dilke et al., 2003; Hwang and Varner, 2004; Stupack and Cheresh, 2004). Consequently, they represent a target of choice for anti-angiogenic therapy. Snake venoms have been shown to possess unique components able to affect cell–ECM interactions. In particular, some have been demonstrated to alter integrin adhesive properties (Chung et al., 2003; Yang et al., 2005). Among these molecules, snake venom disintegrins have been extensively studied (McLane et al., 2004). Another class of peptides, identified as C-type lectin proteins (CLPs), has also been reported to block cell–ECM interactions (Sarray et al., 2001, 2004). CLPs represent a large family of Ca^{++}-dependent lectins that share primary structural homology in their carbohydrate-recognition domains (Morita, 2004). Snake venom CLPs are proteins of about 30 kDa, comprised of two associated subunits, which display varied, distinct biological activities (Goldstein and Hayes, 1978; Ogawa et al., 2005). CLPs were first characterized for their role in affecting immune system functions, such as inflammation and immunity against tumors and virally infected cells. Several CLPs have also been shown to strongly modulate the platelet aggregation process via the interactions between von Willebrand factor and platelet GPIb (Peng et al., 1993; Yoshida et al., 1993; Yeh et al., 2000; Tai et al., 2004). However, their role in angiogenesis, through their anti-integrin properties, remains to be elucidated.

The ability of some *Macrovipera lebetina* venom-derived CLPs to inhibit tumor cell migration through their anti-integrin properties has already been demonstrated (Sarray et al., 2001, 2004). Consequently, we investigated how lebectin, a previously characterized CLP from *M. lebetina* venom (Sarray et al., 2004), can modulate the angiogenic properties of endothelial cells in vitro and in vivo. As an in vitro model, we used human brain endothelial cells (HBMEC) to represent this important therapeutic target for the specific treatment of brain tumors which do not respond to conventional treatments, such as chemotherapy and radiotherapy. We compared the anti-angiogenic effects of lebectin with those of lebecetin, another *M. lebetina* venom-derived CLP (Sarray et al., 2001, 2003). Our results strongly suggest that lebectin is a potent inhibitor of both in vitro and in vivo angiogenesis. Lebectin could thus be used as an anti-angiogenic agent in the treatment of angiogenesis-related diseases.

Materials and Methods

Chemicals

Venom was collected from *M. lebetina* snakes in the serpentarium of the Institut Pasteur, Tunis. Lebectin and lebecetin were purified as previously described (Sarray et al., 2001, 2003, 2004). Trypsin, penicillin, and streptomycin were from Invitrogen (Burlington, ON). Fibronectin was from Roche (Mississauga, ON). Type-I collagen was extracted from rat tail tendon by a standard method (Silver and Trelstad, 1980). Vitronectin was prepared as previously described (Yatohgo et al., 1988). EDTA, laminin-1, sodium dodecyl sulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Matrigel™ was from BD Biosciences Labware (Bedford, MA).

Cells and culture media

RPMI 1640 medium was from Gibco (Burlington, ON) and heat-inactivated fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT). Immortalized human brain microvascular endothelial cells (HBMEC) were maintained in RPMI 1640, 10% heat-inactivated FBS, 10% NuSerum (BD Biosciences Labware), endothelial cell growth supplement (ECGS) (30 μg/ml), heparin (5 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids, vitamins, penicillin, and streptomycin (100 U/ml). Cells were incubated at 37°C, air/CO_2 (95%/5%).

Flow cytometry analysis

Cell surface expression of integrin subunits on HBMEC was determined by flow cytometry as described (Rigot et al., 1998). As a control, non-specific immunoglobulin G (IgG) was used for each experiment. To standardize our experimental conditions, all samples were prepared using the same cell suspension. Rat mAb 69.6.5, against αv integrin, was produced as previously described (Lehmann et al., 1994). Mouse mAbs Gi9 (anti-α2), C3VLA3 (anti-α3), HP2/1 (anti-α4), Lia1/2 (anti-β1), SZ22 (anti-αIIb), and rat mAb GoH3 (anti-α6) were from Immunotech (Marseille, France). Mouse mAbs FB12 (anti-α1), P1D6 (anti-α5), JBS5 (anti-α5β1), LM609 (anti-αvβ3), P1F6 (anti-αVβ5), 10D5 (anti-αVβ6), and 3E1 (anti-β4) were from Chemicon (Temecula, CA). Goat anti-mouse FITC-conjugated antibodies were obtained from Jackson Immunoresearch (West Grove, PA). Sheep anti-rat FITC-conjugated antibodies were from Sigma (St. Louis, MO).

Covalent crosslinking and co-immunoprecipitation

HBMEC cell monolayers were incubated with 50 μg/ml of lebectin or lebecetin in RPMI containing 15 mM HEPES, pH 7.3, and 0.1% BSA. After 5 h at 4°C, cells were washed twice with ice-cold PBS. Two milliliters PBS were added to cells and the reaction was initiated by adding 40 μl 100 mM DSP, dissolved in dimethylsulfoxide just before use. The reaction was performed at room temperature and was stopped 20 min later by the addition of 1 ml PBS containing 60 mM ammonium acetate. Cells were rinsed twice with PBS and then lysed in 1 ml 50 mM Tris-HCl pH 8, 200 mM NaCl, and 1% Triton X100 (RIPA buffer) containing 0.5% BSA and a mixture of proteinase inhibitors (1 mM PMSF, 500 U/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 1 mM iodoacetamide, and 1 mM ortho-phenantrolin). Clarified cell lysates were incubated with 5 μl of anti-lebecetin antiserum (which recognizes both lebecetin and lebectin) overnight at 4°C. After addition of protein G-agarose beads and incubation for 1 h, the suspension was centrifuged, and pellets were washed three times with RIPA buffer, three times with RIPA buffer/500 mM NaCl, and once with PBS. Immunoprecipitated proteins were solubilized in Laemmli sample buffer, heated at 100°C for 5 min, and submitted to SDS–PAGE under reducing conditions. The gel was then blotted onto a nitrocellulose sheet and probed overnight at 4°C with anti-αv or anti-α5 antibodies (1:1,000). Blots were then revealed by a light-based ECL system using HRP-conjugated secondary antibodies.

Cell adhesion assays

Adhesion assays were performed as previously described (Rigot et al., 1998). Briefly, 96-well plates were coated with purified ECM protein solutions for 2 h at 37°C and blocked with a solution of PBS/0.5% BSA. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, added to precoated wells, and allowed to adhere to the substrata for 2 h at 37°C in serum-free medium. After washing, adherent cells were fixed with 3.7% formaldehyde, stained with a solution of 0.1% crystal violet/MetOH 20%, and lysed with 1% SDS. Absorbance was then measured at 600 nm. For adhesion assays on antibodies, 96-well plates were coated with 50 μl of rabbit anti-rat IgG (50μg/ml) overnight at 4°C. Wells were washed once with PBS and 50 μl of anti-integrin blocking antibodies were added for 5 h at 37°C. Antibodies were used as follow: anti-α3 (2.5 μg/ml), anti-α5 (1/200), anti-α6 (10 μg/ml), anti-αv (10 μg/ml), anti-αvβ3 (10 μg/ml), and anti-β1 (10 μg/ml). Wells were then blocked with a solution of PBS/0.5% BSA. Adhesion assays were performed as above, except that HBMEC cells were pretreated with lebectin (10 μg/ml) for 30 min at room temperature and allowed to adhere for 45 min at 37°C.

Haptotaxis migration assays

The lower face of Transwells (8 μm pore size; Costar, Acton, MA) was precoated with type I collagen, laminin-1, vitronectin, or fibronectin, at a concentration of 10 μg/ml, for 2 h at 37°C. The Transwells were then assembled in a 24-well plate (Falcon), the lower chambers were filled with 600 μl of serum-free medium, and 100 μl of HBMEC (5×10^5 cells/ml) were inoculated into the upper chamber of each well. The plate was then placed at 37°C in 95% air and 5% CO_2 for 2 h. Cells which migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/20% methanol (v/v). Five random microscopic fields were photographed at a magnification of 200× and counted using the Northern Eclipse analysis software (Empix Imaging, Inc., Mississauga, ON).

Morphogenic differentiation assay

Matrigel™ was plated in 96-well plates and allowed to gel for 2 h at 37°C prior to cell seeding. Cells were harvested as a single cell suspension by treatment with 0.53 mM EDTA in PBS pH 7.2, at a final concentration of 10^6 cells/ml. HBMEC cells (2×10^4) were added atop the Matrigel™ in serum-free media after a 30 min pretreatment with either lebectin or lebecetin, and incubated at 37°C for 18 h. Capillary-like structures were analyzed microscopically and photographs (50×) taken with a Retiga 1300 camera connected to a Nikon Eclipse TE2000-U microscope. The capillary-like structures formed in the gel were quantified by analysis of digitized images to determine the segment length of the capillary-like network, using Northern Eclipse analysis software (Empix Imaging, Inc.). Results are expressed as a percentage of control cells.

Cell proliferation assay

HBMEC cells were seeded at 1.5×10^4 cells/cm^2 in the presence or absence of 5 μg/ml lebectin or lebecetin. At daily intervals, some HBMEC cells were fixed with 3.7% formaldehyde, stained with a solution of 0.1% crystal violet/MetOH 20%, and lysed with 1% SDS. Absorbance was then measured at 600 nm. All assays were performed in triplicate and data are the means of at least three independent experiments.

Chicken CAM assay

The chicken chorioallantoic membrane (CAMs) were prepared using 8-day-old chick embryos. Filter disks were soaked in 0.9% NaCl alone (control) or containing 0.2 μg of lebectin or 10 μg of lebecetin. After incubation for 72 h, CAMs were photographed with a digital camera. To quantify CAM angiogenesis, we measured the total length of vessels per photographic field. All experiments used five CAM and data are the means of three independent experiments.

In vivo Matrigel™ plug assay

All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, Montréal, QC, Canada). The Matrigel™ implantation assay was based on the method of Passaniti et al. (1992) with some modifications. Prior to injection, heparin was incubated with or without fibroblast growth factor-2 (FGF-2) for 5 min then diluted in phenol red-free Matrigel™ on ice at a final concentration of 0.0025 U/ml heparin and 250 ng/ml FGF-2. Lebectin, lebecetin, or water control alone were then added and the samples were kept on ice until injection. Crl:CD-1® -nuBR nude mice (Charles River Laboratories, Lassalle, QC, Canada), between 5 and 10 weeks old, were subcutaneously injected under anesthesia in the ventral midline region of the right flank with 0.5mL of Matrigel™ alone or Matrigel™ containing FGF-2 with or without lebectin or lebecetin (5 μg). After 7 days, the mice were euthanized and the Matrigel™ implant harvested, washed with PBS, and examined macroscopically for signs of hematoma. Any implant with visible signs of hematoma was excluded from analysis. The remaining implants were immediately frozen and lyophilized overnight. The weight of the dry Matrigel™ implant was determined and the implants were resuspended in 0.4 ml of 0.1% Triton X-100 for 1 h, disrupted by vigorous pipetting, and centrifuged at 14,000*g* for 15 min to remove particulates. The concentration of hemoglobin in the supernatant was then directly determined by absorbance at 405 nm and compared with a standard curve of purified hemoglobin (Sigma-Aldrich, Oakville, ON) as previously described (McMahon et al., 2001).

Statistical analysis

Results are expressed as mean ± SEM and analyzed with Student's *t*-test. Only significant differences ($P < 0.05$) are indicated in the figures.

Results

Basal motility properties of HBMEC

HBMEC have been previously used to study the *Escherichia coli* invasion of brain microvascular endothelial cells or as an in vitro model for lymphocyte transendothelial migration (Prasadarao et al., 1999; Reddy et al., 2000). However, the intrinsic motility properties of HBMEC have not been characterized to date. Consequently, we first determined the basal adhesive properties of HBMEC on various purified ECM proteins. As shown in Figure 1A, HBMEC firmly adhere to fibronectin and vitronectin but do not adhere to type I collagen. Moreover, these cells display a strong adhesion to laminin-1, but only at high matrix concentrations (over 5 μg/ml). We considered fibronectin to be the optimal choice of adhesion substrate for HBMEC because, at a low fibronectin concentration (1 μg/ml), the cells already display full adhesion to this ECM protein. In parallel, we used modified Boyden chambers to characterize the migratory properties of HBMEC towards various ECM purified proteins, including type I collagen, fibronectin, vitronectin, and laminin-1. In agreement with the adhesion results, HBMEC displayed a high rate of migration towards fibronectin, vitronectin, and laminin-1 at the concentration used (10 μg/ml). However, HBMEC did not migrate towards type I collagen (Fig. 1B).

Integrin expression in HBMEC

Amongst the better characterized groups of cell surface adhesion receptors are integrins. These transmembrane proteins play a crucial role during cell motility processes such as

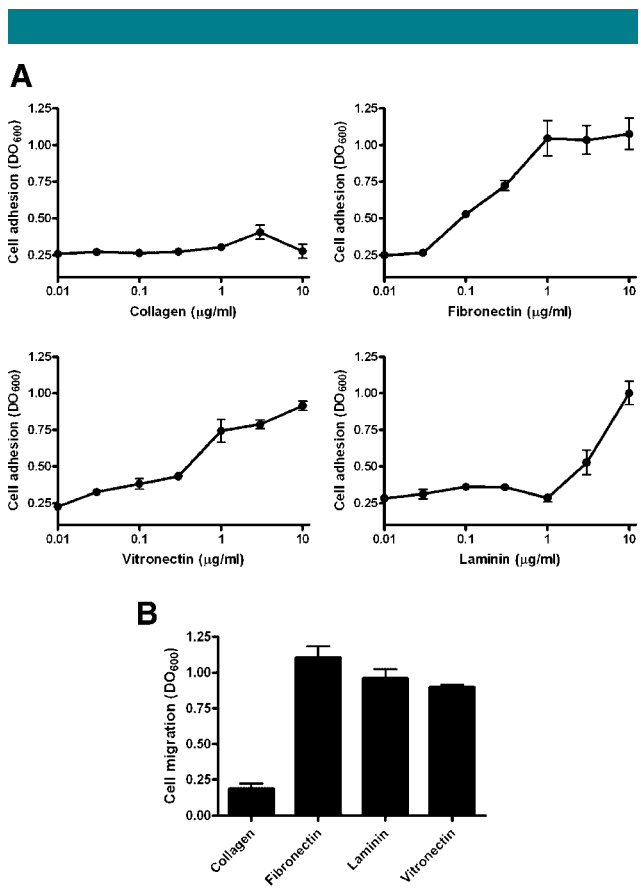

Fig. 1. Basal motility properties of HBMEC. A: Cells (5×10^4) were seeded on 96-well plates previously coated with various concentrations of different, purified ECM proteins, and allowed to adhere for 2 h at 37°C. Cells which adhered were fixed, stained, and absorbance at 600 nm was measured. B: Haptotaxis assays were performed as described in the Materials and Methods. The lower faces of the Transwells were previously coated with either type I collagen, fibronectin, laminin-1, or vitronectin. Cells that migrated to the underside were fixed, stained, and counted. Bar graph represents a mean quantification ± SEM of at least three independent experiments.

cell adhesion and migration. By flow cytometric analysis, we identified the main integrins expressed at the HBMEC cell surface. As shown in Figure 2, HBMEC express high levels of the classical endothelial integrins (αVβ3, αVβ5, and α5β1), which are the major receptors for fibronectin and vitronectin in endothelial cells. They also express a large set of other integrin subunits such as α2, α3, α6, β1, and β4. However, α1, α4, αIIb, and αVβ6 integrins were not detected by FACS analysis, suggesting that their expression levels in HBMEC are low or nonexistent.

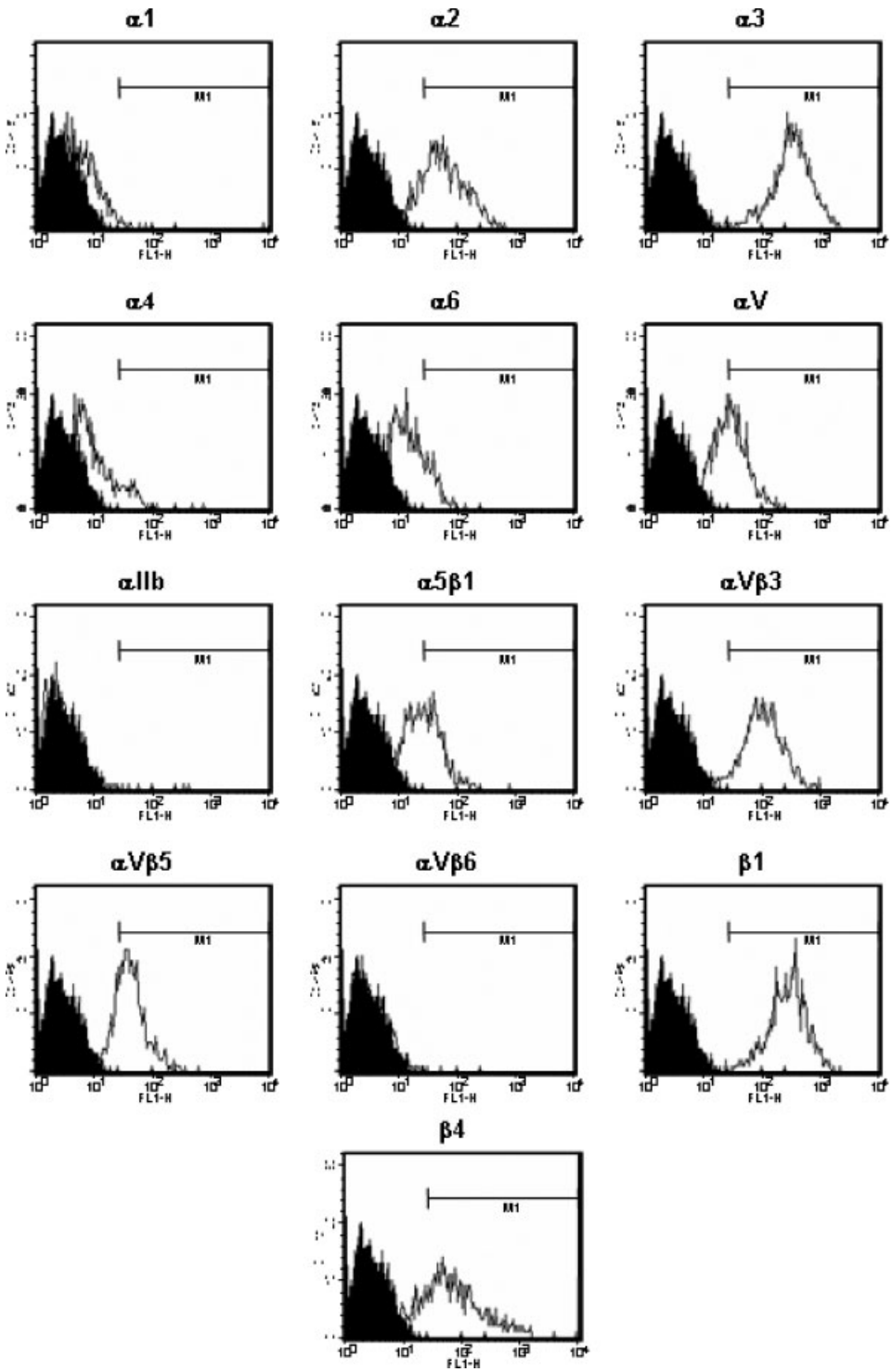

Fig. 2. Expression of integrin subunits at the HBMEC surface. Flow cytometry analysis was performed using various antibodies directed against specific integrin subunits as described in the Materials and Methods. Analysis was repeated three times.

Lebectin inhibits HBMEC adhesion by blocking the adhesive functions of both α5β1 and αVβ3 integrins

We previously reported that lebectin can inhibit tumor cell adhesion on various substrates by impairing cell/ECM interactions through an integrin-dependent mechanism (Sarray et al., 2004). Consequently, we tested the effects of various concentrations of lebectin against HBMEC cell adhesion to fibronectin and vitronectin. Low concentrations of lebectin strongly inhibited cell adhesion on fibronectin but did not affect the adhesive properties of HBMEC cells to vitronectin (Fig. 3A). However, at a high concentration of lebectin (50 μg/ml), HBMEC cell adhesion to vitronectin was also impaired (Fig. 3A). In parallel, we investigated the effect of lebecetin, another related *M. lebetina* venom-derived CLP (Sarray et al., 2003), under the same conditions. We observed that the inhibitory effect of lebecetin on HBMEC adhesion to fibronectin is less than that of lebectin (Fig. 3A). The IC_{50} of lebecetin-mediated inhibition of HBMEC adhesion to fibronectin is ≈600-fold higher than the IC_{50} of lebectin. More precisely, the IC_{50} values for inhibition of HBMEC adhesion on fibronectin for lebectin and lebecetin are, respectively, 0.016 μg/ml (0.52 nM) and 10.5 μg/ml (350 nM). In contrast, HBMEC adhesion on vitronectin is unaffected by lebecetin, even at high concentrations (Fig. 3A).

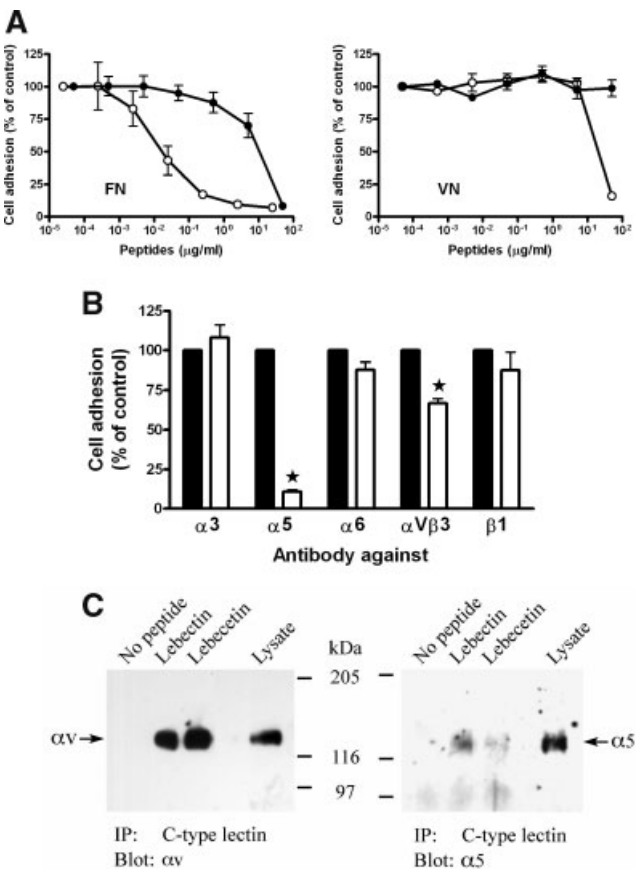

Fig. 3. Lebectin inhibits HBMEC adhesion by blocking α5β1 and αVβ3 adhesive functions. A: HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. Cells (5×10^4) were then allowed to adhere to fibronectin (FN) or vitronectin (VN) as described in Figure 1. B: HBMEC (10^6 cells/ml) were treated (white bars) or not (black bars), with 10μg/ml of lebectin for 30 min at room temperature and allowed to adhere on various anti-integrin antibodies. Adhesive cells were fixed, stained, and absorbance at 600 nm was measured. ★ indicates statistically significant differences ($P < 0.05$) compared with the control assay using Student's *t*-test. C: HBMEC (10^6 cells/ml) were incubated with 50 μg/ml of lebectin or lebecetin for 5 min, lysed and the peptides were co-immunoprecipitated using an anti-lebecetin antiserum. α5 and αV integrins were detected by Western blot.

HBMEC adhere on fibronectin through α5β1 integrin and on vitronectin mainly through αVβ3 integrin (Isogai et al., 2001). Because lebectin inhibits HBMEC adhesion on both fibronectin and vitronectin, we hypothesized that this C-type lectin could interact with α5β1 and αVβ3. To address this issue, HBMEC were treated with lebectin for 30 min and allowed to adhere on various anti-integrin antibodies. At 10 μg/ml, lebectin does not modify HBMEC adhesion on anti-α3, anti-α6, or anti-β1 integrin antibodies (Fig. 3B). However, HBMEC adhesion on an anti-α5 integrin antibody was almost completely inhibited (90%), which demonstrates that lebectin can efficiently block the adhesive functions of the α5β1 integrin (Fig. 3B). We also tested the effect of lebectin on the HBMEC adhesion on an anti-αVβ3 antibody. Our results show that lebectin decreases HBMEC adhesion by 33% on this specific antibody (Fig. 3B). These results demonstrate that lebectin can inhibit the adhesive properties of both α5β1 and αVb3 integrins in HBMEC but displays a stronger effect against α5β1 integrin.

In order to ascertain whether the venom peptides block HBMEC adhesion through a specific interaction with integrins, HBMEC were treated with either lebectin or lebecetin for 5 h. Venom peptides were co-immunoprecipitated and separated by SDS–PAGE. α5 and αV integrins were then detected by Western blot. As shown in Figure 3C, both peptides interacted with α5 and αV integrins since both are co-immunoprecipitated. It should be noted that the interaction of lebecetin with αV integrin was greater than for lebectin, despite the stronger effect of lebectin on αV-mediated HBMEC adhesion on vitronectin. In contrast, lebectin interacted much strongly with α5 than did lebecetin. This interaction pathway may explain the stronger effect of lebectin on α5-mediated HBMEC adhesion on fibronectin, compared to lebecetin.

Lebectin inhibits HBMEC migration on fibronectin

Cell migration can be considered as a finely regulated process including successive steps of cell adhesion and deadhesion. Because lebectin potently blocks HBMEC cell adhesion on fibronectin, we also examined its effects on cell migration towards the same substrate. We tested the ability of various concentrations of lebectin to inhibit integrin-dependent migration of HBMEC cells using haptotaxis assays towards fibronectin in modified Boyden chambers. Lebectin readily inhibited HBMEC cell migration (Fig. 4A). Furthermore, lebectin again exhibited a stronger inhibitory effect than did lebecetin (Fig. 4A,B). More precisely, the IC_{50} values for inhibition of HBMEC migration on fibronectin by lebectin and lebecetin, are, respectively, 0.015 μg/ml or 0.49 nM, and 26.1 μg/ml or 870 nM (Fig. 4B).

Lebectin blocks HBMEC tubulogenesis

Cell adhesion and migration are integral parts of the angiogenic process and affect tubulogenesis. Since HBMEC tubulogenesis can be inhibited by specific anti-αV and anti-α5 antibodies (data not shown), we hypothesized that lebectin-mediated impairment of HBMEC adhesion and migration could therefore affect tubulogenesis. HBMEC were treated with various concentrations of venom peptides and in vitro tubulogenesis assays were performed on Matrigel™. As shown in Figure 5A,B, lebectin strongly inhibited in vitro tubulogenesis. As previously shown for adhesion and migration assays, we observed stronger inhibition by lebectin ($IC_{50} = 0.012$ μg/ml or 0.39 nM) than by lebecetin ($IC_{50} = 2.45$ μg/ml or 80 nM) (Fig. 5B).

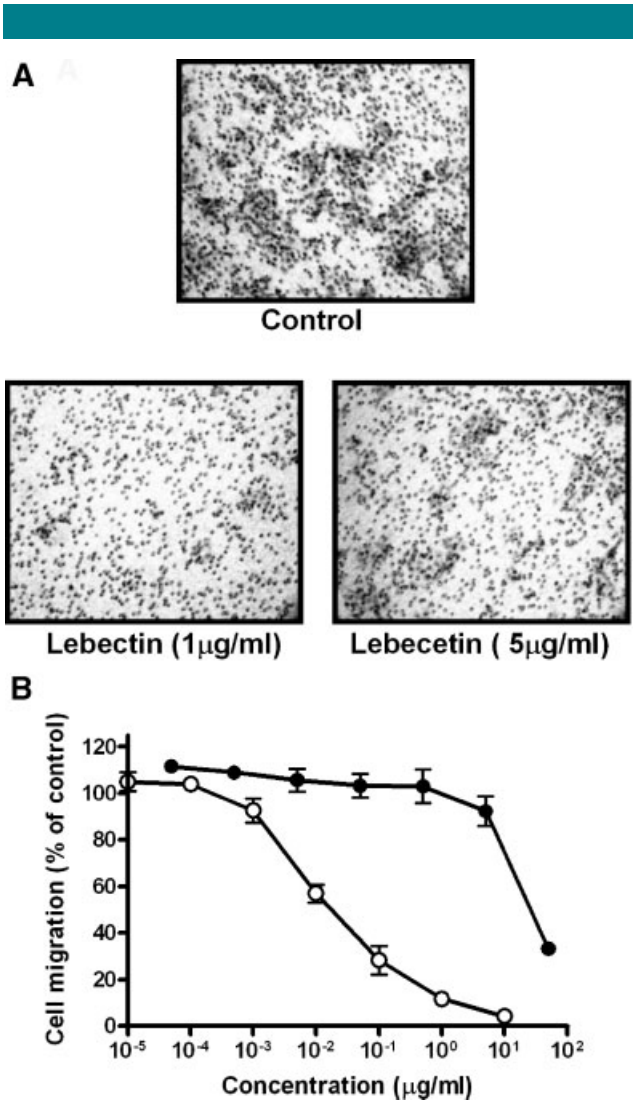

Fig. 4. Lebectin inhibits HBMEC migration. HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. HBMEC (5×10^4 cells) were seeded on top of fibronectin-coated Transwells and allowed to migrate for 2 h at 37°C. A: Representative data are shown. B: Cells that had migrated were fixed, stained, and counted as described in the Materials and Methods. Graph represents a mean quantification ± SEM of at least three independent experiments. Results are expressed as percentage of control cells.

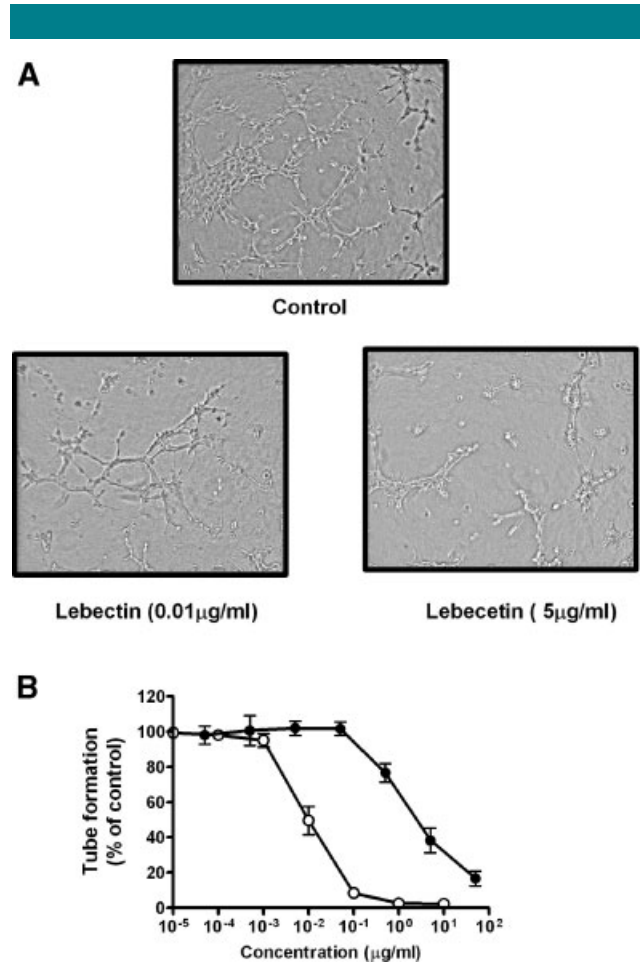

Fig. 5. Lebectin strongly inhibits HBMEC tubulogenesis on Matrigel. HBMEC (10^6 cells/ml) were treated with various concentrations of lebectin (open circles) or lebecetin (closed circles) for 30 min at room temperature. Morphogenic differentiation assays were performed as described in the Materials and Methods. Cells (5×10^4) were added to the Matrigel™-coated wells in serum-free medium and allowed to form capillary-like structures for 18 h at 37°C. A: A representative tubulogenesis assay is shown after treatment of HBMEC with varying concentrations of lebectin or lebecetin. B: The capillary-like structures formed in the gel were quantified and a mean quantification is presented. Graphs represent a mean quantification ± SEM of at least three independent experiments.

Lebectin inhibits HBMEC proliferation

In order to stabilize new vasculature during the angiogenic process, endothelial cells need to actively proliferate. Because lebectin strongly inhibited HBMEC angiogenesis in vitro, we investigated its effect on HBMEC proliferation. As expected, lebectin strongly inhibited HBMEC proliferation during a 5-day period (Fig. 6). Furthermore, lebectin again presented a greater inhibitory effect than did lebecetin. After 5 days, lebecetin inhibited HBMEC proliferation by 56% whereas lebectin inhibited HBMEC proliferation by 84% (Fig. 6). The apoptotic state of HBMEC cells in the presence or absence of peptides was tested after 4 days using the PI/Annexin-5 assay. The results of this assay were negative (data not shown). This suggests that the lower number of cells observed in wells containing peptides is not due to greater cell death.

Lebectin displays anti-angiogenic activity in vivo

To pursue further the characterization of the anti-angiogenic properties of lebectin, we performed two different in vivo angiogenesis assays. First, we looked at the effects of lebectin on angiogenesis, using a chicken CAM assay. Lebectin (0.2 μg) strongly reduced vasculature development (Fig. 7A). The total vessel length was reduced by 51% compared to the untreated conditions. A strong reduction in the number of new capillaries and branching, without affecting mature blood vessels, was also observed (Fig. 7A). This strongly suggests an anti-angiogenic activity of lebectin in vivo. In accordance with our in vitro results, the lebecetin-mediated inhibition of angiogenesis was less than that seen with lebectin, with only 31% inhibition, even though the concentration used was 50-fold higher.

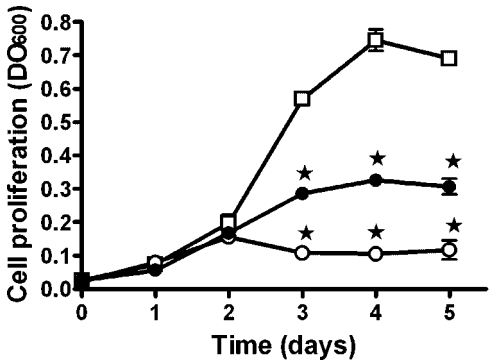

Fig. 6. Lebectin reduces HBMEC proliferation. HBMEC (2000 cells/well) were allowed to grow in 96-well plates for 5 days in 10% serum-containing medium with various lebectin (open circles) or lebecetin (closed circles) concentrations. As a control (open squares), cells were incubated with 10% serum-containing medium alone. Each day, three wells were washed twice with PBS, fixed, and stained. Absorbance at 600 nm was then measured. ★ indicates statistically significant differences ($P < 0.05$) compared with the control assay using Student's *t*-test.

In order to test the ability of lebectin to antagonize FGF-2-induced angiogenesis, we performed Matrigel™ plug assays in nude mice. To quantify the angiogenic index, we measured the levels of hemoglobin in the Matrigel™ plugs, as previously described (McMahon et al., 2001). FGF-2-containing Matrigel™ plugs appeared to be highly vascularized, compared to control plugs (Fig. 7B). More precisely, we measured a sevenfold higher amount of hemoglobin in the FGF-2-containing plugs. When lebectin was added to FGF-2-containing plugs, a strong decrease in vascularization was observed. When hemoglobin was measured, we observed a decrease of 43% in plug vascularization. This result clearly shows that lebectin can antagonize FGF-2-mediated angiogenesis in vivo. The effect of lebecetin on the FGF-2-induced angiogenic process was also tested. As shown by Figure 7B, no effect of lebecetin could be observed on the induced angiogenesis when lebecitin was present at the same concentration as was used for the peptide (5 μg). Consequently, we can conclude that lebectin, but not lebecetin, is able to block normal as well as induced angiogenesis in vivo.

Discussion

Angiogenesis is implicated in numerous diseases such as rheumatoid arthritis, psoriasis, and cancer (Folkman, 1995a). Therefore, the characterization of new anti-angiogenic agents is of considerable utility for the development of anti-angiogenic therapies. Numerous active molecules have been discovered in the plant and animal kingdoms and studied for potential use in medicine. Most notably, it has been shown that several snake venom-derived peptides can affect the angiogenic process. Among these peptides, disintegrins have been widely characterized (McLane et al., 2004). For instance, it has been shown that disintegrins derived from the venoms of *Agkistrodon contortrix* (contortrostatin) (Golubkov et al., 2003), *Agkistrodon halys brevicaudus stejneger* (Adinbitor) (Wang et al., 2004), or *Vipera lebetina obtusa* (Obtustatin) (Marcinkiewicz et al., 2003) exhibit anti-angiogenic activities. In the present study, we describe the anti-angiogenic activity of a newly discovered CLP, lebectin, derived from *M. lebetina* snake venom.
Our data demonstrate that lebectin displays anti-angiogenic activity at very low concentrations, both in vitro and in vivo.

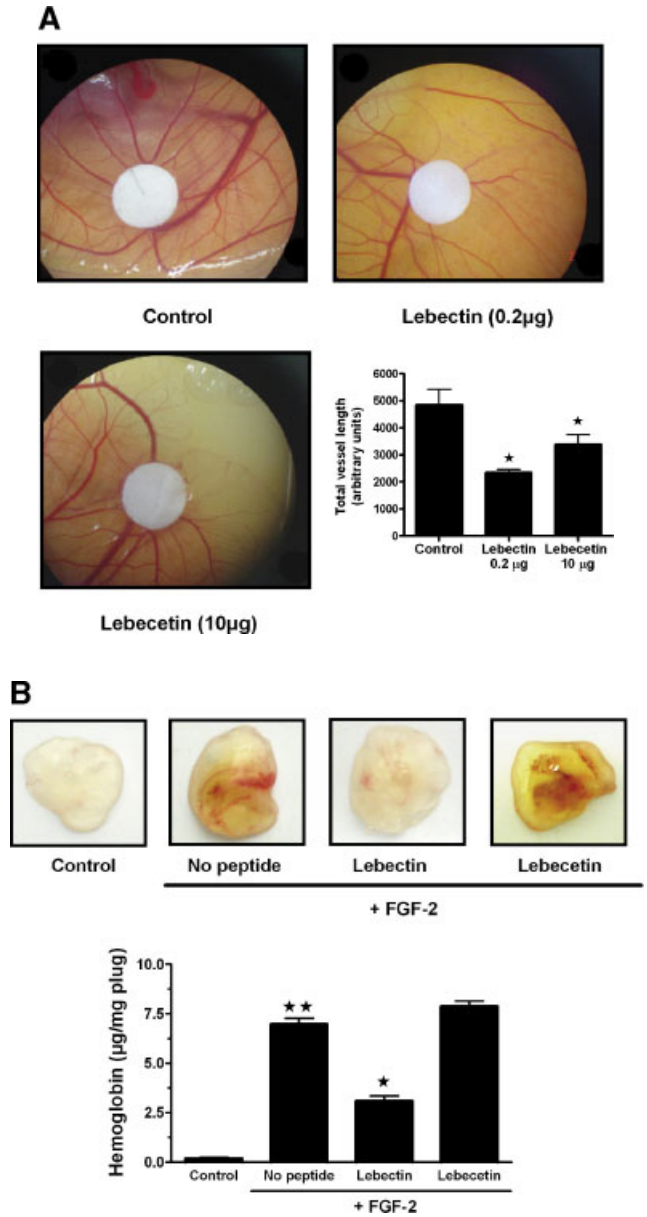

Fig. 7. Lebectin blocks in vivo angiogenesis. Anti-angiogenic properties of lebectin were monitored in vivo by CAM assays and Matrigel™ plug assays in nude mice. A: Chicken CAM were untreated (control) or treated with lebectin (0.2 μg) or lebecetin (10 μg) and a quantitative measurement of vessel density was performed by counting vessel length per embryo. B: Crl:CD-1®-nuBR mice were injected subcutaneously with 0.5 ml of Matrigel™ alone or with Matrigel™ containing FGF-2 (125 ng) with or without lebectin or lebecetin (5 μg). After 7 days, the mice were euthanized and the Matrigel™ plugs excised. Representative Matrigel™ plugs from each of the different conditions were photographed. Neovessel formation was quantified by measurement of hemoglobin in the Matrigel™. Two mice were used per group and the experiment was repeated twice. The data are presented as mean ± SEM. Statistically significant differences, as compared to control FGF-2 conditions (as well as between the two control conditions), are indicated by ★ for $P < 0.05$; ★★ for $P < 0.001$ (Student's t-test). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Lebectin, used at a concentration less than 1 nM, efficiently inhibits several key steps of the angiogenic process in vitro, such as endothelial cell adhesion, migration, and tubulogenesis. Moreover, our results clearly demonstrate that low concentrations of lebectin efficiently block angiogenesis in vivo in two different assays. Thus, lebectin presents the best anti-angiogenic efficacy yet described for snake venom-derived peptides (Golubkov et al., 2003; Marcinkiewicz et al., 2003).

We characterized integrin expression at the HBMEC cell surface by flow cytometry. In addition to the classical endothelial integrins ($\alpha V\beta 3$, $\alpha V\beta 5$, and $\alpha 5\beta 1$), we also demonstrated that $\alpha 6$ and $\beta 4$ are expressed in HBMEC. Because the $\beta 4$ subunit only interacts with the $\alpha 6$ integrin subunit, it seems likely that the heterodimer $\alpha 6\beta 4$ is present at the HBMEC cell surface as a laminin receptor. Moreover, the high expression of $\alpha 2$, $\alpha 3$, and $\beta 1$ indicates the presence of $\alpha 2\beta 1$ and $\alpha 3\beta 1$, which could also participate in adhesion to laminin or to various collagens.

In a previous study, we hypothesized that lebectin acts through the blocking of integrin-dependent processes involving the $\alpha 5\beta 1$ integrin (Sarray et al., 2004). Our present data confirm that low concentrations of lebectin interacts with and impairs $\alpha 5\beta 1$-mediated HBMEC adhesion to fibronectin, as well as $\alpha V\beta 3$-mediated HBMEC adhesion to vitronectin. We clearly observed interaction between lebectin and both $\alpha 5\beta 1$ and αV integrins. Despite the fact than lebectin can interact with both $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins, it exhibits a higher affinity for $\alpha 5\beta 1$ integrin than for $\alpha V\beta 3$ integrin since lower concentrations of lebectin are needed to inhibit the $\alpha 5\beta 1$ adhesive properties. This was confirmed by the finding that lebectin readily inhibited cell adhesion to anti-$\alpha 5\beta 1$ antibodies while only partially affecting adhesion to anti-$\alpha V\beta 3$ antibodies.

The co-immunoprecipitation results clearly demonstrated that lebecetin interacts more strongly with αV integrin than does lebectin. Contrary to this, lebectin interacts more strongly with $\alpha 5$ integrin subunits than does lebecetin. Therefore, it would be expected that lebecetin would have a greater inhibitory effect on αV-mediated HBMEC cell adhesion on vitronectin than lebectin, and that lebectin would display a stronger inhibitory effect on $\alpha 5$-mediated HBMEC adhesion on fibronectin. Our data clearly demonstrated that lebectin actually blocked HBMEC cell adhesion and migration on vitronectin better than did lebecetin. The structural differences between these two CLPs might explain these differences since lebectin presents a homodimeric structure whereas lebecetin displays a heterodimeric structure. In fact, the specific structure of lebecetin could favor interaction with specific integrins at the endothelial cell surface without affecting their functions. A full study of the structural domains of lebectin and lebecetin, which interact with integrins at the cell surface, could thus explain the different interaction potentials and anti-angiogenic activities of these two CLPs. The interaction between lebectin and integrins still remains to be clarified and its characterization is currently in progress in our lab.

$\alpha 5\beta 1$ has already been reported to play a critical role in the development of new blood vessels. One study demonstrated that $\alpha 5$-null mouse embryos displayed a marked decrease in the complexity of the vasculature than was seen in wild-type embryos (Francis et al., 2002). The heterodimer $\alpha 5\beta 1$ has also been shown to be expressed in growing vessels but its expression disappears in mature vessels (Milner and Campbell, 2002). Recent work revealed the reexpression of $\alpha 5\beta 1$ integrin in tumor vessels, which was not present in stable blood vessels in adult (Kim et al., 2000). Because low concentrations of lebectin inhibit angiogenesis through the blockade of the $\alpha 5\beta 1$ integrin, lebectin should thus preferentially affect endothelial cells in growing vessels, which actively participate in the tumoral angiogenic process. Our results showed that lebectin does not affect stable, established blood vessels on CAMs but strongly reduces the number of new capillaries and vascular branching. Moreover, lebectin does not affect the viability of stable confluent endothelial cells in vitro (data not shown). Consequently, low concentrations of lebectin may specifically target activated endothelial cells and block neoangiogenesis, without affecting stable vasculature.

Previous results demonstrated the anti-tumoral potential of lebectin (Sarray et al., 2004). Remarkably, the concentrations of lebectin used to block tumor cell adhesion (over 10 μg/ml) are about 100-fold higher than the concentrations needed to display anti-angiogenic activity ($\approx$0.1 μg/ml). Thus, endothelial cells seem to be more sensitive to lebectin than are various tumor cells, suggesting that lebectin is a better anti-angiogenic agent than an anti-tumoral one. This may be explained by the higher expression of $\alpha 5\beta 1$ and $\alpha V\beta 3$ integrins in endothelial cells compared to tumoral cells. Lebectin could thus be very useful for the specific treatment of brain tumors. Conventional therapies, such as chemotherapy and radiotherapy, fail to strongly affect brain tumor cells due to the presence of the blood–brain barrier and the intrinsic multidrug-resistant phenotype of these type of tumors (Graham and Cloughesy, 2004; Matsutani, 2004). Anti-angiogenic therapies have been presented as a new hope for the treatment of brain tumors (Purow and Fine, 2004a,b). According to our present results, we can hypothesize that lebectin could be used with high efficiency for the inhibition of the brain angiogenic process. Therefore, it is conceivable that lebectin could open new avenues for the treatment of brain tumors.

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