Comano's (Trentino) thermal water interferes with tumour necrosis factor-α expression and interleukin-8 production and secretion by cultured human psoriatic keratinocytes: Yet other mechanisms of its anti-psoriatic action

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Abstract. Thermal balneotherapy with Comano's spa water (CW; Trentino, Italy) is beneficial for psoriasis and other skin disorders but its operative mechanisms are largely unknown. Previously, we showed that CW interferes with the production and secretion of IL-6 and various VEGF-A isoforms and with CK-16 expression by cultured human psoriatic keratinocytes. In this study, confluent cultures of epidermal keratinocytes isolated from the lesional areas of 9 psoriatic patients were exposed for 11-13 days to DMEM, whose chemicals had been dissolved in either deionised water (DW-DMEM, controls) or CW (CW-DMEM, treated cells), in order to assess the expression and secretion of TNF- α and IL-8 by such cells. The results gained by means of immunocytochemistry, Western immunoblotting (WB), and ELISA assays showed that CW exposure significantly downregulated the intracellular levels of TNF- α , a key inducer of IL-8, IL-6, and other chemokines. However, no assayable TNF- α secretion occurred in keratinocyte-conditioned DWand CW-DMEM samples. Moreover, the intracellular levels and secretion rates of IL-8 were also markedly reduced in the protein extracts and conditioned media of CW-DMEMincubated keratinocytes. Notably, the most effective

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inhibition of IL-8 secretion was elicited by a 25% CW fraction in the DMEM. Altogether, our findings indicate that by attenuating at lesional skin sites the deregulated production and secretion of a cascade of several cytokines and chemokines (e.g. TNF- α , IL-8, IL-6, and various VEGF-A isoforms), and by offsetting the keratinocytes' abnormal differentiation program entailing CK-16 expression, CW balneotherapy may beneficially influence 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. Albeit genetically founded, the pathogenesis of psoriasis remains unclear (1). Currently, psoriasis is believed to be a T lymphocyte-driven disorder (2). However, the formation of tortuous, dilated, inflamed, and hyper-permeable venous limbs of capillary plexuses in the upper dermal papillae precedes the plaque's epidermal hyperplasia and dermal infiltration by inflammatory cells (i.e. neutrophils, T lymphocytes, monocytes) (3-6). It has been suggested that psoriasis is an angioproliferative ailment due to the local release of angiogenic molecules by the epidermis (7-10). Local fibroblast activation and increased production and release by keratinocytes of several cytokines and chemokines, such as interleukin-1 (IL-1), tumour necrosis factor- α (TNF- α), IL-6, IL-8, IL-20, vascular endothelial growth factor-A (VEGF-A) isoforms, endothelial cell stimulating angiogenesis factor (ESAF), transforming growth factor- α (TGF- α), amphiregulin, and platelet-derived endothelial cell growth factor/thymidine phosphorylase (TP), are also typical features of the disease (9,11-18).

Several lines of evidence indicate that IL-8 is a C-X-C chemokine partaking in the psoriatic cytokine network (15,19-24) and being thus involved in the pathogenesis of psoriasis (15,19-26). In psoriasis, IL-8 acts as a factor promoting epidermal hyperplasia (27), as a strong chemo-

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Abbreviations: CW, Comano's thermal water; DW, deionised water; IL-8, interleukin-8; sIL-8, secreted IL-8; TNF- α , tumour necrosis factor- α , WB, Western immunoblotting

Key words: chemokines, Comano's thermal water, tumour necrosis factor- α , cytokines, human keratinocytes, interleukin-8, psoriasis

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

^aThis water is hypotonic as its dry residue amounts to only 190 mg/l.

attractant for T lymphocytes, neutrophils, basophils, and mastcells (28-32), and as a stimulant of dermal neoangiogenesis (20). Psoriatic lesional skin contains high amounts of IL-8 mRNA and protein (19,22,24,25). IL-8 synthesis on the part of epidermal, dermal and infiltrating inflammatory cells is stimulated by several cytokines, such as IL-1, TNF- α , GM-CSF, and IFN- γ (19,26,33). However, keratinocytes and neutrophils are IL-8's main sources (22,24). A heightened expression of IL-8's receptor also occurs in psoriatic lesional epidermis (23,26). Thus, an autocrine loop involving IL-8 and its receptor favours the setting off of the psoriatic epidermal hyperplasia (26,27). Serum levels of IL-8, like those of TNF- α , IL-6, IL-12, IL-18 and IFN- γ , are much higher in patients with active psoriasis than in healthy controls (34,35).

As mentioned above, psoriatic lesional skin overexpresses TNF- α , a key cytokine involved in the pathogenetic mechanisms sustaining several inflammatory skin illnesses (12,15,20,33,35-38). Resident dermal cells (fibroblasts, mast-cells) and infiltrated antigen-presenting cells (APCs), macrophages, and T lymphocytes also express TNF- α (38). The early-occurring TNF- α overproduction and hyper-secretion in psoriasis vulgaris, and in its recalcitrant generalised pustular (von Zumbusch) form (39-41) favours the cellular infiltration of epidermal and dermal layers by eliciting the expression of intercellular adhesion molecules such as ICAM-1, V-CAM-1, and E-selectin by the keratinocytes and endothelial cells (42). In synergism with IFN- γ and IL-1, TNF- α acts as a proinflammatory agent by up-regulating cytokines and chemokines such as IL-8 (19,20,43), IL-6, IL-1, leukemia inhibitory factor (LIF), GM-CSF (43), and TGF- α (19). Moreover, TNF- α stimulates the production of arachidonate cascade derivatives

such as leukotriene B_4 (LTB₄) and prostaglandin E_2 (PGE₂), and of oxygen radicals (superoxide anions) (43). TNF- α expression is up-regulated by several cytokines, such as IL-1, IL-2, GM-CSF, and IFN- γ (43).

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 (44). Other dermatoses also cared for with CW include contact dermatitis, seborrhoeic dermatitis, lichen planus, and palmo-plantar keratosis (44). Previous in vivo studies showed the effectiveness of CW balneotherapy in the treatment of psoriasis, since it both significantly lessened hyper-keratosis, acantosis, and dermal papillomatosis and improved skin hydration (44). It must be recalled here that the permeability barrier of normal epidermis is severely disturbed in psoriatic skin (45-47), and that bathing in hypotonic salt solutions triggers antiinflammatory effects in lesional skin sites (48). However, most of 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.

In previous studies (49,50), we showed that exposure to CW interferes with IL-6, VEGF-A isoform, and CK-16 expression by human psoriatic keratinocytes. To further clarify the mechanisms involved in the therapeutic effectiveness of CW balneotherapy in psoriasis, in this study we investigated CW's effects on TNF- α and IL-8 production and secretion by epidermal keratinocytes isolated from lesional skin biopsies and kept in confluent cultures. Here, we will show that the addition of CW (in total or partial stead of DW) to the growth medium remarkably hinders the hyper-expression of TNF-a and the heightened production and release of IL-8 on the part of the psoriatic keratinocytes. Therefore, our previous (49,50) and present findings are consistent with CW being endowed with a complex phenotype- and cytokine/chemokine-regulating potential that translates into valuable anti-psoriatic therapeutic benefits.

Materials and methods

In vitro cell culture. Human epidermal keratinocytes were isolated from skin biopsies taken, after informed consent, from 9 psoriatic 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 incubating the isolated epidermal sheet in trypsin solutions (0.25% w/v), suspensions of keratinocytes were obtained. Trypsin's action was next inhibited by adding an excess of serum, and the cell suspensions were soon spun down at 600 rpm for 10 min at 4°C. The supernatants were decanted, the pellets resuspended, and the living cells counted in a Neubauer chamber. Keratinocytes were next seeded into plastic flasks pre-coated with a feeder-layer of preirradiated 3T3-J2 cells. To expand the keratinocytes' 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

foetal bovine serum (FBS; 10% v/v; BioWhittaker Europe, Belgium), antibiotics (solution of penicillin-streptomycin 1% w/v; BioWhittaker), epidermal growth factor (EGF; 0.1 μ g ml⁻¹; PeproTech, UK), insulin (20 ng ml⁻¹; PeproTech), and hydrocortisone (0.5 μ g ml⁻¹; 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

liferated 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. Once cultured *in vitro*, such keratinocytes kept steadily secreting into the medium, as determined by ELISA assays, amounts of IL-8 at least 6-fold greater than do normal keratinocytes (51).

Experimental protocol. Psoriatic keratinocytes were detached from the culture flasks by a mild trypsin treatment and then seeded at 1.0×10^6 cells into wells containing 2.0 ml of either DMEM medium, whose chemical constituents had been dissolved in DW (controls in DW-DMEM), or in one of three different CW-DMEM media, in which DW had been totally (100%) or in part (50% or 25%) substituted with CW. Between days 3 and 11-13 of experimental treatment, the cultured keratinocytes and/or the cell-conditioned media were sampled and their respective contents of TNF- α and IL-8 determined.

Immunocytochemistry. At chosen time points, psoriatic keratinocytes exposed to either DW- or 100% 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 then incubated for 1 h at 37°C with anti-IL-8 mouse monoclonal antibody (final dilution 10 µg ml⁻¹; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Next, keratinocytes were washed three times with PBS-BSA (1%) and incubated for 1 h at room temperature in the dark with specific secondary antibodies (1:100 dilution) conjugated with Alexa Fluor-488 or -555 (Molecular Probes, Invitrogen Corporation, 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). After 3, 7, and 11 days in vitro, psoriatic keratinocytes kept in 100% CW- or in DW-DMEM were scraped into cold PBS and sedimented at 200 x g for 10 min. The sedimented cells were homogenized in T-PERTM 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 (52) using bovine serum albumin as a standard. Equal amounts (10 or 20 μ g) of proteins from each cell lysate or cell-conditioned DW- or CW-DMEM (25 μ l) were boiled in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% w/v SDS, 5% w/v ß-mercaptoethanol, 10% v/v glycerol, 0.002% w/v bromphenol blue) and electrophoresed in 10% w/v SDSpolyacrylamide gel. The separated proteins were blotted onto a nitrocellulose membrane (0.45 μ m; Bio-Rad Laboratories, Hercules, CA). To immunodetect IL-8 the blots were probed

with the same specific primary antibody as used for immunocytochemistry at a final dilution of 1.0 μ g ml⁻¹. To detect TNF- α the blots were probed with an anti-TNF- α goat polyclonal antibody (Santa Cruz) at a final dilution of 1.0 μ g ml⁻¹. Blots were next incubated with alkaline phosphatase-conjugated anti-mouse or anti-goat IgG (Santa Cruz), and stained with BCIP/NBT liquid substrate reagent (Sigma). Developed blots were photographed with an Olympus 3300TM digital camera, and the determination of the M_r and the densitometric analysis of each specific protein band were carried out using SigmagelTM software (Jandel Corp., Erkrath, Germany).

ELISA assays of IL-8 and TNF- α . Human psoriatic keratinocytes were cultured for up to 13 days in four different growth media containing DMEM components dissolved in the following percent fluid fractions: (i) CW 100%/DW 0%; (ii) CW 50%/DW 50%; (iii) CW 25%/DW 75%; and (iv) CW 0%/DW 100% (control medium). Keratinocyte-conditioned samples of the four kinds of growth media were taken at days 3, 6, 8, 10, and 13 of culture and stored at -80°C to be subsequently assayed for their IL-8 and TNF- α content. To this aim, specific commercial ELISA kits were used (CLB, Amsterdam, The Netherlands). The tests were performed according to the instructions of the manufacturer. The sensitivity of the assays for both IL-8 and TNF- α was 4 pg ml⁻¹. The results were expressed as secretion values per duplicate cultures per 10⁶ keratinocytes.

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

Results

CW's effects on intracellular levels of IL-8. WB analyses revealed that human psoriatic keratinocytes produced an IL-8 precursor endowed with a molecular mass of 51 kDa (Fig. 1B) (53). Both confocal microscopy (Fig. 1A), WB observations (Fig. 1B), and densitometric determinations (Fig. 1C) showed that by days 3 and 5 after the onset of the experiments in the 100% CW-DMEM-incubated keratinocytes the intracellular levels of IL-8 had significantly fallen (in WB specimens: at day 3, -86%, p<0.001; at day 5, -76%, p<0.001) with respect to the DW-DMEM-kept (untreated) cells. At day 7, intracellular IL-8 levels became alike in both CW-treated and untreated keratinocytes (Fig. 1A-C), possibly because the untreated cells kept dumping significant amounts of IL-8 into the medium (Fig. 2). Finally, by day 11, in CW-DMEM-kept keratinocytes intracellular IL-8 levels were found to have risen 3.9-fold (p<0.001 in WB specimens) with respect to 7-day values, thus becoming 3-fold higher (p<0.001) than in DW-DMEMincubated cells (Fig. 1A-C). Thus incubating keratinocytes in CW-DMEM medium brought about an early deep cutback followed by a belated intracellular surge of intracellular IL-8 levels. As assessed from the values of the surface areas under the respective curves in Fig. 1C, between days 3 and 11, total IL-8 levels in CW-DMEM-exposed keratinocytes were 56% lower (p<0.001) than in their DW-DMEM-kept counterparts. Conceivably, these observations were the result of an early remarkable and persistent down-regulation of IL-8 production,



Figure 1. Exposure to CW 100%-DMEM medium elicited an early, deep down-regulation and a late surge of the intracellular IL-8 levels in cultured otherwise IL-8-overproducing and hyper-secreting psoriatic epidermal keratinocytes. (A) Psoriatic keratinocytes incubated in CW 100%-DMEM exhibited a decrease up to day 7 and a discrete rise at day 11 of the fluorescent signal specifically related to IL-8 with respect to their untreated (i.e. DW-DMEM-kept) counterparts. The confocal pictures are representative of 9 distinct experiments. WB observations (B) and densitometric analyses (C) showed an early decrease followed by a delayed increase in the intracellular IL-8 levels of CW-treated keratinocytes vs. parallel untreated keratinocytes. The immunoblot shown in B is representative, and the points on the curves in C are means \pm SEM of 9 experiments. *p<0.001 between time-corresponding DW- and CW-exposed points.



Figure 2. Fractions of CW ranging from 25% to 100% in the DMEM medium deeply and progressively curtailed the hyper-secretion of IL-8 on the part of cultured psoriatic keratinocytes bringing it somewhat closer to within normal range values (i.e. 50- to 150-ng ml⁻¹ per 10⁶ cells) (51). Points on the curves are means from 9 experiments carried out in duplicate. Between day 6 and 13 all values pertaining to CM-treated keratinocytes exhibited a statistical significance of p<0.001 vs. the values of time-corresponding DW-DMEM-incubated (control) cells. sIL-8, IL-8 secreted into the growth media.

onto which a progressively stronger hindrance of IL-8 secretion was lately superimposed.

CW's effects on IL-8 secretion. When untreated (i.e. incubated in DW-DMEM), the psoriatic keratinocytes released from the third day onwards increasing amounts of IL-8 (≤3.8 ng ml⁻¹ per 10⁶ cells) into the medium (Fig. 2). However, once keratinocytes were exposed to DMEM containing various percentages (i.e. from 25% to 100%) of CW fractions, the IL-8secreting activity was strikingly and progressively decreased from day 6 onwards (e.g., at day 8, from -38% to -54%; and at day 13, from -74% to -87%; p<0.001 vs. parallel untreated controls in both instances) (Fig. 2). Most interestingly, a nearly maximum inhibitory effect on IL-8 secretion was achieved by keeping keratinocytes in CW 25%/DW 75%-DMEM (Fig. 2). Hence, as assessed from the values of the surface areas under the respective curves in Fig. 2, an exposure between days 3 and 13 to CW fractions ranging from 25% to 100% in the DMEM similarly reduced from -47% to -51% (p<0.001) the total amount of secreted IL-8 (sIL-8) with respect to that dumped by untreated keratinocytes into the DW-DMEM.

CW's effects on TNF-a expression and secretion. The results of preliminary WB observations and corresponding densitometric assessments showed that after a 5-day exposure to 100% CW-DMEM, the density of the TNF- α -specific 45-kDa



Figure 3. Exposure to CW persistently down-regulated the expression of TNF- α by human lesional keratinocytes cultured *in vitro*. WB observations (A) and densitometric analyses (B) showed that a strong and persistent down-regulation of TNF- α took place in CW-DMEM-kept keratinocytes with respect to DW-DMEM-incubated keratinocytes. By estimating the surface areas under the curves it can be concluded that between days 3 and 11 the incubation in CW-DMEM reduced by -54% on average (p<0.001) the expression of TNF- α by cultured psoriatic keratinocytes with respect to that by untreated cells. The immunoblot shown in A is typical, and the points on the curves in B are means ± SEM of 9 experiments. *p<0.001 between the values pertaining to time-corresponding keratinocyte samples kept in either DW- or CW-DMEM.

protein band had diminished by -66.3% (p<0.001) with respect to that of parallel DW-DMEM-incubated keratinocytes (Fig. 3A and B). Moreover, a 7-day exposure to 100% CW-DMEM elicited the largest decrease in the density of the TNF- α -specific protein band (day 7, -72.9%; p<0.001) vs. that of time-corresponding DW-DMEM-kept keratinocytes (Fig. 3A and B). By day 11 CW-treated keratinocytes were found to express TNF- α moieties again at the day 5 levels, which were still 44% lower than those of their untreated (DW-DMEM-kept) parallel counterparts (Fig. 3A and B). Therefore, as assessed from the values of the surface areas under the respective curves in Fig. 3B, between days 3 and 11 the exposure to CW curtailed by -54% (p<0.001) the total expression of TNF- α by cultured human epidermal keratinocytes with respect to untreated DW-DMEM-kept counterparts.

On the other hand, by using TNF- α -specific ELISA assays we were unable to detect any measurable amount whatsoever of secreted TNF- α in any of the keratinocyte-conditioned samples of DW- or CW-DMEM media tested (data not shown).

Discussion

In this study, we tested CW's effects on the up-regulated production and secretion of IL-8 on the part of psoriatic keratinocytes kept in pure *in vitro* cultures, i.e. in the complete absence of T cells (54). Our findings show that the addition of CW (instead of DW) to the DMEM significantly curtailed both the heightened intracellular levels and secretion rates of IL-8 by these cells. Notably, IL-8 hyper-secretion was cut

down by incubating the psoriatic keratinocytes in a DMEM whose CW fraction was as little as 25%. This increasingly effective inhibition of IL-8 release by CW went so far as to elicit a late discrete intracellular IL-8 accumulation even though its production had concurrently been down-regulated. The operative mechanisms underlying these IL-8-interfering effects elicited by CW components in psoriatic keratinocytes remain to be elucidated. Nuclear factor (NF)- κ B is a transcription factor that modulates the expression of genes encoding cytokines such as IL-8, IL-6, colony stimulating factors, adhesion molecules, and immune receptors (55). Notably, TNF- α partakes in the activation of NF- κ B, thereby acting as a significant modulator of the expression of several pro-inflammatory compounds in psoriasis (12,15,19).

In keeping with this view, the present results show that CW exposure also significantly interferes with the expression of TNF- α by the cultured psoriatic keratinocytes. Hence, it seems conceivable to ascribe the previously observed interfering effects of CW on IL-6 production and secretion (50) and the presently reported hindering of IL-8 synthesis and release by CW to an up-stream inhibition of TNF- α expression by the same CW components.

It is well established that TNF- α plays a major role in psoriasis pathogenesis and progression (40). Clinical improvements of psoriasis associate with a fall in lesional skin and serum TNF- α levels (36,56,57), whereas clinical aggravations of psoriatic area severity index (PASI) scores concur with surges in serum and lesional skin TNF- α concentrations (40,60). Neutralisation of TNF- α by infliximab, an anti-TNF- α -specific chimerical monoclonal antibody, or by etanercept, a soluble p75 TNF receptor fusion protein that binds both TNF and lymphotoxin (LT), is known to cause a rapid and complete remission of psoriasis even in its recalcitrant von Zumbusch form (39-41) and, when associated with methotrexate, of psoriatic arthritis (58-61).

Reportedly, several cytokines, including IL-1, IL-2, GM-CSF, and IFN- γ up-regulate TNF- α expression (43). Most interestingly, the results of preliminary work carried out in our lab indicate that CW exposure also significantly downregulates IL-1 expression in cultured psoriatic keratinocytes, thereby justifying the partial drying up of the tumultuous cytokine cascade sustaining clinical psoriatic manifestations (15,40) (manuscript in preparation).

On the other hand, it is conceivable to ascribe the observed absence of any TNF- α secretion by the cultured psoriatic keratinocytes to a lack of activity on the part of the TNF- α converting enzyme (TACE) in such cells (62,63). TACE, also named ADAM 17, is a member of a disintegrin and metalloproteinase (ADAM) family of proteinases and its mRNA is hyper-expressed in lesional psoriatic skin and in cultured normal keratinocytes (63-65). When active, TACE cleaves pro-TNF- α moieties inserted on the plasmalemmal surface, thereby discharging the mature, soluble TNF- α molecules into the environment. However, little is known about the mechanisms modulating TACE's activity. It may depend upon protein kinase C activation (66) or manifold signalling pathways (67,68). Further study will establish the reasons why TACE, though being hyper-expressed (64), does not function in pure cultures of psoriatic epidermal keratinocytes.

In conclusion, our previous (49,50) and present observations support the view that CW balneotherapy may elicit its beneficial effects by interfering with an improper local production and secretion of several chemokines and cytokines, including TNF- α , IL-8, IL-6, and various VEGF-A isoforms, and by attenuating some facets, such as CK-16 expression, of the psoriatic phenotype, which altogether locally underlie the epidermal hyperplasia and dermal neoangiogenesis, inflammation, and leukocyte infiltration phenomena proper of psoriatic illness.

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