Angiogenic Switch as a Molecular Target of Hepatocellular Carcinoma

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Angiogenesis and Hepatocarcinogenesis

Angiogenesis involves the sprouting of new blood vessels from pre-existing ones and is essential for tumor development and progression.\(^1\) Direct experimental evidence shows that the tumor growth and metastasis require new blood vessels. An avascular tumor rarely grows to a size larger than 2-3 mm\(^3\), but once a tumor becomes vascularized the progression of tumor growth is rapid and aggressive. Such a critical event for tumor progression has been named "angiogenic switch".\(^2\)\(^,\)\(^3\) The typical phenomenon of "angiogenic switch" is well recognized in the progression of human hepatocellular carcinoma (HCC).\(^3\)

It is generally accepted that intensive neovascularization is recognized in HCC of the moderately or poorly differentiated type, but not of the well differentiated type.\(^4\) Well differentiated HCC grows slowly and rarely metastasizes, while moderately or poorly differentiated HCC grows rapidly and aggressively metastasizes. While such clinicopathological phenomenon is frequently recognized, the key factor of the switch to the angiogenic phenotype has not been clarified in HCC tumors. According to several previous studies,\(^5\) major angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor are overexpressed in non-cancerous cirrhotic livers as well as cancer tissues. Therefore, other factors regulating the angiogenesis should play a critical role in the angiogenic switch of HCC.

Identification of an Angiogenic Switch Ggene of HCC

To identify an angiogenic switch gene(s) of HCC, we have utilized a differential display method for analysis of the difference of genetic expression between hypovascular HCC and hypervascular HCC.\(^6\) A specific gene detected in only tumor tissues of hypervascular HCC was extracted, subcloned for sequencing, and substantially identical to an "Angiopoietin-2" gene (GenBank AB009865). Angiopoietin-2 has been isolated as a ligand of vascular endothelial-specific receptor Tie2.\(^7\) While low concentration of Angiopoietin-2 functions as an antagonist of Angiopoietin-1, high concentration of Angiopoietin-2 also functions as the agonist that stimulates the Tie2 receptor tyrosine kinase.\(^8\) Analysis on the transgenic or knock-out mice suggested that Angiopoietins may play an important role in vascular remodeling such as vessel morphogenesis and maintenance between the endothelium and supporting cells. RNA expression analysis revealed that Angiopoietin-2 gene is highly expressed only in the tumor tissues of hypervascular HCC, but not in normal tissues or even in hypovascular HCC tumors, indicating that Angiopoietin-2 is one of the angiogenic switch genes in HCC.\(^9\)

Immunohistochemical analysis demonstrated that Angiopoietin-2 protein is highly expressed in moderately or poorly differentiated HCC tissues, especially in the marginal regions adjacent to the tumor capsule.\(^10\) Well
differentiated HCC tissues shows no strong expression of Angiopoietin-2. It is interesting that the overexpression of Angiopoietin-2 protein is significantly related not only to the dedifferentiation, but also to the peliotic change and capsular invasion of HCC cancer cells. Such clinicopathological features might be mediated by Angiopoietin-2 signal transduction pathways, and/or regulating factors for Angiopoietin-2 expression.²

**Molecular role of Angiopoietin-2 in HCC Angiogenesis**

The biologic effect of ectopic of Angiopoietin-2 expression in a human HuH7 cell line derived from well differentiated HCC was assessed (Figure 1A).³ HuH7 HCC cells endogenously express Angiopoietin-1 but not detectable Angiopoietin-2 transcripts. Analysis of in vitro growth curves of stable transfectants demonstrated that cell proliferation was not effected by Angiopoietin-2 expression. On the other hand, in vivo dramatic effects were observed in a nude mouse model of intraperitoneal inoculation. After inoculation of Angiopoietin-2 transfected HuH7 clones, the lethal intraperitoneal bleeding was recognized in all of the inoculated mice within 3 weeks (Figure 1B). At the time of death, all mice had developed large tumors on the liver, and significant hemorrhage within the tumors (Figure 1C). In contrast, there was no hemorrhage or tumor development in the parental or mock-transfected HuH7 cells. These results suggested a pathogenic role of Angiopoietin-2 expression in the establishment and growth of hypervascular HCC tumors.

The signal transduction pathways of Angiopoietin-2 were analyzed using human umbilical vascular endothelial cells that expresses endogenous Tie2 protein on the surface.⁴ Recombinant Angiopoietin-2 protein activates Tie2 receptor tyrosine kinase, and enhances the interaction of the Tie2 protein to p85 subunit of phosphatidylinositol-3 kinase (PI3K) as well as to several adaptor proteins Grb2, Grb7, and Grb14.⁵ In this context, we have previously isolated and characterized a human Grb7 cDNA sequence,⁶ and demonstrated that it serves as a substrate of focal adhesion kinase during the cellular invasion process.⁷ This finding raises the possibility that Grb7 participates in Tie2-mediated endothelial cell migration.⁸ Additionally, we have demonstrated that Grb2 could stimulate the cell proliferation, and PI3K might induce the cell survival.⁹,¹⁰ Further analysis of Angiopoietin/Tie2 signaling cascade may contribute to the understanding of the functional significance of this pathway. It is a formal possibility that Angiopoietin-2 activate Tie2 in fundamentally different ways via stimulation of signaling pathways which result in varied biological responses.

**Molecular Targeting Therapy for Angiogenic HCC**

Since Angiopoietin/Tie2 signals play a critical role in angiogenic switch, we developed molecular targeting therapy for the HCC tumors.¹¹ The structure of Tie2 protein is composed of an extracellular domain that binds to Angiopoietin ligand, a transmembrane region, and an intracellular domain that carries two tyrosine kinases.¹² We have constructed an expression vector of soluble Tie2 ectodomain (sTie2) lacking the regions of transmembrane and tyrosine kinases, by inserting a stop codon just prior to the Tie2 cDNA transmembrane region.¹³ The product of sTie2 cDNA is a secreted protein that binds to Angiopoietin protein and inhibits the interaction between Angiopoietin ligand and endogenous Tie2 receptor, resulting in inhibition of tyrosyl phosphorylation of the Tie2 protein. The exogenous sTie2 inhibits the Angiopoietin-2-induced expression of the anti-apoptotic protein Survivin, followed by serum deprivation to induce apoptosis in endothelial cells. Thus sTie2 may function as an inhibitor of vascular endothelial cell survival.
Then, in vivo effects of sTie2 on murine HCC was investigated. The electroporation-mediated gene transfer method was used with C3H-derived MH134 HCC cells. Mock gene transferred MH134 cells grew rapidly in the subcutaneously inoculated flank of C3H mice after 14 days. In contrast, sTie2 gene transfer and expression significantly inhibited MH134 HCC growth in vivo. Immunohistological analysis of HCC tumors revealed that the vascular endothelium, expressing CD31, was substantially reduced at day 14 following sTie2 gene transfer. Additionally, areas of necrotic tissue were present and prominent in sTie2-treated tumors probably as the result of poor neovascularization. These observations suggest the targeted inhibition of Angiopoietin/Tie2 signaling substantially induces tumor dormancy on the experimental animal model system of HCC.

Finally, we are now analyzing newly synthesized compounds that inhibited specifically tyrosine kinase of the Tie-2 receptor protein, in collaboration with Dr. Herbert Waldmann, Max Planck Institute for Molecular Physiology (Dortmund, Germany). A candidate compound has been found to inhibit the growth of vascular

![Image A: Transfectants](image_a.png)

![Image B: Angiopoietin-2 Expression](image_b.png)

![Image C: HCC Tumor Development](image_c.png)

**Figure 1.** Ectopic expression of Angiopoietin-2 in human HCC cells. (A) Expression of Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) in Huh7 clones stably transfected with Angiopoietin-2 gene (Huh7-Ang2-C1, C2, C3 and C4) compared to parental and mock-transfected Huh7 cells as demonstrated by the RNase protection assays. (B) Representative example of HCC tumor development in a nude mice inoculated with Huh7-Ang2-C1 cells (hematoxylin-eosin staining, 40x). (C) Characteristic of tumor growth in nude mice following intraperitoneal inoculation with 1X10^6 Ang2-transfected Huh7 HCC cells. Demonstration of the intraperitoneal bleeding phenomenon observed in nude mice at 15 days after inoculation with Huh7-Ang2-C1 (left) or Huh7-Ang2-C3 (right) cells.
endothelial cells in vitro (data not shown). Molecular targeting therapy against the Angiopoietin/Tie2 signal transduction cascade is a promising approach for HCC treatment. To realize the full therapeutic potential of modulation of the Angiopoietin/Tie2 pathway will require a greater understanding of the biological effect of this signaling cascade in the context of HCC tumor progression.\textsuperscript{22}

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**References**

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