Activated Ras Enhances Insulin-Like Growth Factor I Induction of Vascular Endothelial Growth Factor in Prostate Epithelial Cells

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Abstract

Mutations in the three closely related RAS genes, *HRAS*, *KRAS*, and *NRAS* are among the most common mutations found in human cancer; reaching 50% in some types of cancer, such as colorectal carcinoma, and 10% in prostate cancers. The activated Ras proteins produced by these mutations can, among other cellular changes, increase vascular endothelial growth factor (VEGF) production. Moreover, tumors bearing RAS gene mutations are more vascular than tumors without RAS mutations. We find that, in prostate epithelial cells, the introduction of an activated *HRAS* causes cells to produce VEGF in response to insulin-like growth factor I (IGF-I). In comparison, cells lacking an activated Ras are unable to produce VEGF in response to IGF-I. This effect of Ras may occur through stabilization of a second messenger protein, insulin receptor substrate 1, that mediates PI 3-kinase–dependent signaling. Because IGF-I is a paracrine/endocrine hormone that has been associated with increased risk for several types of cancer, these results suggest a novel interrelationship between oncogenic conversion of a cellular gene such as *HRAS*, and IGF-I produced locally for normal tissue homeostasis. (Cancer Res 2005; 65(6): 2085-8)

Introduction

Insulin-like growth factor I (IGF-I) serves as a powerful proliferative and survival factor for many types of cells, and epidemiologic data suggest that individuals in the upper quartile for circulating IGF-I levels may be at higher risk for developing colon, breast, prostate, or lung cancer (1). Although IGF-I has been primarily studied as a survival and growth factor, IGF-I has also been implicated in tumor angiogenesis through stimulation of vascular endothelial growth factor (VEGF) production in colon and pancreatic carcinoma cells (2–4). This effect is mediated by second messenger pathways that involve the docking protein insulin receptor substrate 1 (IRS-1; ref. 5) and PI 3-kinase that increase synthesis of HIF-1α (2). *RAS* gene mutations are common in both pancreatic and colon carcinomas, which are known to harbor activating mutations in *RAS* (6–9). Likewise, RAS gene mutations are found in at least some of the cell lines used to study VEGF production in response to IGF-I. However, it is not clear whether activating mutations in *RAS* genes lead to an increase in VEGF production in response to IGF-I. Thus, the potential interrelationships between Ras, IGF-I, IRS-1, and VEGF production have not been fully elucidated. To examine these interrelationships, we examined the consequences of an activated *Ras* in a prostate epithelial cell line (CPTX-1532; ref. 10). Previous work indicates that prostate epithelial cells produce low levels of VEGF in response to IGF-I (11). We find that the introduction of an activated Ras into CPTX 1532 cells alters the response to IGF-I, increasing VEGF production, through a stabilization of the IRS-1 protein.

Materials and Methods

Cell Culture. The CPTX-1532 cells were derived from a primary prostate tumor and have no detectable defects in the epidermal growth factor (EGF) or IGF-I second messenger pathways. The prostatic origin of these cells was confirmed by the induction of PSA and PMSA by interleukin 10, although the cells do not express the androgen receptor (data not shown). We have previously used the CPTX-1532 cells to elucidate pathways important for the regulation of the IRS-1 protein (12, 13). CPTX-1532 cells and derivative lines were maintained in keratinocyte medium with supplements and passaged every 3 to 4 days using a seeding density of $1 \times 10^4/cm^2$. Derivative lines were prepared through retroviral transduction.

Vascular Endothelial Growth Factor Measurements. Measurements of VEGF were done by standard ELISA assays, employing a kit produced by Zymed Laboratories (San Francisco, CA). For these measurements, cells were seeded at $2 \times 10^4/cm^2$ in 10-cm^2 culture dishes and allowed to attach for 24 hours. Cells were then washed thrice with PBS and placed into serum-free DMEM (1.5 mL; Mediatech, Inc., Herdon, VA) plus or minus the following factors; IGF-I (20 ng/mL purchased from Intergen, Inc., Purchase, NY), EGF (20 ng/mL purchased from Intergen), or the combination of IGF-I plus EGF (20 ng/mL each). Medium was collected after 72 hours and any floating cells were removed by centrifugation. Medium was stored at −80°C until use. The ELISA results presented were done in triplicate using 100 μL of sample and the measurement is representative of three independent experiments.

Endothelial Cell Migration. Human bone marrow endothelial (HBME) cells were seeded on a collagen coated membrane at $1 \times 10^4/cm^2$ in DMEM plus 5% fetal bovine serum and allowed to attach for 24 hours. Conditioned medium from cells treated as described under VEGF measurements was placed into the lower chamber after the 24-hour attachment period. HBME cells that migrated through the membrane were scored visually at 24 to 48 hours following addition of conditioned medium. All experiments were done in triplicate and the results presented are representative of a minimum of five independent observations.

Western Blotting. Western blot analysis was done using standard procedures, employing antibodies to human IRS-1 (Upstate Biotechnology, Inc., Saranac Lake, NY), β-actin (Sigma, St Louis, MO), HIF-1 α (BD BioSciences, PharMingen, San Diego, CA), and HIF-1 β (BD BioSciences PharMingen) and Alexafluor 680 labeled secondary antibodies (Molecular Probes, Inc; Eugene, OR) that were visualized using a Licor Odyssey Fluorescent Imager. Relative levels of proteins of interest were compared using the Quantity One imaging software (Bio-Rad, Hercules, CA).

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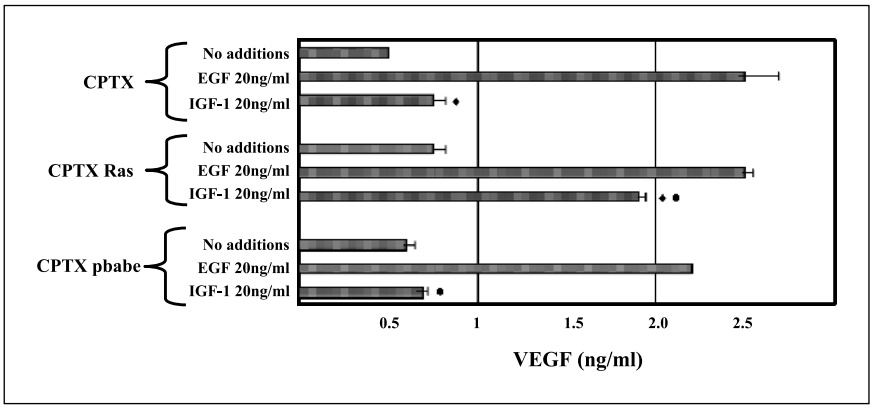

Figure 1. VEGF production in response to EGF and IGF-I. CPTX-1532, CPTX-1532 pbabe, and CPTX-1532 Ras cells were seeded and following a 24-hour attachment period, exposed to either EGF or IGF-I at 20 ng/mL. The level of VEGF in the medium was measured 72 hours later using an ELISA assay. The cells under these conditions do not proliferate and the cell number at the end of the experiment is similar in all groups. Representative of three independent experiments. In every experiment, there was a dramatic increase in the amount of VEGF produced in response to IGF-I following the introduction of an activated Ras. The difference in IGF-I stimulated VEGF production was significantly greater in the CPTX-1532 Ras cells ($P > 0.0001$, Student's t test) compared with either CPTX-1532 or CPTX-1532 pbabe cells (● or ◆).

Results

An activated *HRAS* (Val^{12} mutation) was introduced into the CPTX-1532 cells using a retroviral system (14). The resulting cell lines (termed CPTX-1532 Ras) were unable to form colonies in soft agar and did not have a significant reduction in apoptosis under normal growth conditions. To assess the ability of the CPTX-1532 Ras cells to induce angiogenesis, we first examined VEGF production. The parental CPTX-1532 cells and cells containing an empty plasmid (termed CPTX-1532 pbabe) can be induced to produce VEGF (~3-fold induction) following exposure to EGF, but not IGF-I, at concentrations that we have shown to be physiologically relevant (ref. 15; Fig. 1). In contrast, in CPTX-1532 Ras cells, VEGF production could be stimulated by the addition of either EGF or IGF-I. The difference between the CPTX-1532 or CPTX-1532 pbabe cells and CPTX-1532 Ras cells in VEGF induction by IGF-I were highly significant. Statistical analysis done on each of the experiments conducted revealed a $P < 0.0001$. These results indicate that the presence of an activated Ras alters the cell response to IGF-I, inducing the cells to produce significant amounts of VEGF.

One of the first steps in angiogenesis is endothelial cell migration, a process known to be influenced by VEGF. The effect of a ras mutation on the ability of prostate cancer cells to recruit endothelial cells was examined using a Boyden chamber assay. HBME-1 cells (16) were plated in the upper chamber and their migration towards conditioned media previously exposed to CPTX-1532, CPTX-1532 pbabe, and CPTX-1532 Ras cells was examined. The HBME-1 cells showed strong migration towards conditioned media from CPTX-1532 previously exposed to EGF but not to media from CPTX-1532 or CPTX-1532 pbabe cells exposed to IGF-I. However, endothelial cells migrated towards media from CPTX-1532 Ras cells when the cells were previously exposed to either EGF or IGF-I (Fig. 2). In multiple trials, IGF-I treatment induced a strong chemoattractant effect in CPTX-1532 Ras cells but not in CPTX-1532 or CPTX-1532 pbabe cells and this difference was highly significant ($P < 0.001$). A critical role for VEGF A in this endothelial migration was confirmed in these experiments using a neutralizing antibody to VEGF A, which significantly inhibited migration in all cases (data not shown). In sum, the results suggest that the prostate epithelial cells expressing an activated Ras produce VEGF A when exposed to IGF-I, which in turn induces migration of endothelial cells. This response is absent in prostate epithelial cells without an activated Ras.

We have previously shown that the IGF-I receptor docking protein IRS-1 is stabilized by EGF (12). Because IRS-1 is important for IGF-I induced synthesis of VEGF (5) and Ras is strongly activated by the EGF receptor, we examined the influence of an activated Ras on IRS-1 stability. The steady-state levels of IRS-1 decline in CPTX-1532 cells following exposure to IGF-I (12). We compared IRS-1 levels in CPTX-1532 cells that expressed an activated Ras and parental cells, in response to increasing amounts of IGF-I and found that the CPTX-1532 Ras cells maintained IRS-1 levels at much higher concentrations of IGF-I than the parental cells (Fig. 3*A*). In multiple experiments, higher levels of IRS-1 were consistently observed in CPTX-1532 Ras cells compared with either parental CPTX-1532 or CPTX-1532 pbabe cells. Statistical analysis of the results of several

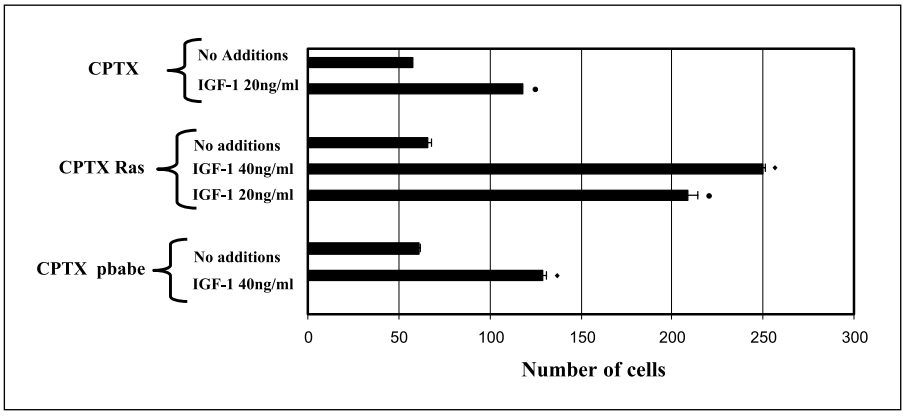

Figure 2. Induction of endothelial cell migration by growth factor stimulated cells. The migration of HBME cells in response to medium from cells treated as in Fig. 1 was tested. In this case, CPTX-1532 and CPTX-1532 Ras cells were treated with two concentrations of IGF-I, 20 and 40 ng/mL. The medium from these cells was used as a chemoattractant in a 10-cm^2 well, whereas endothelial cells were seeded at $1 \times 10^4/cm^2$ onto a collagen-coated transwell membrane suspended in the well. Cells that migrated through the membrane were scored after 24 hours. Representative of five independent experiments in which IGF-I-mediated migration of HBME cells was always significantly greater in response to CPTX-1532 Ras cell conditioned medium. Statistical analysis indicates that the HBME migration induced by IGF-I differs significantly ($P < 0.0001$) between CPTX-1532 Ras and either CPTX-1532, or CPTX-1532 pbabe (● or ◆).

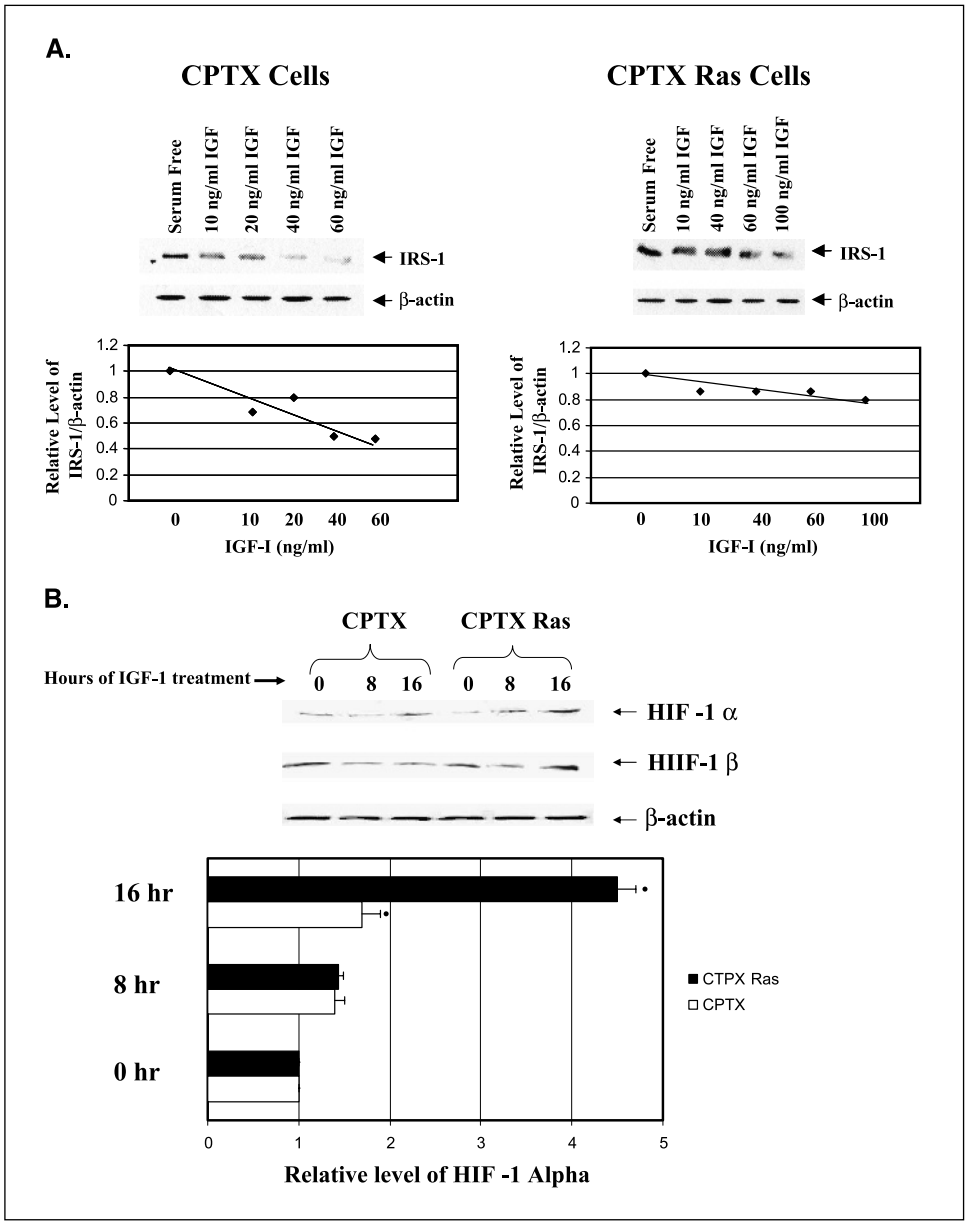

Figure 3. Levels of IRS-1 and HIF-1 α in cells harboring an activated Ras. *A,* CPTX-1532 and CPTX-1532 Ras cells were treated with differing concentrations of IGF-I for 16 hours and the level of IRS-1 protein was determined. CPTX-1532 cells are known to down-regulate IRS-1 through increased degradation following exposure to IGF-I. A comparison of IRS-1 levels in CPTX-1532 and CPTX-1532 Ras cells reveals higher levels of IRS-1 in CPTX-1532 Ras which are maintained in the presence of relatively high levels of IGF-I, up to 100 ng/mL. A quantification of the data is presented below the Western blot and depicts the level of IRS-1 relative to β-actin. A similar experiment was done thrice with consistent results. *B,* level of HIF-1 α and HIF-1 β following stimulation with IGF-I. Cells were treated with IGF-I (20 ng/mL) for the times indicated. Total proteins were extracted and HIF-1α and HIF-1β levels were measured by Western blot analysis. ●, values significantly different between CPTX-1532 and CPTX-1532 Ras cells ($P < 0.05$). Repeated in four independent experiments with similar results.

similar experiments using a single concentration of IGF-I indicates that the differences in IRS-1 protein levels between CPTX-1532 or CPTX-1532 pbabe cells and CPTX-1532 Ras cells is highly significant ($P < 0.001$).

Because IGF-I-mediated VEGF production occurs through increased synthesis of HIF-1 α, we examined HIF-1 α in cells expressing activated Ras. Consistent with increased IGF-I signaling, these cells express higher levels of HIF-1 α than either parental cells or cells transfected with an empty expression vector. The levels of HIF-1 β were relatively constant (Fig. 3*B*). Because IGF-I signaling acts primarily on HIF-1 α, with little effect on HIF-1 β, the results suggest that Ras activation positively regulates the IGF-I-dependent second messenger pathways leading to increased HIF-1 α synthesis. Given the important role of IRS-1 and HIF-1 α in IGF-I-mediated VEGF production, these observations provide a likely mechanism for the increased VEGF production by the CPTX-1532 Ras cells.

Discussion

Normal prostate epithelial cells have been found to produce VEGF in low amounts in response to IGF-I (11). This result is confirmed in our experiments (see CPTX cells in Fig. 1); however, the amount of VEGF that is produced is greatly increased when cells express an activated Ras. This increase in VEGF expression is likely to be significant in tumor formation, because tumor growth requires recruitment of new blood vessels to prevent a hypoxic state. This concept is supported by the finding that cells harboring an activated Ras produce VEGF sufficient to significantly increase endothelial cell migration compared with parental cells (see Fig. 2). The differential stimulation of endothelial cell in these assays is not the result of IGF-I or EGF addition to the CPTX medium, because both the parental and Ras-expressing lines receive equal amounts of growth factors for VEGF stimulation.

Activating mutations in RAS genes have been shown to up-regulate VEGF and this increase in VEGF expression is essential

for tumorigenicity in some cancer cell lines (9). Although it has been suggested that this type of "activating" mutation in a critical regulatory gene would act in conjunction with epigenetic and environmental influences to promote tumor progression, (17) the molecular mechanisms of such interactions have remained elusive. The present report suggests that activating mutations in RAS genes do not universally lead to constitutive expression of VEGF but may alter the cellular response to local factors such as IGF-I, inducing a proangiogenic response. Accordingly, strategies targeting the IGF-I receptor decrease vessel density in animal models of both pancreatic (4) and colorectal carcinoma (18). The interaction between IGF-I and RAS gene mutations may represent a form of molecular cooperation between the activation of an oncogene and normal host characteristics, leading to an increased risk for tumor growth and metastasis.

The stabilization of IRS-1 in the presence of activated Ras suggests that PI-3 kinase mediated signaling is responsible for the increased VEGF production in these cells. This is consistent with previous work from multiple laboratories demonstrating that the PI-3 kinase–dependent signaling can increase VEGF expression (19) and increase the stabilization of IRS-1 by EGF in prostate epithelial cells (12). Furthermore, the results suggest that the activation of Ras alone may not provide stimulation of the PI-3 kinase–dependent signaling in prostate epithelial cells sufficient to induce VEGF production, but that additional stimulation, such as that provided by the activated IGF-I receptor, may be required.

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References

1. LeRoith D, Roberts CT Jr. The insulin-like growth factor system and cancer. Cancer Lett 2003;195:127–37.
2. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002;277:38205–11.
3. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. J Biol Chem 2002;277:27975–81.
4. Stoeltzing O, Liu W, Reinmuth N, et al. Regulation of hypoxia-inducible factor-1α, vascular endothelial growth factor, and angiogenesis by an insulin-like growth factor-I receptor autocrine loop in human pancreatic cancer. Am J Pathol 2003;163:1001–11.
5. Neid M, Datta K, Stephan S, et al. Role of insulin receptor substrates and protein kinase C-ζ in vascular permeability factor/vascular endothelial growth factor expression in pancreatic cancer cells. J Biol Chem 2004;279:3941–8.
6. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M. Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature 1987;327:298–303.
7. Carter BS, Epstein JI, Isaacs WB. Ras gene mutations in human prostate cancer. Cancer Res 1990;50:6830–2.
8. Wong-Staal F, Dalla-Favera R, Franchini G, Gelmann EP, Gallo RC. Three distinct genes in human DNA related to the transforming genes of mammalian sarcoma retroviruses. Science 1981;213:226–8.
9. Okada F, Rak JW, Croix BS, et al. Impact of oncogenes in tumor angiogenesis: mutant K-*ras* up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. Proc Natl Acad Sci U S A 1998;95:3609–14.
10. Bright RK, Vocke CD, Emmert-Buck MR, et al. Generation and genetic characterization of immortal human prostate epithelial cell lines derived from primary cancer specimens. Cancer Res 1997;57:995–1002.
11. Burroughs KD, Oh J, Barrett JC, DiAugustine RP. Phosphatidylinositol 3-kinase and mek1/2 are necessary for insulin-like growth factor-I-induced vascular endothelial growth factor synthesis in prostate epithelial cells: a role for hypoxia-inducible factor-1? Mol Cancer Res 2003;1:312–22.
12. Zhang H, Hoff H, Sell C. Insulin-like growth factor I-mediated degradation of insulin receptor substrate-1 is inhibited by epidermal growth factor in prostate epithelial cells. J Biol Chem 2000;275:22558–62.
13. Zhang H, Hoff H, Sell C. Downregulation of IRS-1 protein in thapsigargin-treated human prostate epithelial cells. Exp Cell Res 2003;289:352–8.
14. Morgenstern JP, Land H. A series of mammalian expression vectors and characterisation of their expression of a reporter gene in stably and transiently transfected cells. Nucleic Acids Res 1990;18:1068.
15. Pietrzkowski Z, Sell C, Lammers R, Ullrich A, Baserga R. Roles of insulinlike growth factor 1 (IGF-1) and the IGF-1 receptor in epidermal growth factor-stimulated growth of 3T3 cells. Mol Cell Biol 1992;12:3883–9.
16. Lehr JE, Pienta KJ. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J Natl Cancer Inst 1998;90:118–23.
17. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759–67.
18. Wu Y, Yakar S, Zhao L, Hennighausen L, LeRoith D. Circulating insulin-like growth factor-I levels regulate colon cancer growth and metastasis. Cancer Res 2002;62:1030–5.
19. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med 2003;54:17–28.