Headpin: A Serpin with Endogenous and Exogenous Suppression of Angiogenesis

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Abstract

Headpin is a novel serine proteinase inhibitor (serpin) with constitutive mRNA expression in histologically normal oral mucosa but with lost or down-regulated expression in head and neck squamous cell carcinoma. Several serpin family members are similarly lost in multiple cancer types and hold tumor suppressor functions including the inhibition of angiogenesis. However, the functional significance for the loss of headpin expression in cancer is not known. Using immunohistochemical analysis of invasive squamous cell carcinoma and matched normal squamous mucosa of patient specimens, headpin expression was lost or down-regulated in the vast majority of tumor specimens. We investigated the functions of exogenous recombinant headpin and endogenously expressed headpin related to angiogenesis. In a rat corneal assay of neovascularization, recombinant headpin protein blocked in vivo angiogenesis mediated by interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF). In assays of cellular events in angiogenesis, headpin blocked the invasion, migration, and tube formation of endothelial cells. In light of our findings of nuclear subcellular localization of headpin, we investigated the expression and secretion of angiogenic factors and found reduced mRNA, protein, and promoter activities of IL-8 and VEGF. Finally, using a murine flank tumor model, headpin expression reduced growth and microvessel density in tumors derived from headpin-expressing UMSCC1 cells relative to those from vector control cells. These findings of nuclear regulatory functions of a serpin in the inhibition of angiogenesis bring new understanding to the cellular and molecular mechanisms of serpins. Therefore, this novel serpin targets diverse mechanisms against tumor angiogenesis on which to base therapeutic strategies. (Cancer Res 2005; 65(24): 11501-9)

Introduction

The serine proteinase inhibitor (serpin) family includes members with tumor suppressor functions that oppose tumorigenesis, proliferation, angiogenesis, invasion, and metastasis in a broad range of cancers. Several highly conserved serpin genes, including plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2), squamous cell carcinoma antigen 1 and 2 (SCCA-1 and SCCA-2), maspin, and headpin, cluster to chromosome 18q21.3 to q23 (1). This locus is a site of frequent loss in multiple cancer types (2–5). Moreover, the loss of serpin expression has been correlated with advanced tumor progression (6–9).

We initially identified headpin as a head and neck serpin that shows strong constitutive expression in histologically normal oral mucosa but profoundly reduced mRNA expression in head and neck squamous cell carcinoma (HNSCC; ref. 10). The gene was independently discovered by Abts et al. (11) on the basis of differential expression in UV-modulated keratinocytes. We then showed the inactivity of the functional promoter region of headpin in head and neck carcinoma lines, consistent with dysregulation of transcription as an explanation for the differential expression of headpin (12). Subsequently, we and others investigated the interaction of recombinant headpin (r-headpin) protein with target enzymes and found that r-headpin inhibits the cysteine proteinases cathepsin K and L (13, 14).

A paradigm for serpin function in cancer is based on the inhibition of proteinases in the cascade of extracellular matrix degradation, a critical pathway in angiogenesis, tumor invasion, and metastasis. For instance, by reacting to form a stable complex with urokinase plasminogen activator, the extracellular forms of PAIs employ a suicide substrate-like inhibition mechanism (15). However, the role of serpins in cancer has proved more complex on the basis of the findings of several studies investigating mechanisms of serpin function. For example, Darnell et al. (16) have shown a novel intracellular function of PAI-2 in the inhibition of retinoblastoma protein degradation. Additionally, the tumor suppressor functions of maspin seem to involve complex non-inhibitory mechanisms associated with the cell surface and cytoplasm in addition to extracellular secretion (17–19). Moreover, recent studies in which immunohistochemical analysis was done have shown the nuclear and cytoplasmic localization of maspin in specimens from patients with breast, lung, and ovarian cancers (20–22). The correlation of nuclear localization of maspin with favorable clinicopathologic features in these cancers suggests that serpins regulate nuclear functions.

Therefore, although the role of serpins in cancer is known, the cellular and molecular events responsible for tumor suppression are not well understood. Because of the diverse involvement of serpins in cancer progression, these proteins offer promising therapeutic potential by targeting multiple mechanisms against tumor growth, invasion, and metastasis. However, an improved understanding of the cellular and molecular mechanism of serpins in cancer is requisite to developing serpin-based strategies of tumor suppression.
To gain a clearer understanding of the mechanisms whereby serpins function in tumor suppression, we sought to determine the biological and functional significance of the loss of headpin expression in HNSCC. We investigated the tumor suppressor effects of headpin on angiogenesis by studying the effects of r-headpin protein on effector cells of the tumor microenvironment and by returning the expression of headpin to head and neck cancer cell lines. We found that headpin localizes to the nucleus and regulates the transcription of mediators of cell proliferation. Furthermore, headpin functions intracellularly to regulate the transcription of key angiogenic factors and extracellularly to block several steps in endothelial cells vital for angiogenesis.

Materials and Methods

Cell cultures, reagents, transfection, and cell extracts. Vector and headpin stable clones in UMSCC1 and parental Tu138 cell lines were maintained in DMEM-F12 with 10% FCS. Human umbilical vein endothelial cells (HUVEC) were maintained in Medium 131 with microvascular growth supplement from Cascade Biologies (Portland, OR). Primary normal oral epithelial cells were explanted and cultivated in keratinocyte growth medium (Clonetics, Walkersville, MD) as previously described (23). SF9 insect cells were maintained in Insect-XPRESS from Cambrex BioScience (Walkersville, MD). Actin antibody and propidium iodide were obtained from Sigma (St. Louis, MO); antibodies against headpin and CD31 and endothelial assays of tube formation, migration, and invasion assay chamber from BD BioScience (Bedford, MA); vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) from Peprotech (Rocky Hill, NJ); VEGF and IL-8 ELISA kits from R&D Systems, Inc. (Minneapolis, MN); and luciferase assay kits from Promega, Inc. (Madison, WI). Cell lysates were resolved on 10% SDS-PAGE unless mentioned otherwise. Transient transfection studies were done in UMSCC1 cell null for headpin expression using a Lipofectamine 2000 kit from Invitrogen (Carlsbad, CA) in accordance with the instructions of the manufacturer. A fragment containing the complete coding sequence of headpin was subcloned in pCDNA3.0 and isolated clones were selected and maintained in 0.4 mg/mL G418. Stable transfection studies were done using a fragment containing the complete coding sequence of headpin subcloned into expression vector pTRE2hyg and cotransfected with the pTet-Off and pTRE2hyg headpin plasmid. Clones were selected and maintained in 0.4 mg/mL G418, 0.05 mg/mL hygromycin, and 1 μg/mL doxycycline. Unless mentioned otherwise, cell extracts were made in NP40 lysis buffer.

Immunohistochemical analysis of patient specimens. Consent was obtained from all patients with HNSCC for the use of specimens of tumor and adjacent normal mucosa in accordance with a protocol approved by the Institutional Review Board of The University of Texas M.D. Anderson Cancer Center. Sections were deparaffinized and rehydrated with successive washes of xylene and decreasing concentrations of ethanol in water. Antigen retrieval was done by heat and the slides were processed in an autostaining machine with LSAB plus kit (Dako, Carpinteria, CA). Endogenous peroxide and protein blocking steps were done and followed by primary antibody incubation with anti-headpin or control mouse immunoglobulin G (IgG) at 1:150 dilution in antibody diluent for 1 hour. Secondary staining was done with link-horseradish peroxidase and streptavidin with 3,3’-diaminobenzidine (DAB) and counterstaining with hematoxylin. Slides were visualized and digitally imaged with a light microscope and polychromatic camera (Leica Microsystems, IL). Tumor specimens were compared with the corresponding adjacent normal mucosa specimens from the same patient.

Rat corneal angiogenesis assay. Angiogenesis was studied in vivo in an assay of neovascularization in the rat cornea as previously described (24). All animals were handled in accordance with the M.D. Anderson Cancer Center Department of Veterinary Medicine and Institutional Animal Care and Use Committee. Experiments were done in duplicate for each condition with at least 12 animals per experiment. Image analysis was carried out with Image Pro Plus software (Media Cybernetics, Silver Spring, MD) to determine the area of neovascularization by calculating the total area of new blood vessels within the cornea of each image as previously described (24). The total vascularization was determined by summing the pixel values of that area within each image containing new vessels. Experiments using at least six animals were done twice using VEGF and thrice using IL-8 as the mediator of angiogenesis.

Endothelial cell assays. HUVECs were grown to 80% to 90% confluence in DMEM supplemented with 5% fetal bovine serum (FBS). Before the assays, cells were resuspended in DMEM containing 0.1% bovine serum albumin. In assays of endothelial invasion, cells were seeded in the top chamber (150,000 per 250 μL) of 24-well plates with a Matrigel-coated, fluorescence-blocking membrane insert (BD Bioscience). Culture medium (5% FCS) alone or with various concentrations of headpin (750 μL) was placed in the bottom chambers. In assays of endothelial migration, cells were starved in basal growth medium for 2 to 3 hours at 37°C and seeded in the top chamber (150,000 in 250 μL) of 24-well plates with a fibronectin-coated, fluorescence-blocking membrane insert (BD Bioscience). Serum-free medium with VEGF at 20 ng/mL alone or with various concentrations of headpin (750 μL) was placed in the bottom chambers. In assays of endothelial tube formation, cells were seeded (20,000 in 50 μL) into 96-well plates coated with a Matrigel (BD Bioscience) and incubated with 50 μL of serum-free basal growth media alone, with VEGF or IL-8 at 20 ng/mL, or with headpin. Cells were allowed to invade or migrate for 22 ± 1 hours at 37°C. Following incubation, cells were labeled with 4 μg/mL of calcein AM (Molecular Probes, Eugene, OR). Migrating or invading cells and endothelial tube lengths were measured by direct fluorescence of cells with a plate reader at 485-nm excitation and 530-nm emission. Fluorescence values were corrected for background and expressed relative to the cells migrating through an uncoated insert membrane to determine the percent invasion or migration. The plates were visualized and digitally imaged with a fluorescence microscope and polychromatic camera (Leica Microsystems). All experiments were repeated at least twice.

ELISA and promoter analysis. VEGF and IL-8 concentrations were determined using VEGF and IL-8 Quantiglo ELISA kits (R&D Systems) following the instructions of the manufacturer. Samples from three different experiments were analyzed in duplicate. Promoter assays were done after transient transfections of the respective reporter constructs (VEGF-Luc and IL-8-Luc, 200 ng) in the presence or absence of headpin (pCDNA3.0, 1 μg). Samples were lysed and measured by using a Promega assay kit (Promega Biosciences, Inc., San Luis Obispo, CA). Results were presented after normalization with β-galactosidase. All experiments were repeated.

Real-time reverse transcription-PCR. Total RNA isolated with Trizol (Invitrogen; 2 μg) was reverse transcribed by Superscript II (Invitrogen). Primers were purchased from Applied Biosystems (Foster City, CA) as Assays-on-Demand gene expression products. Real-time PCR (in triplicate) was done using an ABI Prism 7900HT machine. Reverse transcription-PCR (RT-PCR) for IL-8 (NM_000584) used primers 5′-CTCCAAAACCTTTCCACCACCAAT and 5′-AACCTTTCAACACACCCTGCAG; for VEGF-C (NM_005429), 5′-AACCTTCAATGTTGCTGCTCATA and 5′-GCTGGCAGAGACGTCATAATG; for actin, 5′-CCTCCCTGGGAGAGTACCGAC and 5′-GACAGCATCTGTTGGCGTACAG; and for headpin, 5′-GTCCAGGCGAATGGAAAGGA and 5′-GGGTGATTTGCGTGGAACAT.

Mouse flank tumor model. Female athymic nude mice, ages 6 to 8 weeks (Harlan Sprague-Dawley, Inc., Indianapolis, IN), were used in all experimental flank tumor procedures and cared for in accordance with the Institutional Animal Care and Use Committee and the Department of Veterinary Medicine of M.D. Anderson Cancer Center. Twenty to thirty mice per experiment were inoculated with s.c. injection with UMSCC1 vector control cells or headpin-expressing clones (5 × 10^6 cells). The mice were monitored for body weight and tumors were measured weekly. The animals were euthanized and the tumors were removed, photographed, weighed, and bisected. Mean tumor volumes in cubic millimeters for each group were calculated by the formula $V = \frac{4}{3} \pi r^3$, where $r$ represents length and $w$ is width. For H&E staining, tissues were fixed in formalin and embedded in paraffin. For immunohistochemical analysis, tissues
were placed in optimum cutting temperature compound and frozen on dry ice.

**Immunohistochemical staining of animal specimens.** Frozen mouse tumors were sectioned at 5 µm and fixed in cold acetone for 10 minutes and washed with PBS. Immunostaining was done by using an automated staining machine (DAKO). For CD31 staining, endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 5 minutes followed by incubation with protein blocking solution (1% normal goat serum and 5% normal horse serum) for 5 minutes. A primary antibody using a purified rat anti-mouse CD31 (Invitrogen) was incubated with a biotinylated antimouse secondary antibody [Animal Research Kit (ARK), DAKO] diluted 1:200 for 30 minutes. Slides were incubated with a primary-secondary antibody for 30 minutes followed by streptavidin-ARK for 30 minutes. Slides were then washed and incubated with DAB substrate and counterstained with hematoxylin.

**Quantification of immunohistochemical staining.** The mean number of vessels in the specimens was determined by counting vessel-like structures consisting of endothelial cells that were stained with anti-CD31 antibody in 50× fields from each of four quadrants of tumors of approximately equal sizes. Image analysis was carried out with Image Pro Plus software (Media Cybernetics) to determine the staining area for proliferating cell nuclear antigen or CD31 by calculating the total area within each image containing cells staining positively. The staining intensity for CD31 was determined by summing the pixel values of the area within each image containing cells staining positively.

**Statistical analysis.** In corneal assays of angiogenesis, the areas of neovascularization and total vascularity were analyzed as numerical dependent variables to determine the mean and median differences between the control right and test left corneas of each animal. Differences between groups were compared with a Wilcoxon matched pairs test. In mouse flank tumor experiments, the numerical dependent variables of tumor volumes, tumor weights, vessel counts, CD31 staining area, and CD31 staining intensity were analyzed for differences between groups with a two-tailed Wilcoxon rank-sum test. A P < 0.05 was considered statistically significant for all tests. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, Inc, San Diego, CA) and NCSS software programs (NCSS, Kaysville, UT).

**Results**

**Headpin expression is lost in head and neck squamous cell carcinoma.** We did immunohistochemical analysis on matched specimens of normal oral mucosa and squamous cell carcinoma of various upper aerodigestive tract sites and stages from 60 unselected patients. In specimens of histologically normal mucosa, intense staining was present in epithelial cells throughout the suprabasal layers (Fig. 1A, top left, inset). Normal squamous epithelial cells stained for headpin in a nuclear as well as a cytoplasmic pattern (Fig. 1A, top left). Conversely, in the vast majority of specimens of invasive carcinoma, only weak staining was present in a few cells scattered within the tumor (Fig. 1A, top right, arrow). Further investigation revealed in vitro expression of headpin in normal oral epithelial cells but not in the majority of established HNSCC lines. Figure 1B shows the presence of headpin protein on a Western blot of cell lysates from normal oral epithelial cells but not from the HNSCC lines Tu138 and UMSSC1. These results show that headpin expression is constitutive in normal mucosa but down-regulated or lost in HNSCC, in parallel with our previous findings for mRNA transcription.

**Recombinant headpin blocks in vivo angiogenesis.** In exploring potential secretory pathways of headpin function in the extracellular matrix, we showed by immunoprecipitation-Western blotting that headpin proteins are present in conditioned media from headpin-expressing UMSSC1 cells (Fig. 2A). This finding suggests that headpin interacts with effector stromal cells of the tumor microenvironment. Among the consequences of such interactions, maspin has been shown to block in vitro and in vivo angiogenesis (25).

We determined the effects of headpin on angiogenesis in vivo with r-headpin (purified from a baculovirus insect cell system; Fig. 2B) using a rat corneal assay of neovascularization. In this assay, the growth of new blood vessels into the avascular cornea from the limbus was studied by microsurgically implanting the cornea with r-headpin carried in a vehicle of inert hyaluron polymer. After 6 days, animals were euthanized and perfused with...
India ink for microscopic visualization of harvested corneal specimens. After our initial findings revealed that r-headpin alone had no effect on corneal angiogenesis, we studied its effects on neovascularization mediated by the angiogenic factors IL-8 and VEGF. R-headpin blocked corneal neovascularization mediated by IL-8 in 92% of the experimental animals and showed concentration-dependent inhibition optimally at 6 μmol/L (data not shown). The typical angiogenic response to IL-8 is shown by the dense ingrowth of ink-filled capillary loops from the limbus into the cornea in a representative image from an animal in which the cornea of the right eye was implanted with IL-8 alone (Fig. 2C, top left). Conversely, dramatic inhibition of that response is shown by the absence of vessels in the cornea of the left eye of the same animal in which r-headpin was added to IL-8 in the cornea (Fig. 2C, top right). Likewise, r-headpin also blocked corneal neovascularization mediated by VEGF (Fig. 2C, bottom, right and left).

Corneal specimens were analyzed in a computer-assisted comparison (24) of the test left eye (r-headpin plus cytokine) with the control right eye (cytokine alone) of each animal. The area of neovascularization in corneas implanted with r-headpin plus IL-8 was reduced by a mean of 44.8% [95% confidence interval (95% CI), 20.5-69.2%] from that in control corneas implanted with IL-8 alone ($P = 0.026$; Fig. 2D, top left). Furthermore, the area of neovascularization in corneas implanted with r-headpin plus VEGF was reduced by a mean of 60.0% (95% CI, 37.6-81.3%) from that in control corneas implanted with VEGF alone ($P = 0.003$; Fig. 2D, top right). The total vascularity in the corneas implanted with r-headpin plus IL-8 was reduced by a mean of 50.0% (95% CI, 16.3-83.7%) from the total vascularity in the control corneas implanted with IL-8 alone ($P = 0.05$; Fig. 2D, bottom left). Similarly, in corneas implanted with r-headpin plus VEGF, the total vascularity was reduced by a mean of 60.7% (95% CI, 36.6-84.9%) from that in the control corneas implanted with VEGF alone ($P = 0.003$; Fig. 2D, bottom right). These findings show that r-headpin profoundly blocks the in vivo angiogenesis mediated by both VEGF and IL-8 in a rat corneal assay as assessed quantitatively by objective measures of neovascularization.

**Headpin blocks angiogenic events in endothelial cells.** The inhibition of any one of the stepwise events in angiogenesis can interrupt new vessel formation in vivo (26). To identify the cellular mechanisms by which headpin blocks in vivo angiogenesis, we sought to determine the effects of exogenous r-headpin on these events in vitro.

To elucidate host endothelial cells recruited for tumor neovascularization by various angiogenic stimuli to invade the extracellular basement membrane, we studied the invasion of HUVECs across a microporous membrane coated with Matrigel. The addition of headpin at 100 nmol/L to 5% FBS reduced the relative endothelial cell invasion by 77.9% (95% CI, 71.0-84.9%; $P < 0.05$; Fig. 3A, left). The qualitative difference in postinvasion

![Figure 2](image-url)
fluorescent-labeled cells is shown in the absence and presence of r-headpin (Fig. 3A, middle and right, respectively).

To investigate endothelial migration, we studied the movement of HUVEC across a fibronectin-coated microporous membrane. Headpin blocked VEGF-mediated HUVEC migration in a concentration-dependent manner and reduced relative migration by 53.5% (95% CI, 29.0-78.0%) at 100 nmol/L and by 107.5% (95% CI, 97.6-117.5%) at 1 μmol/L, headpin (P < 0.05 for each condition; Fig. 3B).

Finally, to investigate the differentiation of HUVEC into the capillary-like structures of precursory vessels, we studied the tube formation of HUVEC in a matrix of Matrigel. The formation of endothelial tubes is shown by the early organization of cells into networks under basal growth media conditions, which is then followed by the assembly of lumen-containing structures in growth media containing VEGF at 20 ng/mL (Fig. 3C, left and middle, respectively). The addition of 100 nmol/L headpin to the latter condition blocked the earliest steps in cell organization (Fig. 3C, right). R-headpin reduced the relative tube length by 91.6% (95% CI, 79.6-103.6%) as measured in pixel units by fluorescent labeling (Fig. 3D). Likewise, r-headpin blocked IL-8-mediated tube formation and reduced the relative tube length by 92.7% (95% CI, 83.2-102.2%). These results show that headpin acts on endothelial cells to block several critical steps in angiogenesis and provide a framework for understanding the cellular mechanisms for the in vivo inhibition of angiogenesis by headpin.

**Headpin down-regulates the transcription of key mediators of angiogenesis.** To investigate the biological significance of the down-regulation or loss of headpin expression, we generated headpin-expressing clones in HNSCC lines. We used a mammalian expression vector encoding headpin cDNA and isolated clones in UMSCC1 cell lines that stably express the headpin protein. Western blot analysis showed headpin expression in cell lysates of multiple clones of each cell line but not of corresponding vector cells (data not shown). In identifying the subcellular localization of headpin protein, we found nuclear as well as cytoplasmic distribution of headpin in both normal oral epithelial and headpin transfectants of the UMSCC1 cell line by confocal immunofluorescence microscopy. Moreover, Western blot analysis detected headpin in the nuclear and cytoplasmic fractions of each of headpin-expressing clones C2 and C6 of UMSCC1 but not in vector clone cells (Fig. 4A). These findings are consistent with the observations of our immunohistochemical analysis of headpin expression in patients specimens and suggest a biological function of headpin in nuclear regulation that is lost in cancer cells. Thus, we sought to identify the functions of headpin in the nuclear regulation of HNSCC cells.

To explore the direct effects of headpin on the angiogenic potential of tumor cells, we examined VEGF and IL-8 production in relation to headpin expression. By RT-PCR of total mRNA, we found a reduced message of both VEGF and IL-8 in each of two headpin-expressing clones of UMSCC1 cells relative to vector cells (Fig. 4B, left). Furthermore, by real-time PCR, we found a 3.5-fold reduction in IL-8 mRNA levels in headpin transfectants relative to vector cells (Fig. 4B, right). To evaluate these findings at the protein level, we did ELISA on conditioned media to determine secreted VEGF and IL-8 levels. In headpin-expressing clones, protein levels were reduced by up to 90% for VEGF and 80% for IL-8 (Fig. 4C). To further investigate the mechanism by which headpin might regulate the production of VEGF and IL-8, we did promoter assays in which luciferase reporter constructs were transiently transfected in UMSCC1. In the presence of headpin, the relative promoter activity was reduced by 64% and 75% for VEGF and IL-8, respectively (Fig. 4D). These findings suggest that headpin expression regulates the transcription of both IL-8 and VEGF, key angiogenic factors up-regulated in the tumor microenvironment.

**Headpin expression reduces tumor growth potential and microvessel density in vivo.** To examine the changes in growth characteristics of tumors in which headpin expression was returned to the microenvironment, we inoculated mice with headpin-expressing clones of UMSCC1 and vector control cells. The growth of tumors derived from headpin transfectants was profoundly reduced from that of vector controls. The disparity in gross tumor size is shown by the bulky, lobulated, hypervascular mass of a typical xenograft derived from vector control cells, in contrast to the small, discrete, avascular mass of a typical xenograft from headpin transfectants (Fig. 5A, left, top, and bottom, respectively). The ex vivo mean weight of tumors from headpin transfectants was reduced by 80% compared with that from vector clones (n = 15 in each of two groups; Fig. 5B).

We then analyzed the H&E staining of tumor specimens to identify mechanisms that might explain the reduced growth of tumors derived from headpin transfectants. Despite the disparity in tumor sizes, no difference in tumor differentiation or microscopic pattern of growth was found between tumors derived from headpin transfectants and vector cells. However, tumors derived from headpin transfectants showed less cellularity and more frequent central necrosis (Fig. 5C, left, top and bottom).

To investigate the effects on angiogenesis of returning headpin expression to the tumor microenvironment in vivo, we determined the relative microvessel density of excised tumors by staining with anti-CD31 to detect endothelial cells. There was a distinct difference in the staining pattern between endothelial cells forming vessels in a representative tumor derived from vector cells in contrast to that of the sparse staining of scattered endothelial cells in a tumor derived from headpin transfectants (Fig. 5C, right, top and bottom). In tumors derived from vector cells, a mean of 63.5 microvessels composed of positively staining cells was counted per 50× field (95% CI, 39.8-87.2 vessels) in each of four quadrants. Conversely, in tumors from headpin transfectants, a mean of 31.5 microvessels was counted per field (95% CI, 20.1-42.9 vessels). Therefore, in tumors derived from headpin transfectants, the relative microvessel density was reduced by 50% from that of tumors of comparable size derived from vector cells (Fig. 5D, left). Image analysis of CD31 staining revealed similar results, with a 45% reduction in the area of CD31 staining and a 45% reduction in the intensity of CD31 staining in tumors derived from headpin transfectants compared with those from vector cells (Fig. 5D, middle and right). These results confirmed and extended our in vitro findings of headpin expression in the regulation of angiogenesis.

**Discussion**

Our findings that r-headpin inhibits endothelial cell invasion, migration, and tube formation in vitro and angiogenesis in vivo support the notion that this serpin functions in the microenvironment as a secreted protein. However, a lack of consensus remains about the mechanism of action of serpins in the extracellular...
environment. Indeed, in vitro investigations of recombinant maspin protein suggest diversity in the interaction between serpins and effector cells. For example, Zhang et al. (25) found that mutation of the reactive site loop of maspin had no influence on the inhibitory effect of maspin on angiogenesis either in vivo or in vitro. In contrast, the inhibitory effects of exogenous recombinant maspin on tumor cell motility and invasion are neutralized by an antibody to the reactive site loop peptide of maspin (27). Therefore, although the antiangiogenic inhibitory functions of serpins seem to be independent of their antiprotease activity, other inhibitory functions seem to be dependent on the antiprotease activity of the reactive site loop. Determining the relationship of the antiprotease activity of headpin to its antiangiogenic functions will be important in elucidating the mechanisms of headpin.

Moreover, our findings challenge the dogma of serpin function in the extracellular matrix by suggesting an additional biological role for serpins in nuclear regulation. More importantly, alteration in the nuclear function of serpins may result in unfavorable tumor progression. The concept of serpin function is currently based on the interaction between serpins and target proteases localized in the cytoplasm or extracellular matrix. We showed that headpin protein was subcellularly localized in the nucleus and cytoplasm of headpin transfectants. Moreover, the similar pattern in the subcellular distribution of headpin in normal mucosa suggests a nuclear function of headpin in the regulation of normal cells.

Although the nuclear localization of serpin proteins has been reported, these studies were not designed to study serpin function. Rather, immunohistochemical analysis of tumor specimens was used to investigate serpin proteins as markers for diagnosis and prognosis in various primary cancers. Nonetheless, the observations of those studies have raised important functional implications. In the case of maspin, a consensus is that the finding of nuclear expression is favorable in some cancers. For example, the nuclear expression of maspin was significantly associated with good prognostic factors in breast cancer (20), increased survival and longer duration of remission after resection of lung cancer (21), and a low malignant potential ovarian cancer (22). These findings are consistent with our observations that reexpression of headpin inhibits transcription and secretion of the proangiogenic factors IL-8 and VEGF. Some insight into the nuclear mechanism of headpin can be gained from the recent findings of Goulet et al. (28) that cathepsin L, a principal protease target of headpin (13), localizes to the nucleus and activates the transcription factor CCAAT-displacement protein/Cut homeobox through proteolytic cleavage. Thus, our future work aims to investigate the interaction of headpin with proteases required for the activation of nuclear transcription factors regulating the expressions of IL-8 and VEGF.

Our findings of headpin reexpression in the inhibition of angiogenesis are supported by experimental observations from the study of other serpins. These include reduced tumor angiogenesis resulting from maspin overexpression in a transgenic mouse model (29) and in a model of bone metastasis (30).

**Figure 3.** Headpin blocks angiogenic events in endothelial cells. A, a representative invasion assay of HUVECs shows a 78% reduction in relative invasion in the presence of headpin at 100 nmol/L in 5% FBS compared with 5% FBS alone (left; P < 0.05, two-tailed Wilcoxon rank-sum test). Columns, mean of triplicate wells; bars, SD. Middle and right, postinvasion cells labeled with fluorescent calcein AM in the absence and presence of headpin, respectively. B, a representative migration assay of HUVECs shows a 54% and 108% reduction in relative migration in the presence of headpin (HP) at 100 nmol/L and 1 μmol/L, respectively, in 0.1% FBS containing VEGF at 20 ng/mL compared with 0.1% FBS containing VEGF at 20 ng/mL alone (P < 0.05, two-tailed Wilcoxon rank-sum test). Columns, mean of triplicate wells; bars, SD. C, representative tube formation assays of HUVECs show early organization of cells into networks in basal growth media condition with 5% serum (left) and assembly of lumen-containing structures in media containing VEGF at 20 ng/mL (middle), but interruption of the earliest steps in cell organization by the addition of 100 nmol/L headpin to either condition (right). D, measurement of cells labeled with fluorescent calcein AM showed a 92% reduction in relative tube length in the presence of 100 nmol/L headpin compared with 5% FBS containing VEGF (P < 0.0001, two-tailed Wilcoxon rank-sum test). Columns, mean of triplicate wells; bars, SD.
Moreover, our findings extend the understanding of the molecular mechanisms underlying serpin functions in angiogenesis to the level of transcriptional regulation. The down-regulation of both the promoter activity and the secreted protein levels of VEGF and IL-8 show that headpin functions as an endogenous regulator at the point of synthesis of key angiogenic factors. Indeed, many angiogenic functions are attributed to both VEGF and IL-8, including the stimulation of endothelial cell invasion, migration, and proliferation (31). Equally important is the tight balance between proangiogenic and antiangiogenic factors in the tumor microenvironment that governs the response of endothelial cells in angiogenesis (32). Therefore, down-regulating the synthesis of these mediators can critically shift this balance of factors to interrupt events required in the formation of new vessels.

Our investigational approach was based on the findings that headpin expression is constitutive in normal oral mucosa but down-regulated or lost in specimens from multiple upper aerodigestive tract sites and cell lines derived from HNSCC. We have yet to investigate differential expression of headpin in tumors of other histologic types arising from the mucosa of the upper aerodigestive tract. However, we previously showed by Northern blot analysis of multiple tissue types that headpin expression was also detected in normal skin but not in other epithelial tissues including lung, colon, and small intestine (10). Therefore, although the loss of headpin expression might occur in skin squamous or basal cell carcinoma, the relevance remains to be determined for other cancer types such as breast and prostate carcinoma.

In summary, we have shown the nuclear subcellular localization and functions of headpin, a head and neck serpin in squamous cell carcinoma. We found that headpin functions both as an intracellular protein to regulate the expression and secretion of angiogenic factors and as a secreted protein to inhibit invasion, migration, and tube formation in endothelial cells. As a consequence, the loss of headpin expression can result in an imbalance of mediators in favor of angiogenesis and tumor growth in HNSCC. Our findings have thus brought a new understanding of the cellular and molecular mechanisms of serpins in cancer on which to base therapeutic strategies for tumor suppression.

Figure 4. Headpin down-regulates the transcription of key mediators critical for angiogenesis. A, Western blot detected headpin expression with mouse monoclonal antibody in cytoplasmic (Cyt) and nuclear (Nuc) fractions of clones C2 and C6 of UMSCC1 but not of vector clone cells. HaCaT total cell lysates were used as a positive control. Additional controls confirm the purity of nuclear and cytosolic fractions by Western blot detection of CCAAT binding factor B (WB:CBF-B) in nuclear but not cytoplasmic fractions and of glyceraldehyde-3-phosphate dehydrogenase (WB:GAPDH) in cytoplasmic but not nuclear fractions. B, left, RT-PCR of total mRNA detected both IL-8 and VEGF messages in vector clone V1 (lane 1) but not in the headpin-expressing clones C2 and C6 (lanes 2 and 3). Actin mRNA was used as a control of equal loading. Right, real-time PCR of total mRNA shows a 3.5-fold reduction in IL-8 mRNA in headpin-expressing clones C2 and C6 relative to vector clone V1 (P < 0.05 for clones C2 and C6, two-tailed Wilcoxon rank-sum test). C, ELISA of conditioned media of headpin-expressing clones C2 and C6 detected 10.0- and 9.1-fold reductions, respectively, in VEGF and IL-8, respectively, relative to vector clone V1 (P = 0.12, two-tailed Wilcoxon rank-sum test). D, reporter assay using transient transfection of full-length luciferase promoter constructs showed a 64% and 75% reduction in promoter activity for VEGF and IL-8, respectively, in the presence of pCDNA 3.0-headpin relative to vector pCDNA (P < 0.05, two-tailed Wilcoxon rank-sum test). Columns, mean of triplicate values; bars, SD. D, reporter assay using transient transfection of full-length luciferase promoter constructs showed a 64% and 75% reduction in promoter activity for VEGF and IL-8, respectively, in the presence of pCDNA 3.0-headpin relative to vector pCDNA (P < 0.05, two-tailed Wilcoxon rank-sum test). Values are normalized to β-galactosidase; columns, mean of duplicate values in relative light units (RLU); bars, SD.
Figure 5. Headpin expression reduces tumor growth and microvessel density in vivo. A, representative images of mice with flank tumors. Top, a bulky, lobulated mass typical of tumors derived from vector cells; bottom, a small, discrete nodule typical of tumors derived from headpin-expressing UMSCC1 cells. B, weight and representative images of ex vivo mouse flank tumors. The mean weight of tumors from headpin-expressing clones of UMSCC1 cells was reduced by 80% relative to those from vector clones. Columns, mean tumor weight of 15 animals in each group; bars, SE. P = 0.016, two-tailed Wilcoxon rank-sum test. C, representative micrographs of mouse flank tumors stained with H&E (left) and anti-α-CD31 (right). Left, H&E staining shows a similar differentiation and microscopic pattern but less cellularity and more necrosis in a tumor derived from headpin transfectants (bottom) relative to a tumor from vector cells (top). Right, α-CD31 staining shows positive staining of endothelial cells forming vessels in a tumor derived from vector cells (top) in contrast to sparse staining in a tumor from headpin transfectants (bottom). Bar, 50 μm. D, image analysis of tumors stained with α-CD31. In tumors from headpin transfectants, the mean number of vessels per headpin function (HPF) was reduced by 50% (left), the mean area of CD31 positive staining was reduced by 45% (middle), and the mean intensity of CD31 positive staining was reduced by 45% (right) relative to tumors from vector cells. Columns, mean of 50 × fields from four quadrants of each tumor; bars, SD. P = 0.029 (left) and P = 0.05 (middle and right), two-tailed Wilcoxon rank-sum test.

References


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