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The clinical and biological effects of thalidomide in patients with myelodysplastic syndromes

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Summary. Thirty patients with myelodysplastic syndromes (MDS) were treated with thalidomide at 100 mg/d p.o., increased as tolerated to 400 mg/d for 12 weeks. Levels of apoptosis, macrophage number, microvessel density (MVD), tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were determined in the serum, bone marrow (BM) plasma and BM biopsies before and after therapy. Pretherapy biological characteristics of MDS patients were compared with similar studies performed in 11 normal volunteers. Ten patients demonstrated haematological improvement in the erythroid series, six becoming transfusion independent. Responders had a higher pretherapy platelet count ($P < 0{\cdot}048$) and lower BM blasts ($P < 0{\cdot}013$). Median time to response was 10 weeks, and four remain in remission beyond a year. Pretherapy MDS BMs showed higher MVD ($P < 0{\cdot}001$) and TGF-β ($P < 0{\cdot}03$) and higher serum TNF-α ($P < 0{\cdot}008$) compared with normal control subjects. After therapy, only BM TGF-β decreased significantly ($P < 0{\cdot}002$). Pretherapy haemoglobin was directly related to serum VEGF ($P < 0{\cdot}001$) in responders and inversely related in non-responders ($P < 0{\cdot}05$), suggesting the possibility that angiogenesis may be a primary pathology in the former and a consequence of anaemia-induced hypoxia in the latter. We conclude that thalidomide has important clinical and biological effects in at least a subset of MDS patients, but the precise mechanism of its action remains unknown and requires further study including a larger number of patients.

Keywords: thalidomide, myelodysplastic syndromes, vascular endothelial growth factor, transforming growth factor beta.

A group of four prognostically and biologically variegated haematopoietic disorders linked by the common presentation of cytopenia and monoclonal, dysplastic, hypercellular bone marrow (BM) has been assembled under the heading of myelodysplastic syndromes (MDS). The clinical and morphological manifestations, response to therapy as well as the clinical course of refractory anaemia (RA), RA with ringed sideroblasts (RARS) or with excess of blasts (RAEB) and chronic myelomonocytic leukaemia (CMMoL) tend to be quite distinct from each other. The French–American–British (FAB) morphological classification (Bennett *et al*, 1982) alone failed to provide accurate information regarding survival and risk of transformation for individual MDS patients. Recognition of rather marked differences in survival within morphologic subtypes of MDS led to the development of the International Prognostic Scoring System (IPSS) based on the severity of disease, as judged by the number of cytopenias, cytogenetic abnormalities and percentage of BM blasts (Greenberg *et al*, 1997). Further precision in prognostication is likely to be achieved by including some of the signature biological characteristics of individual patients into the system of classification. It is therefore critical to develop an understanding of the subtle biological differences between MDS patients and to determine how these differences translate with reference to treatment outcome and natural history of the disease.

The only curative treatment for MDS is bone marrow transplant (Deeg *et al*, 2000), generally restricted to patients younger than 55 years of age, whereas most MDS patients are elderly. In the past, many therapeutic approaches have been attempted in MDS, including the administration of recombinant haematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage CSF (GM-CSF) and erythropoietin alone or in combination (Ganser & Hoelzer, 1992; Negrin *et al*, 1993;

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Zeigler *et al*, 1993), chemotherapeutic agents (DeWitt *et al*, 1995; Economopoulos *et al*, 1996; Beran & Kantarjian, 1998) and differentiating agents such as retinoids and cholecalciferols (Koeffler *et al*, 1988; Morosetti & Koeffler, 1996). None have proved to be completely satisfactory, so that supportive care continues to be recommended for most patients who are not participating in experimental trials. The search for better therapies in MDS has acquired a greater urgency as the incidence of the disease appears to be on the rise.

One possible explanation for the ineffective haematopoiesis may be attributed, in part, to cytokine-mediated excessive intramedullary apoptosis of haematopoietic cells (Raza *et al*, 1995; Yoshida & Mufti, 1999). Tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and transforming growth factor beta (TGF-β) appear to be important proapoptotic cytokines (Zoumbos *et al*, 1991; Verhoef *et al*, 1992; Mundle *et al*, 1996; Raza *et al*, 1996; Shetty *et al*, 1996; Kitagawa *et al*, 1997; Gersuk *et al*, 1998; Allampallam *et al*, 1999). A novel anticytokine therapeutic approach has been tested based on the premise that neutralization of the proapoptotic cytokines in MDS patients should lead to the suppression of excessive intramedullary apoptosis and an improvement in the peripheral cytopenias. Some of the earliest trials in this area were conducted with pentoxifylline, ciprofloxacin and dexamethasone (PCD) and met with encouraging results (Raza *et al*, 1998, 2000). The cytoprotective agent amifostine was used alone (List *et al*, 1997) and in combination with PCD (Raza *et al*, 2000), and also provided good palliation to at least a subset of MDS patients. Direct attenuation of TNF-α was attempted with the soluble TNF receptor Enbrel (Immunex), and improvements were noted in both platelet counts and absolute neutrophil counts (Raza, 2000). None of these approaches has been entirely satisfactory, and the search for more effective therapies continues.

Another potentially useful therapeutic strategy would be to target neo-angiogenesis, a phenomenon that appears to be universally present in a variety of human cancers. Angiogenesis is the formation of new vessels from the existing vascular bed and is modulated by several cytokines and growth factors, including vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), TNF-α and TGF-β, the mechanism being only partly known (Fox *et al*, 1996). Increased angiogenesis is common in many pathological conditions, in both neoplastic and non-neoplastic diseases (Battegay, 1995; Folkman, 1995). In solid tumours, there is an inverse relationship between tumour vascularity and prognosis (Chaudhry *et al*, 1999; Giatromanolaki *et al*, 1999; Strohmeyer *et al*, 2000). Recent data indicate the importance of angiogenesis in haematological diseases such as leukaemia (Perez-Atayde *et al*, 1997), lymphoproliferative diseases (Vacca *et al*, 1995; Ribatti *et al*, 1996) and multiple myeloma (Vacca *et al*, 1994, 1999; Rajkumar *et al*, 1999; Ribatti *et al*, 1999). Increased angiogenesis in myelodysplastic bone marrow biopsies has also been demonstrated compared with normal marrows, and a correlation between angiogenesis and progression to leukaemia has been suggested (Pruneri *et al*, 1999). More recently, high levels of VEGF have been found in abnormally localized precursor cells in BM biopsies of CMMoL patients associated with an adverse prognosis (Bellamy *et al*, 2001). On this basis, it can be postulated that anti-angiogenic therapy could play a role in delaying or even preventing disease progression.

One potentially useful drug in this context is thalidomide because of its anti-TNF, anti-angiogenic and immune-modulating activities (Keenan *et al*, 1991; Moreira *et al*, 1993; D'Amato *et al*, 1994; Turk *et al*, 1996; Kenyon *et al*, 1997; Singhal *et al*, 1999). All three effects are clearly desirable in MDS patients. Thalidomide was introduced into the clinic in Europe in the 1950s as a sedative. Because of its antiemetic properties, however, some pregnant women began to take thalidomide for the treatment of morning sickness. The drug was eventually withdrawn from the market in the 1960s when its teratogenic effects were discovered. The recent return of thalidomide results from its broad spectrum of pharmacological and immunological effects (Hales, 1999). We have been using thalidomide to treat patients with MDS since 1998. The preliminary clinical results have shown improvement in haematological parameters in at least a subset of these patients, mainly resulting from an erythroid response with a decrease in red cell transfusions and an increase in haemoglobin levels (Raza *et al*, 1999). The mechanism of action of thalidomide is not completely understood.

The purpose of the present study was to investigate the mechanism of action of thalidomide by examining its effects on a number of biological parameters in MDS patients. The analysis follows 12 weeks of treatment and involves an investigation of the degree of apoptosis, number of macrophages, angiogenesis (microvessel density, MVD) and levels of a variety of both angiogenic and proapoptotic cytokines including VEGF, bFGF, interleukin 6 (IL-6), TNF-α and TGF-β in the serum, BM plasma and BM biopsies of MDS patients. The results indicate that thalidomide may be a useful palliative agent for a subset of low-risk MDS patients, and that it has significant effects on a variety of biological parameters in both serum and bone marrows of these individuals.

MATERIALS AND METHODS

The study was carried out on 30 patients with a confirmed diagnosis of MDS. Morphological classification was performed according to the FAB proposal on all the patients. Bone marrows and peripheral blood samples from 11 normal, healthy donors were studied simultaneously and served as a control group for the pretherapy biological characteristics of MDS patients. The normal donors signed an informed consent approved by the Institutional Review Board (IRB) of the Rush Presbyterian St Luke's Medical Center before donating their blood and marrows for studies.

Clinical studies. After signing an informed consent form approved by the IRB of Rush Presbyterian St Luke's Medical Center, all patients participated in the study MDS 98–21 entitled 'A pilot study of thalidomide in patients with myelodysplastic syndromes'. The clinical results of the study in its entirety (83 patients) are reported in a separate

paper (Raza *et al*, 2001). This paper presents the clinical and biological studies on a subset of the study patients. Every patient had a pretherapy bone marrow aspirate and biopsy examination performed at the Rush Cancer Institute (RCI). All samples were reviewed at the central facility by a haematopathologist at RCI/Rush Presbyterian St. Luke's Medical Center to confirm the diagnosis of MDS. Each patient started by taking 100 mg of thalidomide p.o. hs and increased the dose as tolerated to 400 mg p.o. at bed time (hs) over the next several weeks. Celgene Corporation (NJ, USA) provided the drug free of charge for the patients. In the present study, no premenopausal woman of child-bearing age was included. Newly diagnosed as well as previously diagnosed patients were eligible, as were both primary *de novo* and secondary MDS cases. Patients belonging to all subtypes of MDS, as per the FAB classification, and to all risk categories according to the IPSS were eligible. Patients were required not to have received any therapy for MDS for at least 4 weeks before starting thalidomide except for supportive care with transfusions. No other treatment for the primary disease, such as growth factors, could be administered to study patients while they were on thalidomide. Pyridoxine at 100 mg p.o. qds was prescribed for every patient as prophylaxis against peripheral neuropathy. Weekly complete blood counts (CBCs) with differentials were obtained and, upon completion of 12 weeks of therapy, the patients returned to RCI for a response evaluation, at which time all the pretherapy studies were repeated. In case of any evidence of a partial or complete response, or stable disease judged by the principal investigator, thalidomide was continued at the maximum tolerated dose for up to 1 year. Therapy was stopped in non-responding patients at this time, and they were taken off study.

The clinical end-point of the study was to determine the efficacy of thalidomide in those patients who were able to complete at least 12 weeks of therapy at the maximally tolerated dose.

Response criteria. Response criteria outlined in the report of an International Working Group (IWG) to standardize response criteria for MDS (Cheson *et al*, 2000) were applied by an independent team (Global Therapeutic Development) to assess responses. Minor modifications had to be made to these criteria, as it was a retrospective analysis. The modified criteria used are outlined below in italics.

Complete remission (CR). Bone marrow evaluation: repeat BM showing $< 5\%$ myeloblasts with normal maturation of all cell lines, with no evidence for dysplasia. When erythroid precursors constituted $< 50\%$ of BM nucleated cells, the percentage blasts were based on all nucleated cells; when there were 50% or more erythroid cells, the percentage blasts were based on the non-erythroid cells.

Peripheral blood evaluation (absolute values must last at least 2 months):

- Haemoglobin: > 11 g/dl (untransfused patient not on erythropoietin);
- Neutrophils: $0{\cdot}1 \times 10^9$/l or more (not on a myeloid growth factor);
- Platelets: 100×10^9/l or more (not on a thrombopoietic agent);
- Blasts: 0%;
- No dysplasia.

Partial remission or PR (absolute values must last at least 2 months): all the CR criteria (if abnormal before treatment), except bone marrow evaluation; blasts decreased by 50% or more over pretreatment, or a less advanced MDS FAB classification than pretreatment. Cellularity or morphology was not relevant.

Stable disease: failure to achieve at least a PR, but with no evidence of progression for at least 2 months.

Failure: death during treatment or disease progression characterized by worsening of cytopenias, increase in the percentage of BM blasts or progression to an MDS FAB subtype more advanced than pretreatment.

Disease transformation: transformation to acute myeloid leukaemia (AML), 30% or more blasts.

Cytogenetic response. requires 20 analysable metaphases using conventional cytogenetic techniques. Major: no detectable cytogenetic abnormality if pre-existing abnormality was present. Minor: 50% or more reduction in abnormal metaphases.

Pretherapy assessments: baseline CBC with which improvements were compared was standardized using a mean value of the 4 weeks before the start of therapy for all patients.

During therapy: responses were assessed at 12, 16 and 20 weeks of therapy. Absolute values closest to the 12 weeks and 16 weeks were used, and responses had to be sustained for at least the subsequent 8 weeks. With regard to packed red blood cell transfusions (PRBC) and transfusion independence, the same 4-week time period was used before treatment to determine transfusion dependence and to obtain a baseline monthly requirement. Subsequent transfusions were reviewed at the 12-, 16-, 20-, 24- and 28-week time points. If a patient received transfusion from d 0 to week 12, they were not considered transfusion independent; however, if the patient did not receive any transfusion at week 16 and sustained that independence for another 8 weeks, they were then considered to be transfusion independent. Patients were called late responders if they showed haematological improvement (HI) after 20 weeks of therapy.

Haematological improvement: all improvements must last at least 8 weeks. For a designated response (CR, PR HI), all relevant response criteria must be noted on at least two successive determinations at least 1 week apart after an appropriate period following therapy.

Erythroid response (HI-E). Major response: for patients with pretreatment Hb < 11 g/dl, > 2 g/dl increase in Hb; for transfusion-dependent patients, transfusion independence. Minor response: for patients with pretreatment Hb < 11 g/dl, 1–2 g/dl increase in Hb; for transfusion-dependent patients, 50% decrease in PRBC requirements.

Platelet response (HI-P). Major response: for patients with a pretreatment platelet count $< 100 \times 10^9$/l, an absolute increase of 30×10^9/l or more; for platelet transfusion-dependent patients, stabilization of platelet counts and platelet transfusion independence. Minor response: for patients with a pretreatment platelet count $< 100 \times 10^9$/l,

Table I. Detailed characteristics of 10 responding patients.

Patient no.	FAB		BM cellularity		PRBC dependent		Responses				Response type	Response duration in days	Comment
	Before	After	Before	After	Before	After	Reduction in PRBC (%)	Increase in Hb	Platelets	ANC			
937	RA	RA	20	30	Y	N	100	Yes	No	No	HI-E major, 100% PRBC tx reduction at week 16,minor Hb response at week 16	200	Died of lymphoma
1011	RA	RARS	99	70	Y	N	100	Yes	No	No	HI-E major (PRBC), Tx independent at week 16,major (Hb)	422*	Continues in remission
277	RA	RA	90	N/A	Y	N	100	No	No	No	HI-E major, 100% PRBC tx reduction	454*	Continues in remission
963	RA	RARS	80	90	Y	N	100	No	No	No	HI-E major	227	Stopped responding
400	RARS	RARS	70	70	Y	Y	50	No	No	No	HI-E minor, 50% PRBC tx reduction	306	Stopped after 1 year due toside-effects
460	RARS	RARS	50	50	N	N		Yes	No	No	HI-E major (Hb)	620*	Continues in remission
1016	RA	RA	30	30	Y	N	100	Yes	No	No	HI-E major	527*	Continues in remission
340	RARS	RA	80	90	Y	Y	50	No	No	No	HI-E minor, at 16 week and post 50% reduction in PRBC tx	168	Stopped responding
333	RARS	N/A	50	N/A	Y	Y	50	No	No	No	50% PRBC reductionfrom week 20 to weeks 24 and 28	239	Stopped due to side-effects
123	RA	RA	80	90	Y	N	100	Yes	Yes	Yes	Late responder, trilineage	210	Late response after thalidomide stopped

RA, refractory anaemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; HI-E, haematological improvement, erythroid series; HI-P, haematological improvement, platelets; Tx, transfusion; PRBC, packed red blood cells.

Patient no., unique Rush Cancer Institute (RCI) number.

*Continuing response.

a 50% or more increase in platelet count with a net increase $> 10 \times 10^9$/l but $< 30 \times 10^9$/l.

Absolute neutrophil response (HI-ANC). Major response: for ANC $< 1{\cdot}5 \times 10^9$/l before therapy, at least a 100% increase or an absolute increase of $0{\cdot}5 \times 10^9$/l, whichever is greater. Minor response: for ANC $< 1{\cdot}5 \times 10^9$/l before therapy, ANC increase of at least 100% but absolute increase $< 0{\cdot}5 \times 10^9$/l.

Biological studies. Peripheral blood (PB) and bone marrow (BM) biopsy samples were obtained from 11 normal healthy volunteers and 30 MDS patients before and after 12 weeks following therapy with thalidomide.

Measurement of bone marrow angiogenesis. The angiogenesis studies were carried out on paraffin-embedded BM biopsies in 30 patients with MDS and 11 control subjects. Two BM biopsy samples were obtained from MDS patients, one immediately before starting therapy and the other after 12 weeks of thalidomide treatment. All blood vessels were highlighted by staining endothelial cells with an anti-factor VIII murine monoclonal antibody using the standard peroxidase–anti-peroxidase technique. Briefly, after deparaffinization and hydration, the sections were incubated for 30 min in 3% H_2O_2 to inactivate endogenous peroxidase activity. For antigen retrieval, the slides were placed in a 0·1 mol/l citrate buffer at pH 6·0 and boiled twice for 5 min each. The primary antibody (M616; Dako, Glostrup, Denmark; diluted 1:30) was applied to tissue sections for 1 h, followed by biotinylated secondary antibody for 30 min. The biotinylated antibody was detected using an avidin–biotin–peroxidase conjugate and diaminobenzidine tetrachloride. Slides were counterstained with haematoxylin. MVD enumeration was performed according to the method of Perez-Atayde *et al* (1997). The number of vessels in 20 high-power fields (HPF) was counted using the entire BM core, each field representing an area of 0·72 mm^2, and the median was calculated. The area with the highest microvessel count was designated as a hot-spot.

Detection of the cytokines in the microenvironment. Levels of two cytokines, TNF-α and TGF-β, were determined immuno histochemically using a semi-quantitative technique in the BM biopsies as follows. The BM biopsy tissues were fixed in Bouin's solution and embedded in plastic using glycol methacrylate. The sections were then labelled individually for each cytokine using the respective antibodies: rabbit anti-human TNF-α polyclonal antibody (Genzyme item no. ip300; 1:40) and mouse anti-human TGF-β (1:70) by methods described previously (Goyal *et al*, 1999). All the slides were observed blindly on a televised screen by several investigators. A subjective quantitative scale was formulated to determine the degree of positivity of the various cytokines as follows: negative (0), low (1–3), intermediate (4–6) and high (7–8).

Detection of macrophages. Macrophages were detected immunohistochemically using a specific monoclonal antibody (CD68; Dakopatts, Denmark) by the method described previously (Goyal *et al*, 1999).

Measurement of apoptosis using in situ end labelling (ISEL). ISEL of fragmented DNA was carried out on plastic-embedded BM biopsies as described in earlier studies (Mundle & Raza, 1995; Raza *et al*, 1995). Briefly, the sections were first pretreated with sodium chloride–sodium citrate (SSC) solution at 80°C and with 1% Pronase [1 mg/ml in 0·15 mol/l phosphate-buffered saline (PBS); Calbiochem, La Jolla, CA, USA] followed by incubation with a mixture of dATP, dCTP, dGTP (0·01 mol/l; Promega, Madison, WI, USA), bio-dUTP (0·001 mol/l, Sigma) and DNA polymerase I (20 U/ml, Promega) at 19°C. Incorporation of bio-dUTP was finally visualized using an avidin–biotin–peroxidase conjugate (Vectastain Elite ABC kit; Vector, Burlingame, CA, USA) and diaminobenzidine tetrachloride. Dark brown nuclear staining indicated cells undergoing apoptotic death. The stained slides were examined by a group of observers on a television screen attached to the microscope. A subjective rating scale from 0 to 8+ was formulated to determine the extent of apoptosis as described before (Mundle & Raza, 1995).

Determination of cytokine levels in serum and bone marrow plasma. Peripheral blood was collected in sterile tubes before and after therapy, centrifuged at 2000 ***g*** for 10 min and stored at −40°C. Levels of serum VEGF, bFGF, IL-6, TNF-α and TGF-β were determined using a quantitative enzyme-linked immunosorbent assay (ELISA) technique (Quantikine; R and D Systems, Minneapolis, MN, USA). BM aspirate plasma was collected before and after treatment using heparin as an anticoagulant. The plasma was collected in the same way as described above and stored in aliquots at −40°C. Levels of plasma IL-6, bFGF, TNF-α and VEGF were determined using a quantitative ELISA technique as above.

RESULTS

Eleven normal age-matched volunteers and 30 patients with a confirmed diagnosis of MDS were treated with thalidomide

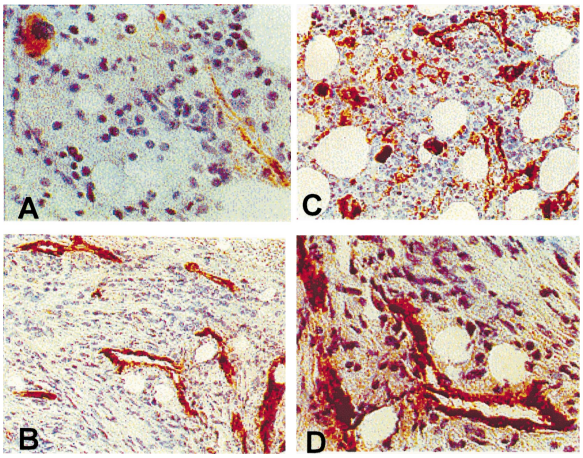

Fig 1. Angiogenesis as seen in a normal bone marrow biopsy after factor VIII staining at a magnification of 20× (A) and increase in microvessel density in MDS patients with factor VIII staining primarily seen in large vessels, sinusoid-like vessels and small endothelial sprouts at a magnification of 20× (B), 20× (C) and 100× (D).

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