Brief Report: Pre-treatment Angiogenic Cytokines Predict Response to Chemoimmunotherapy in Patients with CLL

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Abstract

Serum levels of pro-(vascular endothelial growth factor [VEGF]) and anti-(thrombospondin-1 [TSP]) angiogenic cytokines were prospectively measured in a phase II trial of chemoimmunotherapy (CIT) for chronic lymphocytic leukemia (CLL) patients(n=56). Pretreatment VEGF levels were lower among patients who achieved complete remission (CR) or nodular partial remission (nPR) relative to those with partial remission (PR) or stable/progressive disease (median 122.0 pg/ml vs. 246.8 pg/ml; p=0.03). VEGF:TSP ratio was lower (anti-angiogenic phenotype) among patients who achieved CR/nPR. The pretreatment VEGF:TSP ratio also correlated with overall survival (p=0.008). A pro-angiogenic profile appears associated with diminished response and inferior survival in CLL patients receiving CIT.

Keywords

Chronic lymphocytic leukaemia; angiogenesis; therapy; VEGF, prognostic factors

INTRODUCTION

Combination chemotherapy has significantly improved response rates and progression-free survival (PFS) for younger patients with chronic lymphocytic leukaemia (CLL) (Eichhorst, *et al* 2006). More recent studies suggest that combining the anti-CD20 monoclonal antibody, rituximab, with chemotherapy improves response rates and PFS.(Hallek 2008, Kay, *et al* 2007a, Keating, *et al* 2005) Although overall response rates with such chemoimmunotherapy (CIT) are ≥ 90% (Byrd, *et al* 2003, Hallek 2008, Kay, *et al* 2007a, Keating, *et al* 2005), multi-centre trials suggest that only 40–50% of patients experience a

complete remission (CR).(Byrd, *et al* 2003, Hallek 2008) Importantly, the extent of remission has been shown to predict survival, with those achieving a CR after CIT having a longer time to salvage therapy and overall survival(OS).(Kay, *et al* 2007a, Keating, *et al* 2005)

Clinical and biological characteristics that predict response to therapy can identify patients who are more or less likely to benefit from a given type of treatment. In addition to their value in patient counseling, such biological markers can (i) provide insight into specific mechanisms of treatment resistance (ii) identify alternative pathways that may be targeted to enhance treatment efficacy, and (iii) identify individuals who may benefit from a modified or alternative treatment strategy. To date, few biological parameters have consistently identified CLL patients unlikely to benefit from CIT (e.g. 17p- by fluorescent *in situ* hybridization [FISH]).(Kay, *et al* 2007a) Thus there remains a need for biomarkers that can identify the subset of CLL patients who are unlikely to respond to CIT and who may benefit from an alternative or targeted treatment strategy.

Angiogenesis is an important regulator of the malignant potential of solid tumours and haematological malignancies.(Shanafelt and Kay 2006) CLL patients have been found to have detectable levels of pro and anti-angiogenic cytokines in the serum and abnormal neovascularization in the marrow and lymph nodes. *In vitro* studies also found that CLL B-cells synthesize and secrete both pro and anti-angiogenic cytokines (Kay, *et al* 2002) and possess a VEGF-based autocrine pathway (Kay, *et al* 2002) that facilitates leukemia cell survival partly through up-regulation of anti-apoptotic proteins including MCL and XIAP. Interactions between CLL B-cells and their microenvironment were found to alter the secretion of angiogenic factors that result in enhanced leukemic B-cell resistance to apoptosis.(Kay, *et al* 2007b) Inter-patient variation in markers of angiogenesis also appear to have potential prognostic implications (reviewed in (Shanafelt and Kay 2006)). Although several small studies suggest that pro-angiogenic cytokine levels(Gora-Tybor, *et al* 2002, Smolej, *et al* 2007) and marrow micro-vessel density(Molica, *et al* 2007) decrease among responding patients after purine nucleoside analogue-based treatment, no study has yet evaluated the ability of pretreatment angiogenic cytokine levels to predict response. We hypothesized that serum levels and ratios of pro- versus anti-angiogenic cytokines would predict response to CIT. To this end, we prospectively measured angiogenic cytokine levels at study entry among patients enrolled in our phase II trial of pentostatin, cyclophosphamide, and rituximab(PCR)(Kay, *et al* 2007a) to test this hypothesis.

METHODS

Between March 2002 and July 2005, 64 eligible patients were enrolled in a phase II trial of PCR as initial therapy for CLL. Approval for this study was obtained by the Mayo Clinic and the Ohio State University institutional review boards with informed consent of participants obtained in accord with the Declaration of Helsinki. Details of the study, treatment schedule, and supportive care measures were previously reported.(Kay, *et al* 2007a) Briefly, patients received pentostatin(2 mg/m^2), cyclophosphamide(600 mg/m^2), and rituximab(375 mg/m^2) intravenously on day 1 once every 21 days for 6 cycles. For the first cycle only, rituximab was given on a thrice weekly schedule as previously described.(Kay, *et al* 2007a)

To test our hypothesis that serum levels of angiogenic cytokines would predict response to CIT, we examined serum levels of pro-(vascular endothelial growth factor [VEGF] and fibroblast growth factor [bFGF]) and anti-(thrombospondin-1 [TSP]) angiogenic cytokines for each patient immediately prior to treatment. VEGF (isoform 165) and bFGF were measured using Quantikine kits (R&D Systems, Minneapolis, MN) and TSP using the

Accucyte assay (CytImmune Sciences Inc, Rockville, MD) according to the manufacturer's instructions. Other molecular and biological prognostic parameters, including CD38, ZAP-70, *IGHV* mutation status, and recurrent cytogenetic abnormalities, as assessed by FISH were also performed on baseline study samples as previously reported.(Kay*, et al* 2007a)

To evaluate the relationship between serum angiogenic cytokine levels and Rai stage(0–II vs. III–IV), known prognostic factors(CD38, ZAP-70, *IGVH* mutation, FISH) or clinical outcome, the Wilcoxon rank sum test or Kruskal-Wallis test was used depending on whether continuous variables were being compared between 2 groups or more than 2 groups. OS and time to salvage treatment (TTT) were calculated from the first day of PCR therapy. TTT was defined as the time from the first date of PCR therapy to the date of initiation of next therapy. Univariate Cox models were used to assess the correlation of serum angiogenic cytokine values with OS and TTT. Overall, statistical significance was declared for p<0.05. As the comparisons were hypothesis generating in nature, a correction for multiple comparisons was not employed.

RESULTS/DISCUSSION

The overall response rate in the PCR clinical trial was 91%, with 41% CR, 22% nodular partial remission (nPR), and 28% partial remission (PR).(Kay*, et al* 2007a) With respect to conventional prognostic parameters (CD38, ZAP-70, *IGHV* mutation status, FISH), no characteristic other than the presence of 17p- predicted response to treatment.(Kay*, et al* 2007a) Median TTT after PCR for all patients was 34 months. Median TTT was approximately 51 months among patients achieving CR, 32 months among patients achieving nPR, and 16 months among patient attaining a PR (Figure 1A). This result was not unexpected and was similar in trends to the findings of other CIT trials demonstrating a relationship between response and time to progression.(Keating*, et al* 2005, Tam*, et al* 2008) On updated long-term analysis of OS after PCR, the median survival was 66 months for all treated patients. Median survival by response type is shown in Figure 1B.

Overall, 56 of 64(87%) patients underwent baseline angiogenic serum cytokine evaluation (Table I). To better evaluate the presence of angiogenic switching in CLL B-cells, ratios of VEGF:TSP and bFGF:TSP were calculated for all patients, with higher ratios suggestive of a pro-angiogenic phenotype. We next evaluated the relationship between VEGF, bFGF, TSP, VEGF:TSP ratio, and bFGF:TSP ratio and other prognostic parameters. Angiogenic cytokine levels and the VEGF:TSP and bFGF:TSP ratios had no relationship with disease stage at study entry. The median VEGF:TSP ratio was significantly lower (anti-angiogenic) in *IGHV* mutated patients compared to unmutated patients (p=0.008). In addition, levels of bFGF and bFGF:TSP were significantly correlated with FISH defects (p=0.01, 0.04, respectively); a more pro-angiogenic phenotype was observed among patients with trisomy 12. Although these findings reinforce the concept that more aggressive disease in CLL based on prognostic parameters can be associated with a more pro-angiogenic phenotype, no significant relationships were found between baseline angiogenic cytokine values and CD38 or ZAP-70 status.

To evaluate whether elevated levels of pro-angiogenic cytokines may identify patients less likely to respond to CIT or have a shorter clinical benefit, we evaluated the relationship between baseline serum VEGF, bFGF, TSP, VEGF:TSP ratio, and bFGF:TSP ratio and response to PCR therapy. The baseline serum VEGF level was significantly lower among patients who achieved a CR or nPR than those attaining PR, stable disease or progressive disease (median 122.2 pg/ml vs. 246.8 pg/ml; p=0.03). The VEGF:TSP ratio was also significantly lower (anti-angiogenic phenotype) among patients who achieved CR or nPR

relative to those with a PR, stable disease or progressive disease (median 0.08 vs. 0.19; p=0.04). No difference in response to PCR therapy was observed based on baseline serum bFGF levels, TSP levels, or bFGF:TSP ratios.

Finally, we evaluated the relationship of baseline serum VEGF, bFGF, TSP, VEGF:TSP ratio, and bFGF:TSP ratio with TTT and OS. The VEGF:TSP ratio was significantly correlated with OS (p=0.008, Hazard Ratio =1.26 [95% confidence interval: 1.06–1.49]). This correlation with OS persisted in two factor multivariate Cox models adjusting for stage, absolute lymphocyte count at the time of study entry, *IGHV* mutation status, CD38 status, FISH results, or ZAP-70 status (all p<0.01). No other significant relationships between baseline angiogenic cytokine levels and TTT or OS were observed.

The present analysis has several important limitations. Angiogenesis is a complex process regulated by an intricate balance of numerous pro- and anti-angiogenic molecules.(Ribatti, *et al* 2007) In this regard, our use of 2 pro-angiogenic and 1 anti-angiogenic cytokines is a gross simplification of this continuum where other unmeasured cytokines undoubtedly play an important role in this process. Future studies exploring the relationship between response to treatment and angiogenic phenotype using more comprehensive profiling of angiogenic cytokines will be enlightening. Since the relationship between the VEGF:TSP ratio and response is not absolute, the lack of a relationship between baseline VEGF:TSP and time to progression in this small series is not surprising. Given the lack of association with TTT however, it is surprising that the VEGF:TSP ratio was associated with OS. Although we are unable to explain this unexpected result, it is possible that patients with more angiogenic phenotypes relapse at the same rate as those with less angiogenic phenotypes but are less likely to respond to salvage therapy when relapse occurs.

In summary, the results of this prospective analysis are consistent with the hypothesis that a relationship exists between pre-treatment serum angiogenic cytokine levels and response to CIT in patients with CLL. Specifically, a pro-angiogenic phenotype (higher pro-angiogenic factors and lower anti-angiogenic factors) at the time of treatment correlated with a poorer response to CIT and a lower likelihood of achieving a CR or nPR. This finding supports previous *in vitro* studies suggesting that angiogenic cytokines are important for the survival of CLL B cells and that these cytokines may up-regulate levels of anti-apoptotic proteins known to influence response to therapy.(Shanafelt and Kay 2006) Combining anti-VEGF therapy with chemotherapy has been proven to prolong OS in patients with colon and non-small cell lung cancer and to improve PFS in patients with metastatic breast cancer(Hurwitz, *et al* 2004; Sandler, *et al* 2006). While additional studies are needed to confirm the present findings with other CIT regimens and in larger patient populations, the results suggest that a pro-angiogenic profile is associated with diminished response and inferior survival in CLL patients receiving CIT. Furthermore, the findings suggest that the addition of anti-angiogenic therapy and, in particular, anti-VEGF therapy may enhance the efficacy of CIT in CLL patients and encourage further clinical testing of this strategy.

Acknowledgments

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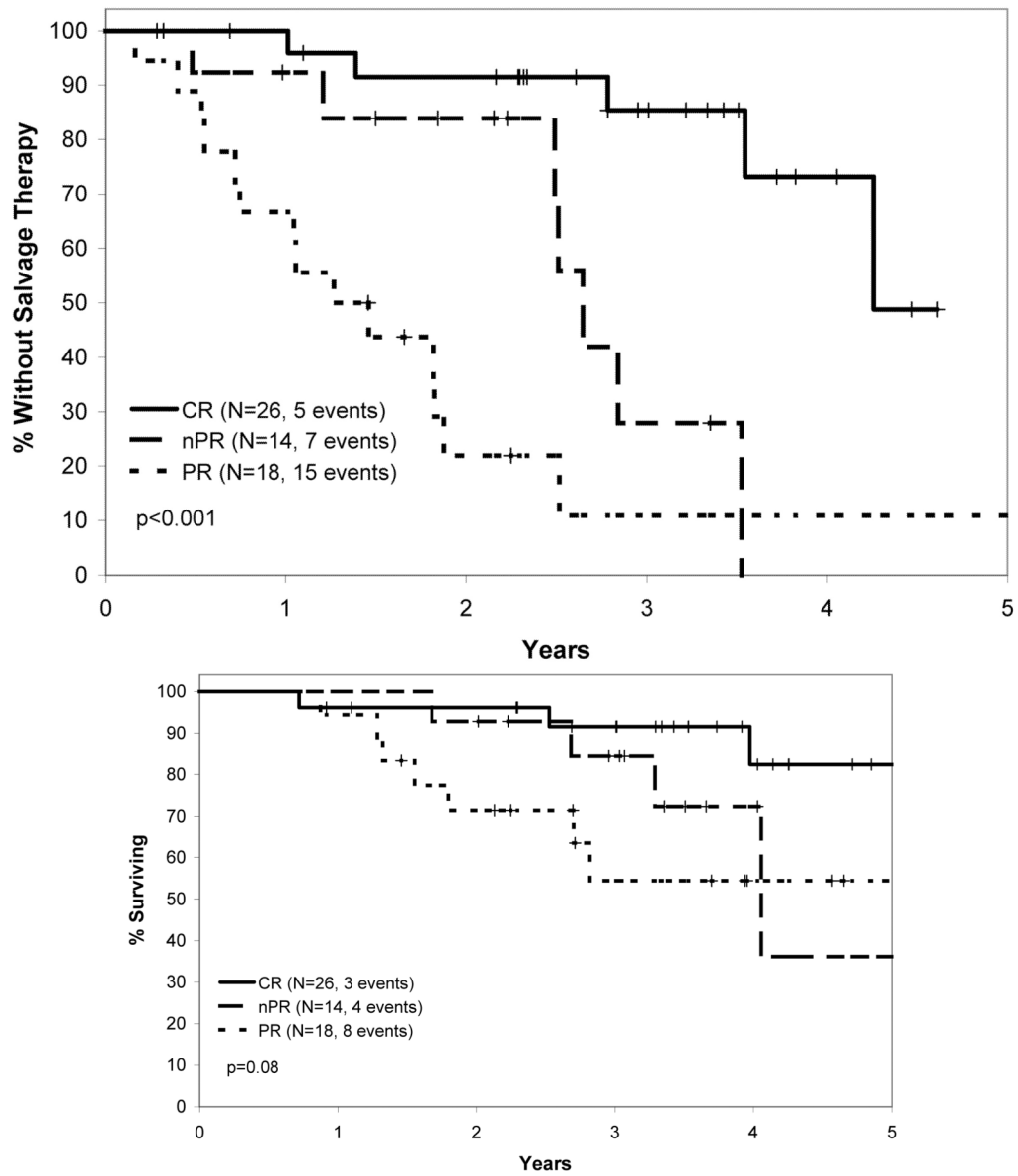
Figure 1.
Figure 1A: Time to Salvage Therapy After PCR (pentostatin, cyclophosphamide, rituximab)
Therapy.
Time from first date of PCR therapy to the date of next therapy based on response to PCR.
Log rank p value shown.
Figure 1B: Overall Survival After PCR Therapy.
Time from first date of PCR therapy to death based on response to PCR. Log rank p value
shown.

Table 1

Baseline Serum Levels of Pro and Anti-Angiogenic Cytokines Table shows the median VEGF(pg/ml), bFGF(pg/ml), and thrombospondin levels(pg/ml) for patients enrolled on phase II trial of PCR as first line treatment for CLL(N=56). Additional columns show the ratio of VEGF:TSP and bFGF:TSP where smaller ratios indicate a more "anti-angiogenic" phenotype and higher ratios indicate a more "pro-angiogenic" phenotype.

	N	VEGF median (Q1, Q3)	bFGF median (Q1, Q3)	TSP median (Q1, Q3)	VEGF:TSP median (Q1, Q3)	bFGF:TSP median (Q1, Q3)
All patients	**56**	**172 (93, 378)**	**6.6 (1.6, 24.2)**	**1395 (771, 3715)**	**0.0914 (0.0524, 0.2654)**	**0.0036 (0.0009, 0.0161)**
IGHV Mutation Status						
Mutated	16	151	4.5	1708	0.0593[1]	0.0022
Unmutated	39	226	7.4	1277	0.1291[1]	0.0054
CD38 Status						
Negative	37	177	3.7	1455	0.0867	0.0029
Positive	19	154	8.4	1277	0.1103	0.0061
FISH						
13q-	20	137	2.4[2]	1982	0.0789	0.0013[3]
Normal	6	184	11.5[2]	1284	0.1479	0.0095[3]
+12	13	226	35.5[2]	1434	0.1291	0.0171[3]
11q-	12	152	2.2[2]	1836	0.1014	0.0026[3]
17p-	3	253	5.7[2]	1137	0.1867	0.0050[3]
ZAP-70						
Negative	33	154	5.7	1991	0.0867	0.0028
Positive	17	119	8.4	973	0.1103	0.0062
Response						
CR	24	111[4]	7.1	1278	0.0847[5]	0.0043
nPR	13	245[4]	7.8	1991	0.0787[5]	0.0062
PR	14	237[4]	3.1	1385	0.1718[5]	0.0023
Stable or progressive disease	5	253[4]	15.7	1355	0.1867[5]	0.0063

[1] mutated vs. unmutated; p=0.008(Wilcoxon rank sum)

[2] FISH p=0.01(Kruskal-Wallis)

[3] FISH p=0.04(Kruskal-Wallis)

[4] CR or nPR *vs* PR, stable or progressive disease; p=0.03(Wilcoxon rank sum); CR vs nPR, PR, stable or progressive disease; p=0.02(Wilcoxon rank sum)

[5] CR or nPR vs. PR, stable or progressive disease; p=0.04(Wilcoxon rank sum); CR vs nPR, PR, stable or progressive disease; p=0.18(Wilcoxon rank sum)