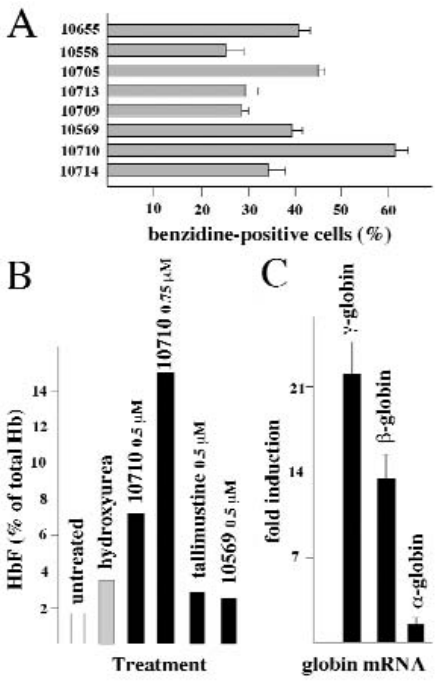

Figure 2. A. Effects of tallimustine analogs on differentiation of K562 cells. K562 cells were cultured in the presence of concentrations of tallimustine analogs that caused 50% inhibition of cell growth; after 6 days the proportion of benzidine-positive (Hb-containing) cells was determined as previously described (results are the means ± SD of three independent experiments).[2] B, C. Production of HbF (B) and globin mRNA (C) by normal human erythroid cultures. Cells were grown according to the two-phase liquid culture protocol[4,5] with no drug (untreated), with HU (150 μM) or with the indicated concentrations of 10710, 10655 and 10569. Hemoglobins were analyzed by HPLC and the % HbF determined (B). Accumulation of mRNA was measured by quantitative real-time RT-PCR assay (C).[6] The results (mean ± SD) of the fold increase of globin mRNAs induced by 0.75, μM 10710 (compared to untreated cells) in three experiments are summarized.

in normal erythroid progenitors. This agent, with its four pyrrole rings, was more active than tallimustine[2] or hydroxyurea, the drug currently used for HbF enhancement in β-thalassemia and sickle-cell disease.[3] In addition to increasing γ-globin mRNA synthesis, this agent also increased β-globin mRNA, but only slightly increased α-globin mRNA.

Since these DNA-binding molecules are cytotoxic,[2] chemical modifications will be required to reduce their toxicity. In this respect, our study should be considered as a basis for further efforts to develop agents for hematologic diseases in which the induction of both γ-globin and β-globin genes could be clinically relevant.

Cristiano Chiarabelli, Nicoletta Bianchi,* Monica Borgatti,* Eugenia Prus,° Eitan Fibach,° Roberto Gambari*#*

**Laboratory for the Development of Pharmacological and Pharmacogenomic Therapy of Thalassemia, Biotechnology Center, Ferrara University, Ferrara, Italy; °Department of Hematology, Hadassah University Hospital, Jerusalem, Israel; #Department of Biochemistry and Molecular Biology, Ferrara University, Ferrara, Italy*

Funding: this work was supported by CNR PF Biotecnologie and by Associazione Veneta per la Lotta alla Talassemia (Rovigo). We thank S.I.T. ULSS 18, Rovigo (Servizio di Immunoematologia e Trasfusione: Prof. Rocco Potenza, Dr. Francesco Chiavilli, Dr. Stefano Modonesi).

Acknowledgments: the experienced technical assistance of Mrs. Aliza Treves (Hadassah) is greatly appreciated.

Key words: tallimustine, erythroid differentiation, β-thalassemia, fetal hemoglobin.

Correspondence: Prof. Roberto Gambari, Department of Biochemistry and Molecular Biology, Ferrara University, Via L.Borsari 46, 44100 Ferrara, Italy. E-mail: gam@unife.it

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Bill Wood, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Wood and the Editors. Manuscript received January 27, 2003; accepted June 2, 2003.

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In vitro study of stromal cell defects in myelodysplastic syndromes

The long term bone marrow culture (LTBMC) system was used to examine *in vitro* characteristics of stromal cells in myelodysplastic syndromes (MDS): confluence after four-week culture, activation of caspase-3, production of tumor necrosis-α, interleukin (IL)-1β and vascular endothelial growth factor (VEGF), and density of endothelial (CD31) cells. Low confluence, associated in some cases with caspase-3 activation and increased angiogenesis, was the most frequent abnormality.

haematologica 2003; 88:827-829
(*http://www.haematologica.org/827.htm*)

The ineffective hematopoiesis that characterizes MDS has been attributed to an accelerated apoptosis in hematopoietic progenitors.[1] Altered interactions between stromal and hematopoietic cells have also been reported, but discrepancies exist between studies regarding the implication and functional

Table 1. Clinical data and culture results.

	Clinical data				Cultures results									
							Immunocytochemistry		VEGF production (pg/mL)				Cytokines (pg/mL)	
	Sex	Diagnosis	Age (years)	Karyotype	Stromal growth RPMI	Stromal growth MyeloCult	% of cleaved caspase-3 cells	% of CD31 cells	RPMI medium week 1	RPMI medium week 4	MyeloCult medium week 1	MyeloCult medium week 4	TNF-α	IL-1β
Normal marrows														
NM 1	F		38		3	3	ND	ND	20	490	20	300	145	180
NM 2	F		33		3	3	5%	5%	50	4835	70	2170	0	0
NM 3	F		27		3	3	0%	2%	15	170	140	1800	0	40
NM 4	F		42		3	3	0%	10%	200	220	75	2710	50	0
NM 5	M		36		3	3	15%	15%	20	2800	35	1800	190	185
range values					*3*	*3*	*0-15%*	*2-15%*	*15-200*	*170-4835*	*20-140*	*300-2710*	*0-190*	*0-185*
MDS patients														
MDS 1	F	RA	83	46XX, del(5)(q13q34)	3	3	10%	50%	515	9125	ND	ND	215	55
MDS 2	M	RA	80	46XY	2	ND	0%	5%	150	3780	ND	ND	0	11
MDS 3	M	RA	76	46XY, +8[4]/46	1	1	100%	20%	135	675	240	2670	115	720
MDS 4	M	RA	77	46XY	2	3	10%	ND	20	340	120	590	0	0
MDS 5	M	RA	76	46XY,+8[2]/46	3	3	0%	15%	305	7580	2600	6380	260	450
MDS 6	F	RA	55	46XX, t(2,5)(p25,q13)[2]/46, XX [28]	2	ND	15%	20%	20	2230	ND	ND	25	95
MDS 7	M	RA	90	46XY	1	3	ND	50%	65	65	165	1350	0	0
MDS 8	F	RA	87	46XX	3	3	0%	10%	195	6555	ND	ND	0	33
MDS 9	M	RA	81	45X,-Y[9]/46,XY[16]	3	3	ND	ND	80	515	550	3255	0	0
MDS 10	M	RA	89	46XY	0	2	0%	1%	300	480	1240	2500	480	590
MDS 11	M	RA	66	46XY	0	2	ND	ND	780	1320	2250	1400	260	800
MDS 12	M	RAS	72	46XY	3	3	ND	ND	1175	7010	2940	10600	ND	ND
MDS 13	F	RAS	75	46XX	1	ND	0%	15%	20	180	ND	ND	0	0
MDS 14	M	RAEB	73	46XY	3	ND	0%	5%	390	4925	ND	ND	250	320
MDS 15	F	RAEB	73	46XX,del(9)(q22q32),del(13)(q13q21)[5][cp7]/46,XX[10]	3	ND	ND	30%	800	2800	ND	ND	0	3
MDS 16	M	RAEB	76	46X, del(20)(q11)[9]/46 XY[16]	3	3	1%	2%	765	2095	2290	1420	ND	ND
MDS 17	M	RAEB-t	71	46XY	2	ND	0%	25%	855	4805	1070	2305	0	14
MDS 18	M	CMML	91	46XY	3	ND	0%	ND	235	3565	485	2590	45	128
MDS 19	M	CMML	83	46XY	3	3	0%	0%	690	5040	1100	3875	75	48
MDS 20	F	CMML	67	46XX	1	1	30%	30%	30	2000	20	580	0	15
range values							*0-100%*	*0-50%*	*20-1175*	*65-9125*	*20-2940*	*580-10600*	*0-480*	*0-800*

Stromal growth at week 4 was assessed as described. Cleaved caspase-3 and endothelial CD31 expression were evaluated by an immunocytochemical technique on cytospins obtained after trypsinization of adherent RPMI layers. Expression was noted as percentages of positive cells which exhibited strong immunostaining. VEGF levels (pg/mL) were established on RPMI or MyeloCult culture supernatants, after the first (week 1) and the fourth week (week 4) of culture, in normal marrows (NM) or MDS samples. TNF-α and IL-1β productions (pg/mL) were measured at week 1 of RPMI or Myelocult cultures. ND = not done.

status of MDS stromal cells.[2-5]

To investigate the alterations of adherent layers from MDS LTBMC, we used two *in vitro* culture systems of bone marrow mononuclear cells (BMMNC) prepared as described.[6] Five normal and twenty MDS marrow samples were obtained with informed consent. According to FAB recommendations,[7] the diagnoses were: refractory anemia (RA) in eleven cases, RA with sideroblasts (RAS) in two cases, RA with excess of blasts (RAEB) in three cases; RAEB in transformation (RAEB-t) in one case and chronic myelomonocytic leukemia (CMML) in three cases.

A first culture medium consisted of RPMI (Eurobio, Les Ulis, France) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (100 U /mL), allowed the development of a fibroblastic stromal layer without hematopoietic cells.[8] In a second culture condition, allowing the development of a stromal layer with hematopoietic progenitors,[9] cells were grown in MyeloCult HT5100 medium (StemCell Technologies, Meylan, France). Cell confluence was assessed weekly and scored from 0 to 3 corresponding respectively to a stromal layer covering from < 25%, 25-50%, 50-75% and >75% of the area of the culture dish.

We used immunocytochemistry, as previously described,[10] to analyze the expression of cleaved caspase-3 (rabbit antibody, Cell Signaling, Beverly, MA, USA), and CD31 endothelial marker (JC70A, Dako, Glostrup, Denmark) on adherent RPMI stromal cells, at week 4. Vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF-α), and interleukin-1β (IL)-1β levels were assessed in both types of cultures by ELISA. All kits were purchased from R&D Systems and procedures followed the manufacturer's instructions (Systems Europe, Oxon, United Kingdom).

After four weeks, confluence of adherent layers was observed in all normal cultures, in both systems. In MDS a score of 3 was reached in 10/20 RPMI and 9/13 MyeloCult cultures. Two layers exhibited lysed figures (grade 0) in RPMI. We did not observe significant differences between patients with RA/RAS and RAEB-t/CMML. However, in RA/RAS samples, there was a trend towards better stromal growth in MyeloCult than in RPMI medium ($p = 0.09$, *Fischer's method*).

In MDS the mean percentage of cells with cleaved caspase-3 expression was 11±26% (5±7% for normal stromal cells). Nine of 15 studied cases were negative, 4/15 had between 1 and 15% positive cells, and 2 cases showed higher staining (respectively 30% and a majority of positive cells). These last cases also exhibited a low (score 1) confluence in

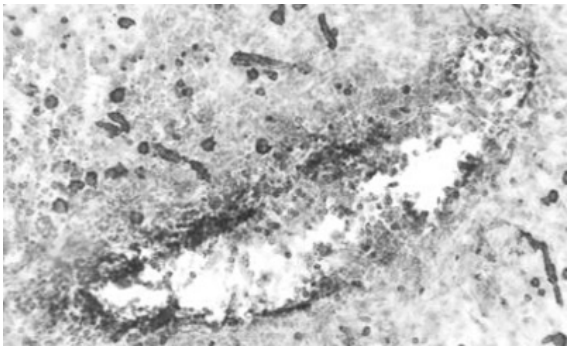

Figure 1. Immunocytochemistry with a CD31 monoclonal antibody. The image shows MDS adherent stromal layer, obtained in MyeloCult culture, which exhibits neoformation of a blood vessel.

both RPMI and MyeloCult cultures.

CD31 expression was not significantly different between normal and MDS stromal cells (respectively, 8 ± 6% and 18 ± 16% positive cells), but in MDS (n=15) this expression was more heterogeneous : 5 cases with weak expression (<10% of CD31-positive cells), 5 cases with intermediate expression (10-20% of positive cells), and 5 cases with a CD31 expression higher than 20% including two adherent layers with 50% of CD31-positive cells (two RA subtypes). Interestingly, for these two cases we observed *in vitro* formation of blood vessels in MyeloCult cultures (Figure 1).

We also observed a weak correlation between RPMI and MyeloCult VEGF concentrations at weeks 1 (p<0.001, r=0.84, Spearman test), and 4 (p<0.05, r=0.61). MDS samples tended to have higher VEGF production in both culture systems as compared to normal marrows, particularly at week 1: respective means were 376 ± 351 pg/mL versus 61±79 in RPMI medium (p=0.02, Mann-Whitney test), and 1159±1032 pg/mL versus 68±46 in MyeloCult medium (p=0.009). At week 4, concentrations were respectively 3254±2761 pg/mL versus 1703±2064 (p=0.22), and 3040±2745 pg/mL versus 1756±895 (p=0.43). We did not observe any correlation between VEGF levels and CD31 expression, particularly for the five cases exhibiting more than 20% $CD31^+$ cells.

Finally, in five samples (4 RA and 1 RAEB) we observed a high production (>200 pg/mL) of IL-1β as compared to production in normal supernatants, and a correlation between TNF-α or IL-1β production (p<0.0001, r=0.89), but no correlation between higher cytokine concentrations and a weak confluence or cleaved caspase-3 expression.

In conclusion, our *in vitro* study is the first to underline some abnormalities associated with myelodysplastic stromal layers: stromal growth was abnormal in 30-50% of cases regardless of the culture system used, and was partially associated with activation of caspase-3. In line with *in vivo* observations on biopsy sections reporting increased angiogenesis, we observed neovessel formation in two cases. However, this was not correlated with *in vitro* VEGF output.

Delphine Boudard, Annie Viallet,° Simone Piselli,* Denis Guyotat,*° Lydia Campos*°*

**EA3063 "Mort Cellulaire et Néoplasies", Faculté de Médecine, Saint-Etienne, France; °Département d'Hématologie, CHU de Saint-Etienne, France*

Key words: myelodysplastic syndromes, stromal cells, caspase-3, VEGF

Funding: supported by grants from Comité Départemental de la Lutte contre le Cancer de la Loire.

Acknowledgments: we thank Daniel Licari for his helpful comments regarding cell cultures.

Correspondence: Dr. Delphine Boudard, MD, Faculté de Médecine J.Lisfranc, laboratoire «Mort Cellulaire et Néoplasies», 15, rue Ambroise Paré, 42023 St-Etienne Cedex 02, France. Phone: international +33.4.77421425. Fax: international +33.4.77421489. E-mail: delphine.boudard@univ-st-etienne.fr

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor David T. Bowen, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Bowen and the Editors. Manuscript received April 22, 2003; accepted June 10, 2003.

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