Abstract. Thermal balneotherapy with Comano’s spa water (CW; Trentino, Italy) is used for psoriasis and other skin disorders but its mechanisms of action are mostly unknown. Previously, we showed that CW can interfere with the expression and secretion of various VEGF-A isoforms by cultured human psoriatic epidermal keratinocytes. In this study, confluent cultures of IL-6-hypersecreting keratinocytes isolated from 6 psoriatic patients were exposed for 11-15 days to DMEM, the chemicals of which had been dissolved in either deionised water (DW-DMEM, controls) or CW (CW-DMEM, treated cells). As detected by means of immunocytochemistry, Western immunoblotting, and ELISA assays, the intracellular levels and secretion rates of IL-6 were drastically curtailed in the CW-DMEM-incubated keratinocytes and in their cell-conditioned media. A nearly maximal inhibition of IL-6 release had already been induced by a CW fraction in the DMEM as low as 25%. CW exposure also promptly, intensely, and persistently down-regulated the expression of cytokeratin-16 (CK-16), a marker associated with keratinocyte psoriatic phenotype. Hence, CW balneotherapy may beneficially affect the clinical manifestations of psoriasis via an attenuation of the local deregulation of several cytokines/chemokines, including IL-6 and VEGF-A isoforms, and of a concurrent, abnormal cell differentiation program entailing the expression, amongst other proteins, of CK-16.

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). 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 (7-12). Local fibroblast activation and increased keratinocyte production and release of several cytokines/chemokines, such as interleukin-1 (IL-1), IL-6, IL-8, IL-20, vascular endothelial growth factor-A (VEGF-A) isoforms, endothelial cell stimulating angiogenesis factor (ESAF), tumour necrosis factor-α (TNF-α), amphiregulin, transforming growth factor-α (TGF-α), and platelet-derived endothelial cell growth factor/thymidine phosphorylase (TP), are also included in the typical features of the disease (10,13-20).

IL-6 is a multifunctional cytokine of the haemopoietins family that also comprises leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, granulocyte-colony stimulating factor (G-CSF), ciliary neurotrophic factor (CNTF), and cardiotrophin (referenced in refs. 17,21). All haemopoietins share the gp130 signal-transducing subunit (21). IL-6 acts as a growth factor for keratinocytes either directly via its specific receptor signalling (22-24) and/or indirectly by inducing the production and release of keratinocyte growth factor (KGF) by dermal fibroblasts (25). Non-activated normal keratinocytes
express low levels of IL-6-specific mRNA and release only tiny amounts of IL-6 (26). IL-6 expression is significantly heightened at psoriatic lesional skin sites (27-33), chiefly in transitional zones in which moderate epidermal cell hyper-proliferation occurs (31). IL-6 levels are increased even in the supernatants of lesional psoriatic skin (34). However, actual levels of IL-6 production and secretion may vary in psoriatic skin samples and supernatants from different patients according to disease stage and genetic background (1,35,36). In fact, IL-6 concentrations in lesional skin go up with the worsening or go down with the improvement of the clinical signs of psoriasis (29,31,36). Moreover, IL-6 production by keratinocytes is induced by IGF-II via the activation of NF-κB (37), augmented by LIF (38) and by several other cytokines (24), IL-6 also may enhance the proliferation of human adult dermal fibroblasts and/or upregulate the production of collagen, glycosaminoglycans (GAGs), interstitial collagenase, and stromelysin-1 by such cells (24,41,42), thereby favouring collagen, glycosaminoglycans (GAGs), interstitial collagenase, and stromelysin-1 by such cells (24,41,42), thereby favouring the inflammatory processes related not only to psoriasis but even to wound healing and hypertrophic burn scarring (26,31,34,41,43-45). The increased circulating levels of IL-6 detected in psoriasis are thought to mediate, via the induction of other cytokines (e.g. IL-2) and adhesion molecules (e.g. ICAM-1), both the proliferative and functional activities of B, T, and natural killer (NK) cells, thereby modulating the systemic immune responses of the host in psoriasis and other pathologies (26,31,34,46,47).

**Materials and methods**

**In vitro cell culture.** For this work human epidermal keratinocytes were isolated from skin biopsies taken, after informed consent, from 6 psoriatic patients. After rapid transfer to 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 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 were seeded into plastic flasks pre-coated with a feeder-layer of preirradiated 3T3-J2 cells. To expand the keratinocyte

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Table I. Components of Comano’s water.

<table>
<thead>
<tr>
<th>Ions</th>
<th>mM</th>
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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>
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</tr>
<tr>
<td>Bicarbonate</td>
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</tr>
<tr>
<td>Chloride</td>
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</tr>
<tr>
<td>Sulfuric acid</td>
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</tr>
<tr>
<td>Silicon</td>
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</tr>
<tr>
<td>Fluorine</td>
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</tr>
<tr>
<td>Lithium</td>
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<tr>
<td>Aluminum</td>
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</tr>
<tr>
<td>Manganese</td>
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</tr>
<tr>
<td>Iron</td>
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</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.*
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 μg ml⁻¹; PeproTech, UK), insulin (20 ng ml⁻¹; PeproTech), and hydrocortisone (0.5 μg 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 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-6 several fold greater than did normal keratinocytes (55).

Experimental protocol. IL-6-hypersecreting psoriatic keratinocytes were detached from the culture flasks by a mild trypsin treatment and then seeded at 1.0 x 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 15 of experimental treatment, the cultured cells and/or the cell-conditioned media were sampled and their respective contents of IL-6 and/or CK-16 were 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 incubated for 1 h at 37°C with an anti-CK-16 IgG mouse monoclonal antibody (final dilution 10 μg ml⁻¹; Chemicon International, Inc.) or with an anti-IL-6 rabbit polyclonal 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
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-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 (56) 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 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 IL-6 and CK-16, the blots were probed with the same specific primary antibodies as used for immunocytochemistry at a final dilution of 1.0 μg ml⁻¹. Blots were next incubated with alkaline phosphatase-conjugated anti-mouse or 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).

ELISA assay of IL-6. Human psoriatic keratinocytes were cultured for 15 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). Cell-conditioned samples of the four kinds of growth media were taken at days 3, 5, 7, 10, 12, and 15 of culture. The concentrations of IL-6 in the media were determined using Sigmagel™ software (Jandel Corp., Erkrath, Germany). To this aim, a specific commercial ELISA kit was used (CLB, Amsterdam, The Netherlands). The tests were performed according to the instructions of the manufacturer. The sensitivity of the assays for IL-6 was 0.5 pg ml⁻¹. The results were expressed as daily secretion values per duplicate cultures.

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

Effects of CW on intracellular levels of IL-6. WB analyses revealed that human psoriatic keratinocytes produced IL-6 species endowed with molecular masses close to 45 kDa (Fig. 1B), suggesting that the original 23- to 25-kDa IL-6 protein moiety underwent significant post-translational modifications (N- and O-glycosylations and phosphorylations) within the epidermal epithelial cells (57). Both confocal microscopy (Fig. 1A), WB observations (Fig. 1B), and densitometric determinations (Fig. 1C) showed that by day 3 after the onset of the experiments the intracellular levels of IL-6 had significantly fallen (-73%, p<0.001 in WB specimens) in the 100% CW-DMEM-incubated keratinocytes with respect to the DW-DMEM-kept (untreated) cells. Next, between days 5 and 7, intracellular IL-6 levels became close in both untreated and CW-treated keratinocytes (Fig. 1A-C). Finally, by day 11, intracellular IL-6 levels were found to have risen (+59%, p<0.01 in WB specimens) in CW-DMEM-incubated keratinocytes with respect to untreated ones (Fig. 1A-C). Thus, an early deep cutback of intracellular IL-6 levels was induced by incubating keratinocytes in CW-DMEM medium, whereas a tardy discrete intracellular IL-6 surge was the likely upshot of a quite strongly hindered secretion of IL-6, the production of which had meanwhile been down-regulated.

Effects of CW on IL-6 secretion. When untreated (i.e. incubated in DW-DMEM), the psoriatic keratinocytes released massive amounts (up to >50 ng ml⁻¹ per 10⁶ cells) of IL-6 into the medium from the third day onwards (Fig. 2). However, when exposing keratinocytes from day 5 onwards to DMEM containing various percentages (i.e. from 25% to 100%) of CW fractions, the IL-6 hyper-secretory activity of the same cells was strikingly and progressively cut down (e.g., at day 5, from -69% to -76%, p<0.001 vs. parallel untreated keratinocytes; at day 15, from -88% to -96%, p<0.001 vs. parallel untreated cells) (Fig. 2). Most interestingly, a nearly maximum inhibitory effect on IL-6 secretion had already been achieved by exposing keratinocytes to CW 25%/DW 75%-DMEM.
Hence, a persisting exposure to CW fractions ranging from 25% to 100% in the DMEM similarly brought IL-6 secretion rates down to within the range of normal values (i.e. ~6- to 8-ng ml⁻¹ per 10⁶ keratinocytes) (55).

Effects of CW on CK-16 expression. Observations under the confocal microscope revealed that after 3 days of exposure to 100% CW-DMEM the intensity of the fluorescent signal specifically related to CK-16 had remarkably weakened in the cytoplasm of psoriatic keratinocytes with respect to parallel controls kept in DW-DMEM (Fig. 3A). The results of WB observations and of corresponding densitometric assessments showed that a strong and persistent down-regulation of CK-16 took place in CW-DMEM-incubated keratinocytes with respect to DW-DMEM-incubated keratinocytes. The immunoblot shown in B is typical, and the points on the curves in C are means ± SEM of 6 experiments. *p<0.001 between the values pertaining to time-corresponding keratinocyte samples kept in either DW-DMEM or CW-DMEM.

Discussion
In this work we tested CW's effects on IL-6 hyper-production and hyper-secretion by psoriatic keratinocytes kept in pure in vitro cultures, i.e. in the complete absence of T cells (58). Our results show that the addition of CW (instead of DW) to parallel DW-DMEM-incubated keratinocytes (Fig. 3B and C). Moreover, after an 11-day exposure to 100% CW-DMEM, the density of the CK-16-specific protein band had been reduced merely to one-tenth (p<0.001) that proper of parallel DW-DMEM-kept keratinocytes (Fig. 3B and C). Thus, a lasting exposure to CW severely hindered the expression of CK-16, a marker of the psoriatic phenotype (48,49), by the human epidermal keratinocytes.
the DMEM significantly curtailed both the heightened intracellular levels and secretion rates of IL-6 by these psoriatic keratinocytes. Notably, IL-6 hyper-secretion was cut down to within the range of normal values (55) by incubating the keratinocytes in DMEM whose CW fraction was as little as 25%. This strong inhibition of IL-6 release went so far as to elicit a late discrete intracellular accumulation of IL-6 even though IL-6 production had also been down-regulated. The operative mechanisms underlying these IL-6-interfering effects elicited by CW components in keratinocytes remain to be elucidated. Collectively, our findings suggest that, by interfering with IL-6 hyper-production and hyper-secretion by the psoriatic keratinocytes, the exposure to CW significantly hinders the mitogenic, proinflammatory, and proangiogenic actions sustained by the occurrence of an IL-6 surplus within psoriatic skin lesions (10,17,22,30,31,59).

The programmed expression of cytokeratins (CKs), which is determined by the location and functioning of the keratinocytes, is commonly taken as a set of phenotypic markers related to the stages of development and differentiation of the epidermal cells (60). The proliferating keratinocytes residing in the basal layer produce CK-5, CK-14, and low amounts of CK-15; the differentiating keratinocytes placed in the suprabasal layers express CK-1, CK-2, and CK-10; conversely, like CK-6 and CK-17, CK-16 is uniquely expressed in activated, hyper-proliferating keratinocytes such as those harboured in psoriatic lesions (48,60). Therefore, CK-16 is usually referred to as a marker associated with the psoriatic phenotype (49,61). The present results show that exposure to CW elicits a massive and persistent down-regulation of CK-16 expression in cultured human adult psoriatic keratinocytes. Thus, the whole of the interferences brought about by CW exposure on VEGF-A isoforms (54), IL-6, and CK-16 expression suggest that CW has the ability to shift the keratinocytes’ psoriatic phenotype towards a somewhat more normal pattern. Further studies should establish whether CW down-regulates other cytokines or chemokines, such as IL-1, IL-8 and TNF-α (17), and markers, such as CK-6, CK-17, and SKALP/elafin (49,62,63), all of which are strongly expressed by the psoriatic keratinocytes. However, our previous (54) and present findings clearly demonstrate that CW acts via identifiable and measurable biological mechanisms, thereby excluding that CW actions fall within the compass of placebo effects.

In conclusion, our previous (54) and present observations support the view that CW balneotherapy may elicit beneficial effects by interfering with an improper local production and secretion of several chemokines and cytokines, including IL-6 and VEGF-A isoforms, and by attenuating some facets, such as CK-16 expression, of the psoriatic phenotype, which altogether underlie the epidermal hyperplasia and dermal neo-angiogenesis, inflammation, and leukocyte infiltration phenomena proper of local psoriatic illness.

References


