ผลของสารสกัดสมุนไพร (จีพีโอ1986) ต่อการเกิดหลอดเลือดใหม่
ในหนูนูดไมซ์ที่ได้รับการปลูกถ่ายเซลล์มะเร็งตับ

นางสาว นภัสนันท์ เดือนศักดิ์

EFFECTS OF HERBAL EXTRACT (GPO1986) ON TUMOR

ANGIOGENESIS OF HEPATOCELLULAR CARCINOMA

CELLS (HepG2)  IMPLANTED IN NUDE MICE

Miss Naphatsanan  Duansak

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Physiology
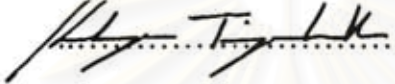
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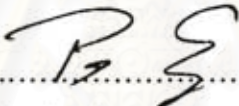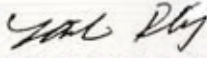Chulalongkorn University

Academic Year 2007

Thesis Title      EFFECTS OF HERBAL EXTRACT (GPO1986) ON TUMOR ANGIOGENESIS OF HEPATOCELLULAR CARCINOMA CELLS (HepG2) IMPLANTED IN NUDE MICE

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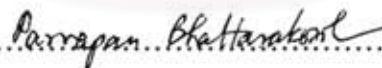Thesis Co-advisor      Ponthip Wirachwong, Ph.D.

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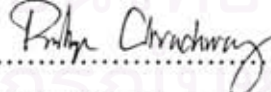
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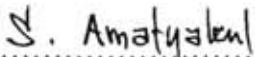
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นภัสนันท์ เดือนศักดิ์ : ผลของสารสกัดสมุนไพร (จีพีโอ1986) ต่อการเกิดหลอดเลือดใหม่ในหนูนูดไมซ์ที่
ได้รับการปลูกถ่ายเซลล์มะเร็งตับ (Effects of Herbal Extract (GPO1986) on Tumor Angiogenesis of
Hepatocellular Carcionoma Cell (HepG2) Implanted in Nude Mice)
อ.ที่ปรึกษา : รศ.ดร. สุทธิลักษณ์ ปทุมราช, อ.ที่ปรึกษาร่วม : รศ.ดร. ภาวพันธ์ ภัทรโกศล และ
ดร. พรทิพย์ วิรัชวงศ์ ,137 หน้า

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาผลของสารสกัดสมุนไพร (จีพีโอ1986) ต่อการเกิดหลอดเลือดใหม่ในหนูนูด
ไมซ์ที่ได้รับการปลูกถ่ายเซลล์มะเร็งตับ (HepG2) และเพื่อศึกษาผลของสารสกัดสมุนไพร ต่อ angiogenic biomarkers คือ ระดับ HIF-
1α และ VEGF

ใช้หนูนูดไมซ์เพศผู้พันธุ์ BALB/C อายุ 8-10 สัปดาห์ในการทดลอง หลังจากการใส่ dorsal skin-fold chamber หนูนูดไมซ์
จะถูกแบ่งเป็น 2 กลุ่ม คือกลุ่ม HepG2 และกลุ่มควบคุม โดยกลุ่มHepG2 ได้รับการปลูกถ่ายเซลล์มะเร็งตับ (HepG2) (2x10$^6$ เซลล์ใน
30 ไมโครลิตร) บน dorsal skin-fold chamber ส่วนกลุ่มควบคุมได้รับ normal saline แทน หลังจากนั้นแต่ละกลุ่มจะถูกแบ่งเป็น 3
กลุ่มย่อย คือ กลุ่มที่ไม่ได้รับการรักษา กลุ่มที่ได้รับการรักษาด้วยสารสกัดสมุนไพร ขนาด 640 และ 3,200 มิลลิกรัมต่อน้ำหนักตัว
หนึ่งกิโลกรัมตามลำดับ วันที่ 2, 7, และ14 หลังการปลูกถ่ายเซลล์มะเร็ง ภาพหลอดเลือดขนาดเล็กบริเวณการปลูกถ่ายเซลล์มะเร็งถูก
ศึกษาภายใต้กล้อง Fluorescence videomicroscopy โดยนำภาพหลอดเลือดขนาดเล็กที่บันทึกมาหาค่าความหนาแน่นของหลอดเลือด
ขนาดเล็กเพื่อศึกษาการเกิดหลอดเลือดใหม่ที่เกิดจากการปลูกถ่ายเซลล์มะเร็งตับ หลังจากนั้นได้ทำการเก็บเลือดเพื่อวิเคราะห์การ
แสดงของ VEGF โดยวิธีการ enzyme immunoassay และเก็บชิ้นเนื้อบริเวณที่ปลูกถ่ายเซลล์มะเร็งตับเพื่อวิเคราะห์การแสดงออกของ
HIF-1α โดยวิธีการอิมมูโนเคมี

ผลการทดลองจากภาพวิดิโอแสดงการขยายตัวของหลอดเลือด การยอมให้สารผ่านเข้าออกทางหลอดเลือดเพิ่มขึ้น การ
คดงอของหลอดเลือด 2 วันหลังจากการปลูกถ่ายเซลล์มะเร็งตับ วันที่ 7 หลังจากการปลูกถ่ายเซลล์มะเร็งตับ พบว่าเกิดหลอดเลือด
ใหม่จากการแสดงลักษณะการงอกออกของเซลล์เอนโดทีเลียมจากหลอดเลือดดั้งเดิม การวิเคราะห์ด้วยโปรแกรม Image software
(Global Lab Image II software) แสดงให้เห็นถึงความหนาแน่นและเส้นผ่านศูนย์กลางของหลอดเลือดขนาดเล็กในกลุ่ม HepG2
เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในวันที่ 7 และ 14 เมื่อเปรียบเทียบกับกลุ่มควบคุมที่มีอายุเท่ากัน (p<0.05) สารสกัดสมุนไพร (จีพีโอ
1986) ระดับความเข้มข้นสูงทำให้ค่าการเพิ่มความหนาแน่นของหลอดเลือดขนาดเล็กลดลงอย่างมีนัยสำคัญทางสถิติ ส่วนการให้
สารสกัดสมุนไพร (จีพีโอ1986)ระดับความเข้มข้นต่ำ ค่าการเพิ่มความหนาแน่นของหลอดเลือดขนาดเล็กลดลงภายในวันที่ 14 หลัง
การรักษาอย่างมีนัยสำคัญทางสถิติ ทั้งนี้สนับสนุนด้วยผลทางเนื้อเยื่อวิทยาซึ่งในกลุ่มสารสกัดสมุนไพร (จีพีโอ1986) ระดับความ
เข้มข้นสูงพบว่าการฝังตัวของเซลล์มะเร็งลดลงอย่างชัดเจน

กลุ่มควบคุมและกลุ่มควบคุมที่ได้รับการรักษาด้วยสารสกัดสมุนไพรที่ไม่ได้รับการปลูกถ่ายเซลล์มะเร็งตับพบว่าการ
แสดงออกของ VEGF ไม่มีความแตกต่างกันทางสถิติ ในกลุ่ม HepG2 พบว่าระดับการแสดงออกของ VEGF ในวันที่ 7 และ 14
สูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม (p<0.05) และพบว่าระดับการแสดงออกของ VEGF มีความสัมพันธ์กับค่า
ความหนาแน่นของหลอดเลือดขนาดเล็กอย่างมีนัยสำคัญทางสถิติวิเคราะห์โดย Pearson's Correlation (r=0.7, p<0.001)

การแสดงออกของ HIF-1α ไม่พบในกลุ่มควบคุม แต่จะแสดงออกในกลุ่มที่ได้รับการปลูกถ่ายเซลล์มะเร็งตับ ซึ่งจะพบ
มากในวันที่ 2 และ 7 ส่วนวันที่ 14 จะพบว่าลดลงอย่างมีนัยสำคัญทางสถิติ ในกลุ่ม HepG2 ที่ได้รับการรักษาด้วยสารสกัดสมุนไพร
(จีพีโอ1986) ทั้งระดับความเข้มข้นสูงและความเข้มข้นต่ำไม่มีผลต่อระดับการแสดงออกของ HIF-1α ทุกช่วงเวลา

โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดสมุนไพร (จีพีโอ1986) มีคุณสมบัติยับยั้งการเจริญของมะเร็งในหนูนูด
ไมซ์ที่ได้รับการปลูกถ่ายเซลล์มะเร็งตับ (HepG2) ซึ่งผลของสารสกัดสมุนไพร (จีพีโอ1986) ในการยับยั้งการเกิดหลอดเลือดใหม่นั้น
อาจผ่านทางการลดการสร้าง VEGF แต่ไม่มีผลต่อ HIF-1α

สาขาวิชา..............สรีรวิทยา..................... ลายมือชื่อนิสิต..........................
ปีการศึกษา..............2550......................... ลายมือชื่ออาจารย์ที่ปรึกษา..........................
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..........................
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..........................

## 4589698420 : MAJOR PHYSIOLOGY
KEYWORD:TUMOR ANGIOGENESIS/NUDE MICE/HepG2/HIF-1α/VEGF

NAPHATSANAN DUANSAK: EFFECTS OF HERBAL EXTRACT (GPO1986) ON TUMOR ANGIOGENESIS OF HEPATOCELLULAR CARCINOMA CELLS (HepG2) IMPLANTED IN NUDE MICE. THESIS ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D. THESIS CO-ADVISORS: ASSOC. PROF. PARVAPAN BHATTARAKOSOL, Ph.D., AND PONTHIP WIRACHWONG, Ph.D. 137 pp.

The objectives of this study were to examine effects of supplementation of herbal extract (GPO1986) on tumor angiogenesis and two kinds of angiogenic biomarkers (HIF-1α expression and serum VEGF levels) in hepatocellular carcinoma cell (HepG2)-implanted nude mice. Male BALB/c-nude mice 8-10 weeks of age were used for this study. All animals were divided into two groups of HepG2 (HepG2) and control (Con). The animals were further separated into 3 subgroups of vehicle, treated with 640 and 3,200 mg/kg BW of GPO1986. In the nude mice model, a dorsal skin-fold chamber was used, in which HepG2 (American Type Culture Collection (ATCC)] was transplanted. On different time-points (2, 7, and 14 days) after HepG2 inoculation, the microcirculation within the chamber was observed using fluorescence videomicroscopy. Based on the recorded video images, capillary diameter and capillary density (CD) were determined to characterize tumor neovasculaization. VEGF expression was measured in blood withdrawn, using enzyme immunoassay while HIF-1α expression was measured on samples isolated from tumor inside the chamber, using immunohistochemistry.

The experimental results revealed that the video images demonstrated dilation, hyperpermeability, tortousity of host microvessels 2 days after HepG2 inoculation. The tumor angiogenesis was initiated with endothelial sprouting from the host vessel on day 7 after HepG2 inoculation. Based on the recorded video images, the neocapillary density and diameter were measured using a digital image analysis (Global Lab image II software). The image analysis demonstrated that in the HepG2 group, the neocapillary density and diameter significantly increased on day 7 and 14 compared to the aged-matched controls ($p<0.05$). Supplementation of GPO1986 at high dose significantly attenuated the increase of tumor capillary density. However, at low dose of GPO1986, these parameters decreased significantly 14 day after HepG2 inoculation. The histological examination of tissue section within the dorsal skin-fold chamber showed remarkable decrease in tumor deposit with supplementation of GPO1986.

The present VEGF measurement showed that in HepG2 group, the serum VEGF levels increased significantly up to 152 pg/ml on day 14 from 67 pg/ml on day 2. The VEGF levels on day 2 was not significantly different, compared with the control levels, but the serum VEGF levels on both 7 and 14 days were significantly higher than their control levels ($p<0.05$). Using the Pearson correlation, the association of serum VEGF with neocapillary density was significantly correlated ($r =0.70, p< 0.001$).

The HIF-1α staining showed that HIF-1α was expressed markedly 2 and 7 days after HepG2 inoculation, but its expression significantly decreased on day 14. The HIF-1α expression was not influenced by supplementation of GPO1986 at high and low dose any experimental periods after HepG2 inoculation.

In conclusion, herbal extract (GPO1986) was able to suppress the growth of HepG2-implanted in nude mice, partly due to the inhibition of tumor angiogenesis. And this anti-angiogenesis might come from its inhibitory effect on VEGF expression, without any effects on HIF-1α expression.

Field of study:............Physiology............Student's signature....... *Naphatsanan Duansak*

Academic year:..............2007..................Advisor's signature.......................................

Co-advisor's signature. *Parvapan Bhattarakosol*

Co-advisor's signature.......................................

# ACKNOWLEDGEMENTS

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# LISTS OF ABBREVIATIONS

| | | |
|---|---|---|
| ARNT | = | Aryl hydrocarbon nuclear translocator |
| AP-1 | = | Activator Protein-1 |
| bFGF | = | Basic fibroblast growth factor |
| bHLH | = | Basic helix–loop–helix |
| BW | = | Body Weight |
| CD | = | Capillary density |
| cGMP | = | cyclic Guanosine monophosphate |
| CM | = | Conditioned medium |
| COX | = | Cyclooxygenase |
| CRE | = | cAMP Response Element |
| DMBA | = | Dimetylbenza anthracene |
| ECM | = | Extracellular matrix |
| ECs | = | Endothelial cells |
| EGFR | = | Epidermal growth factor receptor |
| ERK | = | Extracellular Signal-Regulated Kinase |
| ERK-MAPK | = | Extracellular signal-regulated kinases-mitogenactivated protein kinases |
| FGFs | = | Fibroblast Growth Factors |
| Flk-1 | = | Fetal Liver Kinase-1 |
| GB | = | Glioblastoma cell |
| GPO | = | Government Pharmaceutical Organization |
| HepG2 | = | Hepatocellular carcinoma cell |
| HIF-1 | = | Hypoxia inducible factor-1 |
| HIF-1$\alpha$ | = | Hypoxia-inducible factor one alpha |
| HRE | = | Hypoxia-Response Element |

| | | |
|---|---|---|
| HUVEC | = | Human umbilical vein endothelial cells |
| IGF-IR | = | Insulin like growth factor-I receptor |
| IL-8 | = | Interleukin-8 |
| i.p. | = | Intraperitoneal |
| KDR | = | Kinase Insert Domain-Containing Receptor |
| MAP | = | Mitogen activated protein |
| MAPK | = | Mitogen activated protein kinase |
| MMC | = | Mitomycin-c |
| MMP | = | Matrix metalloprotease |
| MMPs | = | Matrix metalloproteiases |
| NF-kB | = | Nuclear Factor-Kappa B |
| NK | = | Natural killer |
| NLS | = | Nuclear localisation signal |
| NO | = | Nitric Oxide |
| NOS | = | Nitric Oxide Synthase |
| eNOS | = | Endothelial Nitric Oxide Synthase |
| iNOS | = | Inducible Nitric Oxide Synthase |
| ODD | = | Oxygen-dependent degradation domain |
| PA | = | Plasminogen activator |
| tPA | = | Tissue-Type Plasminogen Activators |
| uPA | = | Urokinase Plasminogen Activators |
| PAI | = | Plasminogen activator inhibitor |
| PAIs | = | Plasminogen activator Inhibitors |
| PAI-1 | = | Plasminogen activator Inhibitors-1 |
| PAS | = | Per-ARNT-sim |
| PDGF | = | Platelet-derived endothelial growth factor |
| PDGFR-$\alpha$ | = | PDGF receptor-alpha |

| | | |
|---|---|---|
| PlGF | = | Placenta-like growth factor |
| pVHL | = | Proline von Hippel-Lindau |
| Sp-1 | = | Specificity protein -1 |
| TAD | = | Transactivation domains |
| TIMPs | = | Inhibitors of matrix-degrading proteolytic enzymes |
| TGF- β | = | Transforming growth factor-β |
| VEGF | = | Vascular endothelial growth factor |
| VEGFR- 1 | = | VEGFreceptor-1 |
| VHL | = | von Hippel–Lindau |
| VPF | = | Vascular Permeability Factor |
| vWF | = | von Willebrand Factor |

# CHAPTER I

# INTRODUCTION

A cancer requires nutrients, in particular a tumor greater than 1–2 mm$^3$ in volume needs its own blood supply for continued growth (Folkman, 1971). The new vessels are usually obtained by angiogenesis, a process defined as the formation of new blood vessels from existing vasculature. Inadequate blood supply in the tumor leads to hypoxia as oxygen diffusion is limited.

The center of the tumor is lack of oxygen, therefore the hypoxia is initiated, causing the expression of several angiogenic factor, for instance, vascular endothelial growth factor (VEGF) (Wenger *et al*., 1997). There is a substantial correlation among VEGF expression, cancer progression and metastasis (Bruick *et al*., 2001). The significance of VEGF expression in the disease prognosis has been reported in gastric, colon, breast, bladder and oesophageal carcinomas, as well as in other malignancies (Takahashi *et al*., 1995, Kitadai *et al*., 1998). Yoysungnoen *et al*. (2005) demonstrated that in HepG2- implanted nude mice, the serum VEGF levels were significantly increased on day 7$^{th}$ and 14$^{th}$ day compared to the age-matched control groups.

Hypoxia inducible factor (HIF)-1 activates transcription and plays a critical role in oxygen homeostasis (Iyer *et al*., 1998). HIF-1 is a heterodimer of HIF-1$\alpha$ and HIF-1$\beta$ subunits. HIF-1$\beta$ has been previously identified as an aryl hydrocarbon nuclear translocator (ARNT) (Wang *et al*., 1995). Both transcription factors contain basic helix–loop–helix (bHLH) and per-ARNT-sim (PAS) domains that are required for dimerisation and DNA binding. It has been reported that these domains control various critical embryogenic and pathogenic events (Wang *et al*., 1998, Crews, 1998, Crews, 1998, Semenza, 2002). Under non-hypoxic conditions, HIF-1$\alpha$ is subject to rapid ubiquitination and proteasomal degradation (Wang *et al*., 1995). The degradation of HIF-1$\alpha$ is affected by the von Hippel–Lindau (VHL) protein (Maxwell *et al*., 1999). Therefore, HIF-1 has

been recognized as an important regulatory protein in the transcription of a large number of genes related to glucose transport, glycolysis, erythropoiesis, cell proliferation/survival and angiogenesis (Wenger *et al*., 1997, Wenger, 1999). In human tumors, overexpression of HIF-1$\alpha$ may activate metabolic and pathogenic pathways that are related to tumor angiogenesis, growth, invasion and metastasis (Koukourakis *et al*., 2002). Tumors derived from cells lacking HIF-1$\alpha$ or HIF-1$\beta$ show significantly reduced vascularization and, in most cases, reduced growth rates compared with parental cells (Bruick *et al*., 2001, Ryan *et al*., 2000, Carmeliet *et al*., 1998, Kung *et al*., 2000). The level of HIF-1$\alpha$ proteins is inversely related to the oxygen tension in both cultured cells (Ravi *et al*., 2000) and *in vivo* (Zagzag *et al*., 2000). Hypoxic oxygen tensions that induce HIF-1$\alpha$ levels have been demonstrated in cancer patients with a poor clinical outcome (Semenza *et al*., 1992, Folkman *et al*., 1989, Guidi *et al*., 1997).

When proangiogenic factors outweigh antiangiogenic factors, endothelial cells (ECs) become activated from their normal quiescent state. This is known as the 'angiogenic switch' (Hanahan and Folkman, 1996). Following the angiogenic switch, breakdown of basement membranes and extracellular matrix (ECM) occurs largely as a result of an increasei in matrix metalloprotease (MMP) activity. These matrix changes promote the migration of ECs to the extravascular space where they proliferate. The cells then organize themselves into tubes and lumens, forming the new capillary network. As this progresses, pericytes are recruited and attached to stabilize the new vessels. Up to this maturation point, ECs are dependent on VEGF for their integrity and survival (Benjamin *et al*., 1998).

The ability to induce and maintain angiogenesis is thus a crucial step in the development of a tumor. Therefore, inhibiting tumor angiogenesis may be a promising therapeutic modality for treatment of cancer (Gimbrone *et al*., 1972; Graham *et al*., 1994).

All current chemotherapy modalities have significantly different from side effects and dose-limiting toxicities (Petrylak,2005, Evans,2005). It is now being increasingly recognized that intervening critical processes of cancer growth and development including

angiogenesis and metastasis with naturally occurring herbal and phytochemical agents. Recently, a number of antiangiogenic agents have entered in clinical trials. In china, many clinical trials focusing on the anticancer effects of herbal formulas have been conducted of them demonstrated that herbs are helpful against cancer, especially in improving survival and quality of life in patients suffering from advanced cancer (Ruan, 2006).

Interestingly, GPO1986, a herbal remedy extracted and formulated by Government Pharmaceutical Organization (GPO) was claimed to be effective in the treatment of patient with mammary tumors. This remedy consists of eight medical plants, i.e. *Canna indica* Linn*., Polygala chinensis* Linn*., Clinacanthus nutans* Lindau*., Ammannia baccifera* Linn*. Acanthus ebracteatus* Vahl*., Mallotus philippensis* Muell. Arg., *Premna herbacea* Roxb. Mold. *and Smilax carbularia Kunth C*. It could increase body weight and survival rate, prolong survival time, suppress tumor growth and decrease the rate of metastasis in experimental animals (Pornsiriprasert, 1988; NaBangchang, 1987). In addition, Wiwatwittaya (1992) and Thisoda *et al*., (1995) have indicated that the immunostimulatory effect of the GPO1986 in tumor-bearing nude mice was exerted via an increase in natural killer (NK) cell activity.

Moreover, In 1999, Akarasereenont and his co-worker have found that GPO1986 could inhibit cell proliferation in human umbilical vein endothelial cells (HUVEC) treated with condition medium from glioblastoma cell culture. The herbal extract could also inhibit cell proliferation in HUVEC treated with 15% serum from glioblastoma patients. However, the effect of GPO1986 on tumor angiogenesis has not been determined yet. Therefore, the present study was designed to study the effect of GPO1986 on angiogenesis in hepatocellular carcinoma cell (HepG2) –implanted nude mice and to examine the effects of GPO1986 on HepG2 angiogenic biomarkers, serum VEGF and HIF-1$\alpha$.

# CHAPTER II

# LITERATURE REVIEWS

## Tumor angiogenesis

Angiogenesis, the process by which capillaries sprout from preexisting blood vessels, is tightly regulated by a large number of pro-angiogenic and anti-angiogenic factors.Tumor cells have an absolute requirement for a persistent supply of new blood vessels to nourish their growth and to facilitate metastasis. Thus, tumor vascularization is a vital process for the progression of a neoplasm from a small localized tumor to an enlarging tumor with the ability to metastasize (Folkman, 1971; Liotta *et al*., 1974). The angiogenic cascade leading to tumor vascularization can be divided into two general phases, the prevascular phase (referred to as the "angiogenic switch") and the vascular phase (Pepper *et al*., 1996; Hanahan and Folkman, 1996). Once tumor cells undergo the transformation to an angiogenic phenotype, these malignant cells are capable of inducing phenotypic changes in ECs as well as in other cell types (Norrby, 1997; Polverini, 1996). At that point, vascular tumors acquire their own blood supply, which permits a rapid rate of growth. While tumors lacking adequate vasculature become necrotic (Brem *et al*., 1976) or apoptotic (Holmgren *et al*., 1995), tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential.

In 1971, Folkman proposed that targeting tumor vasculature could limit tumor expansion and be an efficient threapy for cancer progression. The oxygen diffusion limit from blood vessels is known to be approximately 150 μm and therefore it was proposed that cutting off the blood flow induces the apoptosis of tumor cells, which are dependent on the oxygen and nutrition coming from the blood vessels (Hlatky *et al*., 2002; Kerbel and Folkman, 2002). During tumor growth, the switch to an angiogenic phenotype requires up-

regulation of angiogenic stimulators and likely down-regulation of endogenous angiogenesis inhibitors (Folkman, 1972; Hanahan and Weinberg, 2000; Folkman, 1995; Hanahan and Folkman, 1996; Bergers and Benjamin, 2003; Colorado *et al*., 2000; Volpert *et al*., 2002). This angiogenic phenotype is characterized by tumor expression of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), placenta-like growth factor (PlGF), transforming growth factor-β (TGF- β), platelet-derived endothelial growth factor (PDGF), and others. The pro-angiogenic gene expression is also increased by events such as hypoxia and also by oncogenes such as Ras and Myc and the loss or mutation of tumor supressor proteins such as p53 and PTEN (Folkman, 1995; Kerbel and Folkman, 2002; Carmeliet *et al*., 1998; Folkman, 2003; Fukumura *et al*., 1998; Relf *et al*., 1997; Watnick *et al*., 2003; Rak *et al*.,2000; Yu *et al*., 2002; Pore *et al*., 2003). (Fig. 2.1) (From Yuki and Raghu, 2005).
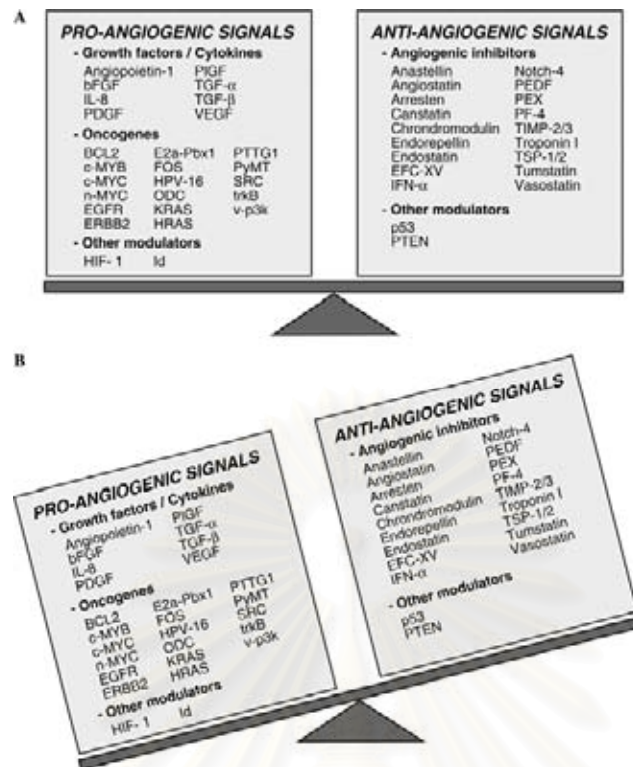
**Figure 2.1** The angiogenic balance. Angiogenesis is tightly regulated by the angiogenic balance (A) and activation of angiogenesis leads to "angiogenic switch" (B). The angiogenic phenotype during tumor growth is characterized by the expression of pro-angiogenic molecules (From Yuki and Raghu, 2005).

Angiogenesis is a complex process involving extensive interplay between cells, soluble factors and extracellular matrix (ECM) components. The construction of a vascular network requires different sequential steps including: (Sandra *et al.*, 2001)

(1) the release of proteases from "activated" ECs

(2) degradation of the basement membrane surrouding the existing vessel

(3) migration of the ECs into the interstitial space

(4) EC proliferation

(5) lumen formation

(6) generation of new basement membrane with the recruitment of pericytes

(7)  fusion of the newly formed vessels

(8)  initiation of blood flow
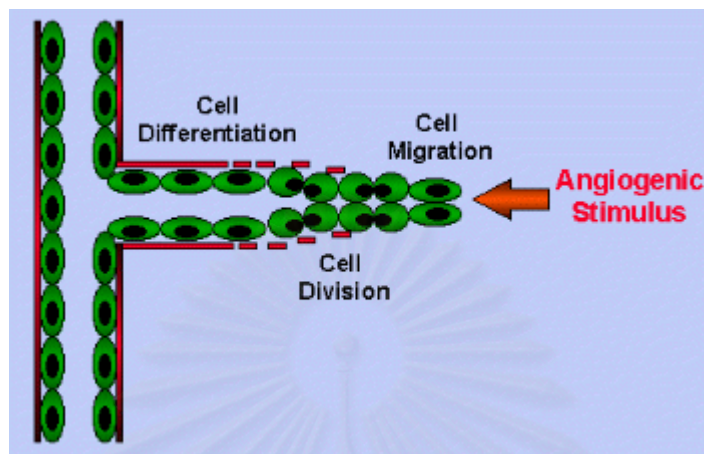


**Figure 2.2**  Angiogenesis process

(From http://www.med.unibs.it/~airc/sandra/process.html)

**Initiation of Angiogenesis**

On receiving a net angiogenic stimulus, ECs in capillaries near the tumor become activated: they loosen the normally tight contacts with adjacent cells (Papetti and Herman, 2002) and secrete proteolytic enzymes (or proteases), leading to a degradation of extracellular tissue (Pepper *et al*., 1990). There are a large number of such enzymes, which may be broadly divided into matrix metalloproteases (MMPs) and the plasminogen activator (PA)/plasmin system (Pepper, 2001). The MMPs are capable of digesting different protein types and may be subdivided accordingly into collagenases, gelatinases, stromelysins, matrelysins and membrane-type MMPs (Vihinen and Kahari, 2002). PAs activate plasminogen which is widely expressed, but inactive, into the broad-spectrum protease, plasmin (Pepper *et al*., 1992). Both of these protease families have an associated class of inhibitors. MMPs are inhibited by tissue inhibitors of metalloproteases (TIMPs) (Jiang *et al*., 2002). PAs are inhibited by plasminogen activator inhibitor (PAI), which also

expresses in fibroblasts and activated EC (Pepper *et al*., 1992). The first target of the proteases produced by the EC is the basement membrane (Pepper, 1997). After the basement membrane has been sufficiently degraded by the protease, the ECs are able to move through the gap in the basement membrane to the ECM. Neighbouring ECs move in to fill the gap and may subsequently follow the leading cells into the ECM (Paweletz and Kneirim, 1989).The first function of the angiogenic growth factors, therefore, is to stimulate the production of proteases by EC (Pepper, 1997). This is a key step in the angiogenic cascade because in the absence of proteolytic activity, the EC are hemmed in by the basement membrane and will be unable to escape from the existing capillary (parent vessel) (Cavallaro and Christofori, 2000).

**EC Migration, Proliferation and Tube Formation**

Following extravasation, the ECs continue to secrete proteolytic enzymes, which also degrade the ECM (Burke and DeNardo, 2001). This is necessary to create a pathway along which the cells can move (Pepper, 2001), and may also release growth factors, such as VEGF, that have been sequestered in the matrix, thus augmenting the angiogenic signal (Hirschi and D'Amore, 1996). The ECs continue to move away from the parent vessel and towards the tumor (Ausprunk and Folkman, 1977), thus forming small sprouts. More EC are recruited from the parent vessel, elongating the new sprouts. These sprouts may initially take the form of solid cell strands, but the EC subsequently form a central lumen, thereby creating the necessary structure for a new blood vessel (Pepper, 1997).

In addition to the angiogenic balance between growth factors and inhibitors, there is a proteolytic balance between proteases and protease inhibitors (Pepper, 2001). A certain amount of proteolysis is necessary to degrade the basement membrane and ECM, allowing EC to move out of the parent capillary and facilitating migration towards the tumor. However, excessive proteolysis is incompatible with angiogenesis because EC migration and tube formation are dependent on the cells' ability to attach to the underlying

substratum (Pepper *et al*., 1992). The secretion of proteases must therefore be precisely regulated. The anti-proteolytic factor, PAI, may play an important role in preventing excessive matrix degradation (Pepper, 2001). EC migration is governed mainly by a chemotactic response to concentration gradients of diffusible growth factors produced by the tumor, which create a potent directional stimulus (Papetti and Herman, 2002). Thus the second key function of the angiogenic growth factors is to induce directed EC migration towards the tumor (Paweletz and Kneirim, 1989). Haptotaxis, cell movement in response to an adhesive gradient, also plays a role EC migration. The effect of haptotaxis, however, is more complicated, and not fully understood, because the EC are continually modifying the adhesive properties of their micro-environment via proteolysis (Pepper, 2001) and the synthesis of new ECM components (Birdwell *et al*., 1978). Perhaps the most important substance involved in cell–matrix adhesion is the ECM component, fibronectin. *In vitro* experimental observations have demonstrated that fibronectin can promote EC migration chemokinetically (i.e. by increasing random, diffusive movement) (Yamada and Olden, 1978; Nicosia *et al*., 1993), and can induce the directional migration of EC up a fibronectin concentration gradient (Bowersox and Sorgente, 1982; Maier *et al*., 1999). However, the situation *in vivo* may not be so straightforward. Some degradation of the ECM is needed to facilitate migration. Although EC may move preferentially up a fibronectin gradient at relatively low concentration levels, their progress may be impeded if the concentration of fibronectin becomes too high. Furthermore, it is possible that by-products of fibronectin proteolysis act as chemoattractants, thus effectively stimulating EC migration towards regions of low fibronectin concentration (Nicosia *et al*., 1993). The effects of haptotaxis are, therefore, far from clear. One possibility is that, at high concentration levels, EC will migrate down a fibronectin concentration gradient to enable them to move through the ECM, whereas, at low concentration levels, EC will exhibit their natural tendency to migrate up a fibronectin concentration gradient to a region of higher cell–matrix adhesion.

In quiescent endothelia, the turnover of EC is very slow, typically measurable in months or years (Han and Liu, 1999). For a short period following extravasation, the low

mitosis levels continue while the initial response is entirely migratory rather than proliferative (Ausprunk and Folkman, 1977). Nevertheless, after this initial period of migration, rapid EC proliferation begins at a short distance behind the sprout tips, increasing the rate of sprout elongation (Paweletz and Kneirim, 1989). In experimental studies, when irradiated ECs (which are incapable of dividing) are exposed to an angiogenic stimulus, the initial response is unaffected and a primitive network of capillary sprouts is formed, but the growth of ECs stops after a few days. Consequently, angiogenesis is not completed (Sholley *et al*., 1984). EC proliferation is therefore necessary for vascularisation to take place, and its stimulation is the third and final key function of the angiogenic growth factors (Han and Liu, 1999).

Sprouts are seen to branch, adding to the number of migrating tips. The sprouts begin by growing approximately parallel  each other, but at a certain distance from the parent vessel, they begin to incline twords other sprouts. This leads to the formation of closed loops (anastomoses), which are necessary for circulation to begin in the new vessels (Paweletz and Kneirim, 1989). This is a crucial event in the formation of a functional vascular network, but the precise stimulus for the change of sprout direction and anastomosis is unknown. In some cases, branching and looping become much more pronounced as the sprouts approach the tumor, producing a dense, highly fused network, with a massive number of sprout tips. This has been termed as the brushborder effect (Muthukkaruppan *et al*., 1982; Sholley *et al*., 1984) and its causes are poorly understood. One possibility is that the higher concentrations of angiogenic factors experienced near the tumor stimulate an increase in EC proliferation and/or vessel branching. Saturation of receptors on the EC surface (see the 'Migration' section) may also have an effect, rendering the EC unable to detect the concentration gradients of the growth factors. If this does occur, it is a transient phenomenon: the receptors eventually recover, allowing the EC to continue to migrate towards the tumor. The capillaries thus reach and penetrate the tumor, vastly improving its blood supply and allowing rapid growth.

**The Vascular Phase**

In physiological angiogenesis, once the target tissue has been vascularised, the expression of angiogenic growth factors ceases. EC migration, proliferation and proteolysis then come to a halt and the newly formed vessels undergo a maturation process (Kraling *et al.*, 1999). Tight cell–cell connections are re-established in the endothelium. Additionally, the EC secrete proteins, such as laminin and collagen, to form a continuous basement membrane (Paweletz and Kneirim, 1989). Finally, peri-endothelial support cells (primarily pericytes in the microvasculature) are recruited (Loughna and Sato, 2001) and the new vessels become a part of the quiescent vascular system. This maturation process does not usually occur in tumor-induced angiogenesis. Despite the fact that capillaries penetrate the edge of the tumor, supplying it with oxygen, there are still hypoxic regions within the tumor, where angiogenic factors can be generated (Sutherland, 1986). In addition, as the newly vascularised areas of the tumor grow, they outstrip their own blood supply and develop hypoxic areas themselves (Holash *et al.*, 1999). The angiogenic switch thus remains turned on and new capillaries continue to grow, extending the blood supply throughout the new rapidly growing and highly heterogeneous tumor. However, continued angiogenesis simply fuels further tumor growth, which in turn demands an improved blood supply. In a highly malignant tumor, the demand for new blood vessels will never be satisfied (Paweletz and Kneirim, 1989).The tumor-related capillaries are not usually able to form mature, stable vessels with a continuous basement membrane, because of the continued production of angiogenic factors (Papetti and Herman, 2002). The new vasculature is irregular, leaky and tortuous (Hashizume *et al.*, 2000) and is constantly being remodelled: some areas of the network regress; some areas undergo robust new angiogenesis, providing a blood supply for previous avascular regions (Vajkoczy *et al.*, 2002).

**Angiogenesis Stimuli**

Angiogenesis is induced when the balance between proangiogenic and antiangiogenic factors is disturbed. In tumors, this imbalance may be caused by either hypoxia (low oxygen tension) or genetic alterations that activate oncogenes and/or inactivate tumor suppressor genes (Rak *et al.*, 2000) (Fig. 2.3).

**Hypoxia**

Using oxygen that diffuses from existing vessels, a tumor grows until oxygen demand exceeds supply. The ensuing hypoxia induces tumor cells to generate, via both increased transcription (regulated by the hypoxia-induced transcription factor, HIF1$\alpha$) and increased mRNA stabilization of a series of proangiogenic factors that function as highly specific growth factors/survival factors for ECs (Dachs *et al.*, 2000). Among these factors are VEGF, FGF, transforming growth factor $\beta$, and tumor necrosis factor $\alpha$. VEGF and FGF are considered to be the most important mediators in tumor angiogenesis. VEGF (VEGF-A) induces proliferation of vascular ECs (Ferrara *et al.*, 1997; Ferrara *et al.*, 1989; Conn *et al.*, 1990; Plouet *et al.*,1989), promotes survival of ECs in newly formed vessels (Alon *et al.*,1995; Gerber *et al.*, 1998; Benjamin *et al.*, 1999; Yuan *et al.*, 1996), and increases blood vessel permeability (Senger *et al.*, 1983, Dvorak *et al.*, 1995). Through increased blood vessel permeability, VEGF allows creation of an extravascular fibrin gel that supports EC growth (Dvorak, 1986; Dvorak *et al.*, 1987). Consequenthy, an in crease in angiogenesis promotes the growth of tumor which repeatedly leads to hypoxia and VEGF production (Fig.2.3)

**Oncogenes and Tumor Suppressor Genes**

In many types of tumor, angiogenesis is not induced by hypoxia, but by genetic alterations. The loss of function of tumor suppressor genes such as *VHL*, *p53*, and *p16INK4a*, or the activation of oncogenes including *ras*, *raf*, *HER2/erbB2 (neu)*, and *src*, results in an increase of VEGF expression and/or secretion (Larcher *et al*., 1996; Kaelin *et al*., 1998; Chiarugi *et al*., 1998; Harada *et al*.,1999; Rak *et al*., 1999; Petit *et al*., 1997) (Fig. 2.3).
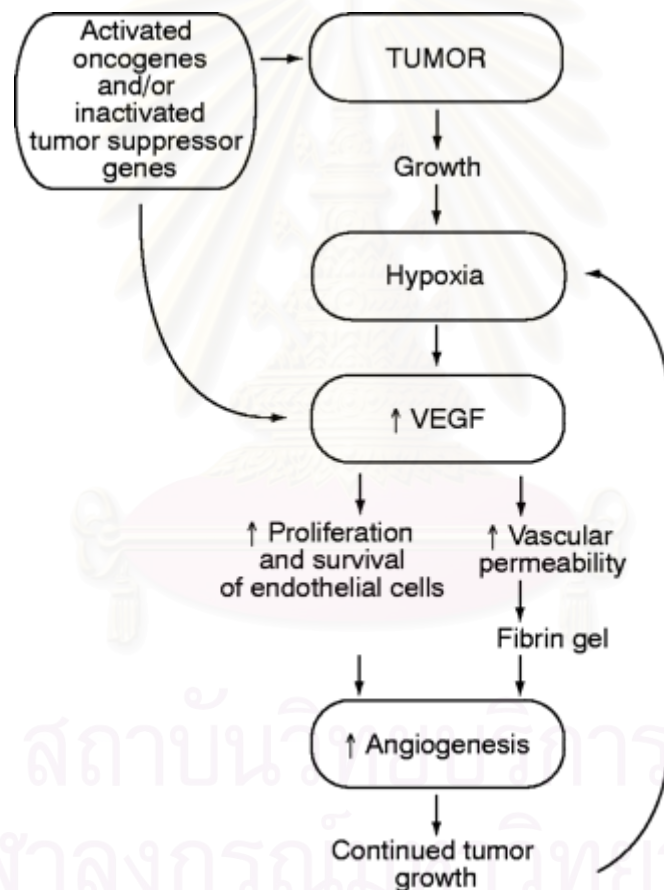


**Figure 2.3** Schematic representation of the triggering mechanisms in tumor angiogenesis: inactivated tumor suppressor genes/activated oncogenes versus hypoxia. In cell culture, these mechanisms can act independently. In human tumors, both mechanisms may modulate angiogenesis (Rak *et al*., 2000).

**Causes of Tumor Hypoxia**

Tumor hypoxia is triggered results from an imbalance between the cellular $O_2$ consumption rate and the $O_2$ supply to the cells (Vaupel  *et al*., 2002). Hypoxia can be caused by a number of factors, most of which are related to perfusion, diffusion, or anemia (Vaupel *et al*., 2001a; Vaupel *et al*., 2002; Höckel and Vaupel, 2001a) :

- Perfusion-related (acute) hypoxia is caused by an  inadequate blood flow in tissues. Tumor microvasculatures frequently have severe structural and functional abnormalities, such as a disorganized vascular network, dilations, an elongated and tortuous shape, an incomplete endothelial lining, a lack of physiological/pharmacological. Perfusion-related $O_2$ delivery leads to ischemic hypoxia, which is often a transient phenomenon.

- Diffusion-related (chronic) hypoxia is caused by an increase in diffusion distances with tumor expansion. This leads to an inadequate $O_2$ supply for cells, distant from the nutritive blood vessels more than 70 μm. Diffusion-related hypoxia may also be caused by deterioration of diffusion geometry, for example, the concurrent versus countercurrent blood flow within the tumor microvessel network.

- Anemic hypoxia is caused by the reduction of  $O_2$ transport capacity of the blood subsequent to tumor-associated or therapy-induced anemia. Experimental studies have shown that the $O_2$ supply to tumors is greatly reduced and hypoxia is intensified at hemoglobin levels below 10-12 g/dl, especially when low $O_2$ transport capacity coincides with a low perfusion rate.   (Vaupel *et al*., 2001a; Vaupel *et al*., 2001b).

Moreover, anemic hypoxia can be caused by carboxyhemoglobin formation in heavy smokers, since carbon monoxide-blocked hemoglobin can no longer transport $O_2$ (toxic hypoxia). Additionally, tumor microvessels may be perfused (at least transiently) by plasma only, which leads to a rapid induction of hypoxia because only a few cells at the arterial end are adequately oxygenated under these conditions (Vaupel *et al*., 2001; Vaupel *et al*.,  2002).

**Cellular Responses to Hypoxic States**

Hypoxia can influence tumor cells either by acting as a stressor that impairs growth or causes cell death (slowing of proliferation, apoptosis, or necrosis) or by serving as a factor that ultimately results in malignant progression and development of resistance to radiation therapy and other cancer treatments (Vaupel *et al*., 2001a; HÖckel and Vaupel, 2001a). To a large extent, the increases in malignant progression and treatment resistance are manifestations of hypoxia-induced proteomic and genomic changes within the tumor cells.

**Proteomic Changes**

Evidence from recent studies suggests that sustained (>6-8 hours) or fluctuating hypoxic stress ($pO_2 \leq 7$ mmHg) can lead to alterations (stimulation or inhibition) of gene expression, as well as posttranscriptional and posttranslational modulations that result in changes in the tumor cell proteome (Vaupel *et al*., 2001; Vaupel *et al*., 2002; HÖckel and Vaupel, 2001a; HÖckel and Vaupel, 2001b). These hypoxia-induced proteomic changes may, in turn, lead to the growth stasis or impairment through molecularly mediated cell-cycle arrest, differentiation, programmed cell death (apoptosis), or necrosis (Moulder and Rockwell, 1987; Durand, 1991; Giaccia, 1996; Riva *et al*., 1998; Haroon *et al*., 2000). Hypoxia-induced cell-cycle arrest at the G1/S check point may be triggered by a hypoxia-inducible factor one alpha (HIF-1α)-mediated activation of the cyclin-dependent kinase inhibitors p21 and p27 (Goda *et al*., 2003). This response seems to be independent of p53, even though p53 accumulates under hypoxia (Koumenis *et al*., 2001). Instead, an increased level of p53 under hypoxic conditions may lead to the alternative activation of apoptosis with Apaf-1 and caspase- 9 as downstream effectors (Soengas *et al*., 1999). However, hypoxia may also induce p53-independent apoptosis pathways involving genes of the BCL-2 family and others. Experimental studies have suggested that hypoxia may act as a

morphogen to induce terminal differentiation of cells, causing necrotic cell death. Overall, the effects of hypoxia-related proteomic changes leading to tumor cell growth stasis or cell death may explain the delayed recurrences, dormant micrometastases, and growth retardation observed in large tumor masses (HÖckel and Vaupel, 2001a; Prehn, 1991; Demicheli *et al*., 1994; Holmgren *et al*., 1995). Alternatively, hypoxia-induced proteomic changes may promote tumor propagation by enabling cells to adapt to nutritive deprivation, or by facilitating proliferation, local invasion, and metastatic spread, thereby permitting the cells to escape their hostile environment. One of the major promoters of tumor cell adaptation to hypoxic stress is the transcription factor HIF-1, which accumulates in response to declining cellular $O_2$ levels (Vaupel *et al*., 2002; HÖckel and Vaupel, 2001a). HIF-1 activates a battery of more than 30 genes, many of which express protein products involved in $O_2$ delivery (e.g., erythropoietin), angiogenesis (e.g., vascular endothelial growth factor [VEGF]), energy preservation (e.g., glucose transporters and glycolytic enzymes), and other processes essential to tumor cell survival, propagation, and spread (Vaupel *et al*., 2002; HÖckel and Vaupel, 2001b). Angiogenesis is an important factor in tumor progression since a tumor usually cannot grow beyond ~1 mm in diameter without an adequate blood supply (Folkman, 1995). A more detailed discussion of HIF-1 and angiogenesis, including their impact on tumor progression, is provided by *Vaupel* (Vaupel, 2004).
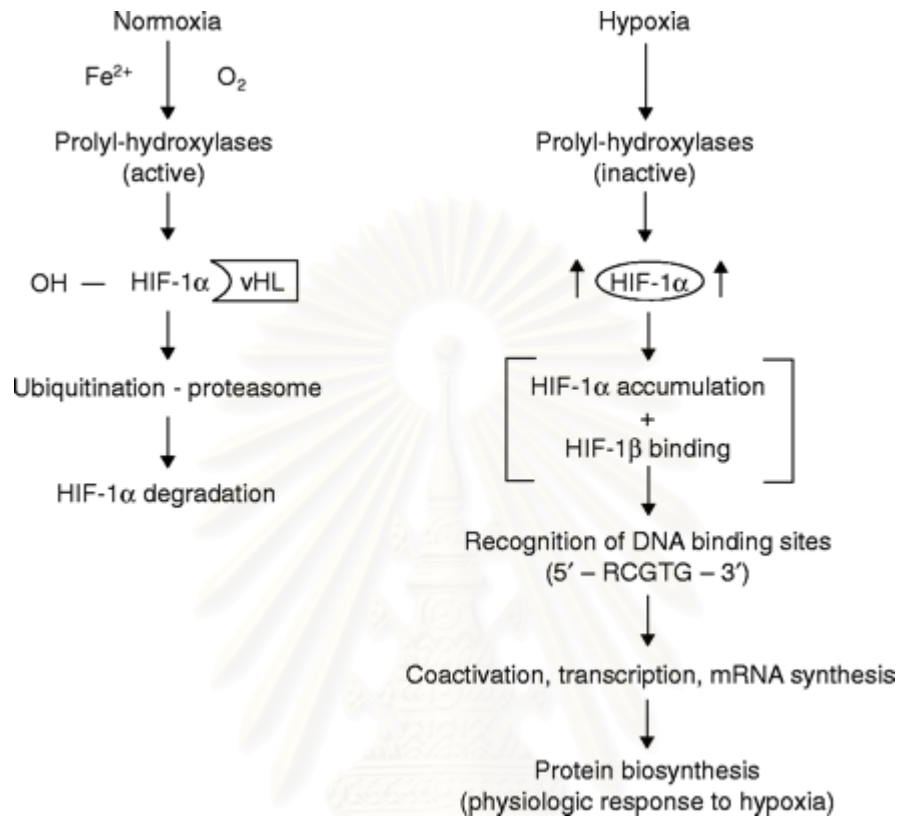
**Figure 2.4** Regulation of HIF-1α by cellular $O_2$ level (From Peter, 2004)

**Genomic Changes**

Mutations in oncogenes and/or tumor suppressor genes are generally thought to be a crucial factor for the development of tumor aggressiveness. Hypoxia ($pO_2 \leq 0.7$ mmHg) promotes genomic instability, thereby increasing the number of mutations (genetic variants). Hypoxia concomitantly exerts a strong selection pressure (Vaupel *et al.*, 2002; HÖckel and Vaupel, 2001a; Höckel and Vaupel, 2001b). Tumor cells adapted to survire under hypoxic conditions (e.g., lower capacity for cell-cycle arrest or apoptosis, greater angiogenic potential) may have growth advantages over nonadapted cells in the hypoxic microenvironment and thire through clonal selection. The expansion of cell clones with

favorable proteomic and genomic adaptive changes can, in turn, exacerbate tumor hypoxia, thereby establishing a vicious circle of increasing hypoxia and subsequent malignant progression. At the clinical level, the consequences of this vicious circle are translated into more local recurrences, locoregional spread, and distant tumor metastases, and greater resistance to radiation therapy and certain forms of chemotherapy (Vaupel *et al.*, 2002).

## Hypoxia inducible -1$\alpha$ (HIF-1$\alpha$)

### HIF-1 Protein Structure

HIF-1, composed of two subunits: HIF-1$\alpha$ and HIF-1$\beta$ is active only as a heterodimer form (Fig. 2.5). While HIF-1$\beta$ is readily found in cells under oxygenated condition, HIF-1$\alpha$ is virtually undetectable in this conditions. For the HIF-1 transcriptional complex to be functional, HIF-1$\alpha$ levels must be induced. The human HIF-1 $\alpha$ gene is located on chromosome14 (14q21-q24) whereas the HIF-1$\beta$ gene is located on chromosome 1 (1q21). Homology is relatively well conserved for the HIF-1 $\alpha$ and HIF-1$\beta$ subunits, with over a 90% similarity between human, rat and mouse. HIF-1$\alpha$ and HIF-1$\beta$ are relatively large proteins in size, being comprised of 826 and 789 amino acids, respectively. Both proteins contain nuclear localization signals and a basic helix-loop-helix motif (bHLH). The basic domain is essential for DNA binding while the HLH domain is responsible for subunit dimerisation. Another common feature of both proteins is the PAS domain. HIF-1$\alpha$ has some interesting unique features including the oxygen-dependent degradation domain (ODD). It has been reported that the region between residues 401–603 is highly oxygen-regulated (Huang, Gu, Schau, & Bunn, 1998). Being a highly labile protein in normal oxygen conditions, specific degradation of HIF-1$\alpha$ in normoxia is triggered through this domain. HIF-1$\alpha$ also contains two transactivation domains (TAD) which are responsible for the transcriptional regulation of HIF-1 target genes. HIF-1$\alpha$' s N-terminal TAD is found between residues 531–575 while a C-terminal TAD is found at the

very end of the protein from residues 813–826. These TADs are also involved in the binding of coactivators such as p300/CBP which are essential for HIF-1's transcriptional activation. HIF-1β also contains a TAD, but this domain was shown to be unnecessary for HIF-1's transcriptional activity. Althoug both subunits are similar, striking differences in oxygen sensitivity and transactivation ability clearly identify HIF-1α as the main functional protein of the HIF-1 complex. Other isoforms of the HIF-1α subunit have also been identified which include HIF-2α and HIF-3α. However, functional knock-down studies have clearly demonstrated the predominance of HIF-1α in hypoxic gene induction.
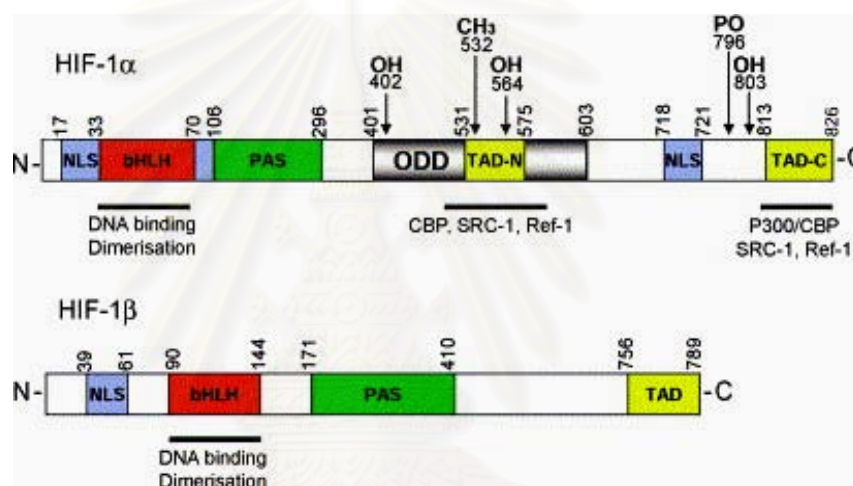


**Figure 2.5**  Protein domains of HIF-1α  and HIF-1β. Functional domains and binding domains with co-factors are shown. Hydroxylation, acetylation and phosphorylation sites mentioned in the text are indicated. bHLH, basic helix-loop-helix; ODD, oxygen-dependent degradation domain; TAD-N and TAD-C, N- and C-terminal transactivation domain; NLS, nuclear localisation signal; PAS, Per-ARNT-Sim. (From Marc-Andr´e *et al*., 2005)

**The Regulation of HIF-1**

HIF-1$\alpha$ protein has a short half-life (t1/2 $\sim$ 5 min) and is highly regulated by oxygen (Salceda and Caro, 1997). The transcription and synthesis of HIF-1$\alpha$ are constitutive and seemly not to be affected by oxygen (Kallio *et al.*, 1997; Wang *et a*l., 1995; Wiesener *et al.*, 1998). However, in normoxia, the HIF-1$\alpha$ proteins are rapidly degraded, resulting in essentially no detectable HIF-1$\alpha$ protein (Wang *et al.*, 1995). During hypoxia, HIF-1$\alpha$ becomes stabilized and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1$\beta$ to form HIF complex (Huang *et al.*, 1996; Kallio *et al.*, 1997). The activated HIF complex then associates with HREs in the regulatory regions of target genes and binds the transcriptional co-activators to induce gene expression (Lando *et al.*, 2002b). Regulation HIF-1$\alpha$ are chiefly controlled by its posttranslational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation (Brahimi-Horn *et al.*, 2005). The modification of HIF-1$\alpha$ occurs within several domains. In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue in its ODD promote interaction of HIF-1$\alpha$ with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex (Masson *et al.*, 2001; Srinivas *et al.*, 1999). pVHL complex tags HIF-1$\alpha$ with ubiquitin and thereby marks it for degradation by the 26S proteasome. In addition, hydroxylation of an asparagine residue in the C-TAD inhibits the association of HIF-1$\alpha$ with CBP/p300 and thus inhibits its transcriptional activity (Lando *et al.*, 2002a).

## Role of HIF-1α in Tumor angiogenesis

The overexpression of HIF-1α has been demonstrated in multiple types of cancer, as well as in their regional and distant metastases, as a result of adaptation of tumor cells to hypoxia. Zhong *et al*., (1999) reported an increased HIF-1α expression in 13 tumor types, including lung, prostate, breast and colon carcinoma. They also suggested that overexpression of HIF-1α can occur very early in carcinogenesis, before histological evidence of angiogenesis or invasion. Detection of HIF-1α in bladder was first reported by Talks *et al*. (2000), who investigated its distribution in bladder adenocarcinoma sample, using immunohistochemistry.

Giatromanolaki *et al*. (2001) have shown that HIF-1α overexpression is a common event in nonsmall cell lung carcinomas, upregulating the angiogenic pathways and associated with poor prognosis. HIF-1α has been related to unfavorable prognosis in woman with cervical cancer and poor response to radiotherapy in patients with orophayngeal and early esophageal cancer (Aebersold *et al*., 2001, Birner *et al*., 2000, Koukourakis *et al*., 2001). Studies in HIF-1α deficient mouse embryonic stem cell have showed both reduced angiogenesis and reduced tumor growth (Ryan *et al*., 1998). In another study (Ryan *et al*., 2000), tumors derived from HIF-1α deficient fibroblasts grew at a slower rate than wild-type counterparts, despite similar angiogenic development.

## Vascular Endothelial Growth Factor (VEGF)

VEGF (also known as vascular permeability factor and VEGF-A) is a tumor-secreted cytokine with critical importance in both normal and tumor-associated

angiogenesis. It was originally described as a protein that increased microvascular permeability to plasma proteins, (Dvorak *et al*., 1979). VEGF has been further characterized with regard to multiple effects relevant to the generation and preservation of tumor vasculature. These effects include induction of EC division and migration (Dvorak *et al*., 1995; Ferrara and Davis-Symth, 1995) promotion of EC survival through protection from apoptosis, (Benjamin *et al*., 1999) and reversal of EC senescence (Watanabe *et al*., 1997). VEGF is a dimeric glycoprotein and a member of the platelet-derived growth factor (PDGF). The superfamily of growth factors includes VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor. The *VEGF* gene, located on the short arm of chromosome (Watanabe *et al*., 1997), is composed of eight exons and is differentially spliced to encode four major isoforms (VEGF121, VEGF165, VEGF189 and VEGF206) (Tischer *et al*., 1991). These isoforms have distinct physical properties relevant to their ability to bind heparin and extracellular matrix, but identical biologic activities when they are free in solution (Houck *et al*., 1992). VEGF165 is the predominant isoform with physical characteristics that result in optimal bioavailability and potency. VEGF exerts its biologic effect through interaction with receptors present on the cell surface. These transmembrane tyrosine kinase receptors include VEGFreceptor-1 (VEGFR- 1; Flt-1) and VEGFR-2 (kinase insert domain-containing receptor/ Flk-1), selectively expressed on vascular ECs , VEGFR-3 (Flt-4), expressed on lymphatic and vascular endothelium, and the neuropilin receptor, expressed on vascular endothelium and neurons (Dvorak, 2002). Upon binding of VEGF to the extracellular domain of the receptor, dimerization and autophosphorylation of the intracellular receptor tyrosine kinases occurs and a cascade of downstream proteins are activated (Fig. 2.6). VEGFR-2 appears to be the main receptor responsible for mediating the pro-angiogenic effects of VEGF. VEGF is the most potent pro-angiogenic protein described to date with biologic effects relevant to tumor angiogenesis. Its suitability as a therapeutic target, however, is dependent on the biology of specific tumor types. VEGF expression is regulated by a number of factors including cytokines (Cohen *et al*., 1996; Matsumoto *et al*., 1997) growth factors (Claffey *et al*., 2001;

Stavri *et al*., 1995; Pertovaara *et al*., 1994; Goad *et al*., 1996; Ryuto *et al*., 1996),

hormones (Wang *et al.*, 2002), hypoxia (Forsythe *et al*., 1996; Levy *et al*., 1995; Shih *et al*., 1998), and tumor suppressor genes (Mukhopadhyay *et al*., 1995; Pal *et al*., 2001)
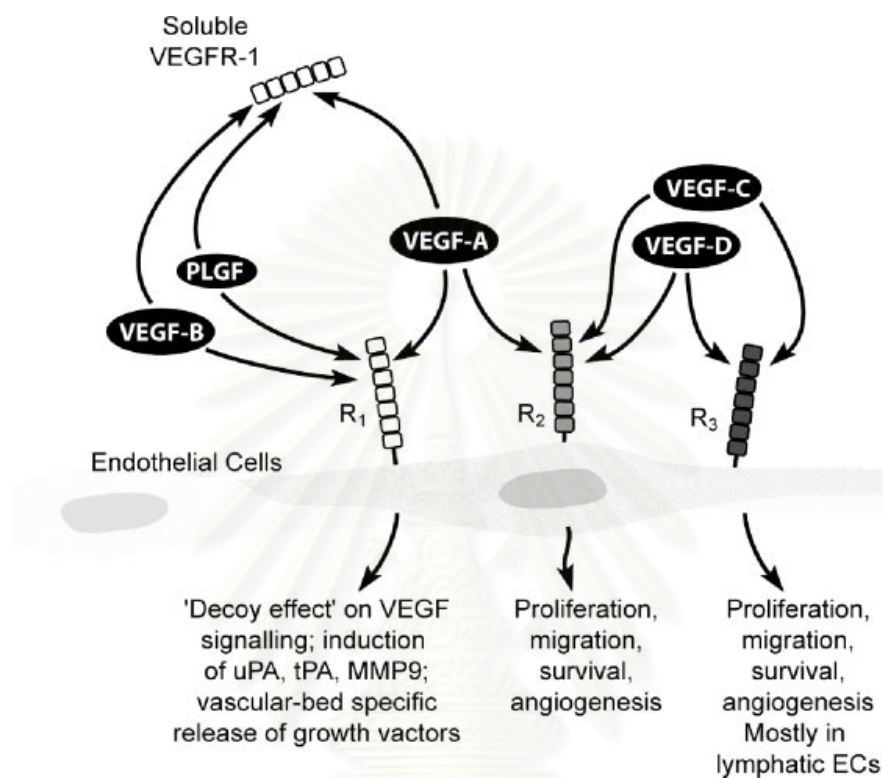


**Figure 2.6**  Role of the VEGFR tyrosine kinases in ECs.

(From Napoleone, 2004)

**Regulators of VEGF expression**

### Hypoxia

Hypoxia has been shown to play an important role in the regulation of VEGF expression. Studies have shown that hypoxia inducible factor-1 (HIF-1) is the key mediator of this hypoxic response and that the product of the von Hippel Lindau (*vHL*) tumor suppressor gene plays an important role (Ferrara *et al*., 2003; Iliopoulos *et al.*, 1996; Maxwell *et al*., 1999). Under normoxic conditions, HIF-1$\alpha$ is rapidly degraded by the

ubiquitin-proteosome pathway a process that is controlled by the *vHL* tumor suppressor gene product (Iliopoulos *et al*., 1996; Maxwell *et al*., 1999; Maxwell and Ratcliffe, 2002). Under conditions of hypoxia, or when *vHL* is absent or mutated, HIF-1α dimerizes with HIF-1β, and this complex translocates to the nucleus and binds to the VEGF promoter, leading to an increase of VEGF transcription.

### Growth Factors and Cytokines

Several studies have demonstrated that growth factors and cytokines can regulate angiogenic factor expression in tumor cell lines and thereby induce angiogenesis indirectly. The importance of the epidermal growth factor receptor (EGFR; ErbB1) and the HER-2/*neu* (ErbB2) systems in VEGF regulation and angiogenesis has been validated in several tumor systems, including colon carcinoma, (Ciardiello *et al*., 2000) pancreatic cancer, (Bruns *et al*., 2000) gastric cancer, (Akagi *et al*., 2003) breast cancer, (Kumar and Yarmand, 2001; Yang *et al*., 2002) glioblastoma multiforme, (Eller *et al*., 2002) non–small-cell lung cancer, (Yano *et al*., 2003) and renal cell carcinoma (Kedar *et al*., 2002). The insulin-like growth factor-I receptor (IGF-IR) is often overexpressed in a variety of human cancers, and has been associated with aggressive disease and formation of metastases. (Reinmuth *et al*., 2002; Akagi *et al*., 1998). Recent experimental model systems have demonstrated the importance of activation of the IGF-IR system in mediating angiogenesis by upregulating VEGF expression in breast, endometrial, pancreatic, and colorectal cancers (Reinmuth *et al*., 2002; Reinmuth *et al*., 2002; Akagi, 1998; Warren *et al*., 1996). Hepatocyte growth factor can induce VEGF expression in normal and tumor cells through activation of c-MET (Van *et al*., 1998). The family of platelet-derived growth factors (PDGFs) modulate angiogenesis *in vivo* by regulating EC survival and pericyte/vascular smooth muscle cell recruitment, as well as by inducing VEGF in several model systems Kotsuji-Maruyam *et al*., 2002; Cao *et al*., 2002; Guo *et al*., 2003; Reinmuth *et al*., 2001). Ferrara *et al*. recently reported on the important role of PDGF in regulating stromal-derived VEGF-A. Using a model that deficient in tumor cell–derived VEGF, these

investigators showed that PDGF-AA expressed by tumor cells is responsible for the recruitment of VEGF-producing, tumor-associated fibroblasts. Disruption of paracrine PDGF receptor-alpha (PDGFR-α) signaling between tumor cells and stromal fibroblasts in this model significantly inhibited angiogenesis and tumor growth. Hence, these findings show that PDGFR-α signaling is essential for the recruitment of VEGF-producing stromal fibroblasts, and highlight the importance of host-derived VEGF for sustaining tumor angiogenesis.

Prostaglandins play critical roles in numerous biologic processes, and certain prostaglandins have recently been implicated in tumor angiogenesis through upregulation of VEGF expression (Gately, 2000). Prostaglandin-endoperoxide synthase (also known as cyclooxygenase [COX]) is the rate-limiting enzyme involved in the oxidative transformation of arachidonic acid into various prostaglandin compounds (Needleman *et al*., 1986). Over the past decade, numerous studies have confirmed an association between COX-2 overexpression and tumor progression and increased angiogenesis (VEGF expression) in several solid malignancies including gastric (Joo *et al*., 2003) colon, (Yao *et al*., 2003; Tsujii *et al*., 1998) prostate, (Liu *et al*., 1999) breast (Costa *et al*., 2002) and pancreatic (Aoki *et al*., 2002; Eibl *et al*., 2003) cancers. In addition, numerous *in vivo* studies have demonstrated that COX-2 mediates VEGF expression in numerous cell lines (Gately, 2000). However, the COX-2 mediated VEGF expression may be tumor dependent, since COX-2 inhibitors do not suppress VEGF expression in all tumor.

**Oncogenes and Tumor Suppressor Genes**

Many oncogenes have been implicated in the process of angiogenesis in solid tumors, in part because of their ability to induce proangiogenic growth factors such as VEGF (Rak *et al*., 2000; Rak *et al*., 2002). The *c-Src* proto-oncogene encodes a protein tyrosine kinase, pp60c-src, that has been implicated in the regulation of VEGF expression and in promoting neovascularization of growing tumors. Additional evidence also suggests that several factors that regulate VEGF expression may depend in part on *c-Src*–mediated

signal transduction pathways in a variety of tumor systems (Ellis *et al*.,Fleming *et al*., 1997). Similarly, the *BCR-ABL* oncogene has been identified as having a key role in the molecular pathogenesis of leukemias, which are considered angiogenesis dependent malignancies (Dias *et al*., 2001). Recent studies in which STI-571 (Gleevec, imatinib mesylate; Novartis, Basel, Switzerland) was used to target *BCR-ABL* showed that VEGF levels in *BCR-ABL*-positive chronic myeloid leukemia cells were reduced (Ebos *et al*., 2002) and transfection of *BCR-ABL* into murine myeloid or human megakaryocyte cells resulted in enhanced VEGF expression. The expression of mutant *Ras* oncogenes is one of the most commonly encountered genetic changes detected in human cancer. Moreover,  the induction of VEGF expression by mutant H- or K-*ras* oncogenes has been reported in various types of cells such as pancreatic cancer, colon cancer, and non–small-cell lung cancer (Konishi *et al*., 2000; Ikeda *et al*., 2001; Rak and Kerbel, 2001; Fekdkamp *et al*., 1999; Okada *et al*., 1998; Zang *et al*., 2001). In addition, genetic disruption of the mutant K-ras allele in human colon carcinoma cells was associated with a reduction in VEGF activity (Rak *et al*., 1995). Like other signaling intermediates, the activation of Ras is also being part of a signaling cascade initiated by certain growth factor receptors such as EGFR (Fekdkamp *et al*., 1999; Maity *et al*., 2000; Casanova *et al*., 2002) and may be one of the key signaling pathways involved in growth factor–induced angiogenesis and VEGF expression. One of the most intensively studied tumor suppressor genes implicated in the molecular pathology of many solid malignancies is *p53*, and several studies have implied that *p53* has a central role in the regulation of VEGF in malignant tumors. Direct interaction of the *p53* protein with the transcription factor Sp1 prevents transcriptional activation of the VEGF promoter in breast cancer cells (Olofsson *et al*., 1998) and inhibits the hypoxic induction of Src kinase. In studies with colon and endometrial carcinoma, stable transfection of wild-type p53 resulted in decreased VEGF expression (Fujisawa *et al*., 2003; Bouvet *et al*., 1998). Several studies have also shown that genetic alterations of tumor suppressor genes, such as *p53*, *PTEN*, and *vHL* (Maxwell *et a*l., 1999; Zundel *et al*.,

2000), can induce HIF-1 activity in tumor tissues leading to an increase in VEGF, as described earlier.

## Role of VEGF in Tumor Angiogenesis

VEGF is considered the most potent angiogenic protein which promotes neovascularization and increases vascular permeability (Zhang *et al*., 1995). Elevations in VEGF levels have been detected in the serum of some cancer patients (Kondo *et al*., 1994). Also, a correlation between VEGF expression and microvessel density in primary breast cancer sections has been observed. A postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of VEGF-poor tumors, suggesting that expression of VEGF is associated with stimulation of angiogenesis and with early relapse in primary breast cancer (Toi *et al*., 1994). A similar correlation has been described in gastric carcinoma patients. VEGF positive in tumor sections was correlated with vessel involvement, lymph node metastasis, and liver metastasis. Furthermore, patients with VEGF-positive tumors had a worse prognosis than those with VEGF-negative tumors (Maeda *et al*., 1996). Using several tumor models, Holash *et al*. reported that VEGF was an important regulator in tumor angiogenesis and progression (Holash *et al*., 1999). Recent studies have demonstrated that hypoxia plays an important role in VEGF expression. Jones *et al*. reported that when bladder cancer cell lines were exposed to hypoxic conditions, VEGF protein expression was significantly increased (Jones *et al*., 2001). Likewise, human hepatocellular carcinoma cell lines exposed to hypoxic conditions  exhibited the overexpressed VEGF mRNA and protein (Von Marschall *et al*., 2001).

**GPO1986**

GPO1986* is a herbal remedy consisting of eight medicinal plants as shown in Table 2.1

| Plant | Family | Part Use |
| --- | --- | --- |
| 1. *Ammannia baccifera* | Lythraceae | Whole plant |
| 2. *Canna indica* | Cannaceae | Rhizome |
| 3. *Mallotus philippinensis* | Euphorbiaceae | Stem |
| 4. *Clinacanthus nutans* | Acanthaceae | Leaf |
| 5. *Polygala chinensis* | Polygalaceae | Whole plant |
| 6. *Acanthus ebracteatus* | Acanthaceae | Whole plant |
| 7. *Premna herbacea* | Verbenaceae | Rhizom |
| 8. Smilax corbularia | Smilacaceae | Rhizome |

**Table 2.1** List of Thai medicine plants (GPO1986)

**Note [*]** Pitty patent number 3328 (ส่วนผสมสมุนไพรและสารสกัดสำหรับใช้ในโรคมะเร็ง)

These eight medicinal plants have been extracted and formulated by GPO. This remedy was claimed to be effective in the treatment of patients with mammary tumors. The preliminary screening of anticancer activity of this remedy was considered in the herbal part. Nowadays, there are many systems to examine anticancer activity of the agents. Only two systems *in vitro* (knowledge base) KB system and *in vivo* dimetylbenza anthracene (DMBA)-induced mammary tumors are chosen for identifying anticancer activity of the herbal part of this remedy. The results from primary screening in KB system exhibited ED50 of the herbal extract at more than 30 μg/ ml (criteria of anticancer activity by National Cancer Institute of USA was ED50= 30 μg/ ml). The antitumor activity in rats

bearing malignant mammary tumor comparing between the herbal extract and solvent control was non-significant in reduction tumor weight but significant in increasing survival rate and prolongation of survival time. The herbal extract showed the practically non-toxic effect to the dose of 10,000 mg/kg by oral route (Pornsiripasert, 1986; Na Bangchang, 1987). In 1992, Wiwatwittaya studied the anticancer activity of the extract on serially transplanted human cervical cancer in nude mice. 115 nude mice were used, but after transplantation the tumor took place in only 89 mice. Finally, only 48 mice with the tumor volume of 7-16 mm$^3$ were recruited. They divided into 3 groups. The experimental group was fed daily with the extract solution equivalent to the dose of 2,580 mg/kg for 60 days. The extract solution was prepared by dissolving 2 g of dried extract in 5 ml of sterile water. The control group was fed daily with equivalent amount of water. The third group was injected intraperitoneally with mitomycin-c (MMC) at the dose of 1 mg/kg in day 1 and day 5 every other week. The result showed that the extract could significantly inhibit tumor growth during the 10$^{th}$ - 60$^{th}$ day of treatment, in comparison with the control group. Moreover, the effect of an extract on the effector cells involved in cancer immunity, NK cells and monocyte/macrophages, was studied in 13 breast cancer patients. Treatment of the patients with the extract for 2 weeks significantly increased *in vitro* NK cell cytotoxic activity against K562 erythroleukemic cells. It also increased the release of tumor necrosis factor - α from monocyte/macrophages. The addition of chemotherapeutic drugs to the treatment schedule seemed to decrease these effector cell functions (Thisoda, 1995).

Moreover, Akarasereenont *et al.*, 1999 studied the effect of GPO1986 on proliferation of vascular endothelium. HUVEC and glioblastoma cell (GB) were cultured as standard techniques and grown to confluence until use. Confluent GB was replaced with fresh medium (DMEM) without serum for 24 h. After which time the conditioned medium (CM) was transferred to HUVEC and incubated for 3,6,18 and 24 h. They concluded that conditioned medium from GB cell culture could cause the increased cell proliferation in HUVEC but did not affect COX activity and protein. Interestingly, GPO1986 could inhibit cell proliferation in HUVEC treated with conditioned medium from GB cell culture. This

inhibition was significant when using the herbal extract at the concentration of 9 mg/ml. Serum from GB patient, but not fetal bovine serum, human healthy plasma or human healthy serum, could also cause the increased cell proliferation in HUVEC. However, serum from GB patient did not have an effect on the COX activity and protein.

GPO1986 could also inhibit cell proliferation in HUVEC treated with 15% serum from GB patient. Interestingly, the effect of the herbal extract was enhanced in HUVEC treated with 15% serum from GB patient with significant inhibition at the concentration of 3 mg/ml. However, the effect of GPO1986 on tumor angiogenesis has not yet determined. Therefore, the purpose of this study is to examine the effects of GPO1986 on tumor angiogenesis in HepG2 implanted nude mice by using intravital fluorescence microscopy. Especially, the mechanism of GPO1986 action is also determined by focusing on tumor biomarkers, VEGF and HIF-1$\alpha$.

1. *Ammannia baccifera Linn.*

| | |
|---|---|
| *Family* | Lythraceae |
| *Local name* | Mafai, Yaa raknaa, Acrid weed |
| *Description* | Glabrous, erect or subscandent, 8-65 cm high, often branching, branches usually opposite |
| *Leaves* | 7-70 mm long, 1-16 mm broad, lower leaves usually opposite, cauline ones opposite or alternate, oblong or narrow-elliptic, narrowed at the base, or rounded or subcordate, or subauriculate, usually obtuse or subacute |
| *Flowers* | Distinctly pedicelled, sessile or sub sessile<br>- Calyx: 1-2 mm long, tube hemispheric, teeth 4, broad, triangular, acute, cornua minute or absent<br>- Petals: 0 or minute, stamens as long as the lobes or slightly shorter |
| *Capsule* | Depressed, globule, 1-2 mm diameter, covered up to % or % by the calyx-tube, slightly or much longer than the teeth |
| *Distribution* | Africa, Asia and Australia<br>In Thailand: throughout the country, from near sea level to 1,300 m. Commonly found in rice fields and humid places |
| *Flowering* | December - March |

**Table 2.2**  *Ammannia baccifera* Linn. description (Kirtikar *et al.*, 1935)

**Figure 2.7** *Ammannia baccifera* Linn.

(From http://www.nju.edu.cn/njuc/plantsweb/species/qianqucaike/shuixiancai.htm)

2. *Canna indica* Linn.

| Family | Cannaceae |
|---|---|
| Local name | Phuttharaksaa, Canna, Indian Shot, Canna lily |
| Description | Herbaceous, not usually over 5 ft high |
| Stem | 0.9- 1.2m |
| Leaves | 15-45 by 10-20 cm, lanceolate to ovate, oval, or almost orbicular, caudate-acuminate, veins arching, sheath open above, margins membranous, raceme with the pedicel 30 cm or more, erect, pedicel with a long narrow sheath about the middle |
| Flowers | 5 - 6.3 cm long<br>- Bracts: 1.3 - 2.5 cm, oblong, membranous, obtuse, green<br>- Calyx-segment: 6-8 mm, membranous, obtuse<br>- Corolla-segments: 2.5 cm, erect, narrow, acuminate oblanceolate, greenishor coloured<br>- Staminal segments: longer than the corolla, 3 suberect, spathulate, 1 linear,revolute |
| Fruit | Erect, 1.3 - 2.5 cm long, subglobose or oblong, obscurely 3-lobed, crowned with the calyx-segments, pericarp echinulate, black, thin |
| Seeds | Very many, globule, testa crustaceous, black, shining |
| Distribution | Asia, Latin America, and Pacific islands |
| Flowering | August -October |

**Table 2.3** *Canna indica* Linn. description

from Kirtikar *et al.. 1935*

**Figure 2.8.** *Canna indica* Linn

(From http://www.samunpri.com/modules.php?name=News&new_topic=3&pagenum=3)

3. *Mallotus philippinensis Muell. Arg.*

| | |
|---|---|
| *Family* | Euphorbiaceae |
| *Local name* | Tang-Tuay, Kamala, Kameela, Spoonwood, Rottlera |
| *Description* | A small much-branched tree, branchlets, young leaves, and inflorescence tawny- or rusty- pubescent |
| *Leaves* | Alternate, variable, 7.5 - 15 by 3.2 - 7.5 cm, ovate or ovatelanceolate, acuminate, entire or slightly toothed, glabrous above, pubescent and with numerous orbicular red glands beneath, reticulately veined, base rounded or acute, strongly 3-nerved at the base and with 4-7 pairs of nerves above the basal ones <br> - Petioles: 2.5 - 5 cm long, cylindric, fulvous-pubescent, with 2 small sessile glands one on each side of the summit |
| Flowers | Dioecious, small <br> - The males: clustered, sessile or very shortly pedicellate, in erect terminal spikes which are usually several together and often longer than the leaves <br> - The females: sessile or nearly so, in short spikes <br> - Male flowers: sepals 4 (rarely 5), 3 mm long, lanceolate, acute <br> Buds : globosely ovoid <br> - Female flowers: calyx divided nearly to the base, sepal 3 or 4, thicker than in the male, ovate-lanceolate |
| *Ovrary* | With red glands, 3 celled; styles 3, simple, papillose |
| *Capsules* | 8-13 mm diameter, 3-lobed, loculicidally 3-valved, covered with a bright red powder consisting of minute stellate hairs and fine grains of a red resinous substance soluble in alcohol and ether |
| *Seeds* | 4 mm diameter, subglobose, black |
| *Distribution* | Throughout tropical India, Ceylon, Burma, Malay Islands, Australia, China and Thailand |

**Table 2.4** *Mallotus philippinensis Muell. Arg*. description

**Figure 2.9.** *Mallotus philippinensis  Muell. Arg.*

(From http://www.nationaalherbarium.nl/ThaiEuph/ThMspecies/ThMallotus.htm)

4. *Clinacanthus nutans Lindau.*

| | |
|---|---|
| Family | Acanthaceae |
| *Local name* | Pa-ya-yaw, Sa-Ied-pung-porn |
| *Description* | Scandent shrub |
| *Leaves* | Simple, opposite, lanceolate, 1-3 cm wide, 4-12 cm long |
| *Flowers* | Dull red with green base |
| *Fruit* | Capsule |
| *Distribution* | Throughout Thailand |

**Table2.5** *Clinacanthus nutans Lindau.* description



**Figure 2.10.** *Clinacanthus nutans Lindau.*

(From http://www.phuketjettour.com/herbs/payayu.htm)

5. *Polygala chinensis Linn.*

| | |
|---|---|
| *Family* | Polygalaceae |
| *Local name* | Peek-kai-dum |
| *Description* | Annual, 10-25 cm high, erect, branched from the base, glabrous or pubescent. |
| *Leaves* | Very variable, 1.2 - 3.8 cm long, obovate, sub-orbicular or linear-oblong, rather thick, coriaceous, glabrous, ciliate, mucronate<br>- Petioles: 2 mm long, hairy |
| *Flowers* | Yellow, fading to pink, in axillary or extra-axillary, short, almost capitate, few-flowered racemes, crest of a single tubular appendage multifid only at the apex<br>- Pedicels: very short<br>- Bracts: small, membranous, oblong-ovate, acute, ciliate, persistent<br>- Outer sepals: broadly ovate, acuminate, with broad, membranous, ciliate margins |
| *Wings* | Herbaceous, oblique, ovate-oblong, acuminate, with narrow., membranous margins ciliate towards the base, longer than the capsule |
| *Capsules* | Didymous, orbicular-oblong, strongly ciliate, obliquely obcordate at the apex, narrowly margined |
| *Seeds* | Hairy; strophiole glabrous or nearly so, rounded at the apex, furnished with 3 membranous basal appendages |
| *Distribution* | Throughout India, tropical Asia, Australia |

**Table 2.6** *Polygala chinensis* Linn. description

(from Kirtikar *et al.* 1935)

**Figure 2.11**.   Polygala chinensis Linn.

(From http://www.dld.go.th/nutrition/exhibision/native_grass/other/Polygala%20%20chinensis.htm)

*6. Acanthus ebracteatus*Vahl.

| | |
|---|---|
| *Family* | Acanthaceae |
| *Local name* | Ngeug-pla-mor, sea holly |
| *Description* | Erect, shrubby herbs up to 1.5 m tall with many spiny, usually green stems |
| *Leaves* | Blade dark green, stiff, deeply lobed with sharp spines at each lobe's tip and curved in full sun, flatter in shade; with neatly organised spikes |
| *Flowers* | Spikes at branch tips |
| . | - Petals: white turning brownish when older |
| *Capsules* | Squarish and slightly flattened, exploding when ripe to send their whitish, flat seeds flying up to 2 m from the parent plant |
| *Distribution* | Southeast Asia |

**Table 2.7** *Acanthus ebracteatusVahl*. description

(From http://mangrove.nus.edu.sg/guidebooks/text/1045.htm)



**Figure 2.12**   Acanthus ebracteatus

(From http://www.rspg.thaigov.net/plants_data/use/crop_8-1.htm)

*7. Premna herbacea* Roxb. Mold.

| | |
|---|---|
| *Family* | *Verbenaceae* |
| *Local name* | Hua-Kao- Yen-Tai |
| *Description* | A small under shrub; stem hardly any; flowering branches 2.5 - 10 cm, springing up after the jungle fires |
| *Leaves* | 10 by 5 - 7.5 cm, sessile, obovate, obtuse, mature, pubescent on the nerves, microscopically dotted above, minutely deciduously pubescent beneath, nerves 5 pairs |
| *Corymbs* | 3.8 cm diameter, pubescent, somewhat dense<br>-  Peduncle: 0 - 3.8 cm |
| *Calyx* | 2.5 mm, subequally 5 toothed, closely pubescent; lobes ovate, obtuse |
| *Corolla* | 4 mm greenish white, hairy in the throat, 4-lobed obscurely 2-lipped. |
| *Drupe* | 6 mm diameter, globose |
| *Distribution* | Asia and Australia |

**Table 2.8**  *Premna herbacea* Roxb. Mold. description

(from Kirtikar *et a.* 1935)



**Figure 2.13**.  *Premna herbacea Roxb. Mold.*

(From http://www.rakbankerd.com/02_spiritual/arthitweek_topic_desc.html?data_id=104&ot=2)

*8. Smilax corbulariaKunth.*

| | |
|---|---|
| *Family* | Smilacaceae |
| *Local name* | Hua-Kao-Yen-Nuea, Hua-Kao-Yen-Wok |
| *Description* | Climber 2-4 m long with woody stem, rather densely branched |
| *Leaves* | Highly variable in shape and thickness<br>Blade: elliptic, 3-10 cm long, 1.5 - 5 cm wide, cuneate, rounded or shallowly cordate at base, the apex acuminate tip, coriaceous, fresh-green and shiny on upper surface, strongly glaucous and more or less whitepowdery beneath<br>- Petioles: short, 7 - 15 mm long<br>-Tendrils: developing only on sterile branches and stems |
| *Flowers* | Branches 5-20 cm long, upper leaves reduced to bracts, umbels with peduncles 5-12 mm long, staminate umbels 10-40 flowered, pistillate 8-20 flowered<br>- Staminate: perianth reddish<br>Tepal.s: free<br>- Stamen: 6, nearly sessile; anther elliptic, 1.3 mm long<br>- Pistillate: perianth greenish to yellowish, 1.5-2 mm long<br>- Tepals: oblique |
| *Ovary* | Ellipsoid, contracted at apex, 2 mm long, 1.5 mm wide, capped with 3-lobed stigma |
| *Berries* | Globose, 6-8 mm across, purplish-black, 1-3 seeded |
| *Distribution* | Southeast Asia; Southern China, upper Burma, Thailand and Indonesia |

**Table 2.9** *Smilax corbularia* Kunth. description

**Figure 2.14** *Smilax corbulariaKunth.*
(From http://www.rakbankerd.com/02_spiritual/arthitweek_topic_desc.html?data_id=104

# CHAPTER III

# MATERIALS AND METHODS

## Animals

The BALB/c nude mice were 8-10 weeks old and weighed 20–25 g were purchased from the National Laboratory Animal Center of Salaya Campus, Mahidol University, Thailand). They were allowed to rest for a week after arrival at the Animal Center, Faculty of Medicine, Chulalongkorn University, before being used in the experiment. The animals were kept in a pathogen limited room where temperature was 25±3 °C. The animals were housed one per cage and had free access to tap water and standard pellet food throughout the experiment. For ethical consideration, our experiments were performed following "Home office guidelines on the scientific use of animal (Scientific procedured) Act 1986" and approved by Ethical Committtee, Faculty of Pharmaceutical Science, Chulalongkorn University. All animals were divided randomly into two groups of HepG2 (HepG2) and control (Con). Then they were further separated into 3 subgroups of vehicle, treated with 640 and 3,200 mg/kg BW of GPO1986.

## Preparation of the dorsal skinfold chamber

The dorsal skinfold chamber preparation contains one layer of striated muscle, subcutaneous tissue and skin and allows for intravital microscopic observation of the microcirculation in animal over a time period of two weeks. (Lehr *et al*.,1993)

Under sodium pentobarbital anaesthesia (50 mg/100g body weight i.p.), the mice were placed on a heated pad, and two symmetrical stainless steel frames were implanted on the extended dorsal skinfold of the nude mice, so that they sandwiched the double layer of skin. One layer of skin was then completely removed in a circular area of ~7 mm in diameter, and the remaining layers (consisting of striated skin muscle, subcutaneous tissue

and skin) were covered with a removable coverslip incorporated into one of the stainless steel. All procedures were done under aseptic conditions (Figure 3.1).

A human hepatocellular carcinoma cell lines, (HepG2) 30 µl cell suspension ($2\times10^6$, viability 95-97 %) (Yoysungnoen *et al.*,2005) were inoculated in to the center of the chambers (Figure 3.1). HepG2 were originally obtained from the American Type Culture Collection (ATCC). Passage numbers for HepG2 was between 250-255 were used. Cell passages were prepared at the Research and Development Institute, the Government Pharmaceutical Organization, Bangkok, Thailand.
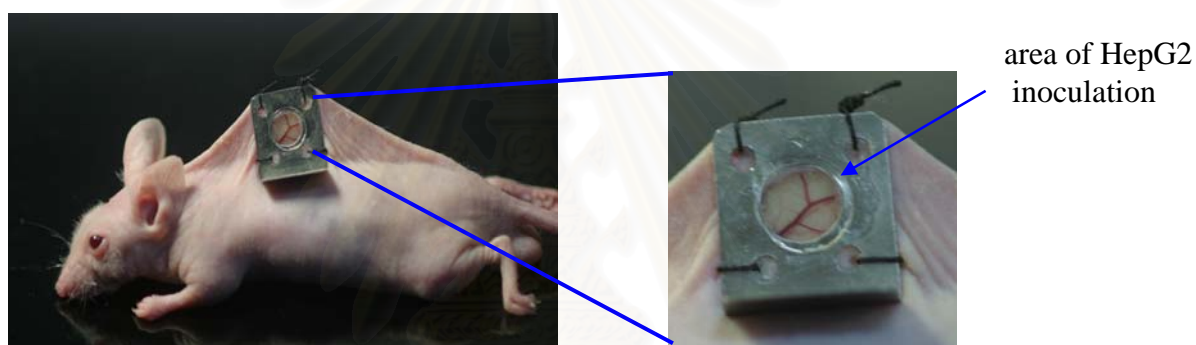


**Figure 3.1** A photograph of the dorsal skinfold chamber implanted on a nude mice.

The treatments were started 24 hours after HepG2 inoculation (Yoysungnoen *et al.*,2005). GPO1986 was dissolved in distilled water and administered by oral gavage once daily, while vehicle group was received distilled water.

## Experimental Protocol

The experiments were performed days 2, 7 and 14 after vehicle or GPO1986 treatments. On the day of experiment, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg BW). A catheter were inserted into a jugular vein for fluorescence tracer application. Then the dorsal skinfold chamber was removed and the skin area around the chamber was fixed properly on a plate for further fluorescence microscopic study (Yoysungnoen *et al.*,2005).

## Intravital fluorescence microscopy

The tumor microvasculature was observed under an intravital fluorescence microscope (Nikon, Japan) equipped with a videocamera (Sony SIT68, Japan). With the use of x 10 and long-distance objectives, 5 min prior to the observation, 0.1 ml of 0.5% fluorescein isothiocyanate (FITC)-labelled dextran 250,000 (Sigma Chemical, USA) was injected intravenously to visualize the intralumen of microvessels. The interested areas within the tumor-bearing chamber were recorded using a video-recorder (Sony SVT-124P, Japan).

## Measurement of microvascular parameters

Quantitative off-line analysis of the videotapes were performed in order to analyze microvascular parameters (capillary diameter and capillary density (CD), using digital image processing software (Global Lab II).

*Capillary diameter*

We first defined capillaries that were newly formed to protrude towords the tumor area, and assigned them capillaries. Capillary diameter was measured with a Global Lab Image II. After play back off-line analysis. A single capillary was chosen for observation. By using the reference point, the diameter measurement was able to confine with the same location. The capillary diameter in micrometer was calculated as the mean of triple measurements from three video frames (Fig. 3.2).



| Frame1: t = 00.00.01 | Frame2: t = 00.00.02 | Frame3: t = 00.00.03 |
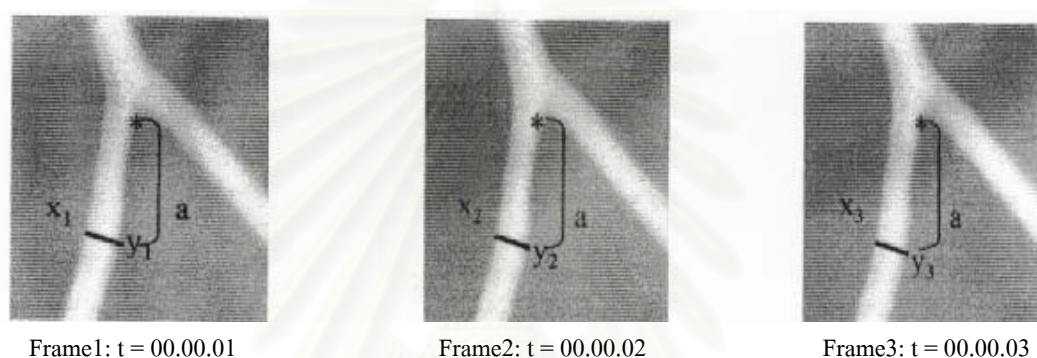
$$\text{mean capillary diameter} = \frac{x_1y_1 + x_2y_2 + x_3y_3}{3}$$

**Figure 3.2** Method for measurement of capillary diameter.

*Capillary density (CD)*

1. From videoimage of each experiment, the videoframes containting clear capillaries metwork were randomly selected.

2. Each videoframe, 2-3 clear window frames approximately 10,000 pixel size was selected to determine CD . The window frame which covered only capillary network no large vessels was selected (Fig.3.3).
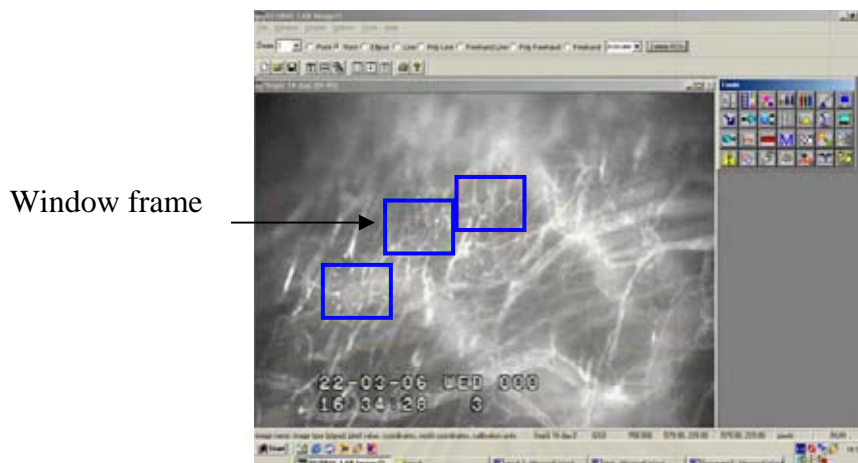
Window frame

**Figure 3.3**   An example of videoimage containing clear capillary network,

which was selected randomly from videoimage of each experiment.

3. Each selected window frame, the minimum and maximum pixel within the capillary were determined. The numbers of pixels located within the capillaries were automatically calculated by using Global lab II software.

4. The ratio of the number of pixels within the capillaries to total number of pixels within the selected window (~ 10,000 pixels) was represented in the percentage, using the "Histogram" functional tool as shown in Fig.3.4.
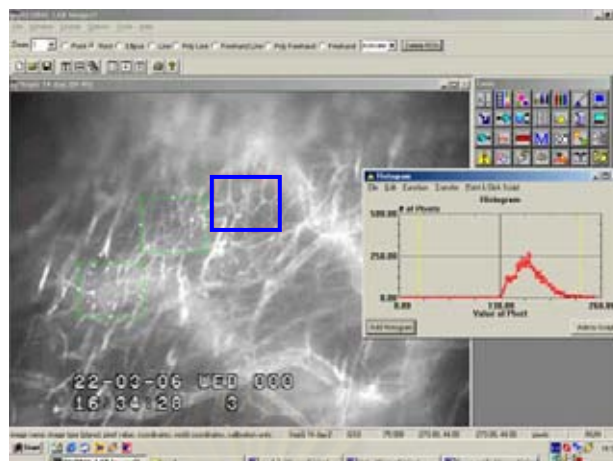
**Figure 3.4** The percentage of capillary density determined by "Histogram" function tool" in one window frame.



% of CD
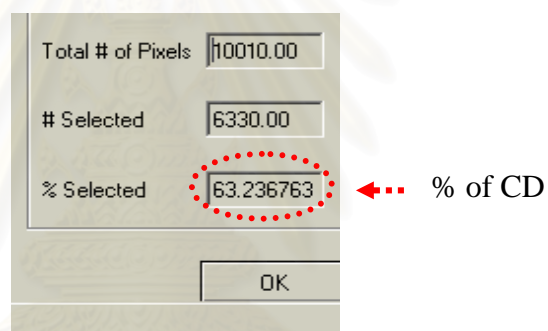
**Figure 3.5** Showing the percentage CD in one window frame determined by "Histogram function tool".

Using the above procedures, we defined the CD as follows;

$$CD = \frac{(\text{number of pixels within the capillaries}) \times 100\ (\%)}{(\text{Total number of pixels within the selected area})} \quad \text{......... Equation 1}$$

Based on the recorded videoimages, the CD was evaluated, and expressed in the percentage (Fig.3.5), for each mouse, the CD was measured at the window frames to provide the average level at different periods after the inoculation of tumor cells. The CD level was used as an index of angiogenesis.

**Histology**

At the end of experiment the tissues samples within the dorsal skinfold chamber were excised and fixed in neutral 10% formalin. The tissue preparation as following :

1.  Tissue was processing: dehydration, cleaning and infiltration.
2.  Paraffin embedding techniques :

    -  Using forceps warmed with a commercial forceps warmer to prevent the paraffin from collected on them and placed it at the bottom of the mold.

    -  Filled the mold with paraffin
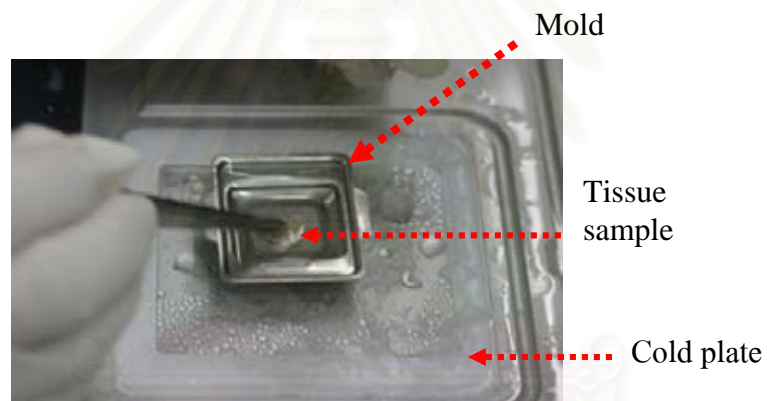


**Figure 3.6**  Paraffin  embedding technique.

-The labeled cassette was placed a top the mold and cool immedialtely by placing

  the mold on the cold plate (Fig.3.7A)

-The paraffin should solidify in about 15 minutes; the mold was then separated

  from the embedding cassette. The tissue and solidified was remain attached to the

  embedding cassette, forming a paraffin block that was ready for sectioning

(Fig.3.7B).
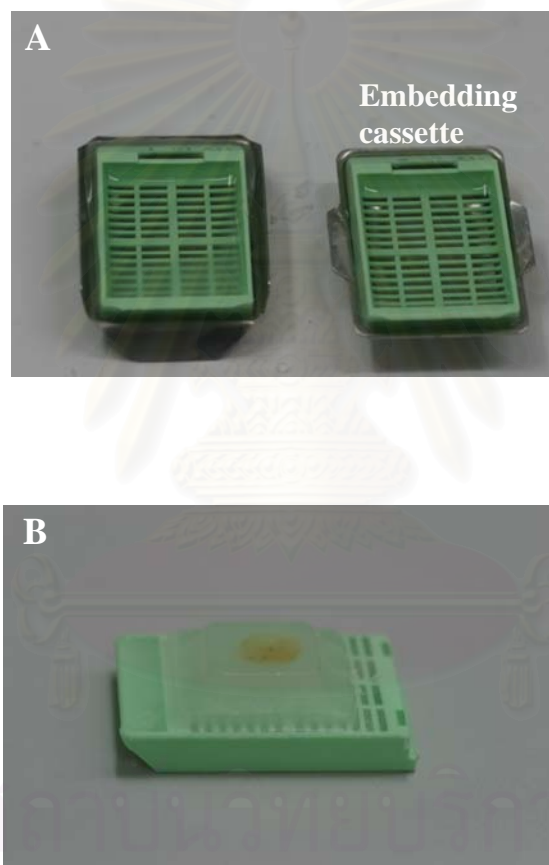


**Figure 3.7** Paraffin embedded the sample tissue.

Embedding cassette (A) and paraffin block (B).

3. Block Sectioning

- The long paraffin slice was refered to as a "ribbons" of 2 μm thick sections were cut from paraffin block on a specialized instrument called a microtome (Shandon Finesse, Thermo Electron corperation) (Fig.3.8).

- The ribbons were floated on a waterbath to spread the sections and individual sections were attached to glass slides, which four sections per one slice (3.9A)

Paraffin block

Parafffin ribbon
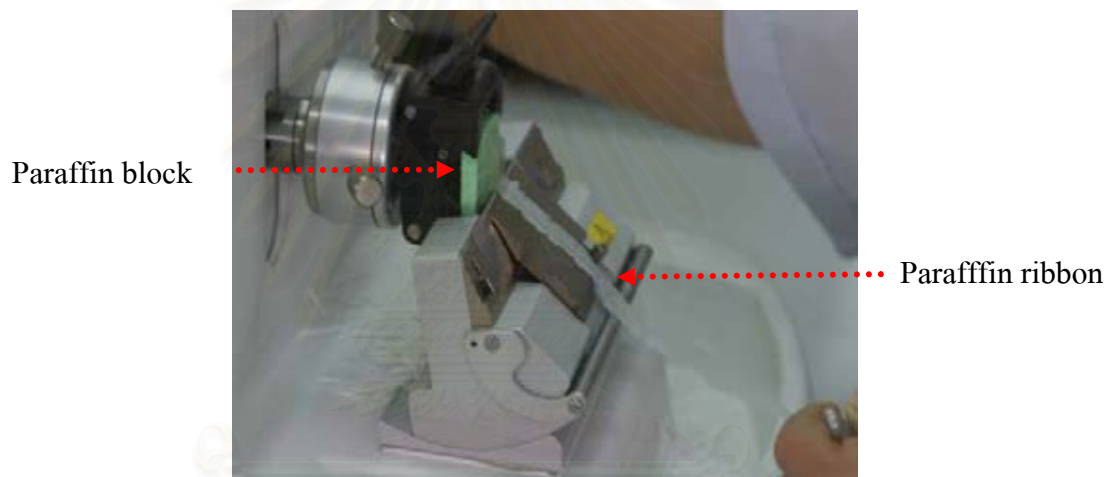
**Figure 3.8** A photograph of microtome (Shandon Finesse, Thermo Electron corperation)

The slide number 30-40 were chosen to study (3.9B). The odd number of slides were stained with hematoxylin and eosin (H&E) according to standard procedures. The even number of slices were studied HIF-1$\alpha$ expression by using immunohistochemistry technique.

**A**



For immunohistochemistry →     ← H&E staining

**B**
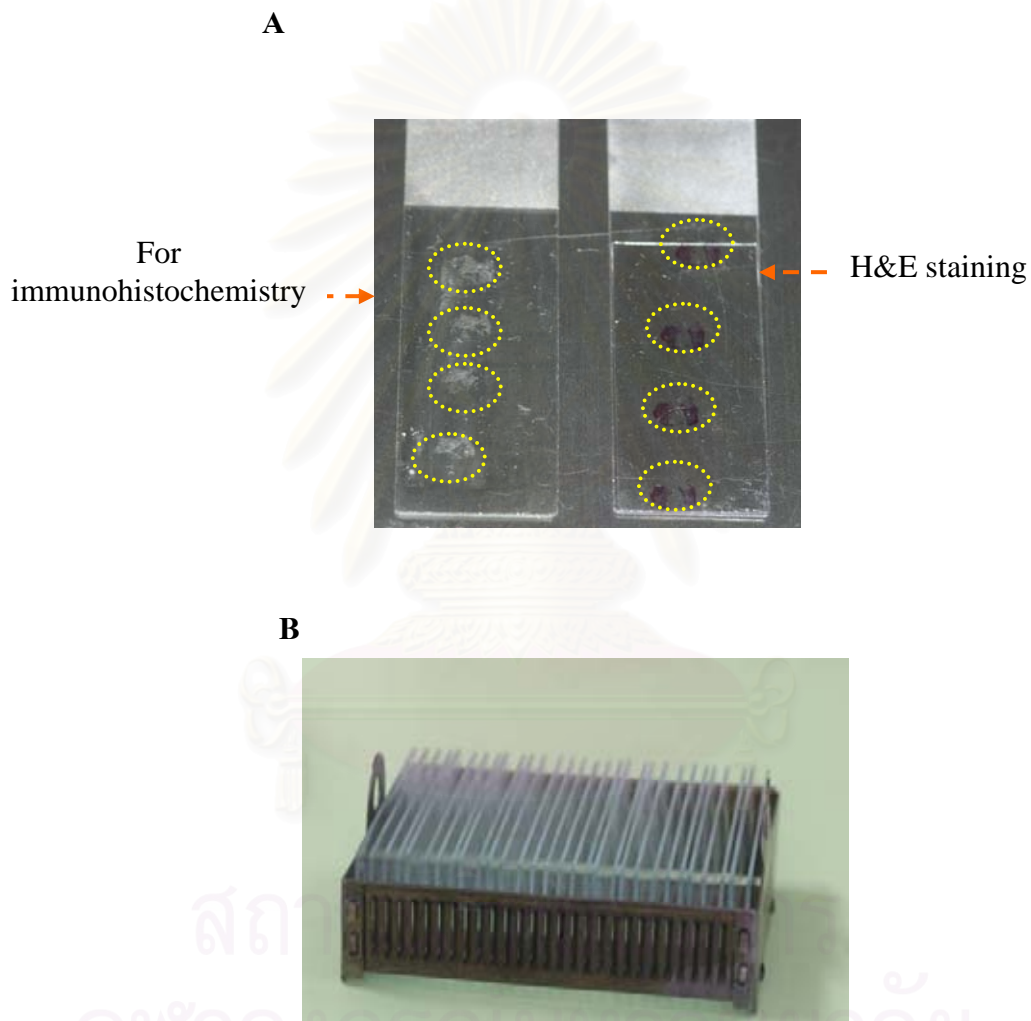


**Figure 3.9** Paraffin sections of the tissues. Sections were attached to glass slides, which four sections per one slice (A). Serial section slices (B)

## Immunohistochemistry Protocol for HIF-1$\alpha$

### *Principle of immunohistochemistry*

Immunohistochemistry or IHC refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in the diagnosis and treatment of cancer. Specific molecular markers are characteristic of particular cancer types. IHC is also widely used in basic research to understand the distribution and localization of biomarkers in different parts of a tissue.Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instancen, and antibody is conjugated to and enzyme, such as peroxidase, that can catalyse a color-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as FITC, Rhodamine, or Texas Red. The latter method is of great use in confocal laser scanning microscopy, which is highly sensitive and can also be used to visualise the interactions between multiple proteins.

Immunohistochemistry (IHC) was performed on 2-$\mu$m thick sections of formalin-fixed, paraffin-embedded tissues IHC for HIF-1$\alpha$ was performed with polymerperoxidase method (EnVision+/HRP, DAKO Cytomation, Denmark) on deparaffinized sections. HIF-1$\alpha$ was detected with a mouse monoclonal antibody [H1alpha67-sub] (ab463) (1:800; Abcam,Cambridge,UK). Sections were deparaffinized and pressure cooker with target retrieval solution (DAKO) for 10 min. The primary antibody reaction was carried out at 4 °C overnight. Subsequent steps was performed according to the manufacturer's instructions. Sections were then washed in PBS and blocked with PBS containing 5% non-fat dried milk for 30 min. 3,3,-diaminobenzidine (DAB)/hydrogen peroxide was used to detect antigen-antibody binding, and slides were counterstained with Mayer's haematoxylin, dehydrated, treated with xylene and mounted onto cover slides for

microscopy (Kimura *et al*., 2004) (Appendix I). HIF-1$\alpha$ immunoreactivity were presented in both nuclei and cytoplasm.

Image analyses were based on analysis of a given area by use of a videotape-recorded at×40 objective lens taken over the tumor area. The centrum of image coresponded to the central part of the tumor based on viusual analysis. From each videoimage, the expression of HIF-1$\alpha$ was then counted automatically by Global Lab II software (options/blob setting tool). Then means of 20 videoimage-frames from each experiment were expressed as cells per area.

Moreover, an independent participant was analyzed by using Global Lab II software to determined the HIF-1$\alpha$ expression. The independent participant, despite having an extensive knowledge with regards to the HIF-1$\alpha$ expression was important to the analysis as it able to derive an unbiased opinion  the result of the experiment.

## Image Analysis HIF-1α (Blob analysis tool)

In this section, an example show to quantify HIF-1α expression by using Global Lab Image II. The operation was as follows:

1. Set the input image. Open a blob analysis tool as shown in Fig. 3.10.
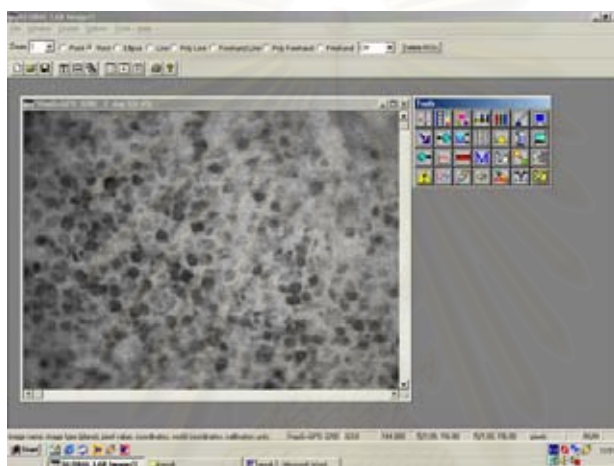


**Figure 3.10.** The image of immunohistochemical analysis of HIF-1α;open.

**Note** that when opened a blob analysis tool, the image in the active viewport was loaded as the input image be default.

2. Set the Active Thresholding Controls checkbox. **Minimum** limit to 0 and the **Maximum** limit to 104 by adjustion the slider control or entering the values manually (Fig.3.11).

**Note** that these settings differ from different images

Threshold ing
checkbox
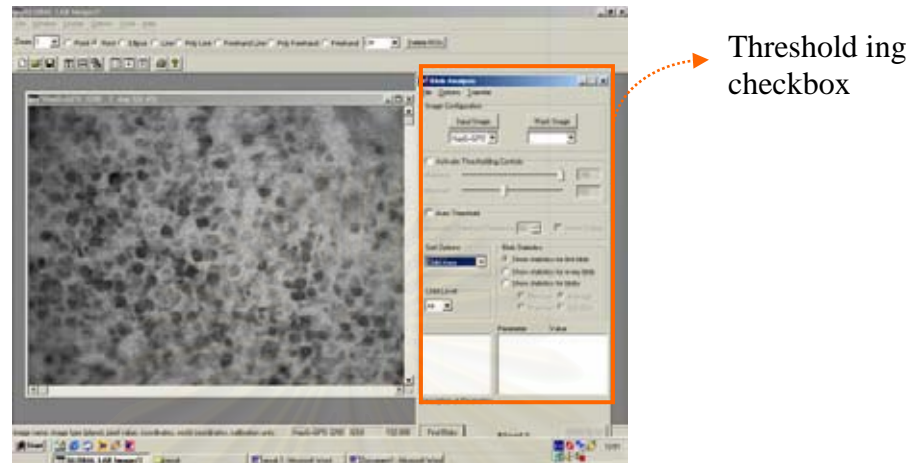
**Figure 3.11.** Active Thresholding Controls checkbox.

3. When all options for the tool were set appropriately, click **Find blobs** to start the blob
analysis and list all the found blobs in the found blobs display list (Fig.3.12).
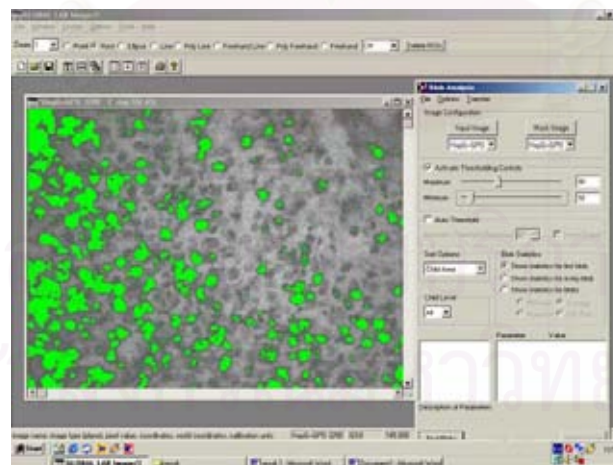


**Figure 3.12** Find blobs for analysis HIF-1α.

4. Look at blob statistics.Click on Blobs in the found blobs display list. Click on the parameter "Parent Area" in the blob parameters and values list (Fig. 3.13).
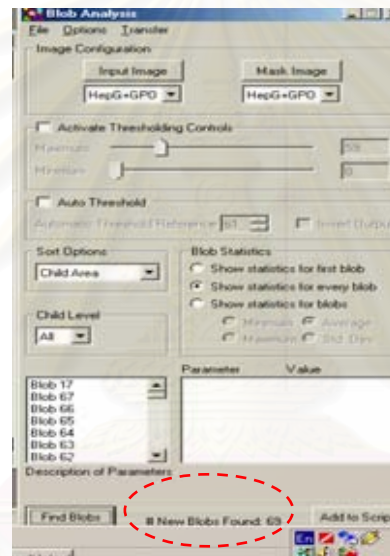


**Figure 3.13.** "Blob analysis" shows statistic values.

## Serum VEGF Immunoassay

*Principle of the assay*

This assay uses the mouse VEGF ELISA kit (R&D system, Abingkon, U.K.) This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse VEGF has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse VEGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse VEGF bound in the initial step. The sample values are then read off the standard curve.

*Sample collection and storage*

Blood sample obtained from the cardiac puncture was collected in a microcentrifuge tube and allowed to clot for 2 hours at room temperature before centrifuging for 20 minutes at approximately 2000 ✕g. Remove serum and stored at -20$^{\circ}$ C until analysis for VEGF was performed.

*Assay procedure*

The experimental protocal as performed by the sequential step as following:-
Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare all reagents, working standards, control, and samples as directed (Appendix II).

2. Remove excess microplate strips from the plate frame, return them to the foil pouch

containing the desiccant pack, reseal.

3. Add 50 µl of Assay Diluent RD1N to each well.

4. Add 50 µl of Standard and  sample to each well. Mix by gently tapping the

   plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at

   room temperature. Plate layouts are provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes.

   Wash by filling each well with Wash Buffer (400 µl) using a squirt bottle.

   Complete  removal of liquid at each step is essential to good performance. After the last

    wash, remove any remaining Wash Buffer by aspirating or

   decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µl of mouse VEGF Conjugate to each well. Cover with a new adhesive strip.

   Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µl of Substrate Solution to each well. Incubate for 30 minutes at room

   temperature. **Protect from light**.

9. Add 100 µl of Stop Solution to each well. Gently tap the plate to ensure thorough

   mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader

    set to 450 nm (correction  wavelength set at 570 nm).

The serial VEGF standard concentrations and the corresponding O.D.'s were demonstrated in Table 3.1

| Standard VEGF concentration (pg/ml) | OD |
|---|---|
| 0 | 0.081 |
| 7.8 | 0.115 |
| 15.6 | 0.150 |
| 31.2 | 0.201 |
| 62.5 | 0.339 |
| 125.0 | 0.576 |
| 250.0 | 1.081 |
| 500.0 | 1.991 |

**Table 3.1** The optical density of serial VEGF standard concentrations
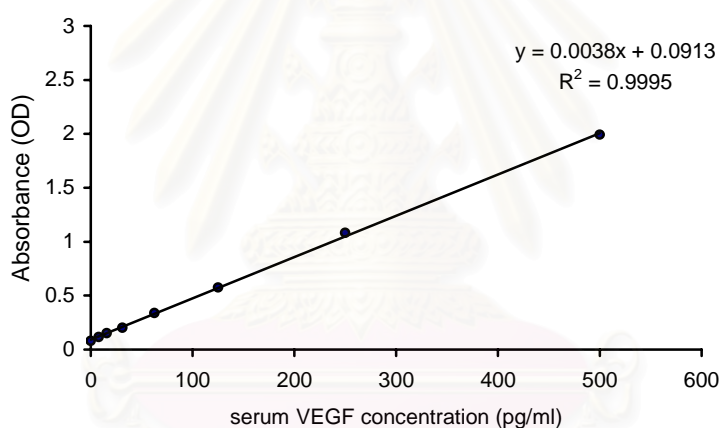


**Figure 3.14** The standard curve of serum VEGF level.

**Calculation of results**

To make the VEGF standard curve, know concentrations of VEGF are plot on the X-axis and the corresponding O.D. on the Y-axis. The standard curve shows correlation between VEGF concentration and the corresponding O.D.'s (absorbance). The concentration of VEGF in unknown samples was evaluated from the standard equation of $x = (y - 0.0913)/0.0038$ as shown in Fig. 3.14.

## Measurement of tumor area

The odd number of slides (31,33,35,37, and 39) were stained with hematoxylin and eosin (H&E)(Fig.3.15) according to standard procedures. The slides were observed the tumor area by using intravital fluorescence microscopy at low magnification (×4). For data analysis, the tumor area was analyzed by quantitative image analysis using Image Pro Plus 6.1 software.
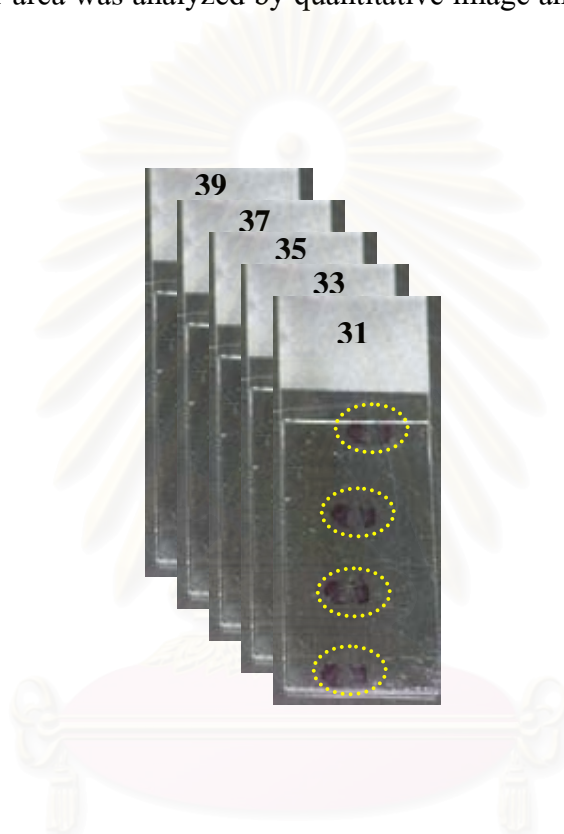


**Figure 3.15**   The section of the slices for tumor area analysis .

1.  Set the input image. Open  a "Measurement tool" as shown in Fig.3.16A.

2. The measurement was calibrated, then draw the line around the tumor area.

3. The area was automatically calculated (mm$^2$) (Fig. 3.16B).

**A**



**B**
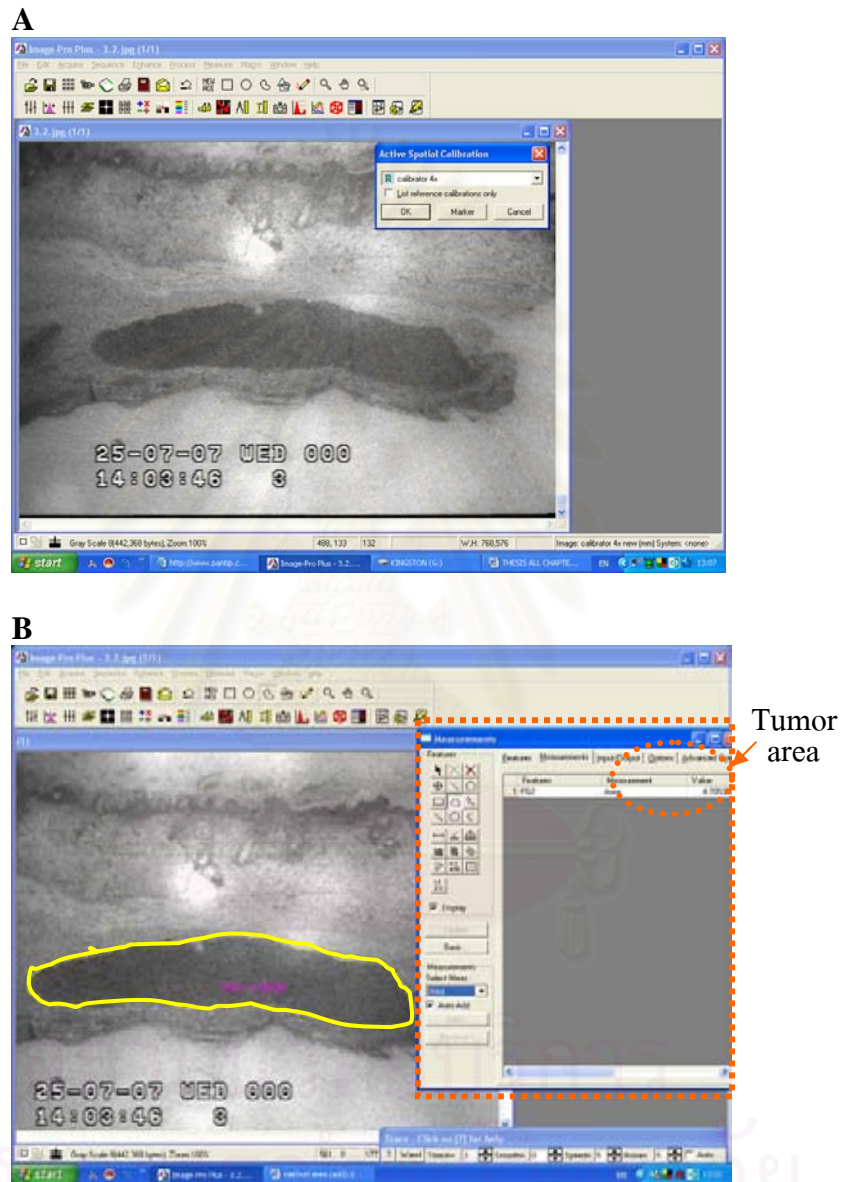


Tumor area

**Figure 3.16**   The "Measurement"  function tool, which was used to dertemint the tumor area (A).The " Measurement" function tool, which used to automatic calculate the tumor area (B).

**Statistics**

Data were first analysed for normal distribution and equal variance. Differences between groups were then calculated by analysis of variance (ANOVA) followed by the appropriate *post hoc* test compensating for multiple comparisons. The relationship between VEGF and CD was made by pearson correlation (*p*-value) and model fit curve (r). All data are given as means ± SEM. Statistical significance was accepted for a value of $p < 0.05$.

# CHAPTER IV

# RESULTS

This chapter is composed of three parts, which demonstrate the effects of herbal extract (GPO1986) on tumor angiogenesis, two kinds of tumor biomarkers (serum VEGF, HIF-1$\alpha$), and tumor growth.

## 4.1 Tumor model

We used HepG2-implanted nude mice model as described in the Materials and Methods (Chapter III). **Table 4.1** shows change of the body weight measured on different time-points after HepG2 inoculation with or without GPO1986 supplementation. When HepG2 cells were inoculated in the mice skin, any toxic effect and body weight loss was not observed.

| Groups | Body weight (g) | | | |
|---|---|---|---|---|
| | Day 0 | Day 2 | Day 7 | Day 14 |
| Con | $23.3 \pm 0.5$ | $23.5 \pm 0.8$ | $25.2 \pm 0.6$ | $25.3 \pm 0.6$ |
| Con-GPO3,200 | $22.3 \pm 0.3$ | - | - | $25.7 \pm 1.2^{ns}$ |
| HepG2 | $22.5 \pm 0.3^{ns}$ | $22.8 \pm 0.8^{ns}$ | $25.0 \pm 0.6^{ns}$ | $25.0 \pm 0.7^{ns}$ |
| HepG2-GPO640 | $22.5 \pm 0.2^{NS}$ | $23.8 \pm 1.1^{NS}$ | $23.9 \pm 0.8^{NS}$ | $23.5 \pm 0.9^{NS}$ |
| HepG2-GPO3,200 | $23.7 \pm 0.6^{NS}$ | $24.2 \pm 0.7^{NS}$ | $24.6 \pm 0.8^{NS}$ | $25.1 \pm 0.6^{NS}$ |

**Table 4.1** Body weight (g) of mice measured on different time-points (2, 7 and 14 days) after HepG2 inoculation with and without GPO1986 supplementation. We did not obtained the data for 2 days, 7 days-control-GPO3,200, because there was no significant difference between 14 days con-GPO3,200 and 14 days control. All values are expressed as mean $\pm$ SEM (n=5). $^{ns}$not significantly different, compared with the control group; $^{NS}$no significantly different, compared with HepG2 group.

## 4.2 Intravital fluorescence videomicroscopy

### 4.2.1 Microvascular changes

Based on intravital fluorescence microscopic images, we examined characteristics of microvascular changes by HepG2 inoculated in mouse skin.

Figures **4.1-4.3** show microscopic images of capillaries observed on different time-periods with and without GPO1986 supplementation. Neocapillarization occurred in the following sequential steps. Three days after inoculation, capillaries in the host tissue, underlying the tumor mass, enlarged. These features are similar to the ones reported by Gullino and Grantham's (1961) . Two days after implantation, there appeared capillary dilation, marked tortuosity and hyperpermeability with extravasations of the fluorescence tracer from the host existing microvessels (**Fig.4.1A**). On day 7, a number of neocapillaries were visible in tumor within the chamber. The host vessels underwent sprouting of endothelial cells (see within the circle of **Fig. 4.1B**). We note that this time-period corresponds to the early stage of tumor angiogenesis onset.
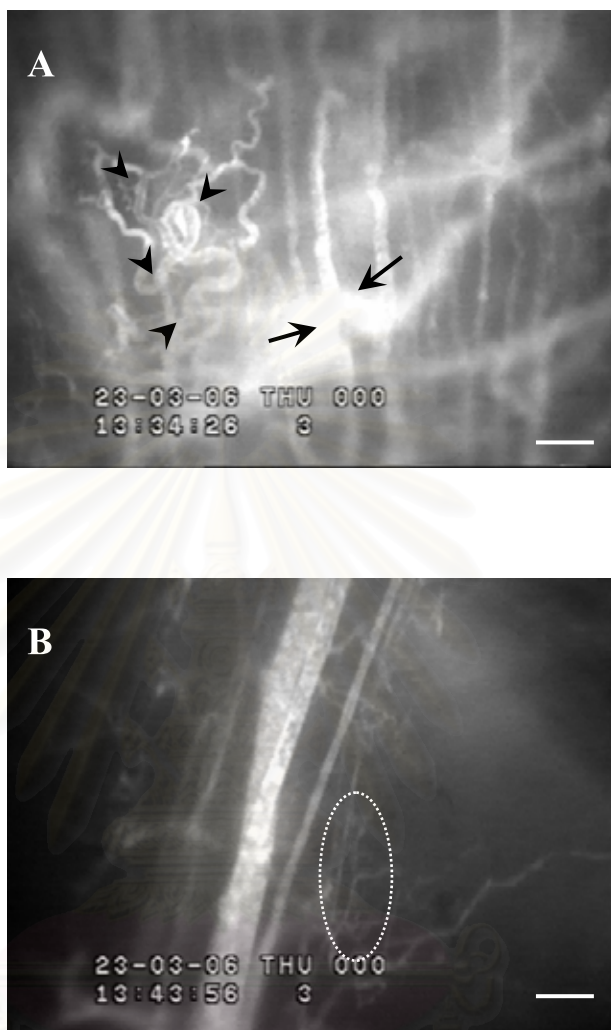
**Figure 4.1** Fluorescence video images of capillaries after HepG2 inoculation. In **A**, arrows indicate dilatation and hyperpermeability in which tortuosity is indicated by arrow-heads (2 days after HepG2 inoculation). In **B**, circle indicates spouting originating from the host blood vessel at the early stage of tumor angiogenesis onset (7 days after HepG2 inoculation). Scale bar: 100 μm.
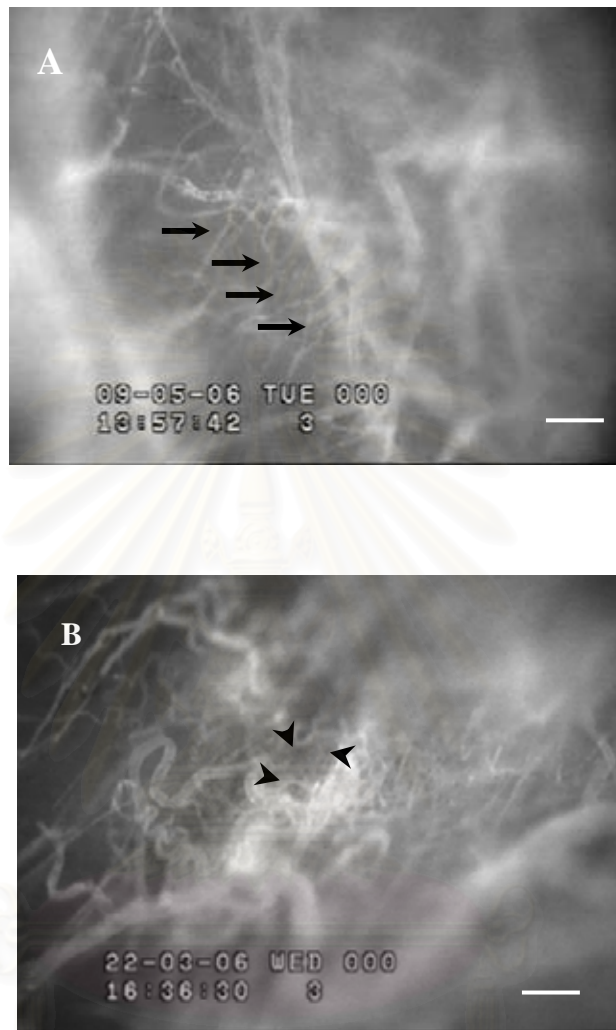
**Figure 4. 2** Fluorescent images of capillaries on day14. Note the formation of capillary network (**A**) and shunts/ loops form (arrow-point **B**). Bar scale: 100μm.
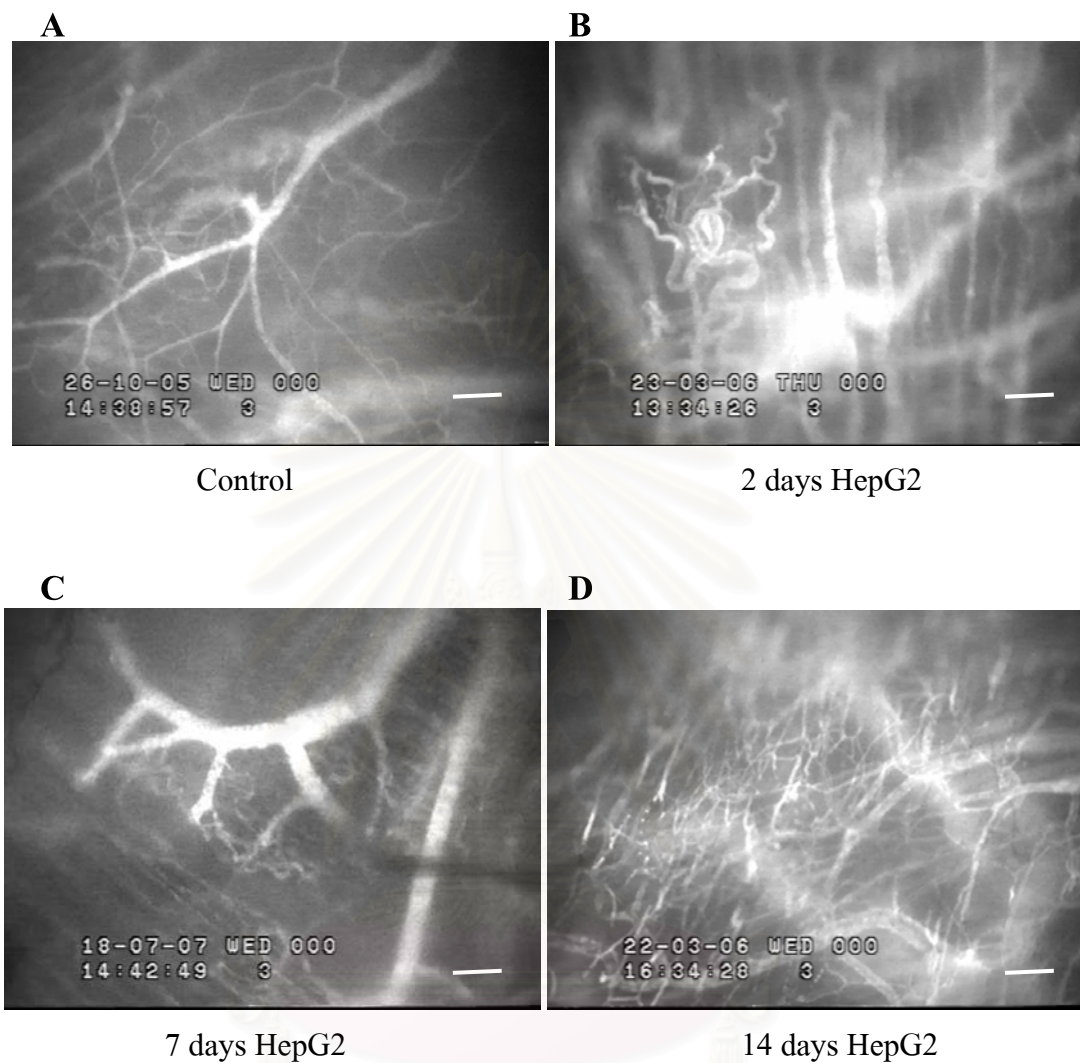
**Figure 4.3** Fluorescent images of capillaries (visualized by FITC-dextran) on day 0 (control), 2, 7 and 14 after HepG2 inoculation. Scale bar: 100 µm.

### 4.2.2 Capillary density

Based on FITC-dextran images recorded on different periods after HepG2 inoculation, we measured capillary density (CD) using Eq. (1). **Table 4.2** and **Fig. 4.4** show the CD levels measured on different time-periods after HepG2 inoculation with and without GPO1986 supplementation. On day 7 and 14 in all HepG2 groups, the CD levels were significantly higher than their control levels ($p<0.05$). Without GPO1986, the CD level increased from 53 % on 7 day to 71 % on day 14. With supplementation of high dose , on the other hand, the CD level decreased from 44 % on 7 day to 42 % on day 14. We note that the capillary density increased with HepG2 inoculation, the increase being suppressed by GPO1986 supplementation.

| Groups | Capillary density (%) | |
| --- | --- | --- |
| | 7 days | 14 days |
| Con | $33.7 \pm 4.4$ | $32.2 \pm 3.4$ |
| Con-GPO3,200 | - | $33.8 \pm 2.9^{ns}$ |
| HepG2 | $53.4 \pm 3.8^{a}$ | $71.3 \pm 1.9^{a}$ |
| HepG2-GPO640 | $47.9 \pm 3.6^{ns}$ | $54.5 \pm 1.6^{b}$ |
| HepG2-GPO3,200 | $44.0 \pm 1.8^{b,NS}$ | $42.2 \pm 4.2^{b,c}$ |

**Table 4.2** Capillary density (%) measured in tumor within the chamber on different time-points after HepG2 inoculation with or without GPO1986 supplementation.

All values are expressed as mean $\pm$ SEM (n=5). $^{ns}$not significantly different, compared with the control group; $^{a}$significantly different compared with Con (p < 0.05); $^{b}$significantly different, compared with HepG2 (p< 0.05); $^{ns}$not significantly different from HepG2; $^{c}$ significantly different, compared with HepG2-640 (p< 0.05); $^{NS}$not significantly different, compared with HepG2-640.
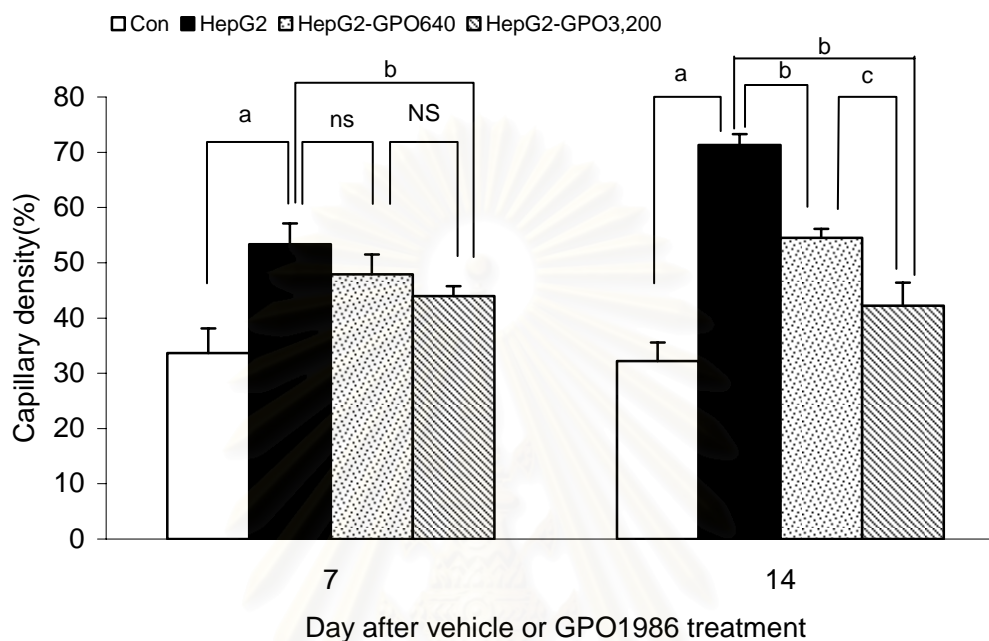
**Figure 4.4** Capillary density (%) in tumor measured on day 7 and 14 (mean ± SEM, n=5). [a]significantly different compared with Con (p < 0.05); [b]significantly different, compared with HepG2 (p< 0.05); [ns]not significantly different from HepG2; [c] significantly different, compared with HepG2-640 (p< 0.05); [NS]not significantly different, compared with HepG2-640.

**4.2.3 Vascular diameter**

**Arteriolar diameter**

**Table 4.3** show arteriolar diameters measured on day 2 after HepG2 inoculation. In control group, the arteriole diameter was $13.3\pm0.2$ µm. In the present experiment, these diameters increased after HepG2 inoculation ($18.1\pm0.6$ µm). However, in HepG2 treated with GPO1986 low dose ($17.1\pm0.6$ µm) and high dose ($17.6\pm0.9$ µm) the significant increased in arteriolar diameter compared to control still remain.

| Arteriole 2 days | | | |
|---|---|---|---|
| Con | HepG2 | HepG2-GPO640 | HepG2-GPO3,200 |
| $13.3 \pm 0.2$ | $18.1 \pm 0.6^{a}$ | $17.1 \pm 0.6^{a}$ | $17.6 \pm 0.9^{a}$ |

**Table 4.3** Arteriole diameters (µm) measured in tumor within the chamber on 2 days after HepG2 inoculation with or without GPO1986 supplementation.

All values are expressed as mean $\pm$ SEM ( n=5); $^{a}$ significantly different, compared with Con (p< 0.05).

**Capillary diameter**

     **Table 4.4** and **Fig. 4.5** show capillary diameters measured on different time-periods after HepG2 inoculation. In control group, the capillary diameter was less than 7 μm. However, in HepG2 group with and without GPO1986 supplementation, the capillary diameters in tumor were greater than 8 μm. In the present experiment, these diameters increased slightly with days after HepG2 inoculation.

| Groups | Capillary diameter (μm) | |
|---|---|---|
| | 7 days | 14 days |
| Con | $6.8 \pm 0.7$ | $6.7 \pm 0.4$ |
| Con-GPO3,200* | - | $6.9 \pm 0.4$ |
| HepG2 | $8.3 \pm 0.4^{a}$ | $8.6 \pm 0.2^{a}$ |
| HepG2-GPO640 | $8.0 \pm 0.3^{ns}$ | $8.4 \pm 0.2^{ns}$ |
| HepG2-GPO3,200 | $7.7 \pm 0.1^{ns}$ | $8.4 \pm 0.5^{ns}$ |

**Table 4.4** Capillary diameters (μm) measured in tumor within the chamber on different time-points after GepG2 inoculation with or without GPO1986 supplementation.

All values are expressed as mean ± SEM ( n=5); $^{a}p < 0.05$ significantly different, compared with Con; $^{ns}$not significantly different, compared with HepG2.

**Figure 4.5** Capillary diameters (μm) on day 7 and 14 (mean ± SEM, n=5). [a]p< 0.05 significantly different, compared with Con; [ns]not significantly different, compared with HepG2.

## 4.3 Tumor biomarkers

### 4.3.1 Serum VEGF

Table 4.5 and **Fig.4.6** show VEGF (pg/ml) measured in blood (serum) 2, 7 and 14 days after inoculation with and without GPO1986 supplementation. In all HepG2 groups, the serum VEGF levels were elevated significantly, compared with their control levels. These VEGF elevations were suppressed greatly by GPO1986 supplementation. In HepG2 group without GPO1986 supplementation, the serum VEGF level increased from 67 pg/ml on day2 up to 152 pg/ml on day 14. In HepG2 with supplementation of high dose, on the

other hand, the serum VEGF level increased from 70 pg/ml on day2 up to 106 pg/ml on day 14. We note that serum VEGF level increased with days after HepG2 inoculation, with suppression by GPO1986 supplementation.

| Groups | Serum VEGF (pg/ml) | | |
| --- | --- | --- | --- |
| | 2 days | 7 days | 14 days |
| Con | $59.58 \pm 4.23$ | $58.98 \pm 9.09$ | $59.16 \pm 4.26$ |
| Con-GPO3,200 | - | - | $54.78 \pm 5.59^{ns}$ |
| HepG2 | $67.14 \pm 4.95$ | $133.43 \pm 9.42^{aa}$ | $152.00 \pm 12.78^{aa}$ |
| HepG2-GPO640 | $75.06 \pm 5.79$ | $130.00 \pm 8.07^{ns}$ | $136.02 \pm 9.22^{ns}$ |
| HepG2-GPO3,200 | $69.71 \pm 7.29$ | $95.32 \pm 13.18^{b,c}$ | $105.55 \pm 8.77^{b,c}$ |

**Table 4.5** VEGF (pg/ml) measured in blood (serum) 2, 7 and 14 days after HepG2 inoculation with and without GPO1986 supplementation.

All values are expressed as mean $\pm$ SEM. $^{ns}$not significantly different, compared with the control group; .$^{ns}$not significantly different, compared with HepG2; $^{aa}$significantly different from Con ($p<0.001$); $^{b}$significantly different, compared with HepG2 ($p<0.05$); .$^{NS}$not significantly different, compared with HepG2 -640; $^{c}$significantly different from HepG2-640 ($p<0.05$).
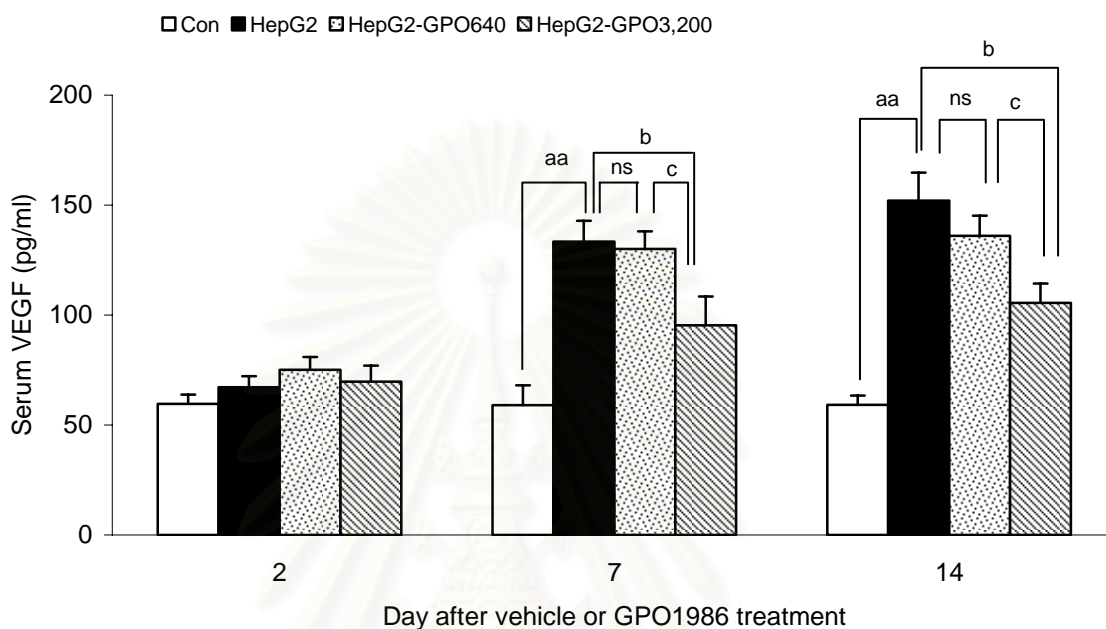
**Figure 4.6** Serum VEGF (pg/ml) of control and HepG2 groups on day 2, 7 and 14 (mean ± SEM). [ns]not significantly different, compared with HepG2; [aa]significantly different from Con ($p<0.001$); [b]significantly different, compared with HepG2 ($p<0.05$); .[NS]not significantly different, compared with HepG2 -640; [c]significantly different from HepG2-640 ($p<0.05$).

**4.3.2 Relationship between serum VEGF and capillary density**

Using the Pearson correlation, we examined whether serum VEGF was correlated with capillary density (CD). In **Fig.4.7** are plotted all data of CD and serum VEGF measured on different time-periods after HepG2 inoculation with and without GPO1986 supplementation. Apparently, the measured CD was correlated well with serum VEGF ($r$= 0.70, $p$ < 0.001 )(y = 0.29x + 19.11)**.** The correlation line was expressed as follows:
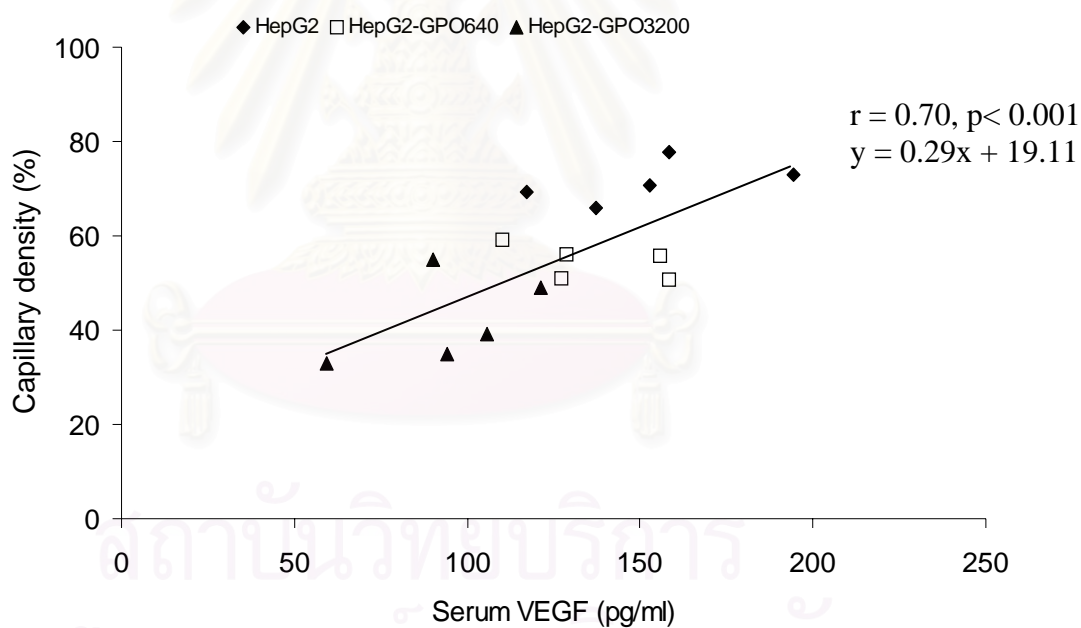


**Figure 4.7**  Relation between CD and serum VEGF on day14 in all groups (Con, Con-GPO3,200, HepG2, HepG2-GPO640 HepG2-GPO3,200).

### 4.3.3 HIF-1$\alpha$

Using immunohistochemical examination of tumor sections, we measured HIF-1$\alpha$ expression in tumor sections on different time-periods after HepG2 inoculation. **Figures 4.8** show immunohistochemical images of HIF-1$\alpha$ positive cells that were visualized with DAB on the background (counter-stained with haematoxylin). Apparently, HIF-1$\alpha$ immunoreactivity was expressed in both nuclei and cytoplasm, whereas HIF-1$\alpha$ staining did not appear in control tissue. HIF-1$\alpha$ was expressed markedly on day 2 and 7, but its expression decreased on day 14. This indicates over-expression of HIF-1$\alpha$ at the early stage of tumor angiogenesis.
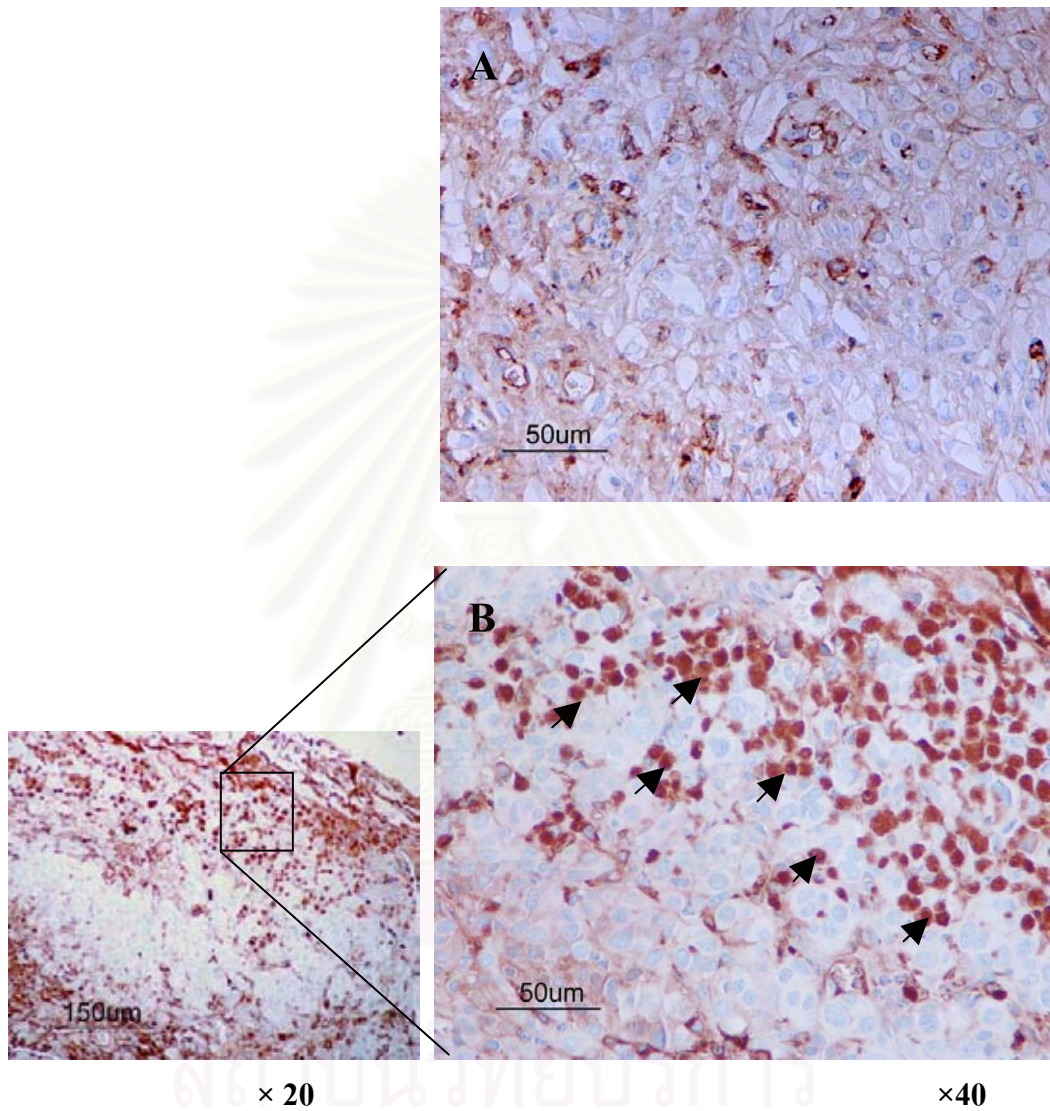
**Figure 4.8** Immunohistochemical images of HIF-1α positive cells. **A**: Normal control.
**B**: Arrows indicate HIF-1α positive cells visualized with DAB and counter stained with
haematoxylin. Scale bar: 50 µm
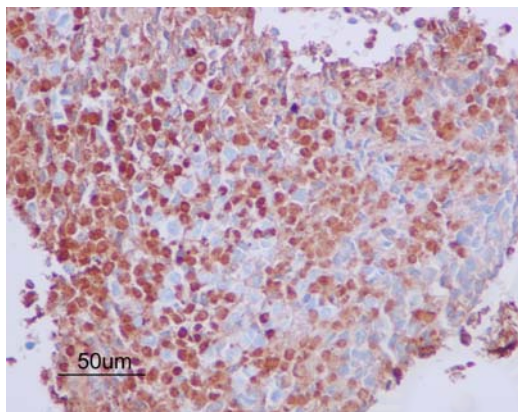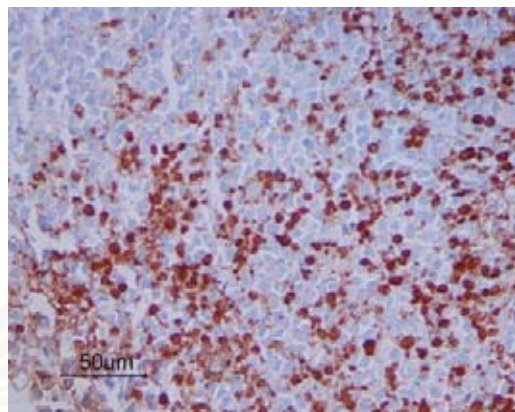
A       HepG2 - 2 days       B       HepG2 – GPO 3,200-2 days
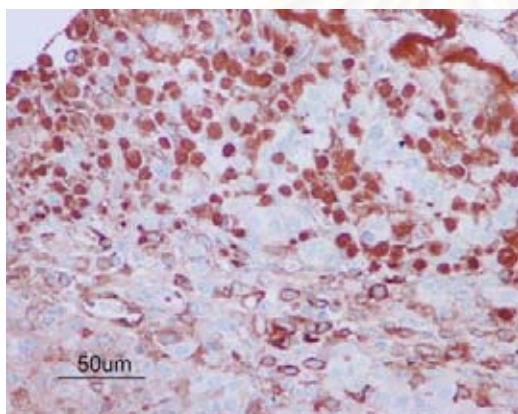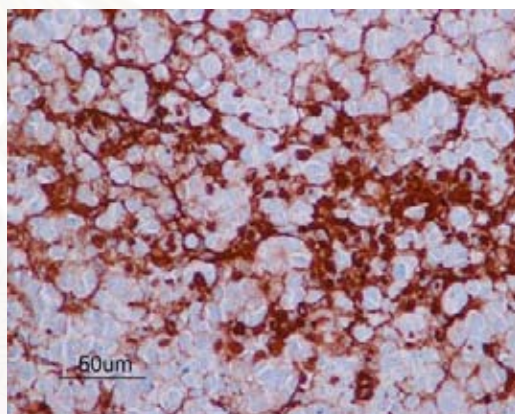
HepG2 – 7  days       HepG2 –GPO3,200 -7 days
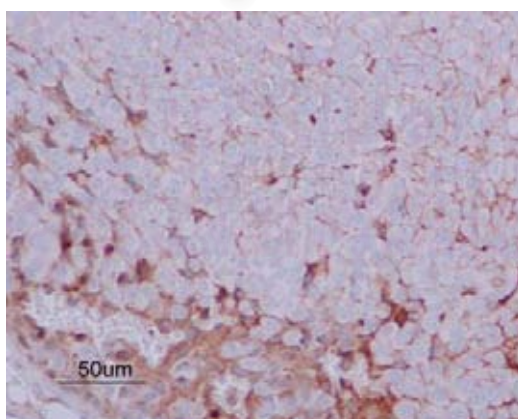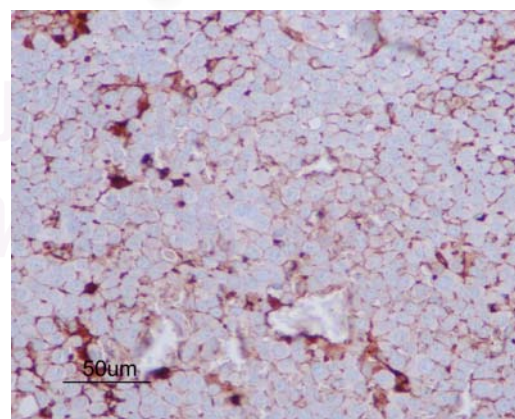
HepG2 - 14 days       HepG2 –GPO3,200 -14 days

**Figure 4.9** Immunohistochemical images of HIF-1α on day 2,7 and 14 in HepG2 (**A**) and HepG2-GPO3,200 group (**B**). Scale bar: 50 μm.

By measuring the number of HIF-1α positive cells, we could examine the expression of HIF-1α in tumor (**Table 4.6** and **Fig. 4.10**). The expression of HIF-1α protein was significantly higher on 2 and 7 day than HepG2 14 day. In HepG2 treated with GPO1986, HIF-1α expression did not decrease at both low and high dose at any time-points (p>0.05).

| Groups | HIF-1α positive (cells/mm$^2$) | | |
|---|---|---|---|
| | 2 days | 7 days | 14 days |
| HepG2 | $746 \pm 66$ | $650 \pm 47$ | $171 \pm 18$ |
| HepG2-GPO640 | $784 \pm 51^{ns}$ | $666 \pm 37^{ns}$ | $168 \pm 28^{ns}$ |
| HepG2-GPO3,200 | $750 \pm 19^{ns,NS}$ | $617 \pm 54^{ns,NS}$ | $184 \pm 23^{ns,NS}$ |

**Table 4.6**  HIF-1α positive (cells/mm$^2$) measured 2, 7 and 14 days after HepG2 inoculation with and without GPO1986 supplementation.

All values are expressed as mean ± SEM (n=5). $^{ns}$not significantly different, compared with HepG2; $^{NS}$not significantly different, compared with HepG2-GPO640.
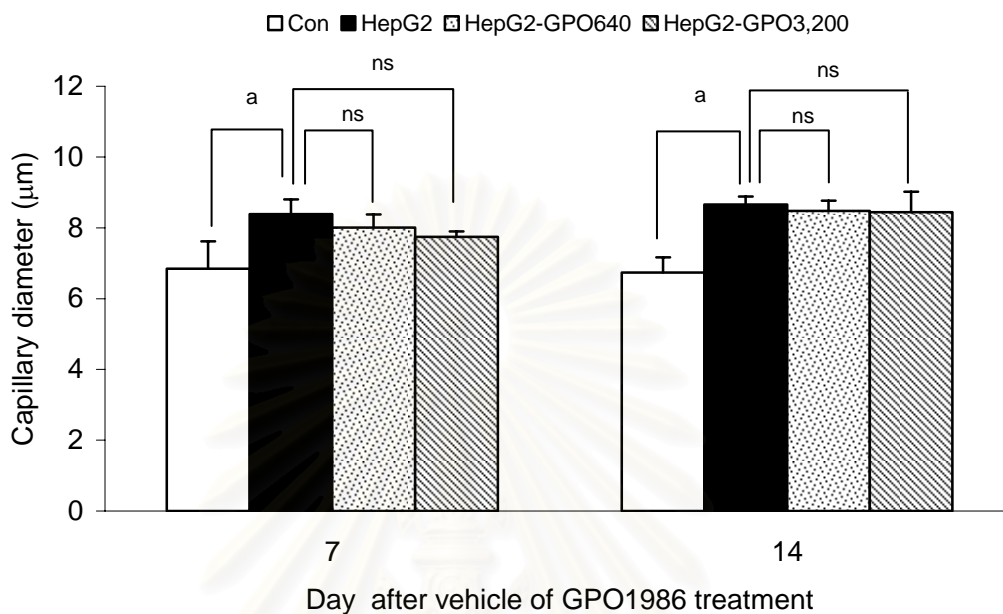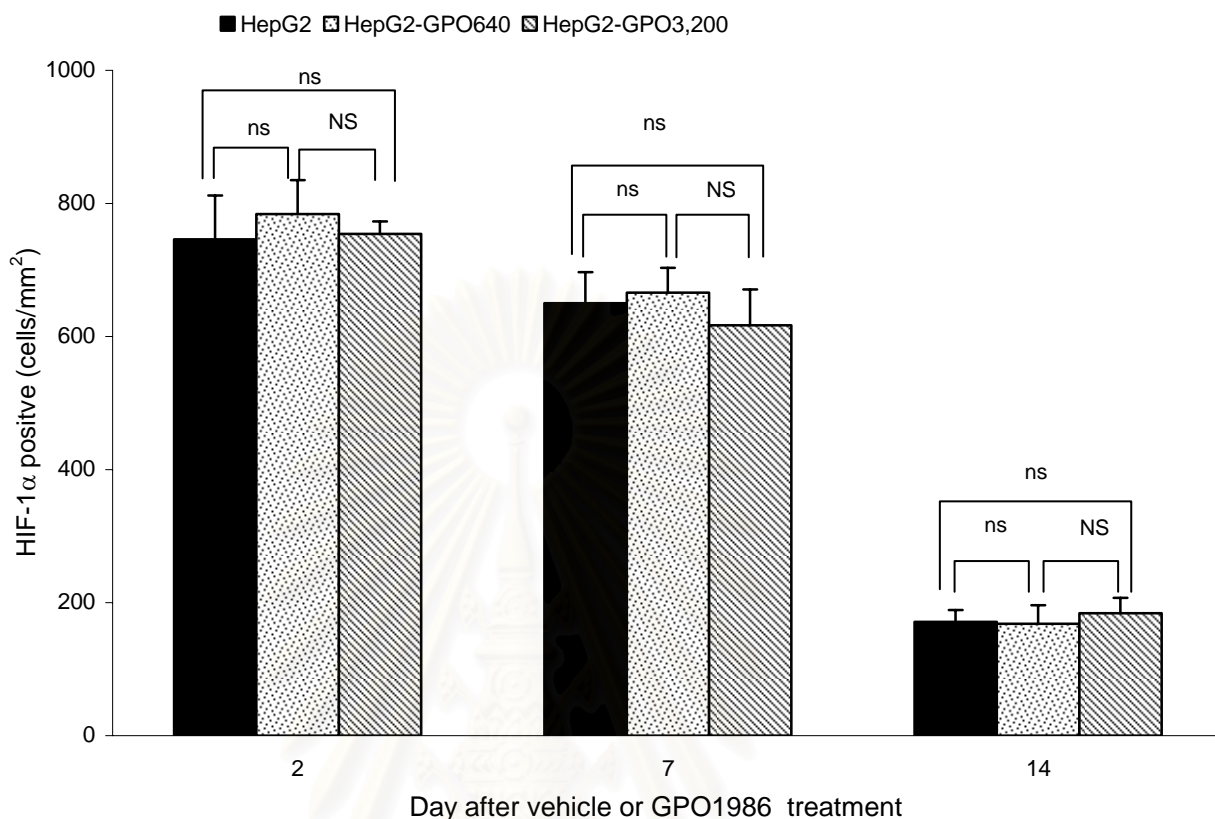
**Figure 4.10** HIF-1$\alpha$ positive (cells/mm$^2$) on day 2, 7 and 14 in HepG2 groups (mean $\pm$ SEM). [ns]not significantly different, compared with HepG2; [NS]not significantly different, compared with HepG2-GPO640.

## 4.4 Tumor growth

We evaluated the tumor growth based on histological examination of the area within the skin-fold chamber on different time-points after HepG2 inuculation. **Figures 4.11, 4.12** show microscopic images of transverse sections to the skin that were stained using hematoxylin-eosin. The borders of tumor area are marked by arrow-heads.

A



Control 14 days

Control-GPO3,200 14 days

B



HepG2  2 days

HepG2-GPO3,200 2 days
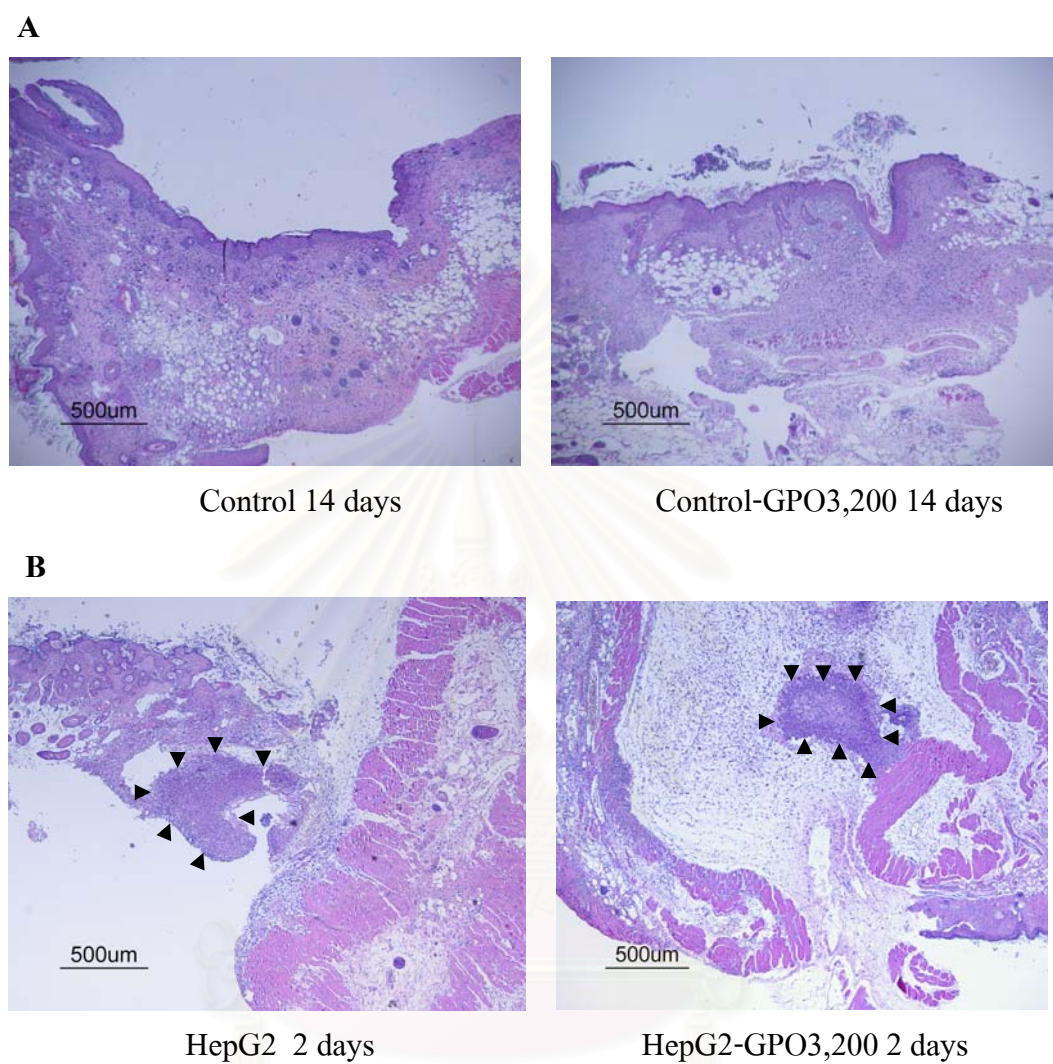
**Figure 4.11** Hematoxylin-eosin stained transverse section of skin within the chamber in control (**A**) and on day 2 of HepG 2 (**B**). Arrow-heads indicates the borders of tumors. Scale bar: 500 μm.

**A**



HepG2  7 days                    HepG2-GPO3,200 7 days

**B**



HepG2  14  days                   HepG2-GPO3,200 14  days
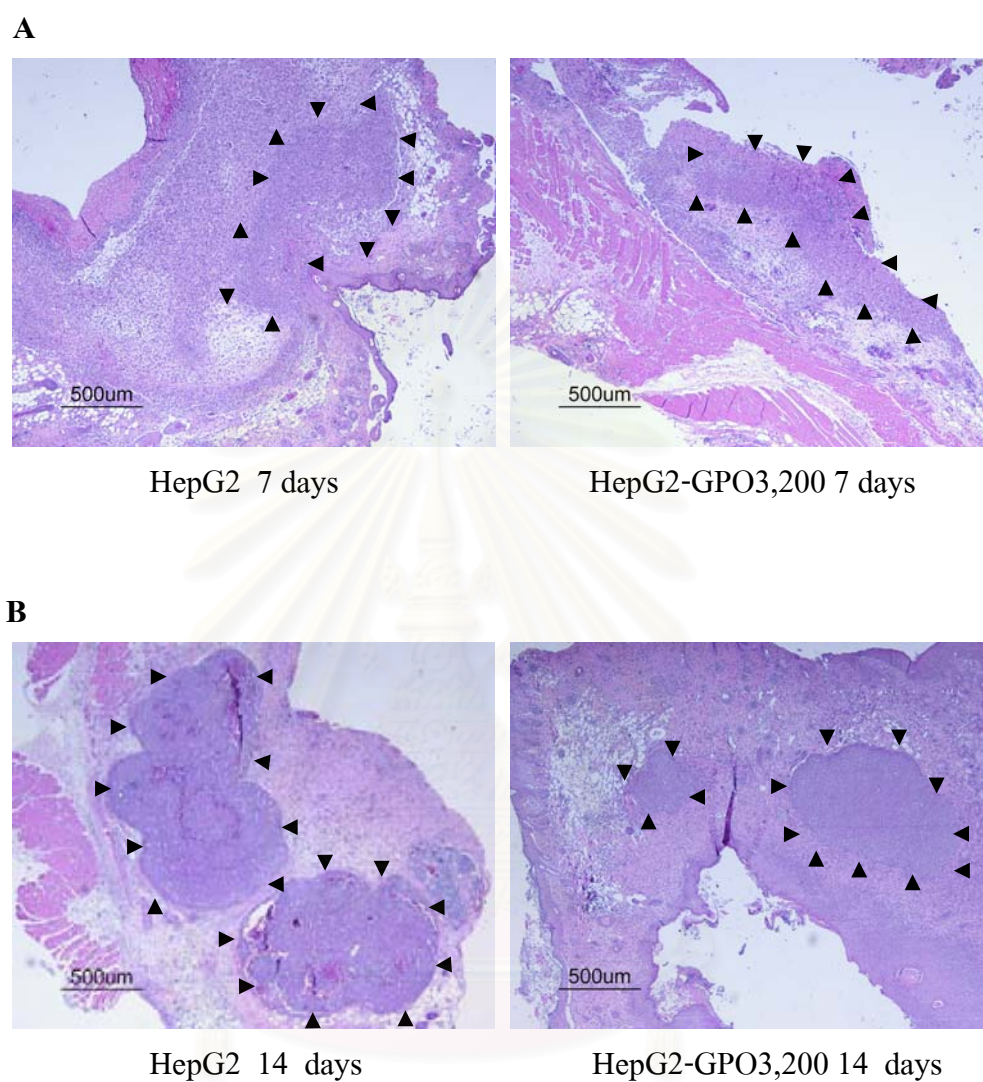
**Figure 4.12** Hematoxylin-eosin stained transverse section on day7 in HepG2 (**A**) and on day 14 in HepG 2 (**B**). (The borders of tumor area are marked by arrow-heads). Scale bar: 500 μm.

**Table 4.7** and **Fig.4.13** show tumor area ($mm^2$) measured on different time-points after HepG2 inoculation with and without GPO1986 supplementation. Apparently, the tumor size decreased significantly with supplementation of GPO1986 at high dose. Without GPO1986 supplementation, the tumor size was approximately 7 $mm^2$ on day 7 and 10 $mm^2$ on day 14. When supplemented with GPO1986 at a dose 3,200 mg/kg BW per day, the tumor size wasapproximately 6 $mm^2$ on both day 7 and 14. We note that the tumor growth was suppressed significantly by GPO1986 supplementation.

| Groups | Tumor area ($mm^2$) | | |
|---|---|---|---|
| | 2 days | 7 days | 14 days |
| HepG2 | $4.30 \pm 0.13$ | $7.29 \pm 0.14$ | $10.24 \pm 0.22$ |
| HepG2-GPO640 | $4.40 \pm 0.22$ | $7.05 \pm 0.25^{ns}$ | $8.57 \pm 0.24^{ns}$ |
| HepG2-GPO3,200 | $4.37 \pm 0.20$ | $5.99 \pm 0.21^{NS}$ | $6.15 \pm 0.24^{b,c}$ |

**Table 4.7** Tumor area ($mm^2$) HepG2 groups 2, 7 and 14 days after HepG2 inoculation with and without GPO1986 supplementation.

All values are expressed as mean $\pm$ SEM (n=5). [ns]not significantly different, compare with HepG2; [NS]not significantly different, compared with HepG2-GPO640; [b]significantly different, compared with HepG2 (p<0.05); [c]significantly different from HepG2-GPO640 (p<0.05).
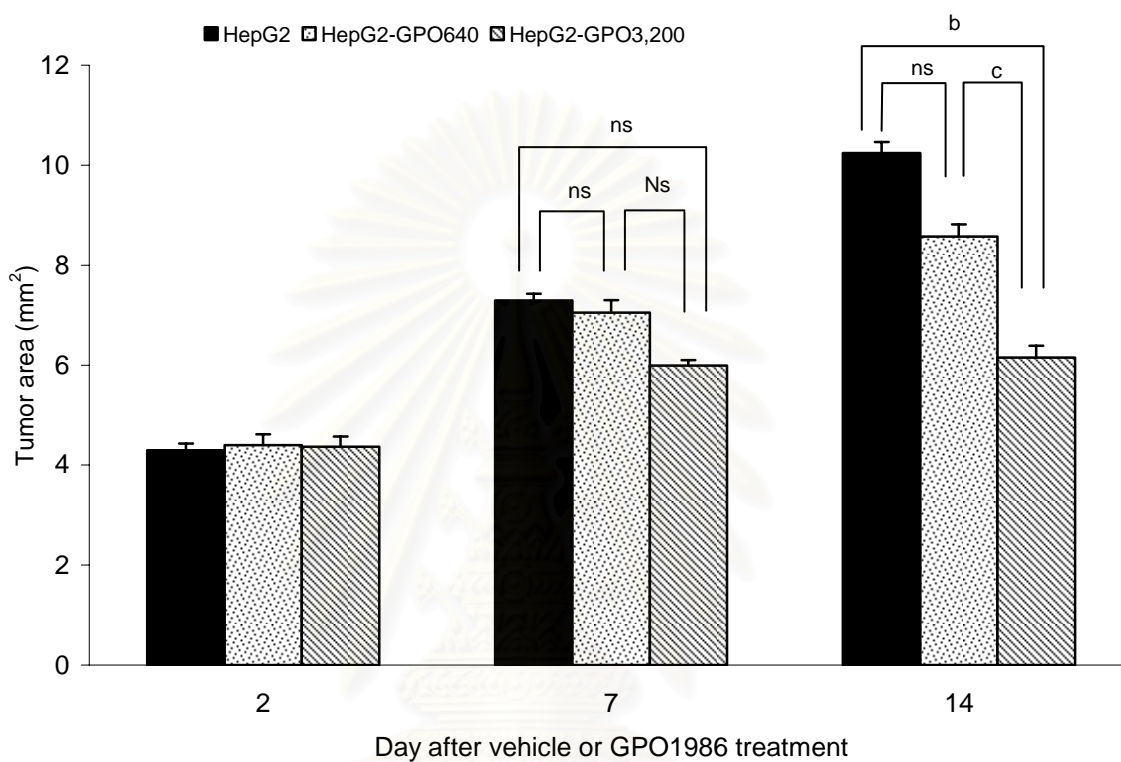
**Figure 4.13** Tumor area $(mm^2)$ on day 2, 7 and 14 in HepG2 groups (mean ± SEM). [ns]not significantly different, compare with HepG2; [NS]not significantly different, compared with HepG2-GPO640; [b]significantly different, compared with HepG2 ($p<0.05$); [c]significantly different from HepG2-GPO640 ($p<0.05$).

# CHAPTER V

# DISCUSSION

We conducted *in vivo* experiment with an aim at the effects of herbal extract (GPO1986) on tumor angiogenesis and tumor angiogenic biomarkers (VEGF and HIF-1α), using HepG2 implanted-nude mice.

## 5.1 Direct microscopic observation of angiogenesis in tumor

The formation of neovessels in tumor-baring nude mice occurred in sequential steps following "two compartment theory" by Folkman *et al.* (1985, 1990). These steps of neovascularization were well described as follows.

### Two days after HepG2 inoculation

Host arterioles and capillaries became tortuous, dilated, and hyperpermeable to macromolecular dextran (lebeled with FITC) (MW = 250,000), as shown in Fig 4.1A. Tumor and endothelial cells might secrete chemotactic signals that can attract each other. It was proposed that a chemotactic substance was endogenous nitric oxide (NO). A number of studies have reported that NO increased from the early phase of tumor angiogenesis. According to Hanahan and Folkman (1996), the NO derived from vascular endothelial and/or tumor cells in tumor area. Moreover, according to Jenkins *et al.* (1995), Orucevic *et al.* (1996) and Fukumura *et al.* (1997), the secretion of NO from tumor and vascular endothelial cells required to maintain blood flow perfusion and also to prevent leukocyte-endothelium interaction in tumor area.

In general, VEGF causes vasodilatation by inducting endothelial nitric oxide synthase (eNOS) and increasing nitric oxide production (Kroll and Waltenberger, 1999). Therefore, the increase in NO production in tumor area could be a cause of vasodilatation as observed in our study. Moreover, this tumor-induced endogenous NO secretion may be

further enhanced through "paracrine effect" of tumor-induced VEGF. VEGF might account for the appearance of hyperpermeability at the early stage as seen in Fig 4.1A.

Hanahan and Folkman (1996) mentioned that microvascular dilatation, tortousity, hyperpermeability and edema appeared as earliest histopathological features. Initially, VEGF was described as a "vascular permeability factor secreted by tumor", because it enhanced permeability in host blood vessels and also stimulated the production of ascites (Senger *et al*., 1983). Since then, it has been postulated that VEGF increases permeability by binding to VEGFR-2 and thereafter by activating guanylyl cyclase to produce *cyclic guanosine monophosphate (cGMP) via a nitric oxide dependent pathway.* Increased cGMP levels probably enhance endothelial permeability by increasing the vesico-vascular organelles, fenestrations and transcellular gaps (Bates and Harper, 2002).

In summary, tumor induces vascular changes, such as vasodilation, tortuosity, and hyperpermeability, at the early stage. VEGF may play important roles in these vascular changes. Figure 5.1 summarizes roles of VEGF. We believe that the microvascular changes would allow endothelial cells to enlongate and form neovascular sprouting, as the following process.
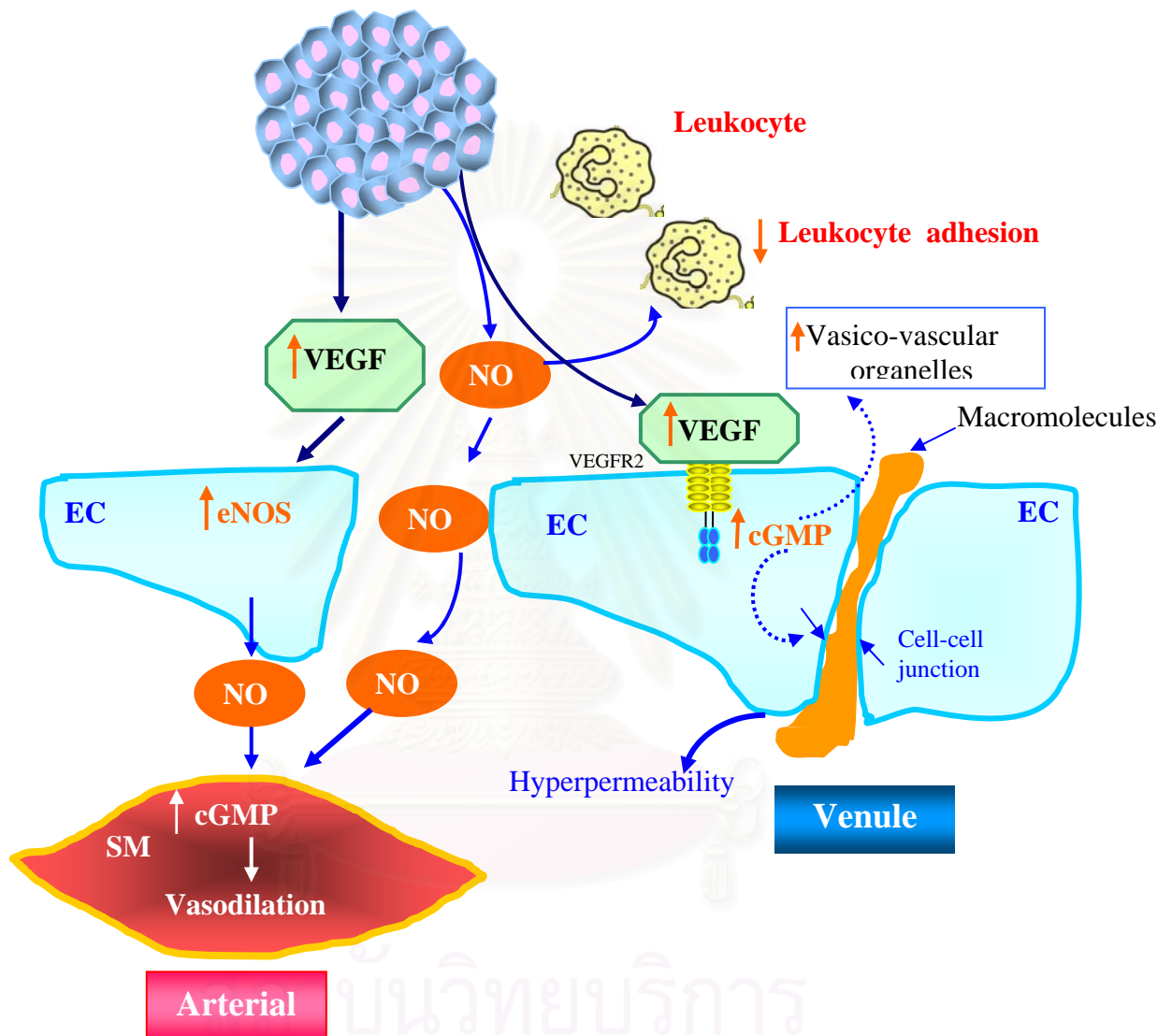
**Figure 5.1** Schematic representation of the possible mechanisms of tumor induced the functional changes in blood vessel. NO was derived from tumor vascular endothelium and/or tumor cells. It could be the underlining cause of vasodilatation. VEGF also causes vasodilatation by induction of eNOS and increasing NO production. Besides, VEGF increases permeability by binding to VEGFR-2 and thereafter activating guanylyl cyclase to produce cGMP via a nitric oxide dependent pathway. Increased cGMP levels probably enhance endothelial permeability by increasing the vesico-vascular organelles, fenestrations and transcellular gaps. Moreover, since NO could prevent leukocyte-endothelial interactions, therefore, it may help to circumvent host immune attack.

*Seven and fourteen days after HepG2 inoculation*

Seven days after HepG2 inoculation, endothelial cells sprouted from the mother vessels, as shown in Fig.4.1B . This is a characteristic process of tumor angiogenesis.

In general, angiogenesis is an essential process for the growth and metastasis of tumor cells. For this reason, the inhibition of angiogenesis is emerging to establish a promising strategy for cancer treatment. Since VEGF plays a key role in angiogenesis, the factor has become a major target for anti-angiogenic therapy.

As shown in Fig 4.1B, the tumor angiogenesis was observed as a large number of newly formed capillary sprouts two days after HepG2 inoculation. Our measured capillary density measured in HepG2 group was significantly higher than the control level on day 7 and 14 (**Table 4.2 and Fig. 4.4**). Moreover, our measured capillary diameter in HepG2 group was significantly greater than the control level on day 7 and 14 (**Table 4.4 and Fig. 4.5**). There are a number of studies (Eddy and Casarett, 1973; Yamaura and Sato, 1974; Patumraj et al., 2005; Yoysungnoen *et al*., 2005) to report an increase of vascular diameter in tumor area.

In the present experiment, the serum VEGF increased markedly 7 and 14 days after HepG2 inoculation (**Table 4.5, Fig.4.6**). Recently, Jian-wei Pan *et al*. (2005) demonstrated that eNOS appeared in tumor cells as well as endothelial cells. When NO was produced by ECs and tumor cells, it might influence both vascular structure and extracellular matrix. Due to the short half-life and narrow effective distance of NO, NO, induced in endothelial cells, might act mainly on the cell itself and its neighboring matters, such as blood ingredients or other vascular structures. This might lead to vascular dilation, which stimulated the endothelial cells to proliferate or move. According to Jian *et al*. (2005), these co-modulations between EC-induced NO and tumor cells should be essential for tumor angiogenesis.

On the other hand, NO, produced in tumor cells, might work mainly on the interstitium, which might change the construction of extracellular matrix. This might stimulate endothelial cells to move or migrate by ion- formed cellular cord.

There are several studies to demonstrate that most effects of VEGF could be achieved through eNOS/NO. In fact, VEGF acts on Flk-1/KDR (Koistinen *et al*., 2001), then act on Ser1177 or Ser1179 in eNOS of ECs. By action of eNOS (Fulton *et al*., 1999), vessels could be dilated, promoting angiogenesis. According to Murohara and co-workers (1998), when eNOS was lacking in eNOS knocked-out mouse, the angiotrophic effect of VEGF could be reduced markedly. Fukumura *et al*. (2001) assumed that VEGF influence not only eNOS but also iNOS. Moreover, VEGF could promote eNOS expression by stimulating endothelium through Flk-1/KDR (Bouloumie *et al*., 1999), while NO could promote VEGF expression directly or through second messages (Wang *et al*., 2001).

In addition to vessel sprouting, there are some pathological processes, including "angiogenic phenotype" that is characterized by an extensive vessel dilatation (Many *et al*., 1984; Bull *et al*., 1992). Previous studies, using a model of multistep tumor-genesis in mouse malignant insulinoma (Ryschich *et al*.,2002) or a model of thyroid hyperplasia (Many *et al*., 1984), have demonstrated that vessel dilatation was the first detectable stage of ongoing angiogenesis, preceding vessel sprouting and cell extended transformation. It was suggested that the observed vessel dilatation, during tumor development and growth, could cause an increase of endothelial surface for the diffusion of oxygen and nutrition. This represents a possible adaptive mechanism to compensate for the insufficient vascularisation (Ryschich *et al*., 2004). In the later session, we will see this oxygen effect on HIF-1$\alpha$ expression.

Endothelial cells in tumors does not show a normal apperarance of the normal cells. In fact, most endothelial cells in tumor are disorganized, loosely connected, branched, overlapping, sprouting and form a defective cellular lining of the vessel wall (Hashizume *et al*., 2000, Donald & Alexander ,2000). As shown in Fig. 4.4 and Table 4.2, capillary

density in HepG2 group CD were significantly than controls, increasing significantly from from on day 7 to day 14.

Furthermore the results of capillary diameter demonstrated that both 7 day and 14 day, the neocapillaries were widen (~20-28%) than controls (day7=8.39±0.41 um, day 14=8.66±0.22 um). These results were agreed with the previous report of Yoysungnoen *et al.* (2005) that 14- day HepG2 tissue perfusion were significantly increased than controls. Besides, Patumraj *et a.l* (2005) also indicated that both mean diameters of arterial and venous vessels were significantly increased with the bifurcation ratio (BR) increased up to 2-3 times (day 7= 2.26±0.05, day 14= 2.7±0.03) as compared to controls (1.63± 0.03).

According to the schematic shown in Fig 5.1, it was possible to make a remark that this increased capillary diameters may be underlined by the ***further increased*** tumor-induced VEGF expression and by other mechanism(s).The previous study reported by Niimi H. (2003) aslo showed that the neocapillary has different characteristics from normal capillary. For instance, it produced different response to topically applied acetylcholine (ACh). Furthermore, the neocapillary pericytes were observed using confocal laser microscopy, based on the fluorescence immunohistochemical images of the neoplastic tissue. Several pericytes, stained with anti-NG$_2$, appeared in the neocapillaries. It was suggested that these pericytes might be recruited in the neocapillaries to regulate blood flow without vascular smooth muscle. Therefore, the dilated neocapillaries may be responsed by these pericytes.

## 5.2 The effects of Herbal extract (GPO1986) on tumor angiogenesis and tumor growth

By using intravital fluorescent microscopy, our study demonstrated that there was a significant increase in the number of CD with the heterogeneous network in HepG2 groups as compare to the controls ( Table 4.2 and Fig.4.4). In particular this increment of capillary density was characterized as a time-dependent  manner  (CD of 14> CD 7 days).  In our

study, it is actually confirmed the fact that angiogenesis has already occurred on day 7 after HepG2 inoculation. A large number of capillary was observed and corresponded with the increase in tumor-area .

In our study, we have demonstrated that the mixture of eight kinds of herbs (GPO1986) could  inhibit angiogenesis which occurred in HepG2 implanted in nude mice model.  The composite formulas usually have greater efficacy than single ingredients. This may be due to the synergistic interactions of the ingredients (Xue and Roy, 2003). In Chinese herbalism, every herb has its own properties. Chinese herbalists believe that illness can be effectively treated by combining herbs based on their various characteristics and the patient's overall status.

From Fig.4.7 the results showed that GPO1986 could inhibit tumor angiogenesis. Interestingly the inhibition of tumor angiogenesis by herbal extract (GPO1986) was a dose-dependent manner. Moreover, the histological examination using hematoxylin – eosin shown in Fig.4.16 - Fig4.17 indicated that the tumor size was decreased by GPO1986 at high dose (GPO3,200 mg/kg BW) ($p< 0.05$).  It  was demonstrated for the inhibition effects of GPO1986 on tumor growth as a result of its anti-angiogenesis property as well. Besides, this high dose of  herbal extract (GPO1986), 3,200 mg/kg BW, used in this study has not been reported for its toxicity (Pornsiriprasert *et al*., 1986).

**5.3 The effects of Herbal extract (GPO1986) on HepG2 angiogenic biomarkers: HIF-1α and serum VEGF levels**

**5.3.1 VEGF**

From our findings (Fig.4.6), VEGF expression was significantly increased in HepG2 groups ($p< 0.001$). Furthermore, there was a correlation between VEGF and CD significantly (Fig. 4.7).

It is well established that VEGF is a critical component of tumor growth, vascularity and metastasis. A recent comprehensive review of the literature reports that the

majority of studies fine a significant correlation between increased VEGF expression and increased tumor microvascular density (Hasan *et al*., 2002). There are a number of reports of positive correlations between serum VEGF levels and tumor progression (Lantzsch *et al*., 2002; Salven *et al*., 1999; Adams *et al*., 2000). Regardless of detection method, there is an increasing body of data to indicate that VEGF expression, both in the tumor and in the circulation, is correlated with poor patient prognosis for neoplasias, such as breast cancer (Gasparini, 2000) and non-small cell line cancer (Giatromanolaki , 2001), as well as cancer in general (Poon *et al*., 2001). VEGF has been found to increase *in vivo* angiogenesis and vascular permeability, *in vitro* proliferation and migration of ECs , protease production in endothelials cells, and expression of intercellular adhesion molecules on ECs. In addition, VEGF overexpression has been detected in many human solid tumors (Shibuya, 1995; Ferrara *et al*., 1997). Furthermore, inhibition of VEGF signal pathway was observed to prevent tumor angiogenesis, suppressing solid tumor growth (Millauer *et al*., 1996; Niethammer *et al*., 2002). Recent study has also reveaed that tumor angiogenesis occurred in HepG2 implanted nude mice comodulated by VEGF and COX-2 (Yoysungnoen *et al*.,2005). During the past few years, Akarasereenont *et al*., 1999 reported that GPO1986 could be effect on *in vitro* endothial cell proliferation.Therefore, our findings have extended the mechanism of this GPO1986 on its inhibit of EC proliferation. Since GPO1986 could decrease VEGF, the essential growth factors for EC survival.

From our study, the inhibitory effect of GPO1986 could decrease VEGF in HepG2 in dose dependent manner ($p < 0.001$). However, high doses of GPO1986 has shown no effect on control' s VEGF (Con-veh=$59.16 \pm 4.26$ and Con-GPO3,200=$54.78 \pm 5.59$). ($p > 0.05$)

### 5.3.2 HIF-1$\alpha$

HIF-1$\alpha$ protein has a short half-life (t1/2 $\sim$ 5 min) and is highly regulated by oxygen (Salceda and Caro, 1997).However, in normoxia, the HIF-1$\alpha$ proteins are rapidly degraded, resulting in essentially no detectable HIF-1$\alpha$ protein (Wang *et al*., 1995). During

hypoxia, HIF-1α becomes stabilized and translocates from the cytoplasm to the nucleus, where it dimerizes with HIF-1β and the HIF complex formed becomes transcriptionally active (Huang *et al*., 1996; Kallio *et al*., 1997). The activated HIF complex then associates with HREs in the regulatory regions of target genes and binds the transcriptional co-activators to induce gene expression (Lando *et al*., 2002b).

In Fig 4.9 the result showed that HIF-1α expression occurred in tumor samples isolated from 2, 7, and 14 day after HepG2 implantation. Immunohistochemical expressions of HIF-1α on different periods after tumor cell inoculation are shown in Fig 4.9. The HIF-1α staining is not observed in normal control tissue (Fig. 4.8A). Within tumors, cluster of HIF-1α positive cells are most dense at the invading edge of the tumor margins. Note that the HIF-1α expression appears in nucleus as well as cytoplasm of the cell. The number of expressed cells per area measured is shown in Table 4.6 and Fig.4.10. Apparently, HIF-1α was expressed markedly on 2 and 7 days after tumor cell inoculation, but its expression was decreased on day 14. This indicates over-expression of HIF-1α at the early stage of neovascularization during tumor progression. Our findings demonstrated that HIF-1α expression turned to decline rapidly within 14 days (85% from day 2 to day 14) where a number of tumor capillary network were increased markedly (~40 % from control to day 14). Our findings are consistent with the previous report by Wang *et al*.,1995. They reported that Hep3B cells deprived of oxygen demonstrated peak expression of HIF-1α mRNA within 2 h; furthermore, when the Hep3B cells were resuscitated with oxygen, HIF-1α mRNA expression decreased to below basal levels (Wang *et al*., 1995). Furthermore, our results also indicated that VEGF were increased significantly during 7- and 14-days (about 50% and 175%, respectively).HIF-1α expression was significantly increase prior to the increase in VEGF. Therefore, it suggested that as an *in vivo* evidence, HIF-1α was a hypoxia-activated transcription factor that may take a major role on regulating VEGF synthesis.

The patterns of immunohistochemical staining in different human tumor cancers suggest that HIF-1α overexpression may result from both physiological (hypoxia) and

nonphysiological mechanisms. It is clear from previous studies that many human tumors have regions of significant hypoxia (Brizel *et al.,* 1996;Hockel *et al*., 1996; Helmlinger *et al*., 1997).This pattern was most obsevious in glioblastamamultiforme in which HIF-1α was detected in viable tumor cells that were closest to areas of necrosis and farthest from blood vessel, as previously demonstrated for the expression of VEGF mRNA in these tumor (Chan *et al*.,1998;Shweiki *et al*., 1992).

It is clear that the hypoxia-mediated increase in HIF-1α plays a critical role in both the establishment and progression of many common cancers through the HIF-1- dependent activation of genes that allow cancer cells to survive and metastasize in the hostile hypoxic tumor environment. HIF-1α is a transcription factor which activates the transcription of the human erythropoietin gene and transcription of the VEGF gene (Forsythe *et al*., 1996). Hypoxia has also been identified as up-regulation the expression of HIF-1α.

In the present study, the positive immunohistochemical analysis of HIF-1α expression was demonstrated since 2-day post-HepG2 inoculation. And the expression was 5 day prior to the existing of tumor-induced neocapillary sprouting. Therefore, it may imply that hypoxic condition was already occurred within 2 days. Since HIF-1α stabilization up-regulates the expression of angiogenic pathway to restore oxygen homeostasis, therefore, HIF-1α protein may be considered as an important factor for the survival and growth of cancer.The level of HIF-1α protein is inversely related to the oxygen tension in culture cells (Jiang *et al*., 1996) and *in vivo* (Semenza *et al*., 2002). Hypoxic oxygen tensions that induce HIF-1α levels have been demonstrated in tumor *in vivo* and are associated with a poor clinical outcome (Brizel *et al*., 1996, Nordsmark *et al*., 1996, Höckel *et al*., 1991).

As shown by the results (Fig4.13), HIF-1α expression seem to decline when the tumor get a number of neo-vascularization, or it may imply that HIF-1α expression decreases according to tumor microenvironment has become normoxic already. Therefore, with the different period and different tumor microenvironment, it may cause the difference HIF-1α expression. Importantly, our results have indicated that herbal extract

(GPO1986) no effect on HIF-1α expression, but herbal extract (GPO1986) can significantly decrease VEGF expression.

Yet, HIF appears not to be sufficient induce maximal levels of VEGF upon hypoxia. In addition to HIF, multiple transcription factor-binding sites for activator protein-1 (AP-1), specificity protein -1 (Sp-1), nuclear factor -kappa B (NF-kB) have been identified within the VEGF promoter (Pages and Pouyssegur, 2005).

Although in hypoxia the binding of HIF to the *vegf* promoter is a key determinant in its expression, two other major transcriptional controls are mediated through the Ras–ERK and PI(3)K–AKT pathways (Rak *et al*., 2000; Pages and Pouyssegur, 2005). VEGF messenger RNA is upregulated by the ERK pathway through the phosphorylation of the transcription factor Sp-1 and its recruitment to the proximal region of the *vegf* promoter (Milanini-Mongiat *et al*., 2002). This regulation is independent of hypoxic stress and reflects the intensity of growth-factor stimulation or oncogenic signals. Transcriptional activation also occurs through ERK-induced phosphorylation of HIF-1α (Richard *et al*., 1999) and the coactivator p300, which might improve the accessibility of RNA polymerase II to the *vegf* promoter. Other levels of regulation of VEGF occur, including the stabilization of the mRNA through the stress-activated kinase p38 (Pages *et al*., 2000) and translation by means of internal ribosome entry site (IRES) sequences present in 5/ non-coding regions of VEGF( Huez *et al*., 1998) and HIF-1α mRNAs (Lang *et al*., 2002) , which are two important attributes for translation of VEGF under nutrient deprivation.

The proposed mechanism for our finding on the anti-angiogenic of herbal extract (GPO1986) as shown in Fig.5.2 (Modified from Pouysségur *et al*., 2006). A rapidly growing tumor mass quickly outstrips its vasculature and thus lacks oxygen and nutrients. This diminished level of oxygen leads to the stabilization and activation of the transcription factor HIF-1α which in turn induces the transcription of VEGF. In addition to HIF, multiple transcription factor-binding sites for AP-1, Sp-1, and activation of NF-kB leads to transcription of targent genes such as those encoding proinflamatory cytokines (e.g.,interleukins 6 and 8, TNF-α) and cyclooxygenase-2 (COX-2). The product of this

gene, together with activated ongogene and inactivated tumor suppressor gene stimulate VEGF expression through the activation of the Ras, MEK and ERK pathway phosphorylation of the transcription factor Sp-1 and its bindings to the proximal region of the promoter of VEGF (Pages and Pouyssegur, 2005).Stimulate the formation of new vessels, that migrate into the tumor mass, a process termed angiogenesis, and thereby reestablish oxygen and nutritional homeostasis(Carmeliet,2003).

In this study, the results showed the anti-angiogenic activity of herbal extract (GPO1986) partly involved the significant decreased in CD and serum VEGF levels than tumors from vehicle-treated mice. **C. nutans**, one of the ingredient in GPO 1986 was shown as anti-inflammatory remedies in Thailand (Dampawan *et al*., 1977;Thongharb and Tejasen, 1977; Tuntiwachwuttikul *et al*., 2004). Therefore, it might have some role on inhibiting. It might be inhibit encoding proinflamatory cytokines (e.g., interleukins 6 and 8, TNF-$\alpha$) and cyclooxygenase-2 (COX-2). Besides, it may involve other possible transcription factor-binding sites, including AP-1, Sp-1, and NF-$\kappa$B, leading to transcription of target genes of those.

In China , **P. herbacea** root has long been used to cure malaria and inflammations (Meng and Chen, 1988). Additionally, it has been reported that **M. philippinensis** downregulated cyclooxygenase-2 (COX-2) gene, interleukin-6 (IL-6) gene expression. These results suggested that it  has anti-inflammatory and immunoregulatory effects. The main inhibitory mechanism of these compounds may be the inactivation of NF- kB. (Daikonya *et al*., 2004).

Figure 5.2 showed our hypothesis described our findings that 1). The observed VEGF expression show in our results is an upstream important mediator for tumor angiogenesis.  2). Since GPO 1986 could inhibit VEGF expression, therefore, herbal extract (GPO1986) could attenuate tumor growth probably through its tumor anti-angiogenesis.
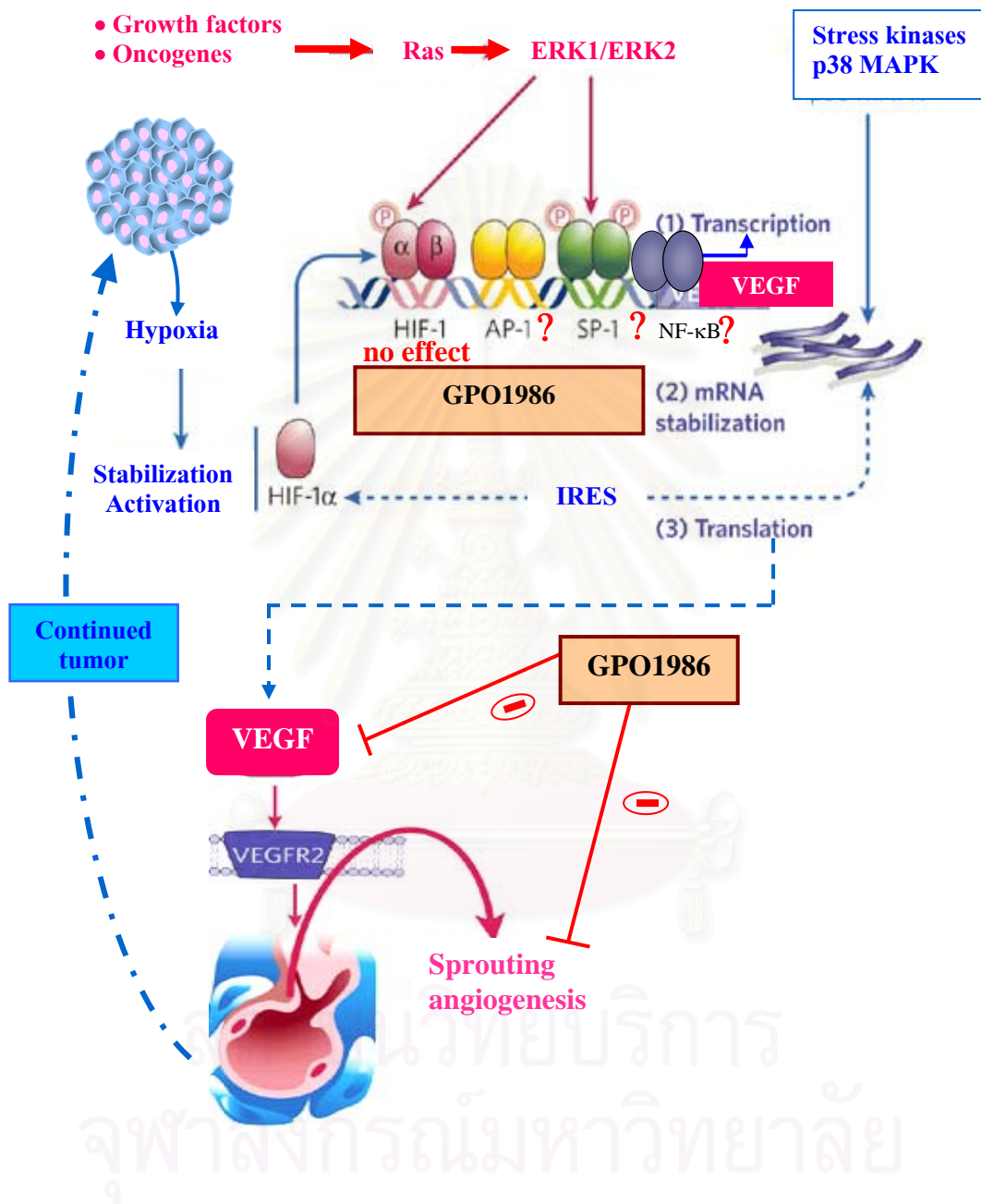
**Figure 5.2** The proposed mechanism for our findings on the anti-angiogenesis of herbal extract (GPO1986) (Modified from Pouysségur *et al*., 2006).

# CHAPTER VI

# CONCLUSION

We have examined effects of herbal extract (GPO1986) on tumor angiogenesis using biomarkers (HIF-1$\alpha$ expression and serum VEGF) in HepG2-impalnted nude mice. The effects of GPO1986 on tumor development could be summarized as follows:

1. Intravital fluorescent microscopic observation showed that capillary various abnormalities of host capillaries, such as dilatation, tortuosity and hyperpermeability, appeared at the early period (2 days) after HepG2 inoculation, prior to the intiation of tumor angiogenesis.

2. Capillary density increased significantly and time-dependently after HepG2 inoculation. In tumor, angiogenesis already occurred 7 days after HepG2 inoculation, associated with increase in capillary diameter.

3. In HepG2 groups, serum VEGF level increased significantly 7 and 14 days after HepG2 inoculation. This increase was suppressed by supplementation of GPO1986.

4. HIF-1$\alpha$ expression declined when a number of neocapillaries were formed in tumor. Supplementation of GPO1986 showed no effect on the HIF-1$\alpha$ expression.

5. The growth of tumor cells (HepG2) was fully suppressed by GPO1986 supplementation, associated with reduction in capillary density. It is hypothesized (or indicated) that reduction of VEGF expression might reduce tumor angiogenesis and then suppress growth of tumor cells.

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# APPENDIX

# APPENDIX I

## Solutions and Reagents

<u>1. Phosphate buffer saline (PBS), 30x</u>

| | | |
|---|---|---|
| Nacl, AR (analytic reagent) | 526 | g |
| $NaH_2PO_4. H_2O$, AR | 82.8 | g |
| DW | 1,600 | ml, heat |
| 5N NaOH | 120 | ml |

Make up to   2,000 ml  with DW

<u>2. Working PBS, pH 7.4</u>

| | | |
|---|---|---|
| 30x PBS | 70 | ml |
| DW | 1930 | ml |
| Triton X | 100 $\mu$l: 1,000 ml | |
| NHS (normal horse serum) | 1,000 $\mu$l : 1,000 ml | |

<u>3.Citrate buffer, pH 6.0</u>

| | | |
|---|---|---|
| Citric acid monohydrate (MW 210.14) | 2.1 | g |
| Distilled water | 900 | ml |
| 2M NaOH | 13 | ml |
| Distilled water | 87 | ml |
| Total volume | 1,000 | ml |

<u>4. DAB Tris-HCl buffer, pH 7.4</u>

4.1. Tris-HCl buffer, pH  7.4

Stock A:     2.42 g Trizma base (sigma T1503) in 100 ml DW

Stock B:       1.7 ml cone HCl in 100 ml  DW

     To make Tris-HCl buffer, pH 7.4


Stock A            2.5 ml

Stock B            2.07 ml

     Make up to 10 ml   with DW


4.2. DAB (sigma D.5637), 3,3' – Diaminobenzidine Tetrahydrochloride, anhydrous


DAB   50      mg/ml  in  DW

Store below 0°C (freezer)


4.3. Working DAB Tris-HCl buffer, pH  7.4 (make up fresh)


| | | |
|---|---|---|
| DAB | 1 | microtube |
| Tris-HCl buffer | 10 | ml |
| 30 % $H_2O_2$ | 10 | μl |
| 1 M   imidazole | 100 | μl |

**Protocol**

**1. Deparaffinize and hydrate the tissue section**

1.1 Dry slides at 60 °C overnight.

1.2 Put the slides into a rack for IHC

1.3 Dip the rack into 3 consecutive stain jars containing xylene to remove

paraffin 10 minutes every step

1.4 Dip the rack into 95% ethanol to remove xylene

1.5 Rinse the slides with tap water to remove ethanol for 3 minutes

**2. Retrieve antigen**

2.1 Place the slice in a coplin jar containing citrate buffer, pH 6.0. Make sure slides

are fully covered with solution.

2.2 Operate the microwave oven for 3 minutes

2.3 Let cool slowly at room temperature for at least 20 minutes before proceeding

to the next step.

**3. Block endogenous peroxidases**

3.1. Circle all sections with DAKO pen.

3.2. Place the slides on a flat level surface. Do not allow slides to touch each other.

Do not allow the sections to dry out at any time.

3.3. Add enough drops of 3% $H_2O_2$ to cover the whole section.

3.4. Incubate for 5 minutes at room temperature.

3.5. Rinse the slides with tap water for 5 minutes.

3.6. Place the slides in PBS. pH 7.4 wash bath for 3 minutes.

## 4. Block non specific background

4.1 Add 3% normal horse serum to cover the whole section.

4.2 Incubation for 20 minutes at room temperature.

## 5. Primary Antibody

5.1 Allow the slides to drain, shake off excess fluid with a brisk motion and carefully wipe each slide around the sections.

5.2 Dilute the primary antibody (HIF-1$\alpha$ mouse monoclonal antibody [H1alpha67 –sup] (ab463) (Abcam, Cambridge, U.K.) 1:800.

5.3 Apply 100 µl primary antibody solution to the appropriate slides, covering the tissue sections.

5.4 Tilt each slide in two different directions, so the liquid is spread evenly over the slide.

5.5 Incubate for 60 minutes at room temperature in humidified chamber.

5.6 Rinse gently with PBS from a wash bottle. Place the slide in a PBS wash bath for 3 minutes.

## 6. Secondary Antibody

6.1 Place the slides on a flat level surface again.

6.2 Apply 100 µl secondary antibody solution (Envision, DAKO, Glostrup, Denmark ) to the appropriate

6.3 Incubate for 30 minutes at room temperature in humidified chamber.

6.4 Rinse gently with PBS from a wash bottle. Place the slide in a PBS wash bath for 3 minutes.

## 7. Colorimetric detection

7.1 Mix 1 ml distilled water with reagents (substrate buffer 3,3'-Diaminobenzidine, (DAB) concentrate, hydrogen peroxide: 1ml/each.

7.2 Apply 250 µl DAB substrate solution to each slide.

7.3 Incubation for 10 minutes at room temperature.

7.4 Wash the slide with PBS buffer.

7.5 Rinse the rack with tap water for 1 minute.
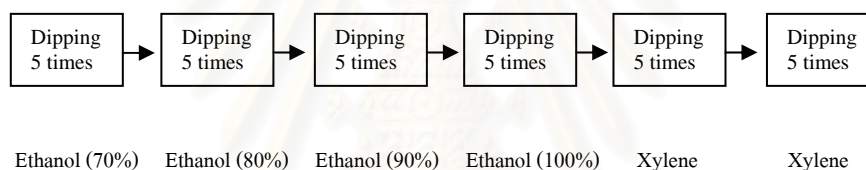
## 8. Counter stain with Hematoxylin)

8.1 Dip the slides in the rack into Hematoxylin for 5 minutes

8.2 Rinse the slides with tap water for 5 minutes.

8.3 Dip the rack into a jar containing 0.1% HCl 3 times and, then, into

tap water 5 times

8.4 Dip the rack into 0.1% NH4OH  3 times and, then, into tap water 3 times

8.5 Dip the rack in ethanol as follow:

| Dipping 5 times | → | Dipping 5 times | → | Dipping 5 times | → | Dipping 5 times | → | Dipping 5 times | → | Dipping 5 times |
|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol (70%) | | Ethanol (80%) | | Ethanol (90%) | | Ethanol (100%) | | Xylene | | Xylene |

## 9. Mounting

9.1 Drop 2–3 drops of  permount medium onto the slide

9.2 Put a cover glass onto the slide.

# APPENDIX II

# ELISA

### Reagent preparation

**Bring all reagents to room temperature before use.**

**Mouse VEGF Kit Control** - Reconstitute the Kit Control with 1.0 ml deionized or distilledwater. Assay the Control undiluted.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 ml Wash Buffer Concentrate into deionized or distilled water to prepare 625 m of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 μl of the resultant mixture is required per well.

**Mouse VEGF Standard** - Reconstitute the mouse VEGF Standard with 5.0 ml of Calibrator Diluent RD5T. Do not substitute other diluents. This reconstitution produces a stock solution of 500 pg/ml. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

The condition of serum VEGF analysis , a standard curve was generated by 6 serial concentration of 7.8, 15.6, 31.2 , 62.5 , 125, 250, 500  pg/ml from Lyophilized Standard. The following protocal recommended by manufacture was used to make up the kit standard in the serial dilutions.

1. Label polypropylene tubes #2-6

2. Pipette 200 µl of Calibrator Diluent RD5T into each tube.

3. Use the stock solution to produce a 2- fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted mouse VEGF Standard serves as the high standard (500 pg/ml). Calibrator Diluent RD 5T serves as the the zero standard (0 pg/ml).
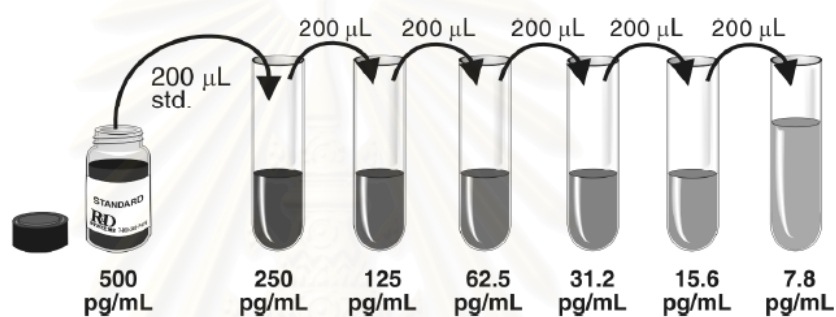


| Initial Volume (µl) | 200 | 200 | 200 | 200 | 200 | 200 | 400 |
|---|---|---|---|---|---|---|---|
| Concentration (pg/ml) | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 |
| Standard Number | #1 | #2 | #3 | #4 | #5 | #6 | #7 |

**Figure A** Serial dilution of VEGF standa

# LISTS OF PUBLICATIONS

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Duansak N, Bhattarakosol P, Wirachwong P and Patumraj S. Hypoxia-inducible factor-1α (HIF-1α) expression and CD in hepatocellular carcinoma cell (HepG2) implanted in nude mice. 24ᵗʰ European Conference on Microcirculation, Amsterdam, Netherland 2006.

Naphatsanan Duansak, Pornprom Yoysungnoen, Jutamard Somchaichana, Parvapan Bhattarakosol , Ponthip Wirachwong, and Suthiluk Patumraj. Correlation between hypoxia-inducible factor and vascular endothelial growth factor expression under tumor neovascularization in hepatocellular carcinoma cell-implanted nude mice. *Asian Biomedicine*,2007 (in press).

# BIOGRAPHY

| | |
|---|---|
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