Methylenetetrahydrofolate reductase C677T and A1298C gene variants in adult non-Hodgkin's lymphoma patients: association with toxicity and survival

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ABSTRACT

Background and Objectives

Common methylenetetrahydrofolate reductase gene variants (*MTHFR* C677T and A1298C) have been described to have opposite effects on cancer patients. They may reduce cancer susceptibility and increase drug-related toxicity when folate antagonists (e.g. methotrexate) are utilized. We analyzed 110 patients with high-grade non-Hodgkin's lymphoma (NHL), 68 of whom were eligible for a chemotherapy combination containing methotrexate (MACOP-B) and 42 for chemotherapy without methotrexate (CHOP).

Design and Methods

Patients were genotyped by polymerase chain reaction and stratified by *MTHFR* variants. These data were related to the toxicity (WHO grade GO-4) that the patients suffered and their survival. Overall 64 cases (58.2%) developed some form of toxicity and 23 (20.9%) had grade 3/4 toxicity.

Results

When considering toxicity of any grade (grade 1-4), the 677TT genotype was significantly over-represented among cases with mucositis (OR=4.85; 95%CI, 1.47-15.97; p=0.009) and those with hepatic toxicity (OR=3.43; 95%CI, 0.99-11.86; p=0.052). Sub-analyses in the group treated with MACOP-B showed a slight increase in the risk of developing mucositis (OR=5.22; 95%CI, 1.20-27.27; p=0.03), and a strong increase in the risk of hepatic toxicity (OR=7.08; 95%CI, 1.38-36.2; p=0.019) and thrombocytopenia (OR=7.69, 95%CI 1.0-58.94; p=0.05). Interestingly, compared to the risk of developing toxicity of any grade, the risk of developing severe (grade 3/4) mucositis was almost doubled in the whole group of cases with 677TT (OR=8.13; 95%CI 1.61-41.04; p=0.011) and dramatically increased in the MACOP-B-treated cases with this gene variant (OR=24.6; 95%CI 2.49-87.41; p=0.001). There were significant results for 1298CC cases exclusively for mucositis (any grade, OR=5.33; 95%CI, 1.25-22.70; p=0.023 and OR=9.15; 95%CI, 1.14-73.41; p=0.037; for the whole group and the MACOP-B-treated group, respectively). Similarly, the risk of 1298CC patients developing severe mucositis increased (OR=9.24; 95%CI, 1.47-58.0; p=0.017 and OR=11.53; 0.93-143.18; p=0.057; in the whole group and in the MACOP-B-treated group, respectively). Event-free survival analysis revealed a lower probability of event-free survival at 5 years for 677T-carriers (log-ranks, p=0.05 and p=0.07 in the whole group and in the MACOP-B-treated group, respectively). More significant results were obtained when 1298CC cases were excluded from the reference group (log-ranks, p=0.03 and p=0.04, respectively). No significant associations were found in the CHOP-treated group.

Interpretation and Conclusions

Our data suggest that *MTHFR* gene variants play a critical role in NHL outcome, possibly by interfering with the action of methotrexate with significant effects on toxicity and survival. Genotyping of folate pathway gene variants might be useful to enable reduction of chemotherapy toxicity and/or to improve survival by indicating when dose adjustments or alternative treatments are necessary.

Key words: non-Hodgkin's lymphoma, *MTHFR* SNP, toxicity, survival, pharmacogenetics.

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Methylenetetrahydrofolate reductase (MTHFR) converts methylenetetrahydrofolate to methyltetrahydrofolate, the major circulating form of folate, so providing methyl groups for methionine synthesis. Methylenetetrahydrofolate and its derivates are essential for purine and pyrimidine synthesis. Therefore, the activity of MTHFR plays an important role in both DNA synthesis and methylation, which are critical processes for rapidly growing malignant and non-malignant cells. Two common single nucleotide polymorphisms (SNP) have been described to affect the activity of the MTHFR enzyme, a C to T nucleotide transition at position 677 and an A to C nucleotide transversion at position 1298.[1,2] Reduced enzyme activity has been reported in 677TT and 1298CC homozygotes as well as in combined carriers and to a lesser extent in heterozygous individuals.[1-4] Several studies have investigated *MTHFR* gene variants and disorders involving folate metabolism,[5-9] and recently there has been growing interest in the pharmacogenetics of antifolate drugs.[10,11] In particular, it was reported that individuals carrying *MTHFR* polymorphisms may have a lower susceptibility to develop solid or hematologic cancers.[12-18] On the other hand, a few recent studies suggested that carrier patients may have exacerbated toxicity when treated with antifolate drugs[19-21] or reduced survival, possibly through interference with the action of methotrexate.[22,23] Such a dualism has also been reported for additional folate metabolizing gene polymorphisms.[14,23-25] Overall, relationships between clinical outcome and folate pathway gene variants in lymphomas have been poorly investigated.[26,27] Methotrexate, an antifolate chemotherapeutic agent, is widely used, alone or in combination with other drugs, in the treatment of a number of solid, hematologic malignancies[28-31] as well as non-malignant disease.[32,33] In particular, the MACOP-B combined scheme is a third-generation regimen that is very effective against high-grade non-Hodgkin's lymphomas (NHL).[34,35] Similarly to many other anticancer drugs, methotrexate has little selectivity for cancer cells, thus its effectiveness is limited by toxicity against normal tissues, particularly towards gastrointestinal epithelium, bone marrow and liver.[36,37] A less aggressive drug combination not containing methotrexate (i.e. CHOP) shows similar effectiveness in the treatment of high-grade NHL.[38,39] We previously reported that SNP in the genes of folate metabolizing enzymes play a greater role in acute lymphoblastic leukemia than in NHL.[18] In the present study we investigated the possible effects of two common *MTHFR* gene variants on toxicity and on clinical outcome in a group of 110 NHL patients treated with different chemotherapeutic regimens.

Design and Methods

Study design, selection of patients and their main characteristics

The aim of our study was to investigate any possible role of *MTHFR* genotypes on the clinical outcome of NHL patients and whether there were differences according to the chemotherapeutic regimens used. For this purpose, we analyzed therapy-related toxicity and survival data in the whole group and in the sub-groups of cases treated with MACOP-B or CHOP (*For details see online supplementary Appendix at www.haematologica.org/*).

Chemotherapeutic regimens and toxicity evaluation

After stratification according to age, Eastern Cooperative Oncology Group performance status,[42] and presence of mediastinal mass, the patients were assigned to the MACOP-B or CHOP chemotherapy regimen protocol. (*For details see online supplementary Appendix at www.haematologica.org/*).

Genotype analyses

DNA was isolated from peripheral whole blood by using proteinase-K treatment followed by phenol/chloroform extraction and ethanol precipitation. The genotyping protocol for detection of the *MTHFR* C677T polymorphism used the following primers: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3'.[1] (*For details see online supplementary Appendix at www.haematologica.org/*). The genotyping protocol for detecting the *MTHFR* A1298C polymorphism used the following primers: 5'-GGG AGG AGC TGA CCA GTG CAG-3' and 5'-GGG GTC AGG CCA GGG GCA G-3'.[2] (*For details see online supplementary Appendix at www.haematologica.org/*).

Statistical analysis

Statistical differences between groups were assessed by the Student's *t*-test and the χ^2 test. When appropriate, Yates' correction or Fisher's exact test was applied. Odds ratio (OR) and 95% confidence intervals (95% CI) were used to estimate the risk of developing different grades of toxicity after chemotherapy. Adjusted OR were calculated with logistic regression models, with the dependent variable being the toxicity grade according to WHO criteria subdivided as grades 1-4 or grades 3-4 versus grade 0 (*see specifics in legends to the Tables*). (*For details see online supplementary Appendix at www.haematologica.org/*).

Results

Main clinical characteristics and genotype distributions in the groups of NHL patients

The main clinical characteristics of the groups of patients considered are listed in Table 1. The whole group consisted of 110 patients with high-grade NHL. Among these 110 patients, 68 received MACOP-B treatment and 42 received CHOP treatment. Stratification according to stage differed significantly between the two treatment groups (p=0.027), whereas there were no differences in performance status or in the presence of a mediastinal mass. Among the whole group of patients, 64 (58.2%)

developed toxicity of some grade (hematologic and non-hematologic) and 23 (20.9%) developed severe, grade 3/4 toxicity. The global treatment-related death rate was 3.6% (two cases in the MACOP-B group and two cases in the CHOP group). The global pattern of toxicities differed according to the chemotherapy regimen used, being more severe in the MACOP-B-treated group (p=0.05) probably due to the presence of methotrexate in this chemotherapeutic combination. Accordingly, mucositis was statistically overrepresented in the MACOP-B group with respect to the CHOP group (30.9% vs 7.1%; p=0.007). Conversely, the rate of the other toxicities considered did not differ statistically between the two groups, and neither did the *MTHFR* genotype.

Treatment was adapted in all patients developing severe (grade 3-4) toxicity (11 treated with MACOP-B, 16.2% and 12 treated with CHOP, 28.6%). In detail, these patients experienced lymphocytic toxicity (65.2%), mucositis (39.1%), anemia (26.1%), thrombocytopenia (21.7%) and hepatic toxicity (13.0%). Treatment was temporarily suspended in 40% of cases and the dose of chemotherapeutic agents reduced (by 20%) in 60% of the patients. When stratified by *MTHFR* polymorphisms, performance status revealed a slight 677 genotype dependence only in the MACOP-B subgroup. A worse performance status was observed as the number of 677T alleles increased (test-trend, p=0.051). Conversely, no significant associations were identified for disease stage or presence of a mediastinal mass with particular *MTHFR* genotypes in the whole group or in the two differently treated subgroups (*data not shown*).

Toxicity and MTHFR polymorphisms in the whole NHL group

Among all the patients with NHL who developed toxicity (n=64), the prevalence of hematologic and non-hematologic toxicities was as follows: 24 mucositis (37.5%), 26 hepatic toxicity (40.6%), 42 lymphocytopenia (65.6%), 21 anemia (32.8%) and 18 thrombocytopenia (28.1%). Table 2 shows the different kinds of toxicities stratified by *MTHFR* 677-genotypes in the whole group of NHL cases. Globally, mucositis was significantly overrepresented among 677TT-homozygotes when compared with both the 677CC reference group (OR=2.21; 95%CI, 1.08-8.75; p=0.045) and the group with the other genotypes (OR= 4.85; 95% CI, 1.47-15.97; p=0.009). Hepatic toxicity was slightly associated with the 677TT-genotype when compared with the other genotypes (OR=3.43; 95%CI; 0.99-11.86; p=0.052). It is worth noting that there was an unexpected low prevalence of 677CT cases (11.3%) among patients with mucositis compared to the prevalence of patients with the other 677-genotypes (23.7% and 47.4% for the CC and TT genotypes, respectively). This was responsible for a low, but not statistically significant, OR-value ascribable to the CT-genotype.

The risk of developing severe mucositis (grade 3/4) was double in 677TT-carriers compared to that in patients with the other genotypes (OR=8.13; 95% CI, 1.61-41.04; p=0.011) (Table 3). Conversely, no further increase in risk was observed when only severe hepatic toxicity was considered. No associations between particular 677-genotypes and thrombocytopenia, nor lymphocytic toxicity, or anemia were found (*data not shown*). As far as concerns the A1298C polymorphism, a statistical significance was found only for the occurrence of mucositis in 1298CC homozygotes when compared to patients with other genotypes, yielding an OR of 5.33 (95% CI, 1.25-22.70; p=0.023). When only severe mucositis was considered (Table 3) the risk increased further (OR=9.24; 95% CI, 1.47-58.01; p=0.017). In combined analyses to evaluate the effect of both *MTHFR* variants, the absence of double wild-type carriers with mucositis did not allow any relative risk evaluation. This supports the idea that wild-type alleles are underrepresented among patients developing toxicity.

Toxicity and MTHFR polymorphisms in the two treatment groups

Among NHL patients treated with MACOP-B who developed toxicity (n=45), the prevalence of hematologic and non-hematologic toxicities was as follows: 21 mucositis (46.6%), 18 hepatic toxicity (40.0%), 27 lymphocytopenia (60.0%), 9 anemia (20.0%) and 11 thrombocytopenia (24.4%). Table 4 shows the different kinds of toxicities stratified by MTHFR 677-genotypes in the MACOP-B-treated subgroup of patients. Among MACOP-B-treated patients with the 677TT genotype, the risk of developing any grade of mucositis, hepatic toxicity or thrombocytopenia was about 5 to 7-fold higher than that in patients with the other genotypes. When only grade 3/4 mucositis was considered (Table 3), the risk for 677TT-carriers increased dramatically (OR=24.60; 95% CI, 2.49-87.41; p=0.001). A further increase in the risk value related to the development of more severe hepatic toxicity or thrombocytopenia was not observed. No association between particular 677-genotypes and lymphocytic toxicity or anemia was found. As for the whole group, a low rate of 677CT cases among those with mucositis was responsible for an associated non-significant decreased OR value.

When the A1298C polymorphism was analyzed, only the occurrence of mucositis in the 1298CC homozygotes was statistically significant when compared with toxicity in patients with the other genotypes, yielding an OR of 9.15 (95% CI, 1.14-73.41; p=0.037). When severe mucositis was considered (Table 3) the risk further increased (OR=11.53; 95%CI, 0.93-143.18; p=0.057). Among NHL patients treated with CHOP who developed toxicity (n=19), the prevalence of hematologic and non-hematologic toxicities was as follows: 3 mucositis (15.8%), 8 hepatic toxicity (42.1%), 15 lymphocytopenia (79.0%), 12 anemia (63.1%) and 7 thrombocytopenia (36.8%). It should be noted that a very low percentage of cases of mucositis was observed in patients treated with CHOP in

Table 1. Main characteristics of the patients.

	Total cases n=110	*MACOP-B n=68*	*CHOP n=42*	*p**
Age (mean, SD), years	56.8±17.1	49.9±16.6	67.9±10.9	0.001
Range, years	18-80	18-80	32-80	--
Sex (male/female)	67/43 (60.9)	47/21 (69.1)	20/22 (47.6)	0.04
Stage				
I	16 (14.5)	6 (8.8)	10 (23.8)	0.027°
II	18 (16.4)	16 (23.5)	2 (4.8)	
III	37 (33.6)	22 (32.3)	15 (35.7)	
IV	39 (35.4)	24 (35.3)	15 (35.7)	
Performance status (ECOG)#				
Fully active or ambulatory (0-1)	69 (62.7)	42 (61.8)	27 (64.3)	NS
NS				
Bedridden (≥2)	33 (30.0)	21 (30.9)	12 (28.6)	NS
Mediastinal mass#				
Yes/No	33/69	22/41	11/28	NS
Toxicity				
Mucositis (n, %)	24 (21.8)	21 (30.9)	3 (7.1)	0.007
Hepatic (n, %)	26 (23.6)	18 (26.4)	8 (19.0)	NS
Lymphocytopenia (n, %)	42 (38.2)	27 (39.7)	15 (35.7)	NS
Anemia (n, %)	21 (19.1)	9 (13.2)	12 (28.6)	NS
Thrombocytopenia (n, %)	18 (16.4)	11 (16.2)	7 (16.7)	NS
Overall, grades 1-4 (n, %)	64 (58.2)	45 (66.2)	19 (45.2)	0.05
MTHFR genotype (n, %)				
677 CC	38 (34.5)	22 (32.3)	16 (38.1)	NS°
677 CT	53 (48.2)	36 (52.9)	17 (40.5)	
677 TT	19 (17.3)	10 (14.7)	9 (21.4)	
1298 AA	44 (40.0)	27 (39.7)	17 (40.5)	NS°
1298 AC	56 (50.9)	34 (50.0)	22 (52.4)	
1298 CC	10 (9.1)	7 (10.3)	3 (7.1)	

**p-values are referred to comparisons between the MACOP-B and the CHOP group; #Data are not available for five MACOP-B- and for three CHOP- treated-patients. °p-values are referred to data distribution.*

Table 2. *MTHFR* C677T genotype and toxicity risk evaluation in the whole NHL group (n=110).

MTHFR C677T (n)	*Toxicity grade 0 n (%)*	*Toxicity grade 1-4 n (%)*	*OR (95% CI)*	*P*
		Mucositis		
CC (38)	29 (76.3)	9 (23.7)	Reference	
CT (53)	47 (88.7)	6 (11.3)	0.50 (0.21-1.42)	NS
TT (19)	10 (52.6)	9 (47.4)	2.21 (1.08-8.75)	0.045
TT vs CC+CT*			4.85 (1.47-15.97)	0.009
		Hepatic toxicity		
CC (38)	31 (81.6)	7 (18.4)	Reference	
CT (53)	42 (79.2)	11 (20.7)	1.17 (0.41-3.39)	NS
TT (19)	11 (57.9)	8 (42.1)	2.94 (0.85-10.20)	0.089
TT vs CC+CT*			3.43 (0.99-11.86)	0.052
		Lymphocytic toxicity		
CC (38)	26 (68.4)	12 (31.6)	Reference	
CT (53)	31 (58.5)	22 (41.5)	1.49 (0.57-3.89)	NS
TT (19)	11 (57.9)	8 (42.1)	0.95 (0.25-3.71)	NS
TT vs CC+CT*			0.78 (0.24-2.50)	NS
		Anemia		
CC (38)	31 (81.6)	7 (18.4)	Reference	
CT (53)	42 (79.2)	11 (20.7)	0.98 (0.30-3.18)	NS
TT (19)	16 (84.2)	3 (15.8)	0.54 (0.10-2.98)	NS
TT vs CC+CT*			0.52 (0.12-2.32)	NS
		Thrombocytopenia		
CC (38)	32 (84.2)	6 (15.8)	Reference	
CT (53)	47 (88.7)	6 (11.3)	0.56 (0.14-2.22)	NS
TT (19)	13 (68.4)	6 (31.6)	1.92 (0.38-9.64)	NS
TT vs CC+CT*			2.31 (0.62-8.60)	NS

*OR-values were computed considering the number of MTHFR 677CC cases as the reference. *OR-values were computed comparing the number of MTHFR 677TT cases versus the number of cases with the remaining genotypes for each type of toxicity. p-values above 0.100 are not shown.*

comparison to the percentage among patients treated with MACOP-B (Table 1). This could be strongly associated with the presence/absence of methotrexate in the two different chemotherapy regimens. Finally, in the patients treated with CHOP, there were no significant associations between any type or grade of toxicity and specific *MTHFR* genotypes for either the C677T or A1298C polymorphism (*data not shown*).

Survival and MTHFR polymorphisms in NHL groups

Kaplan-Meier analysis comparing EFS curves at 5 years of follow-up for the two treatment groups (MACOP-B and CHOP) did not show significant difference (p=0.81; Figure 1). When the whole group of NHL cases was stratified according to 677-genotype, Kaplan-Meier analysis showed that 677T-carriers had a lower probability of EFS compared to cases with the 677CC-genotype (log-rank, p=0.05; Figure 2). Accordingly, 677T-carriers were at higher risk of adverse events compared to patients with the 677CC-genotype (HR=1.99; 95% CI, 1.05-3.55; p=0.046).

Although no significant differences in EFS rate were found between patients stratified according to 1298-variant, 1298CC-homozygotes had the lowest probability of survival at 5 years (*data not shown*). For this reason, and taking into account that only 1298CC homozygotes showed significant toxicity patterns, in an exploratory analysis we excluded these cases from the reference group in the subsequent survival analyses. In the whole group, excluding 1298CC-homozygotes, 677T-carriers had a further reduction in EFS probability (log-rank, p=0.03) and the associated risk of developing adverse events increased (HR=2.40; 95% CI, 1.10-5.10; p=0.024).

The pattern of EFS in the MACOP-B-treated subgroup was similar. Again, the significance was higher when 1298CC-homozygotes were excluded from the reference group and the associated log-rank values were p=0.07 and p=0.04 (when the 1298CC homozygotes were or were not included, respectively, in the reference group). Accordingly, the respective HR for adverse events reserved to the 677T-carriers were 2.21 (95% CI, 0.95-5.11; p=0.070) and 2.99 (95% CI; 1.19-9.50; p=0.030). Finally, EFS among the subgroup treated with CHOP did not differ significantly in relation to specific *MTHFR* geno-

Table 3. ***MTHFR*** **C677T and A1298C genotypes and risk of severe mucositis in the whole group of NHL patients (n=110) and in those treated with MACOP-B (n=68).**

MTHFR	*Whole group (n=110)*			*MACOP-B group (n=68)*		
C677T	WHO G0 n (%)	WHO G3-4 n (%)	OR (95% CI; P)	WHO G0 n (%)	WHO G3-4 n (%)	OR (95% CI; P)
CC	29 (90.6)	3 (9.4)	Reference	13 (81.2)	3 (18.7)	Reference
CT	47 (97.9)	1 (2.1)	0.22 (0.02-2.41; NS)	30 (96.8)	1 (3.2)	0.12 (0.01-1.71; NS)
TT	10 (66.7)	5 (33.3)	3.86 (0.84-23.15; 0.080)	4 (50.0)	4 (50.0)	9.40 (0.64-138.80; NS)
TT *vs* CC+CT*			8.13 (1.61-41.04; 0.011)			24.60 (2.49-87.41; 0.001)
A1298C						
AA	32 (86.5)	5 (13.5)	Reference	18 (81.8)	4 (18.2)	Reference
AC	49 (98.0)	1 (2.0)	0.12 (0.01-1.31; 0.082)	27 (96.4)	1 (3.6)	0.02 (0.0003-1.42;0.072)
CC	5 (62.5)	3 (37.5)	6.11 (0.66-56.25; NS)	2 (40.0)	3 (60.0)	24.36 (0.28-2119.33; NS)
CC *vs* AA+AC*			9.24 (1.47-58.01; 0.017)			11.53 (0.93-143.18;0.057)

*OR-values were computed considering the number of 677CC or 1298AA cases as the reference. *OR-values were computed comparing the number of MTHFR 677TT or 1298CC cases versus the number of the cases with respective remaining genotypes. p-values above 0.100 are not shown.*

Table 4. ***MTHFR*** **C677T genotype and risk of toxicity in the MACOP-B-treated group (n=68).**

MTHFR C677T (n)	*Toxicity grade 0 n (%)*	*Toxicity grade 1-4 n (%)*	*OR (95% CI)*	*p*
		Mucositis		
CC (22)	13 (59.1)	9 (40.9)	Reference	
CT (36)	30 (83.3)	6 (16.7)	0.62 (0.34-1.58)	NS
TT (10)	4 (40.0)	6 (60.0)	3.15 (1.05-9.43)	0.042
TT *vs* CC+CT*			5.22 (1.20-27.27)	0.030
		Hepatic toxicity		
CC (22)	17 (77.3)	5 (22.7)	Reference	
CT (36)	29 (80.5)	7 (19.4)	1.02 (0.24-4.30)	NS
TT (10)	4 (40.0)	6 (60.0)	5.34 (1.04-27.21)	0.044
TT *vs* CC+CT*			7.08 (1.38-36.21)	0.019
		Lymphocytic toxicity		
CC (22)	16 (72.7)	6 (27.3)	Reference	
CT (36)	20 (55.5)	16 (44.4)	2.04 (0.62-6.73)	NS
TT (10)	5 (50.5)	5 (50.5)	2.03 (0.29-14.08)	NS
TT *vs* CC+CT*			1.33 (0.27-6.51)	NS
		Anemia		
CC (22)	20 (90.9)	2 (9.1)	Reference	
CT (36)	30 (83.3)	6 (16.7)	1.90 (0.34-10.51)	NS
TT (10)	9 (90.0)	1 (10.0)	0.82 (0.04-16.43)	NS
TT *vs*CC+CT*			0.75 (0.08-7.40)	NS
		Thrombocytopenia		
CC (22)	20 (90.9)	2 (9.1)	Reference	
CT (36)	31 (86.1)	5 (13.9)	1.59 (0.26-9.59)	NS
TT (10)	6 (60.0)	4 (40.0)	4.87 (0.59-40.08)	NS
TT *vs* CC+CT*			7.69 (1.00-58.94)	0.050

*OR values were computed considering the number of MTHFR 677CC cases as the reference. *OR values were computed comparing the number of MTHFR 677TT cases versus the number of cases with the remaining genotypes for each toxicity. °p-values above 0.100 are not shown.*

types (log-ranks p=0.32 and p=0.26, when computing or not the 1298CC cases in the reference group). Likewise, the respective HRs were 1.50 (95% CI, 0.62-4.3; p=0.39) and 1.81 (95% CI, 0.62-5.69; p=0.30). The A1298C polymorphism by itself did not have a significant effect on survival.

Discussion

The great interindividual variability in drug effect and efficacy is one of the major issues in the clinical management of patients with cancer. Resistance and toxicity greatly affect the clinical outcome of treated patients.[10,36] SNP are emerging as important pharmacogenetic prognostic determinants of response to chemotherapy. Recent studies have investigated the toxic effects of antifolate drugs in relation to folate metabolizing SNP in both hematologic and solid cancers.

In the present study, we investigated whether specific *MTHFR* genotypes were associated with survival and toxicity in a cohort of 110 adults with high-grade NHL treated with different pharmacological regimens containing or not methotrexate (MACOP-B and CHOP, respectively). The first outcome of our survey was that patients with NHL who carry the 677TT-genotype had about a 3- to 7-fold increased risk of developing different kinds of toxicities when compared to patients with other genotypes in both the whole group and in the subgroup treated with MACOP-B. This effect of genotype was particularly evident when severe toxicity phenotypes were considered. Indeed, 677TT cases treated with MACOP-B had an approximately 24-fold increased risk of developing severe (grade 3-4) mucositis. Similarly, hepatic toxicity and thrombocytopenia were more strongly related to the 677TT genotype in the MACOP-B-treated group than in the whole group but the risks were quite similar consid-

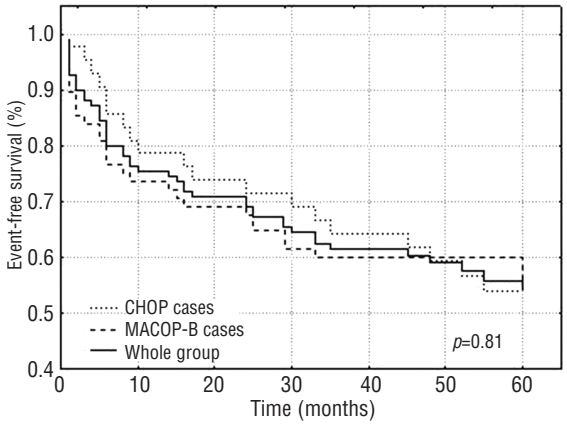

Figure 1. Kaplan-Meier analysis of EFS in the whole NHL group and in the two treatment subgroups.

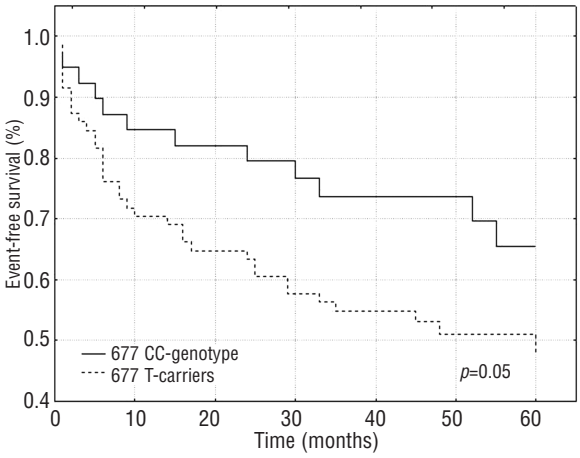

Figure 2. Kaplan-Meier analyses of EFS in the whole group of NHL cases stratified by *MTHFR* C677T genotype.

ering any grade of toxicity or severe toxicity. An unexpected underrepresentation of 677CT heterozygotes was found among patients with mucositis, associated with discordant but non-significant risk results. These data could be explained, in part, by the known partial linkage disequilibrium between 677 and 1298 alleles responsible for an unequal mutual distribution of the two allelic counterparts.[44] As regards the 1298 variant, effects were seen exclusively for mucositis both in the whole group and in the MACOP-B-treated subgroup, with associated risks increased by about 5- and 9-fold, respectively: the risks increased further when only severe mucositis was considered. Conversely, among patients with NHL treated with CHOP, no type or grade of toxicity was associated with *MTHFR* genotypes. Therefore, the role of *MTHFR* variants found in the whole group is mainly ascribable to their effects in MACOP-B-treated patients. Some recent studies showed increased toxicity in 677T-carriers treated with methotrexate[19-21] although other studies did not confirm such an association.[24,26,27] In particular, this association was not observed in pediatric patients with either NHL or acute lymphoblastic leukemia.[23,26] However, in adult NHL, we found strong chemotherapy toxicity associated with the 677TT-genotype. Different methotrexate doses and schemes and also diverse nutritional/folate status between adult and pediatric NHL patients might account in part for these discrepant results. The particularly evident association found in the MACOP-B subgroup could be ascribed to the inclusion of methotrexate in this combination of chemotherapeutic agents. *MTHFR* gene variants may increase sensitivity to methotrexate, perhaps through an imbalance of folate isoforms. Pharmacologically induced low levels of 5-methyltetrahydrofolate and constitutively low availability of this substrate in 677T-carriers, together with predictable effects on homocysteine concentrations, may account for the observed exacerbated toxicity.[21,45] The association between survival and folate pathway gene variants in cancer patients treated with antifolates is less investigated and still controversial. It seems that a diminished survival is present in cases carrying those alleles responsible for an imbalance of folate isoforms.[22,23,26] We found that 677T-carriers had a lower probability of EFS at 5 years of follow-up when compared to patients with the other genotypes, both in the whole group and in the MACOP-B-treated subgroup. Specifically, 677T carriers had an about 2-fold increased risk of adverse events. This was particularly evident in the MACOP-B-treated subgroup and when 1298CC homozygotes were excluded from the reference group. This would imply that the 1298C allele also has negative effects on survival, although previous studies in patients with acute lymphoblastic leukemia did not find such an association.[22] To a lesser extent than 677TT-carriers, individuals with the 1298CC genotype have decreased MTHFR activity and slightly raised homocysteine levels.[2] In addition, because of partial linkage disequilibrium, the coexistence of 677T and 1298C alleles in *cis* is possible, but very rare, supporting the hypothesis that triple mutations (i.e. 677TT/1298AC or 677CT/1298CC) or double homozygous conditions (i.e. 677TT/1298CC) are probably *de novo* recombinant events.[44] This is consistent with the fact that virtually all 677TT subjects have wild-type 1298 alleles. For these reasons, it is hard to observe a clear allele-dosage effect for the 1298 variant being better accounted for in homozygous conditions. This could, in part, justify the difficulty in ascribing effects on survival to the 1298-variant itself and also account for the improved probability of EFS observed in 677-wild-type carriers when the 1298CC homozygotes were excluded from the analysis. Among NHL cases treated with CHOP, no significantly different survival rates or risks were associated with particular *MTHFR* genotypes. This could be explained in part by the very low number of cases investigated, or alternatively, as

for the toxicity data, might be mainly due to the absence of methotrexate in this chemotherapy regimen. Thus, *MTHFR*-dependent survival might partially depend on treatment type and composition. We cannot, however, exclude that different mean ages or gender compositions of the two treatment subgroups might have accounted for the different results. It should be noted that when genotype was not considered very much closer survival profiles were found in the two subgroups (Figure 1). That said, the main purpose of our study was not to compare toxicity or survival patterns between two groups of NHL patients treated with different protocols, but rather to determine whether different *MTHFR*-genotypes have a role in the clinical outcome of such patients or particular subgroups of patients.

How folate unbalancing influences cancer remains to be established. It is currently believed that it may act by altering DNA methylation and/or synthesis.[46-48] Therefore, by affecting folate balance, folate pathway gene variants might modulate cancer risk and influence the effects of chemotherapy. In particular, 677T- and/or 1298C-carriers, who have more 5,10-methylene-tetrahydrofolate may have enhanced thymidylate synthase activity, interfering in turn with the therapeutic target of methotrexate. This might favor residual neoplastic clone expansion. At the same time, 677T- and/or 1298C-carriers, who have less 5-methyl-tetrahydrofolate, may have raised levels of homocysteine, increasing the toxicity of methotrexate. On the other hand, these polymorphisms, as well as other folate pathway gene variants, have been described to protect against the development of cancer.[12,14,16,18,49,50] This means that subjects carrying such variants may have dual but opposite effects from the polymorphism. They may have reduced susceptibility to cancer but increased drug-related toxicity and even reduced survival rates. The same mechanisms (e.g. more efficient thymidylate synthesis) may act beneficially in the healthy subjects but detrimentally in patients with cancer. Such gene variants might be considered *Judas-alleles* acting as friend in the healthy subjects but as a foe in the cancer patients.

In conclusion, our study ascribes *MTHFR* gene variants an important role in the outcome of patients with NHL, possibly by interfering with methotrexate as a part of a chemotherapy combination. We are aware of the limits of our study due to the small sample size and the fact that two gene variants partially account for these complex mechanisms. It is strongly recommended that folate levels are assessed in future studies, because this substrate could affect the efficacy of chemotherapy. Definitive conclusions should nevertheless be drawn with extreme caution, and further larger studies and/or multicenter analyses are needed to address these issues properly and to confirm the present findings.

Authors' Contributions

DG working hypothesis, designed the study, wrote the article and obtained funding support; AO interpreted and analyzed data, ST, LC and FF experimental and molecular biology work, made some important conceptual suggestions; EM and MDP collection, management and analyses of all hematologic and clinical data, DC and AB analyses and assessing of hematologic and non-hematologic toxicity, GG and AP statistical analyses, AC and GLS senior authors, clinical care of patients monitoring therapies, MDM interesting suggestion for the design of the manuscript and the discussion section, revised the manuscript critically. All authors took part in the revision of the manuscript and approved the final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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Variations in clinical presentation, frequency of hemophagocytosis and clinical behavior of intravascular lymphoma diagnosed in different geographical regions

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ABSTRACT

Background and Objectives

This study explored variations in the clinical manifestations of intravascular lymphoma (IVL) on the bases of the association with hemophagocytosis and the country where the diagnosis was made.

Design and Methods

The clinical features of 50 Western patients with IVL were compared with those of 123 patients with IVL diagnosed in Eastern countries (87 diagnosed in Japan and 36 in other Asian countries), previously reported in English literature, and collected by an electronic bibliographic search.

Results

Hemophagocytosis was absent in Western patients, but reported in 38 (44%) Japanese patients (p=0.00001) and in seven (19%) patients from other Asian countries (p=0.002). No clinical differences were evident between patients with hemophagocytosis-negative IVL diagnosed in Western countries, Japan and other Asian Countries. Conversely, Japanese and non-Japanese patients with hemophagocytosis-related IVL more frequently had stage IV disease, fever, hepato-splenic involvement, marrow infiltration, dyspnea, anemia, and thrombocytopenia, and rarely exhibited cutaneous or central nervous system involvement. Lymph node and peripheral blood involvement was uncommon in all subgroups. In Western patients, anthracycline-based chemotherapy was associated with a 52% remission rate, and a 2-year overall survival of 46%.

Interpretation and Conclusions

The clinical features of IVL vary according to the association with hemophagocytosis, regardless of the country in which the diagnosis is made. Western, Japanese and other Asian patients with hemophagocytosis-negative IVL display similar clinical characteristics and should be considered as having *classical* IVL. Patients with hemophagocytosis-related IVL show significantly different clinical features. Both forms have a poor prognosis. Extensive molecular studies are needed to explore whether these clinical differences might reflect discordant biological entities within IVL.

Key words: intravascular lymphoma, hemophagocytosis, cutaneous lymphoma, brain lymphoma.

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Intravascular lymphoma (IVL) is a rare entity characterized by exclusive or predominant growth of neoplastic cells within the lumen of blood vessels. This disorder was recently recognized as a subtype of diffuse large B-cell lymphoma by the World Health Organization (WHO) Classification,[1] although rare forms with a T-cell phenotype also occur.[2] The understanding of IVL is very limited considering that, with a few exceptions,[3,4] literature on this malignancy is almost exclusively represented by case reports and small case series.[5-7] IVL is considered to be a rapidly aggressive and disseminated disease, usually affecting elderly patients, and is associated with a poor performance status (PS), B symptoms, anemia, and elevated serum lactate dehydrogenase (LDH) levels.[4] However, the clinical presentation, behavior and therapeutic outcome are extremely variable; in particular, it has been suggested that there are some differences in clinical and histopathological characteristics between patients diagnosed in Asian and Western countries.[3,4,8,9] A large Japanese study concluded that IVL associated with hemophagocytosis (HPC) is the equivalent of the *Asian variant of IVL*.[4] In Western patients, a *cutaneous variant*, with a favorable clinical behavior, has been described.[4] However, a comprehensive and detailed comparison between relatively large series from Western and Eastern countries has not previously been performed, and clinical and biological differences among these cohorts of patients are still debated. The acknowledgement of the existence of different clinical forms may have diagnostic, prognostic and therapeutic implications and constitutes the rationale for further investigations into the mechanisms of lymphomagenesis, adhesion and dissemination.[10] This paper reports a comparison of presenting symptoms, clinical features, behavior, and therapeutic management of the largest series of patients with IVL diagnosed in Western countries with three potentially different subgroups of previously reported cases of IVL diagnosed in Asian countries, in order to assess whether apparent clinical differences may be driven by the geographical distribution of IVL patients.

Design and Methods

Study group (Western-IVL series)

A questionnaire requesting information about patients' characteristics, clinical presentation, diagnosis, staging, sites of disease, laboratory findings, treatment, objective response, site and date of relapse, second line treatment, survival, salient morphologic features, and autopsy findings was sent to each participating center of the International Extranodal Lymphoma Study Group (I.E.L.S.G.). Criteria for including patients were a histological diagnosis of IVL and no evidence of human immunodeficiency (HIV-1) infection or other immunodeficiency. Thirty centers from ten countries provided retrospectively collected, clinical and pathological data on 50 HIV-negative patients with an *in vivo* (n=38) or *post-mortem* (n=12) histological diagnosis of IVL (from 1985 to 2006), which constituted the Western-IVL series. Preliminary data of a part of this series have been reported previously.[4]

Eastern cases

Features of the Western-IVL series were compared with those of cases of IVL diagnosed in Eastern countries and previously reported in English literature. An electronic search of Medline, Current Contents and Pubmed, updated to March 2006, was performed, including as key words *intravascular lymphoma*, *intravascular lymphomatosis*, *angiotropic lymphoma*, *angioendotheliomatosis*, and *hemophagocytosis*. Each full paper was reviewed and duplicate reports describing the same patients were included just once. We found 123 previously reported cases of IVL diagnosed in Japan (n=87) and other Asian countries (n=36) [see references in the linked file]. These groups were divided accordingly to the country in which the diagnosis was made and to the presence of HPC, which was defined by the morphologic recognition of an excess of mature *benign-looking* histiocytes with phagocytosis of erythroblasts, granulocytes and platelets in histopathological specimens. Therefore, three main comparator groups were considered: 1) Japanese patients with IVL and HPC (J-HPC), 2) Japanese patients with IVL but without HPC (J-IVL), and 3) patients with IVL without HPC diagnosed in Asian countries other than Japan (Eastern-IVL). Additionally, a small subgroup of patients with IVL and HPC diagnosed in Western and Asian countries other than Japan and previously reported in English literature was considered for analysis [see references in the linked file].

Statistical considerations

The distributions of clinical variables among the subgroups of patients were assessed by Fisher's exact test for categorical variables. Survival curves were generated by the Kaplan-Meier method. Overall survival (OS) was calculated from the date of pathologic diagnosis to death or to the last date of follow-up, while event-free survival (EFS) was calculated from the first day of treatment to relapse, progression or death, or to the last date of follow-up. The impact of clinical and therapeutic variables on survival was evaluated using the log-rank test. All the probability values were two-sided, with an overall significance level of 0.05. Analyses were carried out using the Statistica 4.0 statistical package for Windows (Statsoft Inc, 1993, Tulsa, OK 74104, USA).

Results

Study Population

HPC was absent in the 50 Western-IVL cases, but was reported in 38 (44%) of the 87 Japanese patients (p=0.00001) and in seven of the 36 patients (19%) diagnosed in other Asian countries (p=0.002). Accordingly, the three main groups against which to compare the Western-

Table 1. Clinical features in our series (Western), in Japanese cases with (J-HPC) or without (J-IVL) hemophagocytosis and in patients with IVL without hemophagocytosis from Eastern countries other than Japan (Eastern-IVL).

		J-HPC		*J-IVL*		*Eastern-IVL*	
Variable	*Western series*	*series*	*p#*	*series*	*p#*	*series*	*p#*
Number of patients	50	38	49			29	
Median age (range)	68 (34-90)	67 (44-78)	NS	69 (13-82)	NS	62 (34-81)	NS
Male gender	23 (46%)	19 (50%)	NS	24 (49%)	NS	14 (48%)	NS
Previous or concomitant cancer	9 (18%)	4 (11%)	NS	2 (4%)	0.02	2 (7%)	NS
Stage IV	38 (76%)	37 (97%)	0.004	40 (82%)	NS	23 (79%)	NS
Fever	21 (42%)	33 (87%)	0.00001	23 (47%)	NS	18 (62%)	NS
Fatigue	11 (22%)	17 (45%)	0.03	8 (16%)	NS	5 (17%)	NS
Jaundice	0 (0%)	10 (26%)	0.0002	0 (0%)	NS	2 (7%)	NS
Cutaneous lesions	19 (38%)	1 (3%)	0.0001	12 (24%)	NS	3/18 (17%)*	NS
Neurological involvement	21 (42%)	8 (21%)	0.03	26 (53%)	NS	10 (34%)	NS
Hepatic involvement	13 (26%)	25 (66%)	0.0002	15 (31%)	NS	14 (48%)	0.05
Splenic involvement	13 (26%)	29 (77%)	0.00001	10 (20%)	NS	12 (41%)	NS
Bone marrow involvement	15 (30%)	28 (74%)	0.0001	17 (35%)	NS	11 (38%)	NS
Lymphadenopathy	4 (8%)	2 (5%)	NS	2 (4%)	NS	5 (17%)	NS
Peripheral blood involvement	2 (4%)	5 (13%)	NS	0/26 (0%)*	NS	0 (0%)	NS
Pulmonary involvement	9 (18%)	14 (37%)	0.04	13 (27%)	NS	7 (25%)	NS

*NS: not significant; NR: not reported; *Relationship between number of positive cases and number of assessed cases; #p values for comparisons between the Western series and the other subgroups.*

IVL series consisted of 38 J-HPC patients, 49 J-IVL patients and 29 Eastern-IVL patients. Additionally, a small subgroup of patients with IVL and HPC diagnosed in Western (n=5) and Asian countries other than Japan (n=7) and previously reported in English literature was considered for analysis.

Clinical presentation

Comparisons of the patients' characteristics at diagnosis between the Western-IVL series and the three main groups of Asian patients are summarized in Table 1. The number of patients with an *in vivo* diagnosis was 38 (76%) Western-IVL, 35 (92%) J-HPC, 36 (74%) J-IVL, and 16 (55%) Eastern-IVL. No significant differences in age and gender were observed among the subgroups.

The clinical presentation was heterogeneous in Western-IVL patients, with a remarkable deterioration in PS, with an ECOG score ≥2 in 32 cases (64%). PS was rarely reported in Asian series, but the constant presence of systemic symptoms and multi-organ involvement in reported cases suggests that IVL is associated with a poor PS also among Asian patients. As reported in Table 1, with a few exceptions, no significant differences in clinical features were observed among Western-IVL, J-IVL and Eastern-IVL subgroups. Conversely, several significant differences were observed between these three subgroups and patients with J-HPC. Thirty Western-IVL patients (60%) had systemic symptoms, mostly represented by fever, which was present in 21 cases (42%), and associated with other B symptoms in 12 cases. Fever as well as fatigue and jaundice were significantly more common among J-HPC patients (Table 1). Significant differences in terms of Ann Arbor stage of disease and sites of disease were observed. A large majority of J-HPC patients had stage IV disease, which was observed in 76-82% of cases in the other subgroups (Table 1). The most common sites of disease in the Western-IVL series, i.e., skin (38%) and central nervous system (42%), were involved in a significantly lower proportion of cases in J-HPC patients (3% and 21%, respectively). In the J-IVL and Eastern-IVL subgroups, the percentages of involvement of skin and central nervous system were similar to those observed in the Western series (Table 1). Cutaneous lesions were the sole pathologic finding in 12 (24%) Western cases; these patients were considered as having a *cutaneous variant* of IVL. The *cutaneous variant* was diagnosed in 0%, 6% and 0% of J-HPC (p=0.0004), J-IVL (p=0.01) and Eastern-IVL (p=0.002) subgroups, respectively. Involvement of hemolymphatic organs was significantly more common in J-HPC cases: 66%, 77% and 74% of these patients showed liver, spleen and marrow involvement, respectively, which was present in 26%, 26% and 30% of Western-IVL patients (p=0.0002, 0.00001 and 0.0001, respectively). With the exception of slightly more common involvement of liver in Eastern-IVL patients, no significant differences in the involvement of hemolymphatic organs were observed among Western-IVL, J-IVL and Eastern-IVL subgroups (Table 1). There were no signifi-

Table 2. Laboratory findings in our series (Western), in Japanese cases with (J-HPC) or without (J-IVL) hemophagocytic features and in IVL cases from Eastern countries other than Japan (Eastern-IVL).

		J-HPC		*J-IVL*		*Eastern-IVL*	
Variable	*Western series*	*series*	*p#*	*series*	*p#*	*series*	*p#*
Number of patients	50	38		49		29	
Anemia	33 (66%)	32 (84%)	0.05	16/25 (64%)*	NS	19 (66%)	NS
Leukopenia	11 (22%)	11 (29%)	NS	4/25 (16%)*	NS	6 (21%)	NS
Thrombocytopenia	16 (32%)	28 (74%)	0.0003	7/25 (28%)*	NS	14 (48%)	NS
High serum LDH levels	33/39 (85%)*	36/36 (100%)*	0.02	31/33 (94%)*	NS	21/23 (91%)*	NS
High β2-microglobulin	16/19 (84%)*	NR		NR		NR	
Elevated ESR	22/46 (48%)*	NR		NR		NR	
Monoclonal component	7/45 (16%)*	5 (13%)	NS	1/20 (5%)*	NS	NR	
High ALT levels	3 (6%)	10 (26%)	0.02	3 (6%)	NS	1/13 (8%)*	NS
High bilirubin levels	1 (2%)	11 (29%)	0.0008	0 (0%)	NS	2 (7%)	NS

*NS: not significant; NR: not reported; LDH: lactate dehydrogenase; ESR: erythrocyte sedimentation rate; ALT: alanine amino transferase; *Relationship between number of positive cases and number of assessed cases; #p values for comparisons between the Western series and the other subgroups.*

cant differences in lymph-node and peripheral blood involvement among the four studied subgroups, infiltration rates ranged between 4% and 17% and between 0% and 13%, respectively (Table 1).

Histopathological and laboratory findings

In the 38 Western-IVL patients with an *in vivo* diagnosis, the histopathological diagnosis was performed on tissue samples obtained by partial surgical biopsy in 29 cases (skin in 18 cases, central nervous system in five, lung in two, uterus in two, gallbladder in one, and liver in one), visceral resection in six (kidney in three, prostate in two and spleen in one) and bone marrow biopsy in three cases. By definition, all cases from the four subgroups showed large lymphoid cells within vessel lumina (Figure 1). Lymphomatous lesions with infiltration of extravascular tissues (called *extravascular component*) were observed in ten (20%) Western cases, and was significantly more common in samples from lung and kidney of J-HPC cases. All Western-IVL cases but one displayed a B-cell immunophenotype. T-cell immunophenotype was observed in one (2%) Western-IVL case, in one (3%) J-HPC case, in one J-IVL (2%), and in five (17%) Eastern-IVL cases ($p \leq 0.03$ between Eastern-IVL cases and the other three groups; not significant differences among the others).

As reported in Table 2, no significant differences in laboratory findings were noted among Western-IVL, J-IVL and Eastern-IVL subgroups; while a different biochemical profile was observed in J-HPC patients. In fact, anemia (84%) and thrombocytopenia (74%) were significantly more common in J-HPC cases than in the other three subgroups. Thrombocytopenia was associated with anemia, bone marrow infiltration and hepato-splenic involvement in each subgroup. Increased serum LDH levels were observed in all J-HPC patients and in 85%-94% of cases in the other subgroups (Table 2). Increased levels of β_2-microglobulin and elevated erythrocyte sedimentation rates were observed in, respectively, 84% and 48% of Western-IVL cases, while these parameters were not reported for patients in the other subgroups. A monoclonal serum component was reported in 5% - 16% of cases in the studied subgroups (p= not significant). Elevated transaminases and total bilirubin levels were significantly more common among J-HPC cases than in the other subgroups (Table 2).

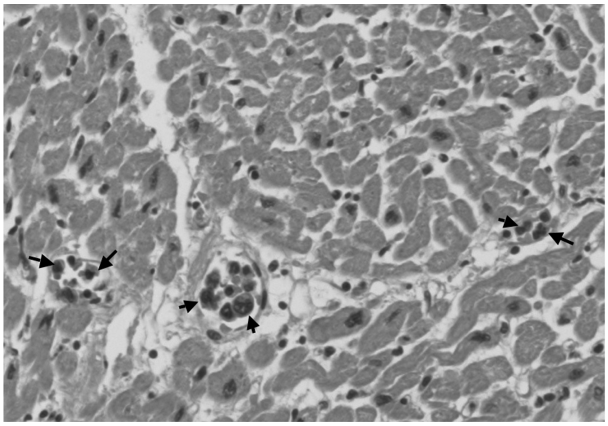

Figure 1. Intravascular lymphoma of the cardiac muscle (hematoxylin and eosin). The growth of neoplastic large cells (arrows) occurs within the lumen of blood vessels. Neoplastic lymphocytes show large nuclei with one or more nucleoli and scant cytoplasm.

Comparison with patients with HPC-related IVL diagnosed outside Japan

The Western-IVL series was also compared with the small group (n=12) of patients with HPC-related IVL diagnosed in Western (n=5) or Eastern (n=7) countries other than Japan and previously reported in English literature

[*see references in the linked file*] to better understand whether clinical features are related to HPC independently of the country of origin of the patient. As reported in Table 3, these patients exhibited similar characteristics to J-HPC patients, while displaying many differences compared to Western-IVL patients. Similarly to J-HPC patients, patients with HPC-positive IVL diagnosed outside Japan more commonly had stage IV disease, fever, thrombocytopenia, and involvement of liver, spleen and bone marrow. A higher prevalence of cutaneous lesions in non-Japanese patients with HPC-positive IVL was the only distinctive feature in comparison with J-HPC patients (Table 3).

Therapeutic outcome

In Western-IVL series, *in vivo* diagnosis was possible in 38 patients, 36 of whom received at least one line of treatment. Seventeen (47%) achieved a complete remission (CR) and four had a partial response (PR), for an overall response rate of 58%; nine patients experienced progressive disease (PD), two had stable disease, and four died of toxicity. There were 23 treatment failures: 11 PD, eight relapses after response and four toxic deaths, All treatment failures but one occurred within the first year of follow-up. Anthracycline-based chemotherapy was administered as initial treatment to 25 patients, resulting in a CR rate of 52%, a median OS of 10+ months and a 2-year OS of 46±10%. The median EFS for the 38 patients with an *in vivo* diagnosis was 6 months (range 1-81), with a 2-year EFS of 32±8%. Seventeen patients are alive at a median follow-up of 28 months, with a 2-year OS of 39±8%. Considering the entire series (including patients with a *post-mortem* diagnosis), the 2-year EFS and OS were 24±6% and 30±7%, respectively.

Discussion

The comparison of our series, the largest one of IVL patients diagnosed in Western countries, with IVL cases from Asian countries strongly suggests the existence of different clinical forms of IVL. This issue does not seem simply to reflect regional differences since IVL cases with similar clinico-pathological characteristics have been reported in Western countries, in Japan and in other Asiatic countries (the Western-IVL, J-IVL and Eastern-IVL groups in this study). Patients from these three subgroups could be comprehensively considered as having the *classical form* of IVL. By contrast, patients with IVL associated with HPC, who have been mostly reported in Japan (the J-HPC group in this study), display clinical features distinct from those observed in the patients with the *classical form*. Patients with IVL and HPC diagnosed in countries other than Japan display similar characteristics to those of J-HPC patients, while Japanese patients with IVL without HPC (the J-IVL group) show similar features to those of Western patients. Taken together, these data suggest that differences in clinical presentation and behavior in IVL

Table 3. Clinical features and laboratory findings in patients with IVL and hemophagocytic features (HPC) diagnosed in Western or Eastern countries other than Japan in comparison with our series (Western) and J-HPC patients

	Non-Japanese pts with IVL and HPC	*Western series*		*J-HPC*	
Variable		*series*	*p#*	*series*	*p#*
Number of patients	12	50		38	
Median age (range)	60 (54-77)	68 (34-90)	NS	67 (44-78)	NS
Male gender	6 (50%)	23 (46%)	NS	19 (50%)	NS
Stage IV	12 (100%)	38 (76%)	0.04	37 (97%)	NS
Fever	11 (92%)	21 (42%)	0.002	33 (87%)	NS
Jaundice	2 (17%)	0 (0%)	0.008	10 (26%)	NS
Cutaneous lesions	4 (33%)	19 (38%)	NS	1 (3%)	0.009
CNS involvement	2 (17%)	21 (42%)	0.02	8 (21%)	NS
Hepatic involvement	7 (58%)	13 (26%)	0.03	25 (66%)	NS
Splenic involvement	7 (58%)	13 (26%)	0.03	29 (77%)	NS
Marrow involvement	9 (75%)	15 (30%)	0.007	28 (74%)	NS
Lymphadenopathy	1 (8%)	4 (8%)	NS	2 (5%)	NS
Lung involvement	3 (35%)	9 (18%)	NS	14 (37%)	NS
Anemia	9 (75%)	33 (66%)	NS	32 (84%)	NS
Leukopenia	4 (33%)	11 (22%)	NS	11 (29%)	NS
Thrombocytopenia	10 (83%)	16 (32%)	0.003	28 (74%)	NS
High serum LDH levels	11 (92%)	33/39 (85%)*	NS	36/36 (100%)*	NS
High ALT levels	5 (42%)	3 (6%)	0.006	10 (26%)	NS
High bilirubin levels	5 (42%)	1 (2%)	0.0008	11 (29%)	NS

*NS: not significant; NR: not reported; CNS: central nervous system; PB: peripheral blood; LDH: lactate dehydrogenase; ESR: erythrocyte sedimentation rate; ALT: alanine amino transferase; *Relationship between number of positive cases and number of assessed cases. #p values for comparisons between the group of patients with IVL and HPC diagnosed in countries other than Japan and the other subgroups.*

might be more related to the concomitant presence of HPC rather than the geographical distribution of the disease *per se*.

This study is the first one comparing large series of IVL patients diagnosed in different geographical regions. The study of cumulative retrospective series and comparison with previously reported cases is so far the only strategy available for studying IVL given the extreme rarity of this disease and the diagnostic difficulties. Conclusions from this study should be viewed with caution, not only because of the intrinsic caveats regarding the methods used, but also considering that cases published in non-English literature were not included in this analysis, which may have introduced some interpretation biases. Nevertheless, the size of the studied subgroups, the high proportion of patients with an *in vivo* diagnosis and the quality of reports in English literature support our conclusions. Moreover, the clinical features observed in the J-HPC and J-IVL subgroups have been confirmed by a recently reported study including the largest series of Japanese patients with IVL (n=96).[11] Our observations suggest that, unlike in Japanese patients,[8] HPC is rarely observed in IVL cases diagnosed in Western countries. Among 321 cases of IVL diagnosed in Western countries, and published in English literature, morphologically confirmed HPC has been reported in only five cases (1.5%).[12-15] However, two of these patients showed HPC features only at relapse and not at initial diagnosis,[14,15] and two others were of Caribbean and Vietnamese origin.[12,16] Comprehensively, the rare cases of HPC-associated IVL diagnosed outside Japan exhibited clinical features similar to those displayed by patients with HPC-associated IVL diagnosed in Japan (J-HPC), i.e., significantly higher rates of advanced disease, fever, hepato-splenic involvement, bone marrow infiltration, fatigue, jaundice, dyspnea, anemia, thrombocytopenia, and altered liver function tests. In contrast, the skin and central nervous system, the most common sites of disease in the classical form of IVL, were rarely involved in J-HPC patients. To the best of our knowledge, there is a single case of cutaneous involvement in J-HPC patients, which was represented by a cutaneous lesion on the torso reported as a cutaneous induration without histological confirmation.[17] Our analysis demonstrates that other characteristics, such as age, gender and rates of lymph-node involvement, peripheral blood dissemination and increased serum LDH levels, overlap substantially between classical and HPC-related forms. Both forms share B-cell immunophenotype in >97% of cases, in contrast to other non-Hodgkin's lymphomas associated with HPC, which are mostly of T-cell lineage.[18] As reported for other hematologic malignancies,[19] the presence of HPC has been proposed as a negative prognostic factor in IVL patients. Unfortunately, the comparison of therapeutic outcomes among the studied subgroups was limited by the fact that data from Asian patients were collected from case reports written by physicians with different expertise and specializations, sometimes with incomplete therapeutic data (complete therapeutic data for 94% of previously reported J-HPC patients, 70% of J-IVL patients and 88% of Eastern-IVL patients) and minimal follow-up. In the largest cumulative series of Japanese patients with IVL,[11] the use of anthracycline-based chemotherapy, the standard strategy against IVL,[11,20] resulted in a 55% complete remission rate and a median OS of 13 months, which are very similar outcomes to those of the current series of Western-IVL patients. In the largest reported series of patients with the so-called Asian variant of IVL, anthracycline-based chemotherapy was associated with a complete remission rate of 53%, and a median OS of 10 months.[3] At the time of analysis, the combination of rituximab and chemotherapy has been reported to have been used in 15 patients (six in our series), resulting in complete remission in 11 of 12 evaluable patients, and no relapses at a median follow-up of 15 months; this combined strategy seems to be advisable in patients with CD20-positive IVL. Consolidation with high-dose chemotherapy supported by autologous stem cell transplantation may improve current outcomes,[21-23] even among J-HPC patients.[9] The application of such a strategy is, however, greatly limited by the high median age and poor PS of IVL patients. Thus, the identification of reliable high-risk predictors constitutes a relevant issue in the management of patients with IVL.

The virtually selective geographical distribution of the *HPC-related form* of IVL as well as its differences from the *classical form* could have environmental causes. In fact, IVL occurs in rural areas of Southwestern Japan, where human T-cell lymphotropic virus type-1 (HTLV-1) is endemic,[8] and associations with some helminthic infections have been proposed in J-HPC patients.[24] Associations between IVL and HTLV-1, Epstein-Barr virus, cytomegalovirus, herpes simplex virus, varicella-zoster virus and human herpes virus-6 have not been detected.[25] In conclusion, differences in clinical features of IVL seem to be correlated to the concomitant presence of HPC rather than to the geographical area where the lymphoma is diagnosed. At least two different clinical forms of IVL seem to exist: a *classical form*, which is the most common presentation in Western countries, with frequent involvement of the skin and central nervous system and less common infiltration of hemolymphoid organs; and a *HPC-related form*, which is remarkably more common in Japan, with an almost constant involvement of hemolymphoid organs, higher rates of advanced disease, fever, respiratory symptoms, anemia, and thrombocytopenia, and virtually always sparing the skin. Despite these clinico-pathological differences, patients with both forms have a poor prognosis when treated with anthracycline-based chemotherapy, and treatment intensification and the addition of rituximab appear advisable. Extensive phenotypic and molecular characterization is needed to test whether these different clinical forms may also have a different biological backgrounds, and, therefore, international co-operative studies are warranted.

Appendix

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Authors' Contributions

Substantial contributions to acquisition of data were made by O Bairey, M Martelli, A De Renzo, C Doglioni, C Montalbán, A Tedeschi, A Pavlovsky, S Morgan, L Uziel, M Ferracci, S Ascani, U Gianelli, C Patriarca, F Facchetti, A Dalla Libera, B Pertoldi, B Horvath, A Szomor. Acquisition of data, analysis and interpretation of data, drafting and revising the article were performed by AJM Ferreri, GP Dognini, E Campo, R Willemze, JF Seymour, E Zucca, F Cavalli, and M Ponzoni. All authors revised the manuscript and approved its final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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A dose exploration, phase I/II study of administration of continuous erythropoietin receptor activator once every 3 weeks in anemic patients with multiple myeloma receiving chemotherapy

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ABSTRACT

Background and Objectives

Continuous erythropoietin receptor activator (C.E.R.A.) is an innovative agent with unique erythropoietin receptor activity and a prolonged half-life, which has the potential for administration at extended dosing intervals. The objectives of this dose-finding study were to evaluate the hemoglobin (Hb) dose-response, pharmacokinetics, and safety of repeated doses of C.E.R.A. given once every 3 weeks to anemic patients with multiple myeloma (MM) receiving chemotherapy.

Design and Methods

This was an exploratory two-stage, open-label, parallel-group, multicenter study. Patients received C.E.R.A. doses of 1.0, 2.0, 3.5, 4.2, 5.0, 6.5, or 8.0 μg/kg once every 3 weeks by subcutaneous injection initially for 6 weeks, followed by a 12-week optional extension period. The primary outcome measures were the average Hb level and its change from baseline over the initial 6-week period, based on values of the slope of the linear regression analysis and the area under the curve. Rates of Hb response (defined as an increase in Hb of ≥2 g/dL without transfusion) and blood transfusion were also evaluated.

Results

Sixty-four patients entered the study. Dose-related increases in Hb levels were observed during the initial 6-week treatment period for C.E.R.A. doses of 1.0-4.2 μg/kg, with a similar response observed at higher doses. At least 70% of patients receiving 2.0-8.0 μg/kg of C.E.R.A. had Hb responses during the 18-week study. The elimination half-life of C.E.R.A. was found to be long (6.3-9.7 days [151.2-232.8 hours]). All doses were generally well tolerated.

Interpretation and Conclusions

Based on its unique, long elimination half-life, C.E.R.A. has been demonstrated to be an effective and well-tolerated treatment of anemia given once every 3 weeks to patients with multiple myeloma receiving chemotherapy.

Key words: anemia, C.E.R.A., chemotherapy, erythropoietin, multiple myeloma.

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Anemia affects more than two-thirds of patients with lymphoid malignancies.[1,2] Cytotoxic chemotherapy may exacerbate this anemia, leading to fatigue, weakness, and reduced quality of life (QoL).[3,4] In multiple myeloma, bone marrow infiltration of the tumor is common and concomitant renal failure is frequently associated with defective endogenous erythropoietin production.[5] Consequently, transfusions are common in patients with multiple myeloma.[6,7]

Recombinant erythropoietic-stimulating agents (ESA) can correct anemia associated with lymphoid malignancies, increasing hemoglobin (Hb) levels, reducing blood transfusions and improving QoL.[1,8-10] The currently used dosing regimens of ESA often involve frequent administration (e.g. three times weekly). A once-weekly administration schedule of epoetin β was shown to correct anemia in patients with lymphoproliferative malignancies.[9] However, further reduction in the frequency of administration is limited by the relatively short half-life of epoetin. A once-weekly schedule of darbepoetin α also proved effective, specifically in patients with lymphoid malignancies,[10] and darbepoetin α has been licensed recently for administration once every 3 weeks. Extended dosing intervals of ESA may provide benefits to patients and physicians. Since many oncology treatments are administered in 3-weekly cycles, once-per-cycle administration may provide optimal convenience and compliance. The development of a new treatment for anemia with improved early and sustained Hb response over current treatments, while allowing coordination with chemotherapy administration, would represent an important advance in the management of anemia. Continuous erythropoietin receptor activator (C.E.R.A.) is an innovative agent with unique receptor activity and a prolonged half-life. It is a chemically synthesized continuous erythropoietin receptor activator, differing from erythropoietin through the integration of amide bonds between amino groups and methoxy polyethylene glycolsuccinimidyl butanoic acid.[11,12] C.E.R.A. is currently in development to provide correction of anemia and stable control of Hb levels at extended administration intervals in patients with cancer. Previous studies in healthy volunteers demonstrated that C.E.R.A. had lower systemic clearance and an increased elimination half-life compared with ESA and superior potency *in vivo* with respect to the magnitude and duration of response.[13,14] Further studies in healthy volunteer demonstrated rapid, dose-dependent increases in reticulocytes following either intravenous (i.v.) or subcutaneous (s.c.) administration.[15]

This phase I/II dose-finding study was designed to examine the Hb dose-response, pharmacokinetics, and safety of multiple doses of C.E.R.A. given once every 3 weeks to anemic patients with multiple myeloma receiving chemotherapy.

Design and Methods

Patients

Patients eligible for inclusion were adults (aged ≥18 years) with a cytologically or histologically confirmed diagnosis of multiple myeloma, with Hb level ≤11 g/dL at screening and who were scheduled to receive systemic anticancer therapy for at least the first 6 weeks of C.E.R.A. administration. Limits were set for levels of serum erythropoietin at screening: ≤70 U/L if Hb was >10 to ≤11 g/dL; ≤100 U/L if Hb was >9 to ≤10 g/dL; ≤180 U/L if Hb was >8 to ≤9 g/dL; and ≤300 U/L if Hb was ≤8 g/dL. Other inclusion criteria were World Health Organization (WHO) performance status grade 0-2, transferrin saturation >20% at screening, and a life expectancy >6 months.

Patients were excluded if they: had received a red blood cell transfusion within 2 months of the first planned dose of study medication or an ESA within 3 months; had known resistance to ESA therapy; had resistant hypertension; had acute or chronic bleeding requiring treatment within 3 months of the study; had grade 3/4 thrombocytopenia (platelet count $<50\times10^9$/L) or thrombocytosis (platelet count $>450\times10^9$/L); had creatinine >2.5 mg/dL, folic acid or vitamin B_{12} deficiency, hemolysis. or epilepsy (uncontrolled or newly diagnosed within the last 6 months); were pregnant or lactating.

Study design

This was an exploratory 18-week, two-stage, open-label, parallel-group, multicenter, dose-finding study. The design and conduct of the study complied with the principles of good clinical practice, in accordance with the Declaration of Helsinki. The study was approved by local ethics committees at each center, and informed written consent was obtained from all patients before enrollment.

Following a screening period of up to 4 weeks, patients were randomized to receive C.E.R.A. 2.0 μg/kg, 3.5 μg/kg, or 5.0 μg/kg (stage I of the study) by s.c. injection once every 3 weeks for 6 weeks. Following a review of the data, two additional groups of patients were enrolled in a sequential manner into stage II of the study: the first group received s.c. C.E.R.A. 6.5 μg/kg and the second received 1.0 μg/kg once every 3 weeks for 6 weeks. After review of the data for these two groups, two further groups of patients were enrolled: the first receiving C.E.R.A. 8.0 μg/kg once every 3 weeks and the second receiving 4.2 μg/kg once every 3 weeks.

During the initial 6-week treatment period, the study medication was administered on day 1 and day 22. Patients attended the clinic weekly, when Hb, hematocrit and reticulocyte levels, electrocardiogram (ECG), safety laboratory and iron parameters, and blood pres-

sure measurements were taken. Blood transfusions were permitted throughout the study. Every reasonable effort was made to avoid transfusions in patients with Hb levels >8.5 g/dL, particularly during the initial 6-week treatment period. The need for blood transfusions was determined at the discretion of the investigator, based on the patient's symptoms and local practice. All transfusions were recorded and specified by type and volume. Following the initial 6-week treatment period, with the agreement of the patient, investigator and sponsor, patients continued to receive the study medication once every 3 weeks for up to 12 additional weeks in an optional extension period. Clinic visits were scheduled every 3 weeks during this period, when Hb, hematocrit and reticulocyte levels, ECG, safety laboratory and iron parameters, and blood pressure were measured. Adverse events were monitored throughout the study and, with regard to serious adverse events, up to 30 days after the final administration of the drug. Safety evaluations also included clinical laboratory tests, ECG, vital signs, and measurement of anti-CERA antibodies. Adverse events were graded according to the National Cancer Institute/National Institutes of Health (NCI/NIH) Common Toxicity Criteria. Tolerability was evaluated based on the number of patients prematurely withdrawn from the study for safety reasons.

Dose adjustments

Limited C.E.R.A. dose reductions were allowed during the initial and extension periods for the following safety reasons: first, if Hb levels increased by >2.5 g/dL in any 6-week period, the next C.E.R.A. dose was decreased by 50% without interruption of dosing; second, if Hb levels exceeded 14 g/dL at any time, treatment was stopped until a Hb level of <13 g/dL was reached and resumed at 50% of the previous dose; third, if a grade 2 or greater acute toxicity occurred which was considered at least possibly related to the study medication (based on experience with ESA in patients with cancer), the treatment could be interrupted if considered necessary by the investigator. Following resolution of the toxicity, the study medication could be resumed at the previous dose. However, if toxicity recurred, the patient was to be withdrawn from the study and followed up until recovery.

Dose increases of C.E.R.A. were only permitted during the extension period and only if Hb levels had not increased by at least 1 g/dL from the baseline value. The dose was only allowed to be increased up to the highest previously tested dose. Thus, at week 7, patients assigned to the C.E.R.A. 2.0 and 3.5 μg/kg groups and 1.0 μg/kg group could have their dose increased to a maximum of 5.0 μg/kg once every 3 weeks. Patients assigned to the 4.2 μg/kg group could have their dose increased to a maximum of 6.5 μg/kg.

Efficacy end-points

The primary efficacy end-point was the average increase in Hb level from baseline up to the end of initial treatment based on the area under the curve (AUC) values and the slope of the linear regression line. The end of initial treatment was defined as the time when the assessment period was complete, a dose change was introduced, or a blood transfusion was given, whichever occurred first. Secondary efficacy end-points included rates of Hb response and blood transfusions and average hematocrit levels and reticulocyte counts during the study. A Hb response was defined as an increase in Hb level of ≥2 g/dL from baseline during the initial 6-week treatment period without blood transfusion.

Pharmacokinetic evaluations

Blood samples were taken for analysis of pharmacokinetic parameters before the first administration of the study medication on day 1 and on days 8, 15, 22 (before the second administration of the study drug), 23, 27, 29, and 32 and before each C.E.R.A. administration during the extension period. The pharmacokinetic parameters of maximum serum concentration (C_{max}), time to C_{max} (T_{max}), elimination half-life ($t_{1/2}$), and $AUC_{22\text{-}43\ days}$ were calculated for each patient. $t_{1/2}$ was estimated from ln(2)/k, where the rate constant of elimination (k) was determined by linear regression on the logarithm of the serum concentration-time data in the post-distribution phase. $AUC_{22\text{-}43}$ days was estimated by the linear trapezoidal rule.

Statistical analyses

This was an exploratory study and no formal sample size calculation was performed. A total of eight patients were planned to be included in each dose group. All efficacy end-points were analyzed based on the intention-to-treat (ITT) population comprising all patients who were randomized (stage I) or enrolled (stage II) and received at least one dose of study medication.

The primary analysis focused on the initial 6 weeks of treatment, with additional analyses for the entire study period (including the extension period). The primary efficacy end-point, change in Hb, was calculated for each patient using a separate linear regression over time with the Hb level as the dependent variable; the mean of all measurements on or before baseline was used as the day 1 value. The regression slopes, which are estimates of the Hb change per day, were multiplied by 42 for standardization and to give an estimate of Hb increases after 6 weeks of treatment. Slopes were summarized using means, standard deviations (SD), medians, and minimum and maximum values. Average Hb changes from baseline during the initial 6-week period were also estimated by an AUC approach. The area under the Hb curve until end of initial treatment was calculated for each patient using the trapezoidal rule. The secondary end-point of change from baseline in

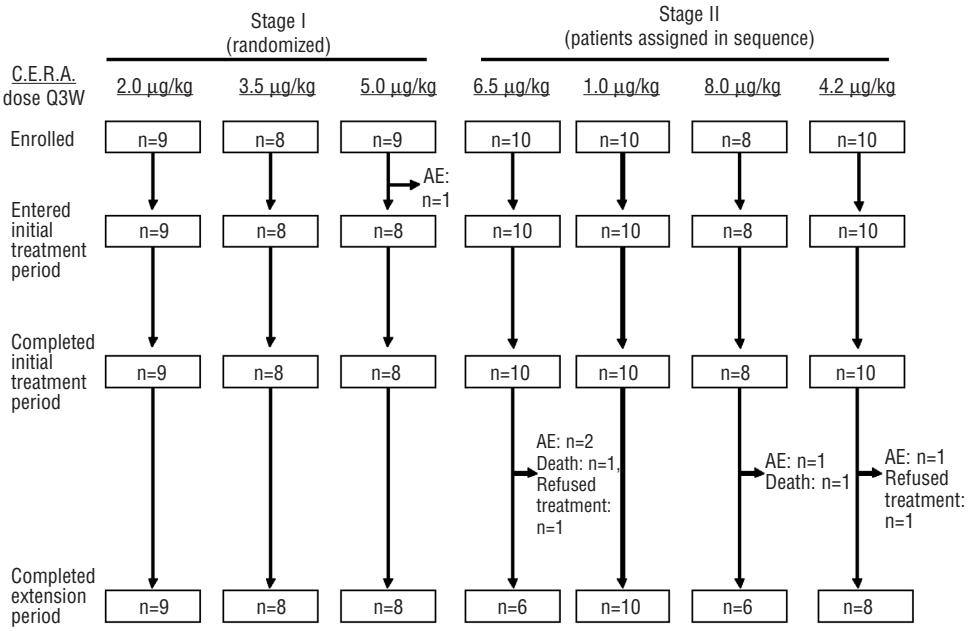


Figure 1. Disposition of patients, including reasons for withdrawal.

AE: adverse event; CERA: continuous erythropoietin receptor activator; Q3W: once every 3 weeks.

hematocrit was analyzed using identical regression and AUC methods as described above. For reticulocyte counts, only the AUC analysis was performed. Descriptive statistics were used to analyze the additional efficacy end-points. Pharmacokinetic end-points were analyzed for all patients who provided evaluable samples. Estimates of pharmacokinetic parameters were performed according to standard non-compartmental methods, using WinNonlin version 4.1 (Pharsight, Mountain View, CA, USA) and based on actual sampling times. Pharmacokinetic parameters were calculated for each patient from the concentration-time data obtained after the second administration of the drug, from day 22 to day 43. All safety data were summarized using descriptive statistics.

Results

Disposition of patients

Sixty-four patients were enrolled at five centers in Poland and the Czech Republic, 26 patients in stage I and 38 in stage II of the study. Similar numbers of patients were assigned to each dose level of CERA (Figure 1). One patient in the 5.0 µg/kg group did not receive the study medication because of an adverse event (renal failure) occurring in the screening period, and was excluded from all analysis populations. The remaining 63 patients all completed the initial 6-week treatment period, entered the extension period, and were included in the ITT and safety analyses (Figure 1).

Fifty-five patients (87%) completed the extension period, and eight patients were withdrawn prematurely (Figure 1). The second dose of CERA in the initial treatment period was withheld from one patient in the 4.2 µg/kg group because of adverse events (fatigue and fungal pneumonia). This patient entered the extension period but subsequently died (for reasons described by the investigator as unrelated to the study medication) on day 44. Of the remaining seven patients, three were withdrawn because of adverse events that were described by the investigator as unrelated to the study medication, two patients died for reasons described as unrelated to the study medication, and two patients refused further treatment (Figure 1).

Baseline characteristics

Baseline characteristics and demographics were similar in all groups (Table 1). Mean Hb levels ranged from 9.8-10.4 g/dL and mean hematocrit from 30.5-32.2% across the dose groups. In accordance with the inclusion criteria, median serum erythropoietin levels were <70 IU/L and the median ratio of observed to predicted $\log_{10}$ serum erythropoietin levels (O/P ratios) were <1 in all treatment groups (Table 1). Most patients had stage 2A or 3A disease based on the Durie and Salmon classification[16] (49% and 35% of patients, respectively) and, in most patients, the serum monoclonal (M) protein was of the IgG type (36 of 63 patients; 57%). Most patients had a WHO performance status of 1 (40 of 63 patients; 63.5%). The most frequently used anticancer therapies were combination regimens with or without anthracyclines (44 of 63 patients; 70%).

Efficacy

Although the number of patients assigned to each dose group of C.E.R.A. was small and there tended to be some variability in the results, the median change in Hb

levels during the initial 6-week period showed dose-related increases in response to C.E.R.A. once every 3 weeks over the dose range 1.0-4.2 μg/kg (Figure 2). The median increase in Hb level was greatest in the 4.2 μg/kg group, being 2.21 g/dL, with similar increases observed for the 5.0, 6.5, and 8.0 μg/kg groups. In contrast, the median increase in Hb level was only 0.03 g/dL in the 1.0 μg/kg group, suggesting a suboptimal response to this dose of C.E.R.A. (Figure 2). The AUC analysis provided a similar trend. After the initial treatment period, Hb levels were maintained or continued to increase and were then maintained for the remainder of the study (Figure 3).

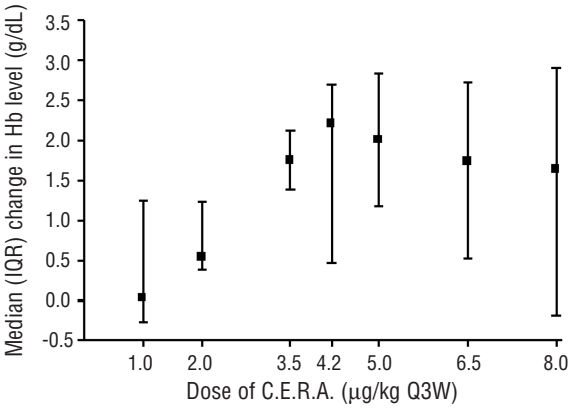
During the initial treatment period, median hematocrit levels remained unchanged in the 1 μg/kg group, but were increased from baseline in the other dose groups: to 33.9% in the 2.0 μg/kg group, to 36-37% in the 3.5-5.0 μg/kg groups, to 43.6% in the 6.5 μg/kg group, and to 38.9% in the 8.0 μg/kg group. AUC analysis of reticulocyte counts showed a progressive increase in the median baseline adjusted AUC over the initial treatment period across the entire range of C.E.R.A. doses examined. The performance status of most patients was maintained or improved over the duration of the study (*data not shown*).

Approximately 60% of patients receiving C.E.R.A. doses of 3.5-8.0 μg/kg had a Hb response during the initial 6-week treatment period, defined as an increase of Hb ≥2.0 g/dL relative to baseline levels without transfusion (Figure 4). During the complete 18-week study period, only 20% of patients in the 1.0 μg/kg dose group demonstrated a Hb response, suggesting that this dose was sub-optimal in anemic patients with multiple myeloma. In comparison, ≥70% of patients assigned to the 2.0 μg/kg or higher dose groups had a Hb response. A Hb response occurred in 78% of patients in the 2.0

Table 1. Patients' characteristics at baseline by treatment group: safety population.

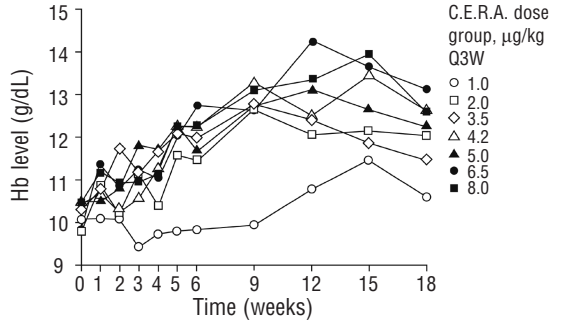
	C.E.R.A. dose group, μg/kg once every 3 weeks						
	1.0	*2.0*	*3.5*	*4.2*	*5.0*	*6.5*	*8.0*
Numbers	10	9	8	10	8	10	8
Sex, n (%)							
male	3 (30)	2 (22)	3 (37)	6 (60)	3 (37)	3 (30)	1 (13)
female	7 (70)	7 (78)	5 (63)	4 (40)	5 (63)	7 (70)	7 (87)
Median age, years (range)	62.5 (41-76)	71.0 (45-84)	64.5 (45-83)	64.5 (52-75)	63.5 (44-81)	70.0 (57-77)	67.5 (53-81)
Median weight, kg (range)	67.5 (55-80)	69.0 (36-89)	68.0 (50-83)	70.0 (54-90)	74.0 (45-97)	63.5 (46-80)	59.25 (55-76)
Median time from initial diagnosis, days (range)	177.5 (34-5382)	225.0 (24-1262)	183.5 (36-2176)	341.5 (15-4166)	195.5 (17-1708)	232.5 (45-2420)	159.5 (13-4136)
Median Hb, g/dL (range)	10.0 (8.7-10.8)	9.7 (6.9-11.5)	10.2 (9.3-10.7)	10.2 (8.1-11.3)	10.4 (9.6-11.1)	9.8 (8.7-11.0)	10.4 (7.8-11.1)
Median hematocrit, % (range)	31.9 (27.6-35.8)	31.2 (22.6-36.1)	31.4 (28.0-32.6)	31.9 (25.7-38.4)	31.6 (29.0-36.2)	31.5 (29.1-35.5)	33.7 (25.5-35.9)
Median reticulocyte count, 10^3/μL (range)	49.5 (31.5-75.5)	37.5 (18.5-88.0)	71.3 (20.0-104.0)	41.8 (17.5-111.0)	40.5 (19.0-78.5)	53.8 (25.0-83.5)	47.8 (25.0-68.0)
Median serum creatinine, μmol/L (range)	72 (59-114)	97 (59-152)	94 (76-210)	89 (74-220)	92 (80-187)	93 (81-184)	82 (57-97)
Median serum iron, μmol/L (range)	15.6 (10.9-22.7)	12.9 (4.5-24.2)	15.7 (11.8-33.8)	12.0 (5.9-20.9)	14.4 (7.9-19.3)	13.0 (6.1-19.7)	10.7 (8.8-16.3)
Median ferritin, μg/L (range)	394 (111-3300)	553 (31-1487)	400 (101-1293)	180 (22-1390)	245 (97-801)	368 (143-1265)	644 (60-1331)
Median transferrin, mg/dL (range)	1.8 (1.4-2.3)	2.2 (1.1-3.1)	1.9 (1.4-2.4)	2.2 (1.3-3.4)	2.2 (1.3-2.6)	1.7 (1.2-2.6)	1.7 (1.6-2.5)
Median TSAT, % (range)	33 (21-59)	23 (12-42)	34 (20-77)	21 (12-38)	28 (13-45)	30 (15-37)	23 (16- 40)
Median serum EPO level, IU/L (range)	51.9 (17.5-161.0)	34.4 (12.3-190.0)	33.0 (15.3-93.6)	38.8 (12.3-121.0)	31.7 (8.2-62.0)	31.8 (11.4-58.9)	42.2 (22.6-70.8)
O/P ratio, median (range)	0.976 (0.705-1.335)	0.811 (0.500-1.106)	0.789 (0.725-0.994)	0.864 (0.668-1.292)	0.824 (0.461-1.004)	0.852 (0.571-1.137)	0.925 (0.669-1.107)

CERA: continuous erythropoietin receptor activator; EPO: erythropoietin; Hb: hemoglobin; O/P ratio: the ratio of observed to predicted $\log_{10}$ serum erythropoietin (EPO) levels; TSAT: transferrin saturation.



C.E.R.A.: continuous erythropoietin receptor activator; Hb: hemoglobin; IQR: interquartile range; Q3W: once every 3 weeks.

Figure 2. **Change in hemoglobin from baseline to end of initial treatment based on regression slope analysis for the 6-week initial treatment period: intention-to-treat population.**

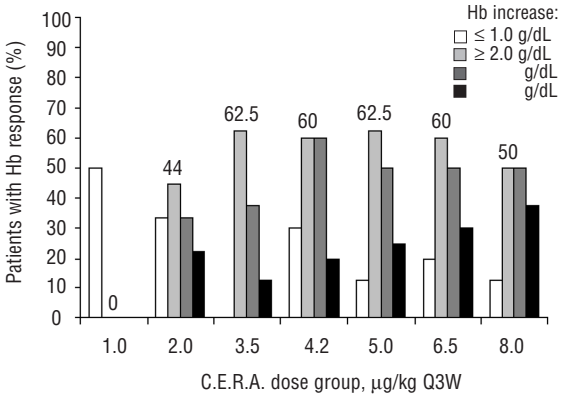


The Hb level shown at time 0 represents the baseline value (please see statistical section for explanation of how the baseline value was determined). C.E.R.A.: continuous erythropoietin receptor activator; Hb: hemoglobin; Q3W: once every 3 weeks.

Figure 3. **Median hemoglobin levels over the entire study period: intention-to-treat population.**

µg/kg group, in 100% in the 3.5 µg/kg group, in 70% in the 4.2 µg/kg group, in 100% in the 5.0 µg/kg group, in 90% in the 6.5 µg/kg group and in 75% in the 8.0 µg/kg group. The percentages of patients who never had a dose modification during the study were 10% in the 1.0 µg/kg group, 33% in the 2.0 µg/kg group, 50% in the 3.5 µg/kg group, 11% in the 4.2 µg/kg group, 50% in the 5.0 µg/kg group, 30% in the 6.5 µg/kg group, and 50% in the 8.0 µg/kg group. Modifications to the C.E.R.A. dose during the extension period generally consisted of reductions, particularly in the higher-dose groups. The exception was the 1.0 µg/kg group, in which nine out of the ten patients had their C.E.R.A. dose increased.

Thirteen patients (21%) received blood transfusions during the study, seven during the initial 6-week treatment period and six during the extension period. In all but three patients (who had transfusions because of anemia-related symptoms within the first 5 weeks of the study at Hb levels of 8.6, 8.8 or 8.9 g/dL), the Hb



Numbers above the bars represent the percentages of patients with a hemoglobin (Hb) response (defined as a Hb increase ≥2.0 g/dL relative to baseline without transfusion during the initial 6-week treatment period). No patients receiving 1.0 µg/kg C.E.R.A. achieved a Hb response. C.E.R.A.: continuous erythropoietin receptor activator; Hb: hemoglobin; Q3W: once every 3 weeks.

Figure 4. **Proportion of hemoglobin responders during the *initial 6-week period* study: intention-to-treat population.**

level at transfusion was ≤8.5 g/dL. There was no clear relationship between the dose of C.E.R.A. and the number and/or volume of blood transfusions given. Sixty percent of patients receiving the 1.0 µg/kg dose, 78% receiving 2.0 µg/kg, 87.5% receiving 3.5 µg/kg, 80% receiving 4.2 µg/kg, 100% receiving 5.0 µg/kg, 80% receiving 6.5 µg/kg, and 75% receiving 8.0 µg/kg remained transfusion-free.

Pharmacokinetics

In total, 54 of the 63 patients (86%) were evaluable for the pharmacokinetic analysis. Mean C.E.R.A. concentration-time profiles over the entire study period are shown in Figure 5. Mean serum concentrations increased with doses up to 4.2 µg/kg. At higher doses, only small further increases were seen; systemic exposure seemed to plateau, especially for C_{max}.

Pharmacokinetic parameters obtained following the second administration of C.E.R.A. (on day 22) are summarized in Table 2. Overall, the median T_{max} ranged from 4.5 to 7.0 days, and median $t_{1/2}$ from 6.3 to 9.7 days (151.2-232.8 hours). These values did not change markedly with dose of C.E.R.A. administered. C_{max} and $AUC_{22\text{-}43\ days}$ increased with C.E.R.A. doses up to 4.2 µg/kg, with no or only small increases at higher doses.

Tolerability and safety

C.E.R.A. was generally well tolerated. Although 78% of patients had adverse events, most were mild to moderate in intensity and were not considered by the investigator to be related to C.E.R.A. therapy, but rather to the underlying disease and its treatment. Also, there did not appear to be any dose-related trends in the incidence and type of adverse events reported. The most frequently reported adverse events across all treatment groups were hypertension, diarrhea and leukopenia.

Table 2. Pharmacokinetic parameters following the second administration of C.E.R.A. (day 22).

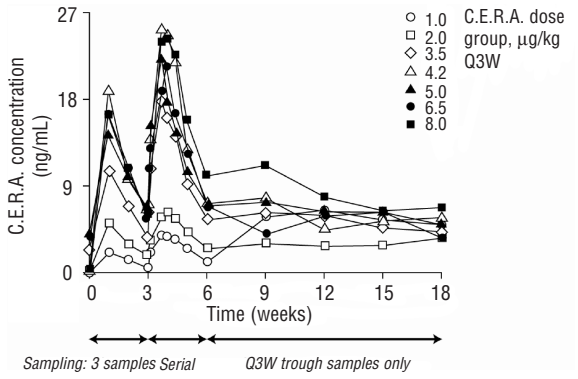
	C.E.R.A. dose group, µg/kg once every 3 weeks						
	1.0	*2.0*	*3.5*	*4.2*	*5.0*	*6.5*	*8.0*
n	10	6*	8	7	8	10	5
Mean (±SD) C_{max} (ng/mL)	4.08±3.85	6.40±2.15	18.4±6.85	26.1±10.4	23.4±6.5	22.5±11.6	25.7±21.7
Median T_{max} (days)	6.85	6.92	4.47	6.85	5.00	6.96	6.87
Mean (±SD) $AUC_{22\text{-}43\ days}$ (day · ng/mL)	57.6±52.4	93.5±29.1	245±85.4	344±112	286±76.0	315±133	380±326
Median $t_{1/2}$ (days)	6.30	9.71	9.51	7.04	8.51	6.72	8.01

**One patient had values below the limit of quantification in all samples. $AUC_{22\text{-}43\ days}$, area under the concentration time curve between days 22 and 43; C.E.R.A.: continuous erythropoietin receptor activator; C_{max}: maximum serum concentration; SD: standard deviation; T_{max}: time to C_{max}; $t_{1/2}$: elimination half-life; Q3W: once every 3 weeks.*

Generally comparable proportions of patients in each treatment group were reported to have suffered each individual event.

Seven adverse events in six patients were considered by the investigator to be related to treatment with C.E.R.A.: in one patient each in the 1.0, 2.0, 3.5, 4.2, 5.0, and 6.5 µg/kg groups (but none in the 8.0 µg/kg group). The adverse events were hypertension of mild to moderate intensity in five patients, which was controlled adequately with antihypertensive medication, pyrexia of mild intensity in one patient, and allergic dermatitis of moderate intensity at the injection site in one patient. The dose of C.E.R.A. was withheld on day 106 in the patient with allergic dermatitis and this event resolved with triamcinolone treatment. Six patients died during the study or during the 30-day follow-up. None of these deaths were considered by the investigator to be related to the C.E.R.A. treatment.

There were no clinically significant changes from baseline in laboratory values assessed during the study and no trend was reported for increasing blood pressure with treatment. Seated diastolic and systolic blood pressures were relatively unchanged throughout the study in all treatment groups, with median changes generally within 5 mmHg. No anti-C.E.R.A. antibodies were detected.



Time 0 points represent the blood samples obtained on the first day of study before the administration of C.E.R.A. C.E.R.A.: continuous erythropoietin receptor activator: Q3W: once every 3 weeks.

Figure 5. Mean serum CERA concentration-time profiles by dose group over the entire study period:

Discussion

Anemia is a common complication of multiple myeloma, being present in approximately two-thirds of patients with this disease at the time of diagnosis.[17] Treatment options include blood transfusions and ESA. Transfusions lead to only transient responses and are associated with several risks and supply issues. In contrast, ESA lead to sustained increases in Hb levels, reduced need for transfusions, and improved QoL in anemic patients with cancer.[18] The use of ESA has been recommended in patients with multiple myeloma.[17] Most ESA regimens involve once-weekly or more frequent dosing. New treatments allowing for less frequent administration and co-ordination of dosing with chemotherapy regimens, together with improved early and sustained Hb response, would be an advantage in the treatment of anemia in the setting of oncology. C.E.R.A. is the first continuous erythropoietin receptor activator being developed for the control of anemia in patients with cancer. Studies in healthy volunteers demonstrated that C.E.R.A. had a prolonged serum half-life, with the potential for administration at extended intervals when compared with the ESA.[13,14] An additional benefit of C.E.R.A. is that it has an approximately 100-fold lower binding affinity for the erythropoietin receptor, driven mainly by a slower association rate,[19] suggesting that it has the potential for a continuous effect on erythropoiesis. This would be beneficial in patients with cancer who are receiving several cycles of chemotherapy.

This dose-exploration study examined the Hb dose-response, pharmacokinetics, and safety of repeated doses of C.E.R.A. given once every 3 weeks in anemic patients with multiple myeloma receiving chemotherapy. Most patients had stage 2A or 3A multiple myeloma, with the IgG type of serum M protein, features consistent with a population of patients with multiple

myeloma. Average Hb change from baseline to the end of initial treatment indicated a dose-related erythropoietic response to C.E.R.A. at doses of 1.0-4.2 μg/kg. The median increase in Hb level was 2.21 g/dL in the group receiving 4.2 μg/kg once every 3 weeks during the initial 6-week treatment period, with higher doses resulting in a similar Hb increase to that in the 4.2 μg/kg group. Moreover, approximately 60% of patients receiving C.E.R.A. 3.5-8.0 μg/kg once every 3 weeks experienced a Hb response (defined as an increase of ≥2.0 g/dL) during the initial treatment period, indicating a rapid onset of effect. This level of response was improved or maintained during the remainder of the study, as a Hb response was demonstrated in ≥70% of patients receiving doses of ≥2.0 μg/kg during the extension period. This study indicated that a dose 1.0 μg/kg once every 3 weeks dose was suboptimal in most anemic patients with multiple myeloma. A comparison of efficacy between dose groups is difficult given the small numbers of patients in each group and the fact that the patients were not randomized into stage II. Nevertheless, doses of 3.5-8.0 μg/kg once every 3 weeks resulted in clinically relevant efficacy, suggesting the suitability of evaluating this dose range in further studies of C.E.R.A. in anemic patients with cancer. Other studies of ESA in lymphoid malignancies allowed dose-doubling if a predefined Hb increase was not reported after ~4 weeks of treatment.[8-10] As the present study was a dose-exploration study, no dose increases were allowed during the first 6 weeks, while only limited dose increases were allowed in the extension period. Modifications to the C.E.R.A. dose during the extension period generally consisted of reductions, particularly in the higher dose groups. The exception was the 1.0 μg/kg group, in which nine of ten patients had their C.E.R.A. dose increased. Very high Hb response rates to C.E.R.A. were observed in the current study (in the range of 70-100% during the entire study period for doses ≥2 μg/kg once every 3 weeks). This level of response to C.E.R.A. compares favorably with the results of studies with ESA in patients with multiple myeloma or other lymphoproliferative malignancies. A double-blind, placebo-controlled study of epoetin α 150-300 IU/kg three times weekly produced a Hb response and/or avoidance of transfusion requirement in 75% of multiple myeloma patients, together with reduced fatigue and improved QoL.[6] Randomized studies evaluating epoetin β in patients with lymphoproliferative malignancies (multiple myeloma, non-Hodgkin's lymphoma and chronic lymphocytic leukemia) showed Hb responses in 67-75% of patients with three times weekly dosing[8,9] and in 72% with once-weekly dosing.[9] A double-blind, placebo-controlled study with darbepoetin α given once weekly to patients with lymphoproliferative malignancies resulted in a Hb response in 60% of patients.[10] However, the extended administration interval of once every 3 weeks used in the present study has not been tested with any ESA specifically in patients with multiple myeloma. The pharmacokinetic parameters of C.E.R.A. given once every 3 weeks demonstrated a long T_{max} of 4.5-7.0 days and a long $t_{1/2}$ of 6.3-9.7 days (151.2-232.8 hours), both values being relatively independent of the dose of C.E.R.A. There appeared to be a plateau effect in serum concentration-time profiles, C_{max} and $AUC_{22-43\ days}$ at doses >4.2 μg/kg. This plateau effect may be a result of the small numbers of patients in each group and the small increments in dose between groups. The values for $t_{1/2}$ were considerably longer than those reported for epoetin β in healthy volunteers (approximately 24 hours)[20] or darbepoetin α in patients with cancer (approximately 61-88 hours, depending on the timing of the chemotherapy).[21] These data provide a scientific rationale for using C.E.R.A. at extended administration intervals. C.E.R.A. was generally well tolerated in the study. Most adverse events were attributed to the underlying disease or chemotherapy rather than to the study drug. There were no dose-related trends in adverse events, although it is difficult to compare the incidence of individual adverse events between the groups because of the small numbers of patients and also because patients were not randomized into the second stage of the study. This was the first study to evaluate C.E.R.A. in patients with cancer; therefore, the adverse event profile, including adverse events possibly related to therapy, is not currently known in these patients. Based on studies of anemia correction using ESA in the setting of oncology, adverse events considered by the investigator to be possibly related to the study medication included five cases of hypertension, which was controlled adequately with antihypertensive medication, and single cases of pyrexia and allergic dermatitis at the injection site. These adverse events occurred at a similar frequency to those reported in studies of ESA in patients with multiple myeloma or other lymphoid malignancies.[6,8] There were no clinically significant changes from baseline in vital signs or body weight. In addition, no clinically significant changes or worsening of pre-existing abnormalities were observed in ECG during the study.

As for other phase I/II studies, limitations of the current study included its small size and the lack of a control group. Nevertheless, the promising results reported here suggest that large, randomized and controlled studies are warranted to investigate further the clinical profile of C.E.R.A. in other malignancies. Results from this study indicate that a dose range of 3.5-8.0 μg/kg may be effective in the setting of oncology, although this needs to be confirmed in bigger trials including other types of cancer. The design of future studies should also allow for increasing the C.E.R.A. dose in the event of an inadequate Hb response.

C.E.R.A., administered once every 3 weeks to anemic patients with multiple myeloma, was generally well tolerated and resulted in a dose-dependent and sustained

increase in Hb levels. Doses of C.E.R.A. of 3.5-8.0 μg/kg appeared to result in clinically relevant efficacy. This supports the use of such doses in future studies in other types of cancer.

This is the first study to have evaluated C.E.R.A. administered once every 3 weeks to patients with cancer. The unique mode of action and pharmacokinetic profile of C.E.R.A. demonstrated in this population of cancer patients support the tested prolonged interval between injections that may enable administration to be synchronized with chemotherapy and improve convenience and flexibility of treatment in patients with multiple myeloma. The results of other studies using C.E.R.A. in the management of anemia in patients with cancer are awaited.

Authors' Contributions

AD: conception and design of the study, acquisition of data, analysis and interpretation of data, determining manuscript content, revising manuscript critically for important intellectual content, final approval of the version to be published; JK, MR, AH, IS: conception and design of the study, acquisition of data, analysis and interpretation of data, revising manuscript critically for important intellectual content, final approval of the version to be published; JEE: analysis and interpretation of data, drafting the article, revising manuscript critically for important intellectual content, final approval of the version to be published.

Conflict of Interest

JEE is an employee of Hoffmann-La Roche. The authors have no other conflicts of interest to report.

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Diminished thrombus formation and alleviation of myocardial infarction and reperfusion injury through antibody- or small-molecule-mediated inhibition of selectin-dependent platelet functions

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ABSTRACT

Background and Objectives

P-selectin has been implicated in important platelet functions. However, neither its role in thrombus formation and cardiovascular disorders nor its suitability as a therapeutic target structure is entirely clear.

Design and Methods

Platelet aggregation was assessed in complementary *in vitro* settings by measurements of static aggregation, standardized aggregometry and dynamic flow chamber assays. Degradation of aggregates was also analyzed under flow conditions using video microscopy. *In vivo*, platelet rolling in cutaneous venules was assessed by intravital microscopy in wild-type mice treated with selectin-blocking compounds as well as in P-selectin-deficient mice. $FeCl_3$-induced arterial thrombosis was studied by intravital microscopy in untreated mice or mice treated with an inhibitor of selectin functions. Finally, inhibition of selectin functions was studied in an ischemia/reperfusion injury model in rats.

Results

Antibody- or small-molecule-mediated inhibition of P-selectin functions significantly diminished platelet aggregation ($p<0.03$) and platelet-neutrophil adhesion *in vitro* ($p<0.01$) as well as platelet aggregate sizes under flow ($p<0.03$). Established aggregates were degraded, either via detachment of single platelets following addition of efomycine M, or via detachment of multicellular clumps when P-selectin-directed Fab-fragments were used. *In vivo*, selectin inhibition resulted in a greater than 50% reduction of platelet rolling in cutaneous venules ($p<0.01$), producing rolling fractions similar to those observed in P-selectin-deficient mice ($p<0.05$). Moreover, inhibition of selectin functions significantly decreased the thrombus size in $FeCl_3$-induced arterial thrombosis in mice ($p<0.05$). In an ischemia/reperfusion injury model in rats, small-molecule-mediated selectin inhibition significantly reduced myocardial infarct size from 18.9% to 9.42% ($p<0.001$) and reperfusion injury ($p<0.001$).

Interpretation and Conclusions

Inhibition of P-selectin functions reduces platelet aggregation and can alleviate platelet-related disorders in disease-relevant preclinical settings.

Key words: platelets, selectins, aggregation and thrombus formation.

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Platelet aggregation plays a major role in hemostasis.[1,2] However, platelet aggregation might also contribute to common pathological conditions including thrombosis, arterial occlusion in myocardial infarction and stroke. Indeed, platelet-related disorders are among the leading causes of death in industrialized countries.[3,4] Several macromolecular components provide a substrate for platelet adhesion, of which fibrillar collagen is considered to be the most thrombogenic compound of the vascular endothelium, supporting platelet adhesion as well as activation.[5,6] The interaction cascade between platelets and collagen involves consecutive steps, with a first phase of adhesion, followed by activation and a second phase of adhesion, secretion, and ultimately aggregation.[7,8] A number or receptors and signaling pathways, including P-selectin, have been implicated in this complex sequence of events, but their exact interactions are partly elusive. P-selectin is a C-type lectin that is expressed in α-granules of resting platelets and is translocated to the cell surface upon activation.[9] The role of P-selectin in thrombotic processes has long been discussed. Previous studies have suggested that P-selectin expression on platelets (it is the only selectin expressed by platelets) may influence the size and stability of thrombi.[10-12] Based upon comparisons between P-selectin-deficient and wild-type mice, it has recently been suggested that P-selectin expressed on activated platelets contributes to myocardial reperfusion injury.[13] However, the exact role of P-selectin in these processes is not clear, and its use as a therapeutic target structure has not been established. P-selectin expressed by endothelial cells is rapidly translocated to the cell membrane upon activation and may also contribute to aggregate formation.[14] Several types of cells, including leukocytes, endothelial cells and platelets, carry P-selectin ligands.[15] Leukocytes are thought to interact with activated platelets and thereby promote thrombosis and vascular occlusion, impairing the blood flow and exacerbating ischemia. Disruption of this binding was shown to be effective in reducing the incidence of re-occlusion in animal models of vascular injury.[11] Moreover, cell-derived microparticles carrying P-selectin glycoprotein ligand-1 (PSGL-1) are thought to circulate in the blood and accumulate in developing platelet-rich thrombi following vessel wall injury, thereby concentrating tissue factor at the site of vascular injury and initiating blood coagulation.[16] Thus, interfering with P-selectin functions might be a means to decrease thrombus formation in some cardiovascular disorders. However, based on partly contradictory results of previous studies,[17,18] the exact roles of P-selectin in platelet aggregation and in platelet-related cardiovascular disorders are still unclear.

Using P-selectin-specific antibodies, their Fab-fragments or efomycine M, a small-molecule compound that inhibits selectin functions,[19] we assessed the role of P-selectin in platelet aggregation *in vitro* as well as in platelet rolling, thrombus formation and myocardial infarction and reperfusion injury *in vivo*.

Design and Methods

Monoclonal antibodies

The P-selectin-specific function-blocking antibodies used were CLB-Thromb/6 (Immunotech, Marseille, France) and AK-4 (BD-PharMingen, Heidelberg, Germany). For functional studies, antibodies were purified by affinity chromatography from the hybridoma cell line WAPS12.2 (ATCC, Manassas, USA). Fab-fragments were generated using the ImmunoPure®-Fab-Kit (Pierce, Rockford, USA).

Platelet isolation

Whole blood from healthy individuals was diluted 4:1 in citrate buffer (pH 6.5) and centrifuged for 20 minutes at 280 g. The resulting platelet-rich plasma (PRP) was then centrifuged for 10 minutes at 500 g, and all supernatant was carefully removed. Platelets were then resuspended in 1/20th of the original volume in Tyrode's buffer (0.1 M $CaCl_2$, 0.1 M $MgCl_2$, 0.5 M HEPES, 10% bovine serum albumin (BSA) and 10% glucose in H_2O, pH 7.35), hereafter referred to as platelet-rich cell-suspension.

Sialyl Lewis^x (sLe^x)-induced platelet aggregation

To study the role of P-selectin in platelet aggregation, 50 μL of platelet-rich cell-suspension were mixed with 20 μL of 1 M $CaCl_2$, 50 μL thrombin (1 U/mL) and 25 μL of 0.5 mg/mL biotinylated polyacrylamide coated with the selectin ligand, sLe^X (Lectinity, Moscow, Russia), in water in the presence or absence of 0.1 mg/mL P-selectin-specific antibodies or efomycine M (99.9% pure).[19] Platelets were incubated at room temperature for 30 minutes while gently shaken and, thereafter, fixed in 1% paraformaldehyde. Platelet aggregation was analyzed microscopically and quantitated digitally using ImageJ.[20]

In vitro adhesion assays

Platelet-neutrophil adhesion

Human platelets and neutrophils were freshly isolated by density gradient centrifugation.[21] After incubation with thrombin (0.25 U/mL, Sigma, Diesenhofen, Germany) for 10 minutes, the platelets were washed and incubated with 10^{-7} to 10^{-4} M of efomycine M for 10 minutes. Platelets were then mixed with neutrophils at a ratio of 10:1 in 96-well plates in 100 μL of culture medium (M199, Gibco/BRL, Karlsruhe, Germany). After 20 minutes, neutrophils with two or more adherent platelets (positive) and one or no adherent platelets (negative) were counted microscopically. Maximum binding was monitored in the absence of inhibitors (negative control); minimum binding was monitored in the presence of 5 mM EDTA to demonstrate calcium-dependence of the interaction (positive control).

Adhesion of neutrophils to srP-selectin

Neutrophils were intravitally labeled with ^{35}[S]-methionine (Amersham, Freiberg, Germany; 0.2 mCi/10^7 cells) for 3 h at 37°C.[19] Each well in a 96-well microtiter plate was coated with 50 µL of srP-selectin (Serotec/Biozol, Eching, Germany; 10 µg/mL) in TBS/$CaCl_2$, and blocked with 1% BSA. ^{35}[S]-methionine-labeled neutrophils (2×10^5/well) were then added to 100 µL of culture medium (RPMI1640, 10% fetal calf serum, 1 mM $CaCl_2$) containing efomycine M (10^{-7} to 10^{-4} M), the P-selectin-specific antibody (2 µg/well), or a mixture of both. The plates were then incubated for 1 h at 37°C, washed gently with TBS/$CaCl_2$, and bound cells were lyzed using 2% sodium dodecylsulfate. Bound radioactivity was quantitated by scintillation counting. Experiments were performed in sextuplicate.

Aggregometry

Light transmission was measured in platelet-rich plasma (160 µL) stimulated with collagen (10 µg/mL) in the presence or absence of efomycine M (0 to 0.3 mM), P-selectin-specific antibodies (6.5 µg) or Fab-fragments of the latter (6.5 µg). Transmission was recorded on a Fibrintimer 4-channel-aggregometer (APACT-Laborgeräte, Hamburg, Germany) for 10 minutes, and was expressed as relative units using plasma to determine 100% aggregation. Aggregation experiments were performed in duplicate for each sample from three donors.

Platelet aggregation under flow conditions

Heparinized human blood from three donors was diluted 1:1 in Hank's balanced salt solution (HBSS)/$CaCl_2$ with or without P-selectin-specific antibodies or efomycine M (0.01 to 0.3 mM). Cover slips (24×60 mm) were coated with 50 µL collagen (Kollagenreagenz Horm, Nycomed, Linz, Austria) at 0.5 mg/mL, dried at 37°C, and then incubated for 1 hour with 0.5% BSA. Transparent flow chambers with a slit depth of 50 µm and a slit width of 500 µm, equipped with the cover slips, were rinsed with HBSS/$CaCl_2$ and connected to a syringe containing the pre-incubated blood. Perfusion was performed using a pulse-free pump at a wall shear rate of 1000 s^{-1}. Microscopic phase-contrast images were recorded in real time. After 10 minutes of perfusion, non-aggregated cells were washed away using HBSS/$CaCl_2$. Images were analyzed off-line using MetaView Imaging software (Universal, Downington, USA). The number and size of aggregates were analyzed using ImageJ.[20]

Degradation of platelet aggregates under flow

Flow chamber experiments were performed as described above, with the difference that 1 mL of whole blood from three donors was perfused to allow the formation of stable aggregates, followed by perfusion with 500 µL of HBSS/$CaCl_2$. Aggregates were monitored microscopically in real time, and the chambers were perfused with 1.5 mL of HBSS/$CaCl_2$ containing efomycine M (0 to 0.3 mM), P-selectin-specific antibodies or Fab-fragments thereof (both at 1 mg/mL). Aggregate degradation was recorded in real-time for 3 minutes. Still images were taken at the beginning and end of the observation period and analyzed using ImageJ.

Platelet preparation for intravital microscopy

Heparinized blood from adult donor mice injected intraperitoneally with efomycine M (5 mg/kg in 1% dimethylsulfoxide, 5% Solutol, 5% ethanol in PBS) or vehicle 1 hour before bleeding was centrifuged at 250 g for 10 minutes, and platelet-rich plasma was gently transferred to a fresh tube. Platelets were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Invitrogen, Karlsruhe, Germany) and adjusted to a final concentration of 200×10^6 platelets/ 250 µL.[22]

Intravital microscopy

All animal experiments were approved by the local authorities. Intravital microscopy of mouse ears was performed as described elsewhere.[23,24] Briefly, wild-type- and P-selectin-deficient mice[23] (Jackson Laboratories, Bar Harbor, USA), both backcrossed for more than ten generations on a C57BL6 background, were anesthetized intraperitoneally using ketamin/rompun, and placed on a homeothermic blanket. A catheter was placed micro-surgically into the right carotid artery for injection of CFDA-SE-labeled platelets, efomycine M (4 mg/kg) or P-selectin-specific antibodies (4 mg/kg). The left ear was gently placed on a microscope slide and covered with glycerin and a cover slip. The cutaneous microcirculation was continuously recorded using a 1/3" DSP 3-CCD camera (Sony, Köln, Germany). Cell behavior in individual vessels was analyzed off-line. Cells were considered non-interacting when they moved at the velocity of the blood flow (V_{free}), whereas lower velocities were defined as rolling. A total of 11 vessels in four wild-type mice and six vessels in three P-selectin-deficient mice treated with efomycine M as well as six vessels in three wild-type animals treated with P-selectin-specific antibodies were analyzed before and after the respective treatments.

$FeCl_3$-induced arterial thrombosis

Four to five-week-old C57BL6 mice were injected intraperitoneally with 5 mg/kg efomycine M (n=18 mice) or vehicle (n=16 mice) 1 hour before they were anesthetized intraperitoneally. One hundred million CFDA-SE-labeled platelets/mouse were injected through the tail vein; efomycine M-treated mice received platelets from efomycine M-treated donors and *vice versa*. The mesentery was externalized through an abdominal incision. Arterioles of 35-60-µm in diameter were visualized microscopically using a CCD camera (Visitron, Puchheim, Germany). After topical application of a filter paper saturated with 20% $FeCl_3$ for 1 minute, arterioles were monitored for 40 minutes or until complete occlu-

sion occurred.[25] Thrombus formation was analyzed using ImageJ.

Tail bleeding time experiments

Wild-type or P-selectin-deficient C57BL6 mice (four mice/group) were injected intraperitoneally with 5 mg/kg efomycine M resulting in serum concentrations of >0.1 mM after 1 h, i.e. concentrations sufficient to inhibit P-selectin and platelets *in vitro*.[19] Mice that received vehicle only served as controls. Ninety minutes after injection, the mice were placed in a restrainer and the distal 5 mm of tail was amputated using a scalpel. The tail was then blotted with filter paper every 10 seconds until the paper was no longer blood-stained.[26]

Myocardial infarction and reperfusion injury

One hour after intraperitoneal injection of efomycine M (0; 0.03; 0.1; 1.0 mg/kg; n=10 animals/group), Lewis rats were anesthetized intraperitoneally with thiopental. For substance administration and blood pressure recording, the left jugular vein and the right carotid artery, respectively, were catheterized with a polyethylene tube. The animals were ventilated through a tracheal tube.

Following thoracotomy, a thin thread (Prolene®, 5-0) was placed microsurgically around the left anterior descending artery with a stitch through the myocardium. Tightening the loop occluded the artery and induced ST-segment elevation on the electrocardiograph. The loop was loosened in order to perfuse the ischemic myocardium. Sham operated animals (negative controls) were treated identically, but the loop was not tightened. After 30 minutes of occlusion and 90 minutes of reperfusion, the heart was removed and perfused via the aorta with TTC-solution (2,3,5-triphenyltetrazolium-chloride 1.5% in a 1:1 mixture of water and PBS) for 15 minutes to differentiate between viable and irreversibly injured myocardium.[27,28] Viable myocardium (bright red) was separated surgically from infarcted tissue (unstained). The infarct size was expressed as a percentage of the weight of the ventricles.

Myeloperoxidase assay

Myeloperoxidase (MPO) is widely used as a marker enzyme for granulocytes.[29-32] Briefly, frozen myocardial samples were immersed in liquid nitrogen, pulverized and homogenized in a N-acetyl-N,N,N-trimethylammonium-bromide-solution (0.5% in phosphate buffer, pH 7.4). Following three cycles of thawing and freezing in liquid nitrogen, the samples were centrifuged for 20 minutes at 4000 rpm. The volume of the supernatant was measured and four samples of 30 µL each were pipetted onto a microtiter plate. The reaction was started with 270 µL of substrate solution (250 µL phosphate buffer, 50 mM, pH 6.0, 10 µL H_2O_2, 10 µL o-dianisidine solution [0.6% in DMSO]), and the extinction was measured over 3 minutes. ΔE/min was calculated as $(E_{t=3min}–E_{t=0})/3$. The MPO concentration was calculated as: (ΔE/min • 1000 • volume [mL])/(8.3 • weight (g)) = MPO (nmol/min • g^{-1} fresh weight)

Statistical analysis

Data are displayed as mean (±SD or SEM as indicated); *p* values were determined using the two-tailed t-test, and *p* values <0.05 (confidence interval of 95%) were considered statistically significant. All statistical tests were two-sided.

Results

Antibody- or small-molecule-mediated inhibition of P-selectin functions diminishes platelet aggregation in vitro

In order to assess the activity of selectin-directed inhibitors on platelet aggregation, thrombin-activated human platelets were incubated with multimeric sLe^x. In addition, P-selectin-specific antibodies or efomycine M were added to the platelet suspension. As expected, platelet aggregation occurred as a result of activation with low-dose thrombin (Figure 1A, first two panels). However, the aggregate size was dramatically increased when sLe^x was added (Figure 1A, third panel, p=0.008 as compared to thrombin alone). The latter effect was completely abolished in the presence of P-selectin-specific antibodies or efomycine M (Figure 1A, last two panels and Figure 1B, p<0.001). These data show that P-selectin plays an important role in platelet aggregation *in vitro* and that P-selectin-specific antibodies or the small-molecule inhibitor, efomycine M, inhibits platelet aggregation. To confirm the effect of P-selectin inhibition on platelet aggregation with a second, independent method, standardized aggregometer experiments were performed. Platelets were isolated and activated with collagen, a potent inducer of thrombus formation.[7,8] This incubation was performed in the presence or absence of efomycine M (0 to 0.3 mM), P-selectin-specific antibodies or the Fab-fragments of these antibodies (Figure 2). Again, it was found that efomycine M significantly inhibited platelet aggregation in a dose-dependent fashion (Figure 2A and B). Surprisingly, the use of whole P-selectin-specific antibodies did not decrease aggregate formation in this system (Figure 2A, C). In order to exclude that the failure of P-selectin-directed IgG to inhibit aggregation was due to potential cross-linking of activated platelets by the intact bivalent IgG molecule, we generated Fab-fragments of this antibody. These fragments significantly reduced the maximal aggregation from 71.3 (±6.4%) in the control samples to 47.0 (±6.7%) in the Fab-fragment-treated platelet suspensions (p<0.03 as compared to controls, Figure 2C). Thus, this system again showed P-selectin-dependent platelet aggregation. Complex pathophysiological processes, such as reperfusion injury