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Title: CGRP and VEGF are Expressed in Lesional but not Uninvolved Skin in Chronic Spontaneous Urticaria

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Abstract

Background

The mechanisms for producing weals in chronic spontaneous (idiopathic) urticaria (CSU) are incompletely understood. Leukocyte infiltration with vascular leakage and expression of the potent vasoactive agents CGRP and VEGF are features of late-phase allergic skin reactions, previously proposed as a model of CSU.

Objective

To measure CGRP and VEGF expression in lesional and non-lesional skin from CSU patients and to compare results with a control group.

Methods

Eight paired biopsies (one from 4- 8 hour spontaneous weals and one from un-involved skin) were taken from eight patients with CSU and nine control subjects and studied by immunohistochemistry and confocal microscopy.

Results

Lesional skin in CSU contained significantly more CGRP+ and VEGF+ cells than non-lesional skin. No significant differences were observed in CGRP and VEGF expression between non-lesional skin and controls. In lesional skin, VEGF and CGRP co-localised to

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UEA-1+ blood vessels. CGRP was also expressed by neutrophils and eosinophils and to a lesser extent by CD90+ fibroblasts, mast cells, CD3+ and CD68+ cells. CGRP and VEGF expression was not related to the duration of disease.

Conclusion and Clinical Relevance

Increased expression of CGRP and VEGF in lesional, but not uninvolved, skin indicates that these potent vasoactive agents may play a role in wealing and tissue oedema in CSU so representing novel targets in therapy.

Introduction

Chronic spontaneous (“idiopathic”) urticaria (CSU) is a common disease associated with recurrent weals resulting from intermittent activation of skin mast cells by unknown mechanisms (1). The condition affects the upper- and mid- dermis and is characterised by a local or generalised red, raised, itchy rash with vasodilatation, increased blood flow and vascular permeability. Functional autoantibodies against the high affinity IgE receptor (FcεR1) have been demonstrated in about one third of patients with CSU, suggesting an autoimmune basis, but the clinical significance of this finding still remains unclear (2, 3).

Although histamine is a key mediator in the wealing process it is unlikely to account solely for prolonged tissue swelling since antihistamines, even in high doses, are often ineffective, particularly in the severe form of the disease. Previous studies, including our own (4-7), have demonstrated infiltration of inflammatory cells into lesional skin in CSU suggesting that in this condition there may be a link between leukocyte accumulation and vascular leakage . In particular we showed that calcitonin gene-related peptide (CGRP), a potent vasodilator, and, vascular endothelial growth factor (VEGF), a permeability as well as angiogenic factor, were both expressed by inflammatory cells infiltrating the allergen-induced cutaneous late phase reaction (LPR) and that the numbers of immunoreactive cells were highest at the peak of the erythema and oedema, i.e. at 4-8 hours (5). The lesions of CSU have many features of the LPR since they peak between 4 and 8 hours and biopsies show the presence of neutrophils, eosinophils and basophils (8). Also, Brain et al (9) showed, in experimental animals and man,

that as little as 15 pmols of CGRP produced erythema which peaked at 5 to 6 hours after injection into the skin. The erythematous component of lesions elicited by CGRP had both the appearance and time course of late-phase allergic reactions.

VEGF, originally termed vascular permeability factor, is a 34-42 kD homodimer. It is required for the growth and differentiation of endothelial cells so stimulating angiogenesis (10). Tedeschi et al have previously described raised plasma levels of VEGF in CSU as well as baseline expression of VEGF by eosinophils in biopsies (11). VEGF is also expressed by eosinophils in biopsies from asthma (12).

In order to provide further evidence that these vasoactive agents contribute to tissue oedema characteristic of established urticarial weals, we have compared lesional and non-lesional skin from CSU, taken from 4 to 8 hour old weals, for the presence of CGRP and VEGF. We have also studied their expression by small blood vessels using the lectin UEA-1 and confocal microscopy and for CGRP have established the cell source(s) of this mediator. Although descriptive, the present report has the advantage that it compares natural exacerbations of the disease (i.e. local, spontaneous, weals) with baseline, uninvolved skin from the same subject. Our overall hypothesis was that CGRP and VEGF would be expressed in lesional, but not in uninvolved, or control, skin indicating that such findings have both pathogenic and therapeutic significance.

Materials and methods

Patients and controls

Eight patients (5 female; 3 male) with chronic spontaneous urticaria (age range 32 to 71 years; mean 51 years) as defined by the EAACI/GA2LEN/EDF/WAO guidelines (13) were recruited from the urticaria clinic of the Department of Dermatology and Allergy at Charité – Universitätsmedizin Berlin. The duration of disease ranged from 9 months to 14 years. On the screening day, subjects were assessed for their suitability to enter the study, and informed consent was obtained. On the study day, patients notified the clinical investigator as soon as they observed an urticarial lesion on the thigh, trunk or upper arm. Biopsies (either spindle-shaped or 4mm punch) were then taken from the lesions and a comparable area of non-affected skin. Biopsies were not taken from linear or asymmetrical wheals. Skin biopsies were also taken from nine healthy subjects (five female and four male), aged between 56 and

76 years (mean age 59 years) who were recruited as controls. Material from these patients have been used in a previous study (7).

None of the patients or controls had a history of concurrent autoimmune, inflammatory (including osteoarthritis and asthma), or infectious disease. None had had anaphylactic episodes or had taken H1 and H2 selective antihistamines for the previous three days. Patients with physical urticarias or urticarial vasculitis were excluded. Additional exclusion criteria included the current or recent (<4 weeks) use of oral corticosteroids or a leukotriene receptor antagonist, or the current or recent (<6 weeks) use of doxepin or other tricyclic antidepressants, or the current or recent (<8 weeks) use of immunosuppressants (e.g. cyclosporine A, azathioprine). One patient took a single dose of 50mg prednisolone as a rescue medication three days before the biopsy. At the time of the study there was no presence of, or prior history of, malignancy. Patients with acute illness, which, in the opinion of the investigator, could pose a threat or harm to the volunteer or obscure interpretation of data were excluded. Also excluded were drug and alcohol abuse, pregnancy and lactation. The study was approved by the Ethics Committee of the Charite – Universitätsmedizin Berlin and the NHS Health Research Authority, UK (ref 07/Q0411/41).

Tissue preparation and immunohistochemistry.

Tissue biopsies were immediately fixed in 4% paraformaldehyde (BDH Chemicals Ltd, Dagenham, UK) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and washed in 15% PBS-buffered sucrose, embedded in optimum cutter temperature compound and snap-frozen in isopentane precooled in liquid nitrogen. Samples were air-freighted to London, UK on dry ice and kept at -80°C until use. Cryostat sections (6 µm) were cut from biopsies, mounted on 0.1% poly-L-lysine-coated slides, dried overnight at 37°C, then stored with silica gel (VWR international, Leicestershire, UK) at -80°C until used.

Immunohistochemistry

Antibody staining was detected by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method, as previously described. (14, 15). Normal human serum (10%) was used to prevent non-specific binding of the second and third layer antibodies. A mouse IgG1

myeloma protein and a rabbit IgG protein were used as negative controls. Monoclonal antibodies against CD31, CD68, mast cell tryptase and neutrophil elastase were purchased from Dako Ltd, Cambridgeshire, UK and anti CD3 from Becton Dickinson, Oxford, UK. Primary antibodies were detected with a second donkey anti-mouse antibody obtained from Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa, USA. APAAP was obtained from Dako Ltd. Positively stained cells were detected with Fast Red (Sigma, Poole, UK). The anti-VEGF was obtained from Abcam, Cambridge, UK and prepared by conjugating residues 50 - 150 of Human VEGF to KLH. The polyclonal rabbit anti-GCRP (Abcam, Cambridge, UK) was prepared using an immunogen linked to the synthetic peptide. Affinity purified polyclonal anti-MBP was obtained from Dr H Kita, Mayo Clinic, Rochester, MN, USA. Double immunofluorescence, using a polyclonal rabbit anti-mouse immunoglobulin and APAAP reagents, was performed as previously reported (15).

Cells were counted in a blind fashion at x400 magnification on an Olympus BX40 microscope (Olympus Imaging & Audio Ltd, Essex, UK) connected with a Zeiss Vision KS300 imaging system (Carl Zeiss, Gottingen, Germany) and total cells expressing phenotypic markers enumerated in entire biopsy sections. Data were expressed as the total numbers of cells per unit area. The mean $\pm$ SD entire cross-sectional area of the cutaneous biopsy sections was $4.2 \pm 0.4 \text{ mm}^2$. The between-observer coefficients of variation for duplicate counts of all markers examined for cutaneous biopsies were 4.4% to 6.8%, respectively.

Immunofluorescence staining for confocal microscopy

Double labelling of sections with the endothelial marker, *Ulex europeus*-1 (UEA-1) lectin, and the other inflammatory markers was carried out using a modification of a protocol used previously (16). Briefly, frozen sections were thawed at room temperature for 30 minutes, then permeabilised and blocked in 0.1% saponin/5% human serum in PBS for 30 minutes at room temperature. Primary antibodies (rabbit anti-CGRP 1/100 or rabbit anti-VEGF 1/200 in 5% human serum/PBS) were applied to sections overnight at 4°C. Slides were washed in PBS 3 X 5 minutes, the secondary antibody (1 $\mu\text{g/ml}$ Alexa Fluor568 goat anti-rabbit (Invitrogen, UK) in 5% human serum/PBS, containing 2 $\mu\text{g/ml}$ Fluorescein Isothiocyanate (FITC)-

conjugated Ulex europeus agglutinin-1 (UEA-1) lectin (Vector Laboratories, UK)) were added to the sections for 2 hours at room temperature in the dark. Slides were washed in PBS 3 X 5 minutes, then coverslips were mounted on sections using VectaShield Hardset mounting medium (Vector Laboratories, UK), and stored at 4°C. To examine the distribution of blood vessels only, one set of sections were stained with UEA-1 alone. Sections were examined using a Leica SP2 confocal microscope system fitted with Argon ion and 561 nm diode lasers, and images were acquired using the Leica confocal software.

Statistics

Data were analysed with the aid of a commercially available statistical package (Minitab for Windows Release 9.2; Minitab Inc.). Significant differences between paired data obtained from lesional and non-lesional biopsies were calculated using the Wilcoxon signed-rank test. The Mann-Whitney *U* test (with Bonferroni's correction) was used to compare differences between groups. For all tests $p < 0.05$ was considered significant.

Results

CSU weals show increased numbers VEGF+ and CGRP+ cells

There were significant increases in cells positive for CGRP ($p=0.02$: approximate 3 fold increase) and VEGF ($p=0.01$: approximate 30% increase) when lesional was compared to non-lesional CSU skin (Fig 1). No significant differences were observed in CGRP and VEGF expression between non-lesional skin and controls. By double immunostaining CGRP in lesional skin was predominantly expressed by elastase+ neutrophils (Fig 2A, C and D), CD31+ cells (Fig 2C and D) and MBP+ eosinophils (Fig. 2B, C and D). Of the CGRP+ cells present in lesional sites approximately 30% were neutrophils, 22% were CD31+ cells and 20% were eosinophils, the remainder being CD90 fibroblasts, mast cells, CD3+ and CD68+ cells (Fig 2C). Conversely, approximately 30% of neutrophils, CD31+ and MBP+ cells were CGRP+, whereas less than 20% of CD90+, mast cells, CD3+ and CD68 + cells were CGRP+ (Fig 2D).

In CSU weals, VEGF+ and CGRP+ cells co-localize to blood vessels

CGRP+ and VEGF+ cells were detected predominantly in the direct vicinity of blood vessels indicating endothelial cell expression of these mediators. Co-localization of VEGF+ cells and CGRP+ cells with skin blood vessels in lesional skin was confirmed by confocal microscopy using VEGF /UEA-1 and CGRP / UEA-1 double staining (Fig. 3 and Fig.4). Some epidermal staining of VEGF and CGRP was observed in urticaria samples and controls. There were no significant relationships between the duration of disease and the expression of CGRP and VEGF.

Discussion

In the present study we show, for the first time, that CGRP is expressed by neutrophils, eosinophils and endothelial cells in lesional, but not uninvolved, skin in biopsies from CSU. We have also confirmed and extended a previous report identifying VEGF in CSU (11) by showing that this cytokine was expressed only in involved skin and to be associated with small blood vessels. We believe our findings may shed light on histamine-independent mechanisms involved in vascular leakage in CSU. Although the numbers of subjects in the present study group was small, the differences in the various markers measured were statistically significant and often substantial.

CGRP is a 37 amino acid peptide resulting from alternative splicing of mRNA from the calcitonin gene (17). It is part of the adrenomedullin, calcitonin, amylin family of peptides and is both an arterial and venous vasodilator (18). CGRP does not induce permeability *per se*, but appears to act synergistically with several mediators of inflammation, including histamine, to produce marked and prolonged oedema (19). The peptide is usually contained within small sensory nerves, and, in the airways, in epithelial neuroendocrine cells (NEC), and is released by a variety of stimuli including heat, chemical and psychological events. Several *in vivo* and *in vitro* studies have demonstrated CGRP production by diverse cells including human airway epithelial cells (15, 20) and T cells (21). In our previous study, we showed that cells infiltrating the human skin during the late phase reaction (LPR) expressed CGRP and that as the reactions resolved so did the numbers of CGRP immunopositive cells (8). This observation was consistent with a role for this peptide in vasoactive events. Using the allergen-induced late-phase reaction as model of CSU, we observed VEGF+ eosinophils,

neutrophils and CD68+ macrophages in biopsies taken six hours after challenge (8). The present study confirms and extends these observations by showing that VEGF also co-localises to endothelial cells. This indicates that in CSU VEGF is associated with both increased permeability and the formation of new vessels. Thus, targeting VEGF or other possible angiogenic factors may be a possible therapeutic approach in CSU since, at least theoretically, this would decrease vessel leakage and control baseline inflammation. In support of this concept is the recent observation that VEGF controls Th2 inflammation through the myeloproliferative leukaemia virus oncogene-P-selectin axis (22). It was previously shown that CGRP regulates the expression of VEGF by human keratinocytes through the CGRP receptor and ERK1/2 MAPK signalling pathway, indicating a tight association between these two vasoactive mediators (23).

We have not excluded a role for other vasoactive mediators in the mechanisms of wealing. For example, using the skin blister technique, LTD4, a permeability factor, was elevated in atopic skin 4-6 hours after allergen challenge (24). Also, leukotriene antagonists are superior to placebo in the treatment of CSU (although considerably less effective than non-sedating antihistamines (9)).

We have recently demonstrated that in CSU, lesional skin has significantly more CD31+ endothelial cells and CD31+ blood vessels, as well as eosinophils and neutrophils, than uninvolved skin, so supporting the view that there is a link between inflammatory cell infiltration and vascular leakage (7). Basophils, macrophages and T cells were also increased in lesional as compared to non-lesional skin but these differences were less marked than with eosinophils and neutrophils. Thus we propose the following scheme that links spontaneous wealing with CGRP, VEGF and vascular leakage. In CSU, as with the LPR, mast cell activation leads to the release of IL-8 and eotaxin, chemotactic factors for neutrophils and eosinophils, respectively (25-27). This, in turn, produces leukocyte-(mainly neutrophil and eosinophil) dependent tissue oedema. Neutrophil-mediated oedema was first described by Wedmore and Williams (28). Although the mechanism remains unclear, it has been proposed that neutrophil-derived vascular leakage factors widen interendothelial gaps allowing plasma to extravasate into the intravascular space. Therefore if neutrophil-mediated oedema is operative in CSU, several leakage factors, including CGRP and VEGF, might be involved. In the present study we found that 30% of neutrophils, CD31+ and MBP+ cells were CGRP+ indicating that both infiltrating inflammatory cells and endothelial cells have the capacity to

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deliver vasoactive mediator to the microenvironment. We found similar endothelial cell expression of VEGF but did not formally study co-localisation of VEGF to other cells in this present study because this mediator is known to be ubiquitous and expressed by various cells type (8). Thus the cells infiltrating lesions in CSU have the potential to produce both vasodilation and leakage. We have shown, furthermore, that inflammatory cytokines such as IL-1, GM-CSF and TNF- α , have the potential to activate epithelial cells (29) and neutrophils (K.Bonner, unpublished) for CGRP expression so supporting the general hypothesis that both infiltrating and structural cells may be sources of vasodilating agents in the inflammation associated with CSU. Some immunostaining of the epidermis was observed in both urticaria (lesional and non-lesional) and controls with anti-VEGF and anti-CGRP. Our impression was that there were no appreciable differences between the groups. However a more detailed assessment of epidermal expression of these mediators is on-going.

There has been controversy regarding the role of CGRP in inflammation and immunity. Earlier studies showed that CGRP inhibited T cell functions, antigen presentation and antibody secretion by B cells (30-32). However, more recently, it has been demonstrated that CGRP may have a pro-inflammatory role in immediate type hypersensitivity. For example, the peptide unregulated both IL-4 production by CD4⁺ cells in vitro (33, 34) and from T cells in the draining lymph nodes in a mouse model of cutaneous type 1 hypersensitivity (35). CGRP has also been shown to be physiologically important in IL-9 production and Th9 cell differentiation in a mouse model of airway inflammation (36). Furthermore CGRP has the potential to activate mast cells by non-immunological mechanisms since mucosal type mast cell lines express the CGRP receptor (37).

In conclusion, we have shown increased expression of CGRP and VEGF in lesional, but not uninvolved, skin raising the possibility that these potent vasoactive agents may serve as therapeutic targets in the wealing and tissue oedema of CSU.

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Legend to Figures

Fig 1. The numbers of CGRP+ and VEGF+ cells in lesional and non-lesional skin compared to controls. The Wilcoxon signed-rank test was used to compare the differences between lesional and non-lesional skin biopsies and the Mann-Whitney *U* test to compare the differences between groups.

Fig 2. Co-localisation of CGRP immunoreactivity to elastase + neutrophils (A) and MBP+ eosinophils (B). The percent of the various cells types co-expressing CGRP are shown in C and the percent of various cell types which are CGRP positive are shown in D.

Fig 3. Co-localization of CGRP immunoreactivity to UEA-1+ vessels. UEA-1 staining in lesional skin (A) is in green, and single CGRP+ cells (B) are in red. Double positive cells in low power (yellow/green) are shown in C and high power in D. Double-positive cells are indicated by the arrows. E is double-staining in non-lesional skin and F is the IgG control in normal skin.

Fig 4. Co-localization of VEGF immunoreactivity to UEA-1+ vessels. UEA-1 staining in lesional skin (A) is in green, and single VEGF+ cells (B) is in red. Double positive cells (yellow/green) (C) are indicated by the arrows. D, E and F represent non-lesional skin shown at a higher power.

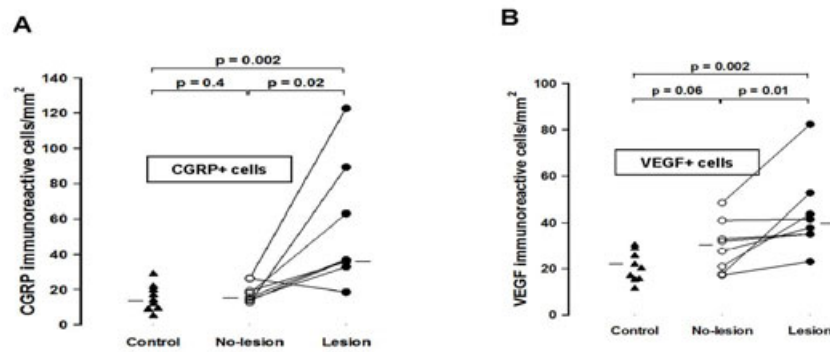


Fig 1

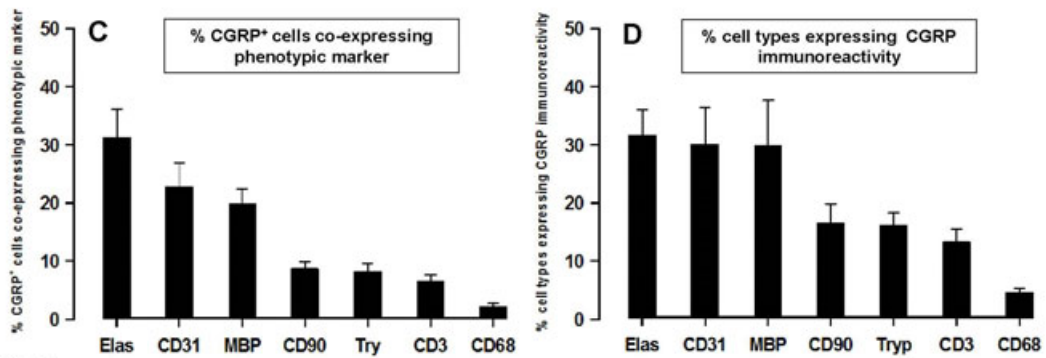
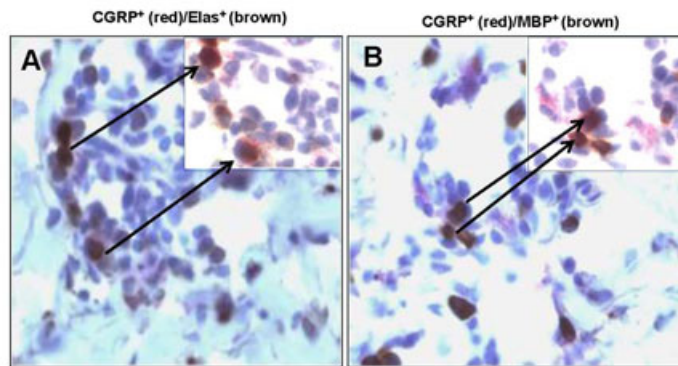


Fig 2

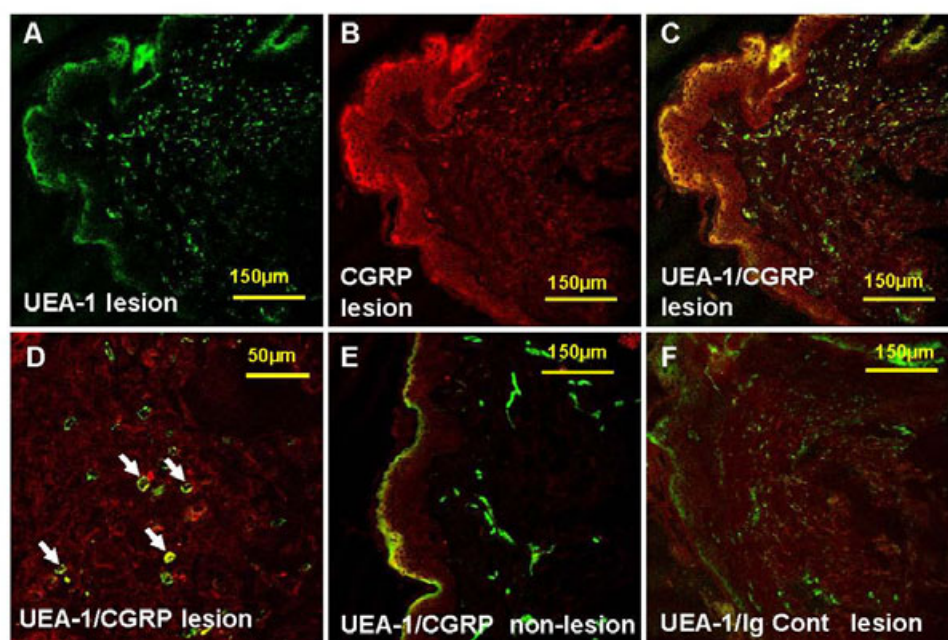


Fig 3

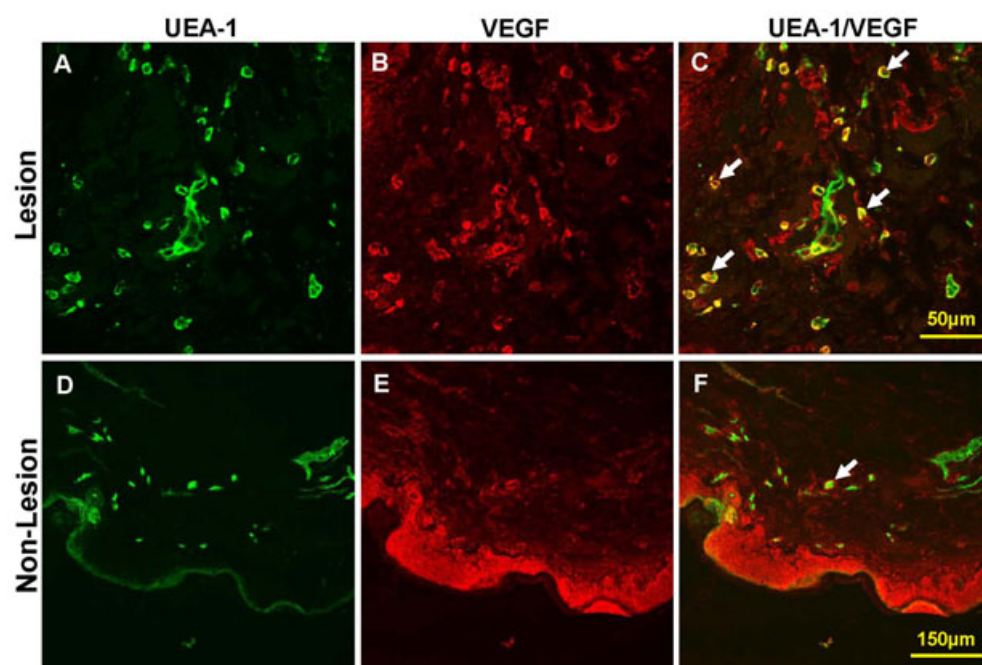


Fig 4