Comano's (Trentino) thermal water interferes with the expression and secretion of vascular endothelial growth factor-A protein isoforms by cultured human psoriatic keratinocytes: A potential mechanism of its anti-psoriatic action

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Abstract. Thermal balneotherapy with Comano spa’s water (CW; Trentino, Italy) is used for psoriasis and other skin disorders but the mechanism(s) of action of this hypotonic water are unknown. Since skin psoriatic manifestations are thought to be angiogenesis-dependent, we assessed CW’s effects on the expression and release of VEGF-A protein isoforms by cultured human lesional keratinocytes isolated from skin biopsies performed in 9 patients. Confluent, psoriatic keratinocytes were exposed for 11 days to DMEM, whose chemicals had been dissolved in either deionised water (DW-DMEM, controls) or CW (CW-DMEM, treated cells). As detected by Western immunoblotting (WB), incubation in CW-DMEM elicited, with respect to DW-DMEM, an increase in intra-cellular and/or cell-bound L-VEGF-A189 and L-VEGF-A165 48 kDa protein isoforms with no concurrent change in L-VEGF-A121 and L-VEGF-A 165 45 kDa proteins. Moreover, WB analysis of the secreted VEGF-A (sVEGF-A) proteins showed that the 20 and 15 kDa bands corresponding to different VEGF-A isoforms were directly and remarkably reduced in keratinocyte-conditioned CW-DMEM vs. DW-DMEM. Thus, CW interferes with VEGF-A isoform expression and secretion by the psoriatic keratinocytes. These effects would reduce all VEGF-A-mediated angiogenic, vessel permeabilising, and chemotactic effects, thereby at least in part explaining the beneficial actions of CW balneotherapy on the clinical manifestations of psoriasis.

Introduction

Psoriasis, a chronic inflammatory dermatosis affecting approximately 2% of the Western population, is clinically marked by relapsing-remitting manifestations of well-defined, symmetrical erythematous plaques covered by scales. Although it has a genetic basis, the pathogenesis of psoriasis remains unclear. Currently, psoriasis is believed to be a T lymphocyte-driven disorder (1). However, at skin lesional sites, early prominent proliferation of a particular subset of endothelial cells lining the venous limbs of capillary plexuses in the upper dermal papillae leads to the formation of tortuous, dilated, inflamed, and hyper-permeable vessels (2,3). As these vascular changes precede the plaque’s epidermal hyperplasia and dermal infiltration by inflammatory cells (i.e. neutrophils, T lymphocytes, monocytes) (4,5), it has been surmised that psoriasis is an angioproliferative ailment depending upon the release of angiogenic molecules by the epidermis (6-11). Increased amounts of several angiogenic cytokines, including transforming growth factor-α (TGF-α), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-20, amphiregulin, platelet-derived endothelial cell growth factor/thymidine phosphorylase (TP), endothelial cell stimulating angiogenesis factor (ESAF), and VEGF-A are produced and secreted by the psoriatic keratinocytes (9,12-19).

VEGF-A, the archetypal and best characterized member of a family of angiogenic growth factors (20,21), acts both as an endothelial cell-specific growth factor and as a chief regulator of angiogenesis and vascular permeability in both physiologic and pathologic conditions (20-23). The human VEGF-A gene, placed on chromosome 6p21.3 (24), contains eight exons (25,26). Alternative splicing of the primary mRNA transcript encoded by the VEGF-A gene at the level of exons 6 and 7...
VEGF-A145, and VEGF-A121 (26-30). Such isoforms exhibit differing receptor affinities and heparin- and heparan sulphate-binding abilities (31), but the details of their distinctive biological functions remain to be elucidated (32). Most VEGF-A-producing cells, keratinocytes included (33), preferentially express VEGF-A189, VEGF-A165, and VEGF-A121 (34). Another isoform, VEGF-A167, is also widely expressed, but often confused with VEGF-A166 (30). Besides, all VEGF-A protein isoforms can be post-translationally modified through proteolysis by plasmin, thereby generating biologically active VEGF-A206 (29). Tissue specific patterns of VEGF mRNA splicing were demonstrated in rats, but the operative mechanisms controlling each splice variant’s expression are not understood (34). Recently, translational regulation of VEGF expression was demonstrated; in fact, the very long 5′-untranslated region (5′-UTR) of VEGF-A mRNA contains two independent internal ribosome entry sites (IRES A and B) that regulate the activity of two different initiation codons, respectively; AUG 1039 and CUG 499. This alternative translation initiation process allows the synthesis of different VEGF-A protein precursors with higher molecular weight called large VEGF-A (L-VEGF) isoforms beside the VEGF-A protein isoforms with a lower molecular weight (35-37). The L-VEGF-A isoforms are the CUG-translated forms and bear an NH2-terminal extension of 206 amino acids that is missing in the AUG-translated forms. The L-VEGF-A isoforms have an exclusive intracellular localization, but can be cleaved into two polypeptides and the resulting COOH-terminal products with the same apparent size as the AUG-translated forms are secreted from the cells just like the AUG-translated forms. Thus, active VEGF-A protein isoforms can be produced either by initiation at the AUG 1039 or through synthesis of L-VEGF-A followed by cleavage of the NH2-terminal extension of 206 amino acids (35-37). Active VEGF-A protein isoforms are produced and secreted as covalently linked homodimers (38), which bind via specific residues coded by exons 3 and 4 to the high affinity tyrosine kinase receptors, VEGF-R1 (flt-1 kinase) and VEGF-R2 (KDR kinase in man, flk kinase in mouse). These receptors are expressed only by endothelial cells (39-42). Dimerization and activation of VEGF-R1 and VEGF-R2 and of the VEGF-R2/VEGF-A165 co-receptor, neuropilin-1 (43), by their ligands is essential for endothelial cell migration, proliferation, differentiation, and survival (44). The importance of the roles of VEGF-A, VEGF-R1, and VEGF-R2 is stressed by the fact that the knockout of each gene, even at single allele level, has lethal consequences due to a defective development of the cardiovascular system in the mouse embryo (45-48).

VEGF-A released from cultured epidermal keratinocytes acts as an effective mitogen in endothelial cells of human dermal microvessels (49). Most importantly, targeted over-expression of VEGF in mouse skin keratinocytes results in an inflamatory condition chronically exhibiting all the traits proper of human psoriasis (50,51). In this model, VEGF-A blockage effectively reversed all the abnormalities observed (51). Being a pleiotropic cytokine/chemokine, VEGF i)

Table I. Components of Comano’s water.*

<table>
<thead>
<tr>
<th>Ions</th>
<th>mM</th>
</tr>
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<tbody>
<tr>
<td>Sodium</td>
<td>0.182</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.026</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.010</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.440</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>6.340</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.047</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>0.144</td>
</tr>
<tr>
<td>Silicon</td>
<td>0.163</td>
</tr>
<tr>
<td>Fluorine</td>
<td>0.048</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.0002</td>
</tr>
<tr>
<td>Aluminum</td>
<td>0.00246</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.00064</td>
</tr>
<tr>
<td>Iron</td>
<td>0.0038</td>
</tr>
<tr>
<td>Copper</td>
<td>0.0017</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.00143</td>
</tr>
<tr>
<td>Strontium</td>
<td>0.00605</td>
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*This water is hypotonic as its dry residue amounts to only 190 mg/l.

stimulates the expression of ICAM-1, VCAM-1, and E-selectin in human umbilical vein endothelial cells (HUVECs) via the activation of transcription factor NF-κB (52); ii) induces the expression of IL-8, a chemokine that powerfully modulates the transcapillary diapedesis of neutrophils (53); and iii) brings about the activation and chemotaxis of VEGF-R1-expressing monocytes (54,55). Hence, VEGF-A may act as a significant causal agent in human psoriasis; a notion having considerable therapeutic implications. In fact, agents used for the topical treatment of psoriasis, such as calcipotriol (a vitamin D3 analogue), retinoids (tazarotene), and cyclosporin A, are able to interfere, amongst other things, with VEGF-A production and release by keratinocytes (56-58).

Comano (Trentino, Italy) spa’s water (CW) is a thermal hypotonic water containing various electrolytes (Table I). The major dermatological diseases so far treated via CW balneotherapy are psoriasis and atopic dermatitis (59). Other dermatoses also cared for with CW include contact dermatitis, seborrhoeic dermatitis, lichen planus, and palmoplantar keratosis (56). Previous in vivo studies showed the effectiveness of CW balneotherapy in the treatment of psoriasis, since it both significantly lessened hyperkeratosis, acantosis, and dermal papillomatosis and improved skin hydration (56). It must be recalled here that the permeability barrier of normal epidermis is severely disturbed in psoriatic skin (60,61), and that bathing in hypotonic salt solutions triggers anti-inflammatory effects in lesional skin sites (62). However, the mechanisms through which the clinical signs of psoriasis (and of the other above mentioned skin disorders) are improved by means of CW balneotherapy have not as yet been clarified. To further understand the mechanism(s) involved in the therapeutic effectiveness of CW in psoriasis, we elected to investigate how CW would affect VEGF-A protein isoform expression.
and secretion on the part of cultured human adult psoriatic keratinocytes. In this report, we will show that the exposure of such cells to CW used instead of deionised water (DW) to dissolve the constituents of the DMEM medium both shifts the protein isoform expression towards the tightly cell-associated L-VEGF-A_189 isoform and diminishes the secretion of soluble VEGF-A proteins into the medium. These findings are consistent with CW being endowed with a complex antiangiogenic and hence anti-psoriatic therapeutic potential.

Materials and methods

Culture of psoriatic keratinocytes. Psoriatic epidermal keratinocytes were isolated from skin biopsy samples taken from 9 patients. After rapidly reaching the laboratory, the biopsies were incubated at 4˚C overnight in a dispase II solution (0.25% w/v; Roche, Milan, Italy). Weak enzymatic digestion allowed the epidermis (as a single lamina) to easily detach from the underlying dermis and subcutaneous tissue. By incubation in a trypsin solution (0.25% w/v), the isolated thin epidermal sheet was carefully and swiftly fragmented. Trypsin’s action was next inhibited by adding an excess of serum, and the suspension of isolated cells was soon spun down at 600 rpm for 10 min at 4˚C. The supernatant was decanted, the pellet re-suspended, and the living cells counted in a Neubauer chamber. Keratinocytes were next seeded into plastic flasks precoated with a feeder-layer of preirradiated 3T3-J2 cells. To expand the keratinocyte population, MCDB153:1 medium [consisting of three parts of Dulbecco’s modified Eagle’s medium (DMEM) and one part of F12 medium; Sigma-Aldrich, Milan, Italy] was used, to which fetal bovine serum (FBS; 10% v/v; Bio-Whittaker Europe, Belgium), antibiotics (solution of penicillin-streptomycin 1% w/v; BioWhittaker Europe), epidermal growth factor (EGF; 0.1 μg/ml; PeproTech, UK), insulin (20 ng/ml; PeproTech), and hydrocortisone (0.5 μg/ml; PeproTech) were added. This medium was replaced every two days with fresh samples of the same medium. Human psoriatic keratinocytes proliferated rapidly starting from minute clusters and formed a single layer of small and highly adherent epithelial cells. They had a mitotic doubling time of approximately 48 h.

Experimental protocol. Psoriatic keratinocytes were detached from the culture flasks by means of a mild trypsin treatment and next seeded at 100% confluence density into wells containing either DW- or CW-DMEM (25 μl) were boiled in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% w/v SDS, 5% w/v β-mercaptoethanol, 10% v/v glycerol, 0.002% w/v bromphenol blue) and electrophoresed in 10% w/v SDS-polyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories, Hercules, CA). To immunodetect VEGF-A protein isoforms, the blots were probed with a rabbit polyclonal antibody (final dilution 1.0 μg· ml⁻¹; Santa Cruz). Blots were next incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz), and stained with BCIP/NBT liquid substrate reagent (Sigma). Developed blots were photographed with an Olympus 3300™ digital camera, and the determination of the Mr and the densitometric analysis of each specific protein band were carried out using SigmaGel™ software (Jandel Corp., Erkrath, Germany).

Statistical analysis. One-way analysis of variance (ANOVA) with post hoc Bonferroni test was used to compare mean values and a significance level of 0.05 was chosen.

Results

Immunocytochemistry. An antibody recognizing the 189, 165, and 121 amino acid protein isoforms of VEGF-A known to be expressed by human keratinocytes (33) decorated subcellular cytoplasmic, but not nuclear, structures of all the human psoriatic keratinocytes cultured for up 11 days in either DW-DMEM or CW-DMEM. Notably, the intracellular or cell-bound amount of the fluorescent material decreased with time in the DW-DMEM-incubated (control) cells, whereas it increased with time in the CW-DMEM-exposed (treated) keratinocytes (Fig. 1).
glycosylation levels), and L-VEGF-A189 (55 kDa) isoforms (Fig. 2A) (35-37, 64). The densitometric analysis of the specific bands showed that, in cells exposed to both media, the amounts of L-VEGF-A189, the most intensely expressed isoform, and VEGF-A48 kDa underwent significant time-related changes (Fig. 2B and C). In fact, L-VEGF-A189 48 kDa levels at day 3 were 4-fold higher (p<0.001) in control (DW-DMEM) than in treated (CW-DMEM) keratinocytes, but the opposite was true 8 days later, when L-VEGF-A189 levels had fallen to one-fourth of their starting values in DW-DMEM-kept (control) cells, yet had increased more than 4-fold in CW-DMEM-exposed (treated) keratinocytes, but the opposite was true 8 days later, when L-VEGF-A189 levels had fallen to one-fourth of their starting values in DW-DMEM-kept (control) cells, yet had increased more than 4-fold in CW-DMEM-exposed (treated) keratinocytes (p<0.001 vs. day 3 values in either instance) (Fig. 2B). Though being identical at day 3, VEGF-A48 kDa levels fell significantly in the keratinocytes grown in DW-DMEM, while increasing in the cells kept in CW-DMEM (Fig. 2C). Evaluating the areas under the respective curves made it clear that, between days 3 and 11, the total intracellular and/or cell-bound levels of L-VEGF-A189 and VEGF-A48 kDa were greater in CW-DMEM-incubated than in DW-DMEM-exposed keratinocytes (Fig. 3A).

Concurrently, the point by point and total intracellular or cell-bound levels of L-VEGF-A165 45 kDa and VEGF-A121 48 kDa were greater in CW-DMEM, while increasing in the cells kept in DW-DMEM (Fig. 2D and E, Fig. 3A).

Effects of exposure to DW-DMEM or to CW-DMEM on secreted VEGF-A (sVEGF-A) proteins. To assess whether the exposure to CW-DMEM changed the secretion pattern of VEGF-A proteins, the dyalised protein fractions of keratinocyte-conditioned media of both kinds were analysed by WB under reducing conditions. In keeping with the findings of Ballanu et al (33), three sVEGF-A monomeric protein bands of 15, 20, and 24 kDa respectively were detected in either group of samples. As VEGF-A48 kDa is known to remain cell-associated (65), the three bands thus detected are likely to correspond to differentially glycosylated VEGF-A165 and VEGF-A121 isoforms (33, 66, 67). These bands were subjected to densitometric analysis (Fig. 4).

The sVEGF-A24 kDa band was the thickest one in either DW- or CW-DMEM, keratinocyte-conditioned media, in both of which, excepting at day 11, it behaved similarly (Fig. 4A and B). There occurred a peak of sVEGF-A 24 kDa protein at day 5 (a 2-fold increase over starting levels: p<0.001), followed by a rapid drop to approximately one-third of the initial level at day 7 (p<0.001 in either instance vs. day 3 values) in both kinds of medium. But, at day 11, the amount of sVEGF-A 24 kDa protein was significantly less (-31%, p<0.05) in CW-DMEM than in DW-DMEM samples (Fig. 4B). However, since this decrease was small and delayed, the total amount of sVEGF-A 24 kDa protein, as assessed by evaluating the areas under the respective curves, did not significantly differ in relation to the type of growth medium considered (Fig. 3B).

The sVEGF-A 20 kDa protein was the second most abundant one secreted into either DW- or CW-DMEM, but its behaviour clearly differed according to the type of medium used (Fig. 4A and C). In DW-DMEM (controls), sVEGF-A 20 kDa protein levels peaked at day 5 (at approximately twice that of starting levels: p<0.001), but rapidly fell to approximately half of its opening values (p<0.001) at days 7 and 11 (Fig. 4C). However, in CW-DMEM (treated cells), sVEGF-A 20 kDa protein levels at day 3 were much lower (-60%, p<0.001) than in DW-DMEM (Fig. 4C). Moreover, sVEGF-A 20 kDa protein levels were also lower at day 5 (-68%, p<0.001)
vs. corresponding DW-DMEM levels and kept falling (at day 11, -55%, \(p<0.001\) vs. DW-DMEM values) (Fig. 4C). Thus, with respect to DW-DMEM medium, the amount of sVEGF-A 20-kDa protein in CW-DMEM was severely reduced and, between days 3 and 11, was significantly less (-56.4%, \(p<0.001\)) than in DW-DMEM samples (Fig. 3B).

The sVEGF-A 15-kDa protein was the least abundant one secreted into either DW-DMEM or CW-DMEM and, just like the sVEGF-A 20-kDa protein, behaved quite differently according to the kind of growth medium employed. In DW-DMEM (controls), sVEGF-A 15-kDa protein peaked at day 5 (a 3-fold surge over starting values; \(p<0.001\)) to fall between days 7 and 9 to levels slightly higher than the initial ones (Fig. 4D). However, in CW-DMEM, sVEGF-A 15-kDa protein levels were lower from the outset than in the alternative medium and remained such up to day 11 (e.g., -70%, \(p<0.001\)) at day 5 (Fig. 4D). Thus, the total amount of sVEGF-A 15-kDa protein released between days 3 and 11 into the CW-DMEM...
was approximately half \( (p<0.001) \) of that released into the DW-DMEM (Fig. 4B). No band with Mr lower than 15 kDa, which would have corresponded to sVEGF-A110, was detected in any of the medium samples examined.

**Discussion**

VEGF fits in the dimeric cysteine-knot growth factor superfamily (68). VEGF-A-producing cells, keratinocytes included, simultaneously express several VEGF-A protein isoforms, amongst which VEGF-A121 and VEGF-A165 appear to predominate in normal tissues (26,33) and in psoriatic scales (67). According to the present findings, cultured human psoriatic keratinocytes express the same three isoforms, i.e. L-VEGF-A189, L-VEGF-A165, and L-VEGF-A121, while secreting active though differently glycosylated VEGF-A165, and VEGF-A121, as normal keratinocytes do (33).

VEGF-A189 retains sequences encoded by both exons 6 and 7 (26), and its binding affinity for heparin and heparan sulphates is higher than that of VEGF-A165 or VEGF-A145 (69). Because of such an affinity, secreted VEGF-A189 is sequestered on heparan sulphate proteoglycans (HSPGs) at the cell surface and thus remains tightly associated to the producing cells (65,69). Moreover, VEGF-A189 is a much less effective angiogenic factor than either VEGF-A165 or VEGF-A121 (70) even because VEGF-A189 receptor-binding sequences are masked when the protein is normally folded. In fact, recombinant VEGF-A189 cannot bind VEGF-R2 receptor and is thereby unable to directly stimulate endothelial cell growth (31).

Our immunocytochemistry and WB findings show that a protracted incubation in CW-DMEM causes a slow yet progressive intracellular and/or cell-bound accumulation of both L-VEGF-A189 and L-VEGF-A165 48-kDa isoforms. It should be noticed that, in quantitative terms, the expression of L-VEGF-A189 by the psoriatic keratinocytes is at least 4-fold greater than that of L-VEGF-A165 48 kDa (Fig. 2B and C, Fig. 3A). Hence, the exposure to CW is likely to favour, by mechanisms at present not understood, the mRNA alternative splicing leading to the synthesis of L-VEGF-A189 and possibly of the highly glycosylated L-VEGF-A165 48-kDa isoform. By itself, this increased expression of tightly keratinocyte-bound VEGF-A189 and VEGF-A165 (65) might result in a lesser direct stimulation of endothelial cell growth (31). Interestingly, a dynamic shift towards the expression of VEGF-A189 has been observed to occur in the human uterus under the effects of progesterone (72). It has been surmised that bound VEGF isoforms might provide a reserve of growth factor available in its biologically active forms, both as endothelial cell mitogens and vascular permeability-enhancing agents, however only after their effective cleavage by heparinase or uPA (65,72).

VEGF-A165 isoform contains 15 basic amino acids encoded by exon 7 and its affinity for heparin is moderate (31,71). VEGF-A165 is actively secreted by the producing cells and most of it (i.e. 50-70%) associates with extracellular matrix (ECM) and cell surfaces due to its interactions with HSPGs (65). On the other hand, VEGF-A121 is a weakly acidic molecule as it is devoid of exon 7-encoded basic amino acids. Since it binds neither heparin (25) nor ECM (69), VEGF-A121 is soluble and rapidly released (65). Notably, VEGF-A121 requires cell surface HSPGs to bind VEGF receptors (73) and, compared to VEGF-A165, is a weaker mitogen for the endothelial cells (71). Our findings show that, at variance with VEGF-A189 and VEGF-A165 48 kDa, VEGF-A165 45 kDa and VEGF-A121 do not accumulate inside keratinocytes.
or remain attached to the surfaces of CW-DMEM-incubated psoriatic keratinocytes. Yet, with respect to the DW-DMEM, the release of differentially glycosylated 24-, 20-, and 15-kDa sVEGF-A proteins (33,66,67) likely pertaining to differently glucosylated VEGF-A165 and VEGF-A121 isoforms (33) into the CW-DMEM is cut by more than half. Therefore, our results indicate that exposure to CW does not favour the synthesis and secretion of both VEGF-A165 and VEGF-A121 proteins. These effects would translate into a diminished direct angiogenic stimulation on the part of psoriatic keratinocytes (31).

Overall, the present results support the view that CW balneotherapy is likely to interfere at the level of psoriatic skin lesions, where the permeability barrier is seriously compromised (60,61), with the protein synthesis and secretion of the three main VEGF-A protein isoforms expressed by the psoriatic keratinocytes. CW exposure would shift the balance in favour of the tightly cell-associated and by itself non-mitogenic (for the endothelial cells) L-VEGF-A189 and L-VEGF-A165 48-kDa isoforms at the expense of the promptly secreted and direct endothelial cell mitogens, VEGF-A165 and
VEGF-A<sub>165</sub> isoforms. Besides locally decreasing the intensity of angiogenic stimulation, CW also induces VEGF-A-elicited alteration of vascular permeability (4); ii) the expression of the neutrophil chemokine, IL-8 (53); iii) VEGF-A’s own chemotactic effects on monocytes (54,55,74); and iv) the expression of ICAM-1, VCAM-1, and E-selectin in human endothelial cells (52).

Further lines of evidence that we are presently gathering show that in vitro exposure to CW-DMEM also interferes with the increased expression and secretion of at least two more angiogenic cytokines, IL-1 and IL-6 (9,12-19), on the part of human psoriatic keratinocytes (unpublished data). Conceivably, such complex anti-angiogenic effects brought about by CW balneotherapy would justify, from a pathophysiological standpoint, at least part of its known beneficial effects on the clinical manifestations of psoriasis (59), and concurrently rule out the possibility that such benefits are of the placebo kind.

References

19. Gimbrone MA Jr, Obin MS, Brock AF, et al: Endothelial inter-
33. Bessar M, Boulard M, Hieblot C, Zanibellato C, Iacovoni JS, Lestalle E and Lestalle E: Endothelial inter-


