

trophoretic mobility shift assay (EMSA), compared to that found in normal marrow (Figure 1). To identify the NF $\kappa$ B family members that bind to the DNA target site, supershift experiments were performed using antibodies specific for p50, p65, p52, c-Rel, and RelB. Figure 1 reveals the presence of p50-p50 homodimers and p50-p65 heterodimers. Although NF $\kappa$ B has been mostly associated with inhibition of apoptosis,<sup>5</sup> it also induces the expression of FasL and tumor necrosis factor (TNF)- $\alpha$ ,<sup>6,7</sup> hence giving NF $\kappa$ B a pro-apoptotic role. We, therefore, analyzed the mRNA levels of these apoptogenic cytokines, as well as the expression of other NF $\kappa$ B-regulated genes in MDS samples with elevated NF $\kappa$ B activity and compared them with MDS and normal marrow samples with low NF $\kappa$ B activity. Figure 2A shows a representative experiment, in which MDS samples 2 (low NF $\kappa$ B activity) and 11 (high NF $\kappa$ B activity), and a normal marrow (sample 5 of controls) were analyzed by RT-PCR. Interestingly, TNF $\alpha$  mRNA levels were clearly upregulated in the high NF $\kappa$ B activity sample, and the expression of FasL was gradually increased in accordance with the level of NF $\kappa$ B activity. Consistent with these data is the observation that TNF $\alpha$  and FasL mRNAs are upregulated in patients with MDS, and that *in vitro* blockade of these cytokines increases the number of hematopoietic colonies.<sup>8</sup> Other NF $\kappa$ B-regulated genes such as TRAIL, and anti-apoptotic members of the Bcl-2 (Bcl-2, Bcl-x<sub>L</sub>, A1) and inhibitor of apoptosis (c-IAP1, and XIAP) families did not modify their expression (Figure 2A). In order to establish a direct correlation between NF $\kappa$ B and upregulation of pro-apoptotic genes, bone marrow cells from MDS sample 11 were cultured in the presence of Bay 11-7082, an inhibitor of NF $\kappa$ B activation. As shown in Figure 2B, the NF $\kappa$ B/DNA binding activity was completely blocked when MDS cells were cultured in the presence of Bay 11-7082. Consistently, after exposure to the inhibitor, the mRNA expression of FasL and TNF $\alpha$  was lost (Figure 2C).

In conclusion, our study reveals the alteration of a transcriptional pathway that may contribute to intramedullary apoptosis in MDS, and opens the possibility of developing therapeutic strategies aimed at avoiding the expression of NF $\kappa$ B-dependent apoptogenic cytokines.

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#### References

1. Greenberg P. Apoptosis and its role in the myelodysplastic syndromes: implications for disease natural history and treatment. *Leuk Res* 1998; 22:1123-36.
2. Parker JE, Mufti GJ, Rasool F, Mijovic A, Devereux S, Pagliuca A. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and

acute myeloid leukemia secondary to MDS. *Blood* 2000; 96:3932-8.

3. Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, et al. Erythropoietin can induce the expression of bcl-x(L) through Stat5 in erythropoietin-dependent progenitor cell lines. *J Biol Chem* 1999; 274:22165-9.
4. Stephanou A, Brar BK, Knight RA, Latchman DS. Opposing actions of STAT-1 and STAT-3 on the Bcl-2 and Bcl-x promoters. *Cell Death Differ* 2000; 7:329-30.
5. Barkett M, Gilmore T. Control of apoptosis by Rel/NF- $\kappa$ B transcription factors. *Oncogene* 1999; 18:6910-24.
6. Kasibhatla S, Genestier L, Green DR. Regulation of fas-ligand and expression during activation-induced cell death in T lymphocytes via nuclear factor  $\kappa$ B. *J Biol Chem* 1999; 274:987-92.
7. Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV.  $\kappa$ B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor  $\alpha$  gene in primary macrophages. *J Exp Med* 1990; 171:35-47.
8. Gersuk GM, Beckham C, Loken MR, Kiener P, Anderson JE, Farrand A, et al. A role for tumour necrosis factor- $\alpha$ , Fas and Fas-Ligand in marrow failure associated with myelodysplastic syndrome. *Br J Haematol* 1998; 103:176-88.

#### Erythropoietin does not modify the prothrombotic effect induced by uremic media on endothelial cells

Recombinant human erythropoietin (rHuEPO) administration has been associated with an increased risk of hypertension and thrombosis in uremic patients. rHuEPO and uremic media independently alter endothelial function both *in vitro* and *in vivo*. We investigated the effect of rHuEPO on endothelial cells cultured in an uremic environment. Results indicate that rHuEPO does not exert an additional activating effect to that caused by the uremic media *per se*.

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Uremic patients suffer from complex hemostatic disorders, with the coexistence of a bleeding tendency<sup>1</sup> and an increased risk of thrombotic complications and cardiovascular events.<sup>2</sup> There is increasing evidence of endothelial dysfunction in uremic patients, characterized by *in vivo* increases in plasma levels of endothelial cell damage markers,<sup>3</sup> impaired endothelium dependent vasodilatation, and *in vitro* increases in the thrombogenic properties of the endothelial cell extracellular matrices (ECM).<sup>4</sup> Treatment with rHuEPO corrects the defect in primary hemostasis frequently observed in uremia, both through an increase in the number of red cells<sup>5</sup> and by improving platelet dysfunction.<sup>6</sup> However, there is some concern about the potential deleterious effects of rHuEPO treatment on blood pressure, on the incidence of thrombotic events, and on the high cardiovascular risk reported in uremic patients.

Endothelial cells (EC) cultured in the presence of uremic sera (uremic EC) were exposed to rHuEPO (n=6), at final concentrations of 0, 3, 10, 50 and 100U/mL, to evaluate changes in tyrosine kinase activity, in the reactivity of the ECM towards circulating platelets, and in the expression of tissue factor (TF) on ECM. Levels of tyrosine phosphorylation of proteins in control EC were almost undetectable, increased significantly when cells were grown in the presence of uremic sera, and did not change after incubation with increasing concentrations of rHuEPO (Figure 1).

Platelet adhesion on ECM-coated coverslips, measured as the

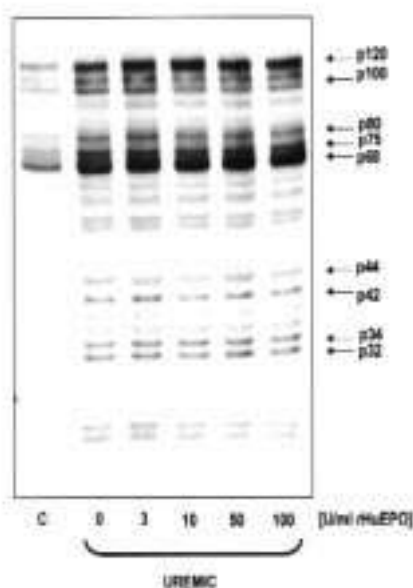


Figure 1. SDS-polyacrylamide gel profiles showing tyrosine-phosphorylated proteins in endothelial cells cultured in the presence of uremic sera, before (0) and after exposure to recombinant human erythropoietin (rHuEPO) at the concentrations indicated. Lane C corresponds to control endothelial cells. Profiles are representative of 1 experiment out of 6.

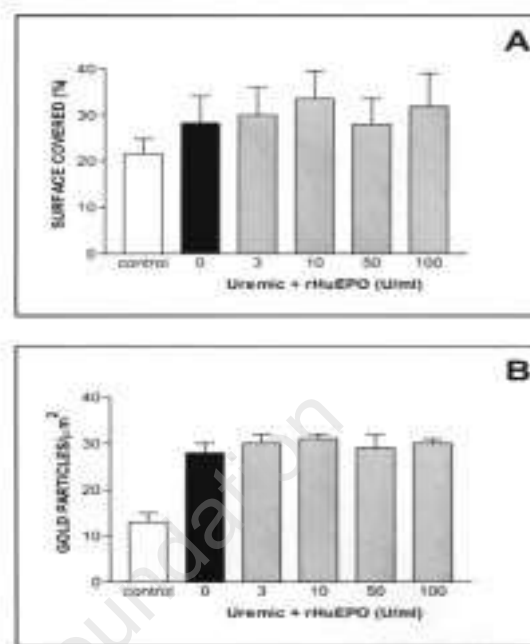


Figure 2. Effect of rHuEPO on the extracellular matrices generated by endothelial cells (ECM) grown in the presence of uremic sera. In panel A, bars represent the surface covered by platelets, expressed as percentage (%SC), after perfusing ECM with LMWH-anticoagulated blood samples, at  $800\text{s}^{-1}$  of shear rate for 5 minutes. In panel B, bars represent levels of tissue factor (TF) expression on the ECM generated. TF was detected by a specific antibody followed by a gold-conjugated IgG. Data are expressed as gold particles per  $\mu\text{m}^2$  (mean $\pm$ SEM, n=6). Data from experiments using control ECM are also included.

percentage of surface covered by platelets (%SC), was evaluated after perfusing low molecular weight heparin (LMWH) anticoagulated blood samples through a parallel-plate chamber.

The %SC on ECM from uremic EC was significantly higher than that observed on ECM from control cells ( $28.3\pm 5.8\%$  vs.  $21.6\pm 3.20\%$ ,  $p<0.05$ ). Incubation of uremic EC with rHuEPO did not significantly modify the %SC on the corresponding ECM (Figure 2A).

Expression of TF on the ECM was assessed by immunogold staining and silver enhancement. ECM generated by uremic EC exhibited a higher presence of TF than that observed on control ECM ( $28\pm 2$  vs.  $13\pm 2$  gold particles/ $\mu\text{m}^2$ ,  $p<0.05$ ). Incubation of uremic EC with rHuEPO did not modify the expression of TF on the corresponding ECM (Figure 2B).

As derived from our present results, rHuEPO does not exert an additional deleterious effect over that observed with the uremic medium. There is a pro-inflammatory state in dialyzed patients, as evidenced by increased levels of the cytokines interleukin (IL)-1, IL-6, tumor necrosis factor (TNF- $\alpha$ ), and C-reactive protein.<sup>7</sup> High levels of circulating cytokines are often associated with anemia caused by hyporesponsiveness to erythropoietin.<sup>8</sup> In a recent work, a direct and causal relationship between cytokine production and erythropoietin resistance was reported in hemodialysis.<sup>9</sup> We previously demonstrated that rHuEPO acts on

endothelial cells, inducing the generation of a more thrombogenic extracellular matrix, and an enhanced presence of tissue factor.<sup>10</sup> These effects occurred following the activation of both the JAK/STAT and the RAS/MAPK signal transduction pathways. Inflammatory cytokines seem to induce the up-regulation of a group of proteins, known as *suppressor of cytokine signaling* (SOCS), which can interfere with the JAK-STAT pathway. This potential inhibitory action could explain the lack of effect in response to rHuEPO in those endothelial cells cultured *in vitro* in the presence of uremic media.

Despite these results, the long-term effects of rHuEPO on the different cell types in the vascular territory in the uremic setting deserve further investigation.

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#### References

1. Remuzzi G. Bleeding in renal failure. *Lancet* 1988; 1:1205-8.
2. Levey SA, Eknoyan G. Cardiovascular disease in chronic renal disease. *Nephrol Dial Transplant* 1999; 14:828-33.
3. Malyszko JS, Malyszko J, Pawlak K, Pawlak D, Buczko W, Mysliwiec M. Importance of serotonergic mechanisms in the thrombotic complications in hemodialyzed patients treated with erythropoietin. *Nephron* 2000; 84:305-11.
4. Serradell M, Diaz-Ricart M, Cases A, Zurbano MJ, Aznar-Salatti J, Lopez-Pedret J, et al. Uremic medium disturbs the hemostatic balance of cultured human endothelial cells. *Thromb Haemost* 2001; 86:1099-105.
5. Moia M, Mannucci PM, Vizzotto L, Casati S, Cattaneo M, Ponticelli C. Improvement in the haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet* 1987; 2:1227-9.
6. Diaz-Ricart M, Estebanell E, Cases A, Lopez-Pedret J, Castillo R, Ordinas A, et al. Erythropoietin improves signaling through tyrosine phosphorylation in platelets from uremic patients. *Thromb Haemost* 1999; 82:1312-7.
7. Stenvinkel P, Heimbürger O, Paultre F, Diczfalusy U, Wang T, Berglund L, et al. Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. *Kidney Int* 1999; 55:1899-911.
8. Jongen-Lavrencic M, Peeters HR, Wognum A, Vreugdenhil G, Breedveld FC, Sweak AJ. Elevated levels of inflammatory cytokines in bone marrow of patients with rheumatoid arthritis and anemia of chronic disease. *J Rheumatol* 1997; 24:1504-9.
9. Allen DA, Breen C, Yaqoob MM, Macdougall IC. Inhibition of CFU-E colony formation in uremic patients with inflammatory disease: role of IFN- $\gamma$  and TNF- $\alpha$ . *J Invest Med* 1999; 47:204-11.
10. Fuste B, Serradell M, Escolar G, et al. Erythropoietin triggers a signaling pathway in endothelial cells and increases the thrombogenicity of their extracellular matrices in vitro. *Thromb Haemost* (in press).