Cancer Chemother Pharmacol (2011) 68:1135–1143
DOI 10.1007/s00280-011-1589-9

ORIGINAL ARTICLE

Aflibercept-mediated early angiogenic changes in aggressive B-cell lymphoma

Martha Romero · Josette Brière · Cédric de Bazelaire · Christophe Lebœuf · Li Wang · Philippe Ratajczak · David Sibon · Eric de Kerviler · Catherine Thieblemont · Anne Janin

Received: 12 November 2010 / Accepted: 9 February 2011 / Published online: 5 March 2011

Abstract
Purpose Diffuse large B-cell lymphoma is the most common and one of the most aggressive lymphomas in adults. The standard, R-CHOP-based treatment needs improvement. There has been recent interest in anti-angiogenic therapy, but its cellular effects in lymphoma are little known.
Methods In five aggressive B-cell lymphoma patients, we analyzed the microvessel and lymphoma cell changes after Aflibercept, an angiogenic inhibitor. Under ultrasonography, we performed two biopsies, one before any treatment and one two hours after Aflibercept, before R-CHOP. Using ultrasonography, immuno-histochemistry, double immuno-fluorescent staining, and electron microscopy, we compared the early changes induced by Aflibercept to the early changes induced by R-CHOP in three control patients.
Results We identified microvessel damage in the five patients treated with Aflibercept but not in the three patients treated with R-CHOP. Two hours after Aflibercept, microvessel damage was focal, with severely damaged microvessel sections close to normal ones in the same area; different stages of microvessel damage were concomitantly found, with an increase in relative necrosis area in three cases. There was no difference in necrosis or relative microvessel area after R-CHOP. For lymphoma cells, the two biotherapies induced similar changes, with increase in apoptosis but not in proliferation.
Conclusion We identified focal microvascular damage, necrosis, and apoptosis of lymphoma cells in aggressive B-cell lymphoma as soon as 2 h after Aflibercept. This suggests that there is more than one mechanism associated with the early effect of anti-angiogenic therapy in lymphoma.

Keywords Anti-angiogenic therapy · Aggressive B-cell lymphoma · Microvascular damage · Tumor cell death

M. Romero · J. Brière · C. Lebœuf · L. Wang · A. Janin (✉)
Laboratoire de pathologie, U728, AP-HP-Hôpital Saint-Louis, 1, Avenue Claude Vellefaux, 75010 Paris, France
e-mail: anne.janin728@gmail.com

M. Romero · J. Brière · C. de Bazelaire · C. Lebœuf · L. Wang · P. Ratajczak · E. de Kerviler · C. Thieblemont · A. Janin
INSERM U728, 75010 Paris, France

M. Romero · J. Brière · C. de Bazelaire · C. Lebœuf · L. Wang · P. Ratajczak · D. Sibon · E. de Kerviler · C. Thieblemont · A. Janin
Université de Paris, VII Diderot, 75010 Paris, France

C. de Bazelaire · E. de Kerviler
Radiology Departement, AP-HP-Hôpital Saint-Louis, 75010 Paris, France

D. Sibon · C. Thieblemont
Hemato-Oncology Departement, AP-HP-Hôpital Saint-Louis, 75010 Paris, France

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common and one of the most aggressive lymphomas in adults, accounting for 31% of non-Hodgkin's lymphomas [1]. The standard of care for patients with DLBCL is based on a cytotoxic regimen, with cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP), and the addition since 2002 of R-CHOP, an anti-CD20 antibody, which has improved response to treatment, overall survival (OS), event-free survival (EFS), and progression-free survival

(PFS) [2, 3]. However, the outcome of these patients remains poor with 5-year OS, EFS, and PFS at 58, 47. and 54%, respectively [4]. Therefore, there is great interest in developing new therapies to improve these results. One of these strategies is to target tumor angiogenesis.

Angiogenesis in lymphoma has clearly been associated with adverse outcome, and particularly the expression of VEGF and VEGF-R in lymphoma cells [5, 6] and high levels of VEGF in blood and in tissue [7]. Moreover, VEGF may play a direct role in lymphoid cell development via an autocrine loop [8]. Angiogenic microvessels, different from normal microvessels, are the proposed targets for anti-angiogenic agents. Anti-angiogenic drugs may act by inhibiting synthesis of angiogenic proteins by cancer cells, neutralizing angiogenic proteins, inhibiting endothelial receptors for angiogenic proteins, or directly inducing endothelial cell apoptosis. There are antibodies and small molecules capable of targeting angiogenic growth factors (VEGF, bFGF) and their receptors (VEGFR and PDGFR) [9]. However, the cellular effects of anti-angiogenic therapy in microvessels in lymphoma have not been studied so far.

Aflibercept, a recombinant fusion protein, with human VEGF receptor extracellular domain fused to the Fc portion of human IgG1, binds to VEGF-A, VEGF-B, PIGF1, and PIGF2, and inactivates circulating VEGF. Recently, Aflibercept (AVE0005, VEGF-Trap) was evaluated in combination with R-CHOP in B-cell lymphoma in a phase I open-label dose escalation trial (GELA-TCD10173-EudraCT number: 2007-003737-16). Using US-guided biopsies before treatment and 2 h after the first injection of Aflibercept, before R-CHOP, we aimed to study the early tumor changes induced by this anti-angiogenic agent in five aggressive B-cell lymphomas. We compared the results with those obtained in three patients biopsied before and 2 h after the injection of R-CHOP.

Design and methods

Patients

Eight patients with newly diagnosed aggressive B-cell lymphoma had superficial lymph nodes. For each patient, two ultrasonography-guided core needle biopsies were performed, at the same time points, (i) before any treatment and (ii) two hours after the first Aflibercept injection, before R-CHOP, for the five patients included in TCD10173 or (iii) two hours after the first R-CHOP injection, for the three control patients.

The 5 patients who received Aflibercept (AVE005, VEGF Trap-Sanofi-Aventis) at the dose of 6 mg/kg, via intravenous infusion, prior to R-CHOP: Rituximab (375 mg/m2 D1) combined with CHOP (Prednisone (p.o) 40 mg/m2 D1 to D5, Doxorubicin (i.v.) 750 mg/m2 D1, Vincristine (i.v.) 1.4 mg/m2 (max 2 mg), Methotrexate (I.T.) 15 mg D1) every 3 weeks for 8 cycles (EudraCT or IND number: 2007-003737-16), were 3 men and 2 women. The 3 control patients treated with R-CHOP alone were 2 men and 1 woman. Approval for these studies was obtained from the Institut Universitaire d'Hematologie-Hopital Saint Louis Institutional Review Board. All patients were informed on the investigational nature of the therapeutic study, and all gave written informed consent, in accordance with the regulations of the Institut Universitaire d'Hematologie-Hopital Saint Louis.

For the 8 patients overall, the median age at the time of diagnosis was 60.6 ± 17 years (range 26–82 years). Performance status ranged from 0 to 2, Ann Arbor stage was I for one patient, II for three patients, III for two patients, and IV for the other two. The number of extra-nodal sites varied from 0 to 2, and the number of nodal sites from 1 to 5. LDH level was high in seven patients. The International Prognostic Index (IPI) was 1 for two patients, 2 for four patients, 3 and 4 for the other two (Table 1).

According to WHO 2008 criteria [10], one case was Burkitt Lymphoma, the other seven cases were diffuse large B-cell lymphoma (DLBCL), centroblastic morphologic variant. Their immuno-histochemical subgroup was established according to the Hans algorithm [11] combining the analyses of CD10, BCL6, and IRF4/MUM1 expression. Six cases were CD10 and BCL6 negative and MUM1 positive. Therefore, they were classified as belonging to the non-germinal center subgroup DLBCL.

US-guided biopsies

The two series of core needle biopsies: (i) before any treatment and (ii) 2 h after the first Aflibercept injection and before R-CHOP for five patients or 2 h after the first R-CHOP injection for three patients, were performed under ultrasonography (US) guidance. All vital structures surrounding the lymph node were first identified under US to ensure a safe procedure. The largest lymph node was then targeted, taking into account optimal access for both the patient and the radiologist. When the first biopsy was performed, the lymph node localization was recorded in order to find it easily for the second biopsy.

The largest dimensions of the lymph nodes in short axis were recorded. Lymph node echogenicity relative to muscles was assessed using a simple scale (4: hyper-, 3: iso-, 2: hypo-, 1: anechogenic). Macro vascularization was quantified using a binary scale (0: no vessel in color Doppler, 1: vessels seen in color Doppler).

Percutaneous biopsies were performed under sterile conditions and local anesthesia, with a 14 gauge

Table 1 Clinical features of the 8 aggressive B-cell lymphoma patients

	Aflibercept					Rituximab		
Patient	1	2	3	4	5	6	7	8
Sex	F	M	M	M	F	M	F	M
Diagnosis	DLBCL	DLBCL	DLBCL	DLBCL	Burkitt L	DLBCL	DLBCL	DLCBL
Age (years)	64	64	75	49	61	26	64	82
Performance status	1	2	0	1	0	1	0	0
Ann Arbor stage	IV	III	II	II	II	III	IV	I
Extranodal involvement (n =)	2	1	1	1	0	0	2	0
Nodal involvement (sites n =)	2	5	1	1	1	1	5	3
LDH	High	High	High	High	High	Normal	High	High
IPI score	3	4	2	1	2	1	2	2

F female, *M* male, *DLBCL* diffuse large B-cell lymphoma, *LDH* lactate dehydrogenase, *IPI* international prognostic index

semi-automated biopsy gun to obtain 20-mm tissue cores (SuperCore^{TM} Biopsy Instrument, Angiotech, FL, USA). A coaxial technique was used in all cases, as it enables multiple samples to be taken without repeating the biopsy procedure. Tilting the device before introducing the biopsy guide enabled the operator to collect multiple lymph node samples in both center and periphery, and eliminate heterogeneous patterns of infiltration or transformation within a given lymph node [12]. For each biopsy procedure, six cores of tissue were obtained, two were AFA-fixed and paraffin-embedded, two were glutaraldehyde-fixed and epoxyresin-embedded, and two were snap-frozen.

Pathological analyses

Microvessel study was performed using two complementary methods: (i) scanned whole slides analyzed using Cell software (Olympus-Tokyo), on five different fields at ×400 magnification. The ratio of CD31-stained (Dako-Denmark) microvessel surface area to tumor surface area provided the relative microvessel area; (ii) ultrastructural analysis, performed on a Hitachi-H7650 electron microscope, focused on endothelial cells, basement membranes and pericytes. For this microvessel study, three cases of reactive lymphoid hyperplasia were included as controls.

Cell proliferation was assessed using the Ki67 (Immunotech-France) index on 5 different fields at ×400 magnification, under a Provis-AX-70 microscope with a 0.344-mm^2 field size at ×400 magnification.

The type of cell death was characterized using electron microscopy, according to the Nomenclature on cell death 2009 [13]. Quantification was performed: (i) for necrosis on scanned whole slides (Dotslide-software) and expressed as the ratio between necrotic areas and tumor area; (ii) for apoptosis of lymphoma cells in five different fields at ×400 magnification on semi-thin sections; (iii) for apoptosis of endothelial cells in five different fields at ×400 magnification using double immuno-fluorescent staining for CD31(Abcam) and cleaved Caspase-3(Cell signaling).

Statistical analyses

Measures were expressed as the mean number per field plus or minus SEM (standard error of the mean). Differences between analyses before and after treatment were assessed using the Wilcoxon signed-rank test. Results for comparisons were regarded as significant if the 2-sided P was < 0.05. Statistical analyses were performed using S-plus2000 software (MathSoft Inc-Berkeley-CA).

Results

Lymph node changes in ultrasonography B mode and Color Doppler

Ultrasonography in B mode for the five patients under Aflibercept showed that enlarged lymph nodes before treatment were cervical in 2 cases, supraclavicular in 2 cases, and axillary in 1 case; the largest short axis diameters ranged from 15 mm to 24 mm (mean 21 ± SEM 1.6 mm). All three patients under R-CHOP had before treatment enlarged cervical lymph nodes; the diameters ranged from 20 mm to 24 mm (mean 21.3 ± SEM 0. 8 mm). No significant change in tumor size was found after treatment in any of the eight patients.

On biopsies performed before Aflibercept, lymph nodes were hypoechogenic in 4 patients and isoechogenic in 1 patient. On biopsies performed before R-CHOP, lymph nodes were hypoechogenic in the three patients. A significant change in echogenicity was observed in only one

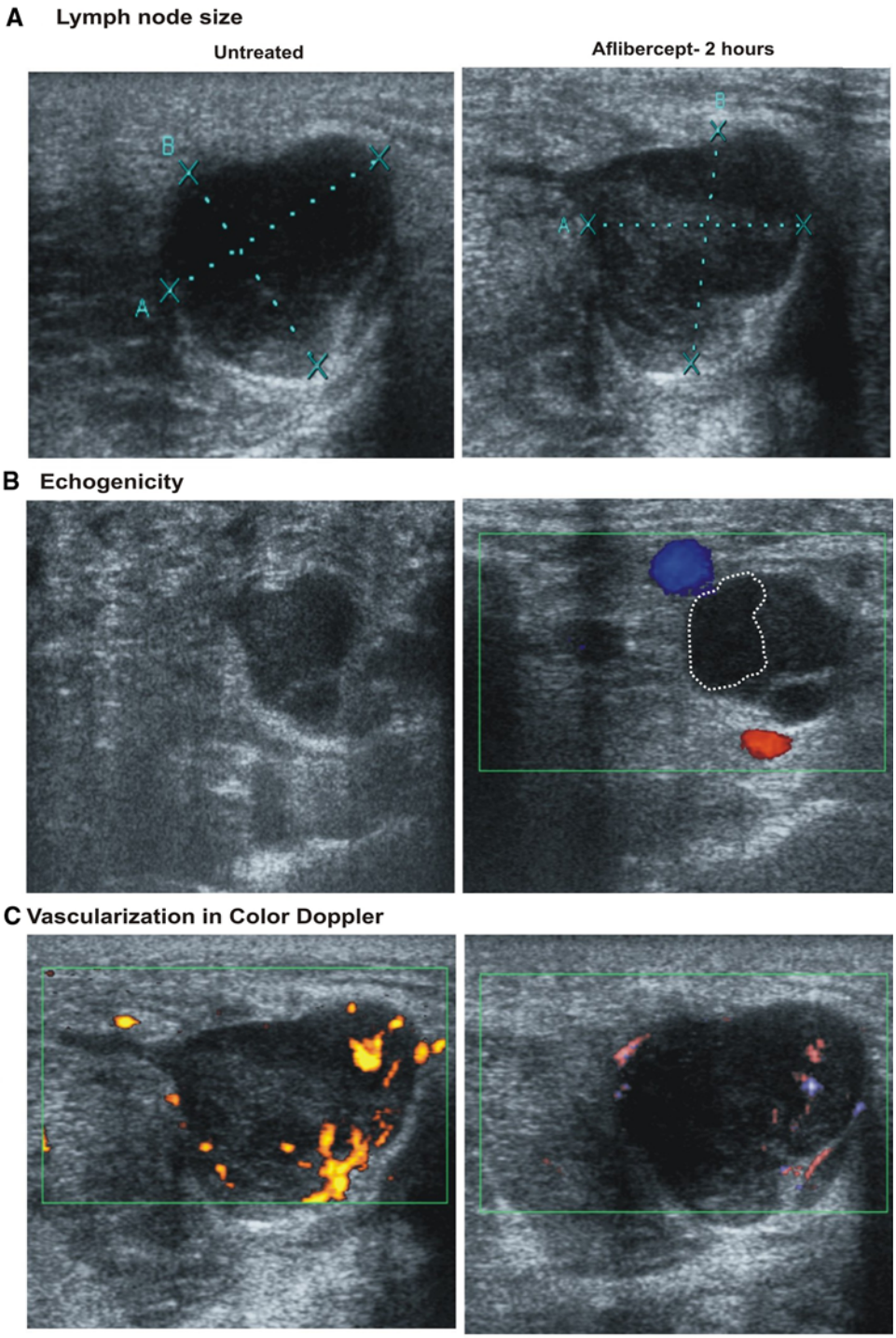

Fig. 1 Lymph node ultrasonographic aspect in B mode and Color Doppler before and 2 h after Aflibercept. **a** No significant tumor size change was found after 2 h of Aflibercept. **b** No significant change in echogenicity was observed after treatment except for one patient (case 4) with a hypoechogenic lymph node and anechogenic areas interpreted as necrosis (surrounded by broken *white lines*). **c** With Color Doppler, no change in vascularization was observed after Aflibercept, with the exception of perfusion decrease in case 2, a patient who rapidly responded to treatment

patient, two hours after Aflibercept (case 4). In this case, a hypoechogenic lymph node displayed anechogenic areas, interpreted as necrosis (Fig. 1).

A significant Color Doppler signal was recorded in one patient before treatment (case 2). In the other patients, lymph nodes displayed weak vascularisation signal. No change was observed after treatment, except in one patient after Aflibercept (case 1) who had a decrease in perfusion.

During the two biopsy procedures performed for each patient, before any treatment and 2 h after treatment, it proved possible to take six different tumor samples without inducing any immediate or delayed side effect.

Table 2 Radiological and pathological features of the 8 aggressive B-cell lymphoma patients

		Aflibercept 2 h					R-CHOP 2 h		
Case		1	2	3	4	5	6	7	8
Lymph node size (mm)	B	23	20	25	15	22	21	24	20
	A	23	18	24	15	20	18	20	19
Echogenicity	B	2	2	2	2	3	2	2	2
	A	2	2	2	1	3	2	1	1
Color Doppler vascularization	B	0	1*	0	0	0	0	0	0
	A	0	0*	0	0	0	0	0	0
Microvessel area CD31 (%)	B	12 (±0.9)*	3.8 (±0.2)*	2 (±0.2)	2.2 (±0.1)	1.9 (±0.1)	4.8 (±0.2)	3.9 (±0.2)	4.8 (±0.2)
	A	3.6 (±0.2)*	1.9 (±0.1)*	1.8 (±0.2)	2 (±0.3)	3.2 (±0.1)	4.7 (±0.1)	4 (±0.3)	4.8 (±0.1)
Cell proliferation Ki67 (%)	B	76.6 (±1.8)	86.8 (±1.16)	85.2 (±1.3)	94.2 (±0.7)	97.8 (±0.13)	76.4 (±0.3)	96.5 (±0.2)	97.5 (±0.2)
	A	64.4 (±1.8)	84.4 (±1)	84.9 (±0.6)	93 (±0.6)	95.6 (±0.2)	65 (±0.4)	78 (±0.2)	97.3 (±0.1)
Necrosis area (%)	B	0.3**	4.2**	0.2	0.1**	0.4	0	0	0
	A	63.4**	78.5**	3.4	32.3**	0.6	0	0	0
Apoptotic cell counts	B	2.7 (±0.4)*	6.8 (±1.1)*	5 (±0.4)*	12.5 (±1.6)*	61.2 (±1.3)*	4 (±0.3)*	43.4 (±1.2)*	37.3 (±2.1)
	A	5.7 (±0.6)*	13.4 (±0.8)*	12.3 (±1.6)*	29.9 (±3.3)*	68.2 (±0.7)*	5.2 (±0.2)*	82.2 (±5.2*	38.9 (±1.8)
Apoptosis/ mitosis ratio	B	0.27 (±0.05)*	1.4 (±0.21)*	0.22 (±0.01)*	1.28 (±0.18)*	8.3 (±1.3)	0.31 (±0.1)*	6.8 (±0.9)*	36 (±4.2)
	A	1.8 (±0.5)*	2.9 (±0.3)*	0.69 (±0.09)*	4.8 (±0.8)*	9 (±0.88)	4.5 (±0.53)*	21.2 (±5.1)*	39.8 (±0.9)

B Before (Aflibercept 2 h for cases 1-5, RCHOP 2 h for cases 6-8), *A* After (Aflibercept 2 h for cases 1–5, RCHOP 2 h for cases 6–8); Echogenicity : *scale 1* anechogenic, *scale 2* hypoechogenic, *scale 3* iso echogenic, *scale 4* hyperechogenic; Vascularization in color Doppler: Binary scale: 0 = no vessel in color Doppler, 1 = vessels seen in color Doppler. * $P < 0.05$; ** $P < 0.01$

Tissue damage was found as early as 2 h after Aflibercept

On lymph node sections, quantitative analyses performed on virtual slides before and after treatment (Table 2) showed a significant difference ($P < 0.01$) for necrosis areas only in patients having received Aflibercept (cases 1, 2, and 4), and not in those having received R-CHOP. However, in this small series of patients, there was no relationship between the extent of necrosis two hours after Aflibercept and the clinical evolution after one year of follow-up.

Systematic ultrastructural study enabled us to demonstrate, as early as two hours after Aflibercept, different stages of vascular damage, with focal alterations in the microvessel network. These microvessel changes were not found two hours after R-CHOP.

Aflibercept-induced microvessel damage was focal, and with different lesional stages

Before any treatment, microvessel changes were present in the eight lymphoma patients, at the ultrastructural level, in the form of: (i) tortuous, enlarged blood vessels (ii) intraluminal bridging (iii) focal thinning of vascular walls (iii) pericytes loosely attached. These changes were not found in reactive lymphoid hyperplasia.

The relative microvessel area before any treatment varied from 1.93 to 11.42% (mean 4.3 ± SEM 1) (Table 2). It showed a significant diminution (more than 50%) after Aflibercept in cases 1 and 2 (Fig. 2a). There was no significant diminution after R-CHOP.

Two hours after Aflibercept, a striking feature in all 5 biopsies was the focal nature of the microvessel damage, with severely damaged microvessel sections close to normal ones in the same area. Different stages of microvessel damage were found (i) signs of endothelial cell activation with cubodial endothelial cells protruding into the microvessel lumen, (ii) partial deletion of the endothelial cytoplasm (Fig. 2b*a*), iii) destruction of all endothelial cells in one microvessel section, with a "ghost-like" aspect of the remaining cytoplasmic structures, (iv) microvessel wall destruction, with disappearance of the microvessel lumen and thrombus formation, without any associated signs of microvessel repair such as endothelial cell mitoses, or circulating endothelial cells. These alterations were not found after R-CHOP. We confirmed endothelial cell apoptosis after Aflibercept, but not after R-CHOP, using double immunofluorescent staining for CD31 and Caspase-3 (Fig. 2b*b*).

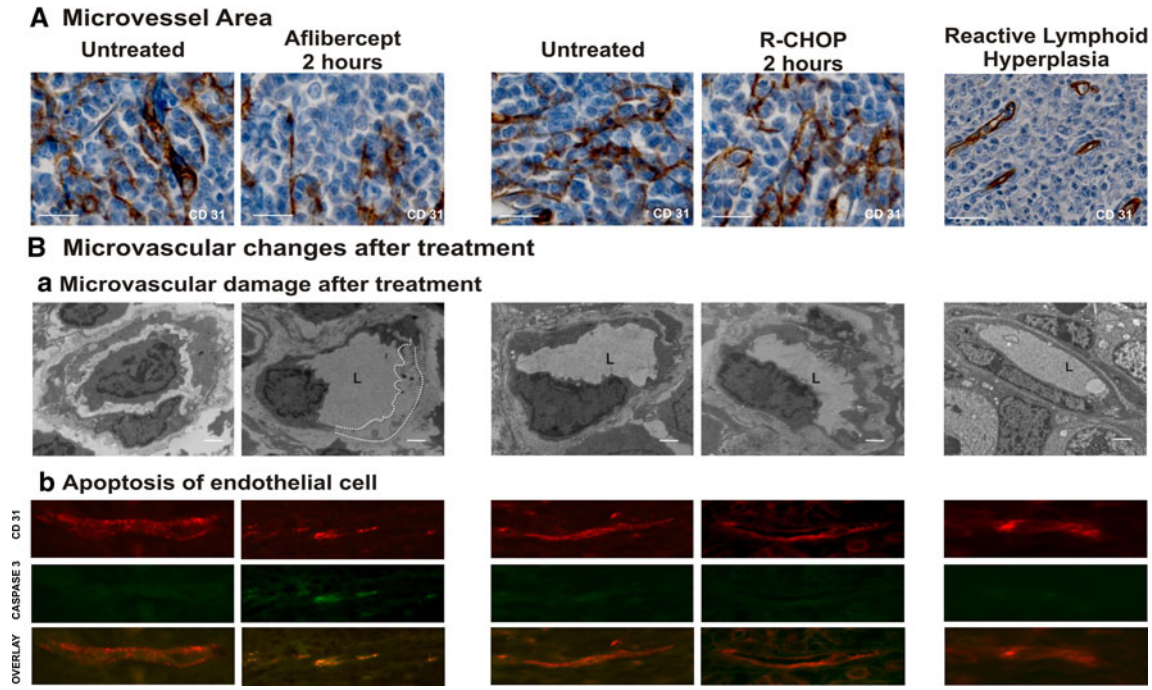

Fig. 2 Early focal vascular damage induced 2 h after Aflibercept in human aggressive B-cell lymphomas. **a** The systematic study of relative microvessel areas showed a significant diminution after 2 h of Aflibercept in two cases, but not after R-CHOP. (CD31 immunostaining, magnification ×400, *scale bar*: 20 μm). **b** Microvessel damage was found in all 5 biopsies after Aflibercept, but not after R-CHOP or in reactive lymphoid hyperplasia: *a* Ultrastructure also shows partial deletion of the endothelial cytoplasm (*broken white lines*) 2 h after Aflibercept, but not after RCHOP or in reactive lymphoid hyperplasia. L.: Lumen, Ultra-thin sections scale bar: 2 μm. *b* Double immunofluorescent staining for CD31 and Caspase-3 shows endothelial cell apoptosis 2 h after Aflibercept but not after RCHOP or in reactive lymphoid hyperplasia

Aflibercept-induced changes in lymphoma cells

Cell proliferation, measured by the Ki67 index (Fig. 3a), was high in all eight lymphoma patients (range 71-98%, mean 87.1% ± SEM 3.3) before treatment. There was no significant difference after Aflibercept (mean 84% ± SEM 5.4), or after R-CHOP (mean 76.7% ± SEM 5.4) (Table 2).

Interestingly, different types of tumor cell death were induced by the two types of treatment (Fig. 3b; Table 2). Necrosis was only found in one DLBCL case before treatment. The relative necrosis area significantly increased in three DLBCL patients after Aflibercept, but it remained unchanged after R-CHOP.

Apoptosis was present before treatment in all eight cases (range 2.7–61.2 apoptotic cells/field at ×400 magnification). The number of apoptotic cells significantly increased in the five patients treated with Aflibercept (range 5.7–69 apoptotic cells/field at ×400 magnification) and in two of the three patients treated with R-CHOP (range 5.7–95 apoptotic cells/field at ×400 magnification). The apoptosis/mitosis ratio was also significantly increased after treatment in the same cases, except for one Burkitt lymphoma treated with Aflibercept (case 5).

Discussion

Microvessel damage was observed as early as 2 h after treatment in the five patients treated with Aflibercept, but not in the three patients treated with R-CHOP. As far as we know, no systematic study of the early effects of anti-angiogenic therapy in human lymphoma has been reported. Previous experimental studies have shown that VEGF-Trap induces a diminution in microvessel density after 24 h in mice with pancreatic tumors [14], and tumor necrosis after 5 days in human Wilms' tumor xenografts [15].

Before any treatment, our systematic ultrastructural study showed microvessel changes in the 8 lymphoma patients but not in the three cases of reactive lymphoid hyperplasia. Heterogeneous patterns of this nature have been described in human B-cell lymphoma [16] and experimental studies have shown that, unlike normal vessels, tumor vessels are not arranged in a hierarchical pattern, but are irregularly spaced and structurally heterogeneous [17, 18]. Two hours after Aflibercept administration, a striking feature in all 5 biopsies was the focal nature of the microvessel damage, with severely damaged microvessel sections close to normal ones in the same area. This type of distribution could be linked to the

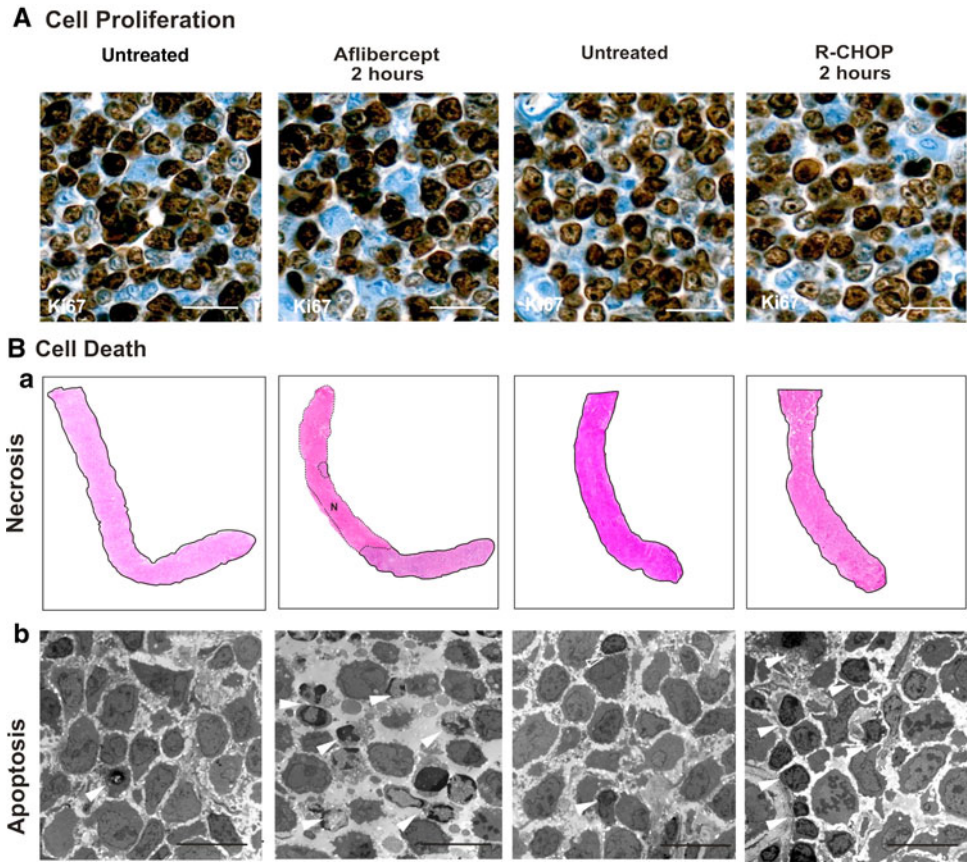

Fig. 3 Lymphoma cell changes induced 2 h after Aflibercept or after R-CHOP treatments. **a** Cell proliferation did not differ significantly two hours after Aflibercept or after R-CHOP. (Ki67 immunostaining, magnification ×400, *scale bar*: 20 μm). **b** Necrosis and apoptosis assessment. *a* Comparison of necrosis area (N, surrounded by *broken black lines*) showed a significant increase in relative necrosis areas two hours after Aflibercept in three patients but not after R-CHOP. *b* Apoptotic cells (*white arrows*) were more numerous two hours after Aflibercept or R-CHOP. Ultrathin sections, *scale bar* 10 μm

tumor microvessel heterogeneity observed before treatment, since immature blood vessels are thought to be more sensitive to anti-angiogenic drugs [19, 20]. This microvessel damage was not found after R-CHOP.

Different stages of microvessel damage were concomitantly observed after Aflibercept. The mildest changes were signs of endothelial cell activation with cubodial endothelial cells protruding into the microvessel lumen. Ultrastructural signs of endothelial activation have not been so far reported in aggressive lymphoma treated with anti-angiogenic drugs. Only VCAM elevation, a biological sign of endothelial cell activation, has been found in mantle cell lymphoma and DLBCL treated with Bevacizumab. In our cases, more severe endothelial cell damage with apoptosis of endothelial cells, confirmed by double immunochemistry, or partial deletion or complete destruction of endothelial cell cytoplasm, were also observed after Aflibercept, but not after R-CHOP. It has been established that endothelial cytoplasm damage leads to exposure of the subendothelial basement membrane, further platelet activation and thrombosis [21]. This is in line with our observation of micro-thrombus formation, together with the disappearance of microvessel lumina and microvessel wall destruction, 2 h after Aflibercept administration.

Quantitative studies also showed the specific effect of Aflibercept on microvessels, with a diminution in the relative microvessel area and an increase in the relative necrosis area in patients treated with Aflibercept but not in patients treated with R-CHOP. Previous reports on the subject of VEGF-Trap-induced lesions, but in experimental models and at later times, have shown a considerable reduction in tumor vasculature and cell proliferation [22]. In the present study, cell proliferation did not differ significantly two hours after Aflibercept. It was thus possible to show that microvessel damage was the initial change in our early biopsied lymphoma cases.

Interestingly, there was also a significant increase of tumor cell apoptosis in all cases two hours after Aflibercept. This early double effect of anti-VEGF therapy on the microvessel network and on tumor cells, which we here identified through direct observation of human lymphoma samples, is in line with experimental data: blocking tumor VEGF-R1 in xenografted human DLBCL has been reported to decrease tumor volume by reducing vascularization and increasing tumor apoptosis [23].

We used US-guided lymph node biopsies, which can be repeated without side effects, [24] with systematic association of glutaraldehyde fixation, cryopreservation, and formalin fixation for tumor samples. This enabled us to establish the diagnoses and to identify early vascular and tumor cell changes that could not have been detected in paraffin or cryocut sections. However, in this limited series

of patients, the correspondence between ultrasonographic and histological analyses was weak. This is in agreement with previous experimental studies using Doppler to quantify changes in blood flow during anti-angiogenic therapy [25]. Contrast enhancement in ultrasound techniques has been recently proposed to assess human tumor vascularization, and to calculate tumor tissue perfusion [26].

In conclusion, we identified early focal microvascular damage, necrosis, and apoptosis of lymphoma cells in aggressive B-cell lymphoma as early as 2 h after Aflibercept. This suggests that there is more than one mechanism associated with the early effects of anti-angiogenic therapy in lymphoma.

Acknowledgments The authors would like to thank Raymond Barnhill, M.D Ph.D, for his critical review of the manuscript, Ms Stéphanie Lefrançois and Mr Sylvain Arien for the electron microscopy technique. MR is a recipient of Assistance Publique-Hôpitaux de Paris grant for cooperation with Hospital Universitario Fundación Santa Fe de Bogotá-Universidad de los Andes, Colombia. WL is a recipient of poste vert Inserm for Pole Franco-Chinois of Shanghai. This work was supported by Inserm, Université Paris VII, INCa, and ANR. Mrs Angela Swaine Verdier reviewed the English language of the manuscript.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ (2006) Cancer statistics. CA Cancer J Clin 56(2):106–130
2. Coiffier B, Lepage E, Briere J, Herbrecht R, Tilly H, Bouabdallah R, Morel P, Van Den Neste E, Salles G, Gaulard P, Reyes F, Lederlin P, Gisselbrecht C (2002) CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med 346(4):235–242
3. Pfreundschuh M, Trumper L, Osterborg A, Pettengell R, Trneny M, Imrie K, Ma D, Gill D, Walewski J, Zinzani PL, Stahel R, Kvaloy S, Shpilberg O, Jaeger U, Hansen M, Lehtinen T, Lopez-Guillermo A, Corrado C, Scheliga A, Milpied N, Mendila M, Rashford M, Kuhnt E, Loeffler M (2006) CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large-B-cell lymphoma: a randomised controlled trial by the MabThera International Trial (MInT) Group. Lancet Oncol 7(5):379–391
4. Feugier P, Van Hoof A, Sebban C, Solal-Celigny P, Bouabdallah R, Ferme C, Christian B, Lepage E, Tilly H, Morschhauser F, Gaulard P, Salles G, Bosly A, Gisselbrecht C, Reyes F, Coiffier B (2005) Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol 23(18):4117–4126
5. Gratzinger D, Zhao S, Marinelli RJ, Kapp AV, Tibshirani RJ, Hammer AS, Hamilton-Dutoit S, Natkunam Y (2007) Microvessel density and expression of vascular endothelial growth factor and its receptors in diffuse large B-cell lymphoma subtypes. Am J Pathol 170(4):1362–1369
6. Gratzinger D, Zhao S, Tibshirani RJ, Hsi ED, Hans CP, Pohlman B, Bast M, Avigdor A, Schiby G, Nagler A, Byrne GE Jr, Lossos IS, Natkunam Y (2008) Prognostic significance of VEGF, VEGF receptors, and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy. Lab Invest 88(1):38–47
7. Salven P, Orpana A, Teerenhovi L, Joensuu H (2000) Simultaneous elevation in the serum concentrations of the angiogenic growth factors VEGF and bFGF is an independent predictor of poor prognosis in non-Hodgkin lymphoma: a single-institution study of 200 patients. Blood 96(12):3712–3718
8. Zhao WL, Mourah S, Mounier N, Leboeuf C, Daneshpouy ME, Legres L, Meignin V, Oksenhendler E, Maignin CL, Calvo F, Briere J, Gisselbrecht C, Janin A (2004) Vascular endothelial growth factor-A is expressed both on lymphoma cells and endothelial cells in angioimmunoblastic T-cell lymphoma and related to lymphoma progression. Lab Invest 84(11):1512–1519
9. Wu HC, Huang CT (2008) Anti-Angiogenic therapeutic drugs for treatment of human cancer. J Cancer Mol 4(2):37–45
10. Swerdlow SH (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th Edition edn. International agency for research on cancer, Lyon
11. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO, Chan WC (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103(1):275–282
12. de Kerviler E, Guermazi A, Zagdanski AM, Meignin V, Gossot D, Oksenhendler E, Mariette X, Brice P, Frija J (2000) Image-guided core-needle biopsy in patients with suspected or recurrent lymphomas. Cancer 89(3):647–652. doi:10.1002/1097-0142(20000801)89:3<647:AID-CNCR21>3.0.CO;2-R
13. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 16(1):3–11
14. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM (2004) Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165(1):35–52
15. Huang J, Frischer JS, Serur A, Kadenhe A, Yokoi A, McCrudden KW, New T, O'Toole K, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Yamashiro DJ, Kandel JJ (2003) Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. Proc Natl Acad Sci USA 100(13):7785–7790
16. Crivellato E, Nico B, Vacca A, Ribatti D (2003) B-cell non-Hodgkin's lymphomas express heterogeneous patterns of neovascularization. Haematologica 88(6):671–678
17. Dvorak HF (2003) Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma. Am J Pathol 162(6):1747–1757
18. Baluk P, Hashizume H, McDonald DM (2005) Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15(1):102–111
19. Kerbel RS (2006) Antiangiogenic therapy: a universal chemosensitization strategy for cancer? Science 312(5777):1171–1175
20. Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307(5706):58–62
21. Pober JS, Min W, Bradley JR (2009) Mechanisms of endothelial dysfunction, injury, and death. Annu Rev Pathol 4:71–95
22. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T,

Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99(17):11393–11398

23. Wang ES, Teruya-Feldstein J, Wu Y, Zhu Z, Hicklin DJ, Moore MA (2004) Targeting autocrine and paracrine VEGF receptor pathways inhibits human lymphoma xenografts in vivo. Blood 104(9):2893–2902
24. de Kerviler E, de Bazelaire C, Mounier N, Mathieu O, Brethon B, Briere J, Marolleau JP, Brice P, Gisselbrecht C, Frija J (2007) Image-guided core-needle biopsy of peripheral lymph nodes allows the diagnosis of lymphomas. Eur Radiol 17(3):843–849
25. Drevs J, Muller-Driver R, Wittig C, Fuxius S, Esser N, Hugenschmidt H, Konerding MA, Allegrini PR, Wood J, Hennig J, Unger C, Marme D (2002) PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging. Cancer Res 62(14):4015–4022
26. Zhu AX, Holalkere NS, Muzikansky A, Horgan K, Sahani DV (2008) Early antiangiogenic activity of bevacizumab evaluated by computed tomography perfusion scan in patients with advanced hepatocellular carcinoma. Oncologist 13(2):120–125