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Short Communication

Changes in VEGF expression and DNA synthesis in hepatocytes from hepatectomized and tumour-bearing mice

Marcela N. García[1]*, Laura B. Andrini*, Ana María Inda*,†, Jorge R. Ronderos‡, Julio C. Hijano§ and Ana Lía Errecalde*

* Cátedra de Citología, Histología y Embriología ''A'', Facultad de Ciencias Médicas, UNLP, Argentina
† CIC, Provincia de Buenos Aires, Argentina
‡ Cátedra de Histología Animal, Facultad de Ciencias Naturales y Museo, UNLP, Argentina
§ Cátedra de Anatomía ''A'' y Cirugía ''E'', Facultad de Ciencias Médicas, UNLP, Argentina

Abstract

Transplanted tumours could modify the intensity and temporal distribution of the cellular proliferation in normal cell populations, and partial hepatectomy alters the serum concentrations of substances involved in cellular proliferation, leading to the compensatory liver hyperplasia. The following experiments were designed in order to study the SI (S-phase index) and VEGF (vascular endothelial growth factor) expression in regenerating liver (after partial hepatectomy) of adult male mice bearing a hepatocellular carcinoma, throughout one complete circadian cycle. We used adult male C3H/S-strain mice. After an appropriate period of synchronization, the C3H/S-histocompatible ES2a hepatocellular carcinoma was grafted into the subcutaneous tissue of each animal's flank. To determine the index of SI and VEGF expression of hepatocytes, we used immunohistochemistry. The animals were divided into two experimental groups: Group I, control, hepatectomized animals; Group II, hepatectomized tumour-bearing animals. The statistical analysis of SI and VEGF expression was performed using Anova and Tukey as a postcomparison test. The results show that in the second group, the curve of SI changes the time points for maximum and minimum activity, and the peak of VEGF expression appears before the first group. In conclusion, in the hepatectomized mice, the increases of hepatic proliferation, measured by the SI index, may produce a rise in VEGF expression with the object of generating a vascular network for hepatic regeneration. Lastly, as we have mentioned, in hepatectomized and tumour-bearing mice, the peak of VEGF expression appears before the one of DNA synthesis.

Keywords: DNA; liver; mouse; tumour; vascular endothelial growth factor (VEGF)

1. Introduction

Cell cycle length may vary in response to specific conditions associated with different stages of the organism's life span. It is regulated by growth factors and other signalling molecules that stimulate or inhibit cellular proliferation (García et al., 2001). There are many techniques to analyse the different phases of the cell cycle. Estimation of SI (S-phase index) can be obtained by detection of labelled nuclei by immunohistochemical methods like Brdu (bromodeoxyuridine), a thymidine analogue used as an indicator of cellular proliferation (Thompson et al., 1999).

On the other hand, partial hepatectomy alters the serum concentrations of substances involved in cellular proliferation, leading to the compensatory liver hyperplasia (Frago et al., 2000; Michalopoulos and Defrances, 2005). In liver regeneration, the development of new capillary blood vessels from existent microvessels by sprouting is a fundamental requirement for cellular proliferation (Taniguchi et al., 2001; Furnus et al., 2003). This complicated process defined as angiogenesis is promoted by VEGF (vascular endothelial growth factor), the most potent and specific growth factor for both angiogenesis and vasculogenesis (Shibuya, 1995; Furnus et al., 2003), the mRNA of which is expressed, after PH (partial hepatectomy), in both hepatocytes and non-parenchymal cells, suggesting that VEGF plays a significant role in liver regeneration (Mochida et al., 1996; Furnus et al., 2003). It has been demonstrated that transplanted tumours could modify the intensity and temporal distribution of the cellular proliferation in normal cell populations (Barbeito et al., 2002; Altun and Ölzapan, 2004; García et al., 2006).

Furthermore, tumour growth and evolution is a complex phenomenon controlled by an intrincate pattern of competing processes (Perez de Castro et al., 2007). Previous studies have demonstrated that many cellular neoplastic populations, like some hepatomas and hepatocellular carcinomas, show circadian variations in the synthesis of DNA (Nash and Echave Llanos, 1971; García et al., 2008). In some cases, the observed rhythm was similar to that of the original cell population (Barbeito et al., 1995), but some undifferentiated hepatocellular carcinomas did not show any mitotic circadian rhythm (Moreno et al., 1985). Some growth factors can 'cross-talk' between the growing tumour and the regenerating liver (Michelson and Leith, 1995). This fact is very important not only for cellular biology knowledge, but also in clinical oncology, considering that hepatectomy is the first treatment for hepatic tumours (Wood and Hrushesky, 1996).

The following experiments were designed in order to study the SI and VEGF expression in regenerating liver (after partial

[1] To whom correspondence should be addressed (email mngarcia@med.unlp.edu.ar).

Abbreviations: HGF, hepatocyte growth factor; SI, S-phase index; TBS, Tris-buffered saline; TGF α and β, transforming growth factor α and β; VEGF, vascular endothelial growth factor.

hepatectomy) of adult male mice bearing a hepatocellular carcinoma, throughout one complete circadian cycle.

2. Materials and methods

2.1. 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 available *ad libitum*, ambient temperature maintained at 22 ± 2°C, alternating light and dark periods restricted to 12 h each with illumination by fluorescent lamps beginning at 06:00 (Echave Llanos et al., 1963).

2.2. Tumour-bearing animals

After an appropriate period of synchronization (15 days), about 70 mg of the C3H/S-histocompatible ES2a hepatocellular carcinoma was grafted into the subcutaneous tissue of each animal's flank. The graft-bearing animals, subsequently divided into lots of five to eight mice each, were then housed for a further 2 weeks under standardization conditions before the lots were separated into the following experimental-protocol groups.

2.3. S-phase index

To determine the index of DNA synthesis of hepatocytes, we used immunohistochemistry. One hour before killing, all animals received an intraperitoneal injection of 50 mg/kg of 5-Brdu (Sigma). Samples of liver were excised and fixed in 10% buffered formalin for 24 h and embedded in paraffin. Sections (5 μm) were placed on silanized slides, dried overnight, deparaffinized in xylene, rehydrated through graded alcohols and washed in TBS (Tris-buffered saline) at pH 7.4. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min. Antigen retrieval was achieved by washing the slides in TBS and irradiating them in citrate buffer, pH 6.0 at 750 W for two cycles of 5 min in a microwave oven. After microwaving, the slides were washed in TBS and incubated with primary antibodies (Bu 20a, 1/100, Dako) for 1 h at room temperature (23–25°C). Envision was used as a detection system with 3′3-diaminobenzidine (Sigma) as the chromogen. The sections were lightly counterstained with Mayer's haematoxylin (Martín and Badrán, 1998).

2.4. Determination of VEGF expression

Deparaffinized and rehydrated sections were microwaved for 10 min in buffer citrate, pH 6. Endogenous peroxidase was blocked for 20 min. The primary antibody against VEGF-A was a mouse monoclonal antibody (Santa Cruz Biotechnology, 1:100 dilution). Bound primary antibody was detected by Envision System (Dako) for 30 min, and the reaction was developed using diaminobenzidine and counterstaining with Mayer haematoxylin. The positive control was a section of liver that had been shown to have a high VEGF content previously, by immunohistochemistry. VEGF staining was seen in the tumour 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.

2.5. Experimental groups

The animals were divided into two experimental groups. Group I (control, hepatectomized animals): these mice were all subjected to a partial (~70%) resection of the liver at 10:00, with the constituent lots being subsequently killed every 4 h begining at 12:00 on the next day of surgery (26 h posthepatectomy) and continuing until 12:00 on the second day after surgery (50 h posthepatectomy). Group II (hepatectomized tumour-bearing animals): after an appropriate period of synchronization (15 days), 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 five to six mice each, were then housed for a further 2 weeks under standardization conditions before the lots were separated. All animals were subjected to a partial (~70%) resection of the liver under the same conditions and at the same times as the first group.

2.6. Statistical analysis

The specimens were then examined microscopically under an oil-immersion objective (at $\times 1.500$) in order to score the total number of labelled nuclei among a minimum of 3000 nuclei. From this data, the SI was calculated and expressed as the number of labelled nuclei per 1000 nuclei. The results are expressed as mean $\pm$ S.E.M. Differences among experimental groups were analysed by Student's *t* test, with $P \leq 0.05$ being considered significant.

The statistical analysis of VEGF expression was performed using Anova and Tukey as a postcomparison test. The marked tumoral cells were recorded by counting 50 areas and the total nuclei every 10 areas. The results were expressed as a percentage of marked cells.

3. Results and discussion

The liver regeneration is not a simple response to one growth factor but rather a delicate and complex interplay of many cellular events. In rodents, after partial hepatectomy, changes in the concentration of substances such as TNF (tumour necrosis factor) α, HGF (hepatocyte growth factor), TGF α and β (transforming growth factor α and β) are involved in cellular proliferation and cause compensatory hyperplasia of the remnants lobes of the liver (Michalopoulos and Defrances, 2005). Particularly in mice and under controlled conditions of light-dark standardization, liver

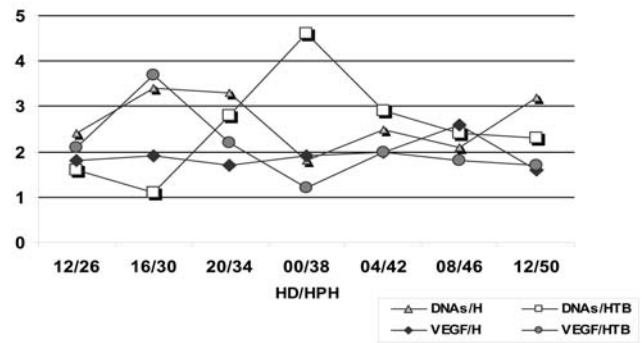

Figure 1 Comparative curves of VEGF expression and DNA synthesis (DNAs) in hepatectomized (H) and hepatectomized tumour-bearing (HTB) mice, over a circadian period

regeneration shows a circadian rhythm in mitotic activity and DNA synthesis on the hepatocyte cell population. The regeneration is not dependent on a small group of progenitor or stem cells; after partial hepatectomy, this process is carried out by a proliferation of all the existing mature cell populations composing the intact organ, but the hepatocytes are the first proliferative cells (Rabes et al., 1976). Previous studies show that the maximum value of SI was found between 30 and 38 h posthepatectomy (Martín et al., 2000; Schibler, 2003; Michalopoulos and Defrances, 2005). Our results shows that the DNA synthesis activity in the control group (hepatectomized mice) has the maximum value (3.4 ± 0.3) at 16/30 hd/hph (hour of day/hour posthepatectomy) and the minimum value (1.8 ± 0.2) at 00/38 hd/hph, with a significant statistical difference ($P<0.01$) (Figure 1 and Table 1), and these are in agreement with what we mentioned before because these clearly show that the peak of SI in control animals was found at 30 h posthepatectomy.

In the tumour-bearing mice group, we found the maximum value (4.6 ± 0.3) at 00/38 hd/hph, and the minimum value (1.1 ± 0.3) at 16/30 hd/hph, with a highly significant statistical difference ($P<0.001$) (Figure 1 and Table 1). It has been demonstrated that transplanted tumours could modify the intensity and temporal distribution of the proliferation in normal cell populations (Barbeito et al., 2002; Altun and Ölzapan, 2004). Moreover, the partial hepatectomy produces significant increases in plasma levels of a number of potential signals mentioned before as: HGF, TGFα and TGFβ, FGF (fibroblast growth factor), and others. Some of these growth factors can 'cross-talk' between the growing tumour and the regenerating liver (Michelson and Leith, 1995). There are few previous papers that analysed the proliferative effects in the liver of tumour-bearing animals; we can mention Ueda et al. (1993) who observed that when the AH109A ascites tumour cells were grafted into subcutaneous flank tissue in rats, there occurs a significantly increased DNA synthesis in the hepatocytes. However, other authors observed that injection with the ES2 and SS1K tumour extract inhibits the hepatic regeneration (Echave Llanos et al., 1963; Badrán et al., 1986). In our study, we have demonstrated that in tumour-bearing mice, the SI curve of the hepatocytes, in regenerating liver, has been desynchronized with a delay of 8 h.

Angiogenesis is a complex process that involves endothelial cell migration, capillary bredding, neovascular remodelling, in addition to endothelial cell proliferation (Karamysheva, 2008). The growth of solid tumours like those analysed 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 tumour cell proliferation decreases with increasing distances from the blood vessels (Vartanian and Weidner, 1996). In our results, we can observe that the VEGF expression in the control group shows the maximum value (2.6 ± 0.1) at 08/46 hd/hph and the minimum value (1.6 ± 0.3) at 12/50 hd/hph, with a significant statistical difference ($P<0.05$) (Figure 1 and Table 2), and in the tumour-bearing mice group, we can see the maximum value (3.7 ± 0.1) at 16/30 hd/hph and the minimum value (1.2 ± 0.2) at 00/38 hd/hph, with a highly significant statistical difference ($P<0.001$) (Figure 1 and Table 2). As we can see in our study, the hepatectomized as well as hepatectomized tumour-bearing animals showed a circadian variation of VEGF expression in hepatocytes, but the presence of transplanted tumour in the host generates modifications in the temporal expression of VEGF. In tumour-bearing mice, the peak of VEGF expression appears 16 h before and with higher values than the control group. Thus, we can affirm that ES2 hepatocellular carcinoma increases, directly or indirectly, VEGF expression in hepatocytes. These results are in agreement with Taniguchi et al. (2001) who observed that the tumour cells synthesized VEGF, which may recirculate and modify indirectly the liver cells' proliferation changing their vascularization.

In conclusion, in the hepatectomized mice, the increases of hepatic proliferation, measured by the SI index, may produce a rice in VEGF expression with the object of generating a vascular network for the hepatic regeneration. Lastly, as we have mentioned, in tumour-bearing mice, the peak of VEGF expression

Table 1 Curve of DNA synthesis activity in two hepatectomized groups: controls and tumour-bearing mice, over a circadian period

Hep, hepatectomized mice group (controls); Hep+TB, hepatectomized and tumour-bearing mice group; (*n*), number of animals; Hday/Hposthep, hour of day/hour posthepatectomy; X, mean.

Hday/Hposthep	Hep (*n*)	Hep+TB (*n*)
12/26	2.4 ± 0.1 (5)	1.6 ± 0.1 (6)
16/30	3.4 ± 0.3 (6)	1.1 ± 0.3 (6)
20/34	3.3 ± 0.4 (6)	2.8 ± 0.4 (5)
00/38	1.8 ± 0.2 (6)	4.6 ± 0.3 (6)
04/42	2.5 ± 0.4 (5)	2.9 ± 0.3 (5)
08/46	2.1 ± 0.1 (6)	2.4 ± 0.2 (5)
12/50	3.2 ± 0.1 (6)	2.3 ± 0.3 (6)
X	2.7 ± 0.2	2.5 ± 0.4

Table 2 Curve of VEGF expression in two hepatectomized groups: controls and tumour-bearing mice, over a circadian period

Hep, hepatectomized mice group (controls); Hep+TB, hepatectomized tumour-bearing mice group; (*n*), number of animals; Hday/Hposthep, hour of day/hour posthepatectomy; X, mean.

Hday/Hposthep	Hep (*n*)	Hep+TB (*n*)
12/26	1.8 ± 0.2 (6)	2.1 ± 0.1 (6)
16/30	1.9 ± 0.2 (5)	3.7 ± 0.1 (6)
20/34	1.7 ± 0.1 (6)	2.2 ± 0.2 (6)
00/38	1.9 ± 0.3 (6)	1.2 ± 0.2 (5)
04/42	2 ± 0.1 (6)	2 ± 0.2 (5)
08/46	2.6 ± 0.1 (6)	1.8 ± 0.1 (6)
12/50	1.6 ± 0.3 (5)	1.7 ± 0.2 (6)
X	1.9 ± 0.1	2.1 ± 0.1

appears before that of DNA synthesis in the hepatocytes. This could be due to the fact that the tumour cells synthesized their own VEGF, which enters general circulation to stimulate the hepatic cells.

Author contribution

Marcela Garcia performed the DNA synthesis experiments and wrote the manuscript. Laura Andrini performed the VEGF experiments. Ana María Inda wrote the manuscript. Jorge Ronderos performed the statistical data analysis. Julio Hijano performed the surgical manipulations of the animals. Ana Lía Errecalde was the research project director.

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