Venous Leg Ulcers And Apoptosis: A TIMP-3-Mediated Pathway?

To the Editor:

Proteolysis of cell surface- and extracellular matrix molecules (ECM) is intrinsically linked to the physiological cell function and fate (Hengartner, 2000; Brinckerhoff and Matrisian, 2002). The regulation of the proteolytic processes is achieved primarily through coordinated interactions between proteinases and their inhibitors. We previously reported that matrix metalloproteinases (MMP) are involved strongly in the pathogenesis of venous leg ulcers (Herouy *et al*, 1998, 2000). Tissue inhibitor of metalloproteinase-3 (TIMP-3) belongs to a family of secreted proteins that regulate the activity of several metalloproteinases and is an inducer of apoptotic cell death (Woessner, 2001). The prodeath domain of TIMP-3 resides in its N-terminal region and apoptosis is induced by the activation of a signaling cascade involving caspase-8 and caspase-9 (Bond *et al*, 2000). Venous leg ulcers account for the majority of chronic wounds and are a common cause of morbidity, with a significant incidence in the population of the western world. The underlying mechanisms leading to such drastic cellular changes of venous ulcerations have been a matter of debate. Pressure-damaged capillary vessels with leakage of fibrinogen, release of toxic metabolites by accumulated leukocytes or cytokine-mediated fibrin cuff formation have been suggested (Herrick *et al*, 1992). Due to the impaired oxygen supply, all three hypotheses are supposed to result into necrotic events; however, venous leg ulcers lack such clinical signs. Although imbalanced proteolytic activity supports strongly the progression of venous leg ulcers, the factors determining cell fate have not been discussed until now. Thus, we investigated in the following experiments whether apoptosis plays a role in the pathogenesis of venous leg ulcers.

Here we determined the protein expression of TIMP-3, FAS/CD95, and BCL-2 by immunohistochemistry, the caspase-8 and caspase-9 protein expression and activity by caspase activity assays as well as the DNA-fragmentation by positive DNA *in situ* nick end-labeling (TUNEL). All analyses were performed in tissue specimens of venous leg ulcers ($n = 24$; mean age 60.2 SD $\pm$ 8.3; mean wound size 23.6 cm^2) and age- and sex-matched healthy skin controls ($n = 24$; mean age 61.7 SD $\pm$ 9.6). All specimens were obtained from different patients within the ulcer ground. All patients received solely compression therapy before biopsy specimens were taken. Staining with anti-TIMP-3 displayed cuffed pattern of vessel staining and an intense signal around collagen bundles in the reticular dermis of venous leg ulcers (Fig 1*a*), whereas weak immunoreactivity was seen in healthy skin (Fig 1*a*). Staining of acute wounds showed no expression of TIMP-3 and were therefore comparable to the condition of healthy skin. Staining with Fas/CD95 showed an intense cellular immunoreactivity in lesions (Fig 1*a*) in comparison with healthy controls (data not shown), whereas BCL-2 showed weak cellular immunoreactivity in leg ulcer lesions in comparison with healthy controls (data not shown). Immunoblotting with caspase-8 and caspase-9 antibodies revealed expression of the procaspase form for caspase-8 and caspase-9 in healthy skin, whereas these forms were less immunoreactive in lesional skin of venous leg ulcers (Fig 1*b*). In these patients, the processed forms as well as the active subunits of these apoptosis regulators were found as prominent bands. Lesional skin displayed increased activity for caspase-8 ($p < 0.001$) and caspase-9 ($p < 0.001$) in comparison with healthy controls measured by caspase activity assays (Fig 1*c*). Moreover, quantification of apoptotic cells by TdT-mediated incorporation of dUTP into fragmented DNA (TUNEL) revealed significantly elevated apoptotic cell death in venous leg ulcers in comparison with healthy controls ($p < 0.001$) (Fig 1*c*). There were no significant differences in the TUNEL assay in healthy skin in comparison with biopsies taken 1.5 cm distance from the edge of the ulcer (data not shown).

Venous leg ulcers are characterized by loss of epidermal and partly dermal cellular structures. These drastic morphological changes were thought to be caused by impaired oxygen supply or reactive toxic metabolites (Herrick *et al*, 1992). Our results suggest that TIMP-3 could contribute strongly to the pathogenesis of this disease through induction of apoptosis in venous leg ulcers by using the caspase activation cascade in Fas-receptor pathway. It is well known that TIMP-3 mediates ligand binding-induced trimerization of death receptors (Fas/CD95), resulting in recruitment of the receptor-specific adapter protein Fas-associated death domain (FADD), which activates caspase-8 (Bond *et al*, 2000). Tumor necrosis factor-α receptors has, however, been also implicated as prodeath receptor in colon cancer cells. Therefore further investigations are required as to how this pathway additionally plays a role to induce apoptosis in venous leg ulcers. Activated caspase-8 propagates the apoptotic signal by breakdown of the BH3 Bcl2-interacting protein. This leads to the release of cytochrome *c* from mitochondria and triggers activation of caspase-9 and a complex with dATP and Apaf-1 (Fig 1*d*). Activated caspase-9 further regulates "downstream caspases" and ultimately induces the programmed death cell via death substrates (Kroemer and Reed, 2000). In summary, we provide here circumstantial evidence that venous leg ulcers could be the

Abbreviations: TIMP-3, tissue inhibitor of metalloproteinase-3; TUNEL, TdT-mediated incorporation of dUTP into fragmented DNA nick end-labeling

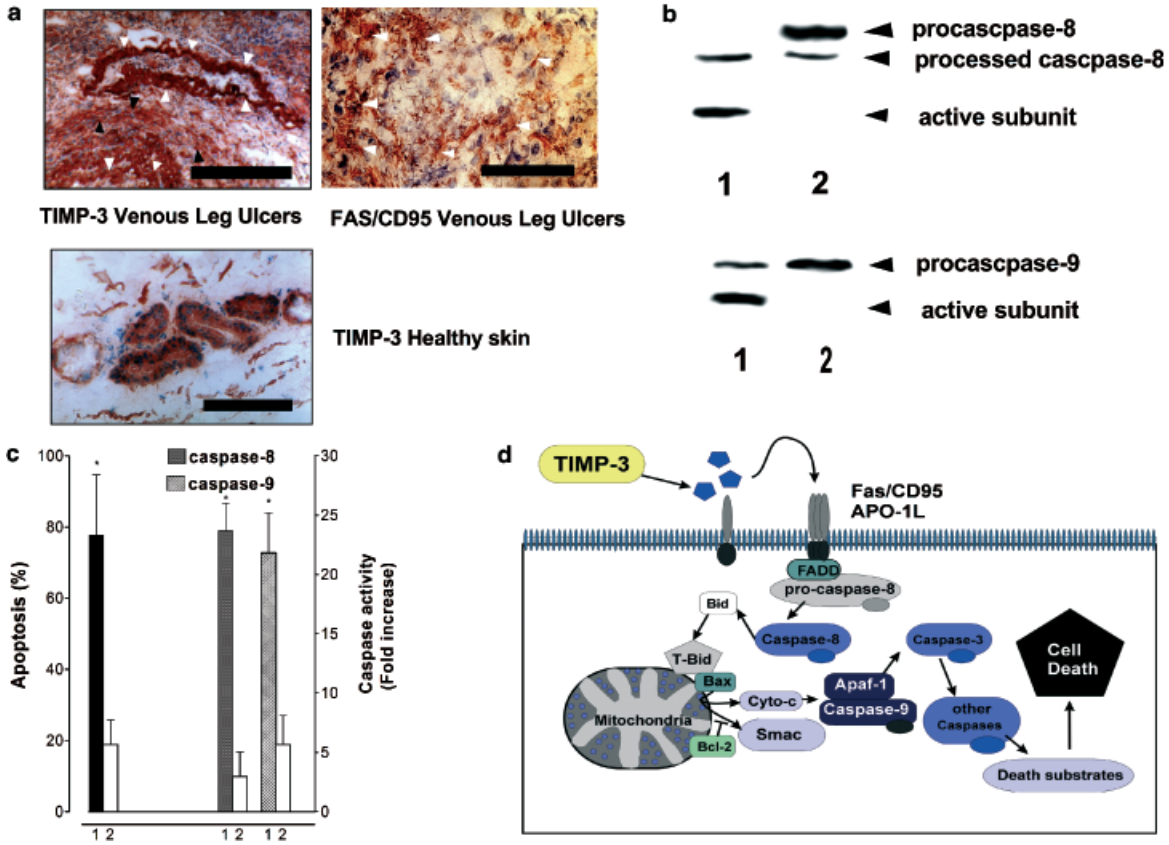

Figure 1
(*a*) Immunhistochemical staining of TIMP-3 and Fas/CD95 in venous leg ulcers. TIMP-3 displays intense immunoreactivity in dermal layers (*black arrowheads*) and in perivascular regions of lesional skin (*white arrowheads*), whereas weak signal was detected in the dermal layer of healthy skin (n = 6 for venous leg ulcers; n = 6 for controls). Fas/CD95 displays intense immunoreactivity in the cellular regions (*white arrowheads*), whereas weak signal was detected in healthy skin (n = 6 for venous leg ulcers; 6 for controls). Representative photographs from one patient is shown. *Scale bar* = 100 μm. (*b*) Protein analysis of caspase-8 and caspase-9 was performed by western blot using primary antibodies. Immunoblotting reveals elevated immunoreactivity for the active subunits of caspase-8 and caspase-9 in venous leg ulcers (1) in contrast to healthy controls (2) (n = 5 for venous leg ulcers; n = 5 for controls). (*c*) Quantification of apoptotic cells in venous leg ulcers (1) in comparison with controls (2) (*left bars*). Apoptotic cell death was measured by TdT-mediated incorporation of dUTP into fragmented DNA (TUNEL), which was measured in a liquid scintillation counter. Rate of apoptosis was significantly elevated in lesional skin in comparison with healthy controls (*t* test $^{*}p < 0.001$). Control TUNEL reactions in the absence of TdT enzyme yielded no staining. Values are given as specific apoptosis ± SD (n = 7 for venous leg ulcers; n = 7 for controls). Quantification of the caspase-8 and caspase-9 activity shows significantly elevated activity levels for both caspases in lesional skin (1) in comparison with healthy controls (2) (*t* test $^{*}p < 0.001$) (*right bars*). (*d*) Pathway for TIMP-3-mediated caspase activation and apoptosis. The extrinsic pathway for caspase activation is mediated by TIMP-3 and is triggered through an FADD-dependent (Fas/CD95) type II apoptotic pathway. These proteins recruit adapter proteins to their cytosolic domains, such as FADD, which then bind pro-caspase-8. The intrinsic pathway is induced by release of cytochrome *c* (Cyto-c) from mitrochondria, which is induced by elevated levels of pro-apoptotic Bcl-2 family proteins such as Bax or Bak. In the cytosol, cytochrome *c* binds and activates Apaf-1, which then binds and activates pro-caspase-9. Active caspase-9 can directly cleave and activate "downstream caspases", triggering apoptosis ultimately.

result of an apoptotic signaling pathway, which might be induced through a TIMP-3-mediated inhibition of proteinase-dependent ECM degradation. These results suggest that controlling programmed cell death may have therapeutic potential in preventing venous leg ulceration.

University of Freiburg has approved the experiments and all patients undergoing the experiments gave their informed written consent. All experiments performed adhere to the Declaration of Helsinki Principles.

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References

Bond M, Murphy G, Bennett MR, Amour A, Knauper V, Newby AC, Baker AH: Localization of the death domain of tissue inhibitor of metalloproteinase-3 to the N terminus. Metalloproteinase inhibition is associated with proapoptotic activity. J Biol Chem 275:41358–41363, 2000

Brinckerhoff CE, Matrisian LM: Matrix metalloproteinases: A tail of a frog that became a prince. Nat Rev Mol Cell Biol 3:207–214, 2002

Hengartner MO: The biochemistry of apoptosis. Nature 407:770–776, 2000

Herouy Y, Hellstern MO, Vanscheidt W, Schöpf E, Norgauer J: FXIII-mediated inhibition of fibrinolysis and venous leg ulcers. Lancet 355:1970–1971, 2000

Herouy Y, May AE, Pornschlegel G, *et al*: Lipodermatosclerosis is characterized by elevated expression ans activation of matrix metalloproteinases: Implications for venous ulcer formation. J Invest Deramtol 111:822–827, 1998

Herrick SE, Sloan P, McGurk M, Freak L, McCollum CN, Ferguson MW: Sequential changes in histologic pattern and extracellular matrix deposition during the healing of chronic venous ulcers. Am J Pathol 141:1085–1095, 1992

Kroemer G, Reed JC: Mitochondrial control of cell death. Nat Med 6:513–519, 2000

Woessner JF Jr: That impish TIMP: The tissue inhibitor of metalloproteinases-3. J Clin Invest 108:799–800, 2001