# Circadian rhythm of VEGF expression in the liver of hepatectomized tumor bearing mice

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In this study we analyzed VEGF-C expression in regenerating liver (after partial hepactectomy) of ES2 hepatocellular carcinoma bearing mice, throughout one complete circadian cycle. The animals were sacrificed every 4 h throughout one complete circadian cycle from 26 to 50 h post-hepatectomy. Tumor samples were processed for immunohistochemistry. The expression of VEGF was assessed according to the percentage of immunoreactive cells in a total of 1000 cells (quantitative analysis). The results show that controls and tumor bearing mice have statistical differences at all analysed time points, but the maximun value of VEGF expression of treated animals is significantly higher ($p < 0.01$) than the control group. We can conclude that when a partial hepatectomy is made with the purpose of eradicating a hepatic tumor, the presence of possible metastasis could release factors related with cellular proliferation that could increase the possibilities of tumoral recidives.

**Keywords:** liver; VEGF; hepatocellular carcinoma; circadian rhythms; mouse

## Introduction

VEGF is the most potent angiogenic factor known (Mattern et al. 1996). It is a glycoprotein with angiogenic, mitogenic and vascular permeability enhancing activity in endothelial cells (Mineo et al. 2004). The VEGF family includes VEGF-A, B, C, D, E, F as well as PLGF (placenta growth factor) (Shibuya 2008). In humans, VEGF-C and D activates VEGFR-2 and VEGFR-3 receptors, both of which are essential for vascular development (Cao et al. 1998).

On the other hand, tumor growth and evolution is a complex phenomenon controlled by an intricate pattern of competing processes (Perez de Castro et al. 2007). Previous studies have demonstrated that many cellular neoplastic populations have circadian variations in their mitotic activity (MA), such as primary human and murine tumors (Colombo et al. 2000), including mammary carcinomas (Echave Llanos and Badrán 1963), hepatomas and hepatocellular carcinomas (Barbeito et al. 1993, 1995). Circadian variations in the synthesis of DNA (DNAs) of some hepatomas and hepatocellular carcinomas have also been found (Nash and Echave Llanos 1971; Garcia et al. 2008). In some cases, the observed rhythm was similar to that of the original

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cellular population (Barbeito et al. 1995), but some undifferentiated hepatocellular carcinomas did not have any mitotic circadian rhythm (Moreno et al. 1985).

Moreover, when a partial hepatectomy is performed on rodents, a compensatory hyperplasia of the remnant lobes is observed (Fausto et al. 2006). Particularly in mice, and under controlled conditions of light–dark standardization, this process involves a circadian rhythm in the MA and DNAs, on the hepatocyte cell population. The partial hepatectomy produces significant increases in plasma levels of a number of potential signals: Hepatocyte Growth Factor (HGF), Insulin-Like Growth Factor (IGF), Transforming Growth Factor $\alpha$ and $\beta$ (TGF$\alpha$ and $\beta$), Fibroblastic Growth Factor (FGF), and others. Some of these growth factors can ''cross-talk'' between the growing tumor and the regenerating liver (Michelson and Leith 1995). There are numerous previous studies that investigated the effect of hepatectomy on the proliferation and growth of the different tumors (Lee 1971; Ono et al. 1986; Hanigan et al. 1990; Sun et al. 1996; Altum and Olzapan 2004). This fact is very important not only in the cellular biology area, but also in clinical oncology, because hepatectomy is the primary treatment for hepatic tumors (Wood and Hrushesky 1996).

These previous studies suggest that tumor-based signals, acting upon the tumor bed stroma, could mimic normal signaling, and, among others things, produce a reciprocal cue beneficial to the proliferating tumor cells (West and van de Rjin 2007). The interaction between tumor cells and their supportive stroma play a crucial role in tumor development and progression (Zhang et al. 2006), in which the angiogenesis is the essential process since solid tumors cannot growth beyond 1–2 mm in diameter without neovascularization (Folkman et al. 1981).

We analyzed VEGF-C expression in regenerating liver (after partial hepactectomy) of ES2 hepatocellular carcinoma bearing mice throughout one complete circadian cycle.

## Materials and methods

### Animals

For these experiments, we used adult (90 days old) male C3H/S-strain mice. Conditions concerning animal management fully respected the policy and mandates of the Guide for the Care and Use of Laboratory Animal Research of the National Research Council. They were subjected to the following standardization conditions: water and food were available *ad libitum*, ambient temperature was maintained at $22 \pm 2°C$, alternating light and dark periods were restricted to 12 h each with illumination by fluorescent lamps beginning at 06:00 h.

### Determination of VEGF expression

Deparaffinized and rehydrated sections were microwaved for 10 minutes in buffer citrate at pH 6. Endogenous peroxidase was blocked for 20 minutes, the primary antibody against VEGF-C (mouse monoclonal antibody; Santa Cruz Biotechnology, California, USA 1:100 dilution). Bound primary antibody was detected by Envision Systen (Dako) for 30 minutes and the reaction was developed using diaminobenzidine, and counter-staining with Mayer hematoxylin. The positive control was a section of liver that had previously been shown to have a high VEGF content, by immunohistochemistry. VEGF staining was seen in the hepatocyte cell cytoplasm.

The expression of VEGF was assessed acording to the percentage of immunoreactive cells in a total of 1000 cells (quantitative analysis). There was >95% agreement between the two observers for the VEGF evaluation. A final score was determined by consensus after re-examination.

### Experimental groups

The animals were divided in two experimental groups: Group I (control, hepatectomized animals): these mice were all subjected to a partial (ca. 70%) resection of the liver at 10:00 h, with the constituent lots being subsequently sacrificed every 4 h beginning at 12:00 h on the next day of surgery (26 h post-hepatectomy) and continuing until 12:00 h on the second day after surgery (50 h post-hepatectomy). Group II (hepatectomized and tumor bearing animals): after an appropriate period of synchronization (15 days), about 70 mg of the C3H/S-histocompatible ES2a hepatoma was grafted into the subcutaneous tissue of each animal's flank. This rapidly growing undifferentiated hepatocellular carcinoma is maintained by subcutaneous serial transplant in male mice. The graft-bearing animals, subsequently divided into lots of 5–6 mice each, were then housed for further two weeks under standardization conditions before the lots were separated. All animals were subjected to a partial (ca. 70%) resection of the liver and sacrificed at every 4 h, under the same conditions and hours as the first group.

### Statistical analysis

The statistical analysis of VEGF expression was performed using ANOVA and Tukey's post-comparison test. The marked hepatocytes were recorded by counting 70 areas and the total nuclei every 10 areas. The results were expressed as a percentage of marked cells.

### Results

As seen in Table 1 and Figure 1, both groups have statistical differences in all analysed time points, but the tumor bearing animals present the maximum value of VEGF expression at 16/30 h (hour of day/hours post-hepatectomy) and the minimum at 20/34 h, meanwhile the other group shows its maximum value at 08/46 h and a minimum at 00/38 h.

The maximum value of VEGF expression of tumor bearing animals is significantly higher ($p < 0.01$) than that of control animals.

### Discussion

Liver regeneration is not a simple response to one growth factor, but rather a delicate and complex interplay of many cellular events. The ES2 hepatocellular carcinoma secretes factors that stimulate the expression of VEGF by hepatocytes in regenerating liver. Relevant factors might include FGF-7, Keratinocyte Growth Factor (KGF), Epidermal Growth Factor (EGF) and TGF-α, which exert mitogenic actions on specific cellular populations. In addition, partial hepatectomy in rodents changes the concentrations of substances such as Tumor Necrosis Factor-α (TNF-α), HGF, TGF-α and TGF-β, which are involved in cellular proliferation and cause

Table 1.    Daily mean values of VEGF expression of ES2a hepatocellular carcinoma of the two experimental groups.

| Lote | Time point (h) | Control (X ± SE) | n | Tumour bearing (X ± SE) | n | p |
|---|---|---|---|---|---|---|
| 1 | 12/26 | 3.4 ± 0.6 | (5) | 4.5 ± 0.6 | (5) | ns |
| 2 | 16/30 | 3.7 ± 0.6 | (5) | 14.3 ± 2.1 | (5) | 0.001 |
| 3 | 20/34 | 2.9 ± 0.5 | (5) | 5.4 ± 2.1 | 5) | ns |
| 4 | 00/38 | 3.2 ± 0.9 | (5) | 1.7 ± 0.3 | (5) | ns |
| 5 | 04/42 | 4 ± 0.4 | (5) | 4.4 ± 1.0 | (6) | ns |
| 6 | 08/46 | 7.1 ± 1.0 | (6) | 3.7 ± 0.7 | (5) | 0.05 |
| 7 | 12/50 | 3.9 ± 1.3 | (5) | 3.0 ± 0.8 | (5) | ns |

Lotes 2 y 4: $p < 0.01$.
Lotes 3 y 6: $p < 0.05$.
h: hour of day/hours post-hepatectomy.
X: means of the samples.
ES: standard error.
n: number of mice per group.
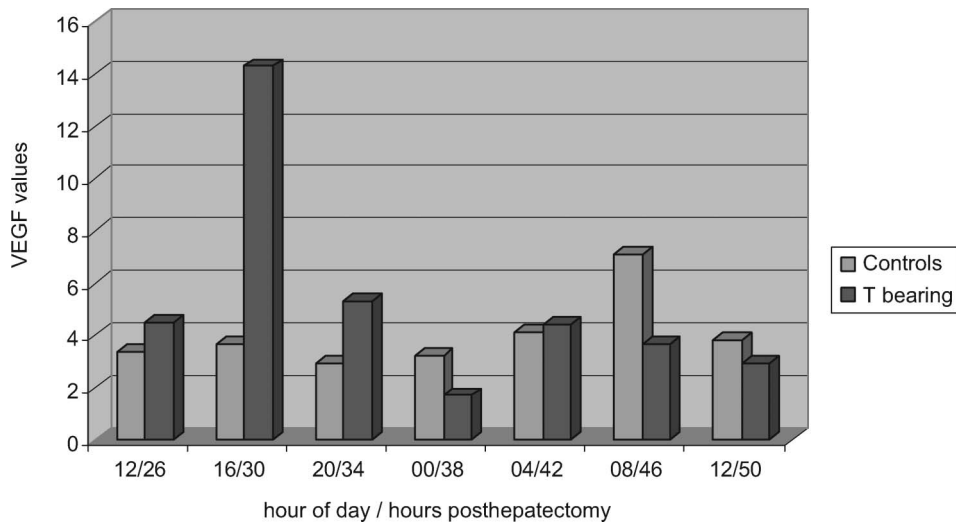p: probability.



Figure 1.    VEGF expression in hepatocytes throughout one circadian rhythm after hepatectomy. Controls: controls mice; T bearing: tumor bearing mice.

compensatory hyperplasia of the remanent lobes of the liver (Dielh and Rai 1996; Kimura et al. 1997; Michalopoulos and Defrances 1997). Some of these factors produced during the hepatic compensatory hyperplasia can alter tumoral cell proliferation. Moreover, the concentration of a factor can increase in the tumors; for example TGF-α is synthesized by numerous hepatocellular carcinomas in the rat (Perez Tomas et al., 1992).

On the other hand, angiogenesis is a complex process that involves endothelial cell migration, capillary bredding, neovascular remodeling, in addition to endothelial cell proliferation (Karamysheva 2008). The growth of solid tumors like those analyzed in this study needs an adequate vascular network for the supply of oxygen and nutrients, and to remove waste products, although it has been established that

tumor cell proliferation decreases with increasing distances from the blood vessels (Vartanian et al. 1994; Vermeulen et al. 1996).

As we can see in our study, both groups have circadian variations of VEGF expression in hepatocytes. But the presence of transplanted tumor causes modifications in the intensity and the temporal expression of VEGF in this cellular population. In transplanted animals the VEGF peak appears before, and with higher values, than the control group. Thus we can affirm that ES2 hepatocellular carcinoma increases directly or indirectly with VEGF expression of hepatocytes.

All the foregoing considerations confirm that beyond an experimental design which takes into account factors such as the moment of grafting, the location of the tumor implant and the origin of the neoplasm, it is not possible to make a general statement regarding the effects of host hepatectomy on tumor growth, because these influences will vary according to the nature of each tumor. The production of growth factors on the part of the host and the neoplastic cells themselves will vary among different tumors (Aaronson 1991; Yeh et al. 2006).

We can conclude that when a partial hepatectomy is made with the purpose of eradicating an hepatic tumor, the presence of possible metastasis could release factors related to cellular proliferation and the possibility of tumoral recidives could increase.

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