

Addendum

Autophagy and Angiogenesis Inhibition

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Original manuscript submitted: 06/25/07

Manuscript accepted: 07/13/07

Previously published online as an *Autophagy* E-publication:

<http://www.landesbioscience.com/journals/autophagy/abstract.php?id=4734>

KEY WORDS

angiogenesis, autophagy, kringle 5, endostatin, Beclin 1, Bcl-2

ACKNOWLEDGEMENTS

This work was supported by a grant from the NIH, CA 104347, the Sparboe Endowment for Women's Cancer Research, the Cancrurables Foundation and an AHC translational grant from the University of Minnesota.

Addendum to:

Kringle 5 of Human Plasminogen, an Angiogenesis Inhibitor, Induces Both Autophagy and Apoptotic Death in Endothelial Cells

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Blood 2007; 109:4793-802

ABSTRACT

Angiogenesis, the process by which new blood vessels are formed is critical for embryonic development and physiological functioning of normal tissues. Angiogenesis also plays a critical role in the pathology of many diseases including cancer, wherein the supply and demand for blood vessels determines the rate of cancer growth. A number of therapeutic strategies are being developed to inhibit pathological angiogenesis. Kringle domains of plasminogen such as kringle 5 (K5) and a proteolytic fragment of collagen type XVIII (endostatin) are well-characterized, potent angiogenesis inhibitors. These inhibitors activate different intracellular signaling pathways to induce apoptosis and inhibit cell proliferation. Recent studies from our group have shown that K5 and endostatin can also induce autophagy in addition to apoptosis in endothelial cells. A common feature of the two treatments was the upregulation of Beclin 1 levels leading to alterations in the Beclin 1-Bcl-2 complex. Angiogenesis inhibitor-induced autophagy in endothelial cells was independent of nutritional or hypoxic stress and initiated even in the presence of endothelial-specific survival factors such as vascular endothelial growth factor (VEGF). Interfering with the autophagic response by knocking down Beclin 1 levels dramatically increased apoptosis of endothelial cells. These findings identify the autophagic response as a novel target for enhancing the therapeutic efficacy of angiogenesis inhibitors.

INTRODUCTION

Strategies that limit angiogenesis are being developed as potential approaches to inhibit cancer growth.¹ One of the approaches to prevent neovascularization of tumor tissues is to induce apoptosis in endothelial cells. Programmed cell death (PCD) of endothelial cells in combination with changes in cell migration culminates in inhibition of neovascularization. Indeed, a variety of angiogenesis inhibitors transduce apoptotic signals either by interfering with integrin-mediated signaling or by affecting Bcl-2 levels.²⁻⁵ Additionally, endothelial cells also activate autophagy when they are treated with angiogenesis inhibitors.⁶ It is becoming increasingly evident that alternative PCD pathways, such as autophagy (PCD II) or necrosis can also be induced in cells in response to the stresses that activate apoptosis. For instance, cells respond to hypoxia, nutrient deprivation and notobiotic agents by inducing autophagy as well as apoptosis (PCD I), although the autophagic response is recognized as a primarily protective cellular response to the stress stimuli under these circumstances.⁷ Accumulating evidence suggests that autophagic and apoptotic pathways are not mutually exclusive but, rather, interact at many levels. The molecular interactions between the autophagy protein Beclin 1 and potent apoptosis regulator proteins, Bcl-2 and Bcl-x_L, are believed to be critical to the cell's decision to survive or to die an apoptotic or necrotic death and are the subject of active investigation.⁸⁻¹¹ We are interested in understanding how autophagy is induced in endothelial cells with response to angiogenesis inhibitors. The identification of signaling pathways that participate in the response, and of the molecules that bridge the two forms of PCD, could lead to novel approaches for enhancing the therapeutic efficacy of angiogenesis inhibitors.

VASCULAR ENDOTHELIUM

The innermost layer of every blood vessel is lined by endothelial cells, which are anchored onto basement membrane composed of collagens and other extracellular matrix components. Mature blood vessels contain additional cellular layers at the abluminal side of the endothelial cell layer, which includes pericytes and vascular smooth muscle cells. In mature blood vessels, endothelial cells are extremely quiescent, dividing about once in

six months. However, in tumor tissues endothelial cells proliferate rapidly in response to angiogenic growth factors secreted into the tumor microenvironment. Increased endothelial cell proliferation also occurs during vessel remodeling in normal tissues such as corpus luteum, endometrium and healing wounds.^{12,13} Tumor angiogenesis is of special interest since it offers a novel target for cancer therapy. Transformed cells can grow to the size of 2 mm in diameter without vascular supply. Passive diffusion of nutrients from nearby blood vessels is sufficient to sustain their viability at this stage. However, further growth is dependent on new vascular supply.

This critical transition from an avascular stage to a fully vascularized phenotype is now recognized as the 'angiogenic switch' that is necessary for tumor growth and invasion. From this point onwards, cancer progression is kinetically linked to the rate of angiogenesis. When the supply fails to match the demand, tumor tissue becomes hypoxic and alters the metabolic needs of the cell. Lower oxygen tension, reduced intracellular glucose levels and a shift in ATP/ADP ratio can then activate many survival pathways to sustain tumor growth. It is well recognized that hypoxia induced factor 1 alpha (HIF-1 α)-mediated transcriptional activation is an important survival pathway. Under hypoxia, proteasomal degradation of HIF-1 α is prevented by the inhibition of prolyl hydroxylases, leading to the stabilization and nuclear translocation of HIF-1 α . HIF-1 α then heterodimerizes with HIF-1 β and transcriptionally activates the expression of vascular endothelial growth factor, glucose transporters and other target genes that are necessary for the survival of tumor cells.¹⁴ Apart from the tumor cells, the vascular compartment of the tumor tissue is also affected by hypoxia and metabolic stress. Previous studies from our laboratory have shown that endothelial cells react to chemotherapy (notobiotic stress) by secreting vascular endothelial growth factor (VEGF), a survival factor for the endothelium. VEGF-mediated autocrine stimulation activates a PI3K/AKT-mediated survival pathway. Interfering with VEGF signaling leads to pronounced inhibition of angiogenesis and tumor growth.¹⁵ Recent studies suggest that endothelial cells respond to angiogenesis inhibitors by activating autophagy.⁶

ANGIOGENESIS INHIBITORS AND AUTOPHAGY

Two major groups of angiogenesis inhibitors are generated by proteolysis. Fragments of the carboxy-terminal, non-collagenous domain (NC1) of collagens type I, IV, XV and XVIII constitute the extracellular matrix (abluminal)-derived angiogenesis inhibitors. Endostatin, one of the well-characterized members of this group, is generated by the proteolysis of collagen XVIII.¹⁶ The second group of endogenous inhibitors arises from the coagulation system (intraluminal). A proteolytic fragment of plasminogen encompassing kringle domains 1–4 (angiostatin), Kringle 5 (K5) and kringle domains of anti-thrombin, and tissue plasminogen activator are examples of the second group.

Angiogenesis inhibitors act by limiting cell proliferation and migration and by promoting cell death, but the molecular mechanism underlying angiogenesis inhibition is not completely understood.

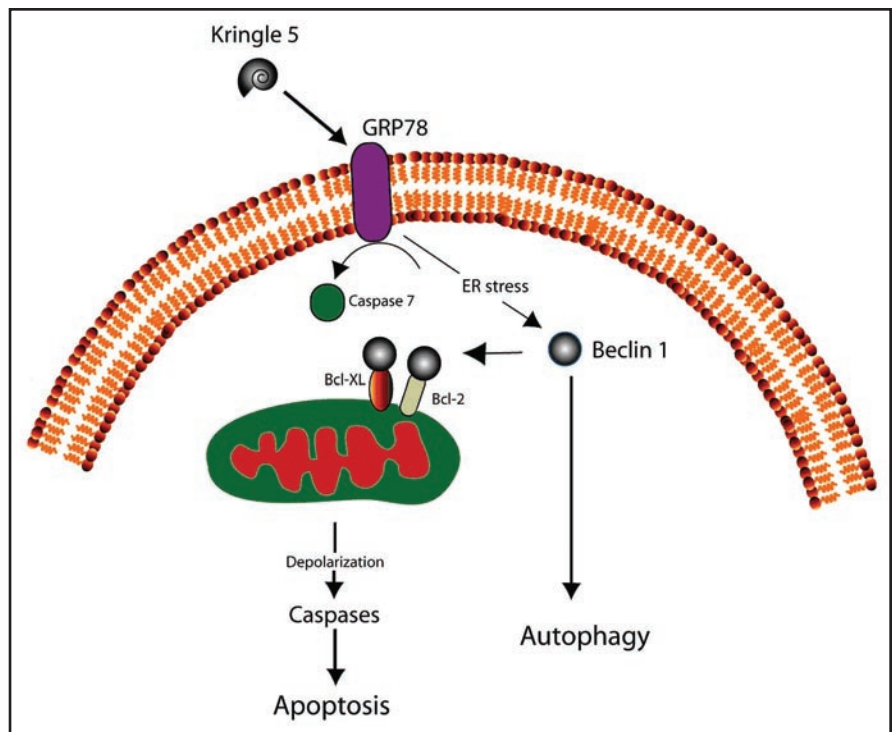


Figure 1. K5 induces both autophagy and apoptosis in endothelial cells.

Endostatin, for example, is known to bind integrin $\alpha 5 \beta 1$ enriched in lipid rafts containing caveolin-1. The integrin pathway probably affects cell migration by modulating RhoGAP and Rho A activity via src kinase.^{3,5} Endostatin also inhibits cell proliferation through interaction with glypican 1 expressed on the surface of endothelial cells. Glypican 1 is a low affinity binding site for endostatin and is believed to participate in the Wnt-1/Frizzled signaling pathway. Treatment of endothelial cells with endostatin promotes cytosolic β -catenin degradation through the Wnt-1 signaling system and inhibits the transcriptional activation of a number of target genes involved in proliferation.⁴ Endostatin treatment decreases the levels of Bcl-2 in endothelial cells^{4,17} and this decrease may underlie its apoptotic effect, although it is unclear whether the decrease is a result of proteasomal degradation of the anti-apoptotic protein or its transcriptional inhibition. Endostatin also activates autophagy in Eahy926 human endothelial cells.¹⁸ We have recently observed that endostatin, while downregulating Bcl-2, also increases levels of Beclin 1, a key autophagy regulator (unpublished). Since a fraction of cytosolic Bcl-2 (or Bcl-x_L) is complexed with Beclin 1 we suggest that the autophagic response to endostatin could in part be due to increased synthesis of Beclin 1 and in part due to its release from a complex with Bcl-2.

Kringle 5 and angiostatin (Kringle 1–4) are endogenous inhibitors of angiogenesis which are generated by the proteolytic cleavage of plasminogen. Angiostatin and K5 bind distinct molecules, ATP-synthase and glucose-regulated protein 78 (GRP-78), respectively, on the endothelial surface.^{2,19,20} GRP-78 is a component of the unfolded protein response and is localized within the endoplasmic reticular lumen and membrane. Its cell surface expression is associated with stress responses such as hypoxia and endoplasmic reticulum stress. The function of GRP-78 is dependent on ATP binding and

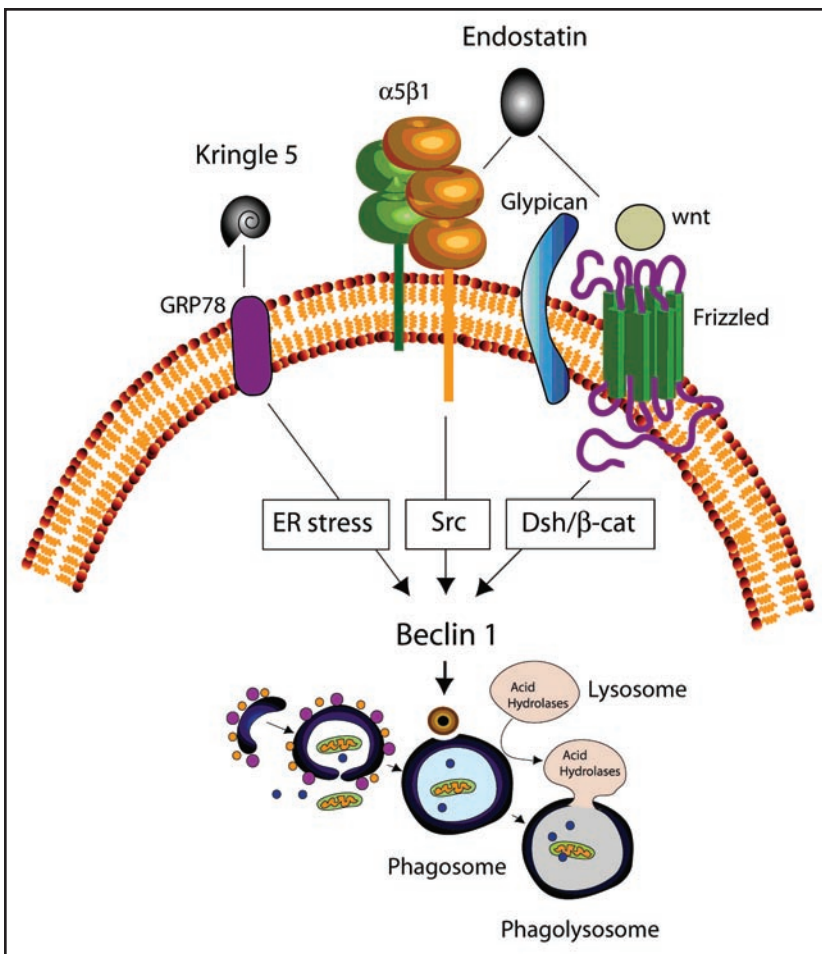


Figure 2. Signaling pathways of angiogenesis inhibitors and autophagy.

mutations affecting this binding lead to dominant negative inhibition.²¹ High affinity binding of K5 to GRP-78 was believed to activate apoptosis by dissociating a complex between the receptor protein and an effector caspase, caspase 7. Our studies also detected caspase-7 activation in response to K5 exposure although this was a late event. These studies suggest that K5-induced apoptosis was more likely to be the result of early cleavage and activation of caspase-3 via an intrinsic pathway. In addition to apoptosis, K5 treatment also elicited an autophagic response in endothelial cells.

Whereas autophagy is normally turned on by nutritional deprivation and environmental stress such as hypoxia, K5-induced autophagy occurred under conditions of normoxia and rich nutrient medium suggesting the involvement of a distinct signaling pathway. As with endostatin, Beclin 1 levels were upregulated and increased levels of anti-apoptotic protein Bcl-2 entered into a complex with Beclin 1 following K5 treatment (Fig.1). However, the total levels of Bcl-2 remained the same while the Beclin 1-bound form increased. This would effectively lead to a reduction in levels of the survival protein and activation of intrinsic apoptotic pathways. In fact, K5-treated endothelial cells showed depolarization of mitochondrial membrane and a decrease in ATP/ADP ratio. The nature of the Bcl-2/Beclin 1 interaction and its effect on the ability of Bcl-2 to function as a survival protein on the outer mitochondrial membrane has been the subject of much speculation. Recent studies suggest that Beclin 1 interacts with a conserved hydrophobic groove in

Bcl-x_L, a close counterpart of Bcl-2, through a BH3-like domain.^{8,9} It is tempting to speculate that stress signals that promote the interaction of activated pro-apoptotic BH3-only proteins with Bcl-2 would result in the release of Beclin 1 and induce autophagy. It has also been suggested that the voltage dependent anion channel, VDAC, located on the outer membrane of mitochondria, is a receptor for K5 and that binding of K5 to VDAC could lead to the channel's closure. This hypothesis was based on a single study showing that VDAC purified from a human prostate cancer cell line could bind K5 when reconstituted into liposomes.²² VDAC, however, seems to play a minor role in mitochondrial depolarization.²²

THERAPEUTIC IMPLICATIONS OF AUTOPHAGY AND ANGIOGENESIS INHIBITION

Pathological angiogenesis is linked to a number of diseases such as cancer, macular degeneration and diabetes-induced retinopathy. In each of these conditions, inhibition of angiogenesis has been shown to block disease progression. Angiogenesis is a complex process involving endothelial cell proliferation, matrix degradation, migration, tube formation, and vessel maturation. A number of therapeutic agents have been developed to inhibit any one of these processes to achieve reduced blood supply to target tissues. Some of the successful strategies that show clinical response include humanized antibodies to VEGF, blocking antibodies to VEGF receptors, kinase inhibitors targeting VEGF receptors, and inhibitors of cyclooxygenase.^{21,23} VEGF is a transcriptionally regulated survival factor for endothelial cells. Blocking VEGF receptor-mediated signaling via the PI3K/Akt pathway leads to cell cycle arrest and programmed cell death.¹

Other categories of endogenously produced angiogenesis inhibitors such as endostatin, K5 and angiostatin have shown great promise in experimental tumor model systems. However, recently concluded Phase II clinical trials using recombinant endostatin showed modest effects in advanced cancers. This dichotomy in effectiveness of endogenous angiogenesis inhibitors has evoked many questions, some of which are pharmacological in nature and that are related to bioavailability and delivery. Others are related to how target cells adapt and respond to a given therapeutic intervention. For example, if an important survival factor is blocked, compensatory activation of redundant pathways may be used by target cells to resist the treatment. Could autophagy induced by angiogenesis inhibitors be a sign of a survival response in endothelial cells? If so, can one block autophagy to improve the therapeutic efficacy of angiogenesis inhibitors? Even though K5 and endostatin affect different intracellular signaling pathways both are found to increase Beclin 1 levels and autophagy (Fig. 2).

Recent studies suggest that the increase in Beclin 1 is transcriptionally regulated. The convergent points in the downstream signaling of K5 and endostatin, leading to increased Beclin 1 levels are currently under investigation. When the increase in Beclin 1 levels was suppressed by shRNA, endothelial cells activated the caspase-dependent intrinsic pathway of programmed cell death. Therefore, Beclin 1 seems to be a novel target for therapeutic intervention, and

methods to block its function can be used to potentiate the effect of angiogenesis inhibitors. Such a strategy can be successful only if the enhanced endothelial cell apoptosis is restricted to proliferating cells of the nascent vasculature. Quiescent endothelial cells of mature blood vessels are normally resistant to many inhibitors including platinum compounds and VEGF receptor-mediated delivery of toxin polypeptides that are capable of enzymatically inhibiting translation. Therefore, it is likely that targeting Beclin 1 will selectively affect proliferating vascular endothelial cells. Preliminary studies with virally-delivered Beclin 1 shRNA showed significant inhibition of angiogenesis in vitro. These studies will have to be validated in vivo to determine whether blocking the upregulation of Beclin 1 in endothelial cells can effectively suppress angiogenesis and inhibit tumor growth. Future studies will unravel the merit of targeting Beclin 1 to improve the therapeutic potential of endogenous angiogenesis inhibitors.

References

1. Folkman J. Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 2007; 6:273-86.
2. Davidson DJ, Haskell C, Majest S, Kherzai A, Egan DA, Walter KA, Schneider A, Gubbins EF, Solomon L, Chen Z, Lesniewski R, Henkin J. Kringle 5 of human plasminogen induces apoptosis of endothelial and tumor cells through surface-expressed glucose-regulated protein 78. *Cancer Res* 2005; 65:4663-72.
3. Wickstrom SA, Alitalo K, Keski-Oja J. Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. *Cancer Res* 2002; 62:5580-9.
4. Hanai J, Gloy J, Karumanchi SA, Kale S, Tang J, Hu G, Chan B, Ramchandran R, Jha V, Sukhatme VP, Sokol S. Endostatin is a potential inhibitor of Wnt signaling. *J Cell Biol* 2002; 158:529-39.
5. Wickstrom SA, Alitalo K, Keski-Oja J. Endostatin signaling and regulation of endothelial cell-matrix interactions. *Adv Cancer Res* 2005; 94:197-229.
6. Bui Nguyen TM, Subramanian IV, Kelekar A, Ramakrishnan S. Kringle 5 of human plasminogen, an angiogenesis inhibitor, induces both autophagy and apoptotic death in endothelial cells. *Blood* 2007; 109:4793-802.
7. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 2004; 23:2891-906.
8. Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinski K, Tavernarakis N, Hickman JA, Geneste O, Kroemer G. Functional and physical interaction between Bcl-xL and a BH3-like domain in Beclin-1. *EMBO J* 2007; 26:2527-39.
9. Oberstein A, Jeffrey PD, Shi Y. Crystal structure of the Bcl-xL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J Biol Chem* 2007; 282:13123-32.
10. Pattingre S, Levine B. Bcl-2 inhibition of autophagy: a new route to cancer? *Cancer Res* 2006; 66:2885-8.
11. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; 122:927-39.
12. Hida K, Klagsbrun M. A new perspective on tumor endothelial cells: unexpected chromosome and centrosome abnormalities. *Cancer Res* 2005; 65:2507-10.
13. Ramakrishnan S, Subramanian IV, Yokoyama Y, Geller M. Angiogenesis in normal and neoplastic ovaries. *Angiogenesis* 2005; 8:169-82.
14. Gordan JD, Simon MC. Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev* 2007; 17:71-7.
15. Wild R, Dings RP, Subramanian I, Ramakrishnan S. Carboplatin selectively induces the VEGF stress response in endothelial cells: Potentiation of antitumor activity by combination treatment with antibody to VEGF. *Int J Cancer* 2004; 110:343-51.
16. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005; 65:3967-79.
17. Hanai J, Dhanabal M, Karumanchi SA, Albanese C, Waterman M, Chan B, Ramchandran R, Pestell R, Sukhatme VP. Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1. *J Biol Chem* 2002; 277:16464-9.
18. Chau YP, Lin SY, Chen JH, Tai MH. Endostatin induces autophagic cell death in EAhy926 human endothelial cells. *Histol Histopathol* 2003; 18:715-26.
19. Chi SL, Wahl ML, Mowery YM, Shan S, Mukhopadhyay S, Hilderbrand SC, Kenan DJ, Lipes BD, Johnson CE, Marusich MF, Capaldi RA, Dewhirst MW, Pizzo SV. Angiostatin-like activity of a monoclonal antibody to the catalytic subunit of F1F0 ATP synthase. *Cancer Res* 2007; 67:4716-24.
20. Veitonmaki N, Cao R, Wu LH, Moser TL, Li B, Pizzo SV, Zhivotovsky B, Cao Y. Endothelial cell surface ATP synthase-triggered caspase-apoptotic pathway is essential for k1-5-induced antiangiogenesis. *Cancer Res* 2004; 64:3679-86.
21. Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS. Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J Biol Chem* 2003; 278:20915-24.
22. Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 2007; 9:550-5.
23. Folkman J. Angiogenesis. *Annu Rev Med* 2006; 57:1-18.