ES subclone. Thus, the induction of HO-1 was merely a sign of oxidative stress. Nitric oxide is a powerful inducer of HO-1,[8] and this was shown in the present study. However, it did not offer any protection against apoptosis induced by oxidative stress. Theoretically, nitric oxide may increase the induced apoptosis, since it may react with the superoxides formed during BSO/etoposide exposure, leading to peroxinitrate radical formation, which itself is toxic for many proteins and enzymes.[9] This was not perhaps the case in this study, however, since nitric oxide did not cause any increase in the apoptosis induced by the other agents.

In conclusion, HO-1 does not seem to confer etoposide resistance in AML cells and, its induction does not protect AML cells from etoposide-induced apoptosis.

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Acknowledgments: we thank Mrs. Raija Sirviö, Satu Koljonen and Terttu Niemelä for technical assistance.

Funding: this study was supported by the University Hospital of Oulu.

Key words: AML, etoposide, BSO, NO, apoptosis, HO-1.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received May 31, 2002; accepted November 12, 2002.

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Expression of vascular endothelial growth factor and angiopoietin-2 in myeloma cells

Angiogenesis is important for myeloma. To understand the mechanism of angiogenesis, we analyzed vascular endothelial growth factor and angiopoietins, new angiogenic molecules, in purified myeloma cells at mRNA and protein levels. Co-expression of these factors was observed. We suggest that these molecules may be significant as prognostic factors.

Haematologica 2003;88:113-115
(*http://www.haematologica.org/2003_01/88113.htm*)

Angiogenesis is important for progression of hematologic malignancies including multiple myeloma (MM).[1,2] The most potent angiogenic factor is vascular endothelial growth factor (VEGF)$_{165}$.[3] It has been reported that the level of VEGF in the bone marrow plasma correlates with the activity of disease. Serum levels of VEGF were higher in a group of patients who showed poor response to chemotherapy.[4] However, it has not been clearly reported that VEGF is produced by myeloma cells.

Recently, it was reported that angiopoietins are essential for angiogenesis. Angiopoietin-1 induces phosphorylation of Tie-2, the common receptor of angiopoietin-1 and 2, and contributes to the recruitment and maintenance of an association between peri-endothelial supporting cells and endothelial cells, resulting in stabilization of vessel structures.[5] In contrast, angiopoietin-2 reduces interactions between supporting cells and endothelial cells, leading to production of new vessel sprouting.[6] It has also been reported that co-expression of angiopoietin-2 and VEGF co-ordinately promotes new vessel sprouting.[7] A correlation between the expression of angiopoietin-2 and angiogenesis has already been reported in certain types of cancer tissues[8] while the production of angiopoietins by myeloma cells is not known.

We determined expression of VEGF and angiopoietins in 39 samples from 34 patients (31 patients with MM, 2 with monoclonal gammopathies of undetermined significance (MGUS) and one with reactive bone marrow plasmacytosis). Plasma cells were purified by negative selection with anti-CD2, CD3, CD4, CD14, CD19, CD66b and glycophorin A-conjugated immunomagnetic beads. The primers utilized for the reverse transcriptase polymerase chain reaction (RT-PCR) are shown in Table 1. Immunohistochemical staining was also performed using LSAB Kit (Dako, Carpinteria, CA, USA) and human polyclonal antibodies against VEGF$_{165}$ (Neomarkers, Fremont, CA, USA) and angiopoietin-2 (KM202).[7]

Expression of VEGF$_{121}$ and VEGF$_{165}$ was detected in, respectively, 30 and 22 samples out of 36 bone marrow samples from patients with MM (Figure 1-A). Twenty-seven of 36 MM samples showed expression of angiopoietin-2 (Figure 1-A). Co-expression of VEGF$_{165}$ and angiopoietin-2 was detected in 18 MM samples, indicating that this is not a rare phenomenon in MM. Since VEGF and angiopoietin-2 were reported to work co-ordinately in angiogenesis, it is possible that these molecules induce angiogenesis in bone marrow of myeloma patients.[7] The two cases of MGUS and a case of reactive plasmacytosis did not show expression of VEGF$_{121}$ or VEGF$_{165}$, while low levels of angiopoietin-2 were detected (Figure 1-B). Absence of VEGF may be a characteristic of plasma cells in MGUS patients and plasmacytosis, indicating that VEGFs are important factors for malignant plasma cells. It could be speculated that plasma cells from MGUS or reactive plasmacytosis do not require as much neovascularization as myeloma cells because of their low proliferation. Further analysis of more cases is needed to clarify this hypothesis. Expression of angiopoietin-1 was not detected at all. The presence of VEGF$_{165}$ and angiopoietin-2 at protein level was confirmed by immunohistochemical staining in representative samples (Figure 1-C).

Table 1. Primers used in RT-PCR.

Primers	*Sequence (5'-3')*	*Product size (base pair)*
human $VEGF_{121}$		
sense	GAAGTGGTGAAGTTCATGGATGTC	408 bp
anti-sense	CGATCGTTCTGTATCAGTCTTTCC	
human $VEGF_{165}$		
sense	GAAGTGGTGAAGTTCATGGATGTC	504 bp
anti-sense	CGATCGTTCTGTATCAGTCTTTCC	
human angiopoietin-1		
sense	GAAGCTGCCAGGTGAGAAAC	391 bp
anti-sense	TTCCAAAGGATTATGGCAGG	
human angiopoietin-2		
sense	GGATCTGGGGAGAGAGGAAC	469 bp
anti-sense	CATCTCTGGCAGGAGGAAAG	
human Tie-2		
sense	TACACCTGCCTCATGCTCAG	488 bp
anti-sense	GCAGAGACATCCTTGGAAGC	
β2-microglobulin		
sense	GAATTGCTATGTGTCTGGGT	408 bp
anti-sense	CATCTTCAAACCTCCATGATG	

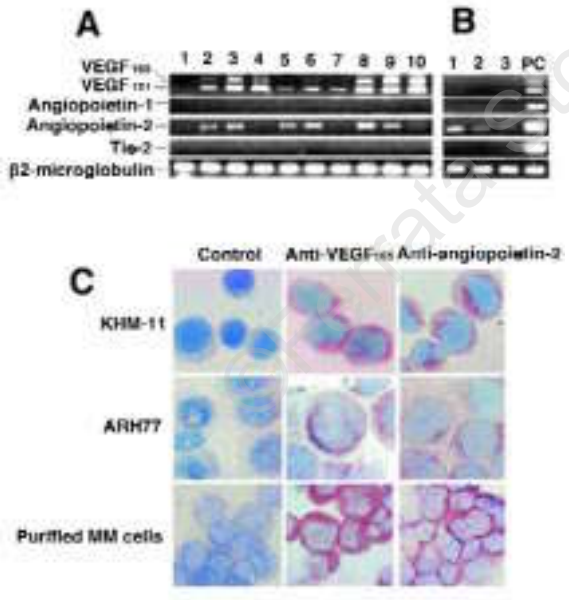

Figure 1. Expression of VEGFs and angiopoietins in MM cells. Panels A and B show representative results of RT-PCR for $VEGF_{121}$, $VEGF_{165}$, angiopoietin-1, angiopoietin-2 and Tie-2 utilizing mRNA from purified myeloma cells (A), plasma cells isolated from MGUS (panel B-lanes 1 and 2) and reactive plasmacytosis (B-lane 3). Panel C shows immunohistochemistry for angiopoietin-2 and $VEGF_{165}$. Note staining for angiopoietin-2 and $VEGF_{165}$ in the cytoplasm of myeloma cell lines (KHM-11 and ARH77) and purified fresh myeloma cells (magnification, × 400).

We examined the expressions of VEGF-receptors, Flt-1 and KDR/Flk-1, by flow cytometry. However, these receptors were not expressed in both cell lines and fresh myeloma cells (*data not shown*). Tie-2, a receptor for angiopoietin-1 and angiopoietin-2, was also not expressed (Figure 1), indicating that VEGFs and angiopoietin-2 are not autocrine factors for myeloma cells but rather directly stimulate surrounding vascular endothelial cells.

Expression of $VEGF_{121}$ was not a prognostic factor (data not shown). However, the survival rate at one year from diagnosis of cases expressing both $VEGF_{121}$ and $VEGF_{165}$ was 35.6 % (n=22) while that of cases expressing only $VEGF_{121}$ was 87.5 % (n=8), suggesting co-expression of $VEGF_{121}$ and $VEGF_{165}$ is a prognostic factor ($p<0.05$, Cox-Mantel test). Survival rate at one year from diagnosis of cases with expression of angiopoietin-2 was 41.2% while that of cases without expression was 77.8 % ($p<0.05$, Cox-Mantel test), suggesting that expression of angiopoietin-2 may contribute to poor prognosis although analysis of more cases in needed to draw a conclusion.

To our knowledge, this is the first report directly describing expression of $VEGF_{165}$ and angiopoietin-2 in purified human myeloma cells. The finding of co-expression of $VEGF_{165}$ and angiopoietin-2 in myeloma cells provides useful information for the development of new strategies targeting angiopoietin-2 as well as VEGF. Therefore, it is anticipated that inhibition of angiopoietin-2 should improve the outcome of anti-VEGF receptor therapy, this latter already being evaluated in clinical trials for multiple myeloma.[9]

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Key words: angiopoietin, VEGF, multiple myeloma, angiogenesis.

Acknowledgments: the authors thank Dr. M. Yoshida (Kumamoto Red Cross Hospital, Kumamoto, Japan) for providing clinical samples. We also thank Dr. M. Morioka (Department of Neurosurgery, Kumamoto University School of Medicine) and Dr. T. Ohtsuki (Kawasaki Medical School, Kurashiki, Japan) for providing anti-angiopoietin-2 antibody, and KMS-12-BM and KMS-12-PE, respectively.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 26, 2002; accepted November 16, 2002.

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The feasibility of reduced-intensity allogeneic hematopoietic stem cell transplantation from a related donor with HLA one-antigen with or without one-allele mismatch

It is still unclear whether reduced-intensity stem cell transplantation (RIST) from an HLA-mismatched related donor is feasible for hematologic malignancies. In the current study on the use of antithymocyte globulin (ATG) in 13 patients, we focused on this issue by evaluating regimen-related toxicities, engraftment, graft-versus-host disease (GVHD), infection, and overall survival. Our results suggest that this procedure may be acceptable for patients without a matched related donor.

Haematologica 2003;88:115-117
(*http://www.haematologica.org/2003_01/88115.htm*)

A total of 13 patients underwent RIST from a serologically HLA one-locus mismatched related donor between March 2000 and September 2002. The characteristics of these patients are shown in Tables 1 and 2. Both HLA antigen and allele matching were generally evaluated, since any disparity in HLA allele typing was considered to be a risk factor in allogeneic hematopoietic stem cell transplantation from an unrelated donor.[1-3] We defined *HLA one-locus mismatch* as any mismatch of one HLA -antigen, with or without a one-allele mismatch.

The conditioning regimen consisted of cladribine (0.66 mg/kg) or fludarabine (180 mg/m^2), busulfan (8 mg/kg), and rabbit anti-thymocyte globulin (ATG: 5 mg/kg in 2 patients, and 10 mg/kg in 16 patients). Infectious prophylaxis procedures have been described previously.[4] Prophylaxis against GVHD was performed with cyclosporine (CSP) alone in the initial 7 patients. Thereafter, short-term methotrexate (MTX) was added to CSP in the subsequent 6 patients because of the observation of severe acute GVHD in the earlier group. Patients who developed grade II-IV acute GVHD were treated with methylprednisolone at a dose of 1-2 mg/kg/day *iv*. Infectious disease was defined as an illness associated with symptoms and signs consistent with an infection, with microbiological documentation of a pathogen. Microbiological documentation consisted of the isolation of a pathogen by culture from a sterile or non-sterile site, or by histologic or immunohistologic evidence. The primary endpoint of this study was the evaluation of engraftment, defined as >0.5×10^9/L absolute neutrophil count (ANC) or >1.0×10^9/L white blood cell count (WBC), and the toxicities associated with the procedure. The secondary end-points included evaluation of the extent of GVHD and infectious episodes. Differences in incidence were evaluated using Fisher's exact test. Actuarial overall survival was estimated by the Kaplan-Meier method.

We found that all of the patients tolerated our RIST regimen and organ toxicities were limited to less than grade II hepatic and stomatitis/gastrointestinal toxicity, except in one patient (UPN 389) who developed a subdural hematoma. The median number of CD 34$^+$ cells infused was 3.6×10^6/kg (range, 2.2 to 7.3×10^6/kg, Table 1) and the median duration of neutropenia was 12 days (range, 7-20, Table 1). Chimerism analysis was performed on days 30, 60, 90, 120, 180, 240, 300 and 360, and we confirmed that 11 of the 13 patients achieved engraftment from this HLA-mismatched transplantation. This result further suggests that our regimen, incorporating ATG, enables successful engraftment by overcoming the HLA barrier that is limited to HLA one-antigen with or without one-allele, as recently reported by Gajewski *et al*.[5] One patient developed primary graft failure and the rapid emergence of recipient-type hematopoiesis on day 17, suggesting that our regimen is not truly myeloablative, and that the RIST procedure, relative to conventional transplantation with a myeloablative regimen, saves patients by retaining the ability of the marrow space to be repopulated by the recipient's own cells.

Table 1. Patients' characteristics.

UPN	*Sex*	*Age*	*Disease*	*Status at transplant*	*Regimen*	*HLA mismatched locus*	*RRT grade*	*CD34*	*Duration of neutr.*
267	M	29	AML	CR	2CdA/Bu/ATG	DRB1 (antigen)	0	2.23	15
295	M	20	RMS	NR	2CdA/Bu/ATG	DRB1 (antigen)	1(hepatic)	7.29	11
312	M	27	RMS	NR	2CdA/Bu/ATG	B (antigen) + A (allele)	1(hepatic)/2 (stomatitis)	2.8	7
326	F	19	MDS	CR	2CdA/Bu/ATG	B (antigen)	2 (stomatitis)	4.38	11
332	M	15	AML	CR	2CdA/Bu/ATG	B (antigen) + A (allele)	0	2.39	–
333	M	66	MDS	CR	2CdA/Bu/ATG	B (antigen)	0	5.09	–
449	M	54	MDS	NR	Flu/Bu/ATG	B (antigen)	1(hepatic)	2.58	12
384	M	53	MDS	CR	Flu/Bu/ATG	DRB1 (antigen)	0	2.43	11
389	M	58	ALL	CR	Flu/Bu/ATG	B (antigen) + A (allele)	2(CNS)	3.39	13
426	F	54	MDS	NR	Flu/Bu/ATG	B (antigen) + DRB1 (allele)	0	6.98	14
434	M	47	MM	NR	Flu/Bu/ATG	B (antigen) +A (allele) + DRB1 (allele)	1(hepatic)	4.03	11
446	F	57	AML	CR	Flu/Bu/ATG	B (antigen)	0	4.04	20
496	F	24	ARCC	NR	Flu/Bu/ATG	A (antigen)	2 (stomatitis)/1 (gastrointestinal)	3.57	13

UPN: unique patient number; RMS: rhabdomyo sarcoma; MDS: myelodysplastic syndrome; ARCC: adrenal cell carcinoma; CR: complete remission; NR: no remission; CD34: CD34 cell dose×10^6/kg; neutr: neutropenia.