Comano's (Trentino) thermal water interferes with interleukin-6 production and secretion and with cytokeratin-16 expression by cultured human psoriatic keratinocytes: Further potential mechanisms of its anti-psoriatic action

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Running title: Comano's water interferes with IL-6 and CK-16 expression

Abbreviations: CK-16, cytokeratin-16, CW, Comano's thermal water; DW, deionised water; IL-6, interleukin-6; WB, Western immunoblotting.

Key words: Chemokines, Comano's thermal water, cytokeratin-16, cytokines, human keratinocytes, interleukin-6, psoriasis.

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 (Chiarini A, *et al*, Int J Mol Med, 17, in press), we showed that CW can interfere with the expression and secretion of various VEGF-A isoforms by cultured human psoriatic epidermal keratinocytes. In this work, confluent cultures of IL-6-hypersecreting keratinocytes isolated from 6 psoriatic patients were exposed for 11-15 days to a 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 (WB), 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 was already brought about 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 keratinocytes' 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 about 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). This has 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 keratinocytes' 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), 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 [see for references: (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 a 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 that are overproduced in psoriasis (i.e. IL-1α, IL-1β, IL-2, IL-4, GM-CSF, TNF-α) (31, 39), and conversely inhibited by treatment with (-)-epigallocatechin-3-gallate, a major green tea polyphenol (40). By means of paracrine and autocrine loops mediated via its specific high-affinity functional receptors (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 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).

Amongst the crucial features a hyper-proliferative skin condition like psoriasis is comprised an abnormal keratinocytes' differentiation program entailing the expression of proteins that are otherwise absent from normal skin, such as cytokeratin-16 (CK-16), CK-6, and

CK-17, and the antimicrobial/elastase inhibitor SKALP/elafin (48). Hence, these proteins or their mRNAs have been used as stand-in markers in drug-screening procedures (48), and CK-16 is usually referred to as a strongly expressed constituent of the psoriasis-associated keratinocytes' phenotype (49).

Comano (Trentino, Italy) spa's water (CW) is a thermal hypotonic water containing various electrolytes (see Table I). The major dermatological diseases so far treated via CW balneotherapy are psoriasis and atopic dermatitis (50). Other dermatoses also cared for with CW include contact dermatitis, seborrhoeic dermatitis, *lichen planus*, and palmoplantar keratosis (50). 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 (50). It must be recalled here that the permeability barrier of normal epidermis is severely disturbed in psoriatic skin (51,52), and that bathing in hypotonic salt solutions triggers anti-inflammatory effects in lesional skin sites (53). 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 a previous work, we showed that exposure to CW interferes with VEGF-A isoform expression and secretion by the human psoriatic keratinocytes (54). To further clarify the mechanism(s) possibly involved in the therapeutic effectiveness of CW balneotherapy in psoriasis, we investigated CW's effects on IL-6 hyper-production and hyper-secretion and on CK-16 expression by epidermal keratinocytes isolated from lesional skin biopsies. Here, we will show that the addition of CW (in total or partial stead of DW) to the growth medium thwarts the heightened production and release of IL-6 and the expression of CK-16 on the part of the psoriatic keratinocytes. These 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. For this work human epidermal keratinocytes were isolated from skin biopsies taken, after informed consent, from 6 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 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 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 $\mu g\ ml^{-1}$; PeproTech, UK), insulin (20 $ng\ ml^{-1}$; PeproTech, UK), and hydrocortisone (0.5 $\mu g\ ml^{-1}$; PeproTech, UK) 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 about 48 hours. Once cultured *in vitro*, such keratinocytes kept steadily secreting into the medium, as determined by ELISA assays (see below) 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 next seeded at 1.0 x10^6 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 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 next 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 an LSM 510 confocal microscope (Carl Zeiss S.p.A., Milan, Italy). Deconvolved fluorescence images were obtained with Huygens Professional Software for Windows (Scientific Volume Imaging b.v., Hilversun, The Netherlands).

Western immunoblotting (WB). After 3, 7, and 11 days of staying *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 (see above) 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 M_r 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 made up with DMEM components dissolved in the following per cent fluid fractions, that is *(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 and stored at -80°C to be subsequent assayed for their IL-6 content. 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. A 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 at least 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-kept keratinocytes with respect to untreated ones (Fig. 1A-C). Thus, an early deep cutback of intracellular IL-6 levels was brought about 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 (see below).

Effects of CW on IL-6 secretion. When untreated (i.e. incubated in DW-DMEM), the psoriatic keratinocytes released from the third day onwards massive amounts (up to and over 50 ng ml^{-1} per 10^6 cells) of IL-6 into the medium (Fig. 2). However, exposing keratinocytes from day 5 onwards to a DMEM containing various percent (i.e. from 25% to 100%) CW fractions, the IL-6 hyper-secretory activity of the same cells was strikingly and progressively cut down (for example, 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 interesting, a nearly maximum inhibitory effect on IL-6 secretion was already achieved by exposing keratinocytes to a CW 25%/DW 75%-DMEM (Fig. 2). Hence, a persisting exposure to CW fractions anywhere from 25% to 100% in the DMEM similarly brought IL-6 secretion rates down to within the range of normal values (i.e. about 6-to-8 ng ml^{-1} per 10^6 keratinocytes) (55).

Effects of CW on CK-16 expression. Observations under the confocal microscope revealed that already after 3 days of exposure to a 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 after a 3-day exposure to a 100% CW-DMEM, the density of the CK-16-specific 48 kDa protein band had diminished by -76% ($p<0.001$) vs. that of parallel DW-DMEM-incubated keratinocytes (Fig. 3B,C). Moreover, after an 11-day exposure to a 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,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.

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 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 a 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 IL-6 notwithstanding that 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. Taken together, 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, just 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 the 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. Obviously, further studies will establish whether CW down-regulates other cytokines/chemokines, like IL-1, IL-8 and TNF-α (17), and markers, like CK-6, CK-17, and SKALP/elafin (49; 62; 63), all of which are strongly expressed by the psoriatic keratinocytes. At any rate, 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 its beneficial effects by interfering with an improper local production and secretion of several chemokines/cytokines, including IL-6 and VEGF-A isoforms, and by attenuating some facets, like CK-16 expression, of the psoriatic phenotype, which altogether underlie the epidermal hyperplasia and dermal neo-angiogenesis, inflammation, and leukocyte infiltration phenomena proper of the local psoriatic illness.

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Figure legends

Figure 1. Exposure to a CW-DMEM medium elicited an early, deep down-regulation and a late, modest rise of the intracellular IL-6 levels in cultured otherwise IL-6-hypersecreting psoriatic epidermal keratinocytes. **A.** Psoriatic keratinocytes incubated in 100% CW-DMEM exhibited a decrease at day 3 and an increase at day 11 of the fluorescent signal specifically related to IL-6 with respect to untreated (DW-DMEM-kept) counterparts. The confocal pictures shown here were deconvolved as indicated in the Materials and Methods and are representative of 6 distinct experiments. **B-C.** WB observations and densitometric analyses of the proteins extracted from keratinocytes exposed to a DW- or a CW-DMEM for 3, 5, 7, and 11 days showed an early fall followed by a late moderate rise of the intracellular IL-6 levels in CW-treated keratinocytes. The immunoblot shown in B is a representative one, and the points on the curves in C are means ± SEM of 6 experiments. **, $p<0.001$ and *, $p<0.01$ between time-corresponding DW and CW points.

Figure 2. Fractions of CW ranging from 25% to 100% in the DMEM medium deeply and progressively curtailed the hyper-secretion of IL-6 on the part of cultured psoriatic keratinocytes bringing it down to within normal range values (i.e. 6-to-8 ng ml^{-1} per 10^6 cells) (55). Points on the curves are means from 6 experiments carried out in duplicate. SEMs, not shown, were within ± 15% of each mean value. Between day 5 and 15 all values pertaining to CM-treated keratinocytes exhibited a statistical significance of at least $p<0.01$ vs. the values of time-corresponding DW-DMEM-incubated (control) cells. sIL-6, IL-6 secreted into the growth media.

Figure 3. Exposure to CW persistently down-regulated the expression of CK-16, a marker of the psoriatic phenotype, by human lesional keratinocytes cultured *in vitro*. **A**. Deconvolved confocal pictures representative of 6 experiments showed that already after 3 days there occurred a sharp reduction in the intracellular CK-16 content of keratinocytes exposed to a 100% CW-DMEM with respect to that of DW-DMEM-incubated (control) cells. **B-C**. WB observations and densitometric analyses showed that a strong and persistent down-regulation of CK-16 took place in CW-DMEM-kept keratinocytes with respect to DW-DMEM-incubated ones. The immunoblot shown in B is a typical one, and the points on the curves in C are means ± SEM of 6 experiments. *, $p<0.001$ between the values pertaining to time-corresponding keratinocytes samples kept in either DW-DMEM or CW-DMEM.

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

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

 # This water is hypotonic as its dry residue amounts to only 190 mg/L

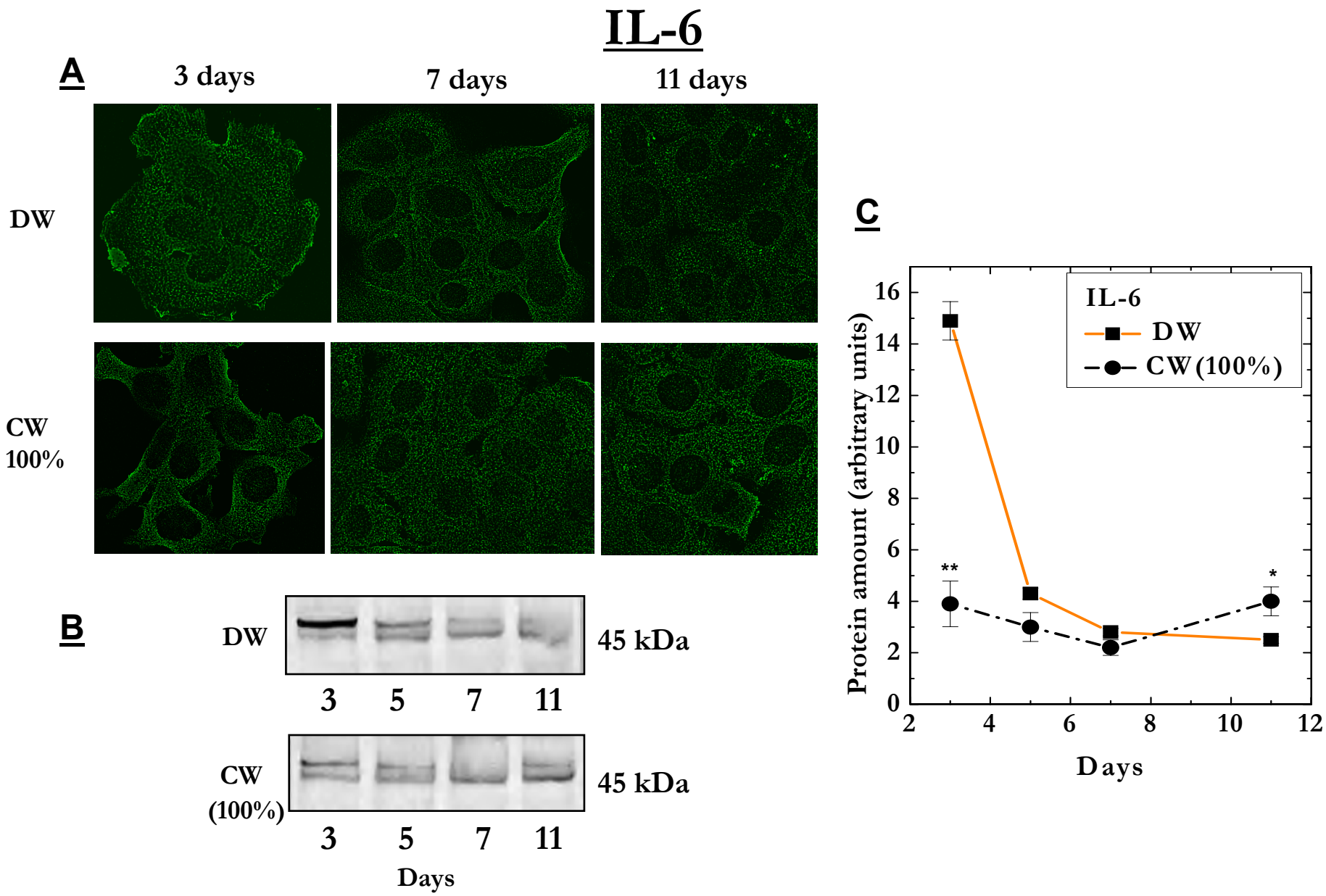
IL-6
A
3 days
7 days
11 days
DW
CW
100%
B
DW
45 kDa
3
5
7
11
CW
(100%)
45 kDa
3
5
7
11
Days
C
IL-6
DW
CW (100%)
Protein amount (arbitrary units)
Days
**
*

Fig. 1

sIL-6

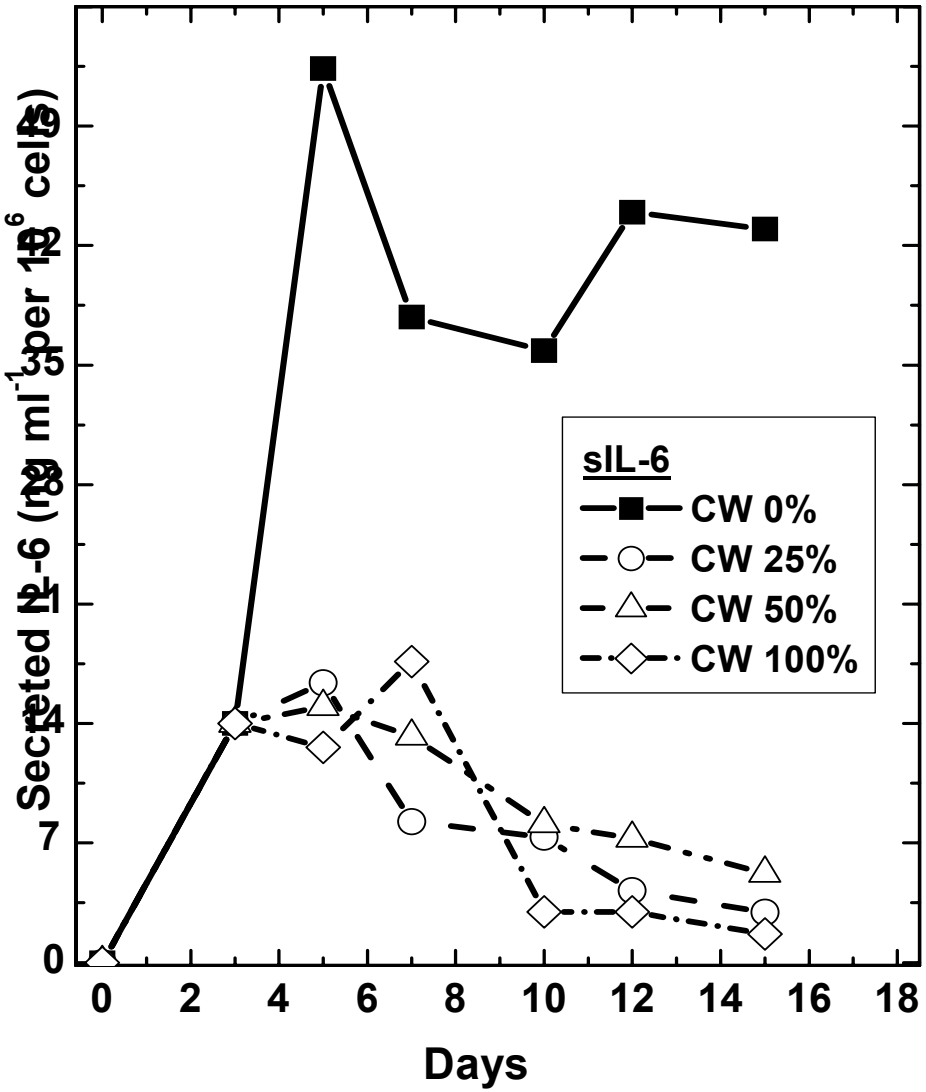

Fig. 2

CK-16

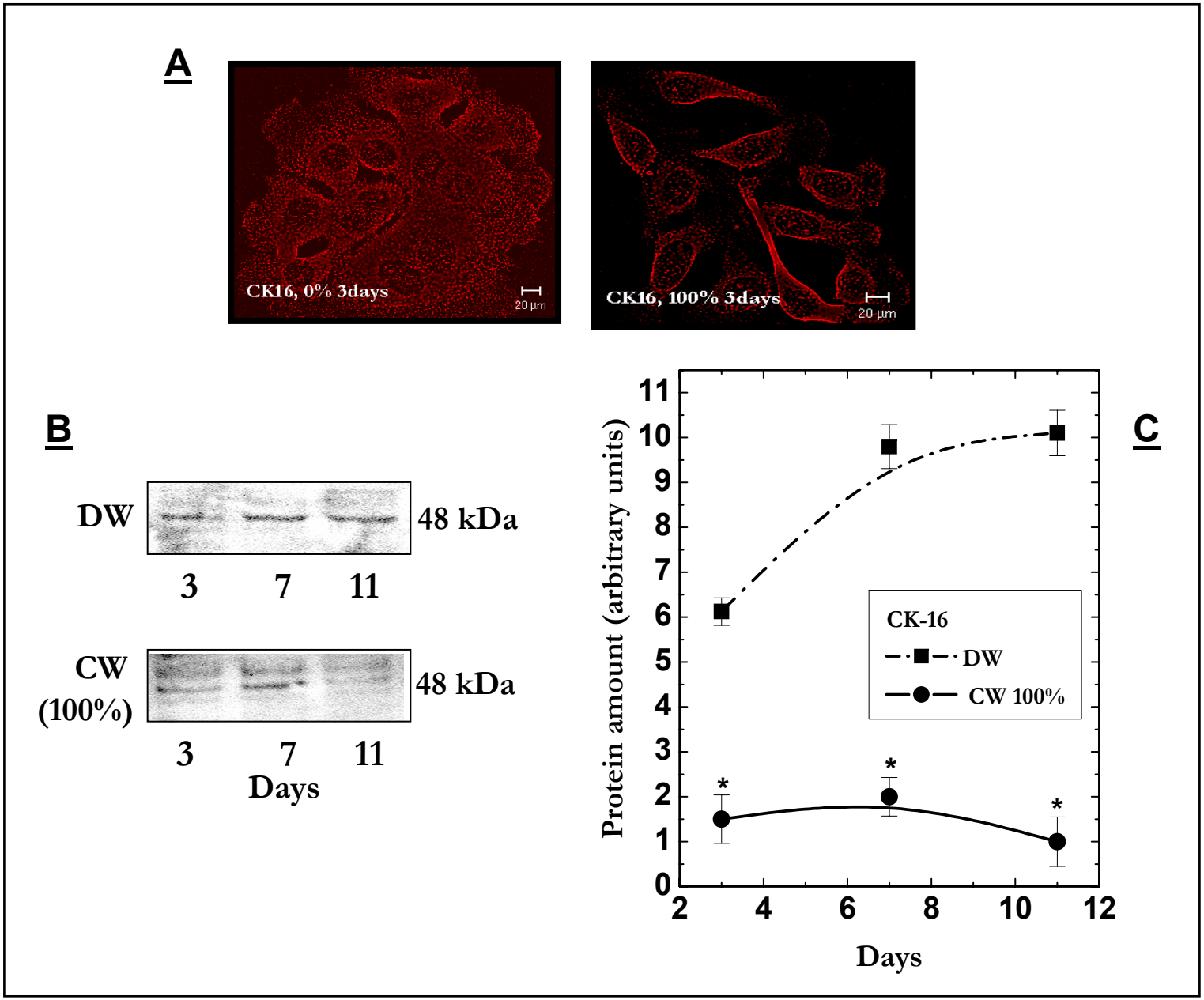
A
CK16, 0% 3days
20 µm
CK16, 100% 3days
20 µm
B
DW
48 kDa
3
7
11
CW
(100%)
48 kDa
3
7
11
Days
C
Protein amount (arbitrary units)
CK-16
DW
CW 100%
Days

Fig. 3