Persistence of TNF α in diabetic wounds

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To the Editor: In the February issue of *Diabetologia*, Graves and colleagues [1] have shown that TNF α concentrations were elevated in the wounds of mice used as a model of diabetes compared with normoglycaemic littermates and that TNF α drove fibroblast apoptosis, hence impairing wound healing. In their study, anti-TNF treatment increased fibroblast density and improved wound healing. Of course, the usual caveat applies to such animal studies (the authors used streptozotocin-induced and *db/db* models of type 1 and type 2 diabetes, respectively) regarding whether they are relevant to human disease, but the results of Siqueira et al. [1] are in close agreement with observations we have made in a human model of dermal wound healing employing cantharidin skin blisters.

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Cantharidin is a blistering agent used in the treatment of molluscum contagiosum and warts [2]. It is a protein phosphatase 1 and 2 alpha inhibitor that causes acantholysis [2]. When applied to healthy skin it can model the proinflammatory and resolving phases of wound healing, depending on the duration of blister: at 16 h blister fluid contains proinflammatory mediators, such as TNF α , IL-8 (also known as CXCL8) and monocyte chemoattractant protein-1 (MCP-1; also known as CCL2), but at 40 h these have substantially diminished to be replaced by wound healing factors, such as TGF β [3, 4]. Early blisters are infiltrated with a predominance of polymorphonuclear leucocytes and monocytes, whereas later skin blisters show evidence of neutrophil apoptosis and differentiation of monocytes into macrophages, characteristics of inflammatory resolution.

To investigate whether the balance of inflammation and resolution was disturbed in persons with diabetes, nine patients with type 2 diabetes mellitus were recruited at the Hammersmith Hospital with informed consent and institutional review board approval, in accordance with the principles of the Declaration of Helsinki as revised in 2000. Twelve healthy individuals were recruited as a control group. Two skin blisters were created on the volar aspect of the forearm by topical application of cantharidin (Cantharone, Dormer Laboratories, ONT, Canada) at 0.1% (vol./vol.) in acetone. One skin blister was harvested at 16 h and one blister at 40 h of duration. Differential leucocyte counts in blister fluid were performed by Kimura's stain and neutrophil apoptosis was determined by flow cytometry, monitoring loss of CD16 staining as an early marker of apoptosis [5, 6]. It was not possible within the ethical constraints of the protocol to investigate keratinocyte or fibroblast apoptotic or proliferative responses. Concentrations of TNF α in blister fluid were determined by ELISA.

Fig. 1 TNF α concentration in blister fluid at 16 and 40 h of blister duration. Two skin blisters were formed on the forearm of healthy controls ($n = 12$, white bars) or persons with diabetes ($n=9$, black bars) by topical application of the blistering agent cantharidin. One blister was harvested at 16 h and the second at 40 h of duration. Blister fluids were analysed by capture ELISA for TNF α levels. Results are expressed as mean concentration (pg/ml) $\pm$ SD. Statistical differences between control and diabetes groups were analysed by unpaired t test. Differences between groups were statistically significant: $p<0.05$ at 16 h and $p<0.01$ at 40 h

There were no significant differences in total leucocyte counts, neutrophils or monocyte infiltrates between diabetes and control groups. However, the pattern of TNF α production in diabetes was deranged: TNF α was significantly lower in the group with diabetes than in the control group at 16 h ($p<0.05$) but significantly elevated at 40 h ($p<0.01$; Fig. 1). Hence, TNF α persisted into the resolving phase in this human model of wound healing. A limitation of the study is that skin blisters could not be extended beyond 40 h since blisters were friable. Hence long-term effects of raised TNF α on wound healing could not be investigated, but from a safety perspective this was beneficial and no adverse events were reported. The proportion of CD16^{low} cells in the neutrophil gate (apoptotic cells) at 40 h was $38.1\pm 9.3\%$ in healthy individuals (mean $\pm$ SD) vs $8.5\pm 1.0\%$ in persons with diabetes.

Hence, our results in this human model of wound healing support the observations made by Siqueira et al. in mice of a persistent inflammatory response in diabetic skin wounds driven by TNF α . TNF α drives apoptosis in

fibroblasts, but in neutrophils it has been shown to promote cell survival [7]. Diminished neutrophil apoptosis seen in resolving skin blisters is consistent with a prolonged inflammatory response in persons with diabetes. Whether impaired neutrophil apoptosis was due to the elevated TNF α levels observed in the skin blisters remains to be established. Nonetheless, the net effect is the same in our human model as observed in diabetic wounds in mice: to extend the net inflammatory response and hence impede wound repair.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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