Blood pressure rise following angiogenesis inhibition by bevacizumab. A crucial role for microcirculation

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Arterial hypertension (HT) has been reported in all studies involving bevacizumab, an antiangiogenic agent designed to target vascular endothelial growth factor (VEGF). The mechanism underlying bevacizumab-related HT is not yet clearly understood. As far as endothelial dysfunction and microvascular rarefaction are hallmarks in all forms of HT, we tested the hypothesis that anti-VEGF therapy could alter the microcirculation in nontumor tissues and, thus, result in an increase in blood pressure (BP).

We used intravital video microscopy to measure dermal capillary densities in the dorsum of the fingers. Microvascular endothelial function was assessed by laser Doppler flowmetry combined with iontophoresis of pilocarpine (acetylcholine analogue). All measurements were carried out in 18 patients before and after a 6-month treatment with bevacizumab (mean cumulative dose: 3.16 ± 0.90 g).

Mean BP was increased after 6 months of therapy compared with baseline, from 129 ± 13/75 ± 7 mmHg to 145 ± 17/82 ± 7 mmHg for systolic BP and diastolic BP, respectively (P < 0.0001). Compared with the baseline, mean dermal capillary density at 6 months was significantly lower (75 ± 12 versus 83 ± 13/mm²; P < 0.0001), as well as pilocarpine-induced vasodilation (P < 0.05).

Thus, bevacizumab treatment resulted in endothelial dysfunction and capillary rarefaction; both changes are closely associated and could be responsible for the rise in BP observed in most patients.

Key words: angiogenesis, capillary rarefaction, endothelial function, hypertension, microcirculation

Introduction

Angiogenesis inhibitors associated with chemotherapy improve the prognosis for patients with several types of cancer [1]; however, adverse effects have been described and several angiogenesis inhibitors have been implicated in the development of hypertension (HT) [2–8]. Early studies with bevacizumab offered the first clue as to the prevalence of HT with angiogenesis inhibitors; serious HT requiring multitherapy was noted in 11%–16% of the bevacizumab-treated cohorts [9], and some dramatic presentations have been reported [10, 11]. The mechanism of angiogenesis inhibitors-related HT remains poorly understood (see review in Kamba and McDonald [12]); in one of the few studies dedicated to this question, Veronese et al. [13] reported that serum catecholamine, renin, and aldosterone levels were not affected in patients receiving antivascular endothelial growth factor (VEGF) therapy, lessening the likelihood that the onset of HT had an adrenergic or a renovascular etiology. Mir et al. [14] suggested that bevacizumab-induced VEGF inhibition could be responsible for cholesterol emboli syndrome which may account for bevacizumab-induced acute complications, including HT, arterial emboli, gastrointestinal perforations (related to mesenteric ischemia), and late-onset renal dysfunction. Whereas the origin of cardiovascular adverse effects of anti-VEGF therapies is still debated, we hypothesized a more general explanation; VEGF both enhances endothelial nitric oxide synthase (eNOS) activity and up-regulates the message and protein levels of the VEGF receptor in human endothelial cells. Nitric oxide (NO) generation is therefore an essential component of the response pattern to angiogenic growth factors [15]; thus VEGF blockade and NO inhibition could result in several common abnormalities. ‘Endothelial dysfunction’ and ‘microvascular rarefaction’ can be observed in most situations with increased cardiovascular risk.

Endothelial dysfunction is a nonspecific injury to the endothelium linked to all cardiovascular risk factors. Endothelial dysfunction disrupts the homeostatic balance, thereby predisposing the vessel wall to vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, prooxidation, thrombosis, impaired coagulation, vascular inflammation, and atherosclerosis. Thus, endothelial dysfunction can be regarded as a marker of cardiovascular risk associated with significant increases in morbidity and mortality [16].
A consistent finding in HT has been that of microvascular rarefaction [17], defined as a reduced spatial density of microvascular networks. Under resting conditions, a part of the microvascular network of most organs remains closed, constituting a flow reversal for adaptation to increased metabolic needs. When merely defined as an abnormally low spatial density of capillaries, rarefaction can be of either functional or structural origin [18]. ‘Functional rarefaction’ would refer to an abnormal prevalence of anatomically existing but noncirculating microvessels. ‘Structural rarefaction’ can be established either by quantitative histology or, in vivo, by the observation of microvascular beds under conditions of maximal vasodilatation.

We hypothesized that the bevacizumab-induced blockade of VEGF, and thus the inhibition of the NO pathway, could induce endothelial dysfunction and microvascular rarefaction in relation to blood pressure (BP) rise.

patients and methods

Twenty consecutive Caucasian patients with metastatic colorectal cancer and eligible to receive bevacizumab concurrently with chemotherapy were enrolled in the study. Bevacizumab (5 or 7.5 mg/kg, mean cumulative dose: 3.16 ± 0.9 g) was administered every 2 or 3 weeks associated with concomitant chemotherapies [FOLFIRI (FOL: folinic acid (leucovorin), F: fluorouracil (5-FU), and IRI: irinotecan (Camptosar)): 6 patients or XELOX (XE: Xeloda® (Capcitabine) and LOX: Oxaliplatin): 14 patients]. The institutional review board approved the protocol and informed consent was obtained for each patient before inclusion. All patients had histologically confirmed metastatic colorectal cancer and decision to initiate bevacizumab chemotherapy was taken by a multidisciplinary staff. Patients were not considered for the study if their life expectancy was <6 months and if they were undergoing any concomitant protocol. African patients were excluded because capillaroscopy is only possible in lighter pigmented subjects [19]. Doses of bevacizumab and chemotherapy were recalculated if a patient’s weight changed by at least 10% during the study. Standard dose modifications of chemotherapy were permitted in patients experiencing treatment-related side-effects. Patients were evaluated at baseline and after 6 months of treatment with bevacizumab.

vascular assessment

‘Intravital video capillaroscopy’ allows visualization of the skin in real time; in combination with video and computer technology, capillaroscopy generates high-contrast numeric images of dermal capillaries [20, 21]. Capillary density is the number of capillaries per unit area of skin. Depending on the skin area under investigation, the capillaries will appear as black dots, if they are perpendicular to the surface, or as lines if the capillaries are lying obliquely (Figure 1).

Capillaroscopy was carried out using a standardized validated technique detailed elsewhere [20]. Briefly, individuals were studied between 9 and 11 a.m. after an overnight fast. The capillaroscopy studies were done in a temperature-controlled quiet room (21–24°C) after the subjects had had 20 min of semisupine rest. Patients were seated with the forearm and hand supported at heart level.

Figure 1. Typical video microscopic image of capillaries in the phalanx skin during venous occlusion. The blue rectangle represents a calibrated 1-mm² surface. The structural capillary density is the number of capillary structures in this surface area.

Video microscopy was carried out with an epi-illuminated optic fiber microscope containing a 100-W mercury vapor lamp light source and a M200 objective (Moritex, micro-ScopernanMS-500C, Tokyo, Japan); final ×200 magnification was used. Microscopic images were transferred for storage and analysis to a PC with a video image converter (Microvision, Evy, France). The skin of the dorsum of the middle phalanx of the nondominant hand was examined. An ~3 × 3 mm skin area on the middle third of the phalanx was defined. Four microscopic fields (1 mm² each) were randomly chosen in this area for recording and measurements. Each field was analyzed offline by two independent investigators (HD and BIL) blinded for any information concerning the patient and time of procedure. For each subject, images were acquired at baseline (to count the total number of visible, i.e. circulating, capillaries). The baseline capillary density represents the number of functional (circulating) capillaries per dermal surface unit. Then, the capillary density was measured during venous congestion (by applying a cuff to the wrist and maintaining a 50-mmHg inflating pressure for 2 min) in order to get the maximal response of all existing capillaries and to assess the structural capillary density. Intraobserver and interobserver repeatability were 4.3% and 5.9%, respectively (data not shown).

laser Doppler flowmetry and iontophoresis of pilocarpine. Endothelium-dependent and -independent vasodilatation of the forearm skin microcirculation was evaluated by iontophoresis and skin heating in combination with laser Doppler flowmetry (LDF) [22]. A laser beam penetrates the skin and a fraction of the light is backscattered by moving blood objects and undergoes a frequency shift according to the Doppler principle, generating a signal proportional to tissue perfusion. Forearm skin blood perfusion was measured by means of a LDF apparatus (Periflux PF5000, Perimed, Stockholm, Sweden) with the following characteristics: 780 nm wavelength, 10 Hz–19 kHz bandwidth, 0.1 s time constant, and 32 Hz sampling frequency. Calibration was carried out using colloidal latex particles whose Brownian motion provides the standard value. The LDF outputs were recorded continuously with an interfaced computer (Dell E521, Round Rock, Texas) equipped with Perisoft dedicated software, allowing measurement of LDF output (mV).

After recording video capillaroscopy images, the LDF probe was applied to the anterior part of the forearm with a plastic holder. The skin temperature was monitored throughout and maintained at 33°C by the same LDF heating probe.

Baseline skin blood perfusion was defined as the mean value recorded during a 4-min time period. In order to investigate the endothelium-dependent vasodilation, iontophoresis of graduated doses of pilocarpine,
Iontophoresis is a noninvasive standard method of drug application that allows the local transfer of electrically charged substances across the skin by using a small electric current. The electrical potential difference actively causes ions in the solution to migrate according to their electrical charge. A battery-powered iontophoresis controller (Periiont 328, Perimed, Sweden) was used to provide a DC for iontophoresis. Pilocarpine was delivered with an anodal current. The battery-powered iontophoresis controller was connected to the LDF apparatus and provided the chosen current to an electrode chamber (PF 383, Perimed, Jarfallan, Stockholm Sweden), allowing the passage of the laser light to the skin.

Pilocarpine (800 µl of 2% solution) was used to fill the chamber. The positive lead of the current source was connected to the chamber, and the negative lead was attached to a conductive hydrogel pad on the wrist, which served as the reference electrode. We used a delivery current of 10 mA and administered three successive doses of pilocarpine for 10 s with an interval of 2 min between each dose in order to achieve a plateau of the response following each delivery of pilocarpine. Lastly, the laser probe was heated to 44°C for 5 min and we recorded the maximal response to local skin heating, i.e. the endothelium-independent maximal vasodilatation. Figure 2 represents typical recordings of laser Doppler flowmetry under skin heating, i.e. the endothelium-independent maximal vasodilatation. The red line represents the skin temperature maintained at 33°C during baseline and pilocarpine administrations and heated at 44°C for recording of the maximal skin flow under local vasodilatation.

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**Tables and Figures**

**Table 1. Patients’ characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD (range)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>59 ± 9 (45–79)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74 ± 75 (42–104)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.4 ± 4.8 (18.8–35.1)</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>78 ± 18</td>
</tr>
<tr>
<td>Serum glucose (g/l)</td>
<td>1.16 ± 0.3</td>
</tr>
<tr>
<td>Bevacizumab cumulative dose (g)</td>
<td>3.16 ± 0.9 (1.57–4.8)</td>
</tr>
</tbody>
</table>

SD, standard deviation.

**Results**

**Patient characteristics**

A total of 20 patients (12 males) with metastatic colorectal cancer and good performance status were enrolled. The main demographic and clinical characteristics of the patients are listed in Table 1. Two patients were not assessed 6 months after inclusion: one died within the period and one was unable to undergo the hemodynamic work-up. At inclusion, three patients were treated for HT (two with an angiotensin-converting enzyme inhibitors and one with a calcium channel blocker). During the 6-month follow-up, a calcium channel blocker was added to the antihypertensive treatment for two more patients. Diagnosis of type II diabetes was known in three patients.

**Changes in blood pressure**

Mean BP significantly increased after 6 months of therapy compared with baseline from 129 ± 13/75 ± 7 mmHg to
145 ± 17/82 ± 7 mmHg for systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively ($P < 0.0001$ for both parameters). Figure 3 shows the individual scatter plot of SBP and DBP at baseline and after 6 months follow-up. At 6 months, SBP increased by at least 10 mmHg in 10 patients, and 7 patients experienced an increase in SBP of at least 15 mmHg. SBP decreased (−1 mmHg) in one patient. DBP increased by at least 5 and 10 mmHg in 10 and 8 patients, respectively. The maximal bevacizumab-associated SBP rise was 41 mmHg and was observed in one of the baseline hypertensive patients. Mean body weight remained stable during the study period (73.7 ± 15.3 versus 74.2 ± 15.3 kg).

changes in capillary density
The capillary density was calculated as the number of capillaries per square millimeters (cap/mm$^2$). Table 2 and Figure 4 show values of structural capillary density (during venous occlusion) at inclusion and after 6 months of treatment. Functional and structural capillary densities at 6 months were significantly lower: 75 ± 12 versus 84 ± 13 (functional) and 81 ± 11 versus 90 ± 13 cap/mm$^2$ (structural) ($P < 0.0001$ for both parameters).

The increase in capillary density during venous occlusion (capillary response) was comparable before and after treatment (7.7% ± 3.4% versus 9.4% ± 3.9%, respectively; $P = 0.10$).

changes in forearm skin perfusion
After 6 months of treatment, the mean value of endothelium-dependent response induced by pilocarpine was significantly lower compared with baseline (347% ± 58% versus 556% ± 84%; $P < 0.05$). Figure 5 shows the individual scatter plot of laser flow response to pilocarpine at baseline and after 6 months follow-up. At 6 months, the endothelial function decreased in most of the patients. The reduction in pilocarpine response was more pronounced in patients with higher endothelium response at baseline condition. In three patients with low baseline indexes of pilocarpine response, the endothelial response was slightly increased at the end of the follow-up period.

correlation between capillary rarefaction and cumulative dose of bevacizumab
Figure 6 shows the relationship between the change in capillary density and the cumulative dose of bevacizumab administrated during the study period ($R^2 = 0.43; P = 0.008$).

discussion
HT is a common feature during treatment with bevacizumab and other antiangiogenic drugs
Drugs that induce HT do so by stimulating pressor responses, increasing extracellular volume, and/or decreasing vascular compliance. The list of compounds capable of increasing BP is quite extensive; it would seem that vascular endothelial growth factor (VEGF) antagonists (or angiogenesis inhibitors) can now be added to an already lengthy list of compounds linked to the onset of a hypertensive state.

In the study of Hurwitz et al. [5], patients were treated with bevacizumab and HT was seen in 22% of cases (versus 8.3% in controls). Recent meta-analysis confirmed that there was a significant dose-dependent increase in risk of HT in patients who received bevacizumab [12]. HT uniformly decreased with the cessation of therapy. Documentation of complete resolution of these adverse effects, however, was not possible because of multiple complications relating to death and commencement of other therapies.

Several lines of reasoning would suggest a likelihood of BP decreasing initially with angiogenic growth factor therapy and increasing with angiogenesis inhibitor therapy. First, a number of studies in animals and humans have noted a drop in BP with angiogenic growth factor administration [4]. In the VIVA Trial (VEGF in Ischemia for Vascular Angiogenesis), infusions of recombinant human VEGF were accompanied by falls in SBP of up to 22% [23]. Secondly, VEGF both
enhances eNOS activity and up-regulates the message and protein levels of VEGF receptors in human endothelial cells; thus, NO generation is an essential component of the response pattern to angiogenic growth factors. Finally, angiogenic growth factors offer a strong stimulus for the construction of new capillaries and the recruitment of endothelial progenitor cells [24, 25]. This enhancement of angiogenesis/arteriogenesis can be expected to decrease vascular resistance [26].

Veronese et al. [13] carefully described the significant increases in BP observed with sorafenib, a specific inhibitor of C-Raf and B-Raf which also inhibits several important tyrosine kinases involved in tumor progression including VEGF [27]. Further, these investigators gathered strong supporting evidence suggesting that activation of HT-producing neurohumoral pathways and/or overt volume expansion are not major contributing factors to sorafenib-related increase in BP. This raises the possibility of other mechanisms, such as microvascular functional and structural alterations resulting in higher BP.

### Table 2. Mean values of SBP, DBP, and capillary density at baseline and after 6 months

<table>
<thead>
<tr>
<th></th>
<th>n = 18</th>
<th>Baseline</th>
<th>6 months</th>
<th>P (paired Student’s t-test)</th>
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<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>129 ± 6</td>
<td>145 ± 6</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75 ± 6</td>
<td>82 ± 6</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Basal capillary density (cap/field)</td>
<td>84 ± 6</td>
<td>75 ± 6</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Maximal capillary density (cap/field)</td>
<td>90 ± 6</td>
<td>81 ± 6</td>
<td>0.0001</td>
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SBP, systolic blood pressure; DBP, diastolic blood pressure.

**Figure 4.** Scatter plot of maximal capillary density (during venous occlusion) at baseline and after a 6-month treatment with bevacizumab.

**Figure 5.** Scatter plot of vasodilatory response to pilocarpine, marker of endothelium function, at baseline after 6 months of treatment with bevacizumab.

**endothelial dysfunction and capillary rarefaction: hallmarks of arterial HT**

The microcirculation in HT is characterized by substantial structural and functional changes. The most consistent finding in individuals with primary HT is the smaller number of arterioles and capillaries compared with those of normotensive control subjects. Studies have reported microvascular structural rarefaction in experimental hypertensive models and tissues, including skeletal muscle, intestine, and skin beds [26]. Interestingly, structural rarefaction can be detected at a very young age (4 weeks) in spontaneously hypertensive rats [28]. Microvascular rarefaction has been indicated to contribute to increased systemic vascular resistance observed in essential HT. By hampering oxygen delivery, they may participate in the pathogenesis of hypertensive end-organ damage. Moreover, dermal capillary density, both at baseline and during venous occlusion, appears to be inversely correlated with brachial BP levels in both normotensive and hypertensive subjects [20].
Data concerning the myocardial microcirculation deserve a separate mention because of the concomitant myocardial hypertrophy. Reduced myocardial capillary density has been largely documented in adult hypertensive animals [29], possibly reflecting an inability of microcirculatory growth (through angiogenesis) to keep up with the progressive increase in myocardial cell size.

The most common tests of endothelial function are on the basis of the noninvasive measurement of the endothelium-dependent vasodilatation in response to pharmacological or physiological stimuli. Noon et al. [22] have investigated the technique of pilocarpine transdermal iontophoresis combined with laser Doppler flow measurement as a noninvasive tool to assess cholinergic dilatation in a large number of patients. This widely validated technique allowed us to estimate the endothelial function and thus to evidence endothelium dysfunction in patients receiving bevacizumab for 6 months. We used pilocarpine as a muscarinic analogue of acetylcholine. Actually, both agents could have different effects in different vascular beds. Patil and Stearns [30] reported that pilocarpine produces vascular relaxation in the rat aorta by its competition with spasmogens like phenylephrine, oxymetazoline, vasopressin, or latanoprost and not by blocking NO-mediated vasodilation. Furthermore, the endothelial function is predominantly mediated by NO in the large arteries. In small resistance arteries, such as those investigated during dermal vasodilatation in response to iontophoresis, vasodilation is predominantly mediated by a dilator prostanoid rather than by NO generation [22].

Finally, some limitations of the present study should be addressed, such as the lack of a clear demonstration of the causative role of bevacizumab in the occurrence of endothelial dysfunction. We cannot exclude that BP rise by itself could induce similar alterations. Moreover, the absence of a control arm in our study does not allow us to determine to what extent reported changes in the present study are due to bevacizumab itself or to cumulative doses of cytotoxic therapy or both.

Hurwitz et al. [5] and others (review in Kamba and McDonald [12]), however, have clearly demonstrated a dose-dependant incidence of HT in patients receiving bevacizumab.

**anti-VEGF therapy deeply alters endothelial function and capillary density**

NO is an important signaling compound synthesized from l-arginine by the NO synthase enzymes. NO plays a crucial role in the vascular homeostasis, including the vasomotor tone and the balance between proliferation and apoptosis in the normal and pathological vessels. eNOS has been implicated in tumor angiogenesis and the maintenance of vasodilation in tumor blood vessels [31–33] and the role of NO in tumorigenesis have been widely investigated [34, 35]. NO has been shown to act subsequent to angiogenic factors such as vascular endothelium growth factor receptor (VEGF) [36, 37], which activates eNOS in vascular endothelial cells [38]. Thus, the therapeutic blockade of VEGF by bevacizumab likely inhibited the NO pathway and then, endothelial function. This is in complete agreement with our present results, demonstrating significantly altered responses of the dermal circulation after treatment with bevacizumab. In a recent communication, Steeghs et al. [39] investigated the increase in BP following the therapeutic inhibition of VEGFR2. As VEGF enhances eNOS activity, the reported decrease in flow-mediated dilatation was interpreted as a reduced bioavailability of NO. For these authors, the likely hypothesis for the profound vascular effects is a reduced number of microvessels (i.e. rarefaction). As the microcirculation is largely responsible for peripheral resistance, the rarefaction associated with VEGFR2 inhibition was attributed to increased peripheral resistance leading to HT. In parallel, there is a close relationship between NO and apoptosis in normal [40] and tumor vessels [41]. Baffert et al. [42] investigated the regression of VEGF-dependent capillaries in a simple vascular network of the mouse trachea. Vessel narrowing and cessation of blood flow were among the earliest changes in tracheal capillaries after inhibition of VEGF signaling. Some normal capillaries regress after VEGF inhibition. The proportion affected decreased with age. Cessation of blood flow was an early sign of regression of capillaries after VEGF inhibition, accompanied by endothelial cell apoptosis. Similar features have been observed in the eye’s pupillary membrane (a transient ocular capillary network) from the young rat where cessation of blood flow precedes endothelial cell apoptosis [43]. According to these results, it may be hypothesized that regression and apoptosis of microvascular endothelial cells could be involved in the dermal capillary rarefaction observed in patients treated with bevacizumab. An important issue from this present study concerns the effect of bevacizumab not only on the pathological and ‘switched’ vessels feeding the tumor area but also on the ‘normal’ arterioles and capillary, far from the tumor zone. Our present hypothesis is reinforced by recent clinical results; Rixe et al. [44] and Maitland et al. [45] reported that in small series of patients with metastatic renal cell carcinoma receiving sunitinib or sorafenib, the appearance of HT was predictive of the antitumoral activity.

The clinical value of the relationship between microvascular rarefaction, endothelial dysfunction, HT, and response to...
treatment remains to be established with other antiangiogenic therapies in future larger and prospective studies.

conclusions

The bevacizumab-induced blockade of VEGF resulted in marked alterations in endothelial function and dermal capillary density. This can be explained by a nonspecific blockade of the NO pathway downstream of the angiogenic factor VEGF. As far as endothelial dysfunction and microvascular rarefaction have been constantly observed in essential HT, bevacizumab-induced increase in arterial pressure can be related to or even explained by capillary rarefaction and alteration in endothelial function in the whole systemic vascular network. We may suggest that larger studies evolving measurements of microvascular structural and functional parameters along with a better evaluation of BP using ambulatory BP techniques in patients undergoing anti-VEGF drugs could confirm the present results. Furthermore, the decrease in dermal capillary density and/or the rise in BP could represent a promising surrogate marker of antitumoral efficacy of angiogenesis inhibitors.

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references