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Original Article

2 Decreased retinoid concentration and retinoid signalling pathways in human atopic dermatitis

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Abstract: Atopic dermatitis (AD) is one of the most common skin diseases. Various features present in AD like inflammation, reduced apoptosis, altered epidermal differentiation and hyperproliferation as well as permeability dysfunction are also regulated by retinoids. The aim of our study is to identify the retinoid signalling pathways and retinoid concentration profiles in AD skin. Human skin biopsies were obtained from healthy volunteers (HS) ($n = 6$) and patients with AD ($n = 6$), with both affected (AS) and non-affected (NAS) skin. The gene expression of retinoid receptors, retinoid-binding proteins and retinoid-metabolizing enzymes was investigated by QRT-PCR. Retinoid concentrations in serum and skin were measured via high performance liquid chromatography mass spectrometry–mass spectrometry. Our results show that the target gene expression of retinoid receptor regulated pathways is significantly decreased in AS and NAS of patients with AD. CYP26A1, transglutaminase 2 and retinoic acid receptor responder 1 decreased in NAS and AS in comparison with HS. The main retinoic acid synthesizing enzyme, retinal dehydrogenase 1, was significantly lower expressed in NAS (0.1%) and AS (1%) in patients with AD. Analysis of retinoid concentration in serum and skin showed comparable all-*trans* retinoic acid (ATRA) and retinol (ROL) concentrations from AD and healthy serum, but strongly reduced ATRA and ROL concentrations in affected and non-affected skin in comparison with healthy skin. Our data indicate that retinoid transport, synthesis, concentrations and signalling are strongly decreased in the affected but also in non-affected skin of patients with AD suggesting a general intrinsic influence on skin retinoid signalling pathway in patients with AD.

Key words: atopic dermatitis – gene expression – retinoids – retinol – vitamin A

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Introduction

Vitamin A and its derivatives, the retinoids, are important for skin physiology (1). Retinoids regulate various effects in differentiation, proliferation, apoptosis, immune regulation, barrier properties (2,3) and sensorial functions (4) in numerous skin cell types. Several skin diseases like psoriasis (5), ichthyosis (6), skin cancers (7), acne (8) and various other dermatoses are related to alterations in retinoid metabolism/signalling. Retinoid-based treatments have been shown to be beneficial for various therapeutic approaches of these skin diseases (9). Topical as well as systemic treatments with retinoids, like retinoic acid (RA) and synthetic RA analogues or derivatives which modify retinoid metabolism, are used already in therapy (1,10–12).

Retinoids mainly mediate their activity via nuclear hormone receptors, the retinoic acid receptors (RAR) with all-*trans* retinoic acid (ATRA) as the major ligand and the retinoid-X receptors (RXR) with 9-*cis* retinoic acid (9CRA) as a potential candidate ligand (13–15). The synthesis, degradation, cellular binding and receptor-mediated binding of ATRA is tightly regulated (16).

Studies reported that vitamin A/retinoids modify the outcome and severity of atopic diseases, mainly the immune phenotype of these diseases (17,18). Skin-specific KO-model of the nuclear retinoid receptors RAR and RXR and combinatory KO-mice models in skin display that retinoid signalling is highly important for the skin phenotype of AD (19,20). In this study, we investigated genes known to be involved in retinoid regulation, metabolism, receptors and signalling of endogenous retinoids (Fig. 1) as well as retinoid target genes regarding their expression profile in skin from healthy volunteers as well as non-affected and affected skin from patients with atopic dermatitis (AD).

The aim of the study was to identify the expression profile from genes involved in retinoid metabolism, regulation and signalling as well as retinoid receptors and retinoid target genes in the skin. This was achieved by a comparable analysis of healthy and diseased skin biopsies and the additional consideration of serum as well as skin retinoid concentrations via sensitive high performance liquid chromatography mass spectrometry–mass spectrometry (HPLC MS–MS) methodology.

Materials and methods

Skin biopsies and serum samples

Skin punch biopsy specimens were taken after obtaining informed consent in involved areas from six patients with AD (two male and four female patients; average age, 31 years), according to the declaration of Helsinki, and from six non-atopic healthy

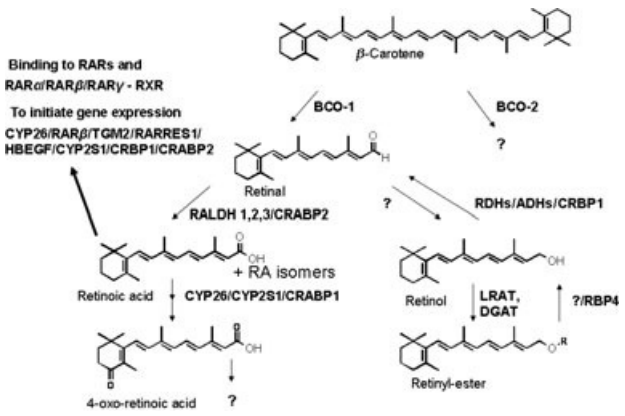


Figure 1. General scheme about retinoid signalling pathway including structures and involved binding proteins and retinoid-metabolizing enzymes: RA, retinoic acid; RDH, retinol dehydrogenase; RALDH, retinaldehyde dehydrogenase; BCMO, beta carotene oxygenase; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; LRAT, lecithin retinol acyltransferase; DGAT, diacylglycerol acyltransferase; RBP4, retinol-binding protein.

volunteers (two male and four female individuals; average age 30 years, see Table 1) characterized by the absence of personal or family history of atopic disease. In case of AD patients, one biopsy of affected skin and one biopsy of non-affected skin were taken from each patient. Epidermis represents ca. 20–30% of the skin biopsy used for analysis. Specimens were immediately frozen in dry ice and stored at −70°C until RNA isolation or HPLC analysis was performed. At the same time, serum samples were drawn from the same patients and were kept at −70°C until analysis. Ethical approval for the study was obtained from the local ethics committee (EA1/168/06), and from each volunteer, a signed informed consent was obtained.

Analysis of mRNA expression

Skin samples were homogenized in Tri® reagent solution, and total RNA was isolated according to the manufacturer's guidelines. The concentration and purity of RNA were measured by means of NanoDrop spectrophotometer (Thermo), and its quality was checked using agarose-gel-electrophoresis. For real-time quantitative PCR (QRT-PCR), total RNA was reverse transcribed into cDNA using the Super Script II First-Standard Synthesis System (Invitrogen). QRT-PCR was carried out in triplicate using predesigned MGB assays ordered from Applied Biosystems, on an ABI Prism 7900. Relative mRNA levels were calculated using either the comparative C_T or standard curve methods normalized to cyclophilin A mRNA. Sequence Detector Software (version 2.1) was utilized for data analysis, and relative fold induction was determined by the comparative threshold cycle method.

Table 1. Clinical and basic demographic data from the healthy and diseased donors

	Healthy volunteers	AD patients
Age in years	30 ± 11	31 ± 14
Gender	67% female	67% female
SCORRAD	0 ± 0	36 ± 11
Total IgE (kU/l)	32 ± 17	1879 ± 764

AD, atopic dermatitis.

High performance liquid chromatography mass spectrometry–mass spectrometry (HPLC MS–MS) analysis:

Concentrations of retinol (ROL) and RAs were determined in human serum and skin biopsies by our HPLC MS–MS method (21). In summary, 100 mg of the skin biopsy (if samples were under 100 mg, water was added up to the used standard weight: 100 mg) or 100 μl serum was diluted with a threefold volume of isopropanol, the tissues were minced by scissors, vortexed for 10 s, put in a ultra sonic bath for 5 min, shaken for 6 min and centrifuged at 13 000 rpm in a Heraeus BIOFUGE Fresco at +4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 μl of methanol, vortexed, shaked, diluted with 40 μl of 60 mM aqueous ammonium acetate solution and transferred into the autosampler and subsequently analysed.

Statistics

Data are shown as mean and standard error mean values of three measurements per data point. Statistical analysis was performed using the program SPSS 16.0. A P value of <0.05 was considered significant.

Results

Expression profiles of retinoid signalling pathways in AD skin samples

First, we investigated the expression profile of genes, which are involved in retinoid homeostasis, retinoid regulation, retinoid metabolism, retinoid receptors and retinoid target genes. These data show that there is a severe dysregulation of retinoid homeostasis, retinoid metabolism and retinoid signalling present in affected and non-affected AD skin.

Dysregulation of retinol homeostasis/retinyl ester synthesis regulation

Retinol-binding protein (RBP) 4 is the carrier protein involved in the transport of ROL, and its mRNA expression was non-significantly down-regulated in AD-affected skin (Fig. 2d). The mRNA expression of diacylglycerol acyltransferase (DGAT) showed a slight decrease in both affected and non-affected skin compared to healthy skin, while lecithin retinol acyltransferase (LRAT) was significantly up-regulated in non-affected skin of AD (Fig. 2a).

Dysregulation of retinal synthesis

Expression levels of beta-carotene 15,15′-monooxygenase (BCMO1) and beta-carotene-dioxygenase-2 (BCMO2) mRNA were not significantly altered in skin with AD compared to healthy skin (Fig. 2a). Retinol dehydrogenases and alcohol dehydrogenases (ADHs) are enzymes which are responsible for converting ROL to retinal. Retinol dehydrogenase (RDH) 2, RDH10 and RDH16 showed a very similar pattern in their mRNA expression. RDH2 was statistically significantly induced in both affected and non-affected AD skin, compared to healthy skin. Augmented mRNA expression of RDH10 and RDH16 was observed in AD-affected skin versus healthy skin. The mRNA expression of ADH 1C was significantly reduced in non-affected and affected AD skin (Fig. 2b).

CRBP 1 is the intracellular carrier of ROL and its mRNA expression showed a significant down-regulation in case of skin with AD, both in non-affected and in affected AD skin (Fig. 2d).

Reduced retinoic acid synthesis in affected and non-affected skin of atopic dermatitis

Retinal dehydrogenase (RALDH) or acetaldehyde dehydrogenase converts retinaldehyde to RA. We observed a significant decrease in RALDH1 mRNA levels in skin with atopic disease, both in

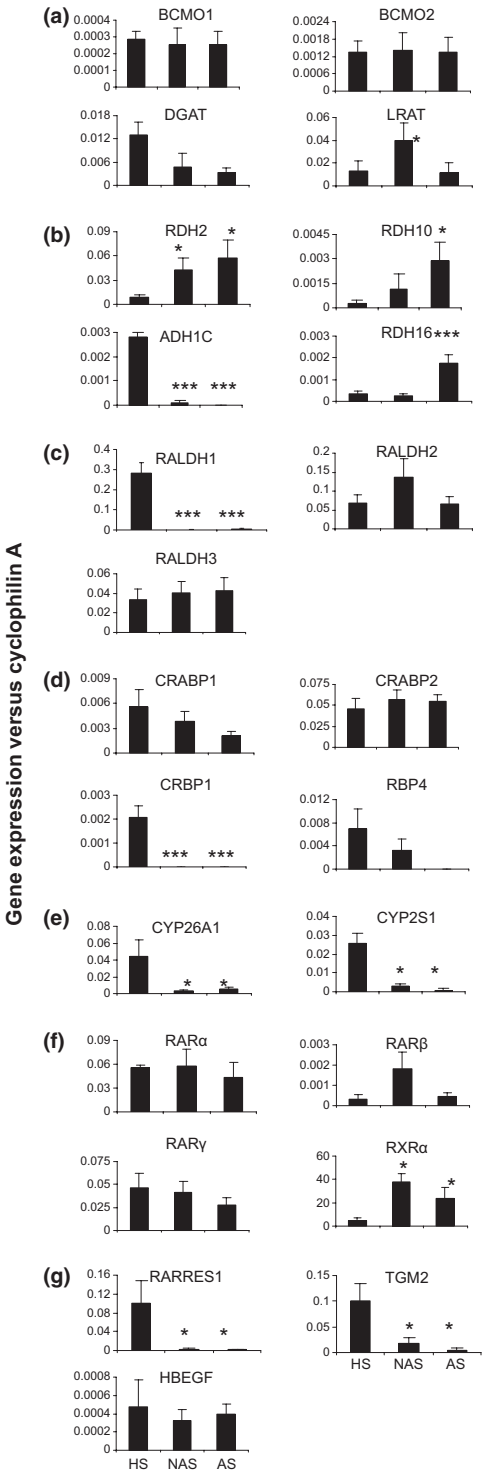


Figure 2. Expression profiles of genes involved in retinoid transport, metabolism and signalling pathway in healthy volunteers as well as in the skin of affected and non-affected atopic dermatitis skin. HS, healthy skin; NAS, non-affected skin, atopic dermatitis; AS, affected skin, atopic dermatitis. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

affected and in non-affected skin, while the mRNA expression of RALDH2 and RALDH3 did not show significant alterations in our experimental setup (Fig. 2c).

CRABP2 is an intracellular RA transporter protein, and its mRNA expression was not altered in skin of patients with AD. Also CRABP1 did not show a significant alteration in diseased skin, in comparison with healthy skin (Fig. 2d).

Reduced expression of RA degradation/metabolism enzymes in affected as well as non-affected skin of atopic dermatitis

mRNA expression level of RA-degrading enzymes was determined and revealed a significant decrease in case of CYP26A1 and CYP2S1, both in affected and in non-affected AD skin. However, mRNA expression of CYP26B1 could not be detected in our experimental setup (Fig. 2e).

Increased expression of RXRα in non-affected skin of atopic dermatitis

RARα mRNA levels did not show any significant alteration in diseased compared to healthy skin, while mRNA expression of RARβ was slightly but non-significantly up-regulated in non-affected AD skin. By contrast, the expression of RARγ was comparable between AD and healthy skin. The expression of RXRα was significantly increased in non-affected AD skin and affected AD skin (Fig. 2f).

Expression of retinoid target genes in atopic dermatitis

The mRNA expression of retinoic acid receptor responder (RARRES1) was significantly decreased in case of AD in non-affected and affected skin, in comparison with healthy skin. Also TGM2 showed a significant decrease both in non-affected and in affected skin of AD. By contrast, HB-EGF mRNA expression was comparable between patients with AD and healthy volunteers (Fig. 2g). Because of strongly reduced retinoid signalling pathways in AD skin, we next analysed ATRA and ROL concentrations in skin biopsies and serum of healthy volunteers and diseased patients.

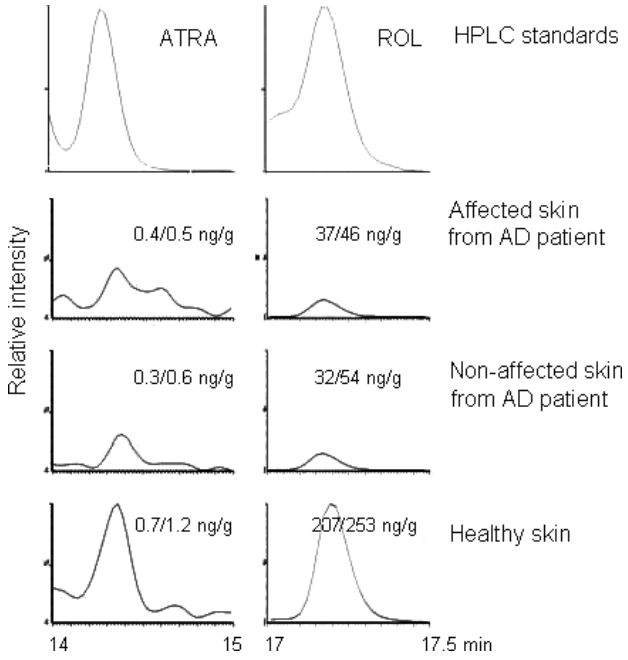
Reduced concentrations of retinol and all-*trans* retinoic acid in AD skin, but not in serum of AD patients

Skin concentrations of ATRA and ROL were strongly reduced in affected (ATRA 0.4/0.5 ng/g; ROL 37/46 ng/g) but also in non-affected skin biopsies (ATRA 0.3/0.6 ng/g; ROL 32/54 ng/g) from an patient with AD in comparison with ATRA and ROL concentrations in healthy skin (ATRA 0.7/1.2 ng/g; ROL 207/253 ng/g) (Fig. 3). Serum concentrations of ATRA 2.8 ± 0.8 ng/ml and ROL 510 ± 217 ng/ml were comparable in healthy volunteers and patients with AD ATRA 2.9 ± 1.0 ng/ml and ROL 573 ± 191 ng/ml (Table 2).

Discussion

In this study, we demonstrated that in affected and in non-affected human tissue biopsies, the retinoid transport, synthesis, concentrations, signalling and homeostasis are severely dysregulated in comparison with skin from healthy volunteers. To our surprise even the skin of non-affected areas of patients with AD displayed dysfunction of retinoid signalling, suggesting an intrinsic disease-specific dysfunction for the regulation of retinoid-binding proteins, metabolizing enzymes, retinoid response target genes expression as well as retinoid concentrations. Interestingly, the mRNA expression of the majority of retinoid response target genes like *CRBP1*, *CYP26A1*, *CYP2S1*, *TGM2* and *RARRES1* was significantly down-regulated, which is in accordance with the decreased level of RA determined in AD skin.

All-*trans* retinoic acid is the major RAR ligand, and its concentration in the mammalian skin is tightly regulated in a specific spatiotemporal manner (19,22,23). Various cell types in the



12 **Figure 3.** High performance liquid chromatography mass spectrometry–mass spectrometry (HPLC MS–MS) chromatogram of all-*trans* retinoic acid (ATRA) and retinol (ROL) using specific MS–MS tracks for ATRA and ROL from representative human skin biopsies of one affected and one non-affected skin biopsy from one patient with atopic dermatitis (AD) and one healthy volunteer. The two values represent the concentrations from the two independent determinations using the skin of two different healthy as well as patient with AD. *Y*-axis of HPLC MS–MS chromatograms of the measured skin samples is the same magnitude for better visualization and comparison.

Table 2. Concentrations of ATRA and ROL in ng/ml in serum of healthy volunteers (healthy, $n = 6$) and AD volunteers (AD, $n = 6$). There were no significant differences between healthy volunteers and patients with AD. For converting concentration into molar, 3 ng/ml of ATRA and 2.86 ng/ml of ROL represent a concentration of 10 nM

	ATRA	ROL
Healthy	2.8 ± 0.8	510 ± 217
AD	2.9 ± 1.0	573 ± 191

AD, atopic dermatitis; ATRA, all-*trans* retinoic acid; ROL, retinol.

skin and especially in the inflamed skin have been shown to be able to synthesize the bioactive RA (24). We found that retinoid response target genes like *RARRES1*, *CRBP1*, *CYP26A1*, *CYP2S1* and *TGM2* are significantly decreased in affected as well as non-affected human skin of AD patients, while the expression of other retinoid targets genes like RARβ, CRABP2 and HB-EGF was not altered. HPLC MS–MS data additionally confirmed that the concentration of ATRA is much lower in affected and in non-affected AD skin in comparison with skin from healthy volunteers; no difference was detected between affected and non-affected AD skin (Fig. 3). This might also be a cause or result of lower delivery of the RA precursor, ROL via RBP4 to the skin, while both ROL levels as well as RBP4 expression are lower in affected AD skin samples in comparison with healthy volunteers (Figs 2 and 3).

The additional analysis of serum concentrations from the same patients and volunteers displayed comparable ATRA levels between patients with AD and healthy volunteers (Table 2). These data suggest a systemic non-RA-based origin for this skin-specific dysfunction of retinoid-mediated signalling in AD. We suggest and partly know already that besides ATRA (the main signalling molecule for retinoid target gene expression), which is much lower in skin of atopic patients, also other relevant and still non-identified bioactive retinoids or/and other retinoid-mediated response pathways involving other retinoid-activated nuclear receptors must be present. Alternative activators of RAR and RXR may be responsible for stable and non-altered expression of the retinoid target genes *RARβ*, *CRABP2* and *HB-EGF* in atopic skin even when ATRA levels are present in much lower concentrations. Identification of novel endogenous RAR as well as RXR ligands is under investigation in our laboratories.

The expression of the major RA synthesizing enzyme in the skin the RALDH1 is significantly decreased in AD skin versus healthy skin (25). This strong down-regulation in affected and non-affected AD skin is suggested to be mainly responsible for lower ATRA concentrations and thereby for the significantly lower retinoid-mediated signalling in the skin of patients with AD.

Deficiency of retinoids/retinoid signalling in the skin or general vitamin A deficiency has been associated to various symptoms also seen in the AD skin phenotype. TH1/TH2 shift (26), altered apoptosis (27), altered skin differentiation and proliferation (28) and increased bacterial skin colonization (29) were associated with vitamin A deficiency or deletion of retinoid receptor-mediated signalling in transgenic skin-specific mouse models (19,20). Whether lower retinoid signalling and lower RA concentration in AD skin is based on an intrinsic abnormality is under examination in various *in vivo* studies in our laboratories.

Remarkable is the reduced gene expression of the retinoid target genes *CRBP1*, *CYP26A1*, *CYP2S1*, *TGM2*, *RALDH1*, *RARRES1* and the *ADH1C* in non-affected AD skin comparably to affected AD skin. We suggest that a general and intrinsic abnormality is responsible for this dysregulation and maybe a result of systemic chronic inflammation. A different expression profile was observed for LRAT and RXRα which are exclusively increased in non-affected AD skin (Fig. 2) confirming also a general intrinsic abnormality responsible for this dysregulation of retinoid signalling (LRAT, RXRα) and maybe of other RXRα-heterodimer-mediated pathways in non-affected AD skin. This increased expression of LRAT and RXRα maybe a response of the non-affected skin on intrinsic chronic inflammation to further enable and balance reduced retinoid signalling. Additionally, the increased expression of RDH2 and RDH10 may be a skin-based response to enable and balance retinoid signalling in the skin.

An altered nutrition with high vitamin A as well as pro-vitamin A carotenoids resulting in significantly higher serum levels of ATRA (30) or increased ingestion of dietary fats which lead to increased expression of various factors/enzymes important for retinoid signalling (31) might contribute also to this altered retinoid signalling in affected as well as non-affected skin of AD patients.

Several approaches using nutritional supplementations with carotenoids and various retinoids as well as systemic inflammation/allergic sensitization are in progress to elucidate why both in

affected as well as in non-affected skin of patients with AD retinoid transport, synthesis, concentrations and signalling are strongly decreased. We suggest that the answer to this question may help to understand the pathogenesis of AD and may lead to strategies for atopy prevention. Based on our observations, we suggest that topical retinoid applications using single or combinations of selective retinoids would be highly beneficial for AD therapy.

In summary, more studies are needed to identify how retinoid transport, metabolism, concentrations and signalling are regulated in the skin and the regulation of key players like RALDH1, which is the major enzyme important for RA synthesis in human skin, in patients with AD in comparison with healthy volunteers. Animal studies using topical as well as systemic application of various retinoids and KO animal models of retinoid-synthesizing enzymes and retinoid receptors are in progress. We conclude that the retinoid signalling pathway is dysregulated in patients with AD based on an abnormal retinoid transport, synthesis and concentrations which might contribute to the pathogenesis of AD, but also offer novel therapeutic approaches.

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References

1 Roos T C, Jugert F K, Merk H F *et al.* Pharmacol Rev 1998: **50**: 315–333.
2 Elias P M, Fritsch P O, Lampe M *et al.* Lab Invest 1981: **44**: 531–540.
3 Elias P M. Dermatologica 1987: **175** (Suppl. 1): 28–36.
4 Aydogan K, Karli N. Clin Exp Dermatol 2007: **32**: 81–84.
5 Saurat J H. J Am Acad Dermatol 1999: **41**: S2–S6.
6 Mevorah B, Salomon D, Siegenthaler G *et al.* J Am Acad Dermatol 1996: **34**: 379–385.
7 Klaassen I, Cloos J, Smeets S J *et al.* Oncology 2002: **63**: 56–63.
8 Rollman O, Vahlquist A. Br J Dermatol 1985: **113**: 405–413.
9 Orfanos C E, Zouboulis C C. Dermatology 1998: **196**: 140–147.
10 Vahlquist A. Dermatology 1999: **199** (Suppl. 1): 3–11.
11 Okuno M, Kojima S, Matsushima-Nishiwaki R *et al.* Curr Cancer Drug Targets 2004: **4**: 285–298.
12 Bissonnette R, Worm M, Gerlach B *et al.* Br J Dermatol 2010: **162**: 420–426.
13 Allenby G, Bocquel M T, Saunders M *et al.* Proc Natl Acad Sci U S A 1993: **90**: 30–34.
14 Reichrath J, Mittmann M, Kamradt J *et al.* Histochem J 1997: **29**: 127–133.
15 Fisher G J, Talwar H S, Xiao J H *et al.* J Biol Chem 1994: **269**: 20629–20635.
16 Chambon P. FASEB J 1996: **10**: 940–954.
17 Rühl R, Garcia A, Schweigert F J *et al.* Int J Vitam Nutr Res 2004: **74**: 279–284.
18 Rühl R, Hänel A, Garcia A L *et al.* Mol Nutr Food Res 2007: **51**: 1173–1181.
19 Chapellier B, Mark M, Messaddeq N *et al.* EMBO J 2002: **21**: 3402–3413.
20 Li M, Messaddeq N, Teletin M *et al.* Proc Natl Acad Sci U S A 2005: **102**: 14795–14800.
21 Rühl R. Rapid Commun Mass Spectrom 2006: **20**: 2497–2504.
22 Everts H B, Sundberg J P, King L E Jr *et al.* J Invest Dermatol 2007: **127**: 1593–1604.
23 Everts H B, King L E Jr, Sundberg J P *et al.* J Invest Dermatol 2004: **123**: 258–263.
24 Spiegl N, Didichenko S, McCaffery P *et al.* Blood 2008: **112**: 3762–3771.
25 Park Y D, Lyou Y J, Yang J M. Exp Dermatol 2007: **16**: 130–134.
26 Cantorna M T, Nashold F E, Hayes C E. J Immunol 1994: **152**: 1515–1522.
27 Trautmann A, Akdis M, Blaser K *et al.* Apoptosis 2000: **5**: 425–429.
28 Gibbs S, Backendorf C, Ponec M. Arch Dermatol Res 1996: **288**: 729–738.
29 Wiedermann U, Tarkowski A, Bremell T *et al.* Infect Immun 1996: **64**: 209–214.
30 Rühl R, Bub A, Watzl B. Nutrition 2008: **24**: 1224–1226.
31 Jeyakumar S M, Vajreswari A, Giridharan N V. Biochem Biophys Res Commun 2008: **370**: 243–247.

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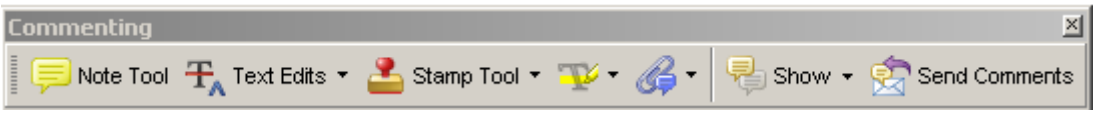

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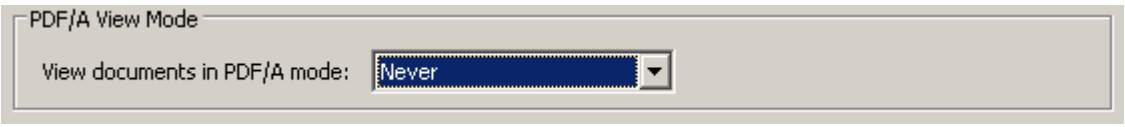

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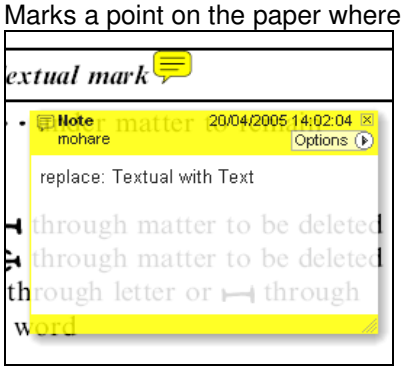

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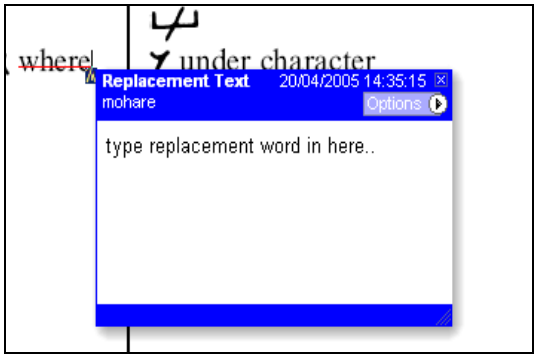

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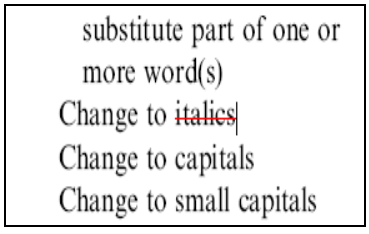
Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

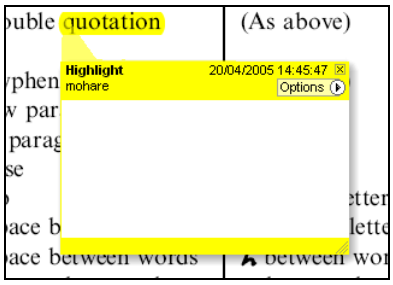
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

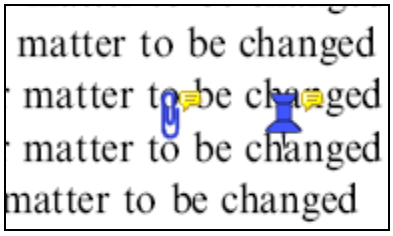


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

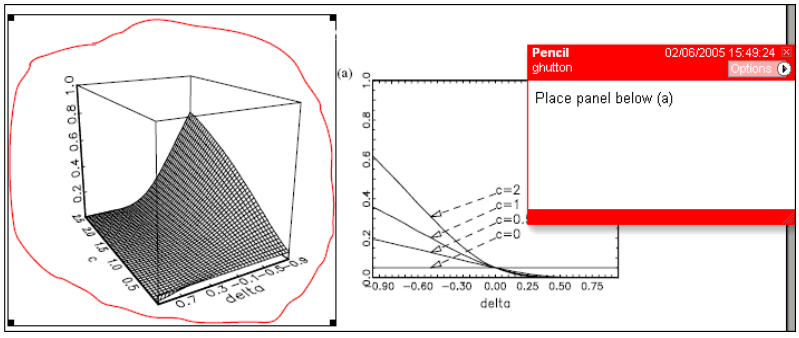


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: