Bevacizumab Impairs Hepatocyte Proliferation after Partial Hepatectomy in a Rabbit Model

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Abstract. *Background: Bevacizumab is used to treat patients with metastatic colorectal cancer, including those who will undergo liver surgery. The effects of this agent on the regenerative capacity of the liver are unclear. We used a rabbit model of partial hepatectomy to assess the effects of bevacizumab on hepatocyte replication and the expression of genes relevant to angiogenesis and proliferation. Materials and Methods: Thirty rabbits underwent 28% hepatectomy. At the end of the procedure, animals were blindly randomized into two groups. A control group was injected i.v. with saline and the other group with bevacizumab at 50 mg/kg. Three rabbits from each group were sacrificed at days 2, 3, 5, 7 and 14 after hepatectomy. Livers were collected and processed. Hepatocyte proliferation was evaluated by Ki-67 immunostaining and apoptosis by caspase-3 activity. Gene expression of Vascular endothelial growth factor (VEGF), Hepatocyte growth factor (HGF) and Inhibitor α of nuclear factor-κB (IκBα) was determined by quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Results: Compared with controls, hepatocyte proliferation in bevacizumab-treated animals was decreased 1.8-fold at day 3, 1.6-fold at day 5 and 2.1-fold at day 14. Neoangiogenesis began after day 5, with a peak of VEGF mRNA evident at day 7 in both groups. Expression of IκBα, a transcriptional target of Nuclear Factor-κB, increased significantly from baseline only in the control group: at day 2, expression was 179% of the day 0 value in controls versus 112% in the bevacizumab group. Expression of HGF and caspase-3 was similar in the two groups and remained stable over time. Conclusion: A single i.v. injection of bevacizumab impaired hepatocyte proliferation in a rabbit model of partial hepatectomy.*

The treatment of colorectal liver metastases (CLM) represents a challenge. Surgical resection is potentially curative, with a reported 5-year survival of 25% to 50% (1), but only 10-20% of patients with CLM are candidates for hepatic surgery (2). There is evidence that neoadjuvant chemotherapy (frequently given in combination with agents targeted at growth factors or their receptors) can render initially unresectable disease amenable to potentially curative surgery. However, it has been suggested that major hepatic resection after such therapy might be associated with increased postoperative morbidity due to a delay in the recovery of hepatic function (3, 4). This could result either from the direct hepatotoxicity of the used agents or, indirectly, from impaired liver regeneration (LR).

LR is a complex phenomenon involving several cell populations (5) and several phases, from a priming phase controlled by interleukin-6 and transcription factors, through a proliferation phase, controlled by growth factors to a growth termination phase with ultrastructural changes. Because of the complex interplay between cell populations and growth factors, *in vivo* models are the most relevant means of studying liver regeneration.

Angiogenesis also plays a fundamental role in LR. While early hepatocyte proliferation leads to the formation of avascular, hypoxic clusters of hepatocytes (6, 7), it is angiogenesis that reconstructs the sinusoidal capillaries that allow the supply of hepatocytes with oxygen and growth factors (8). Neoangiogenesis is mainly controlled by Vascular endothelial growth factor (VEGF). Secretion of VEGF and its two receptors (VEGFR-1 and 2) is increased during LR by hepatocytes, which stimulate sinusoidal endothelial cell proliferation (9, 10). Inhibition of cells and cell signalling that contribute to neoangiogenesis is associated with impairment of liver regeneration, as has been demonstrated with angiostatin (11), TNP-470 (an inhibitor of endothelial

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cells) (8) and rapamycin [an inhibitor of the mammalian target of rapamycin (mTOR) signalling pathway] (12). It is therefore entirely possible that blocking the VEGF pathway could impair LR.

Bevacizumab (Avastin®) is a monoclonal antibody against VEGF approved for first-line treatment of metastatic colorectal cancer (13, 14). Its use in conjunction with conventional chemotherapies increases response and resectability rates (15, 16). Recently, two groups have investigated the effect of bevacizumab on LR after portal vein embolization, with conflicting results (17, 18). The effect of bevacizumab on LR has not been studied in conventional murine models of hepatectomy due to its humanized structure, bevacizumab does not inhibit mouse/rat VEGF. It does, however, inhibit rabbit VEGF, although with a lower affinity than for human VEGF (19). The rabbit represents an alternative to conventional animal models and has been used in pre-clinical studies (20-22). Our preliminary work found that use of hepatic lobe weight was not accurate enough to monitor LR; in the present study, we used a rabbit model involving 28% partial hepatectomy to assess the effect of bevacizumab on LR, focusing on hepatocyte proliferation.

Materials and Methods

Animals. Twelve-week-old female New Zealand White rabbits were purchased from Eurolap (Argentré-du-Plessis, France). Rabbits were maintained in a temperature-controlled room on a 12-hour light-dark cycle, with free access to water and standard chow. Animals were acclimatised to the laboratory conditions for one week prior to the experiments. The experimental protocols followed the guidelines of the Canadian Council on Animal Care and were approved by the Ethics Committee of the Léon Bérard Centre (Lyon, France).

Experimental procedure. The rabbits were anesthetized with a mix of ketamine (1 ml), xylazine (0.6 ml) and glycopyrrolate (0.2 ml). All rabbits underwent resection of the left lateral lobe without hepatic pedicle clamping. A preliminary experiment on 20 rabbits had shown that this type of resection was easily reproducible and equivalent to resection of 27.9% of whole-liver weight.

Immediately after hepatectomy, rabbits (n=30) were blindly randomized to two groups: the experimental group received an intravenous injection of 50 mg/kg bevacizumab and controls *i.v.* saline. This dose was chosen assuming the lowest affinity of bevacizumab for rabbit VEGF and corresponded to 10 mg/kg injected in humans. Bevacizumab was given within 5 min of the end of surgery. Bevacizumab was provided by Roche (Neuilly-sur-Seine, France). To respect the manufacturer's instruction that bevacizumab be administered at a concentration lower than 16.5 mg/ml, the drug was infused in a volume of 9-12 ml over 15 min. All surgical procedures were performed between 9 and 11 A.M. to respect the nycthemeral cycle (23). Any animals dying prematurely were replaced. LR was monitored for 14 days because it appears that LR is almost complete by that time in rabbits (24).

Collection of liver tissue and biological samples. Immediately after hepatectomy, the left lateral lobe was collected. Tissue was divided into three specimens. Two were frozen in liquid nitrogen and conserved at −20˚C. The third was fixed in 10% buffered formalin. Tissues in the remnant liver were collected at the time of sacrifice, with three rabbits from each group being sacrificed at days 2, 3, 5, 7 and 14 after hepatectomy. Biological samples were collected before hepatectomy, the following day and at sacrifice for the measurement of blood cell count, platelet count, prothrombin time, factor V, total bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), gamma-glutamyl transpeptidase (γGT) and alkaline phosphatase (ALP).

Hepatocyte proliferation. Tissues fixed in 10% buffered formalin were used for immunohistological analyses. The 4-μm liver sections were de-paraffinized and endogenous peroxidase activity blocked with H_2O_2. The sections were then incubated with purified primary monoclonal mouse antibody against Ki-67 (Ventana Medical Systems, Tucson, AZ, USA) at 37˚C for 32 min and with secondary biotinylated anti-mouse IgG (BA 2001; Vectorlabs, Burlingame, CA, USA). Streptavidin was used as the chromogen substrate. The proportion of hepatocytes positive for Ki-67 was calculated for five randomly selected high-power (×400) fields. Each slide was photographed. The Ki-67 labeling index was calculated by an observer blinded to the treatment group of the concerned animal.

Caspase-3 activity. Caspase-3 activity was assayed using the caspase 3/CPP32 Fluorimetric Assay Kit (Gentaur Biovision, Brussel, Belgium). Frozen liver tissue was homogenized in 300 μl of caspase-3 lysis buffer. After centrifugation, 100 μg of supernatant proteins were incubated at 37˚C in caspase-3 reaction buffer containing 100 μM of the caspase-3/7-specific fluorogenic substrate Acetyl-Aspartyl-Glutamyl-Valyl-Aspartic acid α-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC). Caspase activity, expressed as fluorescence units per milligram of total protein per minute, was monitored at an excitation wavelength of 405 nm and an emission wavelength of 510 nm, and calculated from a 1 h kinetic cycle reading on a spectrofluorimeter (Infinite F500; Tecan, Männedorf, Switzerland). The protein content of samples was quantified with the Bradford assay. Four samples were analyzed from each sacrificed animal and analyses were carried out in duplicate.

Real-time quantitative RT-PCR. VEGF, Hepatocyte Growth Factor (*HGF*) and Inhibitor-α of Nuclear Factor-κB (*IκBα*) mRNA were analyzed to determine gene expression after hepatectomy for each phase of liver regeneration. VEGF was analyzed in order to determine angiogenic activity, HGF as a marker of the proliferation phase, and IκBα, a transcriptional target of Nuclear Factor-κB (NF-κB), as a marker of the priming phase.

Total RNA was extracted from 50-100 mg of snap-frozen liver tissue using NucleoSpin® RNA II Kit (Macherey Nagel, Düren, Germany). Contaminant genomic DNA was removed by on-column DNA digestion with recombinant DNase I. Total RNA was measured by spectrophotometry (Nanodrop 1000; Thermo Scientific, Wilmington, DE, USA) and quality of RNA was evaluated by 1% agarose gel. RT-PCR reactions were performed with iScript® cDNA Synthesis Kit (Biorad, Ivry-sur-Seine, France). One microgram of total RNA was reverse-transcribed using the following program: 25˚C for 5 min, 42˚C for 30 min and 85˚C for 5 min. Target transcripts were amplified from 50 ng of cDNA in a LightCycler® 2.0 apparatus (Roche Applied Science, Indianapolis, IN, USA), using the LightCycler FastStart DNA Master SYBR

Table I. *Sequence of primers and annealing temperatures used in Polymerase Chain Reactions (PCR).*

Target	Gene name	Primary sequences	Annealing temperature (˚C)
HGF	Hepatocyte growth factor	F 5'-CTTGCTCGTTGTGAAGGTG-3'	
		R 5'- CGTGTTGGAATCCCATTGAC-3'	58
IκBα	Inhibitor α of Nuclear factor-κB	F 5'-GCTGACCTTGGAGAACCT-3'	
		R 5'-CATAGGGCAGCTCATCCTC-3'	58
VEGF	Vascular endothelial growth factor	F 5'-CGAGGAGTTCAACGTCAC-3'	
		R 5'-CTTGTCACATCTGCATTCAC-3'	55
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F 5'-ATGTTCCAGTATGATTCCACC-3'	
		R 5'-CTTCCCGTTGATGACCAG-3'	55

Green I Kit (Roche Applied Science). Amplification was undertaken by initial denaturation at 95˚C for 10 min, followed by 45 reaction cycles (5 s at 95˚C, 5 s at annealing temperature, 4 s at 72˚C). Expression of target genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA, used as the housekeeping gene. The number of target transcripts, normalized to the housekeeping gene, was calculated using the comparative CT method. A validation experiment was performed to demonstrate that the efficiencies of target and housekeeping genes were approximately equal. Amplification specificity was tested by means of melting curves and electrophoresis on 2% agarose gel. Primer sequences were designed with PerlPrimer software (25). The primer sets used are summarized in Table I.

Statistical analysis. All data are expressed as the mean±standard deviation. The Wilcoxon matched-pairs signed-ranks test was used to evaluate differences from baseline within each animal. Differences between the values determined at each time point in the two study groups were assessed using Mann–Whitney *U*-test. *p*-Values less than 0.05 were considered to be significant. Statistical analysis was performed using the R software (v2.11) (Revolution Analytics, Palo Alto, CA, USA).

Results

Surgical data. The mean duration of surgery was similar (31.7±4.4 min in the control group *versus* 32.6±3.8 in the bevacizumab group). Two deaths occurred in each group: the two deaths among control animals were unexplained, while those in the bevacizumab arm were due to gastric perforation and hepatic lobe necrosis. There was one case of wound dehiscence in each group.

Serum parameters. An increase in SGOT and SGPT levels (less than two-fold) was found in both groups at 48 h and 72 h post-surgery. Levels of total bilirubin, γGT, ALP, LDH, prothrombin rate, factor V and platelets were stable and similar in both groups at all time points.

Hepatocyte proliferation during liver regeneration. Hepatocyte proliferation was assessed as the percentage of cells positive for Ki-67. In the early post-hepatectomy period, the proliferation profile in the control and bevacizumab groups was similar: both showed an increase by day 2 in the proportion of Ki-67-positive hepatocytes and a peak at day 3, followed by a decrease. However, the proportion of Ki-67-positive cells was significantly lower in the bevacizumab-treated group than in controls at day 3 (17±10.9% *versus* 30.6±17.2%, $p<0.05$), at day 5 (4.9±2.1% *versus* 7.7±2%, $p<0.003$) and at day 14 (2.2±1.3% *versus* 4.7±2.6%, $p<0.05$). These data suggest that bevacuzimab reduced hepatocyte proliferation from the early phase of LR after partial hepatectomy (Figure 1).

Hepatic cell apoptosis. Apoptosis during LR was determined by caspase-3 activity. Partial hepatectomy did not result in any consistent pattern of change in caspase-3 activity at any time point in either group (Figure 2).

VEGF mRNA. To assess the possible effect of bevacizumab on neoangiogenesis in LR, we evaluated *VEGF* gene expression. At day 7, the normalized expression level of *VEGF* mRNA was significantly greater than baseline in both groups (23.2±14.2 *vs*. 11.4±6.2 in controls and 48±34.3 *vs*. 8.2±5 after bevacizumab treatment) (Figure 3). The level of *VEGF* expression at day 7 in control animals was not significantly different from that in bevacizumab-treated rabbits ($p=0.172$). Indeed, there were no significant differences in *VEGF* gene expression between treatment groups at any time point. By day 14, the expression of *VEGF* mRNA had returned to a physiological level in both groups.

HGF mRNA. To investigate the proliferation phase of LR, we analyzed *HGF* gene expression. Expression of *HGF* mRNA during LR did not change from baseline and showed no significant difference at any time point between the two groups (data not shown).

IκBα. To assess the activation of the transcription factor NF-κB, we analyzed expression of the *IκBα* gene, a direct transcriptional target of NF-κB. IκBα was significantly induced in the control group but not in bevacizumab-treated animals: at day 2, expression was 179% of baseline in

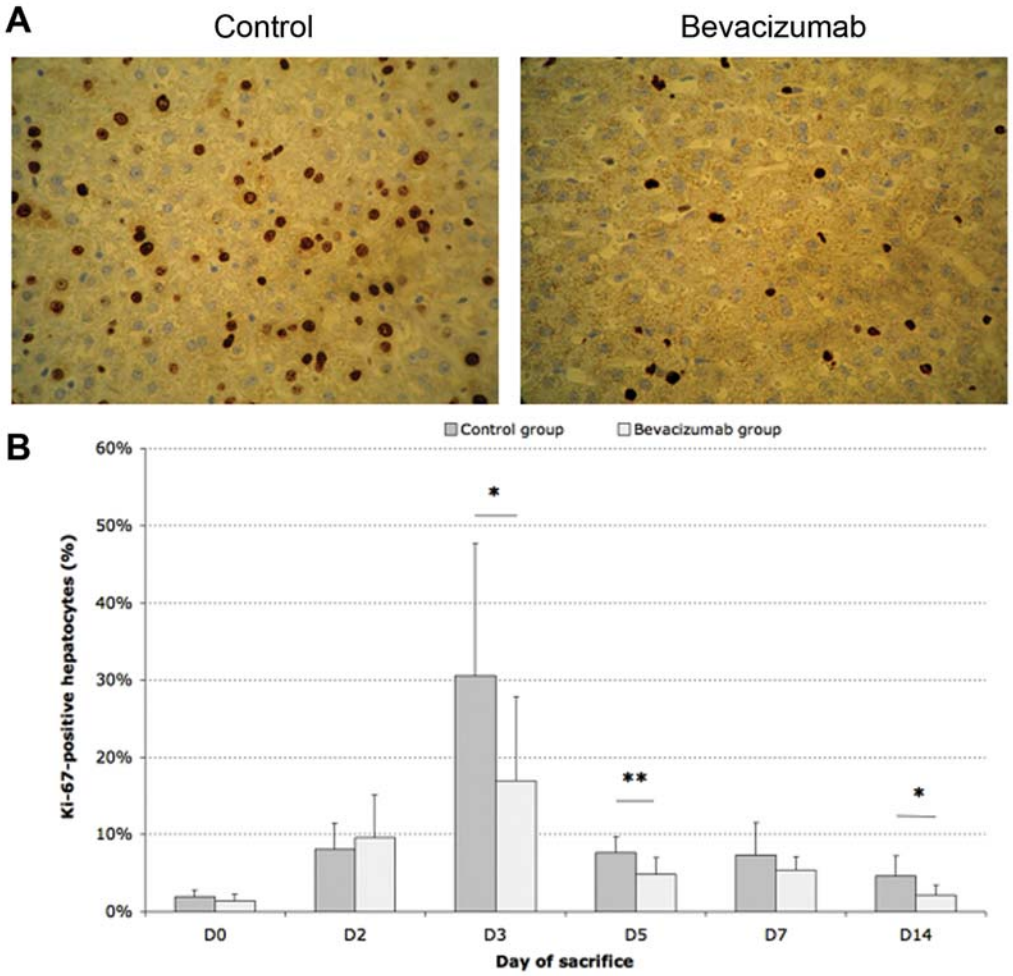

Figure 1. *Effect of bevacizumab on hepatocyte proliferation post-hepatectomy. A: Representative Ki-67 staining of both groups at day 3. A lower proportion of Ki-67-positively stained hepatocytes can be seen in the bevacizumab-treated group. Magnification, ×400. B: Percentage of proliferating hepatocytes determined by Ki-67 immunohistochemistry in both groups. Hepatocyte proliferation was lower in the bevacizumab-treated group at day 3, 5 and 14, *p<0.05, **p<0.005; n=15 in each group.*

control animals but only 112% of baseline in the bevacizumab group. The increase in IκBα gene expression at day 2 was significantly greater in control than in bevacizumab-treated animals ($p<0.05$) (Figure 4). There were no significant differences at any other time point.

Discussion

Bevacizumab is a commonly used antiangiogenic therapy for metastatic colorectal cancer, and may have particular value in patients in whom downstaging can render initially unresectable liver metastases amenable to surgery. The fact that this antibody against VEGF is 93% humanized prevents its evaluation in murine models unless the mice express a humanized form of VEGF (26). Pre-clinical studies were indeed performed with a surrogate marker (muMAb A4.6.1). However, the antibody is active against rabbit VEGF (27), and rabbit models have been widely used in experimental studies (20-22).

We developed an original rabbit model of 28% partial hepatectomy which has the advantages of being standardized and reproducible without clamping of the hepatic pedicle. Our preliminary work found that hepatic lobe weight was not a good tool for monitoring liver regeneration with considerable variation between animals, up to 100% (data not shown). We therefore based our study on hepatocyte proliferation as a means of quantifying liver regeneration. Masson *et al*. had demonstrated that a 30% hepatectomy, similar to our model, was sufficient to induce hepatocyte proliferation in rats (28). Our aim was to study the effect of bevacizumab on LR after hepatectomy. We showed that a single injection of bevacizumab immediately following

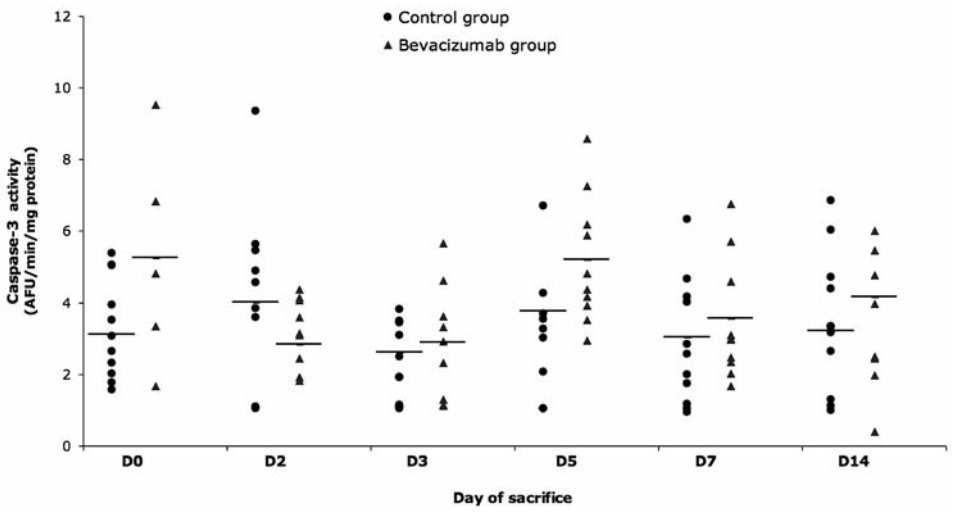

Figure 2. *Effect of bevacizumab on liver apoptosis determined by caspase-3 activity after partial hepatectomy. Caspase-3 activity showed no significant change from the pre-operative value in either group. There were no significant differences between groups at any time point. n=12 samples for each time point and in each group. AFU: Arbitrary fluorescence unit.*

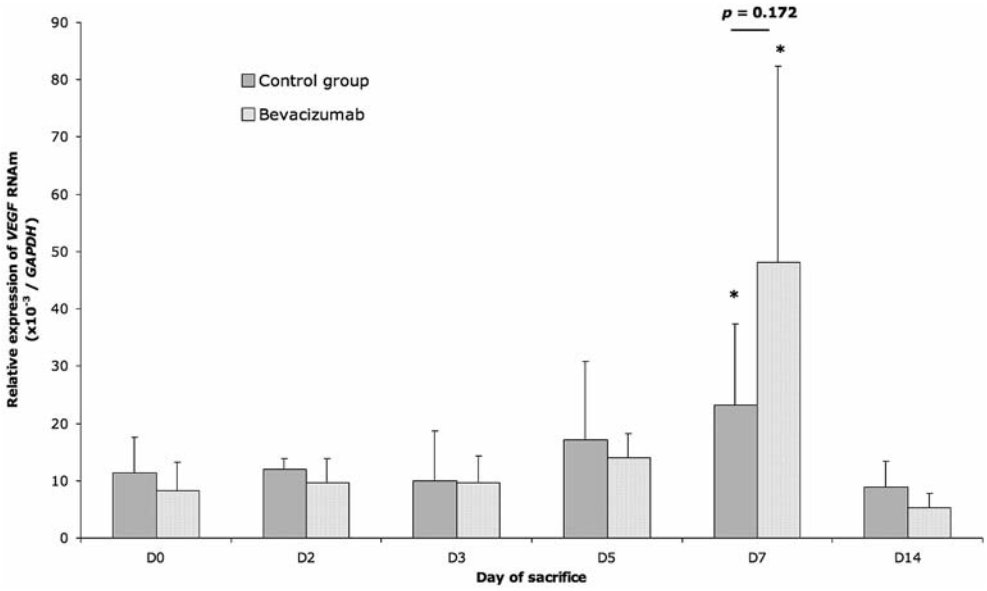

Figure 3. *Normalized expression of Vascular endothelial growth factor (VEGF) mRNA after hepatectomy. No significant difference was found between the two groups. At day 7, expression of VEGF was significantly greater than baseline in both groups. n=6 samples for each time point and in each group, except for D0 with n=30. *p<0.05 compared to baseline.*

surgery was associated with a decrease in hepatocyte proliferation as assessed by Ki-67 immunostaining (29).

Our hypothesis was that bevacizumab would impair regeneration through its antiangiogenic activity during the later stages of LR when new blood vessels are being formed. Thus we expected a delayed impairment in hepatocyte proliferation. However, an effect on proliferation was evident early, and was significant from day 3 after hepatectomy, *i.e.* seemingly prior to neoangiogenesis. We analyzed *VEGF* expression to determine the onset of angiogenesis and showed a significant increase in *VEGF* mRNA from day 7. This confirmed that hepatocyte proliferation was decreased in the bevacizumab-treated group before neoangiogenesis and hence that the effect was independent of the agent's antiangiogenic activity. In an attempt to elucidate drug

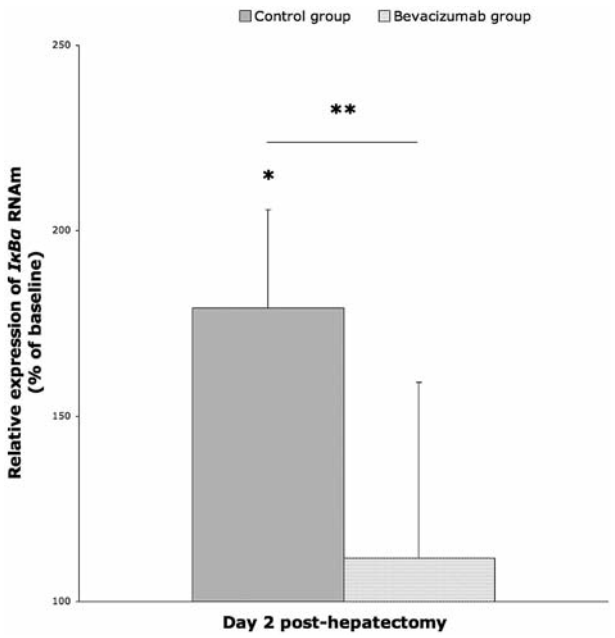

Figure 4. *Expression of Inhibitor α of Nuclear Factor-κB (IκBα) at day 2 relative to baseline in the control and bevacizumab groups. The increase from day 0 was significant for control animals (79±27%) but not for those treated with bevacizumab (12±47%) (*p<0.005). The day 2 level of expression in control animals was significantly higher than that in animals which had been treated with bevacizumab. **p<0.05.*

effects during the earlier priming and proliferation phases of LR, we investigated expression of the gene for *HGF*, the most important mitogenic factor involved in hepatocyte proliferation. We did not find any rise in the expression of *HGF* mRNA, although it is possible that the peak in expression might have preceded day 2, when the first animals were sacrificed. Expression of transcription factors such as NF-κB or Signal Transducer and Activator of Transcription 3 (STAT3) during the priming phase is brief and difficult to measure. The activation of transcription factors is caused by post-translational modifications (30) and cannot be assessed by gene expression. We therefore measured the expression of IκBα, a transcriptional target of NF-κB which reflects its activation (31). We showed an increase in IκBα expression in control animals but not in those administered bevacizumab. Hence impaired hepatocyte proliferation in the bevacizumab group may be caused by a lack of activation of NF-κB.

The rabbit model of hepatectomy did not allow us to study LR at the macroscopic level. However, we showed a clear decrease in hepatocyte proliferation; and the cellular level is the most appropriate tool for monitoring LR since increased liver mass can result from hepatocyte hypertrophy in the absence of mitosis (32).

The effects of VEGF pathway inhibition on LR were investigated by Van Buren *et al*. (33) who found impaired regeneration following administration of an antibody against VEGFR-2. Their aim was to mimic the use of bevacizumab; and the authors argued the mitogenic effects of VEGF were all mediated by VEGFR-2. However, LeCouter *et al*. (34) demonstrated that VEGFR-1 binding to endothelial cells is associated with secretion of hepatotrophic factors such as *HGF* and interleukin-6 (IL-6). VEGFR-1 is able to modify the extracellular matrix (ECM) and is a VEGFR-2 decoy receptor (35). Furthermore, Ding *et al*. (36) showed that blockade of VEGFR-2 clearly impaired LR but this blockade only partially reflects the activity of bevacizumab.

The early effect on hepatic proliferation which we showed here could be caused by the absence of hepatotrophic factor secretion by endothelial cells and by de-regulation of changes to the ECM. Protease remodeling of ECM is essential for the initiation of LR and the destruction of the ECM is necessary to allow division of hepatocytes, and the release and activation of HGF (37, 38). VEGF increases expression of matrix metalloproteinases (39) and of urokinase (40). The early effect we observed could be explained by the partial inactivation of proteases, leading to an environment less suited to neoangiogenesis and the division of hepatocytes.

VEGF and VEGFR-1 are survival factors for endothelial cells both *in vitro* and *in vivo* (41-43), and one of our hypotheses was that blockade of VEGF would impair LR through a pro-apoptotic effect on endothelial cells. However, there was no difference between the two groups in caspase-3 activity (although a limitation was that we studied total liver caspase-3 activity and hence total liver apoptosis, rather than apoptosis specific to endothelial cells).

Bevacizumab was administered at the dose of 50 mg/kg, which, given its lower affinity for rabbit rather than human VEGF, corresponded to the 5-10 mg/kg dose used clinically in colorectal cancer. The pharmacokinetics of bevacizumab in rabbits are described on the European Medicines Agency website (19). At a dose of 10 mg/kg, the initial half-life is short (5.82 hours) but the relatively long terminal half-life of 5.5 days guaranteed the blockade of VEGF throughout the study. Administration was immediately postoperative (rather than in advance of surgery) to avoid interference from rabbit antibodies to bevacizumab which develop between 8 and 11 days after the start of dosing. In patients, an interval of 4 to 6 weeks between the last bevacizumab administration and surgery is recommended. However, the half-life in man is approximately three weeks, so there may still be bevacizumab activity – and potential side-effects – at the time of surgery. Even so, liver surgery after bevacizumab therapy has not been associated with increased postoperative morbidity (44-47).

To the best of our knowledge, our study is the first to demonstrate impaired hepatocyte proliferation caused by bevacizumab. However, the animal model used did not

represent the full range of factors, such as cirrhosis, steatosis or chemotherapy-associated hepatotoxicity, present in patients and which are known to influence the regenerative capacity of the liver (48). Potential interactions between bevacizumab and these factors are not well-understood. For example, sinusoidal obstruction syndrome caused by oxaliplatin (Eloxatin) impairs liver regeneration (49), but bevacizumab might reduce the occurrence and severity of such lesions (50, 51). Establishing the full picture relating to the possible influence of bevacizumab on LR in patients with colorectal cancer undergoing partial hepatectomy is therefore complex and will require well-conducted clinical studies.

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