

An increased rate of thrombosis has been reported in recipients of rHuEPO,<sup>6</sup> and this has been related to the ability of rHuEpo to trigger signaling pathways in endothelial cells, thus increasing their thrombogenicity.<sup>7</sup> Therefore, the combined use of thalidomide and rHuEpo may increase a patients' risk of thrombosis, as suggested by the results of a phase II trial in patients with myelodysplasia.<sup>8</sup> The study was prematurely discontinued, since three out of seven patients experienced deep vein thrombosis or fatal pulmonary embolism. On these premises, we retrospectively analyzed a large group of MM patients receiving thalidomide, to evaluate whether the concomitant administration of rHuEpo increased their risk of thrombosis.

One hundred and ninety-nine patients with MM were treated with thalidomide (Thalomid, Grunenthal, and Myrin, Lipomed) for at least one month at our three Institutions. There were 109 males and 90 females, aged 38-89 years (median, 66) at the beginning of thalidomide treatment. One hundred and forty-nine patients (75%) received thalidomide for relapsed/resistant MM, 36 as maintenance (18%), and the remaining 13 (7%) as first line therapy, alone or in combination with chemotherapy. The median duration of thalidomide treatment was 6.5 months (range, 1-45) and the median overall survival was 12 months. By the time of this analysis, 95 patients had died. Thrombotic events were objectively documented in all cases.

Eighteen patients experienced non-fatal thrombotic complications (Table 1). All thromboses but one occurred in refractory/resistant patients after a median time of 3.7 months from initiation of thalidomide treatment. As a consequence, six patients definitely stopped thalidomide, and three others resumed the drug after adequate treatment with warfarin (aimed at a target PT INR of 2.0-3.0 for three months). Overall, these findings are in line with those reported by other investigators.<sup>4</sup>

Analysis according to the concomitant treatments showed that neither rHuEpo, nor steroids and chemotherapy increased the prevalence, odds ratio, or annual incidence rate of thrombosis (Table 2). Our data are at variance with those recorded in myelodysplastic patients treated with a combination of thalidomide and rHuEpo.<sup>8</sup> The source and dosage of thalidomide cannot explain this difference. Steurer *et al.*<sup>8</sup> used dose-adjusted darbepoietin- $\alpha$ , once weekly, whereas our patients received various commercially available blends of rHuEpo, 10,000 units thrice weekly, or according to their degree of anemia. Although the risk of thrombosis of darbepoietin- $\alpha$  has been reported to be identical to that of rHuEpo in patients with lung cancer,<sup>9</sup> this may not necessarily be the case in other diseases. The correlation between hemoglobin level and risk of thrombosis is well acknowledged. One may, therefore, wonder whether the low prevalence of thrombosis observed in our MM patients receiving both thalidomide and rHuEpo depended on a poor response to rHuEpo. This does not seem likely, as three out of the four patients who developed thrombosis during rHuEpo therapy had hemoglobin levels > 11 g/dL at the time of the event.

Analysis according to the type of thrombosis showed that most events were venous, as expected. However, we also observed three arterial thromboses, none in patients taking rHuEpo. Cavenagh *et al.*<sup>10</sup> reported an excess of cerebral stroke in elderly MM patients treated with thalidomide. These observations underscore the possibility that thalidomide also increases the risk of arterial thrombosis. Seven other thrombotic episodes (six cases of superficial thrombophlebitis, and one transient cerebral ischemic attack) were not included in the present analysis, because they were diagnosed on clinical grounds only. Only two patients were receiving rHuEpo at

the time of these episodes.

In conclusion, our findings seem not to support the hypothesis that rHuEpo increases the thrombogenicity of thalidomide in patients with relapsed/refractory or *de novo* MM.

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## Platelets

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### Reduced plasma membrane Ca<sup>2+</sup>-ATPase function in platelets from patients with non-insulin-dependent diabetes mellitus

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We clearly show that plasma membrane Ca<sup>2+</sup> ATPase (PMCA) activity is lower in platelets from patients with non-insulin-dependent diabetes mellitus (NIDDM) than in those from healthy controls. The lower activity is likely due to reduced PMCA expression and increased tyrosine phosphorylation. These findings provide an explanation for the cellular ionic defects occurring in insulin resistant conditions.

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Non-insulin-dependent diabetes mellitus (NIDDM) is a chronic disease leading to several cardiovascular complications, in which platelet hyperactivity has been shown to be involved.<sup>1</sup> Several factors are associated with platelet dysfunction in NIDDM, including an altered  $\text{Ca}^{2+}$  mobilization.<sup>2</sup> Cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) controls a variety of platelet functions, including aggregation. Agonists increase  $[\text{Ca}^{2+}]_i$  by inducing  $\text{Ca}^{2+}$  release from intracellular stores or  $\text{Ca}^{2+}$  entry.  $\text{Ca}^{2+}$  removal from the cytosol and the maintenance of a low resting  $[\text{Ca}^{2+}]_i$  is mediated by  $\text{Ca}^{2+}$  sequestration into intracellular compartments by SERCA and  $\text{Ca}^{2+}$  extrusion across the plasma membrane.<sup>3</sup> Several studies in different cell types, including platelets, have reported altered  $\text{Ca}^{2+}$  homeostasis in NIDDM subjects,<sup>4,5</sup> however, the precise altered mechanisms are poorly identified and some of the results presented are conflicting. In the present study we investigated the activity of platelet PMCA in NIDDM patients.

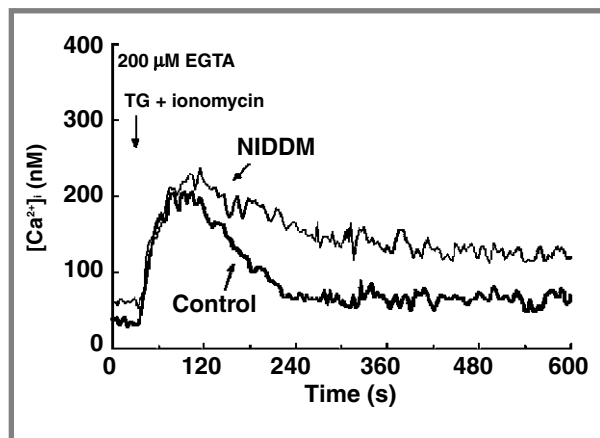
Blood was obtained, in accordance with the Declaration of Helsinki, from 12 healthy and 17 NIDDM subjects randomly obtained from normotensive patients of the Clinical Analysis Laboratory, Cáceres, Spain. Blood glucose concentrations in the NIDDM patients were in the range between 180 and 240 mg/dL. Glycosylated hemoglobin (Hb) levels (HbA1c) of the NIDDM patients, used as an index of metabolic control, were > 6%. Control subjects were normal, age- and gender-matched, healthy people with HbA1c levels of 3.5–5%.

Fura-2-loaded platelets were prepared as described previously<sup>3</sup> and fluorescence was recorded using a spectrofluorimeter with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in  $[\text{Ca}^{2+}]_i$  were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al.*<sup>6</sup> To compare the rate of decay of  $[\text{Ca}^{2+}]_i$ , indicative of PMCA activity,<sup>7</sup> to basal values in control and NIDDM subjects we used the constant of the exponential decay as previously described.<sup>7</sup>

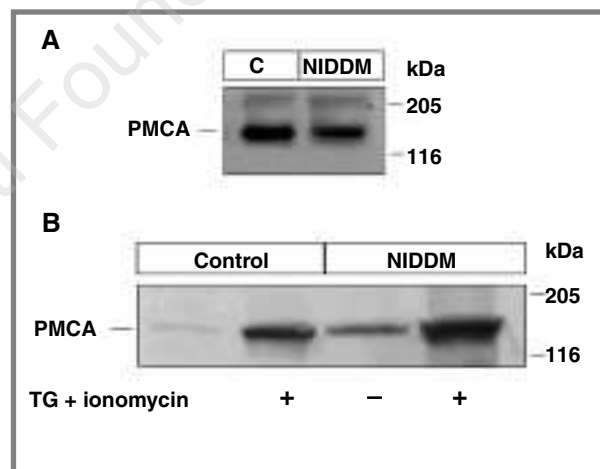
Immunoprecipitation was performed by incubating platelet lysates with 3  $\mu\text{g}$  of anti-PMCA antibody (Affinity Bioreagents, Golden, CO, USA). Immunoprecipitates (15  $\mu\text{g}$ /sample) were resolved by 10% SDS-PAGE.<sup>7</sup> Western blotting was performed using anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY, USA) or anti-PMCA antibodies, as previously described.<sup>7</sup> Statistical analysis was performed using the unpaired Student's t-test. A  $p < 0.05$  was considered to be statistically significant.

Resting platelet  $[\text{Ca}^{2+}]_i$  was significantly higher in NIDDM subjects than in controls (Figure 1;  $p < 0.05$ ;  $n = 10$ –12). When platelets were treated with 1  $\mu\text{M}$  TG, a specific inhibitor of SERCA,<sup>8</sup> plus ionomycin (50 nM), there was a transient increase in  $[\text{Ca}^{2+}]_i$  due to  $\text{Ca}^{2+}$  release followed by  $\text{Ca}^{2+}$  extrusion. Since the peak response was not significantly different in both groups of subjects ( $205.1 \pm 24.8$  nM and  $209.8 \pm 31.8$  nM in platelets from control and NIDDM subjects, respectively (means  $\pm$  S.E.M.)), the amount of  $\text{Ca}^{2+}$  accumulated in the stores must be similar (Figure 1). However, the rate of decay of  $[\text{Ca}^{2+}]_i$  to resting levels was significantly slower in platelets from NIDDM donors (the decay constants were  $0.0051 \pm 0.0006$  in NIDDM patients and  $0.0070 \pm 0.0005$  in controls;  $p < 0.05$ ). These findings suggest that the extrusion mechanisms are altered in platelets from NIDDM patients. We have previously demonstrated that the PMCA is the only system responsible for  $\text{Ca}^{2+}$  extrusion under our experimental conditions;<sup>7</sup> therefore the reduced  $\text{Ca}^{2+}$  efflux might be attributed to decreased PMCA activity.

The mechanism involved in the reduction in PMCA activity in diabetic subjects remains to be elucidated. We focused on two possible factors: PMCA expression and tyrosine phospho-



**Figure 1.**  $\text{Ca}^{2+}$  mobilization by TG + ionomycin in platelets from NIDDM patients and control subjects. Fura-2-loaded human platelets were stimulated with TG (1  $\mu\text{M}$ ) + ionomycin (50 nM) in a  $\text{Ca}^{2+}$  free medium (200  $\mu\text{M}$  EGTA was added). Elevations in  $[\text{Ca}^{2+}]_i$  were monitored using the 340/380 nm ratio and traces were calibrated in terms of  $[\text{Ca}^{2+}]_i$ . Traces shown are representative of ten to twelve separate experiments.



**Figure 2.** PMCA expression and tyrosine phosphorylation in platelets from NIDDM patients and control subjects. **A**, Platelet lysates from NIDDM and control subjects were subjected to SDS/10%-PAGE and Western blotting with the specific anti-PMCA antibody (5F10). The panel shows results from one experiment representative of four others. **B**, Platelets from control (lanes 1 and 2) and NIDDM patients (lanes 3 and 4) were treated in a  $\text{Ca}^{2+}$ -free medium (200  $\mu\text{M}$  EGTA was added) with 1  $\mu\text{M}$  TG + 50 nM ionomycin, as indicated, and aliquots were removed 10 min later and lysed. Whole cell lysates were immunoprecipitated with the anti-PMCA antibody (5F10). Immunoprecipitates were analyzed by SDS/10%-PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody. Molecular masses indicated on the right were determined using molecular-mass markers run in the same gel. The panel shows results from one experiment representative of two others.

rylation, which has been reported to inhibit PMCA activity.<sup>9</sup> PMCA expression was examined by Western blotting. A

PMCA-immunoreactive band of about 140 kDa was detected in platelet lysates; however, the amount of PMCA detected in platelets from NIDDM patients was found to be  $26.7 \pm 7.9\%$  lower than that in platelets from controls (Figure 2A;  $p < 0.05$ ;  $n = 5$ ).

We have previously shown that activation of platelets causes rapid tyrosine phosphorylation of PMCA.<sup>7</sup> Consistent with this, treatment with TG + ionomycin increased the phosphotyrosine content of PMCA by  $418.2 \pm 35.2\%$  of basal (Figure 2B). Here we show for the first time that PMCA tyrosine phosphorylation was significantly higher in platelets from NIDDM patients than in platelets from controls both in resting conditions ( $185.6 \pm 16.8\%$  of platelets from healthy donors) and after stimulation with TG + ionomycin ( $721.8 \pm 45.9\%$  of resting control platelets; Figure 2B,  $p < 0.05$ ;  $n = 3$ ). These findings suggest that platelet PMCA activity in NIDDM patients might be reduced by tyrosine phosphorylation.

We conclude that the lower expression and increased phosphotyrosine content of platelet PMCA in NIDDM subjects result in decreased  $Ca^{2+}$  extrusion, which might explain the altered ionic homeostasis described in insulin-resistant conditions.<sup>10</sup>

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## Stem Cell Transplantation

### Granulocyte-colony stimulating factor after autologous CD34<sup>+</sup> immunoselected peripheral blood stem cell transplantation

Granulocyte colony-stimulating factor (G-CSF) can be administered after a peripheral blood stem cell transplantation with the aim of accelerating neutrophil recovery. In a randomized, single-blind study we studied a new administration schedule of G-CSF in this context.

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High-dose chemotherapy with autologous peripheral blood stem cell transplantation has been used for the treatment of several malignancies because of the more rapid hematopoietic recovery than after autologous bone marrow transplantation (ABMT), and also because it requires less supportive care and a shorter period of hospitalization.<sup>1</sup> CD34<sup>+</sup> cell selection has been developed in order to reduce contaminating neoplastic cells in the graft; on the other hand this procedure eliminates hematopoietic precursors from leukapheresis products, consequently causing a slower hematopoietic recovery than that following unfractionated PBSCT. Since the incidence of infection is proportional to the duration of neutropenia,<sup>2</sup> several measures directed at shortening the duration of neutropenia have been evaluated. Myeloid growth factors (G-CSF and GM-CSF) were introduced into clinical practice to accelerate neutrophil recovery after PBSCT although there is no consensus about their indications and schedules of administration.<sup>3</sup> In order to reduce costs and drug exposure, several researchers<sup>4-6</sup> have evaluated different schedules of G-CSF administration after unmanipulated PBSCT; they compared delayed (day +3 to day +7) to early (day +1) administration; these studies reported contradictory results for the end-points considered, such as time to neutrophil recovery, antibiotic therapy, hospital stay, infections and G-CSF use.

In our experience, G-CSF, administered from day +1 after CD34<sup>+</sup> PBSC infusion, significantly improved granulocyte recovery, approaching the results observed after unmanipulated PBSCT, while its late administration (day +7) induced a significant delay in reticulocyte recovery, a decrease of the high fluorescent reticulocytes (HFR)% peak, a delay in platelet and hemoglobin recovery, and an increase of requirements of packed red blood cell units (pRBCu) and single donor platelet units (SDu).<sup>7</sup>

On the other hand, early daily G-CSF administration resulted in striking and, probably excessive G-CSF increase in serum levels (7-12 fold greater than those in untreated patients in the same setting).<sup>8</sup> Therefore we designed a clinical trial in order to verify the likelihood of a safe reduction of G-CSF combined with the advantages of early (day +1) administration. The trial design was a randomized single blind study evaluating early daily G-CSF administration vs every other day G-CSF, extending the interval between the doses to 48 hours. From April 1999 to September 2002 we enrolled 33 consecutive patients submitted to immunoselected CD34<sup>+</sup> PBSCT. The patients were allocated randomly into two groups in a 1:1 ratio. Group A patients were assigned to receive G-CSF (lenograstim, rHuG-CSF, Chugai-Rhone-Poulenc Rorer) 263 µg/day standard dose from day +1; group B received G-CSF 263 µg/day standard dose from day +1 on alternate days. Given the low number of patients submitted to CD34<sup>+</sup> immunoselected PBSCT, patients were not stratified (Table