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Angiostatic activity of obtustatin as $\alpha 1\beta 1$ integrin inhibitor in experimental melanoma growth

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Abstract

The presented results show the effect of targeting of collagen receptor, $\alpha 1\beta 1$ integrin expressed on the endothelial cells on the development of experimental melanoma and pathological angiogenesis. Obtustatin, a snake venom KTS-disintegrin, was applied as a specific inhibitor of this integrin. This low molecular weight peptide revealed a potent therapeutic effect on melanoma progression in two animal systems, mouse and quail. Its oncostatic effect was related to the inhibition of angiogenesis. Obtustatin inhibited the neovascularization ratio on the CAM embryo of quail, which was pathologically induced by the developing tumor. The i.v. administration of obtustatin completely blocked cancer growth of MV3 human melanoma in nude mice. In B16F10 syngeneic mouse model treatment with the disintegrin revealed a lower effect, although the development of the tumor was significantly reduced for both dosages. The mechanism of obtustatin action is related to the blocking of microvascular endothelial cell proliferation, which undergoes apoptosis in caspase-dependent manner. Summarizing, we present studies of low molecular weight disintegrin, obtustatin as a potential therapeutic compound for treatment of melanoma that contain a high level of vascularization.

Keywords

angiogenesis; melanoma; integrins; disintegrins; apoptosis

Angiogenesis, a neovascularization process is in the focus of research of many laboratories working on the development of anti-cancer pharmacological strategies. The studies proposing targeting the highly developed vasculature in the tumoral tissue were initiated in the laboratory of J. Folkman¹. Since this time, angiogenesis research achieved significant attention by exploration of the basic mechanisms that are involved in this process, as well as providing an attractive target for drug discovery². The very rapid and extensive vascularization process during a tumor progression results in the creation of pathological vessels that significantly differ from the vasculature in normal tissue^{3,4}. In general these vessels may be characterized as immature, tortuous and hyperpermeable formed by improperly organized endothelial cells. Endothelial cells intensively proliferate and migrate during tumoral angiogenesis and cell surface receptors play a significant role in this process. The collagen receptor, $\alpha 1\beta 1$ integrin was shown as an interesting target on the cellular membrane for blocking pathological vessel growth and tumor progression^{5,6}.

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Integrins are a family of receptors expressed on the cell surface, which adhere to multiple ligands and mediate cell-cell and cell-extracellular matrix interaction. These transmembrane glycoproteins are composed of two non-covalently linked α and β subunits^{7,8}. Among them, the collagen receptors are the functional subclass, specifically interacting with various types of collagens. The $\alpha 1$, $\alpha 2$, $\alpha 10$ or $\alpha 11$ subunits of collagen receptors require to be associated with $\beta 1$ subunit for creation of the active complex on the cell surface. All of these α subunits contain the I-domain, which includes approximately 200 amino acid residues, and is localized near the amino-terminal part of this subunit^{9,10}. This domain is highly conserved and plays an important role in ligand binding. $\alpha 1\beta 1$ integrin has been recognized as a specific receptor for collagen type IV and is much less cross-reactive with other collagens including type I and XIII. The selectivity of this integrin is particularly interesting in the context of activity of another collagen receptor, $\alpha 2\beta 1$ integrin. The structure of $\alpha 1$ and $\alpha 2$ subunits is very homologous, however specific interaction for $\alpha 2\beta 1$ integrin is directed to collagen I^{11,12}. Both integrins are not only structural receptors, but play important functions in cell regulation. The cytoplasmic domains of α subunits of both integrins are involved in the promotion of cell-cycle progression¹³. The VEGF-dependent activation of microvascular endothelial cells results in cell proliferation that is supported by $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins and is mediated by the Erk1/2 MAPK signal transduction pathway¹⁴.

Snake venom disintegrins are low molecular weight proteins that interact with certain integrins blocking their functional ability to bind endogenous ligands (for review see 15-17). Structurally they are divided into two groups, monomeric and dimeric molecules, with a molecular weight in the ranging from 4 to 15 kDa. Functional classification includes three groups of snake venom disintegrins based on the active motif present in the integrin binding site, which determines their selectivity¹⁷. The first group includes disintegrins that interact with RGD-dependent integrins ($\alpha II\beta 3$, αv -integrins, and $\alpha 5\beta 1$) and is mainly represented by the disintegrins that contain the RGD tripeptide in their active site. The disintegrins containing the related RGD sequences such as KGD¹⁸, MGD¹⁹ or WGD²⁰ were also assigned to this group. The second group consists of heterodimeric disintegrins containing MLD sequence in the active site. They specifically block the function of certain leukocyte integrins including $\alpha 4$ -integrins and $\alpha 9\beta 1$ ^{21,22}. The last functional group of disintegrins includes selective inhibitors of $\alpha 1\beta 1$ integrin, which contain KTS and related motifs in the active site. Currently two KTS-disintegrins, obtustatin and viperistatin, were isolated from viper venoms²³, and one RTS-disintegrin, jerdostatin, was identified based on the venom gland cDNA²⁴. Structurally, KTS-disintegrins belong to the monomeric short disintegrins, which resemble the previously reported short, RGD-disintegrins such as echistatin or eristostatin²⁵. These disintegrins contain 8 cysteines in their polypeptide chain that are involved in creation of 4 intramolecular disulfide bounds. The 3D structure of obtustatin was recently designed based on NMR coordinates²⁶. Anti-angiogenic activity of obtustatin as well as its inhibitory effect on experimental tumor growth was initially reported²⁷. In this paper, we present complex studies that show the potent pharmacological effect of this disintegrin on angiogenesis and melanoma progression in animal models.

Material and Methods

Cells

Mouse melanoma B16F10 cell line was purchased from ATCC (Manassa, VA), human melanoma MV3 cell line was obtained from Dr. E. Danen (Leiden University, Leiden, The Netherlands). Both cell lines were cultured in DMEM containing 10% FBS. Primary adult dermal human microvascular endothelial cells (dHMVEC) were purchased from Cambrex (Walkersville, MD), and cultured in complete endothelial cell basal media-2 (EBM-2) also from Cambrex. Primary endothelial cells were used in experiments between 5 and 8 passages.

Purification of obtustatin and vpVEGF

Obtustatin was purified from *Vipera lebetina obtusa* venom (Latoxan, France), using a two steps reverse phase HPLC procedure as described previously²³. The purification procedure from the venom of *Vipera palestinae* (gift from Dr. A. Bdolah, Tel-Aviv University, Israel) and characterization of vpVEGF was recently published²⁸. Ethylpyridylated (EP)-obtustatin was prepared by reduction and alkylation of native obtustatin as described earlier²⁷.

Melanoma growth and angiogenesis induction in CAM quail embryonic model

An assay of tumor growth in the quail embryonic CAM system was developed based on the angiogenesis assay described previously²⁹. Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs were purchased from Boyd's Bird Co (Pullman, WA). Eggs were cleaned with ethanol, and maintained at 37°C until embryonic day 3 in incubator without CO₂. The shells were then opened with a razor blade and sterile scissors, the contents transferred into 6-well tissue culture plates and returned to the 37°C incubator. At embryonic day 6, MV3 melanoma cells ($1 \times 10^7/50\mu\text{l}$) were applied on the top of CAM and allowed to grow for 24 hours. Embryos were divided into experimental groups, each containing at least 10 animals, and obtustatin or EP-obtustatin was applied in the amount of 20 μg in 50 μl of PBS on the top of a tumor every day (5 treatments). Control group received a vehicle (PBS) treatment. The experiment was performed until day 12, and then the embryo was fixed with 5 ml of pre-warmed 2% glutaraldehyde, 4% paraformaldehyde in PBS for 48 hours at room temperature. The membranes containing tumor were dissected and transferred onto the glass slide. Tumors were carefully cut out of the membrane and weighed. The rest of the membranes were mounted onto glass slides for evaluation of fractal dimension (D_f) as described earlier²⁹. The area of CAM selected as a square for analysis of vascularization ratio, was localized in the opposite site to the tumor on the membrane. For example, if tumor was developed in the right corner of the CAM, the vascularization tree for analysis was framed in the left corner of membrane.

Melanoma growth in mouse models

The experiments were designed based on previously reported methodology³⁰. Briefly, B16F10 cells (1×10^6) or MV3 cells (1×10^7) were inoculated s.c. on the back of C57BL/6 or CD-1 Nu/Nu mice (Charles River Laboratories, Inc, Wilmington, MA), respectively. Tumors were allowed to grow for 4 days in syngeneic mice and 7 days in nude mice with obtustatin injected i.v. in the tail vein every other day at two doses, 5 mg/kg and 2.5 mg/kg. Control groups were injected with PBS. Each group contained 5 animals. Tumors were measured everyday with a dial caliper, and the volumes were determined using the formula $\text{width}^2 \times \text{length} \times 0.52$. Immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sectioned at a 5- μm thickness. H&E staining was used for a tissue morphology examination. Blood vessels were immunochemically stained by anti-von Willebrand factor antibody with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). TUNEL staining was performed using a colorimetric assay kit (Chemicon, Temecula, CA). Images were analyzed using an Olympus A $\times$ 70 light microscope with 400 $\times$ magnification. Numbers of blood vessels from 10 high-power fields were counted and averaged for the tissue sections labeled with anti-von Willebrand factor. All sections were coded and observed by investigators who were blinded for this protocol to avoid any significant imprecision of vessel density determination. Error bars represent S.D. from 5 animals counted by 3 investigators.

Cell proliferation assay

Cell proliferation assay was performed using BrdUrd kit (Roche, Mannheim, Germany) as described previously³¹.

Cell apoptosis assays

Annexin V detection on dHMVEC following treatment with obtustatin was performed using a reagent kit (BD Biosciences, San Diego, CA), according to manufacturer's instruction. Samples were analyzed within one hour by double color in flow cytometry using EasyCyte flow cytometer (Guava, Hayward, CA).

The experiments for detection of caspase 3 and caspase 8 were performed using a colorimetric ELISA kit (BD Biosciences) and FITC based kit (Chemicon), respectively. ELISA plate was read using an ELISA plate reader EL×800 (BioTek, Winooski, VT) at a 450 nm single wavelength, whereas the plate with fluorescein labeled samples was read using a fluorescent plate reader FL×800 (BioTek).

RNA silencing of caspase 3 gene in dHMVEC was performed in serum-free media by transfection with siRNA (75 pmol, Gene ID# 836, siRNA ID# s399, Applied Biosystem, Foster City, CA) using Lipofectamine (Invitrogen) in Opti-MEM media. After 72 hours of transfection, cells were detached by trypsinization and treated or not with 2 μ M of obtustatin in the media containing 2% FBS. Cells were fixed by 2% paraformaldehyde and stained with TUNEL using a commercial kit (Roche). Detection of apoptotic cells was performed using EasyCyte flow cytometer.

Western blot analysis of releasing caspases 3 and 8 was performed using specific antibodies that recognized cleaved form of caspases and were purchased from Cell Signaling Inc. (Beverly, MA).

Results

Effect of obtustatin on experimental melanoma growth in animal models

Before performing the mouse experiments we established the maximal tolerance dose (MTD) of obtustatin for this specie. This disintegrin was injected once at increasing doses up to 20 mg/kg, i.v. in the tail vein of mice. The behavioral signs and measurement of the body weight did not reveal any toxic side effects during 10 days observation. After this period of time, the paraffin sections of brain, lung, kidney, liver and spleen were prepared and stained with H&E. Analysis of these sections showed no effects at all concentrations up to 7 mg/kg. However, animals in 10 mg/kg and 20 mg/kg groups showed small areas of inflammations and necrotic patches in liver. Small areas of necrosis were also observed in the spleen in some of the animals of the 10 mg/kg group and significant necrotic patches in all animals in the 20 mg/kg group. In all groups we observed no effects in the brain, lung and kidney (data not shown). Based on this data we determined MTD at 7 mg/kg. The treatment doses of obtustatin in the mouse models of tumor development were estimated approximately to be less than 80% and 40% of MTD, at 5 mg/kg and 2.5 mg/kg, respectively. We investigated the oncostatic activity of this disintegrin in two mouse models of melanoma, including induction of tumor by mouse B16F10 cells in syngeneic mice and human MV3 cells in nude mice (Fig. 1). Statistically significant inhibitory effect at both concentrations of obtustatin was observed in all experimental systems. The blocking effect of this disintegrin in mouse melanoma was much lower than that in the nude mice with a human tumor. However, B16F10 tumor is very aggressive and extremely difficult to inhibit by oncostatic agents. Obtustatin showed a decrease of tumor size about 65% after administration at 5 mg/kg, and 40% at 2.5 mg/kg (Fig. 1a,b). These results confirmed that

obtustatin is a powerful anti-tumoral agent having a therapeutic effect within the MTD value. Although determination of MTD was performed only for one dose of animals' treatment, the cumulative effect of obtustatin that may affect MTD was not observed in mice during melanoma experiments, which required multiple injections. Animals did not show any changes in body weight, as well as analysis of organs as described above on the end of experiment showed no differences for 5 and 2.5 mg/kg doses in comparison with non-treated animals. Therefore, based on these observations, we can conclude that used in experiment doses of obtustatin have therapeutic side less effect, although total, cumulative application of disintegrin was higher than calculated MTD. An extremely potent inhibitory effect was observed for obtustatin on human melanoma growth in nude mice. Both concentrations of the disintegrin completely blocked tumor progression during the course of the experiment (Fig. 1c). The tumor weights measured on day 18 showed very low values for the 5 mg/kg group of mice, even lower than the sizes at the beginning of treatment (day 7). In some cases we observed a complete disappearance of cancer. The groups treated with obtustatin at 40% of MTD showed stable suppression of tumor growth that did not exceed the initial size.

The oncostatic effect of obtustatin is highly related to its anti-angiogenic activity. We analyzed the vascularization ratio induced by the tumors in the presence or absence of obtustatin. The paraffin sections of B16F10 melanoma showed a significant reduction of vessel number in the obtustatin treated animals (Fig. 2). Moreover, vasculature in the group of obtustatin revealed different morphology including reduction or disappearance of lumen in comparison with control (Fig. 2a). Significant areas of necrotic tissue were present in obtustatin sections. The necrotic patches in the control group of animals were very limited.

The activity of obtustatin for suppression of tumor growth *in vivo* was confirmed in a quail shell less embryonic assay. We designed this assay based on the angiogenesis model reported previously²⁹. Obtustatin showed a significant decrease of tumor size during five days of treatment (Fig. 3). The shrinkage of melanoma growth in this system could be evaluated by daily observation of tumor size (Fig. 3a), as well as confirmed by a decrease of tumor weight at the end of the experiment (Fig. 3b). The continuation of the experiment beyond day 12 is not possible, because of the high mortality of embryos.

The inhibition of angiogenesis by obtustatin induced by MV3 human melanoma was very easy to observe and quantify in the embryonic quail CAM system. Implanted MV3 cells into the membrane formed a tumor that started to induce angiogenesis in the entire CAM. The pathological vascularization including immature, highly condensed, leaking structures of vessels was observed in the neighboring parts of the membrane. This pathology was possible to observe under the microscope, because broad areas of the developing embryonic CAM in shell less system are transparent. The pathological angiogenesis induced by the tumor was significantly reduced in the obtustatin treated groups (Fig. 4a). Moreover, quantification of angiogenesis index as a fractal dimension (D_f) in the area of membrane placed in the opposite site of the CAM showed a high increase of vascularization for the membrane with a tumor when compared with control embryos without a tumor (Fig. 4b). This angiogenesis was significantly diminished in the group of embryos treated with obtustatin. To eliminate any non-specificity, the separated group of embryos was treated with EP-obtustatin. EP-obtustatin is a refolded form of this protein with reduced disulfide bonds and cysteines blocked with vinylpyridine. This modification resulted in over four orders of magnitude decreasing activity of this disintegrin against $\alpha 1\beta 1$ integrin²⁷. EP-obtustatin at dose 20 μ g showed no activity to inhibit MV3 melanoma-induced angiogenesis in CAM assay.

Effect of obtustatin on pro-angiogenic activity of dHMVEC

Proliferation of endothelial cells is considered as their major activity required for angiogenesis. Newly propagated cells migrate in the surrounding matrix and form new

vessels. We investigated the effect of obtustatin on the proliferation of dHMVEC induced by different agonists. Using BrdUrd assay we found that obtustatin is a potent inhibitor of endothelial cell proliferation induced by 2% FBS, as well as by two types of VEGF (Fig. 5), including human recombinant (hrVEGF) and growth factor isolated from *Vipera palestinae* venom (vpVEGF). Interestingly, under the same concentrations (50 ng/ml) vpVEGF demonstrated significantly higher activity to induce dHMVEC proliferation than hrVEGF. This observation, as well as the independency of anti-proliferative effect of obtustatin from the type of agonist, suggests that this disintegrin does not affect cell surface receptors that are directly involved in interaction with stimulators such as growth factor receptors. Moreover, growing of the cells on the immobilized ECM proteins such as neutral for $\alpha 1\beta 1$ integrin fibronectin, or its natural ligand collagen IV, was not effective to change any anti-proliferative activity obtustatin (data not shown). We hypothesized that obtustatin following binding to $\alpha 1\beta 1$ integrin on the endothelial cell must block proliferation by inducing pro-apoptotic signals.

Effect of obtustatin on induction of apoptosis in dHMVEC

We verified our hypothesis presented above by performing several apoptosis assays. Initially, we looked at the effect of obtustatin on the morphology of dHMVEC. Cells treated with a high concentration of obtustatin (2 μ M) in complete tissue culture medium containing all growth factors and 5% FBS significantly changed their shape and started to detach, showing typical apoptotic morphology (Fig. 6a). The early stages of induction of apoptosis by obtustatin were measured by annexin V assay. Our data revealed that annexin V binds to the surface of dHMVEC following treatment with obtustatin, showing a loss of membrane phospholipid asymmetry and exposure of phosphatidylserine³². Phosphatidylserine is recognized by annexin V and detected by flow cytometry (Fig. 6b). Obtustatin induced apoptosis in dHMVEC by activation of the caspase pathway. Treatment of dHMVEC resulted in a release of caspase 3 in dHMVEC, which was detected by colorimetric ELISA method as well as by Western blot (Fig. 6c). The silencing of caspase 3 gene by siRNA confirmed that $\alpha 1\beta 1$ integrin, following antagonization by obtustatin, transfers the pro-apoptotic signal through a caspase-dependent pathway. dHMVEC with silenced caspase 3 gene were resistant for obtustatin-induced apoptosis that was observed in flow cytometry TUNEL assays and by Western blot (Fig. 6c). The treatment with obtustatin for flow cytometry analysis was performed in suspension after detaching of dHMVEC from the culture. Caspase 8 that is involved in the extrinsic pathway of apoptosis appears to be activated through the binding of obtustatin to $\alpha 1\beta 1$ integrin on dHMVEC. This caspase was detected in the lysate of cells previously treated with obtustatin by fluorescent ELISA and Western blot (Fig. 6d). Vincristine was used as a positive control in the caspase apoptosis assays. Growing of dHMVEC on the neutral for $\alpha 1\beta 1$ ECM such as fibronectin showed no differences in induction of apoptosis by obtustatin that was recognized by caspases 3, 8 assays (data not shown). On the other hand, obtustatin exhibited no apoptotic properties in the melanoma cell lines including human and mouse, MV3 and B16F10, respectively; although both cell lines express $\alpha 1\beta 1$ integrin (data not shown). The apoptosis experiments *in vitro* were verified in the mouse experimental model of B16F10 melanoma. Analysis of paraffin sections of tumoral tissue revealed positive staining of DNA strand breaks (TUNEL reaction) in the endothelial cells of cancer samples isolated from obtustatin-treated animals (Fig. 2a).

Discussion

The molecule of the snake venom disintegrin, obtustatin, contains only 41 amino acids, which is the shortest polypeptide chain among other members of this protein family. It was also first reported to be a low molecular weight inhibitor of $\alpha 1\beta 1$ integrin, which is highly

expressed on intensively proliferating microvascular endothelial cell. This property suggested that obtustatin may be an appropriate compound for the development of new anti-angiogenic therapy, especially for targeting a pathological vasculature in the solid cancers. In this paper, we present primary pre-clinical studies of obtustatin on melanoma progression in animal models, as well as explaining the mechanism of its oncostatic effect.

The inhibition of angiogenesis in oncology is an interesting approach for therapy. We showed in the previous report that obtustatin is a very potent inhibitor of neovascularization process in the chicken CAM assay²⁷. This assay presented obtustatin as a safe therapeutic protein, because the effective dose treatment was not harmful to the chicken embryos. A very important angiostatic activity of obtustatin for cancer therapy was associated with its ability to inhibit tumor-induced angiogenesis in the quail system. This experiment originally designed and developed in our laboratory showed that a progressing tumor increases vascularization of the entire membrane that covers the embryo. Microscopic observation revealed that the majority of CAM vasculature exhibits typical cancer pathology, which includes immature vessels having highly meandering often network-like leaking structure. This unique assay revealed that obtustatin significantly decreased vascularization ratio (Fig. 4a).

Therapeutic effectiveness of obtustatin was particularly remarkable in the mouse experiments. However, these treatments were only effective in the case of i.v. administration. Experiments performed with obtustatin injected i.p. with 2.5 mg/kg dose showed no effect on tumor growth in B16F10 melanoma model, whereas injection of 5 mg/kg using this route exhibited only slightly, close to no significant ratio tumor suppression (data not shown). Interestingly, i.p. injected disintegrin was not detectable by Western blot in the blood plasma tested at variety of time intervals, suggesting that it is completely absorbed by the local tissue in the injection side. This high absorption of obtustatin by the receptors expressed on the tissues influences its half-life time in the blood. We estimated this time for the mice as only 30 minutes, by analyzing presence of this disintegrin in the blood serum by Western blot using anti-obtustatin polyclonal antibody (data not shown). This is a significant limitation for therapy, although fast eliminating drugs from the organisms are easier to control and allow better protection of patients in the case of harmful side effects that may occur during individual response for treatment. The low half-life time of the active pharmaceutical compounds also affects the efficiency of the therapy. These kinds of drugs are usually administrated at a constant rate by the osmotic pump. A good example in cancer treatment is chemotherapeutic drug, 5-fluorouracil (5-FU), which shows a half life time of about 100 minutes³³. We expect that that systemic treatment of mice with obtustatin using an osmotic pump will have a better therapeutic effect including a decrease in the effective doses.

The mechanism of inhibition of melanoma growth by obtustatin is mainly related to its anti-angiogenic activity. Presented here are experiments with dHMVEC, which clearly showed that obtustatin potently blocks proliferation of endothelial cells and this effect is independent from the type of agonist used. Further investigation revealed that obtustatin is a potent inducer of apoptosis in endothelial cells, by activation of the extrinsic pathway that is dependent on caspase 8. This pathway previously described for integrin-mediated death (IMD), may be induced by non-ligation or antagonizing integrins on endothelial cells by soluble ECM (thrombospondin) or degraded ECM such as endostatin or tumbstatin^{34,35}. Anoikis is a type of apoptosis occurring in the cells that lost contact (detached) with ECM, which may be caused by anti-adhesive reagents³⁶. However, obtustatin's pro-apoptotic effect was not related to anoikis, only to its direct interaction with dHMVEC. TUNEL flow cytometry experiments showed that obtustatin induced apoptosis in already detached cells in suspension. Although the extrinsic apoptosis pathway induced by obtustatin was intensively

investigated in this paper, we cannot exclude that the mitochondrial pathway is also activated by this disintegrin. The activation of both pathways was found for small RGD-based peptides that entered endothelial cell in the integrin-independent manner and directly activated caspases³⁷.

Targeting integrins in angiogenesis and cancer therapy development is one of the significant approaches in drug discovery. The most advanced are studies with antagonizing of $\alpha v \beta 3$ integrin, which resulted in clinical trials of humanized monoclonal antibody and synthetic RGD-based cyclic peptides. Even the RGD-containing snake venom disintegrin, contortrostatin was investigated in this context³⁸. Although knockout animals revealed that $\alpha v \beta 3$ integrin is not important for vascularization of experimental tumors and apparently increased this process³⁹, the mechanism of antagonizing of this disintegrin appears to be related to its ability to transfer death signals⁴⁰. The situation with $\alpha 1 \beta 1$ integrin is less complicated. Elimination of this collagen receptor in $\alpha 1$ knockout mice revealed significant reduction of experimental tumor development associated with decreasing angiogenesis^{6,41}. Treatment of the developing tumor in mice using antibodies against $\alpha 1$ and $\alpha 2$ integrin subunits significantly reduced tumor size and vasculature¹⁴. Our initial experiments²⁷ and data presented in this paper qualify $\alpha 1 \beta 1$ integrin as an excellent target for angiogenesis, of which the elimination or blocking appears to be safe with no side effects.

Obtustatin was the first reported exogenous, low molecular weight naturally occurring inhibitor of $\alpha 1 \beta 1$ integrin. Previously, peptide-like inhibitor of this integrin, arresten, was found in the human body as a degradation product of the non-collagenous domain (NC1) of $\alpha 1$ chain of collagen type IV⁴². Recombinant arresten showed a molecular weight of about 26 kDa and blocked angiogenesis and tumor growth in mouse models by regulating HIF-1 α and VEGF expression. However, a mechanism of its angiostatic effect appeared to be associated with inhibition of cell signaling cascade, presumably MAPK without inducing any apoptosis⁴¹. In this context, obtustatin studies revealed that the collagen receptor, $\alpha 1 \beta 1$ integrin may be included to the family integrins, such as $\alpha v \beta 3$, $\alpha 5 \beta 1$, which transfer pro-apoptotic signals.

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Abbreviations

CAM	chorioallantoic membrane
dHMVEC	dermal human microvascular endothelial cells
D_f	fractal dimension
MTD	maximal tolerance dose
vpVEGF	Vipera palestinae vascular endothelial growth factor
hrVEGF	human recombinant VEGF

References

1. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971; 285:1182–6. [PubMed: 4938153]
2. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov*. 2007; 6:273–86. [PubMed: 17396134]

3. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000; 407:249–57. [PubMed: 11001068]
4. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol*. 2000; 156:1363–80. [PubMed: 10751361]
5. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *Am J Pathol*. 1997; 94:13612–7.
6. Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA. Elevated matrix metalloprotease and angiostatin levels in integrin $\alpha 1$ knockout mice cause reduced tumor vascularization. *Proc Natl Acad Sci USA*. 2000; 97:2202–7. [PubMed: 10681423]
7. Sheppard D. *In vivo* functions of integrins: lessons from null mutations in mice. *Matrix Biol*. 2000; 19:203–9. [PubMed: 10936445]
8. Liddington RC, Ginsberg MH. Integrin activation takes shape. *J Cell Biol*. 2002; 158:833–9. [PubMed: 12213832]
9. Dickeson SK, Santoro SA. Ligand recognition by the I domain-containing integrins. *Cell Mol Life Sci*. 1998; 54:556–66. [PubMed: 9676575]
10. Leitinger B, Hogg N. Integrin I domains and their function. *Biochem Soc Transact*. 1999; 27:826–32.
11. Kern A, Eble J, Golbik R, Kuhn K. Interaction of type IV collagen with isolated integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. *Eur J Biochem*. 1993; 215:151–9. [PubMed: 8344274]
12. Dickeson SK, Mathis NL, Rahman M, Bergelson JM, Santoro SA. Determinants of ligand binding specificity of the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *J Biol Chem*. 1999; 274:32182–91. [PubMed: 10542255]
13. Zutter MM, Santoro SA, Wu JE, Wakatuski T, Dockesom SK, Elson EL. Collagen receptor control of epithelial morphogenesis and cell cycle progression. *Am J Pathol*. 1999; 155:927–40. [PubMed: 10487850]
14. Senger DR, Perruzzi CA, Streit M, Koteliansky VE, de Fougères AR, Detmar M. The $\alpha 1(1)\beta 1$ and $\alpha 2(2)\beta 1$ integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. *Am J Pathol*. 2002; 160:195–204. [PubMed: 11786413]
15. Dennis MS, Henzel WJ, Pitti RM, Lipari MT, Napier MA, Deisher TA, Bunting S, Lazarus RA. Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: Evidence for a family of platelet-aggregation inhibitors. *Proc Natl Acad Sci USA*. 1990; 87:2471–5. [PubMed: 2320569]
16. Niewiarowski S, McLane MA, Kloczewiak M, Stewart GJ. Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. *Semin Hematol*. 1994; 31:289–300. [PubMed: 7831574]
17. Marcinkiewicz C. Functional Characteristic of Snake Venom Disintegrins: Potential Therapeutic Implication. *Curr Pharm Des*. 2005; 11:815–27. [PubMed: 15777236]
18. Scarborough RM, Naughton MA, Teng W, Rose JW, Phillips DR, Nannizzi L, Arfsten A, Campbell AM, Charo IF. Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. *J Biol Chem*. 1993; 268:1066–73. [PubMed: 8419315]
19. Marcinkiewicz C, Calvete JJ, Senadhi VK, Marcinkiewicz MM, Raida M, Schick P, Lobb RR, Niewiarowski S. Structural and functional characterization of EMF10, a heterodimeric disintegrin from *Eristocophis macmahoni* venom that selectively inhibits $\alpha 5\beta 1$ integrin. *Biochemistry*. 1999; 38:13302–9. [PubMed: 10529205]
20. Calvete JJ, Fox JW, Agelan A, Niewiarowski S, Marcinkiewicz C. Presence of WGD motif in CC8 heterodimeric disintegrin increased its inhibitory effect on $\alpha 1\beta 3$, $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins. *Biochemistry*. 2002; 41:2014–21. [PubMed: 11827548]
21. Marcinkiewicz C, Calvete JJ, Marcinkiewicz MM, Raida M, Senadhi VK, Huang Z, Lobb RR, Niewiarowski S. EC3, a novel heterodimeric disintegrin from *Echis carinatus* venom, inhibits $\alpha 4$ and $\alpha 5$ integrins in an RGD-independent manner. *J Biol Chem*. 1999; 274:12468–73. [PubMed: 10212222]

22. Bazan-Socha S, Kisiel DG, Young B, Theakston RDG, Calvete JJ, Sheppard D, Marcinkiewicz C. Structural requirements of MLD-containing disintegrins for functional interaction with $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins. *Biochemistry*. 2004; 43:1639–47. [PubMed: 14769041]
23. Kisiel DG, Calvete JJ, Katzhendler J, Fertala A, Lazarovici P, Marcinkiewicz C. Structural determinants of the selectivity of KTS-disintegrins for the $\alpha 1\beta 1$ integrin. *FEBS Lett*. 2004; 577:478–82. [PubMed: 15556632]
24. Sanz L, Chen RQ, Perez A, Hilario R, Juarez P, Marcinkiewicz C, Monleon D, Celda B, Xiong YL, Perez-Paya E, Calvete JJ. cDNA cloning and functional expression of jerdostatin, a novel RTS-disintegrin from *Trimeresurus jerdonii* and a specific antagonist of the alpha 1beta 1 integrin. *J Biol Chem*. 2005; 280:40714–22. [PubMed: 16215260]
25. Marcinkiewicz C, Rosenthal LA, Mosser DM, Kunicki TJ, Niewiarowski S. Immunological characterization of eristostatin and echistatin binding sites on $\alpha \text{IIb}\beta 3$ and $\alpha \text{v}\beta 3$ integrins. *Biochem J*. 1996; 317:817–825. [PubMed: 8760368]
26. Moreno-Murciano MP, Monleón D, Marcinkiewicz C, Calvete JJ, Celda B. The NMR solution structure of the non RGD disintegrin obtustatin. *J Mol Biol*. 2003; 329:135–45. [PubMed: 12742023]
27. Marcinkiewicz C, Wainreb PH, Calvete JJ, Kisiel DG, Mousa SA, Tuszynski GP, Lobb RR. Obtustatin, a potent inhibitor of $\alpha 1\beta 1$ integrin *in vitro* and angiogenesis *in vivo*. *Cancer Res*. 2003; 63:2020–3. [PubMed: 12727812]
28. Brown MC, Calvete JJ, Staniszewska I, Del Valle L, Lazarovici P, Marcinkiewicz C. VEGF-related protein isolated from *Vipera palestinae* venom, promotes angiogenesis. *Growth Factors*. 2007; 25:108–17. [PubMed: 17852405]
29. Parsons-Wingerter P, Lwai B, Elliot KE, Milaninia A, Redlitz A, Clark JJ, Sage H. A novel assay of angiogenesis in the quail chorioallantoic membrane: stimulation by bFGF and inhibition by angiostatin according to fractal dimension and grid intersection. *Microvasc Res*. 1998; 55:201–14. [PubMed: 9657920]
30. Miao WM, Seng WL, Duquette M, Lawler P, Laus C, Lawler J. Thrombospondin-1 Type 1 Repeat Recombinant Proteins Inhibit Tumor Growth through Transforming Growth Factor- β -dependent and -independent Mechanisms. *Cancer Res*. 2001; 61:7830–9. [PubMed: 11691800]
31. Staniszewska I, Zaveri S, Del Valle L, Oliva I, Rothman VL, Croul SE, Roberts DD, Mosher DF, Tuszynski GP, Marcinkiewicz C. Interaction of $\alpha 9\beta 1$ integrin with thrombospondin-1 promotes angiogenesis. *Circ Res*. 2007; 100:1308–16. [PubMed: 17413041]
32. Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, van Oers MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*. 1994; 84:1415–20. [PubMed: 8068938]
33. McDermott BJ, van den Berg HW, Murphy RF. Nonlinear pharmacokinetics for the elimination of 5-fluorouracil after intravenous administration in cancer patients. *Cancer Chemother Pharmacol*. 1982; 9:173–8. [PubMed: 7160054]
34. Stupack DG, Chersesh DA. Get a ligand, get a life: integrins, signaling and cell survival. *J Cell Sci*. 2002; 115:3729–38. [PubMed: 12235283]
35. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res*. 2005; 65:3959–65.
36. Frisch SM, Screaton RA. Anoikis mechanisms. *Curr Opin Cell Biol*. 2001; 13:555–62. [PubMed: 11544023]
37. Aguzzi MS, Giampietri C, De Marchis F, Padula F, Gaeta R, Ragone G, Capogrossi MC, Facchiano A. RGDS peptide induces caspase 8 and caspase 9 activation in human endothelial cells. *Blood*. 2004; 103:4180–7. [PubMed: 14982875]
38. Swenson S, Costa F, Ernst W, Fujii G, Markland FS. Contortrostatin, a snake venom disintegrin with anti-angiogenic and anti-tumor activity. *Pathophysiol Haemost Thromb*. 2005; 34:169–76. [PubMed: 16707922]
39. Taverna D, Moher H, Crowley D, Borsig L, Varki A, Hynes RO. Increased primary tumor growth in mice null for beta3- or beta3/beta5-integrins or selectins. *Proc Natl Acad Sci U S A*. 2004; 101:763–8. [PubMed: 14718670]

40. Cheresh DA, Stupack GD. Integrin-mediated death: an explanation of the integrin-knockout phenotype? *Nat Med.* 2002; 8:193–4. [PubMed: 11875466]
41. Sudhakar A, Nyberg P, Keshamouni VG, Mannam AP, Li J, Sugimoto H, Cosgrove D, Kalluri R. Human alpha1 type IV collagen NC1 domain exhibits distinct antiangiogenic activity mediated by alpha1beta1 integrin. *J Clin Invest.* 2005; 115:2801–10. [PubMed: 16151532]
42. Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, Takahashi K, Volk R, Zamborsky ED, Herman S, Sarkar PK, Ericksen MB, Dhanabal M, et al. Anti-angiogenic cues from vascular basement membrane collagen. *Cancer Res.* 2000; 60:2520–6. [PubMed: 10811134]

FIGURE 1.

Effect of obtustatin on melanoma growth in mouse models. (a) Monitoring of tumor growth in syngeneic (C57BL/6) mice induced by B16F10 mouse melanoma cell line. The i.v. injection of obtustatin was started from tumor post-implantation day 4 and was continued every other day until day 12. Measurements of tumor dimensions were performed everyday. Mice were maintained alive six days after the last injection and at day 18 were sacrificed. (b) Comparison of B16F10 tumor weight dissected from the mice at day 18. The upper panel presents images of representative melanomas dissected from the animals. In the lower plot, the values of tumor weight are presented. (c) Effect of obtustatin on tumor growth in nude mice induced by MV3 human melanoma cell line. Experiment was performed similarly to the syngeneic mice described above starting with i.v. injection of obtustatin at day 7. Left plot presents monitoring of tumor size during progression of experiment, right plot presents

weight of tumors dissected from the animals on day 18. Error bars represent S.D. from analysis of 5 mice per group. The statistic unpaired t-test showed $p < 0.001$ for all experimental groups of obtustatin in comparison with control, vehicle treated animals.

FIGURE 2.

Immunohistochemistry of paraffin sections of B16F10 mouse melanoma. (a) Representative images of tissue sections of control and obtustatin treated B16F10 melanoma. Black arrows indicate vessels, green arrows indicate necrotic patches. (b) Graphic presentation of the number of vessels counted per microscopic (magnification 400 $\times$) field. Error bars represent S.D. from 10 different fields per experimental group.

FIGURE 3.

Effect of obtustatin on human melanoma growth in quail embryonic model. Embryos were grown on 6-well plates until day 6, and MV3 cells at concentration $1 \times 10^7/50 \mu\text{l}$ PBS were applied on the top of CAM. Tumors were allowed to grow for 24 hours and then treated daily with $20 \mu\text{g}$ of obtustatin per $50 \mu\text{l}$ of PBS. Controls received vehicle treatment. On day 12 embryos were fixed, tumors were dissected and weighted. (a) Representative images of embryos (magnification $3.5\times$) showing progression of tumor in the presence or absence of obtustatin. The area of CAM affected by tumor is bordered by black line. (b) Graph presenting tumor weight for obtustatin and control groups of embryos. Each group contained 6 embryos. Error bars represent S.D. from analysis of 6 embryos per group. The unpaired t-test showed $p < 0.001$ for comparison of obtustatin groups with control.

FIGURE 4.

Angiogenic effect induced by a developing human melanoma MV3 tumor on quail embryonic CAM in the presence or absence of obtustatin. (a) Representative images of vasculature (day 12) under different magnifications, occurring on the border of the tumor, obtustatin treated or untreated. Borders of tumors are indicated by black arrows. Control CAM image without the tumor is presented on the top under 25× magnification. (b) Comparison of angiogenic effect induced by MV3 tumor in quail embryonic CAM treated or not with obtustatin or EP-obtustatin. Representative binary images of mid-arterial endpoint fragments of CAMs dissected from embryos are presented on the three top panels; graphic values of angiogenesis index as a fractal dimension (D_f) are presented on the lower plot. The images of CAM were scanned into Photoshop software, mid-arterial endpoints were framed as squares on the opposite to developed tumor areas of membranes and cropped into ImageJ

software. After skeletonization of binary images the D_f value was calculated. (*) $p < 0.001$ for groups developing tumor and control tumor-free CAMs. (**) $p < 0.001$ for groups developing tumor treated or not with obtustatin.

FIGURE 5.

Effect of obtustatin on proliferation of dHMVEC. (a) Proliferation assay induced by 2% FBS in the presence or absence of different concentrations of obtustatin. (b) Proliferation assay induced by 50 ng/ml hrVEGF or vpVEGF in the presence or absence 2 μM obtustatin. BrdUrd color development assay was performed according to manufacturer's instruction (Roche). Error bars represent S.D. from triplicated experiments. (*) Difference between agonist-induced proliferation in the absence and presence 2 μM obtustatin was statistically significant ($p < 0.001$).

FIGURE 6.

Induction of apoptosis by obtustatin in dHMVEC. (a) Morphological changes of dHMVEC in culture treated or not with obtustatin. Cells were cultured in a 6 wells plate until 90% confluence and treated with 2 μ M obtustatin. Incubation was performed in complete EBM-2 media for 24 hours and after washing with PBS cells were fixed 2% paraformaldehyde. Cells were analyzed by contrast phase microscopic observation under 200 $\times$ magnification. (b) Detection of early stage of apoptosis induced by obtustatin in dHMVEC by testing the binding of annexin V-FITC in flow cytometry. Experiments were performed after 16 hours treatment of dHMVEC with obtustatin (2 μ M) in complete EBM-2 media. Cells were detached and stained with annexin V-FITC using reagent kit (BD Biosciences), according to manufacturer's instruction. Samples were analyzed within one hour by double color in flow cytometry. (c) Effect of obtustatin on generation of caspase 3 in dHMVEC. For ELISA cells were cultured in complete media for 16 hours in the presence or absence of obtustatin (2 μ M) or vincristine (50 μ g/ml). Cells were lysed and released caspase 3 was detected using colorimetric ELISA assay kit (BD Bioscience). TUNEL assay for flow cytometry analysis was performed for dHMVEC with silenced caspase 3 gene using specific siRNA. After 72 hours of transfection cells were detached, treated with 2 μ M obtustatin for 2 hours and mixed with TUNEL reagents. Gray areas of plots present FACS histograms for samples treated with obtustatin, whereas black areas control non-treated samples. Western blot analysis for dHMVEC with silenced caspase 3 was performed by treatment of adhered cells with 2 μ M obtustatin for 2 hours, following lysing of the cells and separation of lysate on SDS-PAGE in reduced gel. The proteins from the gel were electro-transferred onto a PVDF membrane and incubated with primary anti-caspase 3 polyclonal antibody. The bands were visualized using chemiluminescent Western detection kit. (d) Effect of obtustatin on generation of caspase 8 in dHMVEC. Detection of caspase 8 was performed using a fluorescence kit (right panel). Cells were treated in the culture with 2 μ M obtustatin for 2 hours, and then FITC-based caspase 8 detection kit reagents were added. Cells were

transferred to 96-well plate, which was read using fluorescence plate reader. Error bars represent S.D. from three experiments. Differences between control groups and apoptosis inducers were statistically significant ($p < 0.0001$). Detection of caspase 8 by Western blot was performed in dHMVEC by treatment of cells in the culture with 2 μM obtustatin by different time points. The lysates of cells were analyzed as described for caspase 3.