Dermal Hyaluronan Is Rapidly Reduced by Topical Treatment with Glucocorticoids

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Skin atrophy is part of the normal ageing process, but is accelerated by topical glucocorticoid (GC) treatments that are widely used in dermatology. Hyaluronan (HA) is one of the most abundant components of the cutaneous extracellular matrix and is involved in tissue homeostasis, hydration, and repair processes, but little is known about the effects of GCs on HA synthesis and stability. Here we examined the regulation of HA metabolism in human skin during GC therapy. Expression of the HA synthesizing enzymes hyaluronan synthase (HAS)-2 and HAS-3 and the HA degrading enzymes HYAL-1, HYAL-2, and HYAL-3 in response to GC treatment was evaluated. HAS-2 expression was markedly suppressed by dexamethasone treatment of cultured fibroblasts and HaCaT keratinocyte cells, and in human skin biopsies taken from volunteers treated with dexamethasone ointment. Consistently, the HA content of cell culture supernatants and in human skin was reduced after dexamethasone treatment. Hyaluronidase expression and activity, on the other hand, was not altered by dexamethasone treatment. These data show that the levels of skin HA rapidly decrease after short-term GC treatment due to a reduction in HA synthesis, while HA degradation is not changed. This may reflect an initiation of skin atrophy in response to topically applied GCs.

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INTRODUCTION

Skin atrophy is partly considered to be a physiological process associated with ageing. However, it is accelerated by glucocorticoid (GC) treatment. GC-induced skin atrophy together with purpura, pseudoscarring, laceration, and dissecting hematoma may lead to significant morbidity and has recently been termed dermatoporosis (Kaya and Saurat, 2007). GCs are widely used to treat inflammatory diseases, and their topical application remains the first-line therapy for many inflammatory skin diseases. Their excellent anti-inflammatory properties are hampered by side effects if application is continued for extended periods (for example, weeks to months). The most prominent irreversible cutaneous adverse effect is atrophy of the skin, and involves both the epidermis and the dermis. The characteristics of GC-induced skin atrophy include increased fragility, tearing and bruising, as well as an increased transparency and a telangiectatic surface (Schacke *et al.*, 2002; Kaya and Saurat, 2007). In addition, thinning of the horny layer results in increased permeability and transepidermal water loss (Schacke *et al.*, 2002).

At the molecular level, GCs have been shown to modulate collagen synthesis in human skin. For example, a decreased synthesis of type I and type III collagens following GC application has been shown in numerous studies (Oikarinen *et al.*, 1998; Nuutinen *et al.*, 2003; Schoepe *et al.*, 2006). A reduction in glycosaminoglycan production in the skin subsequent to treatment with GCs has also been described (Sarnstrand *et al.*, 1982). Hyaluronan (HA) is the major glycosaminoglycan in the skin and one of the most abundant components of the cutaneous extracellular matrix. Although a systematic analysis of HA metabolism in the human skin remains to be performed, a recent study that dealt with possible therapeutic options for skin atrophy reported a reduced HA content in the atrophic skin (Kaya *et al.*, 2006).

Hyaluronan is involved in many biological processes such as tissue homeostatsis, cell proliferation, cell migration, cell differentiation, angiogenesis, tumor biology, and repair processes (Toole, 2004). This linear nonsulfated glycosaminoglycan is composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine ((-β(1,4)-GlcUA-β(1,3)-GlcNAc-)$_n$). Under physiological conditions, high molecular weight HA molecules of 10^5 to 10^7 Da composed of 2,000–25,000 disaccharide units are predominant (Stern, 2003). In the skin, HA metabolism and turnover are rapid, and epidermal HA turns over within 1–2 days

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Abbreviations: GC, glucocorticoids; HA, hyaluronan; HAS, hyaluronan synthase; HYAL, hyaluronidase

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(Stern, 2003; Tammi *et al.*, 2005). We have recently shown that dermal HA is also subject to rapid regulation. For example, during repair processes following acute UVB injury, an increased HA synthesis and turnover could already be detected 3 hours post-UVB irradiation (Averbeck *et al.*, 2007).

Several enzymes are involved in HA metabolism. In mammals, HA is synthesized by three different HA synthases (HAS-1, -2, -3) that possess diverse properties (Stern, 2005). HAS-1 and HAS-2 generate high molecular weight HA ($2–4 \times 10^6$ Da), while HAS-3 is involved in the production of lower molecular weight HA ($0.4–2.5 \times 10^5$ Da). HAS-1 is by far the least active of these enzymes, while HAS-2 and HAS-3 show comparable degrees of activity (Itano *et al.*, 1999). HA catabolism, on the other hand, is mediated by the family of hyaluronidases (HYALs). HYAL-1 is associated with the lysosomal compartment, while HYAL-2 is linked to the plasma membrane by a glycosylphosphatidylinositol link. HYAL-1 generates tetrasaccharides, whereas HYAL-2 cleaves high molecular HA into fragments of $10–20 \times 10^3$ Da (Noble, 2002; Stern, 2003, 2005). HYAL-3 is widely expressed but less well characterized (Stern, 2005).

Limited studies using cultured cells indicate that GCs can influence HA metabolism. Zhang *et al.* (2000) showed a downregulation of HAS mRNA levels after GC treatment of cultured fibroblasts and an osteoblast-like osteosarcoma cell line. Similar results were obtained using fibroblast-like synoviocyte cultures and leukocytes isolated from the synovial fluid of rheumatoid arthritis patients (Stuhlmeier and Pollaschek, 2004b). In both studies, the mRNA levels of HA synthesizing enzymes were assessed *in vitro*. Neither the impact of these changes on the actual HA synthesis, nor the expression and activity of HA-degrading enzymes has been investigated to date. Furthermore, *in vivo* studies that verify findings made using cultured cells are lacking.

In this study, we aimed at investigating the influence of GCs on HA metabolism in human skin. In addition to analyzing GC-treated human skin, we also investigated GC effects *in vitro* on dermal fibroblasts and a keratinocyte-derived immortalized cell line, representative of the major cell types in dermis and epidermis, respectively. The expression of HA metabolizing enzymes was evaluated by quantitative RT–PCR, as well as by measuring the accumulation of HA in cell culture supernatants and in human skin. We found a profound downregulation of HAS-2 mRNA in response to GC treatment that was reflected in a decrease in the HA content of cell culture supernatants and in human skin. In contrast, HYAL mRNA levels and HYAL activity as measured by zymography were not affected by treatment with GCs. Moreover, dermal microdialysis fluid from GC-treated skin did not reveal any increase in low molecular weight HA fragments, which would have been indicative of increased HYAL activity. We conclude that the rapid decrease in HA production following GC treatment is mainly due to a suppression of HAS-2-mediated HA synthesis and not to increased HA catabolism.

RESULTS

Dexamethasone treatment causes a profound downregulation of HAS-2 mRNA

To determine whether dexamethasone treatment affects the expression of HA synthesizing enzymes, cultured fibroblasts and keratinocyte-derived immortalized cells were incubated with 150 nM dexamethasone for 24 hours. The keratinocyte-derived immortalized cell line was used rather than primary keratinocytes because the media required for the culture of primary keratinocytes contains hydrocortisone, which would have hampered our analyses. Human skin was also treated three times daily with 0.1% dexamethasone ointment for 72 hours. The mRNA expression levels of HA metabolizing enzymes in both settings were assessed by quantitative RT–PCR. We used rps26 to normalize expression between the different samples, as ribosomal proteins show particularly stable expression under various conditions and stimuli (Vincent *et al.*, 1993), whereas expression of commonly used housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase and β-glucuronidase is affected by GCs (Nishimura *et al.*, 2006). Indeed, in preliminary experiments we found that rps26 expression was not affected by GC treatment (data not shown), consistent with other studies using human skin that show that rps26 is a reliable housekeeping gene for normalization (Bonnet-Duquennoy *et al.*, 2006).

As we have previously demonstrated that HAS-1 is only expressed at very low levels in fibroblasts, HaCaT and in human skin specimens (Averbeck *et al.*, 2007), we focused our attention on HAS-2 and HAS-3 expression. HAS-2 expression was strongly downregulated after dexamethasone treatment in fibroblasts, the keratinocyte-derived immortalized cell line and GC-treated skin (Figure 1a). HAS-3 expression was not changed significantly (Figure 1b). To investigate the dose-dependency and kinetics of the GC-induced reduction in HAS-2 expression, fibroblasts (Figure 1c) and keratinocyte-derived immortalized cells (Figure 1d) were incubated with 1.5 nM, 150 nM, and 1.5 μM dexamethasone for 3, 6, 24, and 48 hours, respectively. Treatment with 150 nM and 1.5 μM dexamethasone gave a stronger inhibition of HAS-2 expression than 1.5 nM. Incubation with 150 nM gave the maximal inhibition, and this concentration was therefore chosen for further experiments. Although a trend toward stronger effects with longer incubation times or higher GC concentrations was observed, no significant difference between the four investigated time points or the dexamethasone concentrations used could be determined. To test whether cytosolic receptors mediate the GC-induced HAS-2 downregulation, we used RU486 (mifepristone), an archetypal antagonist for cytosolic steroid hormone receptors. In fibroblasts (Figure 1e) and keratinocyte-derived immortalized cells (Figure 1f), we found that at least part of the GC-induced HAS-2 downregulation is mediated by cytosolic steroid hormone receptors, as preincubation with RU486 reduced this effect significantly in keratinocyte-derived immortalized cells. In fibroblasts, the dexamethasone-induced HAS-2 downregulation could not be blocked significantly by preincubation with RU486.

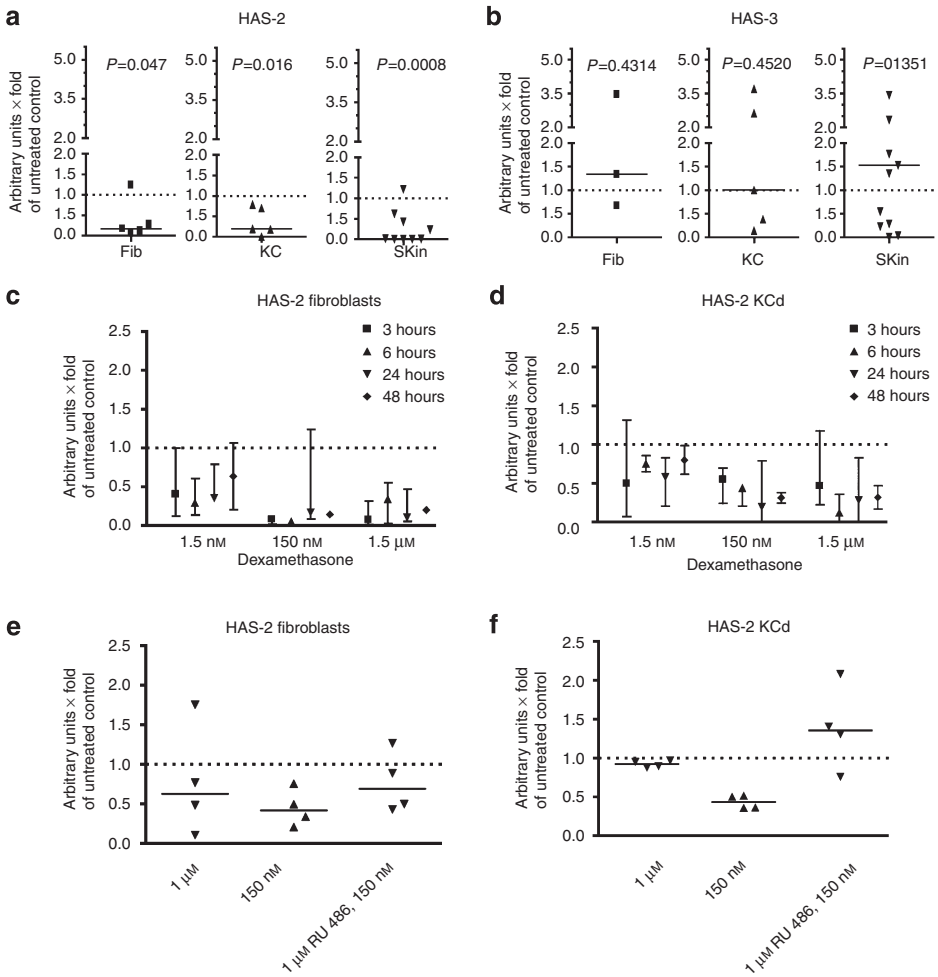

Figure 1. Dexamethasone induces a strong downregulation of HAS-2. (**a** and **b**) Quantitative RT–PCR for HAS-2 (**a**) and HAS-3 (**b**) for fibroblasts (Fib), keratinocyte-derived immortalized cells (KCd), and human skin. Values were normalized to the rps26 housekeeping gene levels and compared with untreated controls (dotted line = 1). RNA was extracted from the cells after 24 hours treatment with 150 nM dexamethasone. Human skin biopsies were taken after either 72 hours of treatment with 0.1% dexamethasone ointment or from an untreated region of the same dermatome. Values are displayed as scatter plots with median values. Indicated *P*-values result from paired *t*-test analysis. (**c** and **d**) Quantitative RT–PCR of HAS-2 RNA levels in fibroblasts (**c**) and keratinocyte-derived immortalized cells (**d**) is shown 3, 6, 24, and 48 hours after treatment with 1.5 nM, 150 nM, and 1.5 µM dexamethasone, respectively. Values of at least three independent experiments are displayed in a category graph as median ± range. (**e** and **f**) Quantitative RT–PCR of HAS-2 RNA levels in fibroblasts (**e**) and keratinocyte-derived immortalized cells (**f**) is shown. Cells were either treated with 1 µM RU486 (clear bars), 150 nM dexamethasone (grey bars), or with 1 µM RU486 for 8 hours followed by 150 nM dexamethasone for 24 hours (black bars). Values are displayed as scatter plots with median values. The significance of differences was determined by analysis of variance (*P*-values Figure 1e: 0.5740; 1f: 0.0184) followed by Tukey's post-test, which revealed a statistical significant difference, as shown in Figure 1f, between dexamethasone-treated cells without and with preincubation with RU486.

HA levels in cell culture supernatants are reduced by dexamethasone

To test if the observed modulation of HAS gene expression affects the amount of HA synthesized by cells, medium conditioned by fibroblasts and keratinocyte-derived immortalized cells treated with 150 nM dexamethasone for 3 and 24 hours was analyzed by ELISA. After 3 hours treatment (Figure 2a), only a weak reduction in the amount of HA released by fibroblasts (left panel, 85 ng ml^{-1}) and keratinocyte-derived immortalized cells (right panel, 57 ng ml^{-1}) could be observed compared with the untreated controls (fibroblasts: 159 ng ml^{-1}, keratinocyte-derived immortalized cells: 68 ng ml^{-1}). After 24 hours treatment (Figure 2b), dexamethasone reduced the amount of HA produced significantly (fibroblasts, 546 ng ml^{-1}; keratinocyte-derived immortalized cells, 352 ng ml^{-1}) compared with untreated cells (fibroblasts, 301 ng ml^{-1}; keratinocyte-derived immortalized cells, 245 ng ml^{-1}).

Topical dexamethasone leads to a rapid reduction in dermal HA

To determine whether our *in vitro* findings are relevant to the *in vivo* situation, we examined whether dexamethasone reduces the HA content in human skin. To address this issue, we examined the amount of HA in human skin samples derived from the same dermatome that had either been treated with 0.1% dexamethasone ointment three times daily for 72 hours or left untreated. Skin sections taken from these samples were stained immunohistochemically with

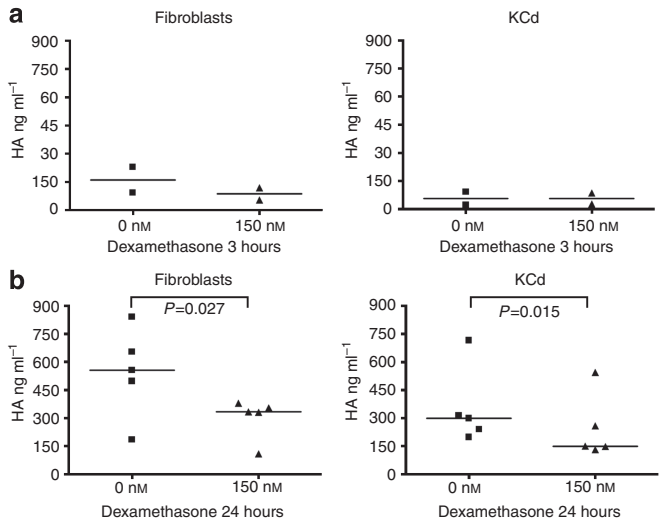

Figure 2. Dexamethasone treatment reduces the HA content in cell culture supernatants. (**a**) HA content of conditioned medium from fibroblasts and keratinocyte-derived immortalized cells (KCd) either treated for 3 hours with 150 nM dexamethasone or left untreated is shown. Values are displayed as scatter plots with median values. (**b**) HA content of conditioned medium from fibroblasts and keratinocyte-derived immortalized cells either treated for 24 hours with 150 nM dexamethasone or left untreated is shown. The HA concentrations were measured by ELISA. Values are displayed as scatter plots with median values. Indicated *P*-values result from paired *t*-test analysis.

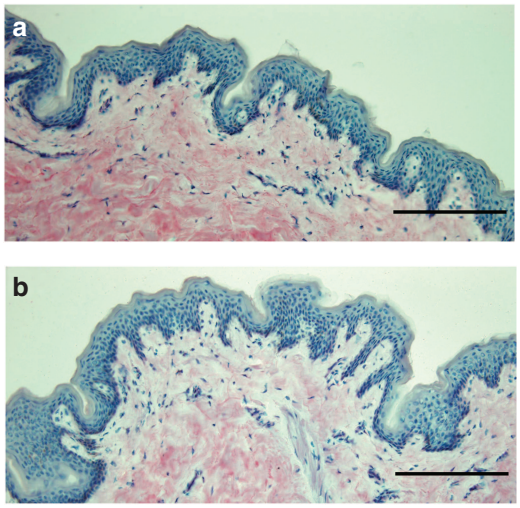

Figure 3. Dexamethasone reduces the dermal HA content *in vivo*. Immunohistochemical staining of HA in human skin treated with 0.1% dexamethasone ointment three times daily (**b**) or left untreated (**a**) from the same individual and from a similar location. HA was detected using biotinylated HA-binding protein and visualized using the avidin-biotin complex technique (red color). Scale bars are 200 µm. The data are representative of that obtained for five individuals.

biotinylated HA-binding protein. A reduced intensity in the HA staining within the papillary dermis after treatment with dexamethasone (Figure 3b) was observed compared with the untreated control (Figure 3a). Specificity of staining was confirmed by earlier digestion of the samples with HYAL (data not shown). These *in vivo* data therefore support our *in vitro* findings, namely that the reduced intensity of the HA staining was consistent with a reduction in HA concentration in cell culture supernatants.

Dexamethasone treatment does not affect HYAL mRNA levels and HYAL activity

Changes in the steady state levels of HA in the skin in response to GCs could be caused by reduced HAS-2 expression, but also by increased expression or activity of HYALs. We therefore also examined the expression of the three major HYALs. As above, RNA was extracted from fibroblasts and keratinocyte-derived immortalized cells after incubation with 150 nM dexamethasone for 24 hours, whereas the expression in the skin was measured after treatment with 0.1% dexamethasone ointment three times daily for 72 hours. Although a slight modulation in the expression of HYAL-1 (Figure 4a), HYAL-2 (Figure 4b) and HYAL-3 (Figure 4c) could be observed, no significant differences were detected between GC-treated samples and untreated controls.

To investigate whether the unchanged HYAL mRNA levels we observed were reflected in the catabolic HYAL activity of the corresponding protein, we used zymography. Conditioned medium taken from fibroblasts and keratinocyte-derived immortalized cells that had been either treated with 150 nM dexamethasone for 24 hours or left untreated were investigated using this method. The degree of HYAL activity is reflected in the density of the lysed band in the zymogram. As can be seen in Figure 5a, no difference in HYAL activity could be observed between GC-treated and untreated samples. The analyzed conditioned medium contained 1% fetal bovine serum, which itself has HYAL activity, so we therefore also performed control zymographies to determine the HYAL activity present in 0.1 and 1% fetal bovine serum. As expected, HYAL activity was measurable in fetal bovine serum, although to a much lower extent than in conditioned medium from the cells (Figure 5b, arrows). The cell-derived HYAL activity therefore accounts for the majority of HYAL activity detected in the conditioned media (Figure 5a). As the activity shown in undiluted supernatants is quite high, we performed zymography of diluted supernatants to detect even small changes in HYAL activity. Analysis of substantially diluted supernatants (1:10 and 1:100) did not reveal changes in HYAL activity in response to GC treatment (Figure 5c).

Levels of dermal low molecular weight HA remain unchanged by dexamethasone treatment

Hyaluronan exerts different cellular effects depending on the length of the polysaccharide chain. Small HA oligomers have numerous biological effects that are not observed with larger HA molecules (Termeer *et al.*, 2002; Fieber *et al.*, 2004; Taylor *et al.*, 2004; Stern *et al.*, 2006). Even though we saw no evidence for a GC-dependent increase in HYAL activity in our previous experiments, we nevertheless wished to confirm that the reduction in HA content in GC-treated human skin is solely due to a reduced HA synthesis. To this end we used intradermal microdialysis limited by a 100 kDa cutoff filter to

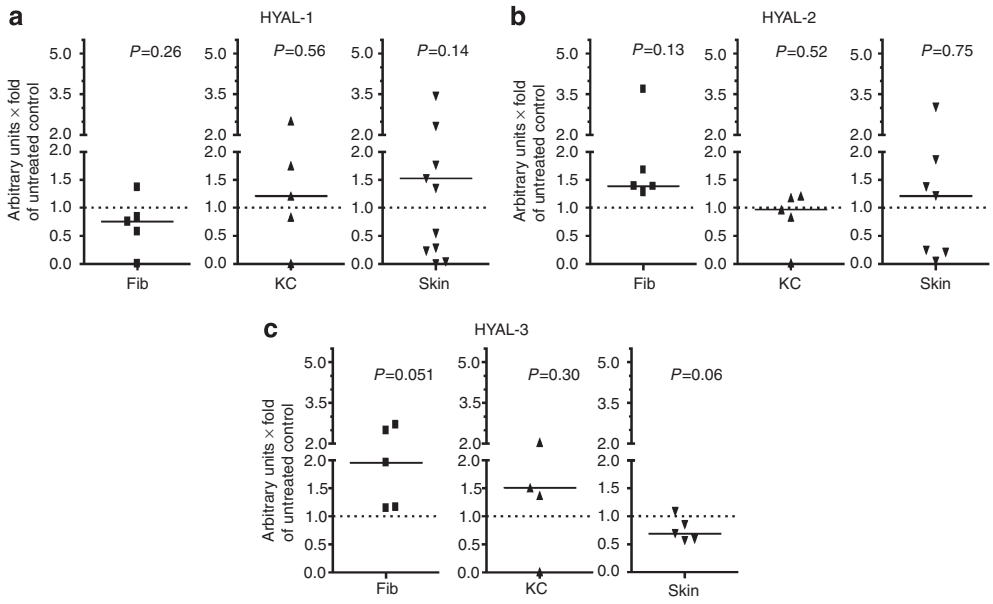

Figure 4. RNA expression levels of HYALs are only marginally changed by dexamethasone. Quantitative RT–PCR for HYAL-1 (**a**), HYAL-2 (**b**), and HYAL-3 (**c**) using RNA extracted from fibroblasts (Fib), keratinocyte-derived immortalized cells (KCd) and human skin. Values were normalized to the rps26 housekeeping gene levels and compared with untreated controls (dotted line = 1). RNA was extracted from the cultured cells after 24 hours treatment with 150 nM dexamethasone. Human skin biopsies were taken after either 72 hours of treatment with 0.1% dexamethasone ointment or from an untreated region of the same dermatome. Values are displayed as scatter plots with median values. Indicated *P*-values result from paired *t*-test analysis.

collect the dermal fluid from GC-treated and untreated skin, and subsequently analyzed the HA content as previously described (Averbeck *et al.*, 2006, 2007). We have already shown that catheters with a 100 kDa cutoff membrane are suitable for the detection of low molecular weight HA fragments (Averbeck *et al.*, 2007). During the sampling interval before GC treatment (16–24 hours), the average HA content of HA fragments was 6.7 ng ml^{-1} (Figure 5d). GC treatment was started after this sampling interval. The skin was treated with 0.1% dexamethasone ointment three times daily and continued for 48 hours. In microdialysis samples taken at later time points after commencement of intradermal microdialysis (40–48 and 66–72 hours), the HA content was 4.1 and 5.9 ng ml^{-1}, respectively. No significant difference could be observed between any of the investigated time points.

DISCUSSION

Glucocorticoids are important anti-inflammatory drugs for the treatment of a variety of diseases, and a detailed knowledge about their side effects is therefore required. Bone and skin atrophy are among the most prominent irreversible side effects of GCs, especially after long-term use (Schacke *et al.*, 2002; Jackson *et al.*, 2007). In the last few decades, great progress has been made in understanding the molecular mechanisms that lead to a reduction in type I and type III collagen synthesis after GC treatment of skin (Oikarinen *et al.*, 1998; Nuutinen *et al.*, 2003; Schoepe *et al.*, 2006). These findings have demonstrated the importance of the extracellular matrix in GC-induced skin atrophy and have revealed important effects such as the reduction in keratinocyte size and proliferation (Delforno *et al.*, 1978; Lange *et al.*, 1997), as well as the antiproliferative effects of GCs on fibroblasts (Ponec *et al.*, 1977). However, little is known about the effects of GCs on glycosaminoglycans. As HA is one of the most abundant components of human skin with rapid turnover due to a high activity of degrading and synthesizing enzymes (Stern, 2003; Averbeck *et al.*, 2007), we aimed in this study at investigating the effects of GCs on HA metabolism and deposition to gain further insight into the effects of GCs on human skin. The major findings of this study are that dexamethasone leads to a rapid reduction of HA levels *in vitro* and *in vivo*. This is consequent to a downregulation of HAS-2, the major HA synthesizing enzyme in cells of the human skin (Averbeck *et al.*, 2007). No effects on HYAL expression or activity were observed.

Our *in vitro* findings are based on experiments using skin fibroblasts and keratinocyte-derived immortalized cells, representative of the major cell types in dermis and epidermis. In our experiments keratinocyte-derived immortalized cells were preferred over primary keratinocyte cultures because of the special keratinocyte media required for the culture of primary cells. As this media contains various supplements including hydrocortisone, a proper investigation of GC effects on HA metabolism in primary keratinocytes is not possible without the risk of manipulation of the observed effects through the necessity of having hydrocortisone in the culture medium. Furthermore, keratinocyte-derived immortalized cells are a well-established model for keratinocytes (Averbeck *et al.*, 2007) and the observed effects in keratinocyte-derived immortalized cells are therefore highly likely to be equivalent in primary keratinocytes.

Downregulation of HAS-2 was observed even at low doses of dexamethasone and occurred as early as 3 hours following GC treatment. While our experiments did not show a

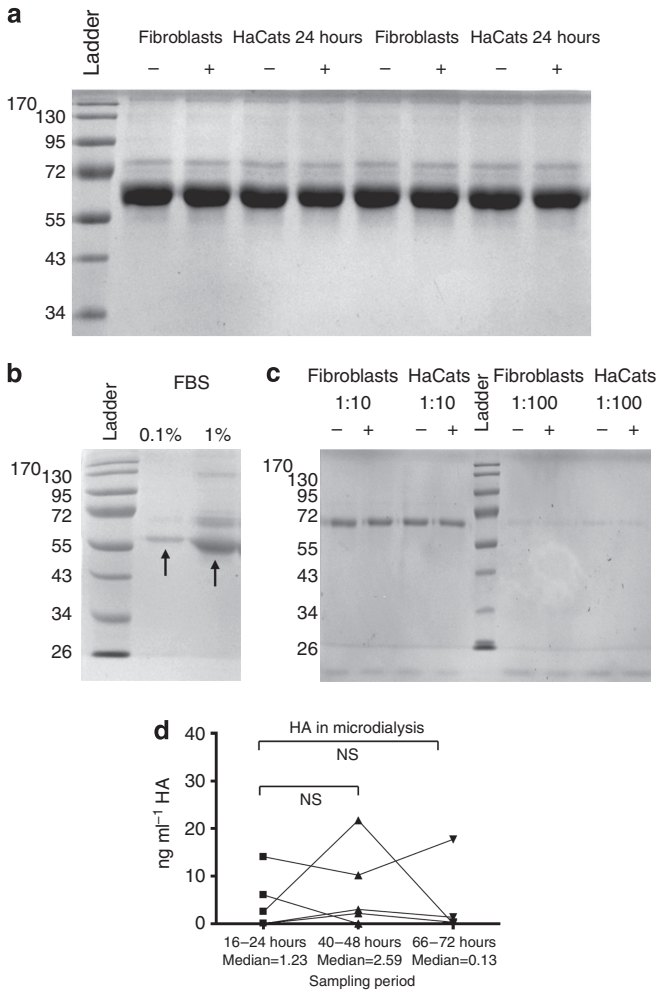

Figure 5. Zymographic analysis shows unchanged HYAL activity after dexamethasone treatment and dexamethasone does not alter the quality of HA in human skin *in vivo*. (**a**) Zymography of conditioned medium taken from fibroblasts and keratinocyte-derived immortalized cells (KCd) that were either treated with 150 nM dexamethasone (+) or were left untreated (−). Equal amounts of protein were analyzed. For size analysis, a protein ladder is shown on the left. HYAL activity in cell culture medium containing 0.1% fetal bovine serum (FBS) and 1% FBS is also shown (**b**). (**c**) Zymography of conditioned, diluted (1:10 or 1:100) medium taken from fibroblasts and keratinocyte-derived immortalized cells that were either treated with 150 nM dexamethasone (+) or were left untreated (−). Equal amounts of protein were analyzed. For size analysis, a protein ladder is shown in the middle. (**d**) HA content of dermal microdialysis fluid samples from human skin. For dermal microdialysis, a 100 kDa cutoff catheter was placed intradermally at a depth of 1 mm in the ventral forearm as controlled by 20 MHz ultrasound. Twenty-four hours following catheter placement, treatment with 0.1% dexamethasone ointment three times daily was initiated, followed by sampling for another 48 hours. Sampling intervals were 16–24, 40–48, and 66–72 hours. HA present in the dermal microdialyis fluid was measured by ELISA. Data are presented as a line graph for each individual patient. The significance of differences between the different sampling periods was determined by Friedman test ($P = 0.7402$).

completely tight correlation between downregulation of HAS-2 and the length of treatment or concentration of dexamethasone used, nevertheless a trend toward a more pronounced downregulation with the highest dexamethasone concentrations used was observed (1.5 μM, Figure 1c and d). HAS-3 mRNA levels were not changed significantly. HAS-1 is by far the least active HAS (Itano *et al.*, 1999) and significant expression in fibroblast-like synoviocytes is only seen, for example, after stimulation with tissue growth factor-β (TGFβ) (Stuhlmeier and Pollaschek, 2004a). In our own studies, we also found that HAS-1 expression in fibroblasts, HaCaT, and human skin samples was very low and at the detection limit (Averbeck *et al.*, 2007, and data not shown).

Reduced HA synthesis and an increased HA catabolism could both lead to a diminished HA content. As HA fragments have proinflammatory, immunostimulatory, and angiogenic properties (Stern *et al.*, 2006; Jiang *et al.*, 2007), expression patterns of HA-degrading enzymes are also of great interest. However, we show here that GCs do not significantly affect the expression of HYALs. Furthermore, the zymographic analyses show that equivalent levels of HYAL activity are produced by GC-treated and untreated cells. Moreover, GC treatment did not lead to a detectable production of low molecular weight HA fragments in human skin, as determined by dermal microdialysis. As HA synthesis is decreased by GCs, one could argue that levels of HA fragments should also be decreased after GC treatment in the dermal microdialysis experiments. However, due to the fact that the baseline levels in the sampling period 16–24 hours (Figure 5c) were already at the detection limit, a reduction in the level of HA fragments could not be measured reliably. Together, these data support the notion that GC treatment reduces steady state HA levels in the skin through reduced HA synthesis but not increased HA catabolism.

The high turnover of HA in the skin means that the reduced HA synthesis capacity we observed should lead to a detectable reduction in the HA content of GC-treated samples. Indeed, a reduced HA content in conditioned medium from GC-treated fibroblasts and keratinocyte-derived immortalized cells was already observed after 3 hours treatment, and became more pronounced after 24 hours. Fibroblasts make up the majority of cells in the dermal compartment of human skin, whereas the epidermal compartment is composed mainly of keratinocytes. Our data therefore suggest that HA synthesis should be reduced in both compartments by GC treatment. Consistently, a decrease in HA content could be observed immunohistochemically in human skin following GC treatment.

A short treatment period (3 days) was sufficient to induce a distinct reduction in steady state HA levels in the papillary dermis of GC-treated human skin. In contrast, it took 3 weeks before a reduction in the synthesis of type I and type III collagens could be observed in response to GC treatment (Nuutinen *et al.*, 2003). HA in the skin is abundant, accounting for half of the total body HA (Stern, 2003). It is therefore significant that reduced HA levels were observed only after 3 days of GC treatment, and raises the question as to whether the decrease in dermal HA might be the initial step in GC-induced skin atrophy and dermatoporosis. If so, the measurement of HA synthesis and deposition could prove to be a useful tool as an early indicator of GC-induced skin atrophy, which in turn could contribute to the development of new GCs with lower atrophogenic potential.

Glucocorticoids exert their effects through different molecular pathways, for example, through the classical cytosolic steroid hormone receptors (Schoepe *et al.*, 2006), through cell-surface steroid hormone receptors (Song and Buttgereit, 2006), and through cross-regulation of other transcription factors through steroid hormone receptors (Schoepe *et al.*, 2006). To our knowledge, the HAS-2 promoter does not contain GC responsive elements. Thus, the classical cytosolic steroid hormone receptor pathways are not likely to account for the observed suppression of HAS-2 through a direct effect on the transcription of HAS-2. However, these receptors can transrepress transcription factors such as AP-1 and NF-κB (Schacke *et al.*, 2002) without GCs exerting a direct effect on the transcription of the investigated gene. The observed inhibition of GC-induced HAS-2 downregulation by RU486 (Figure 1e and f), an antagonist of cytosolic steroid hormone receptors, suggests that such a mechanism may be operative here.

More than one mechanism may be involved in the downregulation of HAS-2 by GCs. GCs, for example, can influence the stability of various mRNAs encoding proteins such as MMP-1, MMP-3, and various integrins (Ing, 2005). Therefore, a destabilization of HAS-2 mRNA would be another possible explanation for the observed downregulation of HAS-2. In addition, cAMP can induce HAS-2 transcription in vascular smooth muscle cells (Sussmann *et al.*, 2004) and GCs are known to affect the cAMP pathway (Thompson, 2008), offering another possible regulatory loop for the observed downregulation of HAS-2. Furthermore, it is known that TGFβ induces HAS-1 and HAS-2 expression (Sugiyama *et al.*, 1998; Dai *et al.*, 2007). We found that TGFβ mRNA and protein levels in fibroblasts, HaCaT cells, and skin were not changed significantly by GC treatment (data not shown), ruling out the possibility that GCs suppress HAS-2 through a direct effect on TGFβ. Nevertheless, GCs suppress the effects of this cytokine by targeting Smad 3 (Song *et al.*, 1999). Moreover, the TGFβ-dependent induction of HAS-2 is mediated through Smad 2, 3, and 4 (Nishitsuka *et al.*, 2007). Thus, the observed GC-induced suppression of HAS-2 could conceivably be mediated in part through effects on TGFβ signaling. Taken together, these observations might explain why the downregulation of HAS-2 in fibroblasts cannot be totally inhibited by RU486 (Figure 1e).

In summary, we could show here that GC treatment induces a rapid loss of dermal HA, which is mainly mediated by a strong suppression of HAS-2 expression without induction of HA degradation. The decrease in dermal HA levels may reflect the initiation of a process that ultimately leads to skin atrophy. These data suggest that a possible link between prolonged HA suppression and irreversible skin atrophy due to long-term GC treatment deserves to be investigated.

MATERIALS AND METHODS

Cell culture

Human skin was obtained from foreskin circumcision operations after informed consent. This study was approved by the institutional review board of Leipzig University. Subconfluent monolayers of human dermal fibroblasts were obtained by outgrowth from the skin sections. For monolayer cultivation, trypsinized fibroblasts were grown in DMEM (Biochrom, Berlin, Germany) supplemented with glutamine (1 ×, Invitrogen, Karlsruhe, Germany), penicillin-G (50 U ml^{-1}; Gibco/Invitrogen, Karlsruhe, Germany), streptomycin (50 μg ml^{-1}; Gibco/Invitrogen), 2 mmol l^{-1} L-glutamine (Gibco/Invitrogen), 0.1 mmol l^{-1} MEM non-essential amino acids (Gibco/Invitrogen) and 10% or 1% fetal bovine serum (PAN Biotech, Aidenbach, Germany). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C, grown to 80–90% confluency and used until the 5th or 6th passage. Keratinocyte-derived immortalized cells (also termed HaCaT cells) were cultivated under the same conditions as described above. Conditioned medium was collected 3 or 24 hours after treatment with dexamethasone (Sigma-Aldrich, Munich, Germany), RU486 (Sigma-Aldrich) or mock treatment, centrifuged at 1,000 × *g*, then stored at −20°C until required.

Human skin biopsies

Gluteal skin biopsies in GC-treated and untreated areas were obtained from nine healthy volunteers (five women and four men of mean age 25.3 years; skin type I–II). All patients gave their informed consent according to the Declaration of Helsinki Principles. The study was approved by the local ethics committee of Leipzig University (no. 168/2007). GC treatment was performed three times daily with 0.1% dexamethasone ointment under occlusion with Tegaderm (3M Deutschland GmbH, Neuss, Germany) for 72 hours. After this time, a 6 mm punch biopsy was taken from the treated area, together with a corresponding untreated control biopsy from the same dermatome. Each biopsy was halved and either conserved in RNAlaterTM (Qiagen, Hilden, Germany) or directly frozen and subsequently used for HA staining.

Quantitative RT–PCR

Total RNA was prepared from cells following standard procedures using the Rneasy kit (Qiagen). RNA from skin biopsies was prepared using Trizol reagent (Invitrogen) and homogenization with a rotorstator homogenizer T25basic (IKA, Staufen, Germany). First-strand cDNA synthesis was performed with 1 μg of total RNA from each sample using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega, Mannheim, Germany) and a GeneTherm thermocycler (Pharmacia, Freiburg, Germany). The cDNA equivalent of 20 ng of total RNA was amplified using a Rotorgene 3000 cycler (Corbett Research, Sydney, Australia). Each reaction mixture contained a 1:50,000 dilution of SYBR green I (Molecular Probes/Invitrogen, Karlsruhe, Germany) 2,500 × in dimethyl sulfoxide (Sigma-Aldrich), 0.25 mmol l^{-1} dNTP mixture (Bioline, Luckenwalde, Germany), 0.5 μmol l^{-1} forward and reverse primers (MWG-Biotech, Ebersberg, Germany), 0.5 U GenTherm DNA polymerase (Rapidozym, Berlin, Germany), 2.5 mmol l^{-1} $MgCl_2$ (Rapidozym) and 2 μl of 10 × PCR buffer (Rapidozym). The amplification consisted of an initial denaturation (95°C, 5 minutes), followed by 40 cycles of 95°C (10 seconds), annealing at 60 °C (20 seconds), extension 72 °C (45 seconds) and fluorescence measurement at 80 °C (15 seconds). After amplification, melt analysis was performed by heating from 70 to 95°C. Primers with the following sequences were used: rps26: forward: 5′-GCAGCAGT CAGGGGACATTTCTG-3′, reverse: 5′-TGCTTCCCAAG-CTGTATGT GAA-3′. Has-2: forward: 5′-CCTCATCATCCAAAGCCTGT-3′,

reverse: 5′-AAACAGTTGCCCTTTGCATC-3′. Has-3: forward: 5′-GTCAGTGGTCACG-GGTTTCT-3′, reverse: 5′-ATTGGCCTCATTCC TGTGTC-3′. Hyal-1: forward: 5′-CAGAGCC-GTGCCCTCTATC-3′, reverse: 5′-ACTTTCTGCCCCTGGATGA-3′. Hyal-2: forward: 5′-CTC ACGGGGCTTAGTGAGAT-3′, reverse: 5′-GTGGTCAATGTGTCCT GGG-3′. Hyal-3: forward: 5′-CCTCCAGTGCCCTCTTCC-3′, reverse: 5′-CTGTCCCAGGATGACCTTGT-3′. The specificity of each primer was ensured by BLAST analysis and visualization of the PCR products on ethidium bromide-stained agarose gels. The ribosomal protein s26 (rps26) was used as a reference gene as previously described (Vincent *et al.*, 1993). Expression of rps26 did not change in response to the dexamethasone treatment (data not shown). The relative level of each mRNA was calculated on the basis of δC_t values. Genes were normalized to rps26 and the fold change of gene expression was calculated compared with the untreated controls, taking the reaction efficiency (E) of each sample into account according to the formula described by Pfaffl *et al.* (2002):

$$\text{Ratio} = \frac{(E_{\text{Targetgene}})^{\Delta CT}\text{Targetgene}^{(\text{control}-\text{Treated})}}{(E_{\text{Referencegene}})^{\Delta CT}\text{Referencegene}^{(\text{control}-\text{Treated})}}$$

The reaction efficiency was determined with a cDNA dilution series of cloned PCR products. For statistical analysis parametric tests (paired *t*-test or analysis of variance) was used (Pfaffl *et al.*, 2002).

HYAL zymography

Zymographic analysis was performed with conditioned medium from dexamethasone-treated or untreated samples. The separation gel (12%) contained 2 ml acrylamide/bisacrylamide, 1.25 ml 4 × separation gel buffer (Tris-HCl 18.8 g l^{-1}, SDS 0.4 g l^{-1}, Na azide 0.1 g l^{-1}, pH 8.8), 1 ml HA solution (0.4 mg ml^{-1}), 0.75 ml distilled water, 5 µl TEMED (tetramethylethylenediamine) and 8 µl ammonium persulfate (0.4 g ml^{-1}). The stacking gel contained 0.83 ml acrylamide/bisacrylamide, 3.08 ml distilled water, 1.25 ml stacking gel buffer (Tris 6.06 g, SDS 0.4 g, Na azide 0.01 g/100 ml, pH 6.8), 5 µl TEMED and 5 µl ammonium persulfate (0.4 g ml^{-1}). The protein content of the conditioned medium was measured and sample volumes equivalent to 5 µg protein were mixed with 2 × sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS 20% glycerol, 0.002% vol/vol bromophenol blue) and loaded onto the gel. Electrophoresis was performed for 30 minutes at 120 V and 45 minutes at 200 V. Subsequently the gel was washed for 1 hour in 3% Triton X-100 (in 50 mM Hepes, pH 7.4) and incubated for 20 hours at 37 °C with Hyal-Assay buffer (0.15 M NaCl in 0.1 M formiate buffer, pH 3.7). Following HA digestion, the bands in the gel were visualized by staining with Alcian blue (0.5% in 3% acetic acid for 2 hours), followed by destaining in 7% acetic acid. Counterstaining was performed with Coomassie Brilliant Blue for 30 minutes.

Dermal microdialysis

Microdialysis experiments were performed as described previously (Averbeck *et al.*, 2006, 2007) . The catheter used in all experiments was the commercially available sterile CMA 60 catheter (Axel Semrau GmbH, Sprockhövel, Germany) customized with a 30 mm polyethylensulphone 100 kDa cut-off membrane. After an initial flush for 5 minutes at a flow rate of 15 µl min^{-1}, the membrane was perfused with Ringer's solution (Na^+ 147 mmol l^{-1}, K^+ 4 mmol l^{-1}, Ca^{2+} 2.25 mmol l^{-1}, Cl^- 155.5 mmol l^{-1}, pH 5–7, osmolarity 309 mosm l^{-1}) at a constant flow rate of 0.3 µl min^{-1} using the CMA 107 microdialysis pump (CMA Microdialysis AB, Solna, Sweden). The microdialysis catheter was inserted into the ventral forearm. Specifically, after anesthesia for 45 minutes with lidocaine-ointment (EMLA, AstraZeneca, Wedel, Germany) the catheter was placed intradermally with an allocated guide cannula at a depth of 0.8–1.2 mm (1,093 µm ± 203.6 ($n = 3$) controlled by 20 Mhz ultrasound (DUB-20, tpm, Lüneburg, Germany). Samples were fractionated in micro vials (CMA) every 8 hours and immediately frozen at −20°C.

HA assays

Microdialysis fluid and supernatants from treated and untreated fibroblast and HaCaT cultures were collected at the indicated time points. The HA content was determined using an ELISA assay (Corgenix, Peterborough, UK). The ELISA assays were performed according to the manufacturer's instructions, with a sample dilution of 1:10. ELISA readings were measured at an extinction of 450 nm with a SynergyHT Reader and analyzed using the Gene5 software (both Biotek, Bad Friedichshall, Germany).

Immunohistochemistry

Cryostat sections (Cryo Star HM560M, Microm International, Walldorf, Germany) were incubated with 1% BSA in phosphate-buffered saline (blocking buffer) for 30 minutes at room temperature, followed by overnight incubation at 4°C with 3 mg ml^{-1} of biotinylated HA-binding protein (Seikagaku, Tokyo, Japan) in blocking buffer. After washing with phosphate-buffered saline, the bound biotinylated HA-binding protein was visualized using the avidin-biotin complex technique according to the manufacturer's protocol (Supersensitive Multilink AP Ready-to-use Detection system, Biogenix, San Ramon, CA). Bound proteins were detected colorimetrically using the New Fuchsin substrate system (Dako, Hamburg, Germany). The sections were counterstained with Mayer's hematoxylin for 2 minutes, washed, dehydrated, and mounted in DePex (BDH Laboratory Supplies, Poole, England). The specificity of the staining was controlled by preincubating the sections with 100 U of HYAL SD (Seikagaku, Tokyo, Japan) for 3 hours at 37°C to remove HA from the tissue (data not shown).

Statistical analysis

Statistical analysis was performed using the Graphpad Prism software (GraphPad Software Inc., San Diego, CA). Indicated *P*-values derive from paired *t*-test analysis if matched pairs are compared. Comparison of more than two groups was performed by Friedman test or by analysis of variance followed by Tukey's post-test (see Figure legends for details).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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