Intense reversal of bone marrow angiogenesis after sequential fludarabine-induction and alemtuzumab-consolidation therapy in advanced chronic lymphocytic leukemia

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ABSTRACT

Background and Objectives
Increased bone marrow (BM) angiogenesis has been demonstrated in several hematologic malignancies. BM angiogenesis is significantly decreased in patients with chronic lymphocytic leukemia (CLL) treated with fludarabine. The anti-angiogenic potential of alemtuzumab in CLL has not yet been investigated. We, therefore, evaluated BM angiogenesis in CLL patients treated sequentially with fludarabine and low doses of alemtuzumab.

Design and Methods
BM microvessel area was sequentially evaluated in 20 patients with advanced B-cell CLL who received, after a clinical response obtained with fludarabine-induction therapy, alemtuzumab, three times weekly for 6 weeks at a dose of 10 mg.

Results
The complete response rate improved from 45% after fludarabine induction to 90% after alemtuzumab consolidation. The extent of BM angiogenesis decreased continuously after either fludarabine or alemtuzumab (p=0.0002; Kruskal-Wallis test). Thirteen out of 20 (65%) patients changed from having a monoclonal to a polyclonal pattern of IgH sequences after alemtuzumab consolidation. A separate evaluation carried out in patients who achieved molecularly undetectable disease, as defined by polymerase chain reaction negativity, and in patients who remained with minimal residual disease after therapy with alemtuzumab showed a significant reduction of BM microvessel area only in the former (p=0.0002). Finally, molecular responses and a significant reduction of BM angiogenesis were more common in patients who received the cumulative planned dose of alemtuzumab (i.e.,180 mg) than in patients who received reduced doses (p=0.007 and p=0.0001, respectively).

Interpretation and Conclusions
Overall, these data demonstrate a decrease in BM vascularity that characterized CLL patients who received low doses of subcutaneous alemtuzumab consolidation therapy after a clinical response to fludarabine induction therapy. Such a finding reflects, at least in part, the molecular response and cumulative dose of alemtuzumab.

Key words: alemtuzumab, antiangiogenesis, CLL, fludarabine, molecular response, prognosis.

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The growth of solid tumors is certainly angiogenesis-dependent, whereas the role of angiogenesis in the growth and survival of hematologic malignancies has only recently become evident. Increased bone marrow (BM) angiogenesis has been demonstrated in multiple myeloma, chronic myeloid leukemia, acute myeloid or lymphoid leukemia, as well as in myelodysplastic syndromes.

Angiogenesis plays a significant pathophysiologic role in the biology of B-cell chronic lymphocytic leukemia (CLL) and has prognostic implications. The potential therapeutic significance of these findings for patients with CLL are currently under investigation and combination regimens which include anti-angiogenic compounds would appear to be a very attractive therapy for these patients.

We have previously demonstrated that BM angiogenesis decreases significantly in CLL patients who receive fludarabine. The anti-angiogenic potential of alemtuzumab (CAMPATH®), an anti-CD52 monoclonal antibody, which has shown impressive results both in refractory CLL and as up-front therapy for this malignancy, has not been investigated yet.

One of the major drawbacks of the treatment of poor-risk CLL is that many patients who achieve a response to therapy have an early relapse. Analysis of minimal residual disease (MRD) in these patients demonstrates that few of them have no detectable CLL after induction therapy, and it is, therefore, very likely that the progression of disease results from the expansion of this residual disease also supported by angiogenesis. A good option in poor-risk CLL patients could, therefore, be to place alemtuzumab after induction therapy with fludarabine to eradicate MRD. Accordingly, Montillo et al. devised a consolidation policy consisting of low doses of alemtuzumab administered subcutaneously after response to conventional chemotherapy, thus offering patients the chance to deepen remissions and eradicate detectable MRD. Here, in a subset of patients from the series treated by Montillo et al., we used immunohistochemistry to quantify bone marrow (BM) microvascular density, focusing on changes of BM angiogenesis induced by the sequential use of fludarabine and low doses of alemtuzumab.

**Design and Methods**

**Patients and treatment plan**

Twenty B-cell CLL patients who had achieved nodular partial remission (nPR) or partial remission (PR) with fludarabine induction therapy according to National Cancer Institute (NCI) criteria formed the basis of this study. Patients in complete remission (CR) were eligible if residual disease could be detected by consensus primer polymerase chain reaction (PCR) analysis.

The patients, selected on the basis of sequential BM biopsies available, represent a subset of 34 CLL patients treated frontline who were previously reported by Montillo et al. The pre-treatment characteristics, response to treatment and molecular responses were equally distributed between subpopulation reported here and the non-reported population, thus excluding any potential selection bias.

Fourteen patients were male and six female; their median age was 51 years (range, 41-60). Laboratory tests usually recommended to validate the diagnosis and assess the extent of disease were performed. Somatic mutation in the expressed immunoglobulin VH-genes and cytofluorimetric evaluation of cytoplasmic ZAP-70 expression were assessed as previously reported.

All patients received up-front therapy consisting of fludarabine given intravenously at a dose of 25 mg/m² from day 1 to 5, every 28 days, for a median number of six cycles (range, 4-13). Treatment initiation required the criteria of active B-cell CLL according to NCI criteria. At least 8 weeks after fludarabine treatment patients were given subcutaneous alemtuzumab, three times weekly for 6 weeks. The dose was escalated from 1 mg as an initial dose to 3 mg for the second dose and 10 mg thereafter. Thirteen patients received the cumulative planned dose of alemtuzumab (i.e., 180 mg). Seven patients, because of mild to moderate side effects, received lower doses of alemtuzumab (median, 164 mg; range, 134-174).

The protocol was reviewed and approved by the Institutional Ethics Committee of the Niguarda Ca’ Granda Hospital (Milan, Italy). All patients signed informed consent before entering the trial.

**Assessment and definition of response**

Before starting therapy, patients underwent a physical examination, which included spleen and lymph node measurements, and were staged according to the Rai and Binet classifications. A whole-body computed tomography scan was carried out in all instances. Before starting with up-front therapy and 4 weeks after completing either fludarabine or alemtuzumab a disease restaging, which included BM aspirate, trephine biopsies and immunophenotyping, was performed. Residual disease was assessed using a consensus PCR methodology to detect the clonality of IgH sequences.

Patients were monitored weekly throughout the study by early pp65 antigenemia tests for cytomegalovirus (CMV) reactivation. They were promptly treated with ganciclovir if more than ten positive cells were found.

Response criteria were those defined by NCI Working Groups. CR required the disappearance of all palpable disease, normalization of the blood counts (polymorphonuclear cells [PMN] >1.5×10⁹/L, platelets >100×10⁹/L, Hb >11 g/dL), BM aspirate lymphocyte percentage <50% and no disease detectable on BM biopsy. A nPR required the same criteria as for CR, although lymphoid nodules could be seen on BM biopsy. A PR required a 50% or more reduction in palpable disease as well as one or more of the remaining features: PMN >1.5×10⁹/L or 50%
improvement over baseline, platelets >100×10⁹/L or 50% improvement over baseline, Hb >11 g/dL or 50% over the baseline without transfusions.¹²

**Immunohistochemistry**

Two murine monoclonal antibodies (MAb) against the endothelial cell marker CD31 (MAb 1A10) and tryptase (MAb AA1, Dako, Glostrup, Denmark) were used. Briefly, 4 µm thick sections were collected on 3-amino-propyl-triethoxysilane-coated slides, deparaffinized by the xylene-ethanol sequence, rehydrated in a graded ethanol scale and in TRIS-buffered saline (TBS, pH 7.6), and incubated overnight at 4°C with MAb 1A10 (1:25 in TBS) and AA1 (1:1500 in TBS), after prior antigen retrieval by enzymatic digestion with ficin (Sigma, St. Louis, MO, USA) for 80 min at room temperature for tryptase, and in a pressure cooker for 90 sec in EDTA buffer, pH 8 for CD31. The immunoreaction was performed with alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako) and Fast Red as the chromogen for CD31, and with the streptavidin-peroxidase complex (LSAB2, Dako) and 3,3’-diaminobenzidine tetrahydrochloride 5% (Dako) as the chromogen for CD31, followed by hematoxylin counterstaining. An unrelated monoclonal IgG1 produced by the P3X63/Ag8 mouse secretory myeloma replacing the MAb served as a negative control.

**Measurement of BM angiogenesis**

Vessels were detected in 6-µm sections of 4% paraformaldehyde-fixed, paraffin-embedded, biopsies by red-staining endothelial cells with an anti-factor VIII-related antigen (FVIII-RA) rabbit antibody (Dako) and a three-layer biotin-streptavidin-peroxidase system described previously.¹⁴ Megakaryocytes also stained with FVIII-RA were easily distinguishable by their morphology and size. Angiogenesis was measured as microvessel area without knowledge of treatment phase. Microvessels (capillaries and small venules) were selected as endothelial cells, single or clustered in nests or tubes and clearly separated one from another, and either without or with a lumen not exceeding 10 µm, though larger neovessels were found in some patients. A double-headed photomicroscope (Axioplan 2, Zeiss, Oberkochen, Germany) was used in the simultaneous identification by two of us (AV and DR) of the microvessels, and each identification was agreed upon in turn. The microvessel area was measured on four to six × 250 fields covering the whole of each of two sections per biopsy within a superimposed square reticle. This was drawn out by KS-300 software (Zeiss) and formed of 22 lines per slide giving 484 intersection points. At × 250 it defined an area of 12.5 × 10⁷ mm² (reference area), whereas each point covered an area of 72.15 µm². The area occupied by microvessels was estimated by using the direct planimetric method of point counting² with slight modifications for the computed image analysis (same software) as described elsewhere,¹⁵ according to which the microvessel area equals the sum of point areas that hit microvessels. Because cellular areas are vascularized and non-cellular areas (fat, dense connective tissue, necrotic and hemorrhagic foci, bone lamellae) are not, and because the latter hampered comparison between sections, they were always omitted from the reference area. Thus, the point areas that hit non-cellular areas were subtracted from the reference area. Residual point areas defined the cellular area only and the microvessel area was measured inside it. Basically, the measurement of the microvessel and cellular areas fitted the following equations: microvessel area [y] = sum of points that hit microvessels [x] · 72.15 µm²; cellular area = 12.5 × 10⁷ mm² – (sum of points that hit possible non-cellular areas [P] · 72.15 µm²). Values of the microvessel area were normalized to those of the cellular area by the equation: [x]/(484 P) = y/100. Analysis of six to ten serial sections from three biopsy samples revealed an intrabiopsy variability of 10% or less (±1.8%) and a variability between the investigators checking neovessels separately of 5.0% or less (±5.2%) for the microvessel area. The area was expressed as means ± 1 SD for each section and biopsy and groups of biopsies. In all patients the microvessel area was assessed at the time of starting fludarabine therapy, and before and after giving alemtuzumab. Twenty-two patients with anemia due to iron or vitamin B12 deficiency served as controls.

**Mast cell counts**

Mast cells were highlighted in every second section adjacent to that stained for microvessels with tryptase, counted in six to eight × 250 fields, covering almost the whole section, inside a square reticle (0.25 mm²), and calculated as means ± 1 SD and median for each group of samples.

**Statistical methods**

The Mann-Whitney test, Fisher’s exact test, Pearson and Spearman correlations and the corrected χ² tests were applied to compare groups. Kruskal-Wallis analysis was used to compare simultaneously the median values of BM microvessel area before starting fludarabine induction, after completing fludarabine induction, and after completing alemtuzumab consolidation.

**Results**

**BM angiogenesis in CLL patients**

BM angiogenesis was homogeneously increased in all CLL patients. Indeed, the median pretreatment BM microvessel area was significantly higher in CLL patients (3.238 mm² x 10⁻²; range, 0.890-4.210) than in controls (0.088 mm² x 10⁻²; range, 0.066-0.126; p<0.00001, Mann-Whitney test). The attempt to correlate the extent of BM angiogenesis with clinico-biological features of prognostic relevance did not succeed. In our cohort of patients
the microvessel area did not reflect either Binet clinical stage ($p = 0.736$) or ZAP-70-positivity ($p = 0.406$).

**Changes of BM angiogenesis after sequential use of fludarabine and alemtuzumab**

After a median number of six cycles of fludarabine (range, 4-13), 11 (55%) patients could be classified as having achieved CR and nine (45%) as having achieved PR (7 nPR and 2 PR). Consolidation therapy with alemtuzumab deepened remission in seven additional patients who moved from PR to CR. Accordingly, the rate of CR increased to 90% ($18 \text{ CR}$; $p = 0.03$; Fisher’s exact test) after treatment with alemtuzumab.

In keeping with the hematologic responses, significant changes of BM microvessel area were observed. The decrease of BM angiogenesis, seen in virtually all patients (Table 1), was a continuous process characterizing the sequential use of fludarabine and alemtuzumab ($p=0.0002$) [Figures 1 and 2 (a-c)]. Interestingly, this conspicuous feature was easily demonstrable in both ZAP-70-positive ($p=0.02$) and ZAP-70-negative patients ($p=0.001$).

Nonetheless, our approach of sequential chemotherapy failed to normalize BM microvessel area. At the time of maximum response, the median microvessel area remained significantly higher in CLL patients than in controls ($1.823 \text{ mm}^2 \times 10^{-2}$ versus $0.095 \text{ mm}^2 \times 10^{-2}$; $p<0.0001$, Mann-Whitney test).

**Changes of BM angiogenesis after sequential therapy with fludarabine and alemtuzumab and molecular response**

Residual disease was assessed using a consensus PCR methodology to detect the clonality of IgH sequences. After completing the therapeutic program, which consisted of fludarabine induction therapy followed by consolidation with low doses of alemtuzumab, 13 out of 20 (65%) patients changed from having a monoclonal to a polyclonal pattern of IgH sequences (Table 1). We wondered whether molecular response could translate into a change of BM microvessel area. A separate evaluation carried out in patients who achieved molecular unde-

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**Table 1. Changes of BM microvessel area, cumulative dose of alemtuzumab and clinical and molecular response of patients included in the study.**

<table>
<thead>
<tr>
<th>Patient</th>
<th><strong>BM MCV area (pre-fludarabine)</strong></th>
<th><strong>BM MCV area (post-fludarabine)</strong></th>
<th><strong>BM MCV area (post-alemtuzumab)</strong></th>
<th>Clinical response after alemtuzumab consolidation</th>
<th>Clonality of IgH sequences after alemtuzumab</th>
<th>Cumulative dose of alemtuzumab (mg)</th>
<th>ABSCT</th>
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**BM MCV (Bone marrow microvessel area) expressed as mm$^2 \times 10^{-2}$; PR: partial response; CR: complete response; n-PR: nodular partial response; ABSCT: peripheral autologous blood stem cell transplantation.**

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**Figure 1. Changes of bone marrow microvessel area after sequential therapy with fludarabine and alemtuzumab.**

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**Figure 2. Changes of bone marrow microvessel area after sequential therapy with fludarabine and alemtuzumab.**

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Effect of alemtuzumab on angiogenesis in CLL

Detectable disease, as defined by PCR negativity (Figure 3A), and in patients who remained MRD positive (Figure 3B) after therapy with alemtuzumab showed a significant reduction of BM microvessel area only in the former ($p=0.0002$).

**Cumulative dose of alemtuzumab, molecular response and the size of reduction of angiogenesis**

We evaluated molecular response and angiogenesis data in patients who received the cumulative planned dose of alemtuzumab (i.e., 180 mg) in comparison to patients who received reduced doses ($p=0.007$). Similarly, a significant decrease of the extent of BM microvessel area was observed among patients who received the cumulative planned dose of alemtuzumab ($p=0.0001$) (Figure 4A) while the same did not occur in those who received lower doses of alemtuzumab ($p=0.09$) (Figure 4B).

**Changes of mast cell numbers after sequential therapy with fludarabine and alemtuzumab**

In B-cell CLL, there is a striking association between mast cell numbers and microvessel counts and both reflect disease-status (Figure 2). We, therefore, wondered whether reduction of the extent of BM angiogenesis observed after sequential therapy with fludarabine and alemtuzumab translated into a change of BM mast cell numbers.

![Figure 2. Sections of bone marrow biopsies stained with CD31 for microvessels (A, before therapy with fludarabine; B, after therapy with fludarabine; C, after therapy with alemtuzumab) and tryptase for mast cells (D, before therapy with fludarabine; E, after therapy with fludarabine; F, after therapy with alemtuzumab) from a representative patient with B-cell CLL.](image)

![Figure 3. Changes of bone marrow microvessel area after therapy with fludarabine and alemtuzumab in patients without (A) and with (B) molecular response.](image)
number. In keeping with the decrease of BM angiogenesis, the reduction of the number of bone marrow mast cells was a continuous process characterizing the sequential use of fludarabine and alemtuzumab ($p<0.05$; Figure 5).

**Discussion**

Evidence of the biological and prognostic importance of BM neovascularization and angiogenic signaling in CLL has heightened interest in the inhibition of angiogenesis as a therapeutic strategy for this disease. It has been previously shown that BM angiogenesis persists after conventional chemotherapy, as well as after autologous BM transplantation in MM. In contrast, a significant decrease in microvessel density was reported in 81 patients with MM who responded to therapy with thalidomide. The effects of imatinib mesylate therapy on angiogenesis have been investigated and compared with those of interferon and hydroxyurea in 98 patients with newly diagnosed Philadelphia chromosome-positive BCR-ABL chronic myeloid leukemia in first chronic phase. First-line therapy with imatinib induced a significant reduction of BM vascularity, generally associated with a complete cytogenetic response. A significant anti-angiogenic effect was also observed after treatment with hydroxyurea, while no changes in the extent of BM angiogenesis were detectable after interferon treatment.

Information on correlations between the efficacy of therapy and changes of BM angiogenesis in CLL are limited. Vascular endothelial growth factor (VEGF) has been measured in both serum and leukemic B-CLL cells and its presence has been found to be crucial in preventing both spontaneous and chlorambucil-mediated apoptosis. In addition, endothelial and stromal cells, constituting the microenvironment, may express VEGF or VEGF receptors and respond to the tumor VEGF by proliferating to generate new vessels and by secreting several mitogenic and angiogenic cytokines which, in a paracrine fashion, stimulate tumor cell growth. Primarily based on the central role of VEGF in the regulation of angiogenesis, most current therapeutic approaches are focused on the neutralization of VEGF or inhibition of VEGF receptor signaling. No completed trials with these agents in CLL have yet been reported.

In one study thalidomide was used either alone or in combination with fludarabine. Although only one of eight patients treated with thalidomide monotherapy had a response, four of eight patients treated with the thalidomide/fludarabine combination responded, one of whom achieved a CR. In a phase I trial of thalidomide in combination with fludarabine in previously untreated patients the overall response rate was 100% among the first nine patients treated, with a CR rate of 55%.

We recently demonstrated that BM angiogenesis decreases significantly in CLL patients who obtain a response to fludarabine therapy thus providing insight into the role of this drug as a potential anti-angiogenic agent. Expanding on these observations, we wondered whether alemtuzumab, an anti-CD52 monoclonal antibody of clinical use in CLL, might further decrease BM vascularity.

One of the major problems of the treatment of poor-risk CLL is that many patients who achieve a response to therapy have an early relapse. It, therefore, appears reasonable to place alemtuzumab after induction therapy in order to consolidate the response and eradicate MRD. Montillo et al. have reported on 34 patients treated after a fludarabine-based regimen with low doses of alemtuzumab given subcutaneously. Although only the result of low-sensitivity MRD assessment were described, more than 50% of patients converted from a clonal B-cell population to a normal polyclonal pattern. On this basis, we wondered whether the pos-
sibility of deepening remission could translate into significant changes of BM angiogenesis. The analysis of a subset of patients in this series described by Montillo et al.\textsuperscript{11} clearly showed that a significant reduction in the extent of BM vascularity after therapy with alemtuzumab occurred mainly in patients who achieved a molecular response. Interestingly, there was a trend for reduction of angiogenesis, also among patients who remained MRD positive, although this did not reach statistical significance, possibly because of the small number of patients analyzed.

The present study provides new information on the impact of the cumulative dose of alemtuzumab on molecular response and reduction of BM angiogenesis: both achievement of MRD negativity and a significant reduction of BM angiogenesis were more common in patients who received the cumulative planned dose of alemtuzumab than in patients who received reduced doses. Interestingly, our observation suggesting a close relationship between molecular response and cumulative dose received has not previously been reported thus far for a monoclonal antibody.

The mechanisms explaining the effect of alemtuzumab on BM angiogenesis are not completely clear; however, it seems conceivable that a therapy targeting B-CLL cells involved in the production of angiogenic cytokines (e.g., VEGF) might interfere with mechanisms of growth and maintenance of BM angiogenesis. In Waldestrom’s macroglobulinemia it has been demonstrated that BM mast cells express CD52, which may represent a potential target of alemtuzumab.\textsuperscript{31} In B-cell CLL there is a striking association between numbers of mast cells and microvessel counts and both increase as the disease progresses.\textsuperscript{19} As shown in the present study, the consistent decrease of bone angiogenesis we observed after therapy with alemtuzumab paralleled the reduction of mast cells, whose role in angiogenesis in B-cell CLL is well established.\textsuperscript{19}

In conclusion, our results qualify the unintended anti-angiogenic activity correlated with both fludarabine and alemtuzumab in B-cell CLL. Since the lack of normalization of BM angiogenesis provides indirect evidence of persistent disease, it is conceivable that angiogenesis continues to be driven by residual leukemic cells which continue to secrete angiogenic cytokines. This possibility provides the rationale for considering the clinical use of consolidation therapy with alemtuzumab after a fludarabine-based regimen, possibly in addition to new thalidomide analogs. The normalization of BM vascularity associated with complete molecular response could guarantee a longer progression-free survival and, possibly, overall survival.

**Authors’ contributions**

Conception and design: SM, MM, DR; provision of study materials and patients: MM, DR, AT, FR, SV, AV, EM; collection and assembly of data: SM, MM, AT, RM. Data analysis and interpretation: SM, MM, DR. Manuscript writing: SM, MM, DR, AV. Final approval of manuscript: all authors.

**Conflicts of Interest**

The authors reported no potential conflicts of interest.

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