ELSEVIER

Experimental Hematology

Experimental Hematology 2014;42:126–136

Endothelium-mediated survival of leukemic cells and angiogenesis-related factors are affected by lenalidomide treatment in chronic lymphocytic leukemia

Rossana Maffei[a,*], Stefania Fiorcari[a,*], Jenny Bulgarelli[a], Lara Rizzotto[b], Silvia Martinelli[a], Gian Matteo Rigolin[b], Giulia Debbia[a], Ilaria Castelli[a], Goretta Bonacorsi[a], Rita Santachiara[a], Francesco Forconi[c,d], Davide Rossi[e], Luca Laurenti[f], Giuseppe A. Palumbo[g], Daniele Vallisa[h], Antonio Cuneo[b], Gianluca Gaidano[e], Mario Luppi[a], and Roberto Marasca[a]

[a]*Division of Hematology, Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, Modena, Italy;* [b]*Division of Hematology, Department of Biomedical Sciences, Azienda Ospedaliero-Universitaria Arcispedale S.Anna, University of Ferrara, Ferrara, Italy;* [c]*Division of Hematology and Transplant, Department of Clinical Medicine and Immunological Sciences, University of Siena, Siena, Italy;* [d]*Cancer Sciences Unit, CRUK Clinical Centre, University of Southampton, Southampton, United Kingdom;* [e]*Division of Hematology, Department of Clinical and Experimental Medicine and BRMA, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy;* [f]*Department of Hematology, Catholic University of the Sacred Hearth, Rome, Italy;* [g]*Division of Hematology, AOU "Policlinico- V. Emanuele", Catania, Italy;* [h]*Department of Medical Oncology and Hematology, Hospital of Piacenza, Piacenza, Italy*

(Received 2 July 2013; revised 27 September 2013; accepted 27 October 2013)

Lenalidomide is an IMID immunomodulatory agent clinically active in patients with chronic lymphocytic leukemia (CLL). We evaluated the activity of lenalidomide inside an in vitro coculture system of endothelial and CLL cells. Lenalidomide was able to inhibit CLL survival advantage mediated by endothelial contact. Moreover, the marked increase of in vitro angiogenesis determined by CLL-derived conditioned media was reduced by lenalidomide. We also analyzed peripheral blood collected from 27 patients with relapsed or refractory CLL being treated with lenalidomide within a phase II trial. Plasma levels of VEGF and THBS-1 decreased, whereas Ang2 and Ang increased during treatment. Patients who respond to lenalidomide showed a more pronounced decrease of VEGF and bFGF than did patients with stable or progressive disease ($p = 0.007$ and $p = 0.005$). Furthermore, lenalidomide reduced circulating endothelial cells and endothelial progenitors by increasing the percentage of apoptotic cells. Conversely, for six matched bone marrow biopsies available before and after treatment, we did not detect any modification in vessel density, suggesting a possible mechanism of vessel normalization rather than regression. In conclusion, our study provides further evidence that the anti-CLL effect of lenalidomide is mediated through the alteration of microenvironmental elements, implying the modulation of several angiogenesis-related factors and disruption of CLL crosstalk with endothelial cells. © **2014 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Chronic lymphocytic leukemia (CLL) B cells show prolonged survival in vivo and therefore accumulate in peripheral blood, bone marrow (BM) and lymph nodes (LNs) of patients. Intrinsic defects of apoptosis regulation and extrinsic survival signals mediated by several microenvironmental elements are both considered as pathogenic factors for CLL [1]. The relevance of external survival factors is exemplified by the fact that CLL cells, despite their long life in vivo, rapidly undergo spontaneous apoptosis under culture condition but can be rescued from death if cocultured with several types of cells, such as mesenchymal stromal cells, follicular dendritic cells, endothelial cells (EC), and nurselike cells (NLCs) [2–5].

*RM and SF contributed equally to this article.

Offprint requests to: Rossana Maffei, Ph.D., Hematology Division, Department of Medical and Surgical Sciences, University of Modena and Reggio Emilia, Via Del Pozzo 71, 41124, Modena, Italy; E-mail: rossana.maffei@unimore.it

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2013.10.007.

0301-472X/$ - see front matter.
http://dx.doi.org/10.1016/j.exphem.2013.10.007

Increasing evidence suggest that angiogenesis can play a role in CLL pathophysiology. Increased serum or plasmatic levels of angiogenic factors, such as vascular endothelial growth factor (VEGF) and angiopoietin-2 (Ang2) were reported in patients with CLL compared with normal control subjects and characterized patients with CLL with poor clinical outcome [6–10]. In addition, CLL-infiltrated BM and LNs contain abnormal vascular elements that are mainly localized in proliferation centers [11]. Higher vascularization was present in patients with CLL with advanced clinical stage and poor outcome [7,12–14]. In vitro studies demonstrated that CLL cells induce increased angiogenesis throughout secretion of VEGF and Ang2 [15]. In addition, physical contact with endothelial cells mediates survival advantage to CLL cells and protects leukemic cells from fludarabine-induced apoptosis [4,11,16].

Lenalidomide is an IMID immunomodulatory agent clinically active in patients with CLL [17–20]. Significant clinical responses in heavily pretreated patients were observed despite high-risk features [18,21]. The exact mechanism of antileukemic activity of lenalidomide remains undefined, but it implies the modulation of microenvironment through the downregulation of critical cytokines and the activation of immune effector cells [22]. Moreover, lenalidomide potently inhibits angiogenesis in in vitro models of angiogenesis by reducing endothelial cell migration [23,24]. Although lenalidomide has been shown to affect angiogenesis in vitro, little is known concerning its antiangiogenic properties in patients with CLL. Ferrajoli et al. [18] reported a reduction of basic fibroblast growth factor (bFGF) levels in plasma of patients with CLL achieving complete or partial responses to lenalidomide treatment. No modification of VEGF plasmatic levels or bone marrow microvessel density was detected.

In this study, we evaluated the effect of lenalidomide on angiogenesis in a cohort of 27 patients with relapsed or refractory CLL treated within a multicenter phase II trial. We also inspected the activity of lenalidomide inside an in vitro coculture system of endothelial cells and CLL cells. Our study provides evidence that the mechanism of action of lenalidomide in patients with CLL is mediated not only through the activation of immune effector cells, but also through the modulation of angiogenesis-related factors and disruption of CLL crosstalk with endothelial cells. Of interest, we identified angiogenesis-related parameters that are associated with clinical response to lenalidomide.

Methods

Patient population

Twenty-seven patients with relapsed or refractory CLL were enrolled in a multicenter phase II clinical trial (Lenalidomide Predicting Response Study) with a protocol (EudraCT NUMBER: 2009–011225–14) approved by the Institutional Review Board. To be eligible for this study, patients were required to have received at least a purine-analogue containing regimen and have experienced relapse or have been refractory to the last therapy. Patients were also required to have adequate performance status: Eastern Cooperative Oncology Group performance status of ≤ 2, creatinine clearance > 60 mL/min and serum creatinine level < 1.5 mg/dL, total bilirubin level < 2 mg/dL, absolute neutrophil count ≥ 2.000/μL, and platelet count ≥ 75/μL. All patients gave written informed consent obtained in accordance with the Declaration of Helsinki before participation in the study. The patients' baseline characteristics are given in Table 1. In this study, lenalidomide treatment schedule starts with 5 mg daily and increases of 5 mg daily every two weeks, up to 25 mg daily or the maximum tolerated dose. Therapy is supposed to be given for 12 courses (1 course = 4 weeks) unless disease progression or excessive toxicity are observed. Side effects were classified according to the National Cancer Institute common toxicity criteria (version 3.0). Responses were assessed and

Table 1. Patients' characteristics

Patient characteristics	Value*
Sex	
Female	7 (26)
Male	20 (74)
Binet Stage (n = 26)	
A	5 (19)
B	9 (35)
C	12 (46)
IGHV status (n = 21)	
Mutated	4 (19)
Unmutated	17 (81)
CD38 (n = 13)	
<30%	4 (31)
≥30%	9 (69)
ZAP70 (n = 13)	
<20%	5 (39)
≥20%	8 (62)
CD49d (n = 11)	
<30%	3 (27)
≥30%	8 (73)
FISH risk (n = 23)	
Low risk	7 (30)
High risk	16 (70)
17p deletion	5 (22)
11q deletion	4 (17)
Trisomy 12	7 (30)
13q deletion	1 (4)
Age (years)	70 (45–83)
WBC count (x10^9/L)	38 (5–177)
%N	10 (2–40)
%L	85 (43–98)
Hemoglobin (g/dL)	12 (8–15)
Platelet count (×10^9/L)	140 (38–325)
β2-Migroglobulin (mg/L)	6 (3–12)
Lactate dehydrogenase (U/L)	457 (187–1406)
No. of previous lines of therapy	3 (1–6)

IGHV = immunoglobulin variable heavy gene; ZAP-70 = zeta-chain-associated protein kinase 70; WBC = white blood cell; FISH = fluorescence in situ hybridization; %N = percentage of neutrophils; %L = percentage of lymphocytes.

* Values are median (range) or n (%).

NOTE. Low risk FISH = no abnormalities or 13q-; high risk FISH = 11q-, 17p- or trisomy 12.