Canstatin Acts on Endothelial and Tumor Cells via Mitochondrial Damage Initiated through Interaction with \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) Integrins

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Abstract

Canstatin, the noncollagenous domain of collagen type IV \(\alpha\)-chains, belongs to a series of collagen-derived angiogenic inhibitors. We have elucidated the functional receptors and intracellular signaling induced by canstatin that explain its strong antitumor efficacy in vivo. For this purpose, we generated a canstatin-human serum albumin (CanHSA) fusion protein, employing the HSA moiety as an expression tag. We show that CanHSA triggers a crucial mitochondrial apoptotic mechanism through procaspase-9 cleavage in both endothelial and tumor cells, which is mediated through cross-talk between \(\alpha_v\beta_3\)- and \(\alpha_v\beta_5\)-integrin receptors. As a point of reference, we employed the first three kringle domains of angiostatin (K1-3), fused with HSA, which, in contrast to CanHSA, act only on endothelial cells through \(\alpha_v\beta_3\)-integrin receptor-mediated activation of caspase-8 alone, without ensuing mitochondrial damage. Taken together, these results provide insights into how canstatin might exert its strong anticancer effect. (Cancer Res 2005; 65(10): 4353-61)

Introduction

Type IV collagen extracellular matrix (ECM) plays a functional role in angiogenesis, the process whereby new capillaries sprout from existing vessel. Proteolytic remodeling of the ECM not only degrades barriers that obstruct vascular cell migration but also exposes cryptic sites within collagen IV that promote novel integrin-ligand interactions required for angiogenesis (1). The type IV collagen supramolecular structure is composed of \(\alpha\)-chains, each containing a noncollagenous (NC1) domain at the COOH terminus which provides the mechanical stability of the ECM structure (2, 3).

ECM is a potential therapeutic target to regulate neovascularization in cancerous diseases as angiogenesis is required for neoplastic tumor growth (4). Systemic injection of the recombinant \(\alpha\)2NC1 domain monomer of type IV collagen, which has been named canstatin, inhibits the in vivo growth of xenografted tumors following daily injection over a period of 15 days as compared with placebo-treated mice. In vitro, the antiproliferative and antiangiogenic effects of canstatin seem to be due to the induction of apoptotic events through disruption of the FAK signaling pathway and induction of Fas ligand expression (5–7).

NC1 globular domain fragments of \(\alpha\)-chains of human collagen represent a new class of antiangiogenic molecules that differ fundamentally in structure to the plasminogen-derived group of antiangiogenic proteins such as angiostatin kringle 1 to 3 (K1-3; refs. 8–11). Thus, we decided to investigate the relative pharmacological properties of one protein from each therapeutic class: canstatin and K1-3.

In this study, to improve the pharmacokinetic properties of canstatin, we investigated in situ the anticancer properties of a canstatin-human serum albumin (CanHSA) fusion protein by adenoviral-mediated gene transfer, which efficiently yields high levels of transgene expression in many different cell types (12). To this end, we constructed a recombinant adenosine (AdCanHSA) encoding canstatin fused with HSA to decrease in vivo clearance of fused protein by increasing its molecular weight (13). The HSA moiety in the fusion protein carries the added advantage of acting as a tag which allows the expression of canstatin to be analyzed in vitro and in vivo. To evaluate the antitumor potential of CanHSA, we compared its features both in vitro and in vivo with that of adenoviral-expressed unfused canstatin (AdCan) and K1-3, either fused (AdK1-3HSA) or unfused (AdK1-3) with HSA (11, 14).

Importantly, to characterize the antitumor properties of CanHSA compared with those of K1-3HSA, we focused our study on the mechanism of action of each protein. Given that angiostatin and canstatin both induce apoptosis in endothelial cells (7, 11, 15, 16), we asked (a) whether tumor cells as well as endothelial cells could be a direct target of canstatin via an integrin-dependent interaction, and (b) whether the signaling pathways leading to induction of apoptosis are different following canstatin or K1-3 treatment. To this end, we explored cell cycle progression and the activities of caspases within tumor and endothelial cells.

Materials and Methods

Construction of the recombinant adenosines. AdCan and AdCanHSA express the NC1 domain of the human \(\alpha\)2 chain of type IV collagen, also called canstatin (GenBank accession GI 8101723), either fused or unfused to HSA moiety. Both canstatin and CanHSA are secreted through a 24-residue prepro signal sequence from HSA (GenBank accession F01107), which was inserted into the NH2 terminus of both proteins. AdK1-3 and AdK1-3HSA express respectively the angiostatin kringles 1 to 3 (K1-3) and K1-3 conjugated with HSA. A recombinant adenosine not containing a transgene (AdCO1) was used as a negative control in this study (11, 14).

Cell lines. MDA-MB-231 and human umbilical vascular endothelial cells (HUVEC) have been previously described (17). PC-3 cells (ATCC ref. CRL-1435) were grown in F-12K supplemented with 10% fetal bovine serum (FBS), 1 mmol/L l-glutamine, and 1.5 g/L sodium bicarbonate (Invitrogen, Carlsbad, CA).

Anticanstatin antibodies. To generate a polyclonal antibody against canstatin, a peptide spanning the NH2-terminal region of canstatin

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(VSI0LYLVKH5QTDQPMC) was synthesized by conventional solid-phase methodology using an Applied Biosystems Model 431A peptide synthesizer. The purity of the peptide was ascertained by high-performance liquid chromatography and its identity verified by mass spectrometry. The synthetic peptide was conjugated to keyhole limpet hemocyanin, and two rabbits were immunized by two intradermal injections of the synthetic peptide-carrier conjugate. Antisera were verified by ELISA for their capacity to react with the casatin synthetic peptide. Antibodies were purified by affinity chromatography.

Western blot analysis of casatin, CanHSA, K1-3, and K1-3HSA expressions in vitro. For casatin and CanHSA expression analysis, cell culture supernatants of MDA-MB-231 were collected 96 hours after adenoviral infection of cell monolayers. Samples (μg) were incubated with the rabbit anti-casatin antibody and were revealed with a peroxidase-conjugated donkey antirabbit antibody (Amersham, Buckinghamshire, United Kingdom).

For CanHSA and K1-3HSA expression analysis, samples were incubated with a mouse anti-HSA antibody (Biovalley, Marne la Vallée, France; ref. ab7793). For K1-3 and K1-3HSA expression analysis, membranes were incubated with an antihuman plasmigen mononuclear antibody (mAb) A1D12 (11). Both of these antibody complexes were revealed by a goat antimouse immunoglobulin G1 (lgG1)-horseradish peroxidase secondary antibody (SouthernBiotech, Birmingham, AL; ref. 1070-05).

For casatin and CanHSA stability analysis in vitro, MDA-MB-231 xenografted tumors were dissected following intratumoral adenoviral injection. Tumor protein extracts (20 μg) were assessed for casatin and CanHSA expression as described above.

Quantification of CanHSA and K1-3HSA in vivo. For quantification of CanHSA and K1-3HSA by ELISA, wells were coated with a mouse anti-HSA mAb described above. After washing, wells were incubated with mouse sera at the appropriate dilution or a HSA solution standard (Sigma-Aldrich, St. Louis). Wells were washed and incubated with a rabbit anti-HSA polyclonal antibody (Sigma-Aldrich; ref. A0433). Antigen-antibody complexes were revealed with an alkaline phosphatase-conjugated goat antimmunoglobulin lgG antibody (Promega, Madison, WI; H + L) and development with an alkaline phosphatase–conjugated substrate. Absorbance at 405 nm was converted to HSA concentration (ng/mL) via a purified HSA calibration curve.

Endothelial cell proliferation, migration assays, and contrast-enhanced color Doppler high-frequency ultrasonography of tumor neovascularization. Experiments were done as described (17).

Immunohistochemistry and quantification of the intratumoral microvessels. We sacrificed six mice per group at an early stage (day 8) following intratumoral injection of each adenovirus before the appearance of necrosis. Paraffin sections of PC-3 tumors were incubated to quantify the number of intratumoral vessels, as described (17). For each animal (n = 6), a representative murine CD31-immunostained histologic sample was analyzed using a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) and a PCO digital camera. Eight fields per animal were digitized (100 ×), excluding fibrotic areas ascertained by HES staining of parallel sections. Images were then analyzed with custom software run on a Linux-Mandrake 8.2 workstation.

In vivo curative and preventive treatment. Human tumors were induced by s.c. injection of 4 × 10^6 MDA-MB-231 or 5 × 10^6 PC-3 cells into the dorsa of nude mice. When tumors had reached 3 to 4 mm in diameter, recombinant adenoviruses (n = 6 for each group) were injected into tumors [2 × 10^10 plaque-forming unit (pfu) per injection, for a total of two injections separated by a 48-hour interval]. Adenoviruses (5 × 10^6 pfu) were injected systemically 24 hours before s.c. implantation of 4 × 10^6 MDA-MB-231 cells (n = 8 for AdCO1, n = 11 for AdK1-3 and AdK1-3HSA, n = 12 for AdCan, and n = 15 for AdCanHSA).

For each experiment, blood samples were collected at days 8, 15, and 20 to quantify antiangiogenic proteins by ELISA.

Immunohistochemistry and apoptotic cell quantification (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling). An in situ cell death detection kit employing the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method (TUNEL; Roche Applied Science, Basel, Switzerland) was used to detect apoptotic cells within paraffin sections. Five fields per animal were digitized (200 ×). The number of stained cells per field was counted and mean values with their SD were calculated.

Immunofluorescence. HUVEC and MDA-MB-231 cells were infected with adenoviruses 24 hours following seeding of cells. Forty-eight hours later, cells were washed, fixed in PBS/3% formaldehyde, washed twice with PBS, and incubated overnight at 4°C in PBS/0.5% FBS. CanHSA and K1-3HSA proteins were detected using the mouse anti-HSA mAb described above. After washing thrice with PBS/0.5% FBS, cells were incubated with a fluorescein-conjugated secondary antibody (Jackson, West Grove, PA; ref. 111-095-144).

Flow cytometry. For flow cytometric analysis, cells were harvested following adenoviral infection [coupled, or not, with small inhibitory RNA (siRNA) transfection] for 48 hours. Cells were stained with fluorescein-conjugated mAbs (10 μg/mL) directed against specific integrins: LM609 (anti-αv(3)), P1F6 (anti-αv(5)), and AMF7 (anti-α3). Normal mouse fluorescein-conjugated IgG1 was used as a negative control antibody.

For cell cycle studies, HUVEC and MDA-MB-231 cells were collected at 96 hours postinfection. Cells were suspended in PBS/0.1% glucose and then fixed with 70% ethanol. After centrifugation, the supernatants were discarded and cellular DNA was stained in a solution containing 100 μg/mL of RNase A and 50 μg/mL of propidium iodide.

Determination of caspase-3, -8, and -9 activities. HUVEC, MDA-MB-231, and PC-3 cells were harvested 72 hours after infection with adenovirus, with or without caspase-3 and -9 inhibitors, during the infection period (R&D Systems, Minneapolis, MN; ref. FMK004, FMK008). Caspase-3, -8, and -9 activities were determined by caspase-3, -8, and -9 colorimetric assays (R&D Systems). Staurosporine, which induces the mitochondrial caspase-dependent apoptotic pathway, was used as an apoptotic positive control (50 nmol/L; Oncogene, Darmstadt, Germany). αv, β3, and β5 integrin gene silencing in HUVEC cells. siRNAs were purchased as designed, synthesized, purified, and annealed oligos from Ambion (Huntingdon, United Kingdom). The target sequence names for αv, β3, β5, and α5 integrins are ITGB3_1, ITGB3_2, and ITGAV_1. Cells at 50% confluence were treated with 50 nmol/L siRNA in Oligofectamine reagent (Invitrogen) according to the instructions of the manufacturer. A nonsilencing siRNA (Ambion), transfected under the same conditions as the anti-integrin siRNAs, was used as control. Four hours following siRNA transfection, cells were infected with AdCO1, AdK1-3HSA, or AdCanHSA. Expression of integrins was analyzed by flow cytometry 48 hours after adenoviral transduction. Detailed above, Caspase-3 and -9 activities were determined following infection of cells for 72 hours as described above.

Statistical analysis. Each experiment was done twice. Statistical analysis was done using the Student’s t test (unilateral and unpaired).

Results

In vitro and in vivo characterization of recombinant adenoviruses encoding casatin, CanHSA, K1-3, and K1-3HSA. Experiments were done to verify expression and secretion of casatin and CanHSA in vitro and in vivo.

In vitro, each protein was detected at the expected MW of 24 and 89 kDa, respectively, from AdCan- and AdCanHSA-infected cell supernatants at 96 hours postinfection (Fig. 1A, 1). No signal was observed from AdCO1-infected supernatants. Similar signals were carried out for AdK1-3 and AdK1-3HSA (Fig. 1A, 3). There was judged to be no quantitative difference in the maximal level expression of all four proteins through the hybridization signal to the shared HSA tag (Fig. 1A, 2).

In vivo, the circulating levels of expressed proteins in blood samples of MDA-MB-231 xenografted mice, following intratumoral or systemic infection of AdCanHSA and AdK1-3HSA, were quantified by ELISA (Fig. 1B). CanHSA and K1-3HSA showed a similar expression pattern in the sera of mice after both local and systemic adenoviral injection.
To determine whether fusion with HSA affects the in vivo stability of canstatin, Western blot analysis of MDA-MB-231 tumor protein extracts was done at days 2, 5, and 9 following intra-tumoral injection of AdCan, AdCanHSA, or AdCO1. CanHSA was the only protein for which a consistent level of expression was detected for 9 days. No signal was observed for unfused canstatin at day 9 (Fig. 1C). Thus, fusion with HSA improves the intra-tumoral bioavailability of canstatin.

**Canstatin has a more prominent antiangiogenic effect than angiostatin.** The proliferation of HUVEC cells was more strongly inhibited by AdCan, AdCanHSA, or AdCO1. CanHSA was the only protein for which a consistent level of expression was detected for 9 days. No signal was observed for unfused canstatin at day 9 (Fig. 1C). Thus, fusion with HSA improves the intra-tumoral bioavailability of canstatin.

Having shown in vitro that the inherent activities of canstatin and K1-3 were not disrupted by fusion with HSA (Fig. 2A), their antiangiogenic effects were quantified in vivo by anti-CD31 immunohistochemistry following injection of viruses into PC-3 tumors. As shown in Fig. 2B, the level of intratumoral vascularization was reduced following canstatin treatment. The thickness of capillary microvessel walls in the K1-3–treated group was thinner than those observed in the CO1-treated group. However, microvessels remained numerous and relatively thick in contrast to the vessels observed in the canstatin-treated samples. The quantification of intratumoral vessels by contrast-enhanced color Doppler high-frequency ultrasonography analysis enabled us to follow the efficacy of treatment over the treatment period by focusing specifically on the functional vessels in the tumor. At the end of the treatment period (day 42), capillary vessels were observed to invade tumors only in the AdCO1-, AdK1-3–, and AdK1-3HSA–treated groups. No intratumoral vessels were detected following injection of AdCan and AdCanHSA (Fig. 2C). The thickness of vessel walls (CD31 staining) treated with K1-3 or K1-3HSA at day 8 was thinner by visual inspection than for the CO1 slice (Fig. 2B), which, however, seemed to contain as many functional vessels as the thicker nontreated vessels (Fig. 2C).

Interestingly, CanHSA induced a strong and long-lasting inhibition of the penetration of functional vessels into the tumor mass as compared with unfused canstatin (90% and 60%, respectively; P < 0.01 as compared with AdCO1; Fig. 2C).

Thus, unfused canstatin has potent antiangiogenic features in vitro and in vivo, but we show its fusion with HSA undisputably improves its in vivo stability.

**Figure 1.** Assessment of the expression level in vitro and in vivo of canstatin, CanHSA, K1-3, and K1-3HSA proteins. A, Western blotting of adenoviral infected MDA-MB-231 cell culture supernatants confirms that expression levels of canstatin, CanHSA, K1-3, and K1-3HSA are similar. B, quantification of CanHSA and K1-3HSA by ELISA against the HSA moiety of the fusion proteins in the sera of mice at several time points following two intratumoral injections of the appropriate adenovirus (1) or systemic injection (2). C, Western blotting of protein extracts of adenoviral-infected xenografted MDA-MB-231 tumors show the increased in vivo stability of CanHSA (1; for 9 days) as compared with unfused canstatin (2).
improves its efficacy in vivo in inhibiting intratumoral vascularization in the weeks following treatment.

CanHSA exhibits a stronger in vivo antitumor effect than canstatin, K1-3, or K1-3HSA. To evaluate whether the strong antiangiogenic activity of CanHSA was sufficient to broadly affect tumor viability, we followed the growth of xenografted tumor cells after curative and prophylactic injection of the recombinant adenoviruses.

Adenoviruses were injected directly into MDA-MB-231 xenografted tumors. Following expression of canstatin and CanHSA, tumor growth was inhibited by 40% (not significant) and 98% (P < 0.003), respectively, as compared with AdCO1. Moreover, CanHSA exhibited better efficacy with 20% total tumor regression than AdCO1- or AdCan-treated group. Similarly, PC-3 tumor growth was inhibited by CanHSA with better efficacy as compared with canstatin and CO1 (72%; P = 10^{-3} at day 19). The antitumor effect of CanHSA is still significant as compared with K1-3 or K1-3HSA (P < 0.04) for both tumor models (Fig. 3A).

To evaluate the efficacy of CanHSA in a prophylactic protocol, we systemically injected each recombinant virus 24 hours before s.c. injection of MDA-MB-231 cells in mice. After 8 weeks of treatment, 77% inhibition of tumor growth was observed with CanHSA as compared with CO1- (P = 0.003) and canstatin-treated groups (P = 0.01; Fig. 3A). These results were also significant as compared with K1-3 (P = 0.005) and K1-3HSA (P = 0.006; data not shown).

The long-lasting effect of CanHSA in inhibiting tumor penetration by intratumoral vessels is also correlated with the stronger antitumor effect of the CanHSA conjugate as compared with that of free canstatin in vivo.

CanHSA induces an apoptotic mechanism in both endothelial and tumoral cells. We showed in situ by TUNEL analysis that CanHSA induced tumor cell apoptosis in MDA-MB-231 xenografts on nude mice (Fig. 3B). The results showed a marked increase of apoptotic cells in the AdCanHSA-injected tumors 30 days postinfection (11 ± 4 cells/field, P < 10^{-5}; for CanHSA as compared with 1.7 ± 0.9 cells/field observed for the AdCO1-treated group). The results obtained for CanHSA are again significant when compared with those obtained for the angiotatin-treated group (P < 10^{-5}) and the canstatin-treated group (P < 10^{-5}).

To determine whether CanHSA acts directly against tumor cells, or indirectly by inducing tumor hypoxia, nuclear DNA fragmentation, a hallmark of cellular apoptosis, was assessed by propidium iodide staining of AdCanHSA-infected MDA-MB-231 or HUVEC cells. CanHSA induced apoptosis in 31% of tumor cells whereas no apoptotic cells were observed after treatment with AdK1-3HSA or AdCO1 (Fig. 3C). Moreover, CanHSA treatment leads to apoptosis in 42% of the endothelial cells whereas only 17% of K1-3HSA–treated cells were observed in apoptotic (sub-G1) phase. No apoptotic cells were detected in the CO1-treated population.

The strong antiangiogenic and antitumor effects of canstatin seem to be due to a direct apoptotic mechanism in endothelial and tumoral cells.

CanHISA interacts with integrins on the endothelial and tumor cell surface. To determine whether CanHSA exhibited a...
direct antitumor effect, we infected MDA-MB-231 and HUVEC cells in vitro with adenoviruses. Then, cells were fixed, but not permeabilized, and the presence of CanHSA and K1-3HSA was detected by immunostaining. Endothelial cells infected by AdCanHSA or AdK1-3HSA were clearly labeled compared with AdCO1-infected HUVEC cells (Fig. 4A). On the contrary, only MDA-MB-231 cells infected by AdCanHSA were labeled. Thus, CanHSA seems to localize at the extracellular surface of both HUVEC and MDA-MB-231 cells.

Endothelial cell migration and adhesion to the ECM are mediated by integrins, especially $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (19, 20), which also play a role in tumor progression (21). To identify the receptor that binds CanHSA, fluorescence-activated cell sorting (FACS) analysis was done using fluorescein-conjugated anti-$\alpha_v\beta_3$ or anti-$\alpha_v\beta_5$ mAbs on AdCO1- or AdCanHSA-infected cells. Initially, we characterized the presence of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on HUVEC and MDA-MB-231 cells following infection with mock adenovirus. $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were detected at high levels on endothelial cells. MDA-MB-231 cells express a low level of $\alpha_v\beta_3$ and a high level of $\alpha_v\beta_5$, supporting the results of Wong et al. (21). After expression of CanHSA by infected cells, we repeated the FACS analysis to assess the level of integrins bound to CanHSA, which would be inaccessible to anti-integrins mAbs. Fifty percent to 66% of the $\alpha_v\beta_3$ or $\beta_5$ integrins on the HUVEC and MDA-MB-231 cells were not bound to mAbs after expression of CanHSA (Fig. 4B). On the contrary, expression of

![Figure 3](image-url)

**Figure 3.** Tumor growth inhibition and detection of apoptosis following infection with AdCan and AdCanHSA. A, MDA-MB-231– (1) or PC-3–derived (2) tumor size on nude mice, following two intratumoral injections of the adenoviruses listed above, was measured once a week. Points, mean of six mice as compared with the AdCO1-treated group; bars, SE. Asterisks, statistical significance. 3, inhibition of tumor growth by CanHSA in comparison with the canstatin-, K1-3–, and K1-3HSA–treated groups highlights significant tumor regression mediated by CanHSA as compared with other treatment options versus AdCO1. 4, s.c. injected MDA-MB-231–derived tumor growth over ~8 weeks following systemic injection of AdCO1, AdCan, and AdCanHSA. TUNEL staining was done on adenoaviral-treated MDA-MB-231 tumors (day 30 postinfection) as previously described in A1. The proportion of apoptotic cells (arrows) per field (five fields per animal) were quantified within sections (200×) from AdCO1, AdK1-3, AdCan, or AdCanHSA–injected tumors. Representative fields of AdCO1 (1), AdK1-3HSA (2), and AdCanHSA (3). B, columns, mean of two mice for each treated group; bars, SD. C, cell cycle analysis. HUVEC and MDA-MB-231 cells were infected with AdCanHSA (1 and 4), AdK1-3HSA (2 and 5), or AdCO1 (3 and 6). At 96 hours postinfection, cells were fixed with 70% ethanol overnight and chromatin was stained with propidium iodide. FACS analysis was done twice for each group.
K1-3HSA impeded binding of anti-\(\alpha_v\beta_3\) mAb only on the endothelial cell surface. For each cell type, normal fluorescein-conjugated isotype control antibody IgG1 was used as a negative control, confirming that the observed signals were specific.

To confirm that CanHSA binds to \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrins at the cell surface, immunoprecipitations were done from AdCanHSA- or AdCO1-infected cell lysates with mAbs against a cell surface. For each cell type, normal fluorescein-conjugated extracts following hybridization with anti-HSA (data not shown). Precipitated AdCanHSA-infected endothelial and tumor cellular blotting revealed bands characteristic of CanHSA from immuno-precipitated mAbs. FACs analysis included a mouse FITC-conjugated IgG1 as negative control (red line). Y-axis, fluorescence intensity.

CanHSA-induced apoptosis is sequential to mitochondrial damage both in endothelial and tumor cells. Antagonism of \(\alpha_v\beta_3\) integrins have been shown to participate in inhibition of cell proliferation and migration as well as apoptosis (23).

To determine whether canstatin induces caspase-dependent apoptotic pathways, caspase-3, -8, and -9 activities in HUVEC and MDA-MB-231 cellular extracts were quantified. CanHSA treatment of HUVEC cells led to a 4-fold increase in caspase-9 activity as compared with CO1-treated cells (\(P = 0.03\)). K1-3HSA-treated cells exhibited no significant activation of caspase-9 (Fig. 5A). Caspase-8 activity was increased less strongly (1.5-fold) by either canstatin (\(P = 0.024\) as compared with AdCO1) or angiostatin. In addition, canstatin induced a 3.5-fold increase in caspase-3 activity as compared with CO1 treatment (\(P = 0.03\)), whereas angiostatin increased this level by only 2.5-fold. Each result was done by comparison to staurosporine, a known inducer of caspase-dependent apoptosis. The specificity of caspase cleavage was shown by addition of selective caspase-9 or -3 inhibitors to the cell medium.

Our results show that canstatin induced two distinct apoptotic pathways in endothelial cells through the activation of caspase-8 and caspase-9 leading to cleavage of procaspase-3 (7). K1-3 activates only the mitochondrial-independent pathway involving cleavage of caspase-8 and -3. CanHSA-treated MDA-MB-231 cells showed an activation of the mitochondrial caspase-9-dependent apoptotic process (increase of 1.7-fold; \(P = 0.04\) as compared with AdCO1; Fig. 5A). Additionally, PC-3 prostate tumor cells express \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrins, and activation of caspase-9 was observed following CanHSA or staurosporine treatment of PC-3 (\(P < 0.01\) as compared with AdCO1; Fig. 5A). No CanHSA-induced caspase-8 activation was detected in either tumor cell type (data not shown).

Caspase-dependent apoptotic events are mediated by binding of CanHSA to \(\alpha_v\beta_3\) and \(\beta_3\) integrins. To further characterize the functional receptors mediating the apoptotic signaling pathways for CanHSA, we used anti-integrin siRNAs to decrease \(\alpha_v\beta_3\) or \(\beta_3\) expression in HUVEC cells to inhibit binding of CanHSA to the cell surface. Incubation of AdCO1-infected HUVEC cells for 48 hours with anti-\(\beta_3\) anti-siRNAs or anti-\(\alpha_v\) siRNAs decreased the level of expression of each integrin population on the cell surface by 50% on average (Fig. 5B). Identical transfection with a random siRNA bearing no homology with any integrin genes had no effect on integrin expression.

The specific inhibition of \(\alpha_v\beta_3\) and \(\beta_3\) integrin expression significantly reduced caspase-9 activity following CanHSA treatment (decrease of 1.8- to 3.8-fold; \(P < 0.01\)) as compared with transfection of siRNA control oligo coupled with CanHSA infection; Fig. 5C).

As previously shown, K1-3HSA increases caspase-8 activity alone (by 2-fold; Fig. 5A). Inhibition of \(\alpha_v\beta_3\) or \(\beta_3\) integrin expression impeded the activation of caspase-8 following K1-3HSA, but not CanHSA, infection (Fig. 5D). Thus, binding of K1-3HSA with \(\alpha_v\beta_3\) integrins induces a caspase-8–dependent apoptotic pathway, whereas interaction of CanHSA with \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrins selectively triggers the caspase-9–dependent pathway.
Efficacy of Canstatin and Mechanism of Action

The soluble NC1 domain fragment of the α2(IV) chain of collagen, called canstatin, is known to potently inhibit tumor-associated angiogenesis (5–7).

Initially, we investigated the in vivo antiangiogenic properties and antitumor efficacy of canstatin using HSA fusion to increase the bioavailability of canstatin following adenoviral gene transfer. This study sought to evaluate the anticancer efficacy of CanHSA compared to that of the K1-3HSA as a reference molecule belonging to the plasminogen-derived class of proteins (10, 11). In vivo, CanHSA strongly inhibited the vascularization of tumors through a stable and long-lasting effect. Our approach provides a solution to maintain an in situ level of canstatin sufficient to strongly impede the growth of cancer and disrupt implantation of cancer cells and, as such, decrease the number of therapeutic injections required and thus, potentially, adherence to the treatment regime. Recently, therapeutic gene transfer became more commercially attractive following the debut of adenoviral-mediated p53 gene transfer for head and neck squamous cancers, called Gendicine (Sibiono GeneTech, Shenzhen, China).

Next, we attempted to delineate the mechanism of action of CanHSA to explain its improved efficacy as compared with K1-3HSA. The exact mechanisms by which α(NC1) domains, in particular canstatin (α,IV NC1), inhibit tumor angiogenesis are not completely understood. We show here that canstatin binds to the endothelial and tumor cell surface in an integrin-dependent manner. Integrins mediate adhesion to matrix proteins of both endothelial and tumor cells (19–21). Remodeling of the ECM promotes novel integrin-ligand interactions required for angiogenesis (1). As such, CanHSA could compete with collagen IV ECM for cell surface integrin binding and reverse the proliferative and migratory effects induced by cell-ECM interactions. Thus, α3β5 and α5β3 integrins would seem to mediate the antiangiogenic and direct antitumor properties of CanHSA. Just two other proteins are known to have a direct effect on tumor cells through interaction with integrins: endostatin (α5β3) and tumstatin (α6β3), which are both also collagen-derived NC1 domain fragments (24–26). Interestingly, Tarui et al. (27) also showed that K1-3 binds only to α3β5 integrins on endothelial cells. Integrin receptors are known to cross-talk with other receptors and their associated pathways (22). Angiogenesis proceeds through two cytokine-dependent pathways: one depending on α3β5 that is largely induced by basic fibroblast growth factor and vascular endothelial growth factor (VEGF), and another one which is potentiated by α5β3 and VEGF alone (19, 20). As such, dual antagonists of both α3β5 and α5β3, such as canstatin, as we have established here, would have a definite therapeutic advantage by blocking the two tumor-induced angiogenesis pathways.

Here, we investigated the potential role of integrins as regulators of apoptosis (23, 28). We have shown that in vitro CanHSA strongly inhibited the migration and proliferation of HUVEC cells. Moreover, our present findings show that these events are mediated by an upstream event involving canstatin binding to α3β5 and α5β3 integrins, which has not yet been reported. Integrin receptor engagement and subsequent clustering lead to transduction of positional cues from the ECM to intracellular signaling phosphorylation cascades that promote cell survival, such as the activation of focal adhesion kinase/phosphatidylinositol 3-kinase (FAK/Pi3K) pathway (23, 29). Phosphorylated FAK promotes integrin-mediated cell proliferation and migration events (29). When cell-ECM interactions are lost, cells undergo a type of apoptosis called

Figure 5. Determination of CanHSA-induced apoptotic pathway and the key receptor(s) through siRNA-mediated silencing of α3, β3, or β5 expression. A. HUVEC (1–3), MDA-MB-231 (4), or PC-3 (5) cells were infected with AdCO1, AdK1-3HSA, or AdCanHSA for 48 and 72 hours, respectively. Whole cell lysates were mixed with reaction buffer and caspase colorimetric substrate as recommended by R&D Systems. A Bradford assay was done to normalize the solution to maintain an

Discussion

The soluble NC1 domain fragment of the α2(IV) chain of collagen, called canstatin, is known to potently inhibit tumor-associated angiogenesis (5–7).

Initially, we investigated the in vivo antiangiogenic properties and antitumor efficacy of canstatin using HSA fusion to increase the bioavailability of canstatin following adenoviral gene transfer. This study sought to evaluate the anticancer efficacy of CanHSA compared to that of the K1-3HSA as a reference molecule belonging to the plasminogen-derived class of proteins (10, 11). In vivo, CanHSA strongly inhibited the vascularization of tumors through a stable and long-lasting effect. Our approach provides a solution to maintain an in situ level of canstatin sufficient to strongly impede the growth of cancer and disrupt implantation of cancer cells and, as such, decrease the number of therapeutic injections required and thus, potentially, adherence to the treatment regime. Recently, therapeutic gene transfer became more commercially attractive following the debut of adenoviral-mediated p53 gene transfer for head and neck squamous cancers, called Gendicine (Sibiono GeneTech, Shenzhen, China).

Next, we attempted to delineate the mechanism of action of CanHSA to explain its improved efficacy as compared with K1-3HSA. The exact mechanisms by which α(NC1) domains, in particular canstatin (α,IV NC1), inhibit tumor angiogenesis are not completely understood. We show here that canstatin binds to the endothelial and tumor cell surface in an integrin-dependent manner. Integrins mediate adhesion to matrix proteins of both endothelial and tumor cells (19–21). Remodeling of the ECM promotes novel integrin-ligand interactions required for angiogenesis (1). As such, CanHSA could compete with collagen IV ECM for cell surface integrin binding and reverse the proliferative and migratory effects induced by cell-ECM interactions. Thus, α3β5 and α5β3 integrins would seem to mediate the antiangiogenic and direct antitumor properties of CanHSA. Just two other proteins are known to have a direct effect on tumor cells through interaction with integrins: endostatin (α5β3) and tumstatin (α6β3), which are both also collagen-derived NC1 domain fragments (24–26). Interestingly, Tarui et al. (27) also showed that K1-3 binds only to α3β5 integrins on endothelial cells.

Integrin receptors are known to cross-talk with other receptors and their associated pathways (22). Angiogenesis proceeds through two cytokine-dependent pathways: one depending on α3β5 that is largely induced by basic fibroblast growth factor and vascular endothelial growth factor (VEGF), and another one which is potentiated by α5β3 and VEGF alone (19, 20). As such, dual antagonists of both α3β5 and α5β3, such as canstatin, as we have established here, would have a definite therapeutic advantage by blocking the two tumor-induced angiogenesis pathways.

Here, we investigated the potential role of integrins as regulators of apoptosis (23, 28). We have shown that in vitro CanHSA strongly inhibited the migration and proliferation of HUVEC cells. Moreover, our present findings show that these events are mediated by an upstream event involving canstatin binding to α3β5 and α5β3 integrins, which has not yet been reported. Integrin receptor engagement and subsequent clustering lead to transduction of positional cues from the ECM to intracellular signaling phosphorylation cascades that promote cell survival, such as the activation of focal adhesion kinase/phosphatidylinositol 3-kinase (FAK/Pi3K) pathway (23, 29). Phosphorylated FAK promotes integrin-mediated cell proliferation and migration events (29). When cell-ECM interactions are lost, cells undergo a type of apoptosis called
results show that canstatin induces a profound activation of spreading and migration and thus apoptosis. Could mediate FAK inactivation, leading to suppression of cell events (3).

Collectively, these data suggest that the binding of canstatin to \( \alpha_5\beta_3 \) and \( \alpha_\beta_5 \) integrins, shown here, could mediate FAK inactivation, leading to suppression of cell spreading and migration and thus apoptosis.

To further document endothelial cell-specific apoptosis, our results show that canstatin induces a profound activation of caspase-9 as compared with K1-3 treatment of cells. This pathway is triggered by both \( \alpha_\beta_3 \) and \( \alpha_\beta_5 \) integrins. Both canstatin and K1-3 induce the mitochondria-independent pathway with a similar increase in caspase-8 activity. Our findings show that canstatin initiates two apoptotic pathways including activation of caspase-8 and -9, both initiators of the downstream apoptotic process leading to activation of caspase-3. Canstatin-activated caspase-8, by down-regulation of Flip levels and up-regulation of Fas/Fas ligand, triggers not only cell death directly through caspase-3 activation but also indirectly through mitochondrial damage via activation of caspase-9 within the apoptosome (7, 31, 32). On the other hand, phosphorylated FAK/PI3K is known to inactivate the mitochondrial apoptotic pathway by inhibition of caspase-9 (30). So, canstatin directly activates procaspase-9 through inhibition of the FAK/PI3K pathway and amplifies the Fas-dependent pathway in mitochondria (ref. 29; Fig. 6).

This dual mechanism explains how canstatin strongly enhances caspase-9 activity and therefore the number of cells entering apoptosis. Mitochondrial disruption and caspase-9 activation can engage in a circular self-amplification loop that accelerates the apoptotic process. Mitochondrial damage seems to be the critical point of no return of the cell death process (33).

Several studies have shown that angiostatin inhibits endothelial cell function by increasing apoptosis (11, 15, 16). However, the moderate reduction in K1-3–treated endothelial cell number seen in our proliferation assays is due to a single activation of caspase-8 without mitochondrial damage following specific interaction of K1-3HSA with \( \alpha_\beta_3 \). Recently a study has described that unligated \( \alpha_\beta_3 \) integrins (but not \( \beta_3 \)) or soluble antagonists of this receptor initiate endothelial apoptosis, called integrin-mediated death, by recruitment of caspase-8 to the plasma membrane independent of interaction of Fas/Fas ligand with the death domain (34). However, CanHSA-induced caspase-8 activity was not disrupted following \( \alpha_\beta_3 \) gene silencing, as we have established following treatment with K1-3HSA. In this case, canstatin would act only via one of two possible mechanisms: Fas-mediated pathway in caspase-8 activity; the second possible mechanism (i.e., integrin-mediated death leading to caspase-8 activity) being abrogated due to siRNA knockdown of \( \alpha_\beta_3 \) (Fig. 6).

Collectively, it would seem that the involvement of distinct apoptotic pathways is correlated with profound cell death and thus a strong antitumor effect.

In our study, we report that canstatin inhibits tumor development in vivo and enhances apoptotic induction in tumor cells through generation of active caspase-9. No caspase-dependent apoptotic mechanism was observed in K1-3–treated tumor cells, as described previously by Lucas et al. As confirmed by other authors, fragments derived from NC1 domains, like tumstatin, could act as a specific antitumor antagonist of \( \alpha_\beta_3 \) integrins in melanoma progression (26).

In conclusion, CanHSA is a powerful anticancer molecule which triggers two distinct programmed cell death pathways in endothelial cells which converge on mitochondrial disruption: the \( \alpha_\beta_3 \)-integrin-FAK/PI3K/caspase-9 pathway and the Fas/Fas ligand/caspase-8/caspase-9 cascade. These two pathways result in amplification of mitochondrial-linked apoptotic events and activation of caspase-3, the central executioner of the apoptotic process.

Our study argues for the delivery in situ of therapeutic gene with mitochondrial-dependent proapoptotic properties to improve antiangiogenic and antitumor features of such selective dual \( \alpha_\beta_3 \) and \( \alpha_\beta_5 \) agonists fused with a carrier protein.
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References

Efficacy of Canstatin and Mechanism of Action

In the article on the efficacy of canstatin and mechanism of action in the May 15, 2005 issue of Cancer Research (1), Céline Bouquet should have appeared in the list of authors. The list of authors should have read as the following: Claire Magnon, Ariane Galaup, Brian Mullan, Valérie Rouffiac, Céline Bouquet, Jean-Michel Bidart, Frank Griscelli, Paule Opolon, and Michel Perricaudet. Dr. Bouquet’s affiliation is UMR 8121 Laboratoire de vectorologie et transfert de gènes, Institut Gustave Roussy, Villejuif cedex, France.

Canstatin Acts on Endothelial and Tumor Cells via Mitochondrial Damage Initiated through Interaction with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ Integrins

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