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Letter to the Editors-in-Chief

Protein Z is not synthesised by human umbilical vein endothelial cells

Protein Z is a vitamin K-dependent plasma glycoprotein that presents structural similarities with some coagulation factors such as factors VII, IX, X and protein C [1].

The physiological function of protein Z has been uncertain for many years until Han et al. demonstrated that protein Z plays an important role in inhibiting coagulation as it serves as cofactor for the inactivation of activated factor X by forming a complex with the plasma protein Z-dependent protease inhibitor [2]. Over the last years, some studies have reported a role for protein Z plasma levels in prothrombotic conditions, such as ischemic stroke and acute coronary syndromes [3–5].

Protein Z is synthesised mainly by the liver as shown by its cloning from that tissue [6]; nevertheless, protein Z has been found in endothelial cells [7] and a recent communication suggested that it might be synthesised also by endothelial cells (Vasse et al., 2003, personal communication).

Therefore, we performed this study in order to discover whether protein Z is produced by human umbilical vein endothelial cells (HUVEC), and if this production is affected by the inflammatory process and by heparin.

HUVEC were purchased and grown in endothelial growth medium-2 with BulletKit (Cambrex Bio Science, Walkersville, MA, USA) at 37 °C with 5% CO_2. After 24 h of initial plating the medium was replaced every day with fresh culture medium and supplemented with growth factors. Furthermore, the cultures were daily examined for growth and monolayer adhesion by using an optical microscope. At the second passage, HUVEC were detached using trypsin for 5 min at 37 °C, shared into 25 flasks and grown up to 90% confluence (approximately 62,500 cells in each flask). Before performing the experiment, the medium was changed in a nutrient mixture without fetal bovine serum and heparin for 12 h.

No stimulation was performed in 10 flasks, whereas the others were activated for 18 h with tumour necrosis factor-α (TNF-α) 25 ng/ml, for 18 h with heparin 1 U/ml and, for the same period, with both. Among the 10 non-stimulated flasks, cells were immediately detached in 5 of them, while in the remaining 5 cells were collected after 18 h. The supernatant was obtained, cells were detached and lysed by sonication, and protein Z levels were quantified in the supernatant and in the cellular lysate by using an ELISA assay, with a detection limit of 1.03 ng/ml (Diagnostica Stago, Asnieres, France). In addition, we measured in the supernatant tissue factor (TF) and tissue factor pathway inhibitor-free (TFPI-free) by ELISA assays (American Diagnostica Inc., Greenwich, USA; Diagnostica Stago, Asnieres, France, respectively) in order to verify cells' response in relation to the stimulation by TNF-α and heparin.

Our experiment showed no detectable values of protein Z in the supernatant both with and without stimulus. Conversely, as expected, the release of TF and TFPI-free was significantly influenced by TNF-α (paired t-test: $p<0.001$) and heparin addition (paired t-test: $p<0.05$), respectively (Table 1).

To date, this study shows that human endothelial cells are not a source of protein Z. In addition, it demonstrates that heparin administration does not influence the release of protein Z by endothelial cells, as happens with TFPI, one of the major haemostatic regulatory molecule synthesised by endothelium.

Actually, the wide distribution of protein Z plasma levels in normal population has suggested a possible regulatory role of inflammatory cytokines on protein Z biosynthesis. In 1999, Undar at al. demonstrated that, in patients with haematological malignancies, an inverse correlation between protein Z and interleukin-6 (IL-6) plasma levels was present, so suggesting a regulation of protein Z synthesis by IL-6 [8]. On the other hand, in 2002, Vasse et al. observed that protein Z biosynthesis by hepatic cells was only weakly

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Table 1 Effect of tumour necrosis factor-α and heparin on protein Z, tissue factor pathway inhibitor-free and tissue factor biosynthesis by human umbilical vein endothelial cells

	No stimulus ($T=0$)	No stimulus ($T=18$ h)	TNF-α 25 ng/ml ($T=18$ h)	Heparin 1 U/ml ($T=18$ h)	TNF-α 25 ng/ml + heparin 1 U/ml ($T=18$ h)
Protein Z (ng/ml)	N.D.	N.D.	N.D.	N.D.	N.D.
TFPI-free (ng/ml)	28.0 ± 0.9	71.1 ± 4.8	72.5 ± 3.4	102.1 ± 7.1*	86.3 ± 0.6*
TF (pg/ml)	69.3 ± 0.9	84.4 ± 0.7	162.4 ± 2.5**	52.7 ± 2.3**	116.9 ± 8.1**

N.D. = not detectable, TNF = tumour necrosis factor, TFPI = tissue factor pathway inhibitor, TF = tissue factor, HUVEC = human umbilical vein endothelial cells.
*$p<0.05$, **$p<0.001$; reference group: no stimulus ($T=18$ h).

affected by some inflammatory cytokines such as interleukin-1, TNF-α, IL-6 and oncostatin M [9].

A possible limitation of the study is the lack of vitamin K in the media for this experiments. Presently, we did not add vitamin K since a previous paper by Fair et al. [10] showed that vitamin K was not essential for the synthesis of another vitamin K-dependent protein, protein S, even though vitamin K addition increased its secretion by endothelial cells.

In conclusion, our experimental investigation shows that protein Z is not synthesised by endothelial cells, even after stimulation with heparin and TNF-α.

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