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Prokineticin 2/Bv8 is expressed in Kupffer cells in liver and is down regulated in human hepatocellular carcinoma

Running title (short title): Prokineticin 2/Bv8 expression in liver Kupffer cells

Justin Monnier, Claire Piquet-Pellorce, Jean-Jacques Feige, Orlando Musso, Bruno Clément, Bruno Turlin, Nathalie Théret. Michel Samson

Justin Monnier, Orlando Musso, Bruno Clément, Nathalie Théret, Michel Samson, INSERM U620 Université de Rennes 1, IFR140, 2 avenue du Pr. Léon Bernard, 35043 Rennes cedex, France
Claire Piquet-Pellorce, INSERM U522, Rennes, France
Jean-Jacques Feige, INSERM U878, Grenoble, France
Bruno Turlin, Service d'Anatomo-pathologie, CHU Pontchaillou, Rennes, France

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Correspondence to: Dr. Michel Samson, INSERM U620 Université de Rennes 1, 2 avenue du Prof. Léon Bernard 35043 Rennes cedex, France. michel.samson@univ-rennes1.fr
Telephone :+33-02-23 23 47 93 Fax : + 33-02-23 23 47 94

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Author contributions:

Justin Monnier, Claire Piquet-Pellorce, Bruno Turlin, Nathalie Théret and Michel Samson designed research and analysed data; Justin Monnier, Nathalie Théret and Bruno Turlin performed research; Jean-Jacques Feige contributed new reagents tools; Orlando Musso provided clinical data; Bruno Clément for administrative organisation; Justin Monnier, Claire Piquet-Pellorce and Michel Samson wrote the paper.

Abstract

AIM: To study the implication of prokineticin 1 (PK1/EG-VEGF) and prokineticin 2 (PK2/Bv8) in hepatocellular carcinoma angiogenesis.

METHODS: The gene induction of PK1/EG-VEGF and PK2/Bv8 was investigated in 10 normal, 28 fibrotic and 28 tumoral livers by using real time PCR. Their expression was compared to the expression of VEGF (an angiogenesis marker), vWF (an endothelial cell marker) and to CD68 (a monocyte/macrophage marker). Furthermore, the mRNA levels of PK1/EG-VEGF, PK2/Bv8, prokineticin receptor 1 and 2 were evaluated by real time PCR in isolated liver cell populations. Finally, PK2/Bv8 protein was detected in normal liver paraffin sections and in isolated liver cells by immunohistochemistry and immunocytochemistry.

RESULTS: PK2/Bv8 mRNA but not PK1/EG-VEGF was expressed in all types of normal liver samples examined. In the context of liver tumor development, we reported that PK2/Bv8 correlates only with CD68 and showed a significant decrease in expression as the pathology evolves towards cancer. Whereas, VEGF and vWF mRNA were significantly upregulated in both fibrosis and HCC, as expected. In addition, out of all isolated liver cells examined, only Kupffer cells (liver resident macrophages) express significant levels of PK2/Bv8 and its receptors, prokineticin receptor 1 and 2.

CONCLUSION: In normal liver PK2/Bv8 and its receptors were specifically expressed by Kupffer cells. PK2/Bv8 expression decreased as the liver evolves towards cancer and did not correlate with HCC angiogenesis.

Key words: Prokineticin, Hepatocellular Carcinoma, PK2/Bv8, Angiogenesis, Kupffer cells, VEGF, Liver.

Monnier J, Piquet-Pellorce C, Feige JJ, Musso O, Clément B, Turlin B, Théret N, Samson M. **Prokineticin 2/Bv8 is expressed in Kupffer cells in liver and is down regulated in human hepatocellular carcinoma.**

INTRODUCTION

In vivo, a solid tumor can not progress beyond a few cubic millimeters without oxygen and nutrients supplied by an adequate vascular support [1]. The creation of novel vascular vessels in the tumor microenvironment resulting from an over expression of pro-angiogenic factors plays a central role in tumor invasion and metastasis [2]. Hepatocellular carcinoma (HCC) present typical neo vascularization [3]. Among the soluble factors responsible for the shift towards tumor neo vascularization, vascular endothelial growth factor (VEGF) is thought to play an important role in HCC. This factor is synthesized essentially during hypoxic stress [4] and promotes the growth, migration, morphogenesis of vascular endothelial cells and increases vascular permeability [5, 6]. Furthermore, the VEGF/VEGF receptor system is thought to be closely related to the histological grade of hepatocellular carcinoma [7] and strongly expressed in well differentiated HCC [8]. Several other potent angiogenic factors have been reported to be involved in HCC angiogenesis, notably basic fibroblast growth factor (bFGF) [9, 10], angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) [11]. Interestingly, inflammatory cytokines like Interleukin 8 (IL-8) and tumor necrosis factor-alpha (TNF-α) have also been shown to be implicated in the maintenance of HCC angiogenesis but also in chronic hepatitis [12, 13].

Prokineticin 1, also named endocrine gland-vascular endothelial growth factor (PK1/EG-VEGF) and prokineticin 2 also known as Bombina variegata 8 (PK2/Bv8) are widely distributed in mammalian tissue and exhibit diverse biological activities. Prokineticins bioactivities includes, smooth muscle contraction in the gastrointestinal tract [14], supporting neuronal survival, pain sensation and circadian rhythms in the central nervous system [15-17]. Prokineticins display also potent cytokine properties, such as inducing bone marrow leukocyte production, and modulating the growth and function of peripheral leukocytes [18-20]. These peptides were also shown to act as angiogenic mitogens, indeed, PK1/EG-VEGF and PK2/Bv8 induce proliferation, migration and fenestration of endothelial cells from adrenal capillaries [21, 22]. In addition, in a mouse model of myocardial infarctions, PK2/Bv8 protected cardiomyocytes by increasing neovascularization [23]. Prokineticins are a family of peptides characterized by a common AVIT motif, and 10 conserved cysteine residues [24-26]. Prokineticins are not structurally related to VEGF and mediate their biological activity by selectively acting on two G

protein coupled receptors, prokineticin receptor 1 and 2 (PKR1 and PKR2), for which they have similar affinities [24, 26]. Activation of PKRs triggers multiple signaling pathways, such as calcium mobilization, phosphoinositol turnover, and activation of Akt kinase and mitogen-activated protein kinase (MAPK) [27].

Numerous studies have evaluated the implication of prokineticins in tumor development. For example, mice implanted with colorectal cancer cell line SW620 stably expressing PK1/EG-VEGF had an increase of angiogenesis and of colorectal cancer metastasis [28]. In ovarian carcinoma, PK1/EG-VEGF was evenly detected in benign, low malignant potential neoplasms or stage I ovarian cancer [29], and no expression was observed in endometrial carcinoma [30]. However, PK1/EG-VEGF was shown to be associated in Leydig cell tumor growth [31] and in neuroblastoma progression[32]. In prostate cancer, both prokineticin 1 and 2 and their receptors were up regulated [32, 33]. Taken altogether, the literature indicates that prokineticins are differentially associated to tumor development. In the present study, we explored the potential implication of both prokineticins in HCC angiogenesis, and identified their cellular origin in liver parenchyma.

MATERIALS AND METHODS

Patients

Liver tissue samples were obtained from 28 patients with HCC undergoing surgical resection of the tumor. Patients with HCC were 25 males and 3 females (59.9 ± 9.0 yr). The etiology of fibrosis (*n*= 8) and cirrhosis (*n*= 20) included hepatitis C (*n*= 22), hepatitis B (*n*= 7) and alcohol abuse (*n*= 14). All tumor samples were taken within the tumor, and only tissue with anatomic pathology features that allow a matching diagnosis with the pathology report of each patient were used for RNA extraction. Matching non tumor livers (*n* = 28) were taken distantly from the tumors. Histological evaluation before their inclusion in the study ruled out the presence of tumor tissue in these samples. Controls were 10 histologically normal liver samples obtained from metastatic livers of colorectal cancer [34]. Access to this material was in agreement with French laws and satisfied the requirements of the local Ethics Committee. The histological stage of fibrosis and the intensity of necro-inflammatory lesions were graded according to the METAVIR score [35]: A0 = no activity, A1 = mild, A2 = moderate, A3 = severe, F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3

= numerous septa without cirrhosis, and F4 = cirrhosis. All tissue sections were routinely analyzed after staining with hematoxylin-eosin-saffran and Sirius red.

Human hepatic cell isolation

Human hepatic cells were isolated from histologically normal specimens of partial hepatectomy from patients undergoing hepatic resection for liver metastases. A 2 step perfusion method using collagenase and pronase, followed by differential sedimentation allowed separation and recovery of hepatocytes (HA) from non-parenchymal cells enriched in sinusoidal cells [36]. From the latter fraction, hepatic stellate cells (HSC) were efficiently purified by a density gradient centrifugation with Nycodenz 13 % (Sigma-Aldrich, St. Louis, MO) and cultured as previously described [37]. Similarly a population enriched in liver macrophages, also known as Kupffer cells, was obtained from the same non-parenchymal cell population by using a density gradient centrifugation with Nycodenz 17 % [38]. The proportion of Kupffer cells present in the Kupffer cell enriched population (KC-Enr) was evaluated by flow cytometry (Becton Dickinson's FACScalibur, Grenoble, France) using an antibody directed against CD14, a typical surface marker of monocyte/macrophage (Immunotech Beckman Coulter, Marseille, France). Kupffer cells were then further enriched from the Kupffer cell enriched population (KC-Enr) by using anti-CD14-Dynabeads according to the manufacturer's indication (Dynal A.S., Oslo, Norway) [38].

Hepatoma cell lines (Hep3B, BC1, B16A2) were cultured in 25% 199, 75% MEM medium (Eurobio, Courtaboeuf, France), supplemented with 1mg/ml albumin, 5µg/ml insulin, 50000 U/L penicillin-streptomycin, 12.5 nM glutamine and 100 ng/ml hydrocortisone. HepG2 cell line was cultivated in MEM α Medium (GIBCO, Paisley, Scotland), to which was added 50000 U/L penicillin-streptomycin and 12.5 nM glutamine.

RNA isolation and RT-PCR

Total RNA was extracted from isolated human liver cells, liver tissue or directly from Kupffer cells linked to the CD14 coated beads using the SV Total RNA isolation Kit ® (Promega, Charbonnieres-lesbains, France) and 1µg of total extracted RNA was subjected to a reverse transcription reaction using high capacity cDNA archive kit® (Applied Biosystem, Foster City, CA). A total of 12.5 ng total complementary DNA was

used as a template for amplification with primers specific for PK1/EG-VEGF, PK2/Bv8 (PK2β and PK2L) and β-actin (Table 1) used at a 250nM final concentration. The PCR reaction performed using PCR master mix from Promega for which the final working concentration included 0.5 units of Taq polymerase, 200μM dNTPs and 1.5mM $MgCl_2$. The PCR reaction was performed using GeneAMP PCR 9700 (Applied Biosystems), after an initial denaturation step at 94°C for 2 min, 35 cycles of PCR were programmed for PK1/EG-VEGF and PK2/Bv8, whereas 25 cycles were performed for β-actin. Denaturation step was performed at 94°C for 30s, annealing at 60°C for PK1/EG-VEGF and PK2/Bv8, and 55°C for β-actin, and extension at 72°C for 1 min. The last extension was at 72°C for 7 min.

For real-time quantitative PCR, the mRNA levels of PK1/EG-VEGF, PK2/Bv8 (PK2β and PK2L), VEGF (VEGF A), vWF, CD68, PKR1 and PKR2 were assayed using the 7000 sequence detection system ABI Prism ® sequence detector (Applied Biosystems), using the double strand specific dye SYBR® Green system (Applied Biosystems). Primer sequences are summarized in Table 1. The PCR condition and cycle were as follows: initial DNA denaturation 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec, follow by an annealing step, and then extension at 60°C during 1 min. Each point was performed in triplicate. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was performed during the PCR cycle and only primers with a unique dissociation peak were selected, followed by migration on a 2% agarose gel to ensure that the PCR product was unique. The PCR products were then sequenced to confirm that the correct cDNA was amplified (data not shown). The amplification efficiency for each primer pair was calculated and presented in Table 1. The expression level of each gene was adjusted by the level of 18S mRNA and expressed as the ratio to 18S mRNA or as the ratio to the average gene expression level in normal liver.

Immunohistochemical staining

Paraffin embedded liver sections were de-parafinised by 2 changes of xylene for 5 min, and re hydrated, in 2 changes of 100% ethanol for 3 min, 2 changes of 95% and 80% for 1 min, and then washed with distilled water. Antigen retrieval was performed using a citrate pH 6.0 buffer, heated at 95-100°C for 40 min; sections were let to rest at room

temperature for 20 min. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide (10 min, RT) and the non specific sites were blocked with PBS/BSA 2% (1 h, RT). PK2/Bv8 protein was detected using rabbit polyclonal anti-PK2/Bv8 antibody (gift of Dr. Feige, INSERM U858, Grenoble, France) at a final concentration of 1.44 µg/ml for 1 h at RT or for 24h at 4°C [39]. CD31, CD34, CD68 and VEGF detection was performed by incubating sections with primary antibodies overnight at 4°C using mouse monoclonal anti-CD31 IgG1 (cloneJC70A, dilution 1:50, Dako, Glostrup, Denmark), mouse monoclonal anti-CD34 IgG1 (clone Qbend10, dilution 1:10 Immunotech, Beckman Coulter), mouse monoclonal anti-CD68 IgG3 (clone PGM1, ready to use, Dako), rabbit polyclonal anti-VEGF (sc-152, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were diluted in Dako's REAL™ Antibody Diluent. Sections were processed with appropriate biotinylated secondary antibody and a streptavidin biotin peroxidase amplification kit (Vectastain, Vector Laboratories, Burlingame, CA). The peroxidase reaction was finally developed with diaminobenzidine (Merck, Whitehouse Station, NJ) and sections were counter stained with Mayer's hematoxylin. Non-immune rabbit serum or control mouse IgG was used instead of primary antibodies as negative controls. In addition, competition experiments were performed by pre-incubating overnight at 4°C PK2/Bv8 antibodies with the appropriate antigen peptides.

Immunocytochemical staining

Enriched Kupffer cells (10^5 cells) were spotted on slides by cytospin (10 min at 400 rpm) and then fixed using 4% paraformaldehyde at room temperature for 10 min. Permeabilization was achieved by treating cells for 10 min at RT with Triton X100 0.1 % and blocking of non-specific sites by 20min incubation at RT with PBS/BSA 2%. PK2/Bv8 protein was detected with mouse anti-PK2/Bv8 anti-serum (dilution 1:50, Abnova, Tapei City, Taiwan) and revealed by TRITC labelled anti-mouse antibody (dilution 1:150, Merck). CD68 protein was detected directly with FITC labeled mouse monoclonal anti-CD68 IgG1 (clone KP1, dilution 1:10, Dako). Primary and secondary antibodies were diluted in appropriate diluent (Dako's REAL™ Antibody Diluent) and incubated at RT for 2hr and 1hr respectively. The cells were counterstained with nucleus dye DAPI (dilution 1:10000, Dako). Sections processed with one or both of the

control primary antibodies (control mouse serum and/or control IgG1) were free of immunostaining in their corresponding color channel. We checked that FITC labeled cells remained negative in TRITC channel.

Statistical analysis

Statistical comparisons for significance were made with Wilcoxon's test for paired samples, and with Mann-Whitney U test for un-paired samples. The correlation between continuous variables was examined by means of Spearman's rank-order coefficients. A level of $P<0.05$ was considered significant. Calculations were made with the commercially available software Statistica.

RESULTS

Expression of PK1/EG-VEGF, PK2/Bv8, vWF and VEGF mRNA in normal and tumoral livers

The mRNA expression of PK1/EG-VEGF and PK2/Bv8 in liver was first evaluated by PCR in 4 normal livers (NL), 7 tumoral livers (HCC) and in their corresponding adjacent non-tumoral tissue (NT) (Figure 1A). THP1, a monocytic cell line which expresses high levels of both PK1/EG-VEGF and PK2/Bv8 transcripts, served as a positive control. Our results show that, in all liver samples analyzed PK2/Bv8 is significantly expressed, whereas PK1/EG-VEGF is barely detectable.

In order to monitor more precisely how PK2/Bv8 varied during HCC progression, we measured the mRNA expression of PK2/Bv8 by real time PCR in a larger cohort of 28 patients (Figure 1B). PK2/Bv8 mRNA is expressed in normal liver (n= 10) at significant levels (average cycle threshold ≈ 28). However, a significant decrease in PK2/Bv8 mRNA expression was detected in HCC relative to normal liver tissues (^{a} $p < 0.05$), while no significant difference was found between HCC (n=28) and peri-carcinomatous liver tissues (NT, n=28). Such expression of PK2/Bv8 was compared to two angiogenesis markers vWF (Figure 1C) and VEGF (Figure 1D). vWF, an endothelial cell marker, showed remarkable over expression in tumor (^{b} $P< 0.01$) and in adjacent liver tissues compared with normal liver (^{b} $P < 0.01$). Similarly, VEGF mRNA levels were induced in HCC and the underlying cirrhotic liver (NT) compared with normal liver (^{b} $P < 0.01$). By

using the spearman's rank order correlation, a significant correlation was observed between VEGF and vWF ($r = 0.6$; ^{b} $P < 0.001$) but not with PK2/Bv8.

The peri-carcinomatous liver tissues were classified according to the METAVIR score. vWF and VEGF were significantly up-regulated in fibrosis and cirrhosis (^{b} $P < 0.01$) compared with normal liver (Figure 1F and 1G) whereas no difference was observed with PK2/Bv8 (Figure 1E). Moreover, PK2/Bv8, vWF and VEGF mRNA expression did not show any significant statistical correlation with necro-inflammatory activity (data not shown).

Immunolocalisation of PK2/Bv8, CD31, CD34 and VEGF in normal livers

Sections of normal livers from 5 different individuals were immunostained with antibodies against: PK2/Bv8, CD31 (a constitutive marker of endothelial cells), CD34 (a marker of endothelial cell in proliferation), and with an antibody against VEGF A (including 165, 189 and 121 variants). PK2/Bv8 protein expression was detected in cells restricted to the hepatic sinusoids and presenting morphology similar to resident macrophages (Figure 2). The specificity of the staining was demonstrated by the inhibition of PK2/Bv8 labeling after anti-PK2/Bv8 pre-incubation with an excess of recombinant PK2/Bv8 peptide. In normal liver, both large capillary endothelial cells and sinusoidal endothelial cells were positive for CD31, whereas CD34 positive endothelial cells were practically un-detectable in the parenchyma, excepted in large capillaries. VEGF protein was detected at low levels in the hepatic parenchyma.

Expression of CD68 mRNA and protein in normal and tumoral livers

Recent reports have demonstrated that PK2/Bv8 has cytokine properties [20] and is strongly expressed by several types of leukocytes including the monocyte/macrophage lineage [19]. We therefore, hypothesized that the liver resident macrophages might be responsible for the expression of PK2/Bv8. The Kupffer cell population expresses the specific marker CD68, and on this basis we investigated the correlation between CD68 and PK2/Bv8 in term of transcripts and tissue localization. The mRNA expression of CD68 was measured by quantitative PCR in the same cohort of patients. As shown in Figure 3A, there was a significant down expression of CD68 in liver HCCs compared to the expression in normal livers (^{a} $P < 0.05$). Thus the expressions of PK2/Bv8 (Figure

1A) and CD68 (Figure 3A) in HCC patients follow a similar down regulation pattern in tumors relative to control livers. Furthermore, using spearman's rank order correlation, CD68 showed a correlation with PK2/Bv8 (r = 0.45, [b] $P < 0.01$). Similar to PK2/Bv8, CD68 did not show any correlation of expression with VEGF or vWF. In addition, CD68 and PK2/Bv8 were immunolocalized in normal liver serial sections (Figure 3B). The specificity of PK2/Bv8 staining was evaluated by the disappearance of labeling when anti-PK2/Bv8 antibodies were pre-incubated with antigen peptide. We showed that most CD68 positive cells were not PK2/Bv8 positive. However, all cells showing positive staining for PK2/Bv8 were positive for CD68 staining.

Expression of PK1/EG-VEGF and PK2/Bv8 in Kupffer cell enriched non-parenchymal cells and in isolated hepatic cells

To further investigate the relationship between Kupffer cell population and PK2/Bv8 positive cells in liver, we studied various sequentially purified liver hepatic cells: hepatocytes (HA), hepatic stellate cells (HSC), and hepatic non parenchymal cell enriched in Kupffer cells (KC-Enr). The proportion of Kupffer cells in the KC-Enr population was evaluated by flow cytometry using an antibody against the cell surface monocyte/macrophage marker, CD14 (Figure 4A and 4B). This CD14 positive cell fraction represents 15% of the total KC-Enr population (representative of two separate experiments). CD14 positive Kupffer cells (CD14+KC) were further enriched using anti-CD14 labeled microbeads.

The mRNA expression of PK1/EG-VEGF and PK2/Bv8 was measured by real time PCR on the various isolated liver cell populations, and compared whole liver expression. As shown in Figure 4C, the mRNA expression of PK2/Bv8 increases gradually as the concentration of Kupffer cells increases. Indeed in the KC-Enr cell population, containing 15% of Kupffer cells, the expression of PK2/Bv8 mRNA is 5 fold higher then in normal liver where Kupffer cells compose roughly 2% of the liver mass [40]. In CD14+ Kupffer cells (CD14+KC), expression of PK2/Bv8 is 35 fold higher than in normal liver. Primary hepatocytes, primary hepatic stellate showed no significant PK2/Bv8 expression (Figure 4C), as did several human hepatoma cell lines; BC1, Hep3B, HepG2, B16A2 (data not shown). Concerning PK1/EG-VEGF mRNA expression, it was barely detectable in all liver samples analyzed as shown in Figure 4C (average cycle threshold ≈ 33). All together

these data strongly suggest that PK2/Bv8 transcript production in liver is associated with Kupffer cells.

Immunocytochemical co-localization of CD68 and PK2/Bv8 in isolated human liver cells

The human non-parenchymal liver cell population enriched in Kupffer cells (KC-Enr) was spotted on slides for a double immunolocalisation using FITC directly labeled anti-CD68 and indirectly labeled TRITC anti-PK2/Bv8. Results are presented in Figure 5, CD68 positive cells are shown in green and PK2/Bv8 positive cells appear in red, appropriate negative controls for both of the antibodies showed no specific staining. Co-immunolocalization of PK2/Bv8 and CD68 is shown in orange and demonstrates that all Bv8 positive cells are also CD68 positive.

Expression of PKR1 and PKR2 mRNA in human isolated liver cells and hepatoma cell lines

In order to assess the cellular target of PK2/Bv8 in liver cells, the mRNA level of their receptors, PKR1 and PKR2, was measured by real time PCR in isolated hepatic cells which includes CD14+ KC cells, hepatocytes, stellate cells, and human hepatoma cell lines BC1, Hep3B, HepG2, B16A2. In comparison to normal liver, PKR1 and PKR2 are most strongly expressed by the CD14 positive Kupffer cell population and expression was very weak in cultured stellate cells, in hepatoma cell lines and undetectable in hepatocytes (Figure 6).

DISCUSSION

PK1/EG-VEGF and PK2/Bv8 are two novel peptides with diverse biological functions [27, 41] and one of such bioactivities is their ability to act as angiogenic mitogens. Their angiogenic activity was first evaluated in an *in vitro* assay by observing their capacity to induce proliferation, survival and migration of bovine adrenal cortical capillary endothelial cells (ACE) [21, 22]. In this report we investigated the angiogenic potential of both PK1/EG-VEGF and PK2/Bv8 in human hepatocellular carcinoma (HCC). Interestingly, the literature shows two reports with divergent results on prokineticin expression in liver. In 2001, M. Li *et al* [14] showed an equivalent expression of

prokineticin 1 and 2 in human liver by northern blot assay, whereas in 2005, Chen and colleagues [42], using RT-PCR observed a strong expression for PK2/Bv8 in human liver and an undetectable expression for PK1/EG-VEGF. Confirming the latter report, our results show a predominant expression of PK2/Bv8 in liver. Thus, we investigated further the expression of PK2/Bv8 in human liver, and its potential role in HCC angiogenesis. Our results show that Bv8/PK2 mRNA levels decrease significantly as the pathology evolves towards cancer.

Most cases of HCC develop on a liver fibrosis/cirrhosis background, which constitutes a very hypoxic environment for liver cells, and drives the production of numerous potent angiogenic factors [43]. In our HCC cohort, we monitored angiogenesis by measuring the expression of the endothelial cell marker; vWF and the angiogenic factor VEGF. Our results show that they are both strongly up-regulated in fibrosis and even more in tumors, confirming that our cohort exhibits a strong angiogenic profile. However, even though our HCC cohort constitutes an angiogenic environment, expression of PK2/Bv8 decreases in fibrosis and even more in tumors, suggesting that in this tissue context PK2/Bv8 is not induced by hypoxia in fibrosis and HCC.

To investigate further why PK2/Bv8, which is expressed at significant levels in normal liver, is down regulated through out the course of HCC development, we identified its cellular origin by several different techniques. Firstly, by immunolocalization on liver paraffin sections, PK2/Bv8 positive cells liver were located in the hepatic sinusoids and showed morphology similar to resident macrophages, otherwise known as Kupffer cells. Secondly, comparison of PK2/Bv8 expression in isolated human primary hepatocytes, hepatic stellate cells, Kupffer cells and hepatoma cell lines, showed that only Kupffer cells express significant levels of PK2/Bv8. Immunostaining on normal liver serial sections of CD68 and PK2/Bv8 showed that all PK2/Bv8 positive cells were equally CD68 positive. Finally, colocalization of PK2/Bv8 with CD68, a Kupffer cell marker, carried out in an isolated Kupffer cell-enriched population showed that all cells positive for PK2/Bv8 were also CD68 positive. Taken all together these data validate the hypothesis that Kupffer cells are the specific source of PK2/Bv8 in liver. Interestingly, it is a well known event that the number of Kupffer cells decreases in cirrhosis and even more in HCC relative to normal liver [44]. In this study, we confirm this previous report and observe that the expression pattern of CD68, a marker for Kupffer cells, decreases in fibrosis and

in HCC compared to normal liver. CD68 and PK2/Bv8 follow a similar down-regulation pattern, which is confirmed by a correlation between the two gene expressions. Thus, these results contribute towards a potential explanation that the down regulation of PK2/Bv8 in HCC is due to the gradual decrease in number of Kupffer cells during the progression of the pathology. However, based on our results we can not exclude a possible regulation of PK2/Bv8 in Kupffer cells by the hepatic micro-environment.

Our results showing that PK2/Bv8 producing cells are Kupffer cells; are consistent with the fact that PK2/Bv8 is expressed by several types of leukocytes including the monocyte/macrophage lineage [14, 19]. Even though PK1/EG-VEGF was not expressed in Kupffer cells it has also been associated to leukocyte lineage. For instance, PK1/EG-VEGF expression was positively correlated to macrophages in corpus luteum regression and follicular atresia of bovine ovaries [45], and to tumor infiltrating lymphocytes of human ovarian carcinoma [29]. All together, PK1/EG-VEGF and PK2/Bv8 appear to be expressed by immune cells differentially according to the tissue. Furthermore, we characterized the cellular targets of PK2/Bv8 in liver by measuring the expression for PK2/Bv8's receptors, called PKR1 and PKR2, in the same panel of liver cells. Interestingly, PKR1 and PKR2 are most strongly expressed by Kupffer cells. Recent reports have demonstrated that PK2/Bv8 has potent cytokine properties, PK2/Bv8 has the ability to stimulate monocyte production, mobilization and differentiation into macrophage like cells [19, 20]. Therefore we can speculate that, in normal liver macrophage physiology, PK2/Bv8 could act by an autocrine manner on the resident macrophage population in the liver sinusoids. PK2/Bv8 might regulate Kupffer cells function and be involved in monocyte recruitment and differentiation of Kupffer cells.

In conclusion, we demonstrate that PK2/Bv8 is expressed in resident Kupffer cells in normal liver and that the expression of PK2/Bv8 decreases significantly as the pathology evolves towards hepatocellular carcinoma.

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COMMENTS:

Background

Liver cancer is one of the most common types of cancer and is a solid tumor showing important angiogenesis. Angiogenesis is an essential step in tumor progression, where the creation of novel blood vessels allows the growth and dissemination of cancer cells. Disrupting cancer blood vessels by blocking the secreted peptides responsible for endothelial cell proliferation has become a novel axis of anti-cancer treatment. Thus, identifying new secreted molecules involved in liver cancer vascularization could provide novel therapeutic targets.

Research frontiers

The aim of this study was to evaluate if two novel secreted peptides PK1/EG-VEGF and PK2/Bv8, described as having potent angiogenic effects, were involved in the vascularization process of human hepatocellular carcinoma.

Related publications

The present study had for goal to evaluate the angiogenic potential of prokineticins in hepatocellular carcinoma. To this effect we cited several articles from other investigators reporting researches on prokineticin action as an angiogenic factor, their implication in several other types of cancer.

Innovations and breakthroughs

This study clearly shows that PK1/EG-VEGF and PK2/Bv8 are not involved in human hepatocellular carcinoma vascular progression. Furthermore, in liver PK2/Bv8, but not PK1/EG-VEGF, was detected and was specifically expressed only by liver resident macrophages, known as Kupffer cells. Moreover, Kupffer cells express high levels of prokineticin receptor 1 and 2 suggesting paracrine/autocrine regulation by PK2/Bv8.

Applications

Even though our results show that PK2/Bv8 is not associated with hepatocellular carcinoma, it brings novel data on Kupffer cell physiology. All together our findings,

corroborate numerous other reports demonstrating that prokineticins are expressed by immune cells, and have potent immunomodulatory activities.

Terminology

Prokineticin 1 (PK1/EG-VEGF) and prokineticin 2 (PK2/Bv8) are small secreted peptides of 8 kda that signal through two receptors that are members of the G-protein coupled receptors superfamily. In addition to its angiogenic activites, prokineticins have also been shown to be implicated in hematopoiesis, reproductive angiogenesis, neurogenesis, ingestive behaviour and hormone release, gastrointestinal motility, circadian rhythms, and pain sensation. Hepatocellular carcinoma (HCC) is the major type of liver cancer; it develops majoritarily on a cirrhotic background, and is highly angiogenic.

Peer review

Although the data are only descriptive, this manuscript describes for the first time in detail the expression and localization of PK2/BV8 in human liver, and in general the manuscript is well written.

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Figure Legends

Figure 1: (A) RT-PCR analysis of PK1/EG-VEGF and PK2/Bv8, and **(B-G)**, real-time PCR analysis of PK2/Bv8, vWF and VEGF in normal liver (NL, *n*=10), non-tumorous liver (NT, *n*=28), and in Hepatocellular carcinoma (HCC, *n*=28).

Figure 2: Immunolocalization of PK2/Bv8, CD31, CD34 and VEGF on normal liver sections.

Figure 3: (A) Expression of CD68 normal liver (NL, *n*=10), non-tumorous liver (NT, *n*=28), and in Hepatocellular carcinoma (HCC, *n*=28) by real time PCR, and **(B)** immunolocalization of CD68, PK2/Bv8, and PK2/Bv8 with blocking peptide on normal liver paraffin embedded serial sections.

Figure 4: (A) Scatter plot and **(B)** histogram plot of CD14+enriched Kupffer cells, and **(C)** expression of PK1/EG-VEGF and PK2/Bv8 mRNA in isolated human liver cells by real time PCR.

Figure 5: Immunocytochemical staining of PK2/Bv8 and CD68 on isolated liver cells enriched in CD14+ Kupffer cells.

Figure 6: Expression of PKR1 and PKR2 mRNA in isolated human liver cells, by real time PCR.

Table 1. Primer sequences for quantitative PCR

Gene		Primer sequence	Primer efficiency %
VEGF	sense	AGGAGGAGGGCAGAATCATCA	99
	antisense	CTCGATTGGATGGCAGTAGCT	
vWF	sense	TGGAGCAGCAAAGGGACGAGA	99
	antisense	TAGGAGGAGGGGCTTCAGGGG	
PK2/Bv8	sense	TACAGCTTTTGGTCCCTTGC	97
	antisense	GATTCCCATCAGTGATTCTGC	
CD68	sense	TAGCTGGACTTTGGGTGAGG	99
	antisense	AAGGATGGCAGCAAAGTAGC	
PKR1	sense	GCTCATGTGCATCCTCACC	99
	antisense	GATCATGCTGTTGCTCAGG	
PKR2	sense	CTTCTTCCCCACTGTGTTCG	99
	antisense	GGTGTTGATCATGCTGTTGC	
EG-VEGF	sense	GGGCTTCAGTGGTTAACTGG	97
	antisense	TGTGACCTGTGACCTTCTGC	
18S	sense	CCATCCAATCGGTAGTAGCG	98
	antisense	GTAACCCGTGGAACCCCATT	
β-Actin	sense	GATGAGATTGGCATGGCTTT	95
	antisense	GAGAAGTGGGGTGGCTT	