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 intracellular and/or cell-bound L-VEGF-A_{189} and L-VEGF-A_{165} 48 kDa protein isoforms with no concurrent change in L-VEGF-A_{121} 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.

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Abbreviations: CW, Comano's thermal water; DW, deionized water; HSPGs, heparan sulphate proteoglycans; IL, interleukin; VEGF-A, vascular endothelial growth factor-A; L-VEGF-A, large VEGF-A; sVEGF-A, secreted VEGF-A; WB, Western immunoblotting

Key words: psoriasis, human keratinocytes, vascular endothelial growth factor-A, cytokines, chemokines, Comano's thermal water

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

(25) produces several mature mRNAs that are translated into many protein splice variants, according to their amino acid numbers (once the signal sequence has been cleaved), VEGF-A_{206}, VEGF-A_{189}, VEGF-A_{183}, VEGF-A_{165}, VEGF-A_{148}, VEGF-A_{145}, and VEGF-A_{121} (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-A_{189}, VEGF-A_{165}, and VEGF-A_{121} (34). Another isoform, VEGF-A_{183}, is also widely expressed, but often confused with VEGF-A_{189} (30). Besides, all VEGF-A protein isoforms can be post-translationally modified through proteolysis by plasmin, thereby generating biologically active VEGF-A_{110} (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 NH_2-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 NH_2-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-A_{165} 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 overexpression of VEGF in mouse skin keratinocytes results in an inflammatory 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) 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).

Table I. Components of Comano's water.[a]

Ions	mM
Sodium	0.182
Potassium	0.026
Magnesium	1.010
Calcium	2.440
Bicarbonate	6.340
Chloride	0.047
Sulfuric acid	0.144
Silicon	0.163
Fluorine	0.048
Lithium	0.0002
Aluminum	0.00246
Manganese	0.00064
Iron	0.0038
Copper	0.0017
Zinc	0.00143
Strontium	0.00605

[a]This water is hypotonic as its dry residue amounts to only 190 mg/l.

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 anti-angiogenic 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; BioWhittaker 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 DMEM medium, whose chemical constituents had been dissolved in DW (controls in DW-DMEM) or CW-DMEM, in which DW had been substituted with CW. Between days 3 and 11 of exposure to either medium, the keratinocytes and the cell-conditioned media were sampled and their contents of VEGF-A protein isoforms were analysed by immunocytochemistry and Western immunoblotting (WB).

Immunocytochemistry. At chosen time points, psoriatic keratinocytes exposed to either DW- or CW-DMEM were fixed with absolute methanol at -20˚C for 10 min, washed twice with PBS and permeabilised in 0.1% Triton X-100 at room temperature for 15 min. Then the cells were washed with PBS-FBS (1%) (Cambrex BioScience, Milan, Italy) at room temperature for 1 h and next incubated for 1 h at 37˚C with a rabbit polyclonal anti-VEGF-A antibody (final dilution 10 $\mu g \cdot ml^{-1}$; Santa Cruz Biotechnology, Inc., Germany). Next, keratinocytes were washed three times with PBS-FBS (1%) and incubated for 1 h at room temperature in the dark with a specific secondary antibody (1:100 dilution) conjugated with Alexa Fluor-488 (Molecular Probes, Invitrogen Corp., Carlsbad, CA). Control cells not exposed to the primary antibody were always run in parallel. The cells were finally examined under an LSM 510 confocal microscope (Carl Zeiss S.p.A., Milan, Italy).

Western immunoblotting (WB). WB was performed on cellular lysates and on dialysed samples of cell-conditioned growth media of either kind. Psoriatic keratinocytes were scraped into cold PBS and sedimented at 200 x g for 10 min. The sedimented cells were homogenized in T-PER™ tissue protein extraction reagent (Pierce Chemical Co., Rockford, IL) containing a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Monza, Italy). The protein contents of the samples were assayed by Bradford's method (63) using bovine serum albumin as a standard. Equal amounts (10 or 20 μg) of proteins from each cell lysate or cell-conditioned 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 ß-mercapto-ethanol, 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 $\mu g \cdot ml^{-1}$; 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 M_r 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 to 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).

Effects of exposure to DW-DMEM or CW-DMEM on intracellular and/or cell-bound VEGF-A protein isoforms. WB analysis under reducing conditions showed that total proteins extracted from cultured human psoriatic keratinocytes kept in either DW-DMEM or CW-DMEM contained L-VEGF-A_{121} (35 kDa), L-VEGF-A_{165} (45 and 48 kDa bands due to different

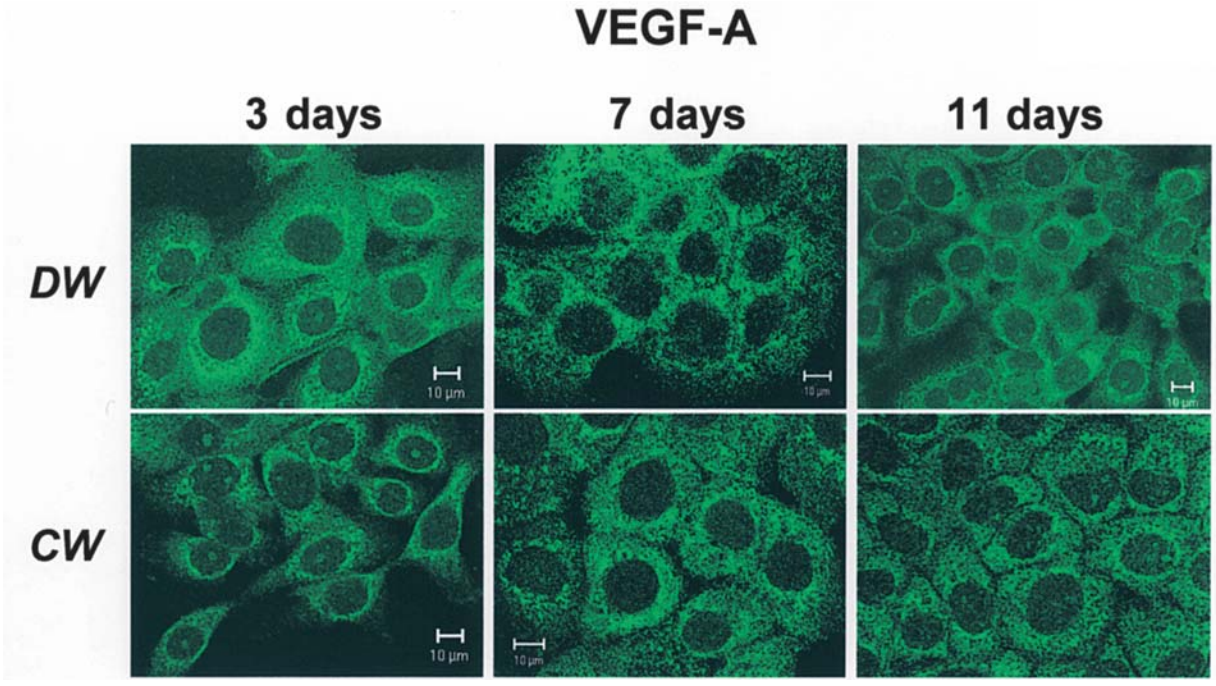

Figure 1. Intracellular and/or cell-bound VEGF-A proteins were down-regulated with time in human psoriatic keratinocytes cultured in DW-DMEM (controls), whereas they were up-regulated when the same cells were incubated in CW-DMEM. After 3, 7 and 11 days *in vitro*, the cells were processed for immunocytochemistry with an anti-VEGF-A antibody (for details see Materials and methods). The pictures shown are representative of 9 experiments.

glycosylation levels), and L-VEGF-A_{189} (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-A_{189}, the most intensely expressed isoform, and VEGF-A_{165} 48 kDa underwent significant time-related changes (Fig. 2B and C). In fact, L-VEGF-A_{189} 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-A_{189} 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-A_{165} 48 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-A_{189} and VEGF-A_{165} 48 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-A_{165} 45 kDa and L-VEGF-A_{121} did not significantly change and did not differ between keratinocytes kept either in DW-DMEM or in CW-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 growth media of both kinds were analysed by WB under reducing conditions. In keeping with the findings of Ballaun *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-A_{189} isoform is known to remain cell-associated (65), the three bands thus detected are likely to correspond to differentially glycosylated VEGF-A_{165} and VEGF-A_{121} isoforms (33,66,67). These bands were subjected to densitometric analysis (Fig. 4).

The sVEGF-A 24 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 lesser (-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%, $p<0.001$)

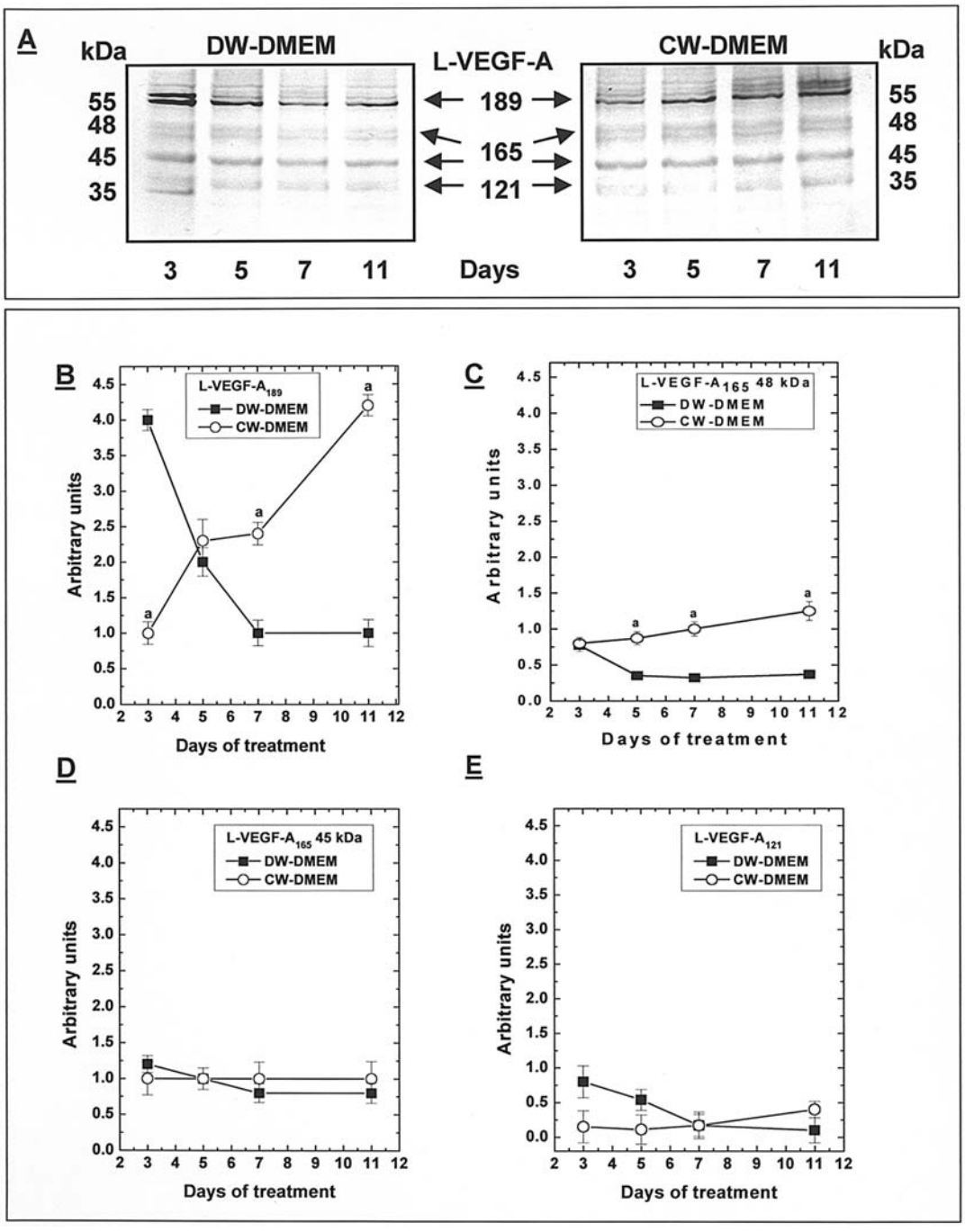

Figure 2. CW up-regulates the levels of intracellular and/or cell-bound L-VEGF-A_{189} and L-VEGF-A_{165} 48-kDa protein isoforms without affecting the intracellular and/or cell-bound levels of L-VEGF-A_{165} 45 kDa and L-VEGF-A_{121} in cultured human adult psoriatic keratinocytes. (A) Immunoblots were prepared from the protein extracts of psoriatic keratinocytes incubated in either CW-DMEM or DW-DMEM and sampled at devised time points. For technical details, consult the Materials and methods. The blots shown are representative of 9 experiments. (B-E) Densitometric analyses of the specific protein bands corresponding to each L-VEGF-A isoform detected in the protein extracts from psoriatic keratinocytes incubated in either CW-DMEM or DW-DMEM samples. Points on the curves are means ± SEM of 9 distinct experiments. Levels of statistical significance vs. corresponding DW-DMEM (controls) values: [a]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

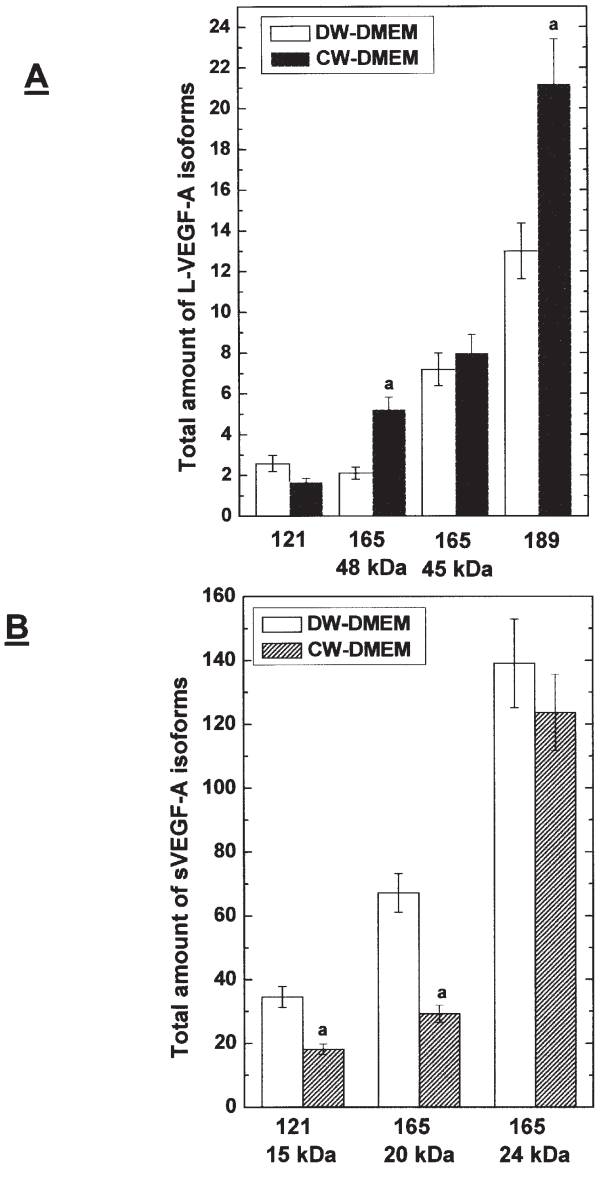

Figure 3. Incubation in CW-DMEM up-regulates the total (3-11 days) intracellular and/or cell-bound expression of L-VEGF-A_{189} and L-VEGF-A_{165} 48-kDa isoforms (A), while curtailing the total (3-11 days) secretion of the 20- and 15-kDa sVEGF-A proteins likely to pertain to differently glycosylated VEGF-A_{165} and VEGF-A_{121} proteins (33) (B). Total values were obtained by evaluating the areas under the corresponding curves of Fig. 2B-E and Fig. 4B-D. Levels of statistical significance vs. corresponding DW-DMEM values: $^{a}p<0.001$.

was approximately half ($p<0.001$) of that released into the DW-DMEM (Fig. 4B). No band with M_r lower than 15 kDa, which would have corresponded to sVEGF-A_{110}, 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-A_{121} and VEGF-A_{165} 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-A_{189}, L-VEGF-A_{165}, and L-VEGF-A_{121}, while secerning active though differently glycosylated VEGF-A_{165}, and VEGF-A_{121}, as normal keratinocytes do (33).

VEGF-A_{189} 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-A_{165} or VEGF-A_{145} (69). Because of such an affinity, secreted VEGF-A_{189} is sequestered on heparan sulphate proteoglycans (HSPGs) at the cell surface and thus remains tightly associated to the producing cells (65,69). Moreover, VEGF-A_{189} is a much less effective angiogenic factor than either VEGF-A_{165} or VEGF-A_{121} (70) even because VEGF-A_{189} receptor-binding sequences are masked when the protein is normally folded. In fact, recombinant VEGF-A_{189} cannot bind VEGF-R2 receptor and is thereby unable to directly stimulate endothelial cell growth (31). To mature and become able to bind VEGF-R2 and act as an endothelial cell mitogen, secreted VEGF-A_{189} must be extracellularly cleaved by urokinase-type plasminogen activator (uPA) or plasmin into VEGF-A_{110} (65,71). However, no 110-amino acid sVEGF-A isoform was released into the growth media conditioned by human normal or psoriatic keratinocytes (33, and present findings).

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-A_{189} and L-VEGF-A_{165} 48-kDa isoforms. It should be noticed that, in quantitative terms, the expression of L-VEGF-A_{189} by the psoriatic keratinocytes is at least 4-fold greater than that of L-VEGF-A_{165} 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-A_{189} and possibly of the highly glycosylated L-VEGF-A_{165} 48-kDa isoform. By itself, this increased expression of tightly keratinocyte-bound VEGF-A_{189} and VEGF-A_{165} (65) might result in a lesser direct stimulation of endothelial cell growth (31). Interestingly, a dynamic shift towards the expression of VEGF-A_{189} 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-A_{165} isoform contains 15 basic amino acids encoded by exon 7 and its affinity for heparin is moderate (31,71). VEGF-A_{165} 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-A_{121} 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-A_{121} is soluble and rapidly released (65). Notably, VEGF-A_{121} requires cell surface HPSGs to bind VEGF receptors (73) and, compared to VEGF-A_{165}, is a weaker mitogen for the endothelial cells (71). Our findings show that, at variance with VEGF-A_{189} and VEGF-A_{165} 48 kDa, VEGF-A_{165} 45 kDa and VEGF-A_{121} do not accumulate inside

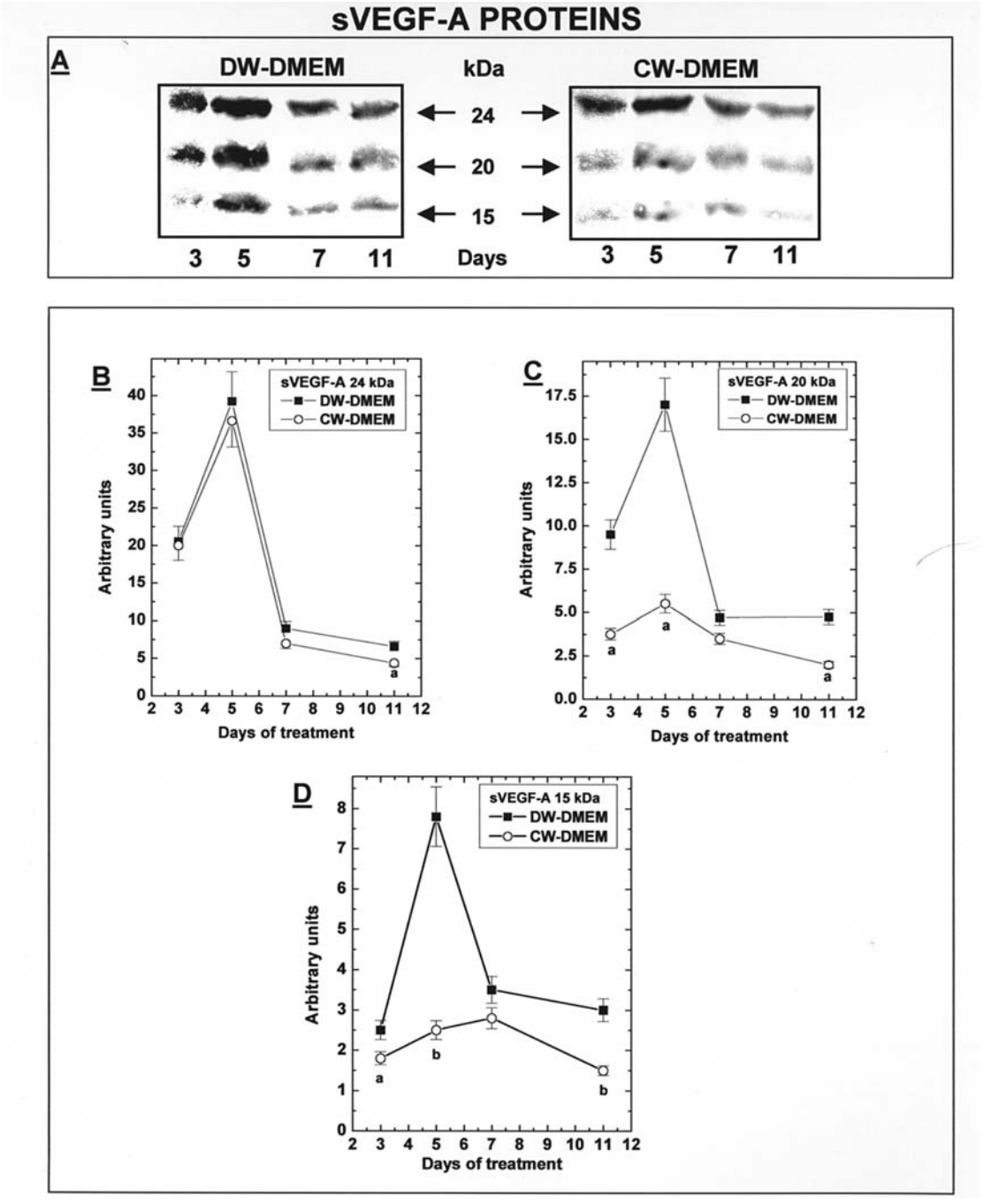

Figure 4. Incubation in CW-DMEM rapidly and markedly reduced the secretion of 20-kDa and 15-kDa sVEGF-A proteins likely to pertain to differently glycosylated VEGF-A_{165} and VEGF-A_{121} isoforms (33). By contrast, 24-kDa sVEGF-A_{165} protein starts decreasing significantly only by the 11th day of exposure to CW-DMEM. (A) Immunoblots of the proteins extracted from keratinocyte-conditioned CW-DMEM or DW-DMEM media sampled at devised time points. Relative masses (M_r) are indicated. The blots shown are representative of 9 experiments. (B-D) Densitometric analyses of the specific sVEGF-A protein bands secreted into keratinocyte-conditioned CW-DMEM or DW-DMEM samples. Points on the curves are means ± SEM of 9 distinct experiments. Levels of statistical significance vs. corresponding DW-DMEM (controls) values; B and C, $^{a}p<0.001$; D, $^{a}p<0.01$; $^{b}p<0.001$.

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-A_{165} and VEGF-A_{121} 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-A_{165} and VEGF-A_{121} 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-A_{189} and L-VEGF-A_{165} 48-kDa isoforms at the expense of the promptly secreted and direct endothelial cell mitogens, VEGF-A_{165} and

$VEGF\text{-}A_{121}$ isoforms. Besides locally decreasing the intensity of angiogenic stimulation, CW also reduces i) 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 patho-physiological 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.

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