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Plasma nitric oxide is associated with the occurrence of moderate to severe acute graft-versus-host disease in hemopoietic stem cell transplant recipients

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Background and Objectives. Nitric oxide (NO) has been implicated as one of the mediators of acute graft-versus-host disease (GVHD) but reports on its measurement during hemopoietic stem cell transplantation (HSCT) in humans are scarce. The present study was conducted to measure the plasma NO in HSCT recipients in order to delineate its relationships with acute GVHD.

Design and Methods. Thirty-nine randomly selected patients undergoing HSCT were recruited. Thirty-one patients received an allogeneic transplant (ALLO) from HLA-identical siblings (n=20), a haploidentical parent (n=1) or matched unrelated donors (n=10). Eight patients received an autologous (AUTO) HSCT. Plasma levels of nitrite/nitrate (NO_2^-/NO_3^-), the end-product of NO, were measured by chemiluminescence and the results were correlated with the occurrence and severity of acute GVHD.

Results. Baseline NO_2^-/NO_3^- levels before HSCT were similar in the ALLO and AUTO patients (17.4 vs 21.1 µmol/L, $p>0.05$). Significant increases in plasma NO_2^-/NO_3^- (> 2 times the baseline level) were found in all 13 patients with acute GVHD ≥ grade 2, in 15 out of 18 patients with acute GVHD grade ≤ 1 and 3 out of 8 patients receiving autologous HSCT. The increase in NO_2^-/NO_3^- among the three groups of patients was significantly different (135.5 vs 56.3 vs 36.6 µmol/L, $p < 0.001$). The average NO production, calculated as the area under the curve, was also significantly differently among the three groups of patients (44.5 vs 30.0 vs 23.8 µmol/L, $p < 0.001$).

Interpretation and Conclusions. Plasma NO in HSCT recipients is quantitatively associated with the occurrence of acute GVHD; its role remains to be determined.

Key words: nitric oxide, stem cell transplantation, graft-versus-host disease.

Graft-versus-host disease (GVHD) is a major cause of morbidity and occasionally mortality following hemopoietic stem cell transplantation (HSCT). Despite the recent improvement in HLA-typing by molecular techniques, moderate to severe acute GVHD occurs in 10 to 50% of patients receiving allogeneic HSCT.[1] At present, there is no reliable parameter by which we can predict the occurrence of acute GVHD and once it occurs, the resulting inflammation triggers the release of various cytokines, forming a vicious cycle that leads to severe end-organ damage.

Nitric oxide (NO) is generated from the oxidation of L-arginine by nitric oxide synthase (NOS) that exists in three isoforms: constitutive endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In addition to vasodilatation and neuronal transmission, NO that is generated by iNOS has been implicated as a mediator of immunologic reactions.[2] The latter has been demonstrated in monocytes/macrophages, neutrophils and T-lymphocytes.[3,4] In animal models of transplantation, serum NO increased during allograft rejection.[5,6] However, reports on the measurement of NO during HSCT in human are scarce and therefore, its role in human acute GVHD and its use in the clinical setting remain undefined.[7,8]

In this study, we measured plasma nitrite/nitrate (NO_2^-/NO_3^-), the stable end products of NO, in patients undergoing HSCT and correlated them with

Table 1. Clinical characteristics and plasma NO_2^-/NO_3^- in allogeneic and autologous stem cell transplant recipients.

	GVHD ≥ grade 2	*GVHD ≤ grade 1*	*Auto transplant recipients*
No. of patients	13	18	8
Male: Female	8:5	9:9	4:4
Median age (range)	40 (27-57)	36 (17-47)	46 (25-67)
Diagnosis-Status			
CML-CP	8	6	0
AML (CR1/CR2)	2	8	0
NHL (CR/PR)	1	1	3
ALL (CR1/CR2)	2	2	0
Others	0	1	5
Regimens			
Bu-Cy	11	14	0
Cy-TBI	2	3	0
CBV	0	1	3
Others	0	0	5
Day 0 Plasma NO2-/NO3- Median (range)	17.4 (7.3-24.6)	17.0 (9.3-58.2)	21.1 (9.6-29.2)
Peak Plasma NO2-/NO3- Median (range)	135.5 (60.2-449.8)	56.3 (25.6-141.9)	36.6 (30.9-51.6)

CML-CP, chronic myeloid leukemia in chronic phase; AML, acute myeloblastic leukemia; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukemia; CR, complete remission; Bu, busulfan; Cy, cyclophosphamide; CBV, cyclophosphamide, BCNU, etoposide.

the occurrence of moderate to severe acute GVHD in order to define the role of NO in the pathogenesis of acute GVHD. Chronic GVHD, defined chronologically as GVHD that occurs three months after transplantation, was not considered in this study. The severity of acute GVHD was graded according to the criteria described by Glucksberg *et al.*[9] and was classified into mild (overall grade ≤ 1 or no acute GVHD) or severe (overall grade ≥ 2). No distinction was made, however, between patients with mild or no GVHD as these patients were often managed conservatively and skin biopsy was not routinely performed.

Design and Methods

Patients

Thirty-nine randomly selected patients undergoing HSCT from June 1999 to April 2000 at Queen Mary Hospital, Hong Kong, were recruited into this study with their consent. Their baseline characteristics are shown in Table 1.

GVHD prophylaxis

In allogeneic HSCT, prophylaxis against acute GVHD comprised methotrexate (15 mg/m^2 on day 1, 10 mg/m^2 on days 3,6 and 11) and cyclosporine A (3 mg/kg intravenously or 8 mg/kg orally day 1–50, tailed off at 6 months). Patients developing acute GVHD received additional immunosuppression according to the discretion of the attending physicians.

Measurement of plasma nitrite and nitrate levels

Fasting plasma was collected from each patient daily and was centrifuged at 2,500 g for 10 minutes at 4°C and stored at –70°C until analyzed. Nitrite/nitrate (NO_2^-/NO_3^-), the stable end-products of NO, was measured as previously described.[10] Briefly, thawed samples (100 μL) were diluted 4-fold with deionized water and deproteinated by zinc sulfate (final concentration of 15g/L). They were centrifuged at 10,000g for 5 minutes at room temperature. Next, 5 μL of supernatant were added to the purge vessel containing 5 μL of saturated vanadium (III) chloride (VCl_3) (Merck, Germany) (in 1 mol/L HCl) and heated in a water bath to 95°C. A constant stream of nitrogen was bubbled through the VCl_3/HCl solution, a gas bubbler containing 1M NaOH, an IFD filter and finally the chemiluminescence nitric oxide analyzer (Sievers 280 NO Analyzer, USA). The sensitivity of measuring NO and its reaction products in liquid samples is 1 picomole.

Statistical methods

Comparisons between groups of data were made using the Kruskal-Wallis test or Mann-Whitney test from SPSS (USA). The contributions of various clinical parameters to the occurrence of acute GVHD were evaluated by binary logistic regression analysis. p values < 0.05 were considered statistically significant.

Results

Temporal profile of plasma NO_2^-/NO_3^-

Figure 1 shows the results of a representative patient with acute GVHD (overall grade III) showing an increase in plasma NO_2^-/NO_3^- that peaked on day 26 after transplantation and preceded the onset of acute GVHD. A significant increase in plasma NO_2^-/NO_3^- was defined as twice the level measured on day 0 and was found in all 13 patients with acute GVHD ≥ grade 2, of whom eight patients had the peak preceding the onset of acute GVHD by three days (median, range 2-4 days), in 15 out of 18 patients with acute GVHD grade ≤ 1 and in 3 out of 8 patients receiving autologous HSCT. For patients in whom the increase in plasma

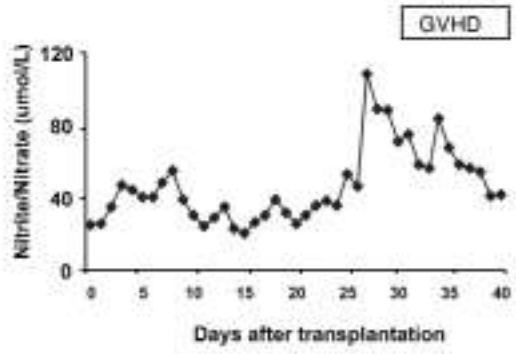

Figure 1. Plasma NO_2^-/NO_3^- (μmol/L) during HSCT in a representative patient with severe GVHD (overall grade 3). The rectangle on top indicates the duration of acute GVHD. Note that the peak level preceded the onset of aGVHD.

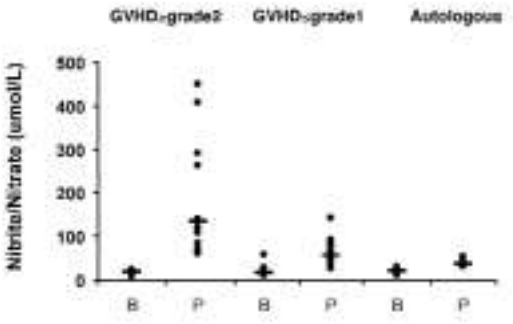

Figure 2. Plasma NO_2^-/NO_3^- (μmol/L) according to the type of HSCT and the severity of GVHD. B, baseline level (day 0); P, peak value. While there was no significant difference in the baseline plasma NO_2^-/NO_3^- levels, patients with GVHD ≥ grade 2 had significantly higher levels compared with those who had GVHD ≤ grade 1 and those who received an autologous HSCT (Kruskal-Wallis Test, $p < 0.01$).

NO_2^-/NO_3^- was less than twice the baseline level, the highest value during the course of HSCT was defined as the peak in subsequent analyses.

Comparison of plasma NO_2^-/NO_3^- between groups of patients

Figure 2 shows the plasma NO_2^-/NO_3^- in patients receiving allogeneic HSCT who (a) developed GVHD ≥ grade 2, (b) GVHD ≤ grade 1, and (c) who received autologous HSCT. There was no significant difference in baseline (day 0) levels of plasma NO_2^-/NO_3^- in these three groups of patients ($p > 0.05$). However, patients with GVHD ≥ grade 2 had significantly greater increase in plasma NO_2^-/NO_3^- than those with ≤ grade 1 or patients who received autologous HSCT (Table 1). The average level of plasma NO_2^-/NO_3^- was calculated by integrating the area under the curve (AUC) during the hospital stay divided by the number of days over which the AUC was obtained. Patients with GVHD ≥ grade 2 had a significantly higher average plasma NO_2^-/NO_3^- than those with ≤ grade 1 or patients who received autologous HSCT (44.5 vs 30.0 vs 23.8 mmol/L, $p < 0.001$). When groups of data were compared separately, the peak increase as well as the average plasma NO_2^-/NO_3^- in patients with acute GVHD ≤ grade 1 was significantly greater than that in patients who received an autologous HSCT (Mann-Whitney test, $p < 0.01$). Using a cut-off at 100 μmol/L, high peak plasma NO_2^-/NO_3^- (> 100 μmol/L) was confined to patients with GVHD ≥ grade 2. An exception was found in patient number 14 (Table 2) who had mild acute GVHD (grade 1) but severe hemorrhagic cystitis requiring prolonged bladder irrigation.

Contributions from other clinical parameters

To see whether clinical parameters other than plasma NO_2^-/NO_3^- might determine the occurrence of acute GVHD in patients receiving allogeneic HSCT, the age of patients and donors, sex mismatched transplantation, the conditioning regimens (cyclophosphamide versus TBI), source of HSCT (siblings vs MUD) and the underlying diagnosis were entered into binary logistic regression analysis together with the baseline and peak plasma NO_2^-/NO_3^- level. Only the peak plasma NO_2^-/NO_3^- level, but none of the other factors, had a significant association with the occurrence of moderate to severe acute GVHD ($p<0.01$).

Discussion

The present study demonstrates that plasma NO_2^-/NO_3^- exhibited a transient increase during HSCT that was associated with moderate to severe GVHD. In particular, all but one patient with peak plasma NO_2^-/NO_3^- greater than 100 μmol/L developed acute GVHD ≥ grade 2. The only patient with grade 1 GVHD had severe hemorrhagic cystitis requiring prolonged bladder irrigation and repeated cystoscopies. Hemorrhagic cystitis has been considered as a manifestation of acute GVHD[11] but is not included in the conventional grading scale. Our results, therefore, are in keeping with those in a murine model demonstrating NO as a mediator of acute GVHD.[12] The source of NO has not been

Table 2. Individual clinical data and plasma NO_2^-/NO_3^- (μmol/L) in the cohort.

Pts	HSCT type	Sex	Age	Dx	Regimen	Plasma No_2^-/No_3^-			GVHD grade	NO peak (day)	GvHD onset (day)
		R	D			Day 0	Peak	AUC			
1	SIB	F	49	CML	Bu-Cy	10.32	139.76	31.96	≥ 2	28	28
2	SIB	M	30	CML	Bu-Cy	19.40	87.48	44.5	≥ 2	22	24
3	MUD	F	41	AML	Bu-Cy	12.12	60.20	32.29	≥ 2	41	43
4	MUD	M	31	CML	Bu-Cy	24.44	70.96	37.9	≥ 2	32	35
5	SIB	M	34	CML	Bu-Cy	17.40	262.92	67.04	≥ 2	28	28
6	SIB	F	57	CML	Bu-Cy	14.16	292.08	77.95	≥ 2	12	14
7	SIB	M	40	CML	Bu-Cy	22.96	449.76	141.58	≥ 2	11	14
8	SIB	M	38	ALL	Cy-TBI	23.88	85.04	39.38	≥ 2	16	14
9	SIB	F	40	AML	Bu-Cy	24.56	108.12	46.82	≥ 2	27	31
10	SIB	M	28	CML	Bu-Cy	16.72	138.72	32.9	≥ 2	24	28
11	SIB	F	27	NHL	Cy-TBI	16.52	407.12	79.98	≥ 2	35	34
12	MUD	M	42	CML	Bu-Cy	19.56	135.52	52.57	≥ 2	36	28
13	SIB	M	48	ALL	Bu-Cy	7.32	120.60	31.56	≥ 2	20	23
14	SIB	F	40	AML	Bu-Cy	12.24	141.88	27.51	≤ 1	34	
15	SIB	F	40	NHL	Big CBV	20.76	81.92	39.28	≤ 1	24	
16	MUD	F	32	ALL	Cy-TBI	9.32	76.76	26.46	≤ 1	38	
17	MUD	M	38	CML	Bu-Cy	26.12	81.52	49.37	≤ 1	27	
18	SIB	F	29	AML	Bu-Cy	15.16	42.88	26.6	≤ 1	32	
19	SIB	F	22	ALL	Cy-TBI	19.28	48.28	29.92	≤ 1	22	
20	SIB	M	47	AML	Bu-Cy	13.48	52.56	32.07	≤ 1	20	
21	SIB	F	40	AML	Bu-Cy	21.44	25.56	21.92	≤ 1	36	
22	SIB	M	32	CML	Bu-Cy	13.32	48.80	30.92	≤ 1	23	
23	Parent	M	17	CML	Cy-TBI	12.28	48.16	25.5	≤ 1	32	
24	SIB	M	36	CML	Bu-Cy	28.68	89.72	55.68	≤ 1	29	
25	SIB	F	36	CML	Bu-Cy	27.12	60.04	30.57	≤ 1	27	
26	SIB	M	42	MM	Bu-Cy	13.48	37.80	30.01	≤ 1	23	
28	MUD	M	28	AML	Bu-Cy	15.56	52.28	26.62	≤ 1	34	
29	MUD	M	38	AML	Bu-Cy	14.81	92.36	27.33	≤ 1	32	
30	MUD	F	19	AML	Bu-Cy	26.36	43.04	24.9	≤ 1	30	
31	MUD	F	28	CML	Bu-Cy	18.48	63.36	37.9	≤ 1	32	
32	MUD	M	37	AML	Bu-Cy	58.20	77.20	37.49	≤ 1	34	
27	Auto	F	62	MM	Mel	28.16	32.48	23.17	-	18	
33	Auto	M	48	NHL	Big CBV	20.20	30.88	22.8	-	17	
34	Auto	F	44	NHL	Big CBV	25.88	40.64	24.38	-	13	
35	Auto	F	30	HD	BEAM	12.80	48.36	32.83	-	16	
36	Auto	M	25	NHL	Big CBV	9.60	34.12	26.56	-	16	
37	Auto	M	56	MM	Mel	17.00	36.08	21.81	-	14	
38	Auto	M	67	MM	Mel	21.92	37.08	22.12	-	23	
39	Auto	F	41	CA Br	CarboPEC	29.24	54.60	32.51	-	14	

SIB, transplant from sibling donor; MUD, matched unrelated donor; Auto, autologous transplant; AUC, Area under the curve divided by the number of days (from admission to discharge) over which the area is measured.

investigated in this study, although activation of iNOS, the enzyme that gives rise to NO, has been demonstrated in Th-1 lymphocytes,[2] monocytes and other tissues,[3,4,13-15] and the former is known to play an important role in the pathogenesis of acute GVHD.[16,17] On the other hand, previous studies using a murine model of GVHD have demonstrated that NO formation might play a suppressive role in T-lymphocyte proliferation.[18] Therefore, whether NO in HSCT patients is a mediator leading to GVHD or whether an increase in NO synthesis plays a protective role in patients with severe GVHD, would have to be investigated by further studies.

It is also interesting to note that a significant (albeit smaller) increase in NO could be demonstrated in patients with GVHD ≤ grade 1 and in patients who received autologous HSCT, suggesting that it may be involved in other processes during HSCT, including chemotherapy-related tissue toxicities,[19] bacterial[20] and viral infection.[21] These possibilities remain to be further investigated.

There are certain limitations in the present study in delineating the role of NO in GVHD. In this cohort, patients who developed GVHD ≥ grade 2 were invariably treated with steroids as a first-line treatment. The observed increase in NO in the present study might be an underestimation as steroids have been shown to inhibit iNOS activity and hence NO production. On the other hand, inhibitors of NO synthesis may be useful in dampening the alloimmune response due to release of NO and whether they can also be useful in a clinical setting in the prophylaxis and treatment of GVHD has not been ascertained in the present study. On the other hand, the results of this study might provide ground for further research into the role of NO in GVHD. In particular, the observation that peak NO levels greater than 100 μmol/L were confined to patients with GVHD ≥ grade 2 and that the peak level preceded the onset of GVHD by 1 to 4 days in the majority of patients suggests early detection of NO might enable pre-emptive intensification of immunosuppression. This would have to be investigated by further studies. In conclusion, the present study demonstrates that plasma NO exhibited a transient increase during HSCT which was associated quantitatively with the occurrence of moderate to severe GVHD.

Contributions and Acknowledgments

CYC: analysis and interpretation of data and drafting the article; PCWF: conception and design of the study and critical revision of the manuscript; AYHL: data interpretation and drafting the article; AKWL: conception and design of the study and critical revision of the manuscript; RL: conception and design of the study and critical revision of the manuscript. All authors approved the final version of the manuscript to be submitted.

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Disclosures

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Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

The correlation between plasma NO and GVHD reported in this study provides ground for further studies into the effects of NO synthesis blockade on the occurrence of GVHD - a potential target of anti-GVHD prophylaxis and therapy.[22]

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