

Spatial distribution of mast cells in chronic venous leg ulcers

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Chronic venous leg ulcers (CVUs) show chronic inflammation but different pathological changes occur in different parts of the ulcer. There is a lack of re-epithelialisation and defective matrix deposition in the ulcer base but epidermal hyperproliferation and increased matrix deposition in the surrounding skin. The role of mast cells in wound healing, inflammation, fibrosis and epidermal hyperproliferation has been extensively studied but less is known about their role in CVUs. In the present study, we investigated the distribution of mast in CVUs with specific consideration of the differences between the ulcer base and the skin surrounding the ulcer.

Both histochemical and immunohistological methods were used to detect the mast cell marker tryptase in frozen sections of CVU biopsies. Mast cells were counted in the dermis of normal skin, in the ulcer base and in the skin surrounding the ulcer. Double immunofluorescence staining was used to study the location of mast cells in relation to blood vessels. In normal skin few mast cells were seen in the dermis but none in the epidermis. However in CVUs there was a significant increase in intact and degranulated mast cells in the surrounding skin and ulcer edge (184 per field, $p < 0.003$) of CVUs and a significant reduction in the ulcer base (20.5 per field $p < 0.05$) in comparison to normal skin (61 per field). In CVUs mast cells showed a characteristic location near the epithelial basement membrane whilst mast cell granules and phantom cells (mast cells devoid of granules) were predominantly seen in the epidermis. In the dermis, mast cells were seen associated with blood vessels.

The marked increase in mast cells in the surrounding skin of CVUs and depletion of mast cells in the ulcer base could implicate mast cell mediators in the pathological changes in CVUs particularly in the epidermal and vascular changes occurring in the surrounding skin.

Key words: Mast cells, tryptase, re-epithelialisation, inflammation, leg ulcers, wound healing, angiogenesis

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There are many theories regarding the pathogenesis of CVUs, most of them focused on inflammatory and vascular changes (Agren *et al.*, 2000; Gostishcher and Khokhlov, 1991; Herrick *et al.*, 1992).

We have previously shown roles for inflammatory mediators such as transforming growth factor-beta, nitric oxide synthase, arginase and cyclooxygenase in the pathogenesis of CVUs (Abd El-Aleem *et al.*, 2000; 2001; Jude *et al.*, 2002).

A major source of these enzymes was inflammatory cells such as macrophages, endothelial cells and mast cells (Abd El-Aleem *et al.*, 2000; 2001; Jude *et al.*, 2002; Maurer *et al.*, 2003; Gordon *et al.*, 1990).

Mast cells secrete various biologically potent mediators known to have crucial roles in inflammation, wound healing, angiogenesis and fibrosis (Artuc *et al.*, 1999; Maurer *et al.*, 2003; Bevan *et al.*, 2004; Iba *et al.*, 2004).

Interestingly, CVUs show a combination of these pathological changes but the possible role of mast cells in these changes has received little attention. Huttunen *et al.*, (2000) have shown a reduction in mast cells in the epithelialising region of CVU using a tryptase histochemical method.

We extended this study to look at the distribution of mast cells in the ulcer bed and the surrounding skin further from the lesion and we have used an immunohistological method, which is more sensitive and gives information on morphological changes in mast cells.

We have also looked at the location of mast cells in relation to the blood vessels in the CVUs.

This provided a detailed immunological and histochemical description of the distribution of mast cells in the different parts of CVUs.

Materials and Methods

Specimens

For this study, we used biopsies taken previously for looking at the expression of inflammatory mediators cyclooxygenases nitric oxide synthases and arginase in CVUs (Abd El-Aleem *et al.*, 2000; 2001). Briefly, seven normal skin and fifteen long-standing non-healing chronic venous ulcer biopsies were used, from patients with a mean age of 55 years. Patients with evidence of diabetes, diabetic ulcers, and clinical evidence of infection were excluded. No patients were taking NSAIDs or steroids or antibiotics. Skin biopsies were taken under local anaesthesia after written consent from the patients. The biopsies were taken to involve part of the surrounding skin, ulcer edge, and ulcer base. Biopsies were snap frozen in iso-propanol in a bath of liquid N₂, then embedded in OCT, cryo-sectioned and thaw mounted on poly-L-lysine coated slides.

Histochemical staining for mast cell tryptase

The following solutions were made; solution A the buffer (100mM Tris HCl pH 7.3), solution B 10mM Carboenzy (Z)-Gly-Prp-Arg-4-methoxy-2-naphylamide (Bachem, Bubendorf, Switzerland) made in solution A, solution C 0.5% Fast Black K salt (Sigma St Louis, USA). The final staining solution consisted of 8 mL solution A + 1 mL solution B + 1 mL solution C. 7m sections were fixed in 0.6% formaldehyde and 0.6% acetic acid for 7 minutes at 4C. Then sections were covered by solution A for 1 hour at room temperature, followed by the staining solution for 30 minutes. Sections were then mounted in polyvinyl alcohol. Staining resulted in deep blue to black staining of mast cells, gray staining of the nuclei and light brown staining of the epidermis and connective tissues. Specimens were viewed using a Leica DRRB microscope and images were captured using a Spot RT Slider digital camera (Image Solutions) using Spot RT software run on a PC.

Immunohistochemical staining for mast cell tryptase

7m sections were air-dried, fixed in acetone for 10 minutes. Endogenous peroxidases were quenched by treatment with 0.5% H₂O₂ in methanol with subsequent washing in Tris buffered saline (TBS) {0.7% Tris HCl + 0.2% Tris base + 0.02% NaCl + 1% Triton X 100}. Non-specific binding was blocked by incubation for 30 minutes in normal goat serum

diluted 1:50 in 0.1% bovine serum albumin in TBS. Sections were incubated with 1:600 anti-tryptase antibody (Chemicon, Temecula, CA, USA) at 4C overnight, washed and incubated for a further 30 minutes with a biotinylated goat anti-mouse secondary antibody (Vector laboratories, Burlingame, USA) diluted 1:2000. Following a further 30 minutes incubation with Vectastain ABC kits, the substrate, diaminobenzidine tetrahydrochloride in distilled water (Sigma, Poole, UK), was added for 8 minutes. Positive cells were labelled brown. For the negative control, the primary anti-serum was replaced with normal goat serum. Haematoxylin was used as a counter stain to show the nuclei. The sections were mounted in Pertex mounting media and images were captured as above.

Double immunofluorescence

Sections were prepared and incubated with antibody to tryptase as described above. Then, they were washed and incubated for a further 30 minutes with a FITC conjugated goat anti-mouse secondary antibody diluted 1:200 in TBS. Sections were incubated with an antibody to von Willbrand factor (Sigma) 1:1000 at 4C overnight. Then, they were washed and incubated with TRITC conjugated goat anti-rabbit secondary antibody 1:100 in TBS for 1 hour at room temperature. Sections were mounted as for histochemistry, viewed using the Leica DMRB microscope operating in fluorescence mode with appropriate filter sets and images were captured as above.

Mast cell counting

Mast cells were counted in 13 adjacent areas from the surrounding skin (1) to the ulcer base (13) to produce data in Figure 1A or in 8 areas either within the ulcer base or surrounding skin or normal dermis for figures 3A and 3B. Cells were counted in the field of a 20x objective (1.19 mm²) using bright field microscopy. In each case 6 sections were counted and the distance between sections were 150 μm. Results were expressed as the mean cell number per field ± SEM.

Statistical analysis

All statistical analysis was performed using the Statistics Package *SIMFIT* (Bardsley, 1993). Statistical significance of the experiments was determined using one way ANOVA test followed by Tukey-Cramer post-hoc test. $p < 0.05$ was considered as statistically significant.

Results

Histochemical and immunohistochemical methods were used to investigate the distribution and the morphological changes in mast cells in CVUs. Mast cells were identified using both a specific histochemical method for tryptase and an immunohistochemical method using an antibody against mast cell tryptase. Both methods stained intact mast cells but the immunohistochemical method stained also mast cell granules and 'phantom cells', which appeared to have degranulated. In the normal skin, few mast cells were seen in the dermis and none were seen within the epidermis or even in contact with it (Figure 2A). However CVUs showed a marked increase in the number of mast cells using both histochemical and immunohistochemical staining methods (Figure 2B-D). In CVUs, mast cells showed a characteristic distribution. They showed an intimate contact with the basement membrane, particularly at the ulcer edge (Figure 2B). They were predominantly seen in the dermis of the surrounding skin and gradually decreased

towards the ulcer edge until they were virtually absent in the ulcer base (Figure 2D). In CVU mast cells showed morphological changes; while most of mast cells were oval with granulated cytoplasm, a proportion of them showed evidence of degranulation having a *phantom appearance* in which the cells were stained but contained no granules (Figure 2E). In the epidermis, mast cells were found in between keratinocytes (KCs) mainly in the more basal layers and mast cell granules were seen in the epidermis using the immunoperoxidase method (Figure 2F). Double immunofluorescence showed a large number of blood vessels in the papillary dermis of CVUs and location of mast cells around blood vessels and occasionally within the vascular lumen (Figure 2G). Quantification of mast cell numbers, identified by both histochemical and immunohistochemical methods, in adjacent fields across the ulcer showed the highest numbers in the surrounding skin declining steadily towards the ulcer edge and reaching the lowest numbers in the ulcer base (Figure 1B). A significant increase in mast cells in the surrounding skin of CVUs was

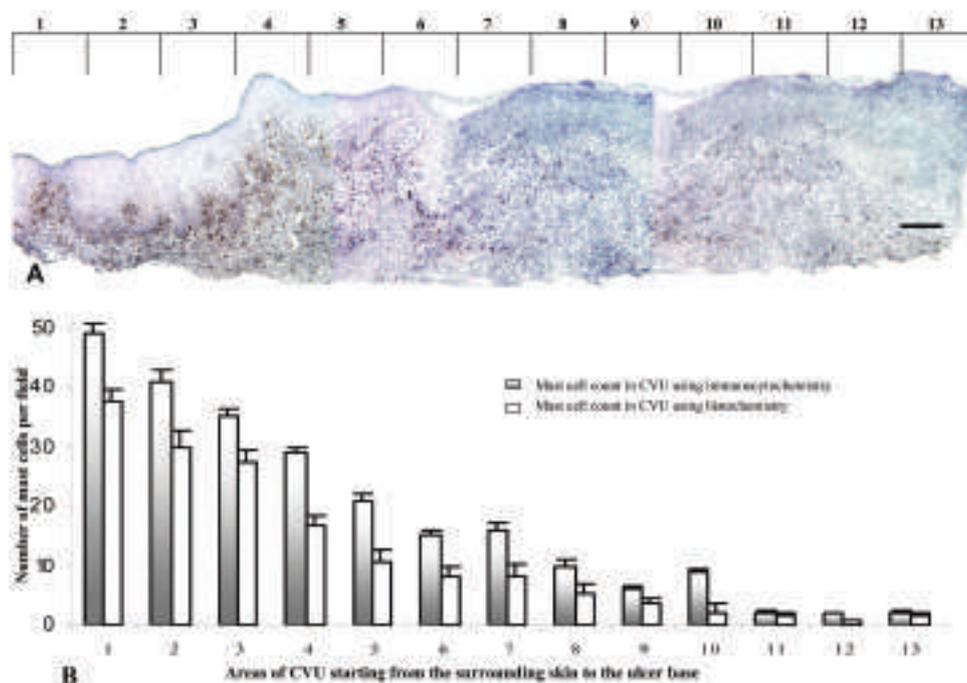


Figure 1. A) Montage of low power sections across a CVU showing how the spatial distribution of mast cells changed. It shows the 13 adjacent areas from the surrounding skin (1) to the ulcer base (13) in which mast cells were counted to produce the data in figure 2B. In the surrounding skin (ss), a large number of mast cells were seen. There was a decline in numbers towards the ulcer edge (ue) and almost complete lack of mast cells in the ulcer base (ub). Scale bar = 400m. B) Graph shows the detailed changes in the number of mast cells across the ulcer in adjacent fields. The highest number of mast cells was seen in the surrounding skin furthest from the ulcer edge (1) and this number gradually decreased towards the base (13). This was shown both by histochemical and immunohistochemical methods.

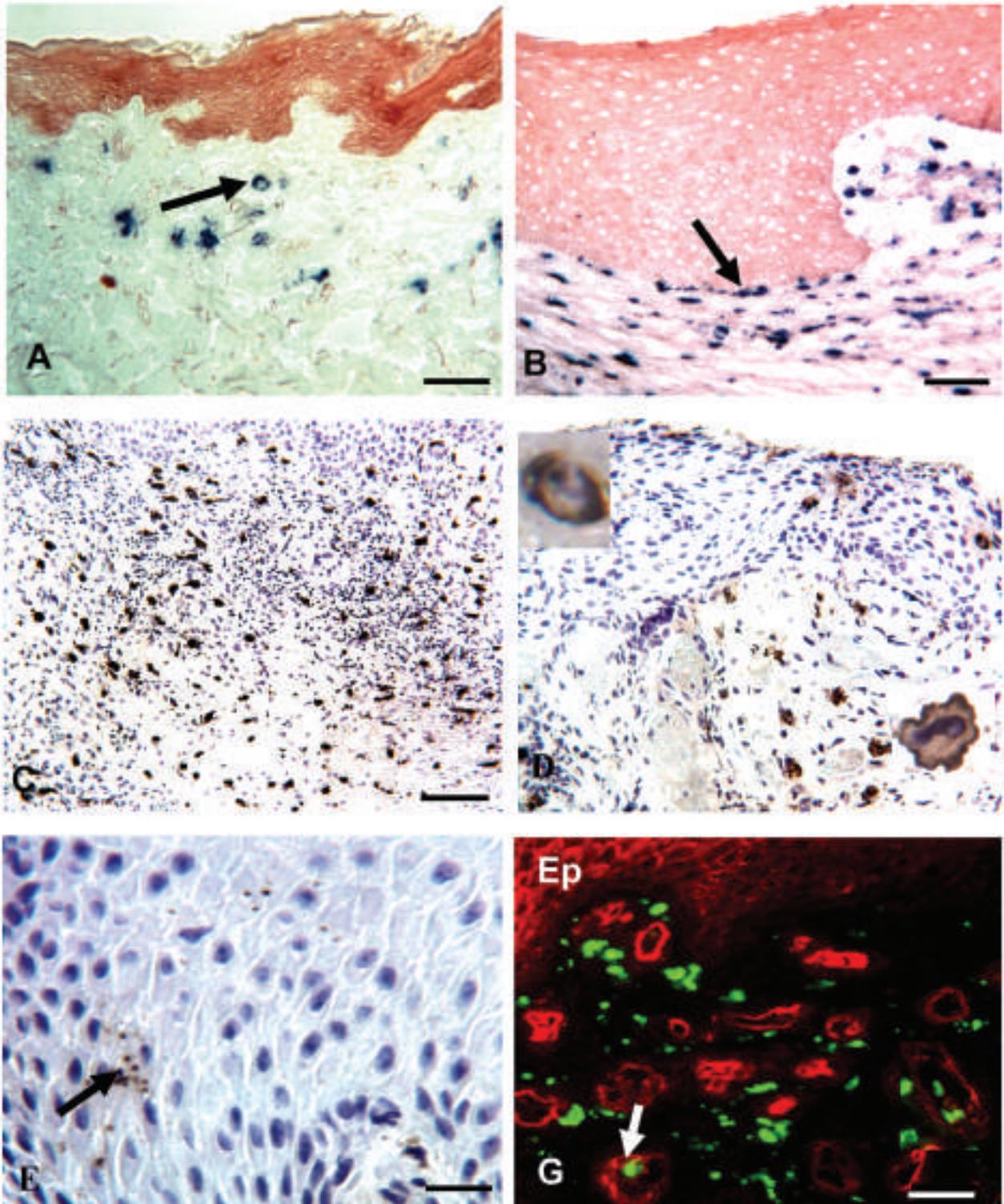


Figure 2. Histochemical staining for tryptase (A, B) A) Mast cell distribution in normal human skin, only a few blue stained mast cells were seen in the dermis. The staining was perinuclear (arrow). B) The surrounding skin of a chronic venous leg ulcer showed densely granulated mast cells located inferior to the basement membrane. Most of mast cells were elongated but towards the ulcer base the morphology became more oval and the location at the basement membrane was no longer evident (arrows). Immunohistochemical staining for mast cells (C-E). C) In the surrounding skin of a chronic venous leg ulcer, a large number of tryptase positive mast cells stained brown were seen throughout the dermis. D) Mast cells were seen in the epidermis and dermis of chronic venous leg ulcers. Some 'phantom cells' weakly staining apparently degranulated mast cells were seen both in the epidermis (top insert) and in the dermis (lower insert). E) Mast cell granules (arrows) were seen scattered in the epidermis of the surrounding skin. F) Double immunofluorescence staining showed localization of mast cells and blood vessels. A large number of blood vessels (red) were seen in the papillary layer of the dermis and a number of mast cells (green) were seen around the vessels. Mast cells were also seen within the lumen of some blood vessels (arrows). Ep = Epidermis. Scale bar A, B = 100m, C = 200m, D = 100m, E, F = 50m.

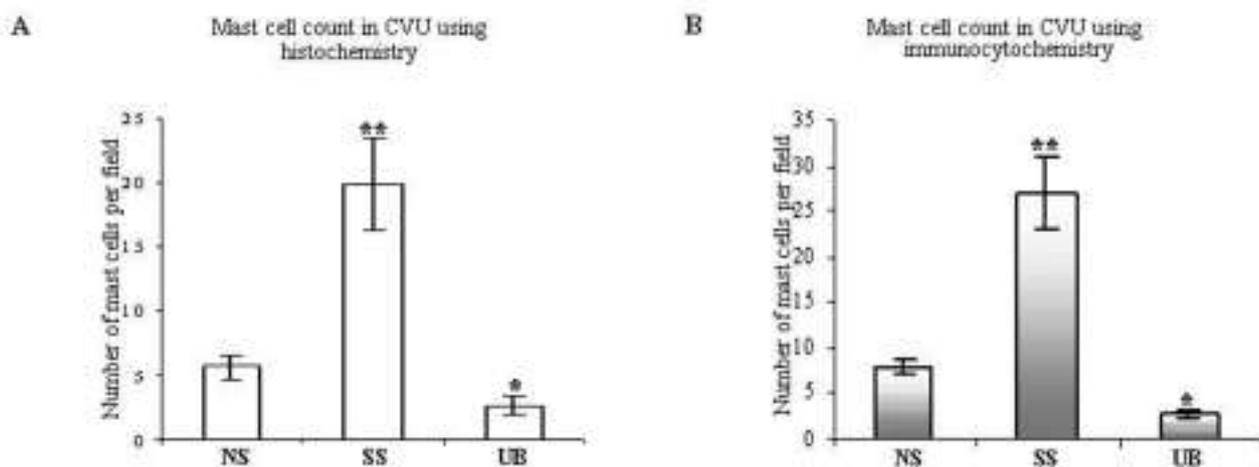


Figure 3. Graphs show a comparison of numbers of mast cells counted in normal skin and ulcers using both histochemical (A) and immunohistochemical (B) methods. There was a significant increase in the number of mast cells in the surrounding skin (ss) by comparison to normal skin (ns) and ulcer base (ub). It also showed a significant reduction of mast cell number in the ulcer base by comparison to normal skin.

found compared to the ulcer base ($p < 0.0004$) or to normal human skin ($p < 0.003$) (Figure 3A, B). The ulcer base showed significant reduction in mast cell number ($p < 0.05$) by comparison to normal human skin (Figure 3A, B).

Discussion

Identification of mast cells was based on morphological characters and both enzyme histochemical and immunological methods for tryptase. Tryptase is good marker for mast cells since it stains specifically (Harvima *et al.*, 1993; Huttunen *et al.*, 2000) and is expressed in all human mast cells whilst only a small percentage express chymase (Hermes *et al.*, 2000; Weber *et al.*, 1995; Algermissen *et al.*, 1994; Harvima *et al.*, 1990; Irani *et al.*, 1986; Nadel, 1991; Harvima *et al.*, 1990). Also, tryptase, unlike chymase, is not affected by the tissue environment particularly by protease inhibitors produced during inflammation (Huttunen *et al.*, 2000; Harvima *et al.*, 1991; Schechter, 1989). Interestingly both the histochemical and immunohistochemical method for tryptase showed a similar pattern of distribution of mast cells in CVUs. Although both methods detected mast cells, the immunohistochemical method was more sensitive in detection of discrete mast cell granules and phantom cells and this led to higher number of mast cells seen using the immunohistochemical method. In this study we have shown that mast cells showed a characteristic distribution in

CVUs; a novel aspect of this study was looking at the quantitative spatial distribution of mast cells along a strip of the ulcer and the surrounding skin. We showed a high number of mast cells in the surrounding skin and this gradually decreased toward the ulcer base where they were less numerous than in normal dermis. In CVUs, mast cells showed close contact with the basal layer of the epidermis and degranulated cells were seen in the epidermis of the surrounding skin. This distribution could implicate mast cells in the pathogenesis of chronic venous leg ulcers and indicate an active role for mast cells in the re-epithelialisation in CVUs. An increase in mast cells has been found in a number of dermatological disorders associated with epidermal hyperproliferation such as psoriasis, lichen planus and chronic wounds (Huttunen *et al.*, 2000; Naukkarinen *et al.*, 1994; Harvima *et al.*, 1991). However, it is not known if the increase in mast cells causes epidermal hyperproliferation or if the epidermal hyperproliferation triggers migration of mast cells. Huttunen *et al.*, (2001) have shown inhibition by histamine of KC growth in cell culture and whole skin culture and they speculated that increased mast cells in chronic wounds could be impairing re-epithelialisation. However, we have shown here that mast cells were absent from the ulcer base where the re-epithelialisation is impaired but increased in the surrounding skin where there is epidermal proliferation. Moreover, it is important to consider that mast cells produce a wide range of mediators, which can be growth promoting or

growth inhibiting under different conditions; histamine stimulates mitosis of psoriatic epidermal cells *in vitro* (Voorhees *et al.*, 1972) and the proliferation of transformed mouse KCs (Katayama *et al.*, 1992), however it inhibits mitosis of human KCs (Flaxman and Harper, 1975). TNF alpha, another mediator of mast cells, causes a hyperplastic reaction of the epidermis after continuous subcutaneous perfusion *in vivo* (Piguet *et al.*, 1990) and it decreases the mitosis of murine tail KCs after intraperitoneal injections *in vivo* (Nagano *et al.*, 1990), and inhibits the growth of cultured normal and psoriatic skin (Detmar, 1990). Therefore, mast cells could be contributing to the epidermal proliferative activity seen in the surrounding skin of CVUs. In this study mast cells or their granules have also been located around or in blood vessels. Mast cells produce VEGF, which is a potent angiogenic mitogen (Artuc *et al.*, 1999), heparin that stimulates endothelial cell migration (Azizkhan *et al.*, 1980), histamine, TNF and tryptase, which stimulate angiogenesis (Marks *et al.*, 1986; Leibovich *et al.*, 1987; Blair *et al.*, 1997). Thus mast cells may also have a role in vascular proliferative activity seen in CVUs.

In conclusion, the marked increase in mast cells in the surrounding skin of CVUs and depletion of the mast cells in the ulcer base could implicate mast cell mediators in the pathological changes in CVUs particularly the epidermal and vascular proliferative changes which are predominant in the surrounding skin. Thus the effect on mast cells of different therapeutic agents used in CVU should be studied.

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