Cartilage Oligomeric Matrix Protein Negatively Influences Keratinocyte Proliferation via α5β1-Integrin: Potential Relevance of Altered Cartilage Oligomeric Matrix Protein Expression in Psoriasis

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In psoriasis, nonlesional skin shows alterations at the dermal–epidermal junction compared with healthy skin. Cartilage oligomeric matrix protein (COMP) is part of the papillary dermis of healthy skin, and its expression has not yet been studied in psoriatic skin. In this study, we found that COMP localization extended deeper into the dermis and formed a more continuous layer in psoriatic nonlesional skin compared with healthy skin, whereas in psoriatic lesions, COMP showed a partially discontinuous deposition at the dermal–epidermal junction. COMP and β1-integrin showed strong colocalization in nonlesional skin, where the laminin layer within the basement membrane is discontinuous. In in vitro models, the presence of exogenous COMP decreased the proliferation rate of keratinocytes, and this proliferation-suppressing effect was diminished by blocking α5β1-integrin. Our results suggest that COMP can interact with α5β1-integrin of basal keratinocytes through the disrupted basement membrane, and this interaction might stabilize the epidermis in the nonlesional state by contributing to the suppression of keratinocyte proliferation. The antiproliferative effect of COMP is likely to be relevant to other skin diseases in which chronic nonhealing wounds are coupled with massive COMP accumulation.


INTRODUCTION
The pathogenesis of plaque-type psoriasis (Psoriasis vulgaris) is only partially understood, and only symptomatic treatment is currently available. In addition to hyperproliferation, altered keratinocyte differentiation, and massive immune cell infiltration, the dermal extracellular matrix (ECM) and the basement membrane (BM) are also affected in healthy-looking nonlesional skin of patients (Bata-Csorgo et al., 1998; Glisák et al., 1993; Mondello et al., 1996; Vaccaro et al., 2002). Nonlesional epidermal keratinocytes have been shown to represent a preactivated state for hyperproliferation (Chen et al., 2001); these cells are more sensitive to stress (Szabó et al., 2014) and to proliferative signals (Bata-Csorgo et al., 1995). Alterations of the ECM that are already present in nonlesional skin also affect the cell attachment modulator fibronectin (FN), which is differentially expressed in nonlesional skin. Previously, we found that fibroblasts as well as basal keratinocytes express high levels of the FN splice variant that contains the extra domain A (EDA + FN) in nonlesional skin (Gubán et al., 2016; Széll et al., 2004). Moreover, some integrins, including the FN-interacting α5β1-integrin, also exhibit an increased expression (Bata-Csorgo et al., 1998; Gubán et al., 2016) in keratinocytes at the dermal–epidermal junction (DEJ). The enhanced EDA + FN and α5β1-integrin production that we observed in psoriatic nonlesional skin may contribute to the induction of keratinocyte proliferation (Bata-Csorgo et al., 1998, 1995; Széll et al., 2004). Furthermore, at the DEJ in nonlesional skin, the laminin layer of the BM is discontinuous and the connection of keratinocytes to the BM is also altered (McFadden and Kimber, 2016; Mondello et al., 1996).

Cartilage oligomeric matrix protein (COMP) is a non-collagenous glycoprotein component of the ECM. The flexible structure of the COMP homopentamer allows simultaneous interactions with multiple cellular and extracellular components (Malashkevich et al., 1996; Mörgelin et al., 1992). COMP is mainly deposited in cartilage, but it is also located in tendons, ligaments, synovium, and skin. In addition, it is expressed in vascular smooth muscle cells, cardiomyocytes, and activated platelets (Müller et al., 1998; Posey et al., 2018; Tan and Lawler, 2009; Wang et al., 2010). COMP is likely to be relevant to other skin diseases in which chronic nonhealing wounds are coupled with massive COMP accumulation.

Abbreviations: BM, basement membrane; COMP, cartilage oligomeric matrix protein; DEJ, dermal–epidermal junction; ECM, extracellular matrix; EDA + FN, fibronectin splice variant containing extra domain A; FN, fibronectin; KRT17, keratin 17; rhCOMP, recombinant human COMP

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In healthy skin, COMP is primarily produced by fibroblasts (Dodge et al., 1998) and localizes to the papillary dermis, where it is believed to take part in ECM stabilization and provide cohesion between the anchoring plaques of the upper dermis and the BM (Agarwal et al., 2012; Farina et al., 2006). Although COMP accumulation in the dermis is elevated in various fibrotic skin disorders (Agarwal et al., 2013; Inui et al., 2011), COMP has not been investigated previously in the context of psoriasis.

COMP modulates cellular behavior via direct interactions with cell surface proteins, including the α5β1 (Chen et al., 2005), α7β1, and αvβ3 (Rock et al., 2010) members of the integrin family. α5β1-integrin modulates processes in psoriasis pathogenesis, including inflammatory responses and keratinocyte proliferation (Bata-Csorgo et al., 1998; Chen et al., 2001; Pellegrini et al., 1996). These results indicate a crucial role for COMP in the pathomechanism of psoriasis.

RESULTS

COMP level is elevated in psoriatic nonlesional skin

Nonlesional skin carries several known alterations of the ECM in the papillary dermis (Bos et al., 1983; Ting et al., 2000). Although COMP accumulation in the dermis is elevated in various fibrotic skin disorders (Agarwal et al., 2013; Inui et al., 2011), COMP has not been investigated previously in the context of psoriasis.

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Here we show that the COMP level is elevated in nonlesional psoriatic skin, where it colocalizes with α5β1-integrin and EDA + FN and has a suppressive effect on keratinocyte proliferation, which is likely mediated through α5β1-integrin. In this way, COMP can override the proliferation-promoting effect of increased EDA + FN and α5β1-integrin, which is associated with the disrupted laminin layer (Bata-Csorgo et al., 1998; Mondello et al., 1996). These results indicate a crucial role for COMP in the pathomechanism of psoriasis.

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Nonlesional skin carries several known alterations of the ECM in the papillary dermis (Bos et al., 1983; Ting et al., 2000). Because COMP previously has been reported to also be present in the papillary dermis, COMP protein accumulation was characterized in nonlesional and lesional skin from patients with psoriasis and skin from healthy individuals. COMP protein was detected with western blot analysis under reducing (Figure 1a and b and Supplementary Figure S1) and nonreducing (Supplementary Figure S2) conditions. Under reducing conditions, we detected elevated COMP monomer and fragment levels in psoriatic nonlesional protein extracts compared with healthy skin (Figure 1a and b and Supplementary Figure S1).

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and formed a more even and continuous layer than observed in healthy samples (Figure 1c and Supplementary Figure S3). In contrast, COMP deposition in lesional skin extended to the upper part of the reticular dermis and exhibited a discontinuously scattered distribution (Figure 1c and Supplementary Figure S3).

Because skin fibroblasts are the major producers of COMP protein (Dodge et al., 1998), we examined the mRNA expression of COMP in primary dermal fibroblasts derived from healthy and psoriatic nonlesional skin and detected elevated COMP mRNA expression in nonlesional fibroblasts (Figure 1d).

COMP colocalization with ITGB1 of basal keratinocytes and EDA + FN is increased and with LAMA1 is decreased in nonlesional psoriatic skin

COMP is known to interact with several members of the integrin cell-surface receptor family, including α5β1-integrin (Chen et al., 2005), whose expression increases together with EDA + FN in nonlesional skin, possibly owing to damaged BM (Bata-Csorgo et al., 1998; Mondello et al., 1996; Ting et al., 2000). To investigate the possible interactions of COMP with proteins in the DEJ that have been altered, confocal microscopic analysis with dual immunofluorescence staining was applied and consecutive sections of the appropriate area were analyzed.

To determine whether COMP accumulation at the DEJ allows interaction with basal epidermal keratinocytes, COMP and β1-integrin (ITGB1) co-immunofluorescence staining was applied. In the papillary dermis, COMP staining partially colocalized with the ITGB1 from basal keratinocytes in healthy and nonlesional skin (Figure 2a and Supplementary Figure S4). However, the colocalization of the two proteins was most prominent in psoriatic nonlesional skin (Figure 2d).

LAMA1 is a component of the BM laminin layer, which is fragmented and occasionally completely missing in nonlesional psoriatic skin (Mondello et al., 1996; Vaccaro et al., 2002). Therefore, to examine whether the damaged BM of nonlesional skin allows the interaction of COMP and ITGB1, LAMA1—COMP dual immunostaining was performed. In nonlesional skin, COMP—ITGB1 double-positive regions exhibited a discontinuous LAMA1 staining pattern (Figure 2b), and the co-occurrence of COMP and LAMA1 was significantly lower in nonlesional skin compared with healthy skin (Figure 2e).

In addition, FN has also been reported to interact with COMP (Di Cesare et al., 2002); therefore, confocal microscopic analysis was also applied to COMP and EDA + FN. In psoriatic nonlesional skin, in which colocalization of COMP and ITGB1 was apparent, partial colocalization of COMP and EDA + FN was observed (Figure 2c), and the intensity of colocalization was significantly higher in nonlesional skin relative to healthy skin (Figure 2f).

COMP negatively influences keratinocyte proliferation via α5β1-integrin in vitro

To investigate whether the possible interaction between COMP and ITGB1 influences keratinocyte cellular behavior, we first performed an impedance measurement—based, real-time cellular analysis of the HPV-KER immortalized keratinocyte cell line. When the culturing plate was precoated with recombinant human COMP (rhCOMP), cells exhibited reduced cell index values in a manner that was dependent on COMP concentration compared with cells grown on uncoated surfaces (Figure 3a). Cell index is influenced by changes in cell proliferation, viability, morphology, and adhesion (Dickhuth et al., 2015). To investigate whether the proliferation rate of HPV-KER cells was affected, a BrdU cell proliferation assay was performed. Precoating the surface with a high concentration (10 μg/ml) of rhCOMP resulted in significantly reduced proliferation rates at 24 and 72 hours compared with cells grown on an uncoated surface. Cell proliferation of primary normal human epidermal keratinocytes was also reduced when the surface was coated with 10 μg/ml rhCOMP (Figure 3c).

To test whether integrins mediate the observed negative effect of COMP on cell proliferation, blocking experiments using anti-ITGA5 and anti-ITGB1 polyclonal antibodies were performed. Blocking of either the ITGA5 or ITGB1 subunit in cells grown on a surface precoated with 10 μg/ml rhCOMP abolished the negative effect of COMP on HPV-KER proliferation, whereas blocking either ITGA5 or ITGB1 alone had no negative effect on these cells (Figure 4a and b and Supplementary Figure S5). Similarly, the negative effect of COMP on the proliferation rate of primary normal human epidermal keratinocyte cells could also be abolished by blocking COMP, ITGA5, or ITGB1, as determined with the BrdU assay (Figure 4c and d) and Ki67 immunofluorescent staining (Figure 4e and f).

COMP has a negative effect on skin wound healing by attenuating keratinocyte proliferation and by compromising keratinocyte migration and activation in ex vivo wound models

To further study the effect of COMP on keratinocytes, an ex vivo three-dimensional skin wound-healing model was applied. Standardized wounded skin samples, with or without rhCOMP treatment (10 μg/ml) (Figure 5a), and unwounded controls were examined at 72 hours after wounding. Immunofluorescent staining revealed COMP localization on the dermal surface of the injured region in COMP-treated wounds, whereas COMP was not detected at the injured region of untreated wounds 72 hours after treatment (Figure 5a). By applying Ki67 immunofluorescent staining to detect proliferating cells, we found a markedly reduced number of Ki67-positive cells in COMP-treated wounds, indicating a decreased rate of proliferation (Figure 5b and c, Supplementary Figure S6a).

Cell migration processes at the wound edge during the closure of injuries require dynamic reorganization of the actin cytoskeleton in the keratinocytes located close to wound margins. To visualize these cells, immunofluorescence staining for actin was applied. In wounds not exposed to COMP, we found that keratinocytes exhibited high levels of actin expression at wound edges, whereas actin expression at wound edges was markedly decreased in COMP-treated wounds, indicating that actin expression was compromised, possibly resulting in a reduction of active cell migration (Figure 5d).

Keratin 17 (KRT17) expression is known to be induced in keratinocytes at wound edges during healing (Mazzalupo
Figure 2. COMP colocalization with basal keratinocyte β1-integrin and EDA + FN increases and with LAMA1 decreases in nonlesional psoriatic skin. Confocal microscopic immunofluorescence analysis of (a) COMP and β1-integrin, (b) COMP and LAMA1, and (c) COMP and EDA + FN colocalization in healthy (first row) and psoriatic nonlesional (second row) skin. Representative images are shown. Dotted lines indicate the enlarged regions. Colocalized pixels of the indicated proteins were calculated with ImageJ software (n = 5). Original magnification: ×63. Bar = 10 μm. The extent of colocalization of (d) COMP and β1-integrin, (e) COMP and LAMA1, and (f) COMP and EDA + FN was calculated using ImageJ/Fiji software. The graphs show mean Pearson's correlation coefficient R ± SEM (n = 5) versus healthy control. *P < 0.05, **P < 0.01 determined by two-tailed Student t-test. EDA + FN, fibronectin splice variant containing extra domain A. COMP, cartilage oligomeric matrix protein.
et al., 2003). Therefore, we performed KRT17 immunofluorescence staining to further investigate the effect of COMP in the ex vivo wound healing model. In rhCOMP-treated wounds, KRT17 expression and re-epithelization were reduced and restricted to a smaller proportion of keratinocytes, compared with untreated control wounds (Figure 5 and Supplementary Figure S6 b and c). This suggests that the presence of COMP compromised keratinocyte activation.

**DISCUSSION**

In psoriasis, the nonlesional skin contains ECM alterations compared with healthy skin. COMP is localized to the papillary dermis of healthy skin (Farina et al., 2006) and, through its interactions with type XII and XIV collagens, contributes to the stabilization of the DEJ (Agarwal et al., 2012). We found that, in psoriatic nonlesional skin, COMP is localized to the papillary dermis and, in contrast to healthy skin, it forms a continuous, more compact, linear layer beneath the basal keratinocytes. Apart from this altered localization, COMP expression was also elevated in dermal fibroblasts from psoriatic samples.

Psoriatic nonlesional skin is more sensitive to stress (Sonkoly et al., 2005; Sze´ll et al., 2016), and abnormalities at the DEJ and the BM are believed to be important in the development of the disease (Bos et al., 1983; Ting et al., 2000). Interruption of the BM (Mondello et al., 1996; Vaccaro et al., 2002) may allow ECM components, normally located directly below the BM, to come in direct contact with basal keratinocytes. COMP reportedly binds directly to the extracellular domain of ITGB1 of both cardiomyocytes (Huang et al., 2013) and cardiac fibroblasts, resulting in the stabilization of ITGB1 by preventing its degradation and, subsequently, improving cellular survival (Huang et al., 2013; Posey et al., 2018). In cartilage, COMP mediates chondrocyte attachment and stabilization partially via α5β1-integrin (Tan et al., 2009). Our confocal microscopic analysis revealed a partial colocalization of papillary dermal COMP and ITGB1 in basal keratinocytes, which indicates the possibility of a direct interaction between these two proteins in vivo. In nonlesional skin, the α5-integrin (ITGA5) subunit is overexpressed in the basal layer of the epidermis, in contrast to healthy skin, where it is present at low levels or completely missing (Bata-Csorgo et al., 1998). Our findings are in line with this observation, as COMP and ITGB1 strongly colocalize in psoriatic nonlesional epidermis and expression of both are upregulated in nonlesional skin. Moreover, the BM is partially discontinuous in psoriatic nonlesional skin (Mondello et al., 1996; Vaccaro et al., 2002), allowing direct interaction. The possibility of this interaction is supported by our finding that, in areas where COMP and ITGB1 were found to have strong colocalization in psoriatic nonlesional skin, the expression of LAMA1, a member of the BM, is reduced or completely absent.

COMP also interacts with α7β1- and αvβ3-integrins (Chen et al., 2005; Rock et al., 2010). Of these proteins, only α7β1 contains a ITGB1 subunit. There is currently no information available about α7β1-integrin expression in basal keratinocytes. Thus, we assumed that, if COMP exhibits a strong interaction with ITGB1, its α-subunit is likely to be ITGA5. In addition to binding to α5β1-integrin, an FN receptor, COMP might also bind to the FN protein itself (Di Cesare et al., 2002). Furthermore, αvβ3-integrin—associated FN and EDA + FN are known to play roles in psoriasis.
Figure 4. COMP influences keratinocyte proliferation via α5β1 integrin. (a, b) BrdU cell proliferation assay of HPV-KER cells cultured on surfaces that were uncoated or coated with rhCOMP protein (10 µg/ml) for 24 and 72 hours following seeding and treated with (a) anti-α5-integrin or (b) anti-β1-integrin subunit antibodies. (c, d) BrdU assay and (e, f) Ki67-positive proliferating cell number determination of NHEKs grown on surfaces that were uncoated or coated with rhCOMP protein (10 µg/ml) for 72 hours following seeding and treated with (c, e) anti-α5-integrin and (d, f) anti-β1-integrin subunit antibody in a combination with COMP protein neutralization. The graphs show mean proliferation/mean number of Ki67-positive cells ± SEM (n = 3) versus uncoated control. *P < 0.05, **P < 0.01 calculated by one-way ANOVA, followed by Tukey’s post-hoc test. COMP, cartilage oligomeric matrix protein; NHEK, normal human epidermal keratinocyte; rhCOMP, recombinant human COMP.
pathogenesis (Bata-Csorgo et al., 1998; Ting et al., 2000). Enriched expression of \( \alpha_5\beta_1 \)-integrin and EDA + FN in nonlesional skin is thought to be due to the incompleteness of the laminin layer (Mondello et al., 1996; Vaccaro et al., 2002). Our confocal microscopic analysis revealed partial colocalization of COMP and EDA + FN in nonlesional skin. These results suggest that, in addition to interacting with EDA + FN, COMP may also affect basal keratinocytes via interactions with both the EDA + FN and its receptor, \( \alpha_5\beta_1 \)-integrin.

Keratinocyte behavior is influenced by ECM proteins through interactions with different cell surface integrins (Hamill et al., 2012; Tjin et al., 2014), and connection of basal keratinocytes to the altered BM could enhance proliferation (Yang et al., 2016). We analyzed the biological relevance of the interaction of COMP and ITGB1 in basal keratinocytes using HPV-KER and normal human epidermal keratinocyte cells in vitro. We found that the presence of COMP resulted in reduced keratinocyte proliferation in both cell types and that this effect was reversible by blocking COMP with a specific antibody.

We also analyzed whether the observed negative effect of COMP on keratinocyte proliferation involves interaction with \( \alpha_5\beta_1 \)-integrin. By partially blocking the function of the ITGB1 or ITGA5 subunits with specific antibodies, the negative effect of COMP on cell proliferation was abolished, suggesting that the negative influence of COMP on keratinocyte proliferation involves \( \alpha_5\beta_1 \)-integrin.

Our in vitro findings were also validated in an ex vivo wound model; exogenous COMP treatment delayed healing of artificial wounds, and this effect was coupled with reduced keratinocyte proliferation and compromised actin expression.
both important aspects of wound healing (Gurtner et al., 2008). In addition, keratinocyte KRT17 expression, considered a hallmark of normal wound healing (Mazzalupo et al., 2003), was also decreased in the presence of COMP. These results suggest that COMP has a negative influence on ex vivo wound healing. In normally healing wounds of healthy donors, COMP was hardly detectable when re-epithelialization was complete (Agarwal et al., 2013). Similarly, in psoriatic lesions, in which keratinocyte proliferation is abnormally increased, COMP was found to be discontinuous or completely absent from the papillary dermis. Although there are no data regarding keratinocyte proliferation or migration in wounds of COMP-deficient mice (Schulz et al., 2016), in human nonhealing wounds, such as venous leg ulcers, the level of COMP is reported to be highly elevated (Agarwal et al., 2013). Our findings are in agreement with this observation.

In conclusion, our study shows that COMP is present at an elevated level in the papillary dermis of nonlesional psoriatic skin and that it possibly reduces keratinocyte proliferation via the α5β1-integrin. These aspects of COMP contribute to the maintenance of the nonlesional, non-hyperproliferative state of psoriatic nonlesional epidermis, despite the overexpression of EDA + FN and ITGA5. Similar interactions may also take place in other skin diseases in which nonhealing wounds are coupled with massive COMP accumulation.

MATERIALS AND METHODS
Skin samples and ethics
Skin punch biopsies (diameter = 6 mm) were collected from healthy volunteers (n = 10; age 18–70 years, Supplementary Table S1), and from patients with psoriasis with moderate-to-severe chronic plaque-type psoriasis from lesional (n = 13) and nonlesional skin areas (n = 13; minimum of 6 cm from lesional region; age 18–70 years [Supplementary Table S1]). Patients with psoriasis did not receive local therapy for at least 4 weeks and had not been subjected to systemic therapy for at least 8 weeks. Skin biopsies were taken from areas of skin that were not exposed to sun. Tissue collection was obtained after written informed consent, in accordance with the rules of the Helsinki Declaration. The study was confirmed by the Human Investigation Review Board of the University of Szeged (PSO-EDAFN-002, 34/2015, 3517, 23 February 2015, Szeged, Hungary; PSO-ECMPR-002 IF-562-5/2016 and 157/2015-SZTE, 3638, 21 September 2015, Hungary).

Fluorescence microscopic analysis
Biopsies were frozen in a cryogenic matrix (Thermo Fisher Scientific, Waltham, MA) or were paraffin embedded and were subsequently cut into 5-μm sections. For fixation and permeabilization, 4% paraformaldehyde followed by 0.25% TritonX-100 (Thermo Fisher Scientific) or commercially available staining buffer set (eBioscience, Santa Clara, CA) were used. For blocking, Tris-buffered saline containing 1% bovine serum albumin and 1% normal goat serum (Sigma-Aldrich, St. Louis, MO) was used, and for frozen samples, which were digested with chondroitinase ABC (SU, 1:100; Sigma-Aldrich), 10% fetal bovine serum (EuroClone, Pero, Italy) and 5% normal goat serum (Sigma-Aldrich) were applied. Samples were incubated with the following primary antibodies: polyclonal rabbit anti-human COMP (1:250), a kind gift from Mats Paulsson and Frank Zucke from the University of Cologne (Agarwal et al., 2012); mouse anti-human ITGB1 (clone: JB1B, 1:100, Abcam, Cambridge, United Kingdom); rabbit anti-human actin (1:100, Sigma-Aldrich); mouse anti-human Kι67 (1:100, Beckton Dickinson, Franklin Lakes, NJ); mouse anti-human KRT17 (ready to use, Dako, Santa Clara, CA); mouse anti-human LAMA1 (clone: LAM-89, 1:100, R&D Systems, Minneapolis, MN); and mouse anti-human FN (EDA + FN, clone: I5-9, 1:500, Abcam). Isotype controls were the following: rabbit polyclonal IgG (Santa Cruz Biotechnology, Dallas, TX) and mouse IgG1 (Beckton Dickinson) antibodies. As secondary antibodies, Alexa Fluor 546—conjugated anti-rabbit IgG and Alexa Fluor 647—conjugated anti-mouse IgG (Life Technologies, Carlsbad, CA) were used. Nuclei were visualized with DAPI (Sigma-Aldrich) staining. Zeiss LSM 880 or Zeiss Axio Imager Z1 microscopes (Carl Zeiss AG, Oberkochen, Germany) were used for visualization. Pearson’s correlation coefficient, R, was calculated using ImageJ/Fiji software.

Cell cultures and examination of cellular properties
Cell proliferation assay. To investigate the effect of COMP on keratinocyte proliferation, a BrdU cell proliferation colorimetric ELISA assay (Abcam) was performed. HPV-KER cells, a stable human keratinocyte cell line that has been characterized in our laboratory (Dans et al., 2018; Erdei et al., 2018; Tax et al., 2016), and normal human epidermal keratinocyte cells were plated at a density of 10,000 cells per well in 96-well plates (Corning, NY) that were uncoated or coated with low- (1 μg/ml) or high-concentration (10 μg/ml) rhCOMP protein (R&D Systems), in three technical replicates. For the blocking of ITGAs and ITGB1, the following antibodies (1 μg antibody for 10⁶ cells) were used: mouse anti-human ITGAs antibody (clone: IA1, Beckton Dickinson) and mouse anti-human ITGB1 (clone: JB1B). Goat anti-human COMP antibody (1 μg antibody for 10 μg/ml rhCOMP protein, R&D Systems) was applied to block the COMP protein. Integrin- and COMP-blocking was applied to cells grown on uncoated plates or plates coated with 10 μg/ml rhCOMP protein. BrdU assay was performed at 24 and 72 hours after blocking, according to the manufacturer’s instructions.

Ex vivo skin wound-healing assay
Healthy skin samples were collected for the ex vivo organotypic wound healing assay. Approximately 1-cm diameter skin pieces were cut and mildly wounded in the middle using a 4-mm punch biopsy blade (Steele Supply Company, St. Joseph, MI). Wounded skin samples and unwounded control samples were incubated for 72 hours at an air–liquid interface on the upper part of transwell cell culture inserts. The dermal part was in contact with DMEM F12 culture media (Lonza Group, Basel, Switzerland) supplemented with 10% fetal bovine serum (EuroClone) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). The middle of the wounds was treated for 72 hours with high-concentration (10 μg/ml) rhCOMP (R&D Systems) diluted in PBS or PBS only as a control. Samples were fixed in formalin and embedded in paraffin for immunofluorescent staining. To determine the rate of proliferation, 50 cells on each wound edge were counted and the proportion of Ki67-positive cells was determined. Re-epithelialization of untreated, control (where only PBS was administered), and COMP-treated wounds were assessed by measuring the area using the ImageJ software.
Statistical analysis

For comparing only two groups, two-tailed Student t-test was performed. One-way ANOVA with Tukey post-hoc test was used to compare more than two groups. Differences were considered statistically significant at **P < 0.01, *P < 0.05. Data were analyzed using R-Studio software, version 3.2.2 (R-Studio, Boston, MA).

Further methods

More detailed information of the materials and methods regarding protein isolation and western blot analysis, cell culture experiments, H&E staining, RNA isolation, and real-time PCR are presented in the Supplementary Materials.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: GG; Formal Analysis: RB, KS, GG; Investigation: RB, ES, JD, BG; Methodology: RB, GG; Supervision: LK, ZBC, GG; Writing - Original Draft Preparation: RB, GG; Writing - Review and Editing: KS, ZBC, GG.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org and at https://doi.org/10.1016/j.jid.2019.12.037.

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SUPPLEMENTARY MATERIALS AND METHODS

Protein isolation and western blot analysis

For preparation of tissue protein extracts, skin biopsies (healthy, psoriatic nonlesional, and psoriatic lesional) were cut into small pieces with a razor blade. Guanidine hydrochloride (6 M, Sigma-Aldrich, St. Louis, MO) solution was used as an extraction buffer for 24 hours at 4 °C under continuous agitation. For protein precipitation, an ethanol-based method was applied. Protein concentrations were measured using Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein extracts (25 μg) were separated on a 4–20% gradient SDS polyacrylamide gel under reducing or nonreducing conditions. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and blocked in 5% nonfat milk powder containing Tris-buffered saline for 60 minutes at room temperature. Membranes were incubated for overnight at 4 °C with goat anti-human COMP primary antibody (1:2,000, R&D Systems, Minneapolis, MN), and rabbit anti-human actin primary antibody (1:2,000, Sigma-Aldrich). Subsequently, membranes were incubated with horseradish peroxidase–conjugated anti-goat (Thermo Fisher Scientific, Waltham, MA), and anti-rabbit (Southern Biotech, Birmingham, AL) secondary antibodies, both diluted 1:2,000 for 60 minutes at room temperature. Signal was visualized with Clarity Max Western ECL Substrate (Bio-Rad Laboratories) on a C-digit blot scanner (LI-COR Biosciences, Lincoln, NE).

H&E staining and light microscopic analysis

To visualize the tissue structure of ex vivo wound model samples, H&E (Leica Biosystems, Wetzlar, Germany) staining was performed according to the manufacturer’s instructions in a Leica ST5020 Multistainer device (Leica Biosystems). The stained samples were visualized with a Nikon eclipse TS100 microscope (Nikon, Minato, Tokyo, Japan).

Cell cultures and examination of cellular properties

Cell cultures. Primary dermal fibroblasts were isolated from healthy and psoriatic nonlesional skin biopsies, and normal human epidermal keratinocytes (NHEKs) were isolated from healthy skin samples. The epidermis was separated from the dermis with overnight incubation at 4 °C in Dispase II (neutral protease, grade II, 2 U/ml, Roche Diagnostics, Basel, Switzerland) (Szabad et al., 2007) solution. Keratinocytes were obtained from the epidermal part after trypsin digestion for 10 minutes at 37 °C (Sigma-Aldrich). NHEK cells were then grown in keratinocyte serum-free medium (Life Technologies, Carlsbad, CA) supplemented with 1% antibiotic/antimycotic solution (Sigma-Aldrich), brain pituitary (Life Technologies, Carlsbad, CA) supplemented with 1% L-glutamine (PAA Laboratories GmbH, Pasching, Austria.

The human immortalized keratinocyte cell line HPV-KER was also used for our experiments. HPV-KER is a stable cell line, generated from NHEKs transfected with the HPV16/E6 oncosine in a pCMV vector. It was established by continuous culturing (Tax et al., 2016). It shows similar responses to NHEK cells in various immune activation protocols (Danis et al., 2018; Erdei et al., 2018). Culture conditions of HPV-KER cells are the same as NHEK cells. Each cell type was cultured at 37 °C in a humidified atmosphere with 5% v/v CO₂.

Real-time, label-free cellular analysis of HPV-KER cells using the xCELLigence system. xCELLigence (ACEA Biosciences, San Diego, CA) is a real-time, impedance measurement–based cellular analysis system, where dimensionless cell index value is calculated (cell index = [impedance at time point n – impedance in the absence of cells]/nominal impedance value). Differences in cell index values could be due to altered cell proliferation rate, viability, morphology, and adhesion (Dickhuth et al., 2015). This system was used to investigate the effect of the COMP protein on keratinocytes. HPV-KER cells were plated at a density of 10,000 cells per well in uncoated 96-well E-plates (ACEA Biosciences) or wells that were coated with low-concentration (1 μg/ml) or high-concentration (10 μg/ml) recombinant human COMP protein (R&D Systems). Impedance measurement was performed every 15 minutes for 140 hours, and a dimension-free cell index value was calculated for every time point. Four technical replicates were performed.

Further investigation of keratinocyte cell proliferation. To further investigate the effect of COMP on the proliferation of NHEK cells, KI67 immunofluorescent staining (mouse anti-human KI67 antibody, 1:100, Beckton Dickinson, Franklin Lakes, NJ) was applied, using integrin and COMP-blocking as described in the main text. Cells were plated at a density of 20,000 cells per well in 8-well chamber slides (SPL Life Sciences, Naechon-Myeon, Pocheon-si, Korea) that were uncoated or coated with high-concentration (10 μg/ml) recombinant human COMP (R&D Systems) in three biological replicates. KI67-positive cells were counted on three randomly selected areas per group, and statistical analysis was performed.

RNA isolation and real-time PCR. Total RNA was isolated from primary fibroblasts from healthy and psoriatic nonlesional skin cultured in 75-cm² cell culture flasks (Corning, NY) and collected at the fifth passage using TRI-Reagent (Molecular Research Center, Cincinnati, OH) as described by the manufacturer. The iScript cDNA Synthesis kit (Bio-Rad Laboratories) was used for cDNA synthesis, and 0.5 μg total RNA was reverse transcribed. RT-PCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories) with the Universal Probe Library system (Roche Diagnostics) using qPCR Bio Probe Mix Lo-ROX (PCR Biosystems Ltd., London, United Kingdom) and the following primers:

with 1 g/liter glucose (Lonzga Group), supplemented with 5% fetal bovine serum (EuroClone), 1% antibiotic/antimycotic solution (Sigma-Aldrich), and 1% L-glutamine (PAA Laboratories GmbH, Pasching, Austria).

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COMP FWD: CACCGACGTCAACGAGTG, COMP REV: TGGTGTTGATAACGGGACT; 18S rRNA FWD: CGCTCCAC CAACTAAGAAGC, 18SrRNA REV: CTCAACACGGGAAACC TCAC. The expression of COMP was normalized to 18S rRNA expression using the ΔΔCt method.

SUPPLEMENTARY REFERENCES


Supplementary Figure S1. COMP monomer and fragment level is elevated in psoriatic nonlesional skin under reducing conditions. COMP monomers and fragments were detected with western blot analysis from healthy, psoriatic nonlesional, and lesional skin under reducing conditions (n = 3). The band intensities of (a) COMP monomer and (b) COMP fragment separately, as well as (c) the level of monomer and fragment together were analyzed with Image Studio software (LI-COR Biosciences) and presented as fold changes normalized to actin. The graph shows mean ± SEM (n = 3), *P < 0.05 versus healthy control, calculated by one-way ANOVA followed by Tukey's post-hoc test. COMP, cartilage oligomeric matrix protein.

Supplementary Figure S2. COMP protein detection under nonreducing conditions. COMP protein was detected with western blot analysis from healthy, psoriatic nonlesional, and lesional skin under nonreducing conditions (n = 3). A representative blot is shown. COMP, cartilage oligomeric matrix protein; H, healthy; L, lesional; NL, nonlesional.
Supplementary Figure S3.
Characterization of COMP deposition in healthy, nonlesional, and lesional skin. Immunofluorescence staining for COMP in (a) healthy, (b) psoriatic nonlesional, and (c) psoriatic lesional skin (n = 10). Zeiss Axio Imager Z1 original magnification, ×20. Bar = 50 μm. COMP, cartilage oligomeric matrix protein.
Supplementary Figure S4. Enhanced colocalization of COMP with β1-integrin in nonlesional psoriatic skin. Confocal microscopic immunofluorescence analysis of COMP and β1-integrin in (a) healthy and (b) psoriatic nonlesional skin using z-stack pictures. Dotted lines indicate the borders of enlarged regions. Colocalized pixels of COMP and β1-integrin were calculated by ImageJ software (n = 5). Original magnification, ×63. Bar = 10 μm. COMP, cartilage oligomeric matrix protein.
Supplementary Figure S5. COMP negatively influences keratinocyte CI via α5β1-integrin. CI measurement of HPV-KER cells cultured on uncoated and rhCOMP-coated (10 μg/ml) surfaces. CI was determined using real-time impedance measurement–based cellular analysis. The graph is representative of three independent experiments, all showing similar results. Mean CI ± SEM of four technical replicas for each group. CI, cell index; COMP, cartilage oligomeric matrix protein; rhCOMP, recombinant human COMP.

Supplementary Figure S6. Reduced proliferation and re-epithelization in the presence of COMP during ex vivo skin wound healing. (a) Reduced number of Ki67-positive cells was detected in wound samples treated with rhCOMP (10 μg/ml) compared with untreated control wounds. The graph shows mean ± SEM (n = 3), *P < 0.05 versus untreated control, calculated by two-tailed Student t-test. (b) Re-epithelization of artificial untreated and rhCOMP-treated (10 μg/ml) wounds on H&E-stained sections (n = 3). Original magnification, ×20. Bar = 50 μm. Representative pictures are shown. Arrowheads indicate the newly synthetized areas. (c) Re-epithelization of untreated and control- and rhCOMP-treated wounds was measured using area measurement of ImageJ software. The graph shows mean ± SEM (n = 3), *P < 0.05 versus untreated control, calculated by two-tailed Student t-test. COMP, cartilage oligomeric matrix protein; rhCOMP, recombinant human COMP.
## Supplementary Table S1. Donor Data and Experiments Applied to Donor Samples

<table>
<thead>
<tr>
<th>Donor Information</th>
<th>Experimental Application List of Donor Samples</th>
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<td>Figure 1b (Western Blot Analysis)</td>
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Abbreviations: IF, immunofluorescence; n/a, not applicable.