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In vivo inhibition of human hepatocellular carcinoma related angiogenesis by vinblastine and rapamycin

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Summary. This paper illustrates the use of the chick embryo chorioallantoic membrane (CAM) assay to determine the single and combined antiangiogenic effects of very low doses of vinblastine (VBL) and rapamycin (RAP) in human hepatocellular carcinoma (HCC). The angiogenic response induced by human HCC biopsy specimens was inhibited by each drug and sinergistically by their combination. Morever, immunohistochemical detection of microvessels with anti-CD31 mAB showed that their area was significantly lower in specimens treated with VBL and RAP in combination. Sinergy on the part of these well-known drugs when used in combination as antiangiogenics at very low doses may be of significance in the designing of new ways of treating HCC.

Key words: Antiangiogenesis, Hepatocellular carcinoma, Rapamycin, Tumor progression, Vessel growth, Vinblastine

Introduction

Angiogenesis occurs in both physiological and pathological conditions, while the transition from the avascular to the vascular phase is a specific clinical turning point in solid tumor growth (Hanahan and Folkman, 1996). Extensive investigation of *in vitro* and *in vivo* relationship between tumor progression and angiogenesis in human hepatocellular carcinoma (HCC) over the last ten years (see Semela and Dufour, 2004, for a review) has shown that a high vascular index correlates with poor prognosis. Aggressive tumor growth thus seems to depend on active angiogenesis. Establishment of an intrinsic network is the prelude to indefinite *in situ* growth and metastasis (Fidler and Ellis, 1994).

Antiangiogenesis management should be effective against many tumor and the development of drugs for this purpose is an attractive approach to cancer therapy (Folkman, 1971). Angiogenesis can be impeded in two ways: administration of antiangiogenic compounds and disruption of new vessels by vascular-targeting agents.

Angiostatic treatment of HCC with tyrosine kinase inhibitors, becacizumab (Avastin), thalidomide, IFN-alpha, IL-12, epidermal growth factor receptor inhibitors, TNP-470, tissue matrix metalloproteinase inhibitors and endostatin has been investigated (see Zacharoulis et al., 2005, for a review).

We have previously studied the antiangiogenic activity of vinblastine (VBL) in both *in vitro* and *in vivo* angiogenesis assays (Vacca et al., 1999). At nontoxic doses, all endothelial cell functions involved in angiogenesis, namely proliferation, chemotaxis, spreading on fibronectin and morphogenesis on Matrigel, were impacted in vitro by VBL in a dose-dependent fashion. In vivo, too, the chick embryo chorioallantoic membrane (CAM) assay documented VBL's dose-dependent antiangiogenic activity.

Guba et al. (2002) showed that the macrocyclic antibiotic rapamycin (RAP), a mTOR inhibitor with immune-suppressant activities, inhibits tumor growth and angiogenesis by decreasing the production of vascular endothelial growth factor (VEGF) and markedly inhibiting the endothelial cell response to stimulation with VEGF.

VBL and RAP employ different antiangiogenic mechanisms. VBL at very low doses strikingly and reversibly impacts certain endothelial cell functions and angiogenesis without nonspecific cytotoxic or necrotic damage (Vacca et al., 1999). RAP directly inhibits tumor cell proliferation by cell cycle arrest and targeted reduction of VEGF (Guba et al., 2002).

We have recently used *in vitro* and *in vivo* assays to show that VBL and RAP are synergistically antiangiogenic when administered frequently at low doses in human neuroblastoma (Marimpietri et al., 2005). The present study provides further information obtained by using the chick embryo CAM to illustrate

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the antiangiogenic activity of VBL and RAP, both singly and in combination, in HCC.

Materials and methods

Patients and collection of tumor samples

Tumor samples were obtained from 10 patients who were operated on for HCC, and were minced with a knife in a sterile RPMI 1640 medium at room temperature to obtain several 1-2 mm^3 fragments for the CAM assay. Care was taken to select fragments free from necrosis or bleeding. The remaining fragments were fixed in Bouin's fluid and processed routinely for histopathology. All histological slides were reviewed without knowledge of clinical data and tumors were graded according to Edmondson and Steiner (1954).

Chemotherapeutics

VBL (Lilly France SA, Saint Cloud, Paris, France) was solubilized in phosphate-buffered saline (PBS) and diluted stepwise to 1.25 pM with the culture medium. RAP (ICN Biomedicals Inc., South Chillicothe Road Aurora, Ohio, USA) was solubilized in DMSO and diluted stepwise to 10 pM with the culture medium. These concentrations are the most angiostatic in the CAM assay (Marimpietri et al., 2005).

CAM assay

Fertilized White Leghorn chicken eggs (30 per group) were incubated at 37°C at constant humidity. On day 3, a square window was opened in the shell, and 2 to 3 ml of albumen was removed to allow detachment of the developing CAM. The window was sealed with a glass, and the eggs were returned to the incubator. On day 8, eggs were treated with: i) 1 mm^3 sterilized gelatin sponges (Gelfoam Upjohn, Kalamazoo, MI) placed on top of the growing CAM, according to Ribatti et al. (1997), and loaded with: 1 ml of PBS as negative control; 1ml of PBS containing 500 ng of recombinant fibroblast growth factor-2 (FGF-2) (R & D Systems, Abington, UK) as positive control; fresh biopsies from patients with human HCC minced in RPMI 1640 to obtain 1-2 mm^3 fragments and grafted onto the CAM alone or admixed with 1.25 pM VBL and 10 pM RAP, administered singly or in combination. CAMs were examined daily until day 12 and photographed under a stereomicroscope equipped with a camera and image analyzer system (Olympus Italia, Italy). At day 12 the angiogenic response was evaluated by the image analyser system as the number of vessels converging toward the grafts.

Immunohistochemistry

A murine monoclonal antibody (MAb) against the endothelial cell marker CD31, which is more sensitive than Factor VIII Related Antigen (Horak et al., 1992) (Mab 1A10, Dako, Glostrup, Denmark), was used to stain the HCC biopsy specimens on the CAM surface. Briefly, the grafts and their underlying and immediately adjacent CAM portions were removed, fixed in a modified acetate-free Bouin's fluid and processed for embedding in paraffin. Eight micrometer histological sections were collected on poly-L-lysine-coated slides (Sigma Chemical, St Louis, MO, USA), deparaffined by the xylene-ethanol sequence, rehydrated at a graded ethanol scale in Tris-buffered saline (TBS, pH 7.6), and incubated overnight at 4°C with the MAb (1:25 in TBS) after antigen retrieval by enzymatic digestion with Ficin (Sigma Chemical) for 30 min at room temperature. Immunodetection was performed with alkaline phosphatase-anti-alkaline phosphatase (APAAP; Dako) and Fast red as chromogen, followed by haematoxylin counterstaining. Negative control included an unrelated monoclonal IgG-1 produced by the P3X63/Ag8 mouse secretory myeloma replacing the antibody (Vacca et al., 1993).

Quantification of microvascular density by computer image analysis

Some sections stained with the anti-CD31 were examined at 400x magnifications under a Axioplan 2 microscope (Zeiss, Hallbermoss, Germany) via a Digital Videocamera TK-C 1381 (JVC, Yokohoma, Germany) to an automatic image analyzer KS300 (Zeiss). The whole image was captured under contrast conditions of stabilized light source camera settings, and white balance reference. Immunoreactive endothelial cells gave a gray level clearly different from unlabeled cells and the cumulative area was determined from the differences between these levels. Areas occupied in a given reference area of 0.00625 mm^2 by CD31+ endothelial cells were evaluated in 6 randomly chosen fields in each section. Mean ± S.D. were calculated for all fields. The statistical significance of the differences between the mean values of $CD31^+$ endothelial cells in the control HCC specimens and those treated with VBL and RAP alone or in combination was determined by Student's t test for unpaired data. The final mean values reported in Table 1 refer to all the samples.

Results

Macroscopic examination on day 12 showed that CAM treated with sponges loaded with FGF-2 (positive control) presented numerous allantoic vessels radiating in a spoked wheel pattern towards the sponges (Fig. 1). Tumor specimens grafted onto CAM were surrounded after 96 h by numerous allantoic vessels that invaded the implants (Fig. 1), whereas these vessels were significantly fewer in specimens treated with the drug combination compared to VBL or RAP alone (Fig. 1). Morphometric confirmation was provided by the significantly enhanced angiostatic effect of the

combination compared with each drug alone (Fig. 2).

Histological observations of HCC samples stained with anti-CD31 showed that the areas occupied by microvessels were less numerous when VBL and RAP were added, as compared to HCC alone (Figure 3) and mainly immunoreactive structures were recognizable as endothelial cell clusters in nests or tubes, both transversally (diameter 3-10 µm) or longitudinally sectioned, with or without a small lumen. Morphometric analysis confirmed the morphological observations. The mean values of the microvascular area occupied by CD31+ endothelial cells were significantly lower in HCC specimens treated with VBL and RAP in

Table 1. Morphometric evalution of endothelial cells (EC) immunoreactive to CD31 in hepatocellular carcinoma (HCC) specimens implanted on the chorioallantoic membrane (CAM) treated with vinblastine (VBL) and rapamycin (RAP) alone or in combination.

	Area expressed in μm^2 occupied by $CD31^+$ EC
HCC	520±20
HCC+ VBL	390±27 *
HCC+RAP	310±35 *
HCC+VBL+RAP	240±30 *
PBS	0
FGF-2	500±40

*: $p < 0.001$ vs HCC

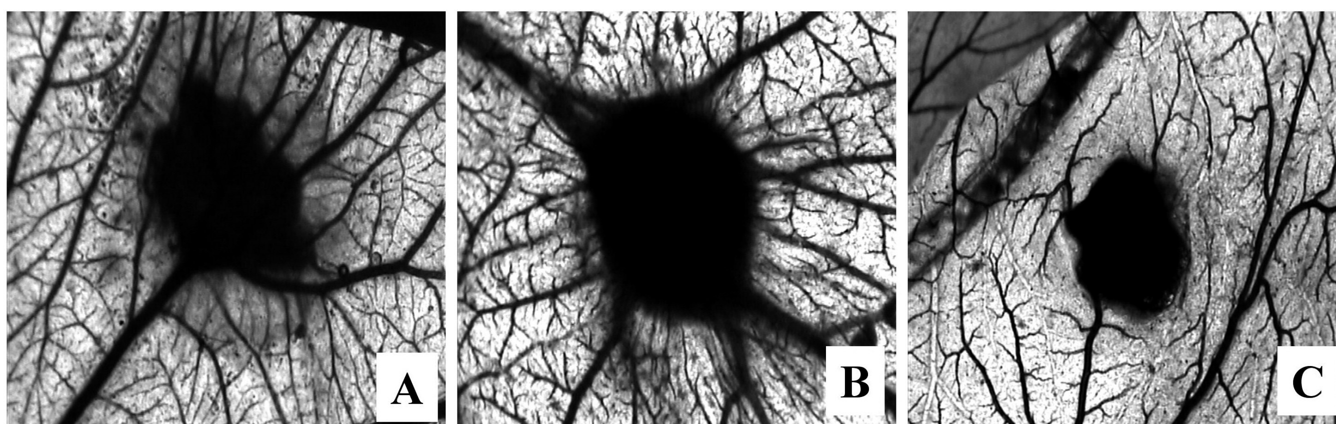

Fig. 1. In vivo CAM assay. Macroscopic observations of the angiogenic response induced by gelatin sponges soaked with FGF-2. Numerous allantoic vessels converge radially in a spoked wheel pattern toward the implant **(A)**. Similar features are recognizable when CAM are treated with HCC biopsy specimens **(B)**. Following treatment with 1.25 pM VBL in combination with 10 pM RAP, fewer vessels invade the specimen **(C)**. x 50

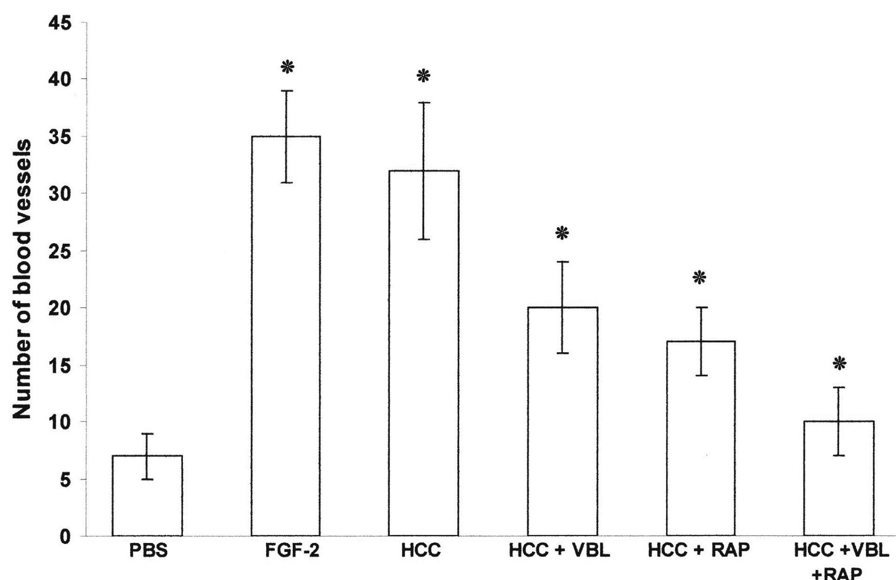

Fig. 2. Morphometric evaluations of the angiogenic response in the CAM assay. The angiogenic response was assessed at day 12 by counting the number of blood vessels entering the grafts, as described in Materials and Methods. HCC biopsy specimens were implanted onto the CAM on day 8 with or without vinblastine (VBL, 1.25 pM) and rapamycin (RAP, 10 pM), alone or in combination. *Statistically significant ($p<0.001$) versus FGF-2.

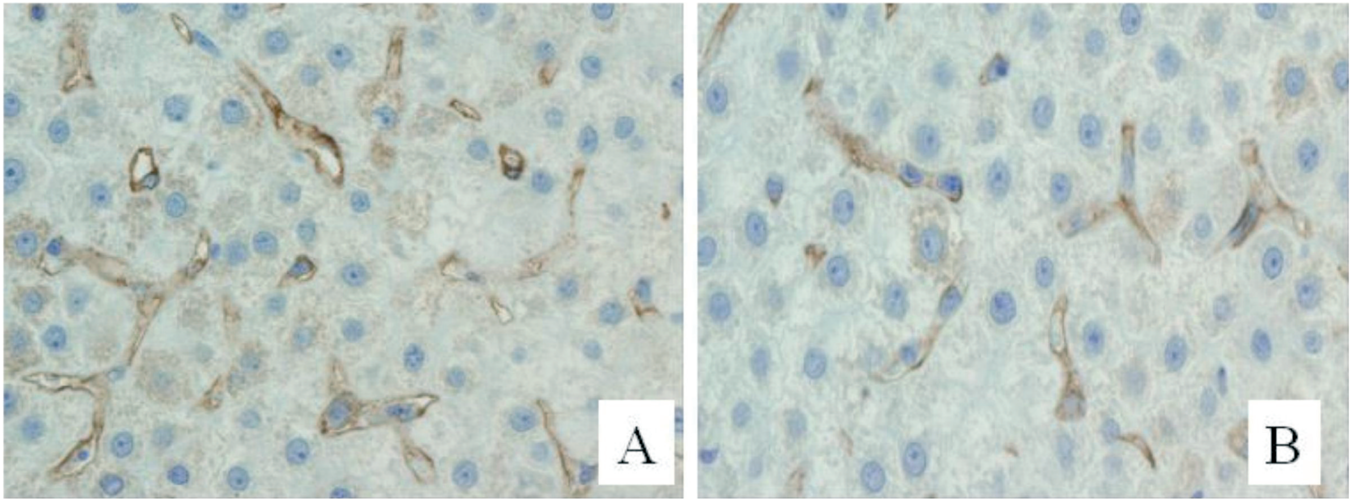

Fig. 3. Immunostaining of HCC specimens implanted onto the CAM surface. Histological sections stained with an anti-CD31 MAb. Note the higher microvascular density of the specimens **(A)** compared to those treated with vinblastine and rapamycin in combination **(B)**. x 400

combination as compared to HCC specimens alone (Table 1).

Tumor cells were disposed in a trabecular pattern (recapitulating liver cell plates), in an acinar pseudoglandular pattern, or can took on a pleomorphic appearance with numerous anaplastic giant cells.

Discussion

The main antiangiogenic agents investigated in clinical trials in HCC are interferon and thalidomide (Zacharoulis et al., 2005). However, the results have been modest. This is the first *in vivo* demonstration that the antiangiogenic effect of a combination of VBL and RAP at very low doses against human HCC is greater that the sum of the effects of the two drugs administered singly. These experimental data agree with the general understanding that antiangiogenic agents targeting a single pathway or growth factor may display preclinical efficacy, but usually confer no clinical benefit. It is clear that combination therapy must be employed.

Tumor angiogenesis does not depend on a single molecule, since many angiogenic inducers and inhibitors are simultaneously expressed. This indicates that targeting a single angiogenic factor may be noteffective. For example, blockade of the VEGF pathway seems too narrow as an approach to inhibit angiogenesis, because a tumor may use a compensatory pathway. It has been suggested that combination of two or more angiogenesis inhibitors may be more effective than single-drug treatment. Combination of inhibitors with different mechanisms of action attacking different biochemical pathways should shut down the multifactor stimulated cascade of biochemical angiogenic processes.

It is not clear which form of combination would be most effective and the most synergistic. The combination of angiogenesis inhibitors with low-dose antiangiogenic chemotherapy constitutes an interesting approach. Enhanced antiangiogenic effectiveness has been reported for the combination of endostatin with angiostatin (Yokoyama et al., 2000), with chemotherapeutic agents (te Velde et al., 2002), and with a VEGFR-2 receptor tyrosine kinase inhibitor (Abdollahi et al., 2003). Bergers et al. (2003) reported that the combination of the tyrosine kinase inhibitors SU5416 (selective for VEGFR-1 and -2) and SU6688 (selective for platelet derived growth factor receptor - PDGFR -) was more effective against the progression of a murine islet cell carcinoma than either agent alone.

Bayless and Davis (2004) have demonstrated that VBL and other microtubule-depolymerizing agents collapse both endothelial cell tubular networks *in vitro* and angiogenic blood vessels *in vivo*, and induce apoptosis after the tube collapses. RAP itself did not induce apoptosis, but produced a cytostatic effect that accelerated anoikis. This combination induced the rapid apoptosis of a higher percentage of cells than each agent alone and the sum of their individual effects.

The possibility that the combination may be responsible for non-angiogenesis-related effects, including direct reduction of tumor cell growth, cannot be ruled out. Even so, our *in vivo* observations indicate that its antiangiogenic activity rapidly inhibits tumor expansion by depriving tumor cells of oxygen and nutrients and limiting their proliferation. Studies to validate the role of the combination of VBL and RAP are needed to assess the prognostic significance of tumor vasculature in HCC.

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