Can Venous Ulcer Size be Associated with the Healing Potential of Serum? Pilot Study

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Abstract: Ulcer size may be associated to slow healing. If this association is true, it could help with diagnosis and subsequent treatment of chronic venous ulcer (CVU). The aim of this work was to study the relationship of ulcer size of CVU patients with the healing potential contained in their serum. This potential was measured directly on patients’ sera through a cytometric technique for the content of Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF). These factors are responsible for revascularization, fibroblast proliferation and collagen deposition. Further, the effect of serum on proliferation and collagen deposition was evaluated in vitro cultures.

The results of the in vitro experiment highlighted the isolated effects of homolateral venous serum factors on Vero cells line not undergoing any inflammation. Ulcer size did not influence the characteristics of sera from CVU patients. In the discussion our results are evaluated in the context of recent research regarding ulcer sizes.

Keywords: CVU, ulcer size, serum, vegf, fgf, proliferation, collagen

1. INTRODUCTION

The prevalence of the venous ulcers in Latin America is estimated between 3 and 6% of the population [1]. In North America and Europe this prevalence is 0.2% [2]. Among Latin-American physicians, there is a consensus on the greater size of ulcers with respect to European and U.S. populations. Large sizes are attributed to lack of prevention, late consultation, delayed treatment and lack of resources. Although statistics are very scarce, this does not diminish its importance as a public health problem [1].

Chronic venous insufficiency is a multifactorial progressive disease that affects the functioning of the venous muscular and valvular-parietal pumps. This valvular incompetence results in edema, induration, fibrosis, skin necrosis, and when a trauma happens a wound appears without tendency to closure. Chronic venous ulcer, results from an imbalance that keeps the wound in the inflammatory phase. The persistent inflammatory response and the unbalanced proteolytic activity overwhelm tissue protective mechanisms [3, 4].

During repair of the cutaneous tissue, Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) are responsible for revascularization, fibroblast proliferation and collagen deposition [5, 6]. VEGF is an endothelial cell mitogen, with a 165-amino-
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acid-long variant representing the major isoform in human tissues. Fibroblasts synthesize and remodel the Extra Cellular Matrix (ECM; 7), and are considered the main producers of procollagen [8]. Collagen provides scaffolds for cellular attachment, migration, and proliferation in wound healing. Ulcer size and patient age may be associated to healing speed, moreover an ulceration surface greater than 20 cm² is indicative of slow healing [37]. If these associations are true, they could help with diagnosis and subsequent treatment.

The aim of this study was to evaluate the in vitro effect of serum from patients with CVU on the wound healing processes of proliferation and collagen deposition. Also VEGF and FGF content was evaluated by a cytometric technique directly on patients sera. The possible modifications of each of the following: proliferation, collagen deposition, VEGF and FGF, with ulcer size, wound duration and patient age were also addressed. We consider that this knowledge will contribute to the understanding of the pathogenesis of CVU.

2. MATERIALS AND METHODS

2.1. Serum Samples

This work was approved by the Bioethics Committee from Universidad Abierta Interamericana, Buenos Aires, Argentina. All patients signed a consent letter prior to their participation. The study was performed including only male patients with refractory venous wounds subjected to 6 months of treatment at the Hospital Municipal de Tigre Buenos Aires, Argentina, between June and December of 2013 evaluated by one physician (R.H.M.). Only male patients were considered to reduce variability due to estrogens, an important factor that would predispose to venous ulcers [38]. The venous ulcer was determined by the clinical characteristics and by Eco Doppler (discarding arterial disease), corresponding to stage C6 active venous ulcer of CEAP (Clinico Etiologic Anatomic Pathologic). Blood from homolateral-to-ulcer femoral veins was obtained. Two weeks prior to blood extraction the studied patients were restricted of medication affecting hemodynamics (venotonic). This was the only intervention for both patients with and without co-morbid conditions (Table 1). All blood samples were tested for Chagas diseases, AIDS, hepatitis B and C, and syphilis before use. The serum samples were blindly handled and inactivated in a water bath at 56°C for 20 min and frozen at 20°C until use. As an experimental control, standardized sera from a pool of healthy men (Sigma Aldrich, H4522) were used.

2.2. Flow Cytometry

Vascular endothelial growth factor (VEGF-165) and basic fibroblast growth factor (bFGF) were measured using the Human Soluble Protein Flex Set Assay kit (BD, Pharmingen, CA, USA). Beads were read in a fluorescence-activated cell sorting equipment AriaII (LANAIS, Buenos Aires). Results were analyzed in BD CBA Analysis Software (BD Biosciences, Cat.No. 550065) obtaining mean fluorescence intensities.

2.3. Cell Line Culture

In vitro experiments with patient serum were performed on a Vero cell line of epithelial cells from African green monkey kidneys, obtained and certified from ABAC (Argentinean Cell Bank). Cells were routinely cultured in RPMI 1640 medium (Sigma, life technology) supplemented with 10% of inactivated fetal calf serum (FCS) (Notocord, Cordoba, Argentina), 1% penicillin-streptomycina (Sigma), 2 mM glutamine (Sigma), and 0.37% Heps (Sigma). They were incubated at 37°C with 5% CO2 (MCO-17AC incubator). After growing for 4 days (80% monolayer confluence), cells were trypsinized and then divided for performing the following two simultaneous experiments.

a) Cell Proliferation

Vero cells were incubated for 48 hrs in 96-multiwell plates at 6x10² cell/ ml, in medium with 2% FCS. They were then incubated for 72 h in medium with 1% of FCS according to the following treatments: either patient or standardized 5% serum. Proliferation was measured by tetrazolium salt colorimetric assay (MTT; 9) in which 2.27 mg of tetrazolium salt (Sigma) in 5 ml of PBS was added to growing cultures and incubated for 1 h at 37°C. The intracellular tetrazolium reactive was dissolved in DMSO (dimethylsufoxide) by shaking at 160 rpm. Colour was measured OD 560 nm with Glomax Multidetetection System (Promega). Each treatment was performed in quadruplicate in each plate and this experiment was performed in three different plates.

b) Collagen Deposition

Vero cells were incubated for 48 h in 24-multiwell plates at 4x10³ cell/ml, in medium with 2% FCS. A 72-hour incubation was then performed according to cell proliferation
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Patients sorted by total area (size) of the ulcer. Hypertension (HT); Chronic pulmonary obstructive disease (CPOD); Postthrombotic syndrome (PS)

3. RESULTS

3.1. Content of Growth Factors in Sera from Patients in a Wide Range of Ulcer Sizes

Sera from patients with CVU presented VEGF-165 (named VEGF) content between 22.5 and 250.54 pg/ml. Ulcers with higher VEGF contents corresponded to both small and large sizes (25 and 225 cm² respectively), while lower contents of VEGF were found in intermediate sizes (50 - 150 cm²) within the range studied (9 - 450 cm²). Neither ulcer size nor wound duration nor patient age (mean 70.2 years old, range 59 – 90, Table 1) were associated with an increase of serum VEGF content. No detectable amounts of bFGF were found, this is, patients' serum levels were below the equipment's threshold (9 pg/ml).

3.2. Effects of Sera from Patients with a Wide Range of Ulcer Sizes on In-Vitro Cultures

a) Cell Proliferation

Vero cells incubated with patient sera (n=10) showed proliferation that was significant when compared with cells incubated with the standardized serum (p < 0.00001). However, this result does not correspond to a comparison between similar, aged-matched healthy individuals. The proliferative effect was very clear in 7 of 10 patient sera (Fig. 2). When proliferation was compared to ulcer size, wound duration, patient age, VEGF content and collagen deposition, no associations were found.

Figure 1: VEGF content in sera from patients with CVU

In the X axis patients are ordered according to ulcer size.

b) Collagen Deposition

The hydroxyproline content of Vero cells cultured with patient serum was not incremented in respect to standardized serum (patient mean 16.76 ± sd 10.31 ug/ml; standardized serum mean 19.35 ± sd 10.53 ug/ml). Moreover, it was reduced in 7 of 12

<table>
<thead>
<tr>
<th>Extending higher ulcer (cm²³)</th>
<th>Age (years)</th>
<th>Evolution time (months)</th>
<th>Comorbidities</th>
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<tr>
<td>9</td>
<td>72</td>
<td>12</td>
<td>HT - CPOD</td>
</tr>
<tr>
<td>25a</td>
<td>73</td>
<td>24</td>
<td>-</td>
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<tr>
<td>25b</td>
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<td>30</td>
<td>77</td>
<td>6</td>
<td>HT-Diverticular disease</td>
</tr>
<tr>
<td>50</td>
<td>59</td>
<td>6</td>
<td>HT-Lymphedema</td>
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<td>80</td>
<td>60</td>
<td>30</td>
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<td>74</td>
<td>46</td>
<td>HT-Arrhythmia</td>
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Figure 2: Effects of patient sera on proliferation and collagen deposition

For each patient the bars show the ratio of Vero cell proliferation and collagen deposition produced with patient serum respect to standardized serum (100%). Bars not seen result from missing determinations. Asterisks indicate significant proliferation when compared with cells incubated with the standardized serum (p < 0.00001).

4. DISCUSSION

It is estimated that 2% of the world population will develop an ulcer at some point in their lives. The typical feature of chronic wounds (CW) is the absence of the granulation tissue that could cover the wound area, restoring tissue integrity [11]. This work shows the isolated effect of serum factors from CVU patients on an epithelial cell line not undergoing any inflammation. This in vitro model avoids the effect of healing on the studied factor function.

In acute wounds, VEGF is up regulated to promote early stages of angiogenesis, and levels remain elevated until epidermal coverage is complete [12]. In fluids of chronic leg ulcers, the expression of VEGF-165 and VEGF receptors is up regulated compared with fluids of uninjured skin of healthy individuals [13, 14]. In the present work the amount of seric VEGF-165 was higher than in standardized sera from healthy patients, resembling the results previously mentioned for fluid levels.

Plasma levels of VEGF in chronic venous disease have been measured in only a few studies [15, 16]. Howlader and Smith(2004) reported higher VEGF levels in patients with C2 to C5 clinical stages in comparison to controls, and showed that statistical significance was only reached in those with healed ulceration (C5). For active venous ulcer patients (C6) with sizes up to 142.4 cm², the VEGF median level was 165 pg/ml (range 21-1315; 18). The results of the present research provide a first report of the seric VEGF content in very large active ulcers of up to 450 cm²; median 90 pg/ml (IQR 66 to 116; range 22.5-229.6). These values are within the range previously described [18]. Both studies support the idea that ulcer size and serum VEGF levels are not related.

In this study we have seen that the cell line behaves differently when incubated in the presence of patient sera or human standardized serum. The observed proliferative effect of sera from patients with CVU indicates the presence of stimulating factors and its potential in tissue formation and wound closure. Since the major proliferative factors stimulating the epithelial cells in CW are epidermal growth factor (EGF; 19) and keratinocyte growth factor (KGF;12), it would be interesting to focus on those factors in future studies.

The results mentioned above indicate that there is an increase of an angiogenic factor (VEGF) and cell proliferation. Both seem to enhance healing capacity. However, it is possible that healing is impaired due to the tissue environment, which is rich in immune cells, proteases and oxidants that cause early breakdown of collagen and growth factors [3,13,20]. Furthermore, the chronic venous wounds would lead to defective angiogenesis and this could also prevent healing. Likewise, it would be valuable to study whether the increment of VEGF in CVU patients favors or not wound healing.

In acute wounds, bFGF shows initially elevated levels, just as a stimulus for angiogenesis, and then declines and remains low (while VEGF remains elevated) until the wound is granulated [6]. In CW, concentrations of bFGF were not detected in serum samples [18]. This agrees with the results of the present study where bFGF was below the detection threshold of the equipment. The observed increased content of serum VEGF along with low levels of serum bFGF matches with inflammatory phase detention expected for this kind of ulcers.

Deficiencies of collagen in CW tissues can be explained by many factors. Oxygen tension is usually below 40 mmHg, causing delay in the hydroxylation of proline and lysine and in subsequent collagen production [19]. Also, the accumulation of degraded collagen in the ECM could inhibit type I procollagen synthesis [21, 22, 23]. Finally, fibroblast senescence is
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Implicated in the wound chronicity [24, 25, 26, 27]. Senescent fibroblasts exhibit an ECM degradative phenotype and down regulate the type I collagen [28]. Moreover, transforming growth factor-β (TGF-b) is the most potent inducer of procollagen I gene expression identified in fibroblasts [27, 28, 29]. In CW tissues, the expression of TGF-b and its receptors is reduced with respect to acute wounds [30, 31]. This reduction could be due to the capture [32] or degradation by proteolytic enzymes [33]. In addition, the present study indicates that systemic causes for limited collagen deposition could be possible given that patient serum impaired normal deposition in Vero cells. At the tissue level, the low collagen content is reflected in the ulcer low ECM support for migration, proliferation and healing.

With regard to age, wound healing in healthy older people includes delayed angiogenesis and slower epithelialization [36]. It is possible that the range we have studied (59 to 90), which is biased towards higher ages, is not large enough to uncover age differences in VEGF content, proliferative effect or collagen deposition.

For Franks et al (1995, [39]) and Margolis et al (1999, [40]), wound size (on ulcers smaller than 30cm²) and wound duration were strong risk factors for the failure of venous leg ulcers to heal. They also studied diabetes mellitus, hypertension and deep vein thrombosis, and found they were not associated with failure of wound healing. A more recent work with comparable ulcer sizes [34], corroborates that the area is associated with non-healing. Moreover in Margolis’ work (1999, [40]), the effect of wound duration on non-healing depends on wound area and vice versa. Margolis concludes that “the risk of not healing initially increases rapidly for patients with large and old wounds, but the magnitude of the increased risk diminishes with the largest and oldest wounds”. In our study, 8 out of 12 ulcers were between 30 and 450 cm². VEGF content in patient sera seems to be consistent with the results previously described, supporting the hypothesis of a non-dependence between healing potential and the size, for ulcers of large areas. To confirm this differential behaviour of ulcer prognosis with size it would be necessary to carry out an exhaustive study, increasing the number of patients, and taking into account the serum factors and the risk factors identified so far. It would be desirable to carry an analysis of wound size in a continuous way. This would give a curve summarizing the behavior of the non-healing risk throughout the whole studied size range, without fixing an arbitrary cut-off point for the analysis. While there are many risk factors involved in the healing potential of the ulcer, the effort to identify one major factor would help to define the therapeutic. In this sense, the size of the ulcer is still relevant.

5. Conclusion

Within this pilot study, ulcer size did not influence the characteristics of sera from CW patients. The systemic effect of chronic ulcer contained in the inflammatory phase is evidenced through the following characteristics of patient serum: 1) presence of VEGF-165; 2) lack of bFGF; 3) presence of cell proliferation; and 4) absence of collagen deposition promotion.

Acknowledgments

We wish to thank the patients who made this study possible. This work was partially supported by a research grant provided by Universidad AbiertaInteramericana (Interamerican Open University), Buenos Aires, Argentina.

References

relationships of


in wound healing.

2001; 127:998–1008


Martin P. Wound healing - aiming for perfect skin regeneration. Science. 1997;27:75-81


Smith RK, Golledge J.A systematic review of circulating markers in primary chronic venous insufficiency. Phlebology. 2014;29(9); 570-579


HowladerMH, Smith PDC. Relationship of plasma vascular endothelial growth factor to CEAP clinical stage and symptoms in patients with chronic venous disease.Eur J Vasc Endovasc Surg. 2004; 27(1);89-93


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[17] HowladerMH, Smith PDC. Relationship of plasma vascular endothelial growth factor to CEAP clinical stage and symptoms in patients with chronic venous disease. Eur J Vasc Endovasc Surg. 2004; 27(1);89-93


[29] Lindahl GE, Chambers RC, Papakrivopoulou J, et al. Activation of fibroblast procollagen α1(I) transcription by mechanical strain is transforming growth factor-β-dependent and involves increased binding of ccaat-binding factor (CBF/NF-β) at the proximal promoter. J Biol Chem.2002;277;6153-6161


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[38] Kucukguven A, Khalil RA. Matrix metalloproteinases as potential targets in the venous dilation associated with varicose veins. *Curr Drug Targets.* 2013; 14(3); 287-324


[40] Margolis DJ, Berlin JA, Strom BL. Risk factors associated with the failure of a venous leg ulcer to heal. *Arch Dermatol.* 1999; 135(8); 920-926


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