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Original article

Correlation between hypoxia-inducible factor and vascular endothelial growth factor expression under tumor neovascularization in hepatocellular carcinoma cell-implanted nude mice

Naphatsanan Duansak[a], Pornprom Yoysungnoen[b], Jutamard Somchaichana[a], Parvapan Bhattarakosol[c], Ponthip Wirachwong[d], Suthiluk Patumraj[a]

[a]Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330; [b]Department of Physiology, Faculty of Medical Science, Naresuan University, Pitsanuloke 65000; [c]The Government Pharmaceutical Organization, Rajtevi, Bangkok 10330; [d]Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Background: In a tumor, hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial cell growth factor (VEGF) are induced to promote angiogenesis for the growth and metastasis of cells. There have been very few studies to examine *in vivo* relation between HIF-1α and VEGF during tumor progression.

Objective: To study the relationship between HIF-1α and VEGF expressions under neovascularization induced by hepatocellular carcinoma cells (HepG2) implanted in nude mice.

Methods: Male BALB/c-nude mice 8-10 weeks of age were used. A chamber was prepared on the dorsal skin in which HepG2 was transplanted to induce a tumor. On the day of the experiment, and on days 2, 7, and 14, microcirculation within the chamber was observed using fluorescence videomicroscopy. Based on the recorded video images, capillary vascularity (CV) was measured to examine tumor neovascularization. VEGF expression was measured in blood (serum) withdrawn, using enzyme immunoassay, while HIF-1α expression was measured on samples isolated from tumor tissue, using immunohistochemistry.

Results: The measured CV significantly increased on day 7 and 14 compared to the aged-matched controls ($p<0.05$). HIF-1α markedly expressed on day 2, and the expression declined on day 7 and 14 post-inoculation. VEGF expression in serum increased more on day 7 and 14 than on day 2. HIF-1α expression decreased with the increase in VEGF expression from 2 to 14 days after HepG2 implantation, showing a reverse correlation.

Conclusion: HIF-1α expression existed prior to both VEGF expression and neovascularization in the tumor. An inhibitor of HIF-1α might be a therapeutic agent for reducing neovascularization via adaptation to hypoxia in tumors.

Keywords: Hypoxia inducible factor 1-α (HIF-1α), hepatocellular carcinoma cell (HepG2), capillary vascularity, tumor angiogenesis, nude mice, vascular endothelial growth factor (VEGF).

Neovascularization is an essential process for tumor growth and metastasis. In the absence of neovascularization, tumors cannot grow larger than 1-2 mm^3 [1-3]. If capillary blood flow is too poor to supply oxygen to tumor cells, the cells should suffer from hypoxia. Such hypoxic conditions may induce the expression of several angiogenic factors such as vascular endothelial growth factor (VEGF) [4]. The VEGF expression induced in tumor cells is also an important factor for the cell growth and metastasis. The prognostic significance of VEGF expression has been reported in various organs such as gastric, colon, breast, bladder and oesophageal carcinomas and other malignancies [5-7].

Under tumor hypoxic conditions, a transcription may be activated by a hypoxia-inducible factor HIF-1, which plays a critical role in oxygen homeostasis [8]. The HIF-1 is a heterodimer that consists of an α and β subunit. Both units belong to the basic-helixloop-helix-Per AhR/ARNT-Sim family of transcription factors [9-12].

Correspondence to: Dr. S. Patumraj, Department of Physiology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; E-mail: suthilukp@yahoo.com

Under non-hypoxic conditions, HIF-1α is subject to rapid ubiquitination and proteasomal degradation [9]. The level of HIF-1α proteins is inversely related to the oxygen tension in both cultured cells [13, 14] and *in vivo* [15]. Under hypoxic conditions, on the other hand, HIF-1α is induced so that tumor cells may adapt to hypoxia. According to Semenza *et al.* [16] and Guidi *et al.* [17], hypoxic oxygen tension *in vivo* might be associated with clinical outcomes including tumor progression.

Both HIF-1α and VEGF expressions may be induced by hypoxia oxygen tension during tumor progression. Under such circumstances, the expression of VEGF should be intimately connected with the expression of HIF-1α. There have been very few studies to examine *in vivo* relations between HIF-1α and VEGF expression in different periods of tumor progression.

In previous studies [18, 19], we established a HepG2-implanted mouse model for the study of angiogenesis during tumor progression. We showed that the tumor capillaries increased significantly in density in different periods (days 2, 7 and 14) after implantation of HepG2 in nude mice. The purpose of this study is to investigate how HIF-1 expression is correlated with VEGF expression during tumor progression using our HepG2-implanted nude mice model.

Methods

HepG2-implanted mouse model

The animal experiment was conducted according to the guideline for experimental animals by (The National Research Council of Thailand 1999).

BALB/c-nude mice (20-25 g; n=30) were used for this study. The mice were divided into two groups: HepG2-implanted mice (HepG2) (n=15) and control mice without HepG2 (Con) (n=15).

The HepG2 mouse model was prepared according to the procedure reported previously [18, 20]. Briefly, a dorsal skin-fold chamber (7 mm diameter) was implanted onto the upper layer of skin in a mouse after anesthesia with sodium pentobarbital (50 mg/100 g BW, *i.p.*). For the HepG2 group, 30 μl HepG2 cells (American Type Culture Collection; 2 X 10^6 cells, viability 95-97 %) were inoculated into the middle area of the chamber. Then, the mice were housed, one animal per cage, with free access to sterile water and standard laboratory chow.

Intravital fluorescence videomicroscopy

On days 2, 7, and 14 after implantation of HepG2, the mice were anesthesized with sodium pentobarbitol (50 mg/kg BW, *i.p*). A catheter was inserted into a jugular vein for injection of fluorescence tracers (0.1 ml of 0.5 % fluorescein isothiocyanate (FITC)-labeled dextran (MW=200,000, Sigma Chemical, USA). The tumor microvasculature was observed under an intravital fluorescence microscope (Nikon, Japan) equipped with a videocamera (Sony SIT68, Japan) and recorded using a video-recorder (Sony SVT-124P, Japan) [21, 22]. The 10× objective lens was used to observe microvessels within the tumor-bearing chamber.

The recorded video image was used to analyze microvascular parameters such as capillary vascularity and diameter.

Evaluation of capillary density

Based on the video image of each experiment, we measured capillary vascularity (CV) that corresponds to capillary density (CD) in [17, 18], as follows:

$$CV = \frac{\text{(Number of pixels within the capillaries)}}{\text{(Total number of pixels within the selected window area)}} \quad 100\ (\%). \qquad (1)$$

In each mouse, we observed four positions on the surface of tumors by moving the microscopic stage. Each video frame (100x100 pixels) was selected to cover no large vessel. After determining both minimum and maximum intensities of pixels located within the capillary network, we counted the total number of pixels over all capillaries in each window, using digital image processing software (Global Lab II). Expressing the CV as percents of capillary area to total area, we obtained the mean CV averaged over four video frames in one mouse.

Immunohistochemistry for HIF-1α

After the *in vivo* experiment, an amount of tumor tissue (approximately 7 mm in diameter) inside the chamber was isolated, fixed with formalin solution and embedded in paraffin. On different days, 2 μm thick sections, in parallel to the skin surface, were prepared of the paraffin-embedded tumor tissue.

A mouse monoclonal antibody [H1alpha67-sub] (ab463) (1:800; Abcam, UK) was used to detect HIF-1α. Sections were deparaffinized and pressure-cooked with target retrieval solution (DAKO) for 10 min. The primary antibody reaction was carried out at 4 °C overnight. Then, the section was washed in phosphate buffer solution (PBS), and then blocked with PBS containing 5 % non-fat dried milk for 30 min. 3,3,-diaminobenzidine (DAB)/hydrogen peroxide was used to detect HIF-1α antigen-antibody binding. The slides were counterstained with Mayer's haematoxylin, dehydrated, treated with xylene and mounted onto cover slides for microscopy [22].

After the immunohistochemical examination, all slides were observed under a fluorescence microscope and recorded on a videotape. On each videoimage, the expression of HIF-1α was counted using Global Lab II software (options/blob setting tool) and expressed as number of cells per area.

Enzyme immunoassay (EIA) for serum VEGF

The blood sample was obtained by puncturing the cardiac ventricle. The blood was collected in a microcentrifuge tube and allowed to clot for half an hour. Then, it was centrifuged at 5,000 rpm for 10 minutes. The separated serum was stored at -20 °C, until VEGF was measured using the Mouse VEGF kit (Chemicon International, Inc., USA).

Statistical analysis

Results were expressed as mean±SEM. One-way ANOVA was used to evaluate the difference of means. Student's *t*-test was used for inter-group comparisons of means. The statistical differences were considered at the probability level (p-value) lower than 0.05.

Results

Figure 1 shows examples of fluorescent video images recorded on days 2, 7 and 14 after HepG2 implantation. On day 2 after tumor cell inoculation, capillary structural changes, such as dilation and tortuosity, appear in association with extravasations of the fluorescence tracer from the host's existing microvessels (**Fig. 1B**). On day 7, neocapillaries are observed in the tumor, together with endothelial cell sprouting in the host vessel (see inside the circle in **Fig.1C)**. These features present the early stage of neovascularization or angiogenesis onset in tumor.

Figure 2 shows capillary vascularity (CV) on the surface of tumor in different periods after HepG2 implantation. In control groups, no significant difference appeared in the CV. The mean density made no change with days, falling around 38 %. In the HepG2 group, the mean density increased significantly from 39.6 % (on 2 day) up to 71.3 % (on day 14). The CV levels on day 2 is not significantly different, compared with the control levels, but the CV levels on both day 7 and 14 are significantly higher than their control levels ($p<0.05$).

Figure 3 (A, B) shows HIF-1α expression examined in the samples of tumor tissue. Immunohistochemical examination of HIF-1α in different periods (2, 7, and 14 days) after tumor cell inoculation is shown in **A.** HIF-1α positive cells were visualized with DAB, while the counter (background) was stained with haematoxylin. The HIF-1α staining was not observed in normal control tissue (data not shown). Within tumors, clusters of HIF-1α positive cells were most dense at the invading edge of the tumor margins. Note that the HIF-1α expression appears in the nucleus as well as cytoplasm of the cell. The number of expressed cells per area measured is shown in B. Apparently, HIF-1α was expressed markedly on days 2 and 7 after tumor cell inoculation, but its expression decreased on day 14. This indicates over-expression of HIF-1α at the early stage of neovascularization during tumor progression.

Figure 4 shows serum VEGF level measured on different days (days 2, 7 and 14) after HepG2 implantation in the control and HepG2 groups. In control groups, no significant difference appeared in the VEGF level. The mean VEGF level did no change with days, being about 67 pg/ml. In the HepG2 group, the mean level increased significantly from 67.1 pg/ml (on 2 day) up to 152.0 pg/ml (on day 14). The VEGF levels on day 2 is not significantly different compared with the control levels, but the VEGF levels on both 7 and 14 days are significantly higher than the control levels ($p<0.05$).

All data of HIF-1α and VEGF measured in HepG2 groups are plotted in **Fig. 5**. Apparently, the HIF-1α expression decreased with increase in the VEGF expression from day 2 to 14 after HepG2 implantation. Interestingly, the VEGF expression reversely correlated with HIF-1α expression under tumor progression as:

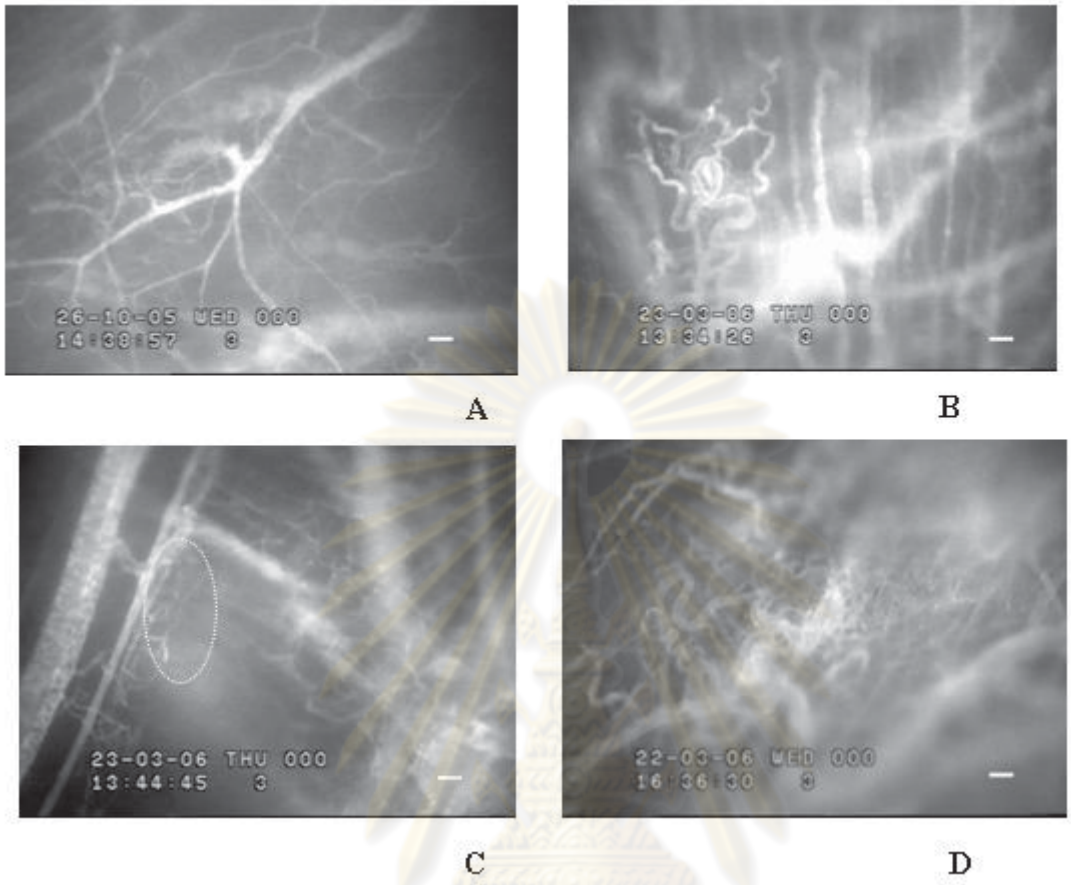

Fig. 1 Fluorescent videoimages of capillary networks recorded in the tumor inside the chamber on different periods from after HepG2 implantation (**A**: control, **B**: on day 2, **C**: on day 7 and **D**: on day 14). Bar indicates 50 μm. Note that extravasation of the fluorescence tracer in **B** and endothelial cell sprouting in **C** (inside the circle).

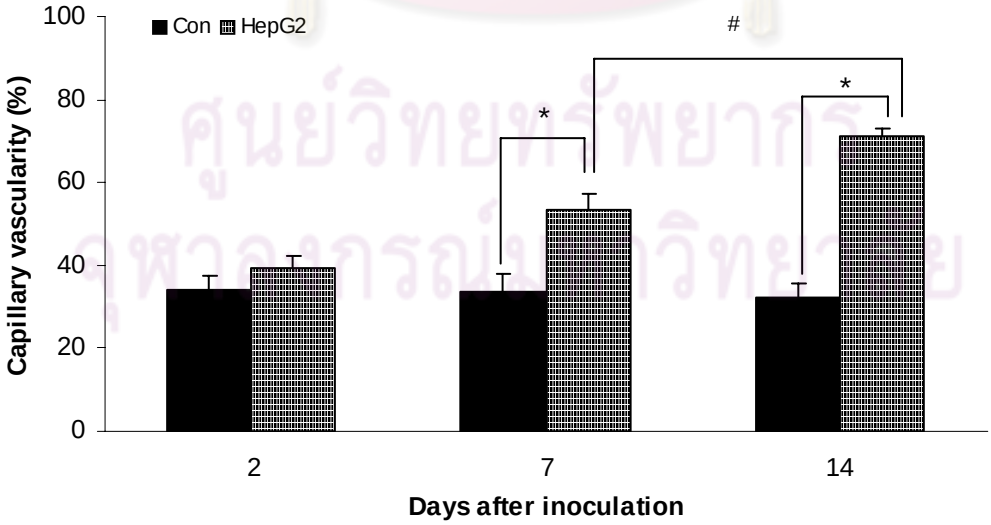

Fig. 2 Tumor capillary vascularity (CV) measured in different periods (days 2, 7 and 14) after HepG2 implantation in the control and HepG2 groups. Values are expressed using Mean ± SEM. *Significantly different compared to corresponding control levels ($p < 0.05$). $^{\#}p<0.05$ *vs* HepG2 on day 7.

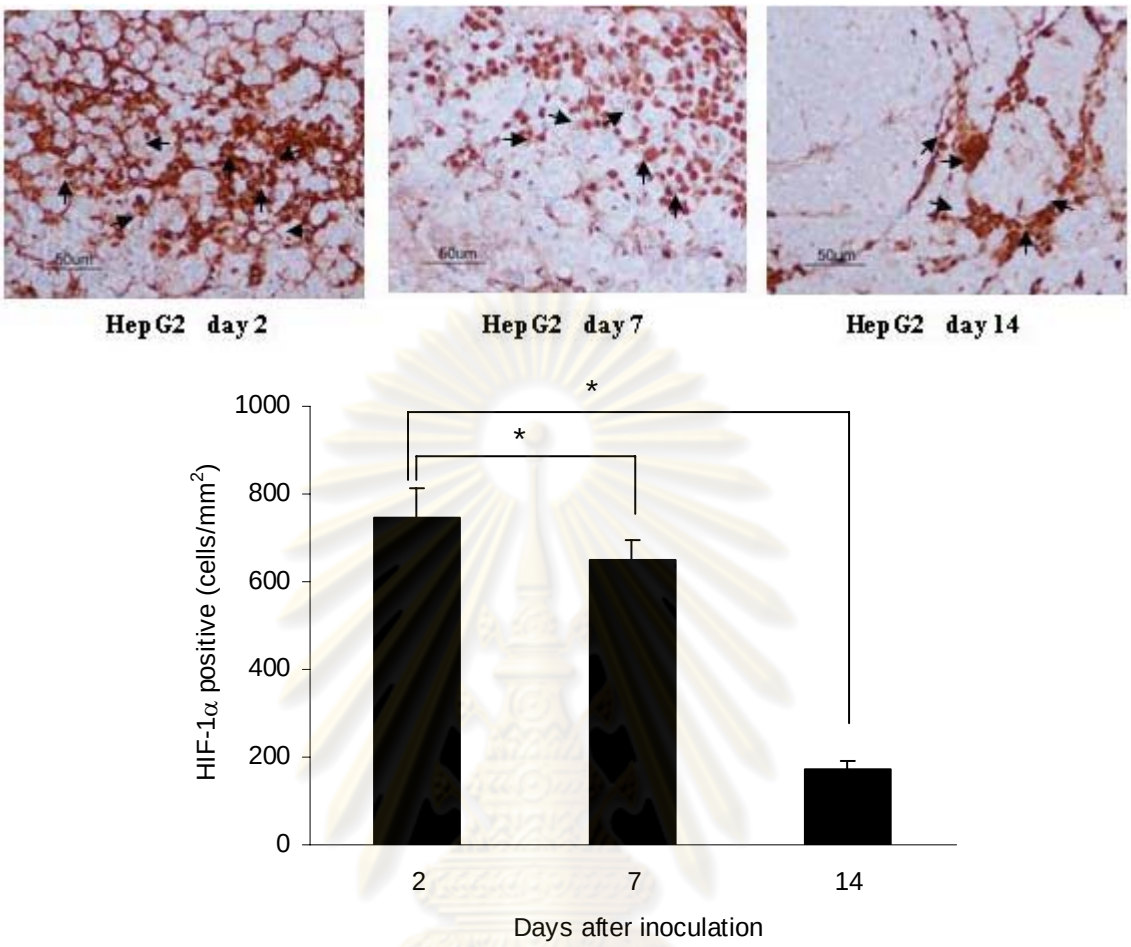

Fig. 3 Immunohistochemical analysis of HIF-1α in HepG2-implanted nude mice on days 2, 7, and 14. **A:** Expression of HIF-1α in the nucleus and cytoplasm of cells. Arrow indicates HIF- 1 α positive cells (visualized with DAB). Brown color corresponds to the counter (background) (X 400; bar: 500 μm). **B:** Number of expressed cells per area in different periods in HepG2 groups. Values are expressed as means ± SEM. *Significant difference between the groups ($p < 0.05$).

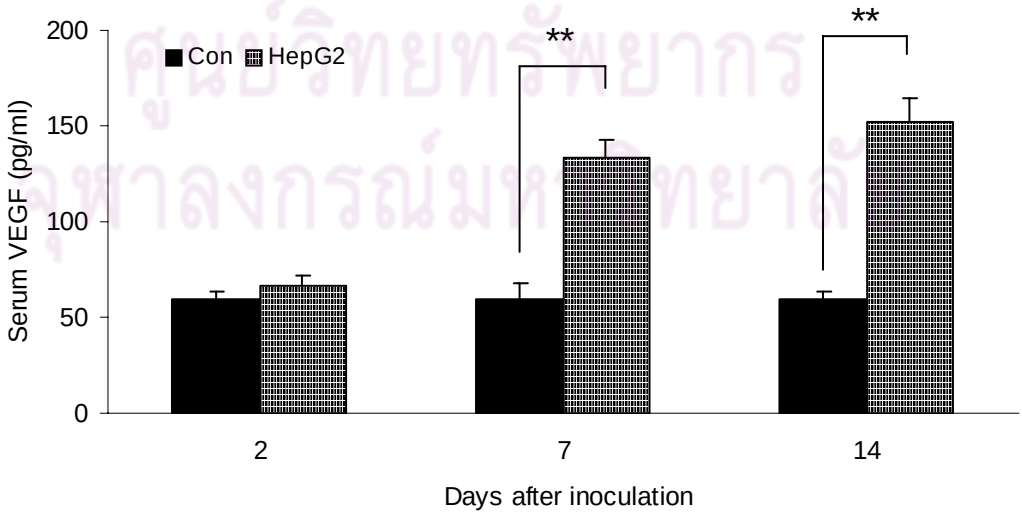

Fig. 4 Serum VEGF (pg/ml) measured in blood withdrawn on different days (days 2, 7 and 14) after HepG2 implantation in the control and HepG2 groups. Values are expressed as means ± SEM. *Significantly different between the groups ($p < 0.001$).

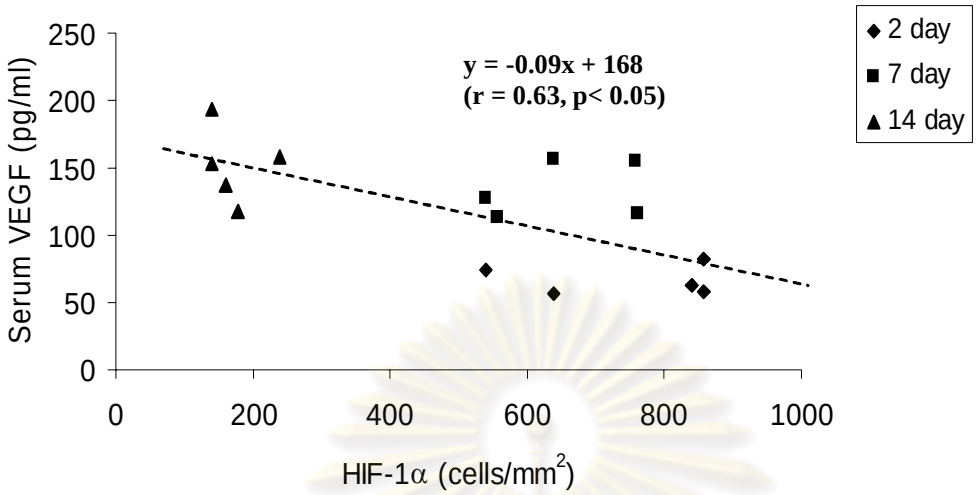

Fig. 5 Relationship between serum VEGF (pg/ml) and HIF-1α (cell/mm^2) in the HepG2 group from day 2 to 14 after HepG2 implantation.

Discussion

We directly observed tumor microcirculation in mouse dorsal skin in different periods from 2 to14 days after tumor cell inoculation, and measured capillary vascularity (CV) in the tumor using video recording. On the other hand, we measured VEGF expression using blood withdrawn from the cardiac ventricle, and also measured HIF-1α expression using tissue samples isolated from the tumor inside the chamber. We note that the measured VEGF expression did not reveal the time course of the level in the tumor. Recently, Tomita *et al.* [24] showed that in tumors induced by colon cancer in syngenetic mice, the VEFG level measured in tumor tissue was much higher (approximately ten times) than that measured in the blood. Therefore, the present VEGF data must be used with caution when we examine the tumor progression.

The present immunohistochemical analysis of HIF-1α expression showed that HIF-1α expression appeared on day 2 post-HepG2 inoculation. This expression was 4-5 days prior to the appearance of neocapillary sprouting in tumor tissue (**Fig. 1C**). Therefore, a hypoxic condition should already occur within 2 days after tumor cell inoculation. In general, HIF-1α stabilization up-regulates the expression of angiogenic pathway so that oxygen homeostasis may be restored. Thus, HIF-1α protein may be an important factor for the survival and growth of cancer. In fact, there have been a number of clinical reports to show poor clinical outcomes associated with hypoxic oxygen tensions in tumors [15, 16, 25-27].

In our experiment, HIF-1α expression declined (approximately 85 %) within 14 days, while the tumor capillary network increased markedly (approximately 40 %). Moreover, VEGF increased significantly during those 7 to 14 days (approximately 50 % and 175 %, respectively). Therefore, the present study provides an *in vivo* evidence to indicate that HIF-1α is a hypoxia-activated transcription factor that is responsible for regulation of VEGF synthesis.

Tumor angiogenesis requires the expression of VEGF. According to Huang *et al.* [28], a major pathway leading to VEGF expression under hypoxic condition is the binding of HIF-1α to hypoxia-response elements in the VEGF promoter. The present result (**Fig. 5**) has presented an inverse relationship between HIF-1α and VEGF expression during tumor progression. Recently, Tanaka *et al.* [29] showed that HIF-1α was activated in preneoplastic hepatocytes during the early stages of hepatocarcinogenesis. Their results agreed in the time-period of HIF-1α expression with our experimental results. Lindgren *et al.* [30] studied the expression of HIF-1α using renal cell carcinoma (RCC). It was indicated that HIF-1α expression was involved in tumorogenesis and progression of RCC, but no association appeared between protein VEGF expression in serum and the HIF-1α expression. They did not show any correlation between VEGF expression and tumor stage and prognosis. In our experiment, HIF-1α expression declined when a number of neocapillaries appeared in the tumor and the tumor microenvironment became normoxia.

HIF-1α expression might be greatly dependent on tumor microenvironment. It is likely that different microenvironments in tumors may cause different results of HIF-1α expression.

In conclusion, within two days after tumor cell inoculation, HIF-1α expression appeared prior to VEGF expression as well as neovascularization in tumor. An inhibitor of HIF-1α might be a better therapeutic agent that halts tumor growth by reducing neovascularization and adaptation-to-hypoxia in tumors. This HIF-1α inhibitor would prevent the serious consequence of VEGF expression, tumor neovascularization, and tumor metastasis.

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