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Dithranol hatása humán keratinociták citokin expressziójára*
The effect of dithranol on the cytokine expression of human keratinocytes

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ÖSSZEFOGLALÁS

A pikkelysömör kezelésében használatos dithranol hatásmechanizmusa teljesen ma sem ismert. A betegség patomechanizmusában az interleukin-8 és az interleukin-10 fontos szerepet játszik. A szerzők azt vizsgálták, hogy miként befolyásolja a dithranol az interleukin-8 és az interleukin-10 termelést, valamint az interleukin-10 receptor kifejeződést humán keratinocitákon. Munkájuk során humán keratinocita (HaCaT) sejtekből 0,1-1 μg/ml dithranol kezelés után 2 és 4 órával az összes RNS-t izolálták. Az RNS reverz transzkripcióját (RT) követően polimeráz láncreakciót hajtottak végre a cDNS-en interleukin-8, interleukin-10 és interleukin-10 receptor specifikus primer párokkal. Immunhisztokémiai vizsgálatokhoz a HaCaT sejteket humán interleukin-10 receptorellenes antitesttel inkubálták. Eredményeik szerint HaCaT sejtekben a dithranol kezelés nem változtatta meg az igen magas interleukin-8 mRNS-szintet, míg az interleukin-10 termelése PCR technikával nem volt kimutatható. Az interleukin–10 receptor esetében a dithranol koncentrációjától függően az mRNS emelkedését találták, amit immunhisztokémiai vizsgálat segítségével fehérjeszinten is igazoltak. Mivel az interleukin-10 receptor a pikkelysömörös lézió keratinocitáinak felszínén csökkent mennyiségben van jelen, a dithranol okozta receptormennyiség-növekedés része lehet a terápiás hatásnak.

Kulcsszavak:
interleukin-8 - interleukin-10 - interleukin-10 receptor - pikkelysömör - dithranol - citokinek

SUMMARY

Dithranol is highly effective in the treatment of psoriasis, however its mode of action is still not well known. Since interleukin-8 and interleukin-10 are involved in the pathogenesis of psoriasis, the aim of our study was to investigate the effect of dithranol on interleukin-8, interleukin-10 mRNA production and interleukin-10 receptor expression of keratinocytes. Cultured HaCaT cells were treated with 0.1-1 μg/ml dithranol for 30 minutes. After 2 and 4 hours total cellular RNA isolated from HaCaT cells was reverse transcribed (RT) to DNA which was subjected to polymerase chain reaction (PCR) with specific primer pairs for interleukin-8, interleukin-10 and interleukin-10 receptor. For immunohistochemistry cultured HaCaT cells were stained with a monoclonal antibody against the human interleukin-10 receptor. Our results showed that dithranol treatment did not change the highly elevated level of interleukin-8 mRNA of HaCaT cells and interleukin-10 mRNA signal with RT-PCR could not be detected in HaCaT cells. Depending on the concentration dithranol increased the mRNA production of interleukin-10 receptors in HaCaT cells. This dithranol induced interleukin-10 receptor upregulation was also observed on the protein level using immunohistochemistry in a dose dependent way. Since the interleukin-10 receptor expression of keratinocytes in psoriatic lesional skin is downregulated, the dithranol induced upregulation of the receptor might be involved in the therapeutic action of the drug.

Key words:
interleukin-8 - interleukin-10 - interleukin-10 receptor - psoriasis - dithranol - cytokines

A pikkelysömörre jellemző gyulladás és epidermális proliferáció létrejöttében a stimuláló hatásoknak és a gátló mechanizmusok elégtelenségének jelentősége van. Az interleukin-8 (IL-8) egy gyulladáskeltő citokin, melyet számos sejttípus termel a bőrben, specifikus receptora (IL-8R) a keratinociták felszínén is jelen van (19). A betegség patogenezisében fontos szerepe van az interleukin-8 (IL-8) (46, 47) és receptora (44) megváltozott szabályozásának. Munkacsoportunk korábbi eredményei azt mutatták, hogy a különböző antipsoriatikus hatású szerek gátolják az IL-8 keratinocitákhoz kötődését (19, 24, 32) és befo-

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lyásolják az IL-8 gén, valamint az IL-8R expresszióját is (30, 44). A p53 mennyisége alacsonyabb a pikkelysömörös plakkokban, szintje emelkedik az antipsoriatikus hatású tacrolimus (FK–506) hatására (30).

A gyulladáscsökkentő citokinek szerepéről még keveset tudunk. Az interleukin-10 (IL-10) a gyulladásos folyamatokat gátolja. Termelését először a T-helper$_2$ (Th_2) sejtekben írták le, hatása a T-helper$_1$ (Th_1) limfociták típusos citokin válaszának csökkentése (12). Újabb eredmények szerint az IL-10 szubkután alkalmazása antipsoriatikus hatású, mely arra utal, hogy a pikkelysömörös lézióban relatív IL-10 hiány áll fenn (1).

Az IL-10 hatását sejtfelszíni receptor segítségével fejti ki (IL-10R). Az utóbbi évek kutatásai tisztázták az IL-10R jelenlétét humán keratinocitákon, és csökkent kifejeződését a pikkelysömörös lézióban (34). További vizsgálatok igazolták, hogy epidermális sejtek felszínén az IL-10R kifejeződést növeli az antipsoriatikus hatású D3-vitamin, a calcipotriol (31) és steroid (34) kezelés. A pikkelysömörös beteg kezelésében használatos dithranol hatásmechanizmus ma sem ismert teljesen (23).

A spontán transzformált humán keratinocita sejtvonal (HaCaT), mely differenciálódás szempontjából hasonló a normál keratinocitákhoz elfogadott modell a hiperproliferatív bőrbetegségek in vitro tanulmányozásában (5, 41).

Jelen munkánkban azt vizsgáltuk, hogyan befolyásolja a dithranol a HaCaT sejtek IL-8 és IL-10 termelését, valamint az IL-10 receptor kifejeződését.

Módszerek

Sejtkultúra

A spontán transzformált humán HaCaT keratinocita sejtvonalat (a sejtekért köszönet dr. N. E. Fusenignek, Heidelberg, Németország) 10%-os borjúszérum albuminnal (FCS), 100 U/ml penicillinnel és 100 µg/ml streptomycinnel kiegészített (Gibco, Németország) Dulbecco modified Eagle mediumban (DMEM) (Gibco, Németország) inkubáltuk 37 °C-on, 5% CO2-t tartalmazó, 100%-os relatív páratartalmú termosztátban.

Az IL-10 termelés vizsgálatához pozitív kontrollként a BCBL-1 sejtvonalat használtuk (16) (a sejtekért köszönet dr. Gáspár Gábornak).

Dithranol kezelés

A szubkonfluens HaCaT sejteket foszfát puffer (PBS) (Gibco, Németország) mosás után 30 percig 0,1-1 µg/ml koncentrációjú dithranolt (Hermal Chemie, Reinbek, Németország) tartalmazó 0,5% FCS tartalmú DMEM oldatban 37 °C-on, 5% CO2-t tartalmazó, 100%-os relatív páratartalmú termosztátban inkubáltuk. A dithranolt közvetlenül felhasználás előtt acetonban oldottuk fel. Kontrollnak acetonnal kezelt és kezeletlenül hagyott HaCaT sejteket használtunk.

RNS-izolálás és szemikvantitatív reverz transzkriptáz-polimeráz láncreakció (RT–PCR)

A dithranol kezelt HaCaT sejteket PBS-mosás után 10% FCS tartalmú DMEM oldatban 37 °C-on, 5% CO2-t tartalmazó, 100%-os relatív páratartalmú termosztátban 75 cm^2-es tenyésztő (Costar) edényekben 2 és 4 órán át inkubáltuk. Dithranol kezelt HaCaT sejtjeinkből és a kezeletlen BCBL-1 sejtekből a TRIzol (Sigma) protokoll szerint az összes RNS-t kivontuk. Az RNS minőségét 1%-os formaldehid-agaróz gélen etídiumbromid jelenlétében ellenőriztük. Az RNS koncentrációt spektrofotométer segítségével határoztuk meg. Az oligo dT15 primerek segítségével végrehajtott reverz transzkripciót (RT) követően polimeráz láncreakcióval (PCR) vizsgáltuk az IL-8, IL-10 és IL-10R mRNS-szintet.

Primerek, szekvenciák

IL-8 sense
5' ATG ACT TCC AAG CTG GCC GTG GCT 3'
IL-8 anti-sense
5' TCT CAG CCC TCT TCA AAA ACT TCT 3'

IL-10 sense
5' ATG CAC AGC TCA GCA CTG CTC TGT 3'
IL-10 anti-sense
5' CTT AAA GTC CTC CAG CAA GGA CTC CTT TAA 3'

IL-10R sense
5' ATG TGG GTG GCA CTG ACC TGT TCT 3'
IL-10 anti-sense
5' AGG GGG ATG CTA CTC CAT TTG TTG 3'

A PCR reakciót a következőképpen végeztük:

35 ciklus: 94 °C 60 másodperc, 59 °C 90 másodperc és 72 °C 90 másodperc az IL-8 esetében, 35 ciklus: 94 °C 60 másodperc, 60 °C 60 másodperc és 72 °C 60 másodperc az IL-10 esetében, és 35 ciklus: 94 °C 60 másodperc, 59 °C 45 másodperc és 72 °C 75 másodperc az IL-10R esetében. A PCR termékeket agaróz gélen etídiumbromid jelenlétében ultraibolya fény alatt tettük láthatóvá. Minden minta esetében párhuzamos ß-aktin PCR reakciót hajtottunk végre, így tudtuk mennyiségileg is összehasonlítani a PCR reakciókban kapott termékeket (szemikvantitatív RT-PCR).

Immunhisztokémia

Az IL-10 receptorellenes antitestet (R & D Systems) és az egér IgG1-t (DAKO) 0,1% Triton X–100-at és 0,5% borjúszérum albumint tartalmazó foszfát pufferben hígítva 1 µg/ml koncentrációban használtuk. A speciális tárgylemezeken (NUNC slide chambers) tenyésztett HaCaT sejteket 2%-os paraformaldehidben fixáltuk 20 percig 4 °C-on, majd 0,1% Triton X–100-at tartalmazó foszfát pufferrel (TBST) mostuk. A tárgylemezeket 30 percig 4°C-on humán IgG-vel inkubáltuk, hogy a sejtfelszíni Fc receptorokat blokkoljuk. Ezután mintáinkat az elsődleges antitestekkel (monoklonális, humán IL-10 receptorellenes) és a megfelelő kontroll antitestekkel (egér IgG1) végzett éjszakán át tartó 4 °C-on történő inkubálás után a biotinnal konjugált másodlagos antitestekkel 1 órán át inkubáltuk. A DAKO Strept ABC komplex kitjével (DAKO A/S, Dánia) streptavidin-biotin peroxidázt adtunk a rendszerhez, peroxidáz szubsztrátként 3-amino-9-etilkarbazolt (AEC, Sigma) használtunk, majd a metszeteket hematoxilinnel festettük.

Eredmények

RT-PCR

Az IL-8 mRNS-szint nem változott 2 és 4 órával a dithranol kezelést követően a kezeletlen és az acetonos kontrollokhoz viszonyítva *(1. ábra)*.

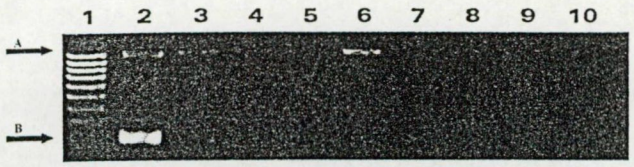
A HaCaT sejtek IL-10 termelésének vizsgálatához pozitív kontrollként a BCBL-1 sejtvonalat használtuk. Eredményeink szerint HaCaT sejtek nem termelnek IL-10-et. Mintáinkban csak a kis mennyiségben jelen levő genomi IL-10 DNS-t detektáltuk, ami méretben különbözött az RNS jelenléte esetén várt terméktől *(2. ábra)*.

Az IL-10 receptor mRNS-szintek a kontrollokhoz képest a dithranol kezelt mintáinkban már 2 óra múlva a koncentrációfüggő módon szignifikáns emelkedést mutattak *(3. ábra)*.

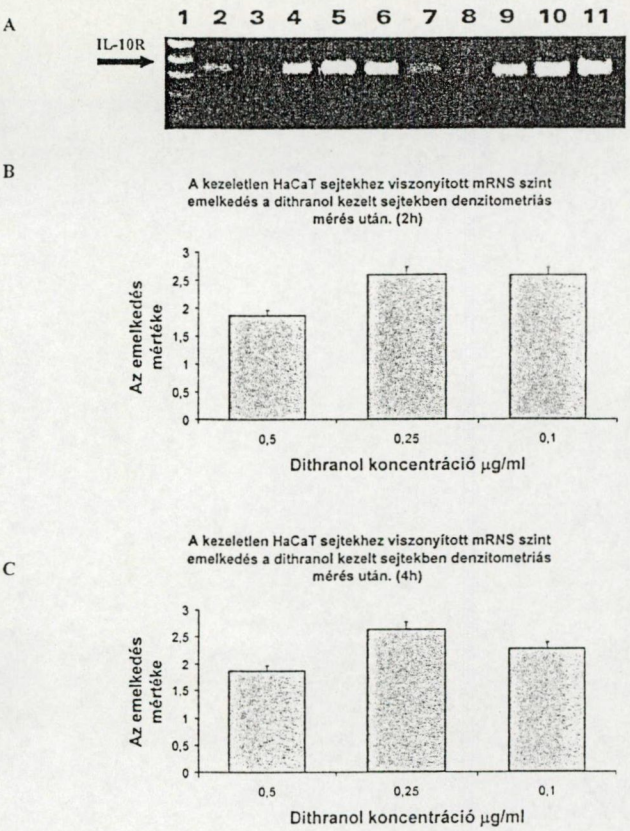
Immunhisztokémia

A humán IL-10 receptorra specifikus monoklonális antitesttel pozitív festést kaptunk a kezeletlen HaCaT keratinociták felszínén. Technikai kontrolljaink esetén igen gyenge diffúz citoplazma festést láttunk, ami arra utal,

1. ábra
Dithranol kezelt HaCaT sejtek
IL-8 mRNS-szint vizsgálata RT-PCR módszerrel
1. sáv: molekulasúly marker, *2. sáv:* pozitív kontroll, *3. sáv:* aceton kezelt sejtek 4h, *4. sáv:* 1 µg/ml dithranol kezelt sejtek 4h, *5. sáv:* 0,5 µg/ml dithranol kezelt sejtek 4h, *6. sáv:* 0,1 µg/ml dithranol kezelt sejtek 4h, *7. sáv:* aceton kezelt sejtek 2h, *8. sáv:* 1 µg/ml dithranol kezelt sejtek 2h, *9. sáv:* 0,5 µg/ml dithranol kezelt sejtek 2h, *10. sáv:* 0,1 µg/ml dithranol kezelt sejtek 2h



2. ábra
Dithranol kezelt HaCaT sejtek
IL-10 mRNS-szint vizsgálata RT-PCR módszerrel
1. sáv: molekulasúly marker, *2. sáv:* pozitív kontroll (BCBL-1 sejtvonal), *3. sáv:* aceton kezelt sejtek 4h, *4. sáv:* 1 µg/ml dithranol kezelt sejtek 4h, *5. sáv:* 0,5 µg/ml dithranol kezelt sejtek 4h, *6. sáv:* 0,1 µg/ml dithranol kezelt sejtek 4h, *7. sáv:* aceton kezelt sejtek 2h, *8. sáv:* 1 µg/ml dithranol kezelt sejtek 2h, *9. sáv:* 0,5 µg/ml dithranol kezelt sejtek 2h, *10. sáv:* 0,1 µg/ml dithranol kezelt sejtek 2h
A nyíl: genomi IL-10 DNS-ről készült PCR-termék
B nyíl: IL-10 mRNS-ről készült RT-PCR-termék

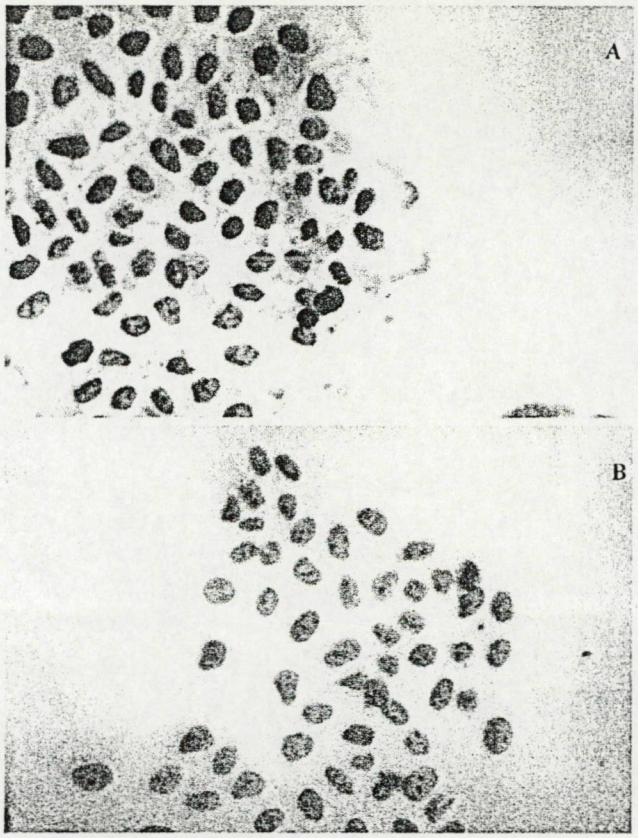


3. ábra
(A) Dithranol kezelt HaCaT sejtek
IL-10 receptor mRNS-szint vizsgálata RT-PCR módszerrel
1. sáv: molekulasúly marker, *2. sáv:* kezeletlen kontroll 4h, *3. sáv:* aceton kezelt sejtek 4h, *4. sáv:* 0,5 µg/ml dithranol kezelt sejtek 4h, *5. sáv*: 0,25 µg/ml dithranol kezelt sejtek 4h, *6. sáv:* 0,1 µg/ml dithranol kezelt sejtek 4h, *7. sáv:* kezeletlen kontroll 2h, *8. sáv:* aceton kezelt sejtek 2h, *9. sáv:* 0,5 µg/ml dithranol kezelt sejtek 2h, *10. sáv:* 0,25 µg/ml dithranol kezelt sejtek 2h, *11. sáv:* 0,1 µg/ml dithranol kezelt sejtek 2h
(B) A kezeletlen HaCaT sejtekhez viszonyított mRNS-szint emelkedés a dithranol kezelt sejtekben denzitometriás mérés után (2h)
(C) A kezeletlen HaCaT sejtekhez viszonyított mRNS-szint emelkedés a dithranol kezelt sejtekben denzitometriás mérés után (4h)

hogy festésünk specifikus volt. Érdekes megfigyelés, hogy a kolóniák széli részén elhelyezkedő sejtek erősebb festést mutattak. Specifikus festődésű sejtjeinkben citoplazmatikus festődést is megfigyeltünk *(4. ábra)*. A humán IL-10 receptorra specifikus monoklonális antitesttel dithranol kezelt HaCaT sejtek immunhisztokémiai vizsgálatát is elvégeztük. A sejtfelszínen a kezeletlen és csak aceton kezelt kontrollokhoz viszonyítva az IL-10 receptor fokozott kifejeződése volt megfigyelhető. Ez a fokozott expresszió koncentrációfüggőnek bizonyult *(5. ábra)*.

Megbeszélés

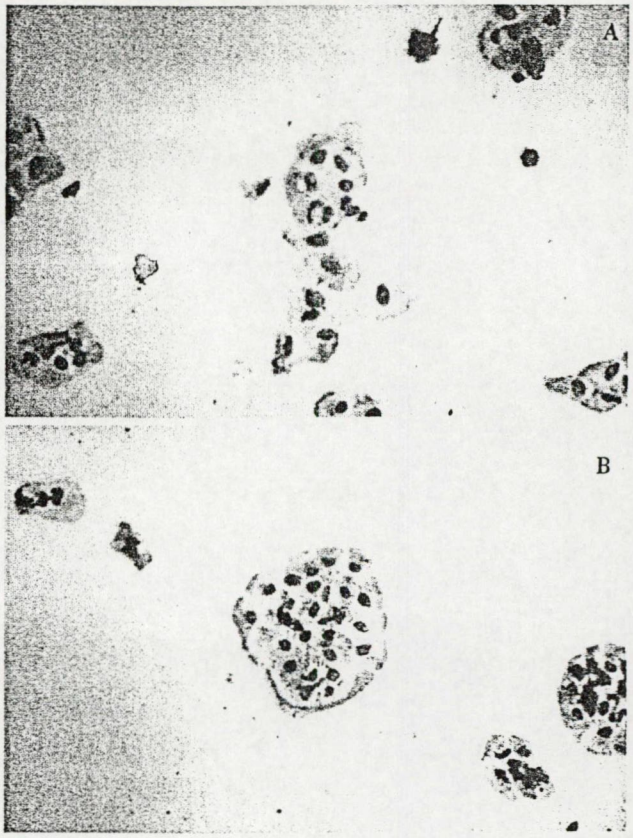
Pikkelysömörös léziós bőrben az IL-8-termelés és IL-8 receptor kifejeződés keratinociták esetében fokozott (6, 32). Az IL-8 kemotaktikus hatással rendelkezik neutrofilekre (29), T limfocitákra (26), bazofilekre (27) és keratinocitákra (33). Fokozza a keratinocita proliferációt (49) és e sejtek HLA-DR kifejeződését indukálja (25). Az IL-8 specifikus receptora segítségével fejti ki hatását (IL-8R), amelynek a kifejeződését az interleukin-1 (IL-1) és tumor nekrózis faktor-alfa (TNF-α) fokozza (20, 21). Pikkelysömörös léziós bőrben az IL-8 mRNS 10-szer nagyobb mennyiségben van jelen a betegség által nem érintett bőrterülethez képest (44). Munkacsoportunk korábbi eredményei arra utalnak, hogy különböző antipsoriatikus hatású szerek közül a cyclosporin, a calcitriol, a calcipotriol és a dithranol az IL-8 keratinocitákhoz való kötődését gátolja (19, 24, 32) míg a tacrolimus (FK–506) az IL-8 gén kifejeződését és az IL-8R expresszióját befolyásolja (30, 44). Egy másik igen fontos citokin, az IL-10 a gyulladásos folyamatokat gátolja (1).



4. ábra

(A) A humán IL-10 receptorra specifikus monoklonális antitesttel pozitív festést kaptunk a kezeletlen HaCaT keratinociták felszínén

(B) Technikai kontrolljaink esetén igen gyenge diffúz citoplazma festést láttunk, ami arra utal, hogy festésünk specifikus volt.



5. ábra

A humán IL-10 receptorra specifikus monoklonális antitesttel dithranol kezelt HaCaT sejtek immunhisztokémiai vizsgálatát is elvégeztük. A sejtfelszínen az IL-10 receptor fokozott kifejeződése volt megfigyelhető. Ez a fokozott expresszió koncentrációfüggőnek bizonyult

(A) 0,1 μg/ml dithranol kezelt HaCaT sejtek

(B) 0,25 μg/ml dithranol kezelt HaCaT sejtek

Termelését először a T-helper2 (Th2) sejtekben írták le, hatása a T-helper1 (Th1) limfociták típusos citokin válaszának csökkentése (12).

A Th2 sejteken kívül monociták/makrofágok, B-sejtek, eozinofilek és hízósejtek képesek IL-10 termelésre (7, 28, 36, 45). Az emberi bőrben a legjelentősebb a makrofágok szerepe az IL-10 előállításában (17). Keratinociták IL-10 termelésével kapcsolatban ellentmondásos irodalmi adatok állnak rendelkezésre (10, 13, 15, 37, 38, 40, 48). Eredményeink azt mutatták, hogy a HaCaT sejtek nem termelnek IL-10-et. Az IL-10 gátolja a monociták/makrofágok (7, 8, 11), dendritikus sejtek (35), Langerhans sejtek (4, 9), és a keratinociták (2) gyulladáskeltő citokin termelését és antigén prezentálóképességét, valamint a keratinocita kultúrákban az interferon-g (IFNg) kiváltotta HLA-DR expressziót, és kismértékben csökkenti a keratinociták növekedési ütemét (34). Az IL-10 hatását specifikus receptorán keresztül fejti ki, amely szerkezeti hasonlóságot mutat az IFNg receptorral (14). Korábbi tanulmányok igazolták, hogy pikkelysömörös lézióban az IL-10 receptor kifejeződése csökkent (34).

A dithranol egy igen hatásos szer a pikkelysömör kezelésében, hatásmechanizmusát azonban még ma sem ismerjük teljesen. A dithranol gátolja a polimorfonukleáris sejtek (neutrofilek) működését (43), és befolyásolja az arachidonsav metabolizmust (3, 42). Közvetlen gátló hatással bír a keratinocita proliferációra (39). Epidermális sejtek bizonyos sejtfelszíni receptorainak kifejeződését is befolyásolja (18, 22).

Eredményeink szerint a dithranol a HaCaT keratinociták IL-8 termelését nem befolyásolja. További vizsgálataink azt mutatták, hogy a HaCaT sejtek nem termelnek IL-10-et. Azonban a humán HaCaT keratinociták rendelkeznek IL-10 receptorral, és ezen sejtek in vitro dithranol kezelése az IL-10 receptor kifejeződésének fokozását eredményezi. Feltételezhető, hogy a dithranol antipsoriatikus hatásának része IL-10 receptor modulálása, melynek a pikkelysömör jövőbeni terápiájában jelentős szerepe lehet.

Köszönetnyilvánítás

A munka az OTKA T–32496 és az ETT-T 05073/99 támogatásával készült.

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Inflammation Research

Dithranol upregulates IL-10 receptors on the cultured human keratinocyte cell line HaCaT

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Abstract. *Objective:* Dithranol is highly effective in the treatment of psoriasis, however its mode of action is still not well known. Since interleukin-8 and interleukin-10 are involved in the pathogenesis of psoriasis, the aim of our study was to investigate the effect of dithranol on interleukin-8, interleukin-10 mRNA production and interleukin-10 receptor expression of the HaCaT keratinocyte cell line which is commonly used in experiments examining the effects of therapeutic drugs on keratinocytes.

Materials and Methods: Cultured HaCaT cells were treated with 0.1-0.5 µg/ml dithranol for 30 minutes. After 2 and 4 h total cellular RNA isolated from HaCaT cells was reverse transcribed (RT) to cDNA which was subjected to polymerase chain reaction (PCR) with specific primer pairs for interleukin-8, interleukin-10 and interleukin-10 receptor. For immunohistochemistry cultured HaCaT cells were stained with a monoclonal antibody against the human interleukin-10 receptor.

Results: Our results showed that dithranol treatment did not change the highly elevated level of interleukin-8 mRNA of HaCaT cells. Interleukin-10 mRNA signal with RT-PCR could not be detected in HaCaT cells. Depending on the concentration dithranol increased the mRNA production of interleukin-10 receptors in HaCaT cells. This dithranol induced dose dependent upregulation of IL-10 receptors in HaCaT cells was also observed on the protein level using immunohistochemistry.

Conclusions: Since the interleukin-10 receptor expression of keratinocytes in psoriatic lesional skin is downregulated, the dithranol induced upregulation of the receptor in our model system might help to reveal the therapeutic action of the drug.

Key words: Interleukin-8 – Interleukin-10 – Interleukin-10 receptor – Psoriasis – Dithranol

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Introduction

Psoriasis a common skin disease is characterized by inflammation and abnormal epidermal proliferation. The highly elevated amounts of stimulatory signals and the failure of inhibitory mechanisms may cause these pathologic features. IL-8 a proinflammatory cytokine is produced by several cell types of the skin and receptors for IL-8 are also present on keratinocytes [1]. An altered regulation for interleukin-8 (IL-8) [2, 3] and its type I receptor (IL-8RA) [4] could be demonstrated in psoriasis. The IL-8/IL8R system is involved in the accumulation of inflammatory cells in psoriatic lesions and in keratinocyte proliferation [5]. Several antipsoriatic compounds inhibit the IL-8 binding to cultured keratinocytes [1, 6, 7] and affect the IL-8 gene and IL-8 receptor expression [4, 8].

On the other hand, the antioncogene p53 is expressed at low levels in psoriatic plaques and can be significantly induced by the antipsoriatic drug tacrolimus (FK-506) [8]. There is only limited knowledge about the role of anti-inflammatory cytokines in psoriasis. Interleukin-10 (IL-10) a negative modulator of inflammatory processes was firstly described as cytokine synthesis inhibitory factor (CSIF) [9]. Recent studies showed that subcutaneous injection of IL-10 has clinical efficiency in psoriasis [10]. The effects of IL-10 are mediated by specific receptors (IL-10R).We could earlier show the presence and functionality of the IL-10 receptor in human keratinocytes, its dramatic decreased expression in psoriatic epidermis [11] and the possibility of raising IL-10 receptor transcript levels by the treatment of epidermal cells with antipsoriatic compounds such as vitamin D3, calcipotriol [12] and steroids [11]. Dithranol is highly effective in the treatment of psoriasis, however its mode of action is still not well known [13].

The HaCaT cell line is widely used in experiments examining the effects of therapeutic drugs on keratinocyte physiology and studying keratinocyte proliferation and differentation [14–17].

In the present report we studied the IL-10 production of human HaCaT keratinocytes [18] and the effect of dithranol on the expression of IL-8 and IL-10 receptor genes in human HaCaT keratinocytes.

Materials and methods

Cell culture

The spontaneously transformed human epidermal cell line HaCaT (kindly provided by Dr. N.E. Fusenig, Heidelberg, Germany) was cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco, Germany) containing 10% fetal calf serum (FCS) (Gibco, Germany). The cells were maintained at 37 °C in a humid 5% CO_2 atmosphere incubator.

As a positive control for the IL-10 RT-PCR studies the BCBL-1 cell line (kindly provided by Dr. G. Gáspár, Szeged, Hungary) [19] was used.

Dithranol treatment

The subconfluent cells were washed with Phosphate Buffered Saline (PBS) (Gibco, Germany) under sterile conditions and were incubated for 30 min at 37 °C in a humid 5% CO_2 atmosphere incubator with increasing concentrations of dithranol (0.1–0.5 μg/ml) (Hermal Chemie, Reinbek, Germany) in DMEM containing 0.5% FCS. Dithranol was always freshly dissolved in acetone and used immediately. Control cells were treated with the solvent (acetone) only or left untreated.

RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Dithranol treated cells were then washed with PBS and were incubated in DMEM containing 10% FCS for 2 and 4 h at 37 °C in a humid 5% CO_2 atmosphere incubator before isolating total cellular RNA according to the TRIzol® (Sigma) protocol. The quality of the RNA was tested in 1% formaldehyde-agarose gel stained with ethidium bromide (EtBr). The RNA concentration was measured using a spectrophotometer. After reverse transcription (RT) with oligo $(dT)_{15}$ primers, semi-quantitative polymerase chain reaction (PCR) was performed with dilutions of our primary RT reaction products (1:1; 1:2; 1:4; 1:8) to determine IL-8 and IL-10R mRNA levels. For our IL-10 mRNA studies we have performed qualitative RT-PCR without diluting the primary RT product.

Primer sequences:

IL-8 sense
5' ATG ACT TCC AAG CTG GCC GTG GCT 3'
IL-8 anti-sense
5' TCT CAG CCC TCT TCA AAA ACT TCT C 3'

IL-10 sense
5' ATG CAC AGC TCA GCA CTG CTC TGT 3'
IL-10 anti-sense
5' CTT AAA GTC CTC CAG CAA GGA CTC CTT TAA 3'

IL-10R sense
5' ATG TGG GTG GCA CTG ACC TGT TCT 3'
IL-10R anti-sense
5' AGG GGG ATG CTA CTC CAT TTG TTG 3'

β-actin sense
5' AGA GAT GGC CAC GGC TGC TT 3'
β-actin anti-sense
5' ATT TGC GGT GGA CGA TGG AG 3'

The following cycling times and temperatures were used to amplify IL-8, IL-10, IL-10R and β-actin transcripts: 35 cycles of 94 °C for 60 s, 59 °C for 90 s and 72 °C for 90 s for IL-8, 35 cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s for IL-10, 35 cycles of 94 °C for 60 s, 59 °C for 45 s and 72 °C for 75 s for IL-10R and 20 cycles of 94 °C for 60 s, 55 °C for 120 s and 72 °C for 105 s for β-actin. After PCR amplification the products were visualized by EtBr-stained agarose gel electrophoresis. Band intensities were monitored by densitometric scanning, standardized against β-actin signals from parallel reactions (semi-quantitative RT-PCR).

The identity of the RT-PCR products in the case of IL-8, IL-10 and β-actin were confirmed by cloning and sequencing. The identity of the IL-10 receptor RT-PCR product was confirmed by direct sequencing.

Immunohistochemistry

Both anti-IL-10 receptor monoclonal antibody (R&D Systems), and mouse IgG_1 (DAKO) were diluted to a working concentration of 1 μg/ml in Tris-buffered saline (Sigma), containing 0.1% Triton X-100 (Sigma) and 0.5% bovine serum albumin. HaCaT cells cultured on 8.6 cm^2 uncoated culture slides were fixed in 2% paraformaldehyde for 20 min at 4°C, and washed in Tris-buffered saline, containing 0.1% Triton X-100 (TBST). Slides were then incubated for 30 min at 4 °C with human IgG (0.5 mg/ml, heat aggregated at 65 °C for 20 min.) (Jackson ImmunoResearch Lab. Inc, West Baltimore Pike, USA) in order to block cell surface Fc receptors. Excess liquid was eliminated without washing, and the slides were incubated overnight at 4 °C with anti-IL-10 receptor monoclonal antibody and negative control reagent (mouse IgG_1), respectively. The antigen-antibody reaction was revealed by using a StreptABComplex Duet (mouse and rabbit) Reagent Set (DAKO). Briefly, the slides were incubated for 1 h at room temperature with the biotin-labeled second antibody (goat anti-mouse/rabbit Ig). After washing, avidin-HRP was added and the incubation was performed in similar conditions as described for the second antibody. The peroxidase was developed with 3-amino-9-ethyl-carbazol (AEC Sigma). Finally, the slides were washed in tap water, counterstained with hematoxylin, rewashed and mounted in DAKO Glycergel® aqeous mounting medium (DAKO).

Results

RT-PCR

The expression of the IL-8, IL-10 and IL-10 receptor (IL-10R) gene was studied in HaCaT cells using semi-quantitative RT-PCR. A housekeeping gene β-actin was used as an internal control. The β-actin RT-PCR signals were constant in all our samples (Fig. 1). The IL-8 mRNA levels did not change after 2 or 4 h of dithranol treatment compared to the untreated or acetone treated cells when RT-PCR was carried out with several dilutions of our primary RT reaction products (1:1; 1:2; 1:4; 1:8) (Fig. 2). To study the IL-10 production of HaCaT cells we used the BCBL-1 cell line (kindly provided by G. Gáspár) as a positive control. We could not demonstrate the IL-10 production of HaCaT cells. We could only detect the small amount of genomic DNA in our RNA samples differing in size from the product originating from the IL-10 mRNA (Fig. 3). The IL-10R mRNA levels were significantly elevated already after 2 h in the dithranol treated cells compared to the controls when RT-PCR was carried out with several dilutions of our primary RT reaction products (1:1; 1:2; 1:4; 1:8). When the primary RT product was used at the dilution of 1:8 we could not dectect any PCR signal. The induction of the IL-10R gene was dose

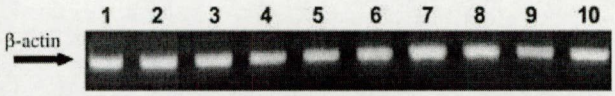


Fig. 1. RT-PCR analysis of β-aktin expression in HaCaT cells treated with dithranol. Lane 1: untreated control 2 h, lane 2: acetone treated cells 2 h, lane 3: 0,1 μg/ml dithranol treated cells 2 h, lane 4: 0,25 μg/ml dithranol treated cells 2 h, lane 5: 0,5 μg/ml dithranol treated cells 2 h, lane 6: untreated control 4 h, lane 7: acetone treated cells 4 h, lane 8: 0,1 μg/ml dithranol treated cells 4 h, lane 9: 0,25 μg/ml dithranol treated cells 4 h, lane 10: 0,5 μg/ml dithranol treated cells 4 h

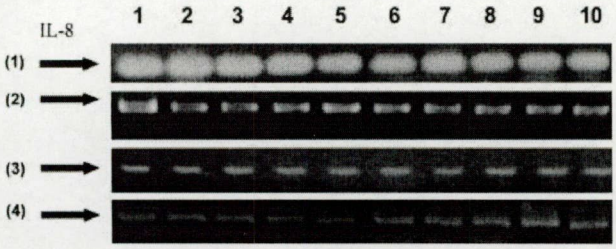


Fig. 2. RT-PCR analysis of IL-8 expression in HaCaT cells treated with dithranol. Lane 1: untreated control 2 h, lane 2: acetone treated cells 2 h, lane 3: 0,1 μg/ml dithranol treated cells 2 h, lane 4: 0,25 μg/ml dithranol treated cells 2 h, lane 5: 0,5 μg/ml dithranol treated cells 2 h, lane 6: untreated control 4 h, lane 7: acetone treated cells 4 h, lane 8: 0,1 μg/ml dithranol treated cells 4 h, lane 9: 0,25 μg/ml dithranol treated cells 4 h, lane 10: 0,5 μg/ml dithranol treated cells 4 h. Arrow (1): undiluted primary RT product RT-PCR, Arrow (2): RT-PCR with 1:2 dilution of primary RT product, Arrow (3): RT-PCR with 1:4 dilution of primary RT product, Arrow (4): RT-PCR with 1:8 dilution of primary RT product.

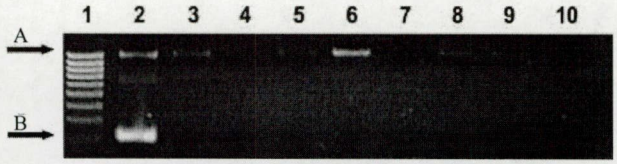
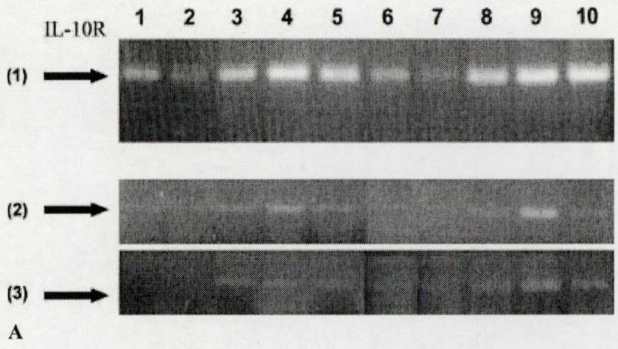


Fig. 3. RT-PCR analysis of IL-10 expression in HaCaT cells treated with dithranol. Lane 1: molecular weight marker, lane 2: positive control, (BCBL-1 cell line), lane 3: untreated control 2 h, lane 4: acetone treated cells 2 h, lane 5: 0,1 μg/ml dithranol treated cells 2 h, lane 6: 0,5 μg/ml dithranol treated cells 2 h, lane 7: untreated control 4 h, lane 8: acetone treated cells 4 h, lane 9: 0,1 μg/ml dithranol treated cells 4 h, lane 10: 0,5 μg/ml dithranol treated cells 4 h. Arrow A: PCR product from genomic IL-10 DNA in the RNA samples. Arrow B: RT-PCR product from IL-10 mRNA.

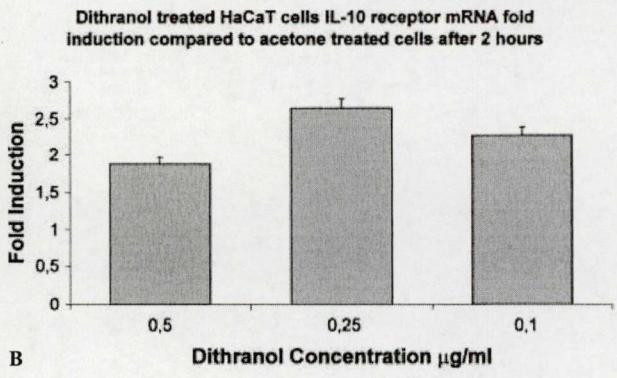
dependent reaching its maximum at 0,25 μg/ml dithranol concentration (Fig. 4).

Immunohistochemistry

Untreated cultured HaCaT cells were stained with a monoclonal antibody against human IL-10 receptor. Relative to the isotype control the antibody clearly stained the cells. A clear membrane localisation was apparent and interestingly it



A



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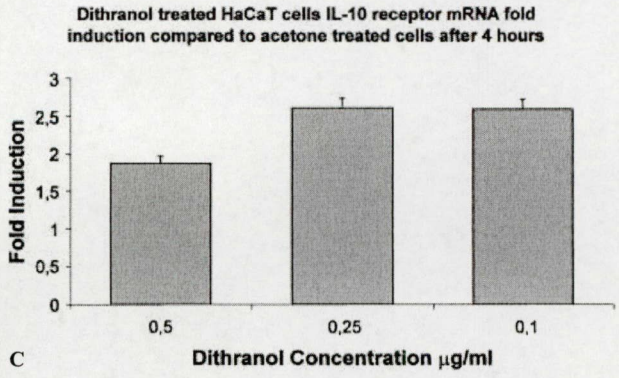

C

Fig. 4. (A) RT-PCR analysis of IL-10R expression in HaCaT cells treated with dithranol. Lane 1: untreated control 2 h, lane 2: acetone treated cells 2 h, lane 3: 0,1 μg/ml dithranol treated cells 2 h, lane 4: 0,25 μg/ml dithranol treated cells 2 h, lane 5: 0,5 μg/ml dithranol treated cells 2 h, lane 6: untreated control 4 h, lane 7: acetone treated cells 4 h, lane 8: 0,1 μg/ml dithranol treated cells 4 h, lane 9: 0,25 μg/ml dithranol treated cells 4 h, lane 10: 0,5 μg/ml dithranol treated cells 4 h. Arrow (1): undiluted primary RT product RT-PCR, Arrow (2): RT-PCR with 1:2 dilution of primary RT product, Arrow (3): RT-PCR with 1:4 dilution of primary RT product. **(B)** Integrated peak areas after densitometric signals plotted as "fold induction" in comparison to unterated cells after 2 h (undiluted primary RT product RT-PCR). **(C)** Integrated peak areas after densitometric signals plotted as "fold induction" in comparison to unterated cells after 4 h (undiluted primary RT product RT-PCR).

seemed that cells localised at the edges of the colonies showed stronger expression. Beside the membrane staining there was some cytoplasmic staining as well (Fig. 5). We have also performed immunocytochemistry on cultured HaCaT cells that were treated with dithranol. At all concentrations

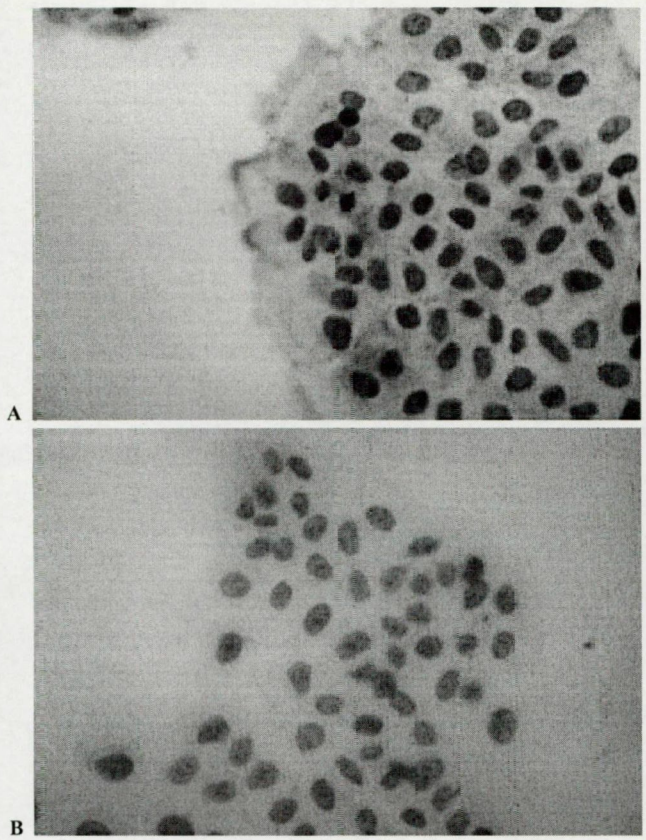

Fig. 5. Immunocytochemistry with a monoclonal antibody against human IL-10 receptor on cultured HaCaT cells. **(A)** HaCaT cells with clear membrane localization of the human IL-10 receptor. **(B)** Isotype control.

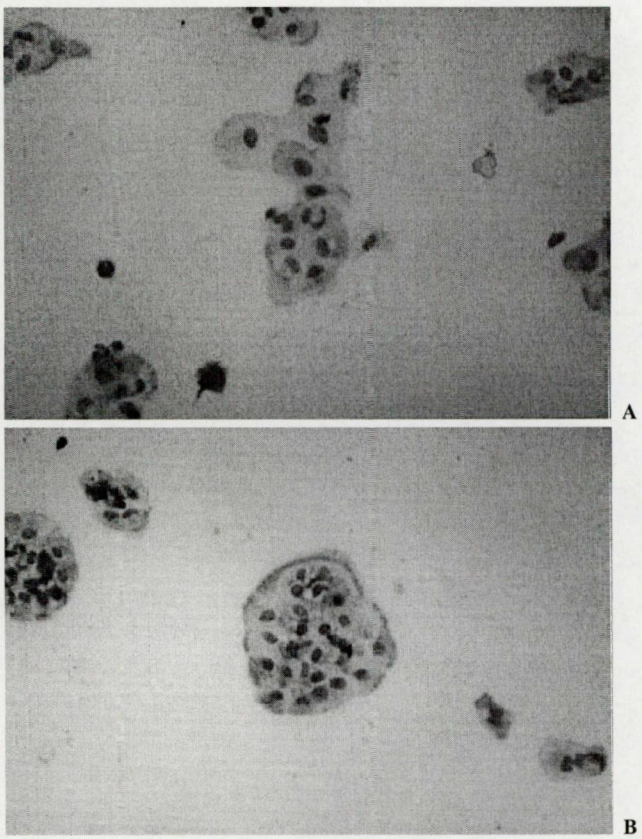

Fig. 6. Immunocytochemistry with a monoclonal antibody against human IL-10 receptor on cultured HaCaT cells that were treated with dithranol. **(A)** 0,1 µg/ml dithranol treated HaCaT cells. **(B)** 0,25 µg/ml dithranol treated HaCaT cells.

tested (0.1–0.5 µg/ml) the dithranol clearly had a cytostatic effect on the cells, that was apparent by the size of the cell colonies relative to the untreated control cultures. With the monoclonal antibody staining there was a slight indication for enhanced expression of IL-10 receptor, predominantly on the cell membrane in the dithranol treated cultures, and this difference seemed to depend on the concentration (Fig. 6).

Discussion

In psoriatic lesional skin the genes for proinflammatory factors as the IL8/IL-8 receptor system are overexpressed [7]. IL-8 is chemotactic for neutrophils [20], T lymphocytes [21], basophils [22] and keratinocytes [23], it stimulates neutrophil degradation and oxidative burst activity [24]. It promotes keratinocyte proliferation [25] and induces HLA-DR expression in keratinocytes [26]. The effects of IL-8 on keratinocytes are mediated through specific receptors, which may be upregulated by interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) [27, 28]. In lesional psoriatic epidermis, IL-8 receptor specific mRNA level was found to be 10-fold higher compared with the uninvolved skin [18]. Our earlier results showed that several antipsoriatic compounds as cyclosporine, calcitriol, calcipotriol and dithranol have inhibitory effects on IL-8 binding to cultured keratinocytes [1, 6, 7]. The IL8/IL8R system can be downregulated when epidermal cells are treated with the antipsoriatic drug tacrolimus (FK-506) in vitro [4, 8].

Another cytokine IL-10 has gained increasing importance as a negative modulator of inflammatory processes [10]. IL-10 was firstly described in T-helper$_2$ (Th_2) cells and characterized by its ability to inhibit the typical cytokine response of T-helper$_1$ (Th_1) lymphocytes [9]. Besides Th_2 cells monocytes/macrophages, B cells, eosinophils and mast cells are able to produce IL-10 [29–32]. In human skin macrophages are the main source of IL-10 [33]. The IL-10 synthesis of keratinocytes is controversally discussed in the literature [34–40]. Our present studies suggest that HaCaT cells are not capable of producing IL-10.

IL-10 suppresses proinflammatory cytokine production and antigen-presenting capacity of monocytes/macrophages [29, 41, 42], dendritic cells [43], Langerhans cells [44, 45] and keratinocytes [46].

We found earlier that in cultured keratinocytes IFN-γ induced HLA-DR expression is inhibited by IL-10. It has a moderate but statistically significant and dose dependent inhibitory effect on the keratinocyte growth rate [11].

The effect of IL-10 is mediated through its specific receptor (IL-10R), which shares structural homologies with the interferon-gamma (IFN-γ) receptor [47]. Previous studies showed that IL-10R expression is decreased in psoriatic epidermis [11].

Dithranol is a highly effective antipsoriatic compound. Its mode of action is still not completely understood. Dithranol inhibits polymorphonuclear leukocyte (PMN) function [48] and modulates the arachidinoc acid metabolism [49, 50]. It has a direct inhibitory effect on keratinocyte proliferation [51]. It is also capable of modulating cell surface receptors of epidermal cells [52, 53].

Our results show that dithranol does not act by regulating the IL-8 production of human cultured HaCaT keratinocytes. We could show that HaCaT cells are not capable of producing IL-10.

In the present work we could demonstrate that HaCaT keratinocytes have IL-10 receptors and these cells in vitro respond to the treatment with the antipsoriatic drug dithranol by the induction of the IL-10 receptor gene.

Since the IL-10 receptor gene is downregulated in psoriatic epidermis [11] the pharmacological modulation of the receptor in our model system [14–17] may be an important target in the future for the therapy of psoriasis.

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II.

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$CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis

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SUMMARY

Psoriasis is a chronic, inflammatory, hyperproliferative skin disease, in which autoimmunity plays a great role. Natural killer T cells (NK T cells), are suggested to be involved in the pathogenesis of different autoimmune diseases. To examine the involvement of $CD3^+CD56^+$ NK T cells in the pathogenesis of psoriasis, we investigated the lymphocyte subpopulations obtained from blood samples of psoriatic patients before and after treatment, and of healthy controls, using two-colour flow cytometry. We found no significant differences between total T cells, total B cells, T helper cells, T cytotoxic cells and NK cells in patients with psoriasis before and after treatment and in controls. Increased percentage of memory T cells and decreased percentage of naive T cells was detected in psoriatic patients compared to controls, but these changes were not statistically significant. The $CD3^+CD56^+$ cells of psoriatic patients were significantly decreased relative to controls. The percentage of $CD3^+CD56^+$ cells increased after different antipsoriatic therapies, but remained significantly lower than those found in controls. $CD3^+CD56^+$ cells of healthy controls were capable of rapid activation, while in psoriatic patients activated NK T cells were almost absent. The decrease in the number of $CD3^+CD56^+$ cells may represent an intrinsic characteristic feature of patients with psoriasis, which is supported by the fact that after treatment NK T cells do not reach the values found in controls. In conclusion our results suggest that $CD3^+CD56^+$ NK T cells could be actively involved in the development of Th1 mediated autoimmune diseases.

Keywords $CD3^+CD56^+$ NK T cells autoimmunity psoriasis

INTRODUCTION

Although the pathogenesis of psoriasis is not yet clear, there are characteristic features of the disease which suggest an immunological mediated process. Several direct and indirect evidences suggest that T cells play a crucial role in the pathogenesis of psoriasis [1–7]. The presence of T helper cells, that secrete type 1 cytokines (IFN-γ, IL-2, TNF-α), was demonstrated in psoriatic skin lesions [8–13]. A type 1 differentiation bias was also observed in circulating blood T cells of psoriatic patients [14]. The existence of an imbalance between Th1 and Th2 cells in psoriasis was supported further by findings which demonstrated that IL-10 was decreased in psoriatic lesions [15]. Moreover, during antipsoriatic therapy an increase in IL-10 mRNA expression was observed in peripheral blood mononuclear cells [16]. IL-10 therapy given either intralesionally or subcutaneously resulted in marked reduction of psoriatic lesions [16,17]. These data suggest that psoriasis is an inflammatory Th1 mediated autoimmune disorder, but the triggering autoantigens are still not identified.

Although the factors that induce the imbalance between Th1 and Th2 cells in psoriasis are unknown, a possible role could be attributed to natural killer T cells (NK T cells) [18]. NK T cells are a heterogeneous T cell population characterized by the co-expression of $\alpha\beta$ or $\gamma\delta$ TCRs and various NK receptors, including CD16, CD56, CD161, CD94, CD158a and CD158b [19–21]. NK T cells have the ability to rapidly secrete large amounts of cytokines following activation [22–24]. NK T cell clones secrete type 1, type 2 or both types of cytokines, which could influence the differentiation of Th0 cells towards Th1 or Th2 cells [25,26]. $CD3^+CD56^+$ cells represent one of the NK T cell populations.

The number of $CD3^+CD56^+$ NK T cells has been shown to be significantly decreased in the peripheral blood of patients with

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rheumatoid arthritis, another Th1 mediated autoimmune disease [27].

In the present study we determined the number of $CD3^+CD56^+$ NK T cells in the peripheral blood of patients with psoriasis (before and after treatment) and in healthy controls. Other lymphocyte subpopulations (total T cells, T helper cells, T cytotoxic cells, T memory cells, T naive cells, B lymphocytes and NK cells) were also analysed and compared in psoriatic patients and healthy volunteers.

Our results show that $CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis relative to the healthy controls. This finding is discussed in relation with the development of a type 1 immune response in psoriasis.

PATIENTS AND METHODS

Patients' profile

Peripheral blood samples of 15 patients with psoriasis (one erythrodermic, two guttate type and 12 chronic plaque psoriasis) were obtained with informed consent for use in this study. The Ethical Committee of the University of Szeged, Hungary approved this investigation. Blood samples were collected before and after treatment from all patients. The patients' characteristics are presented in Table 1. Therapeutic modalities included monotherapy and combined therapeutic regimens (Table 2). The second blood sample was taken at the time when the applied treatment regimen had been completed. At this time the majority of patients was symptom-free or had minimal skin changes (PASI < 4).

The control group consisted of 12 healthy hospital employees (seven females and five males), aged 37·08 ± 7·21 years with informed consent for use in the study. In the control group nobody took any medication and nobody suffered from any known acute or chronic disease.

Reagents

Anti-CD3 FITC, anti-CD3 PE (clone UCHT1), anti-CD19 PE (clone HD37), anti-CD4 FITC (clone MT310), anti-CD8 PE, anti-CD8 FITC (clone DK 25), anti-CD25 FITC (clone ACT-1) and isotype-matched labelled mouse immunoglobulins were obtained from DAKO (Copenhagen, Denmark), anti-CD56 FITC (clone NKH-1), anti-CD45RA PE (clone F8-11-13) were obtained from Serotec (Oxford, UK), anti-CD4 FITC (clone SK3), anti-CD56 PE (MY31) from Becton-Dickinson (San Jose, CA, USA) and anti-CD45RO FITC (clone UCHL1) was obtained from Immunotech (Beckman Coulter, Fullerton, CA, USA).

Table 1. Patient profile

Sex (male:female)	12:3*	
Age (years)	42·35 ± 14·32	(20–67)‡
Age of onset (years)	25·7 ± 10·38†	(9–45)
Disease duration (years)	16·7 ± 9·21	(3–37)
Psoriasis type (chronic: eruptive)	13:2	
PASI score	18·77 ± 12·25	(6–54)
Family history (positive: negative)	6:9	

*Number of patients; †mean ± s.d.; ‡range.

Immunostaining and flow cytometry

Peripheral blood, anticoagulated with EDTA, was collected. Each blood sample (50 μl) was stained with two monoclonal antibodies, one conjugated with FITC and the other with PE (10 μl from each) at room temperature, in the dark for 20 min Erythrocytes were lysed with FACS Lysing Solution (Becton Dickinson San Jose, CA, USA). After two washes with PBS the cells were resuspended in PBS for immediate analyses or were fixed with 2% paraformaldehyde for overnight storage before analyses. Two-colour flow cytometry was performed by using a FACSCalibur cytometer and the data were analysed using Cell Quest software (Becton Dickinson, San Jose, CA, USA). In each stain 30000 events were acquired.

Isolation and stimulation of T cells from peripheral blood

Mononuclear cells (PBMC) were isolated from peripheral venous blood samples of psoriatic patients and healthy controls by Ficoll-Hypaque density gradient centrifugation (Biotech Inc, Piscataway, NJ, USA). PBMC were recovered at the interface and washed in PBS supplemented with 2% fetal calf serum (FCS). T cells were isolated by positive selection using uniform magnetizable polystyrene beads coated with monoclonal antibodies specific for CD3 (Dynabeads M-450 CD3 from Dynal, Oslo, Norway) and a magnetic particle separator (Dynal MPC, from Dynal, Oslo, Norway) following the protocol provided by the manufacturer. Briefly, PBMC were incubated with CD3 monoclonal antibody coated magnetic beads (bead:cell ratio 5:1, Dynabeads concentration 1×10^7/ml) for 6 h in RPMI 1640 culture medium supplemented with 10% FCS, at 37°C in a humid 5% CO_2 incubator. After the incubation time Dynabeads were detached from the cells by pipetting the cell suspension 10 times through an automated pipette. The beads were than removed from the cell suspension using a Dynal MPC. The beads attached to the tube wall while the cells remained in the suspension. This isolation procedure through the CD3 binding results in stimulation of the $CD3^+$ cells [28]. The isolated and stimulated cells were then analysed by two-colour flow cytometry, using monoclonal antibodies for

Table 2. Treatment regimens used in the study

Treatment	Number of patients
Dithranol†	6
PUVA*	6
Dithranol + PUVA‡	1
Dithranol + narrowband ultraviolet B (311 nm)§	1
Re-PUVA¶	1

†Dithranol treatment was used in slowly increasing concentrations starting with 0·5% up to 8% depending on the induced erythema. *Oral 8-methoxypsoralen + UVA (four times a week) was administered from 1 J/cm² up 5 J/cm² depending on the induced erythema. ‡Dithranol treatment with slowly increasing concentrations starting with 0·5% up to 8% was combined with oral 8-methoxypsoralen + UVA (four times a week) from 1 J/cm² up 5 J/cm² depending on the induced erythema. §Dithranol treatment with slowly increasing concentrations starting with 0·5% up to 8% was combined with narrowband ultraviolet B from 0·2 J/cm² up to 2·2 J/cm² (five times a week) depending on the induced erythema. ¶Oral acitretin (0·5 mg/kg body wt) + oral 8-methoxypsoralen + UVA (four times a week) from 1 J/cm² up 5 J/cm² was administered depending on the induced erythema.

detection of CD56, CD25, CD4 and CD8. After 6 h of anti-CD3 antibody stimulation, the CD3 antigen was transiently down-regulated. By growing the cells for further 24 h in culture medium the CD3 antigen was reexpressed on the cell surface, and could be detected using FITC conjugated CD3 antibodies. Vitality of isolated cells was tested by trypan blue exclusion.

Statistical analysis

Statistical analysis of the data was made by ANOVA, Pearson correlation test and Spearman's rank order correlation test. For significant ANOVA values, groups were compared by Tukey's *post hoc* test for multiple comparisons with unequal cell size. A probability level of 0·05 was accepted as indicating significant differences.

RESULTS

Analyses of lymphocyte subsets

The lymphocyte subsets in patients with psoriasis before treatment and in healthy controls were analysed. The percentage of $CD3^+CD56^+$ NK T cells was significantly decreased in the peripheral blood of patients with psoriasis before treatment compared with healthy controls (1·79 ± 1·07% in patients *versus* 5·22 ± 1·74% in controls, $P < 0·0001$) (Table 3). Representative flow cytometric analyses show $CD3^+CD56^+$ NK T cells in a patient before treatment (0,78%) (Fig. 1b) *versus* in a healthy control (6·28%) (Fig. 1a). The absolute number of circulating $CD3^+CD56^+$ NK T cells was also significantly lower in psoriatic patients before treatment than the values found in healthy controls (29·43 ± 17·12 μl in psoriasis before treatment *versus* 120·15 ± 45·35 μl in controls, $P < 0·0001$). Memory T cells ($CD3^+CD45RO^+$) represented a larger (35·36 ± 9·38% in patients *versus* 27·21 ± 7·34% in controls) and naive T cells ($CD3^+CD45RA^+$) a smaller population (37·71 ± 8·34% in patients *versus* 45·00 ± 7·19%) in the peripheral blood of patients relative to controls; however, these differences did not reach statistical significance. Similarly, a slight but statistically not significant increase in the proportion of helper CD4+ T helper cells was observed in patients with psoriasis (44·52 ± 9·05% in patients *versus* 38·97 ± 5·66% in controls $P > 0·05$). There was no difference in the percentages of B lymphocytes, conventional NK cells ($CD3^-CD56^+$), total T cells and T cytotoxic $CD8^+$ cells between the two groups (Table 3).

Table 3. Percentage of lymphocyte subsets (mean ± s.d.) in peripheral blood of patients with psoriasis before treatment, after treatment and of healthy controls

	Psoriasis before treatment (%)	Psoriasis after treatment (%)	Healthy controls (%)
$CD3^+$	72·46 ± 8·59	70·04 ± 8·59	70·78 ± 4·71
$CD19^+$	11·24 ± 4·87	11·5 ± 5·41	13·97 ± 4·63
$CD3^+CD4^+$	44·52 ± 9·05	42·54 ± 8·22	38·97 ± 5·66
$CD3^+CD8^+$	27·12 ± 8·21	26·39 ± 7·18	28·95 ± 7·43
$CD3^+CD56^+$	1·79 ± 1·07	2·68 ± 1·04	5·22 ± 1·74
$CD3^-CD56^+$	10·20 ± 5·69	12·47 ± 6·98	10·30 ± 4·7
$CD3^+CD45RA^+$	37·71 ± 8·34	37·90 ± 8·60	45·00 ± 7·19
CD3+CD45RO+	35·36 ± 9·38	35·13 ± 9·87	27·21 ± 7·34

Comparing the lymphocyte subsets of patients with psoriasis before and after treatment, the only lymphocyte population in which changes were statistically significant was the NK T ($CD3^+CD56^+$) subset. Both the percentage and the absolute cell number of NK T cells were significantly increased in the peripheral blood of patients with psoriasis after treatment (2·68 ± 1·04%, 29·43 ± 17·12 μl *versus* 1·79 ± 1·07%, 58·95 ± 31·56 μl, $P < 0·001$), but did not reach the values found in healthy controls (Table 3). A representative flow cytometric analysis shows the comparison of $CD3^+CD56^+$ NK T cells in a patient before treatment (0·78%) (Fig. 1b) *versus* in a patient after treatment (3·04%) (Fig. 1c). We found no statistically significant changes in the other lymphocyte subsets (Table 3). The antipsoriatic treatments used in this study had no effect on the number of memory and naive T cells (Table 3).

After treatment the absolute cell number of $CD3^+CD56^+$ NK T cells remained significantly decreased in patients with psoriasis compared to healthy controls (58·95 ± 31·56 μl in patients after treatment *versus* 120·15 ± 45·35 μl in controls, $P < 0·001$). The same was observed when the percentage of NK T cells among lymphocytes was analysed in peripheral blood samples (2·68 ± 1·04% in patients *versus* 5·22 ± 1·74% in controls, $P < 0·001$) (Table 3). Representative flow cytometric analyses show $CD3^+CD56^+$ NK T cells in a patient after treatment (3·04%) (Fig. 1c) *versus* in a healthy control (6·28%) (Fig. 1a). Memory T cells remained elevated (35·13 ± 9·87% in patients *versus* 27·21 ± 7·34% in controls) and naive T cells were decreased in the psoriasis group compared to healthy controls (37·90 ± 8·60% in patients *versus* 45·00 ± 7·19% in controls) (Table 3), without reaching statistical significance. Similarly, the number of T helper cells showed a slight, statistically not significant elevation in treated patients compared to controls (42·54 ± 8·22% in patients *versus* 38·97 ± 5·66% in controls $P > 0·05$) (Table 3). No difference between the two groups was found regarding B lymphocytes, NK cells, total T cells and cytotoxic T cells (Table 3).

$CD3^+CD56^+$ NK T cells and patients' profile

To determine whether the number or the percentage of $CD3^+CD56^+$ NK T cells in patients with psoriasis shows any correlation with age, PASI score and disease duration the appropiate correlation test has been applied. The analysis of the possible correlation of NKT cells with age was also performed in healthy controls. Statistical analyses of our data showed a slight, but significant direct correlation between NK T cells and the age of controls ($r_1 = +0·39$). In contrast in psoriatic patients a slight, but statistically significant inverse correlation ($r_2 = -0·31$) was detected (data not shown). We observed that patients with a long-term history of frequent relapses, who did not respond well to treatment, had the lowest NKT cell counts. In these patients the recovery of NK T cells following therapy was slower and generally poor. However, $CD3^+CD56^+$ cells showed no correlation with PASI score and disease duration (data not shown).

Stimulation of peripheral blood T cells with anti-CD3 monoclonal antibodies

To examine the activation status of $CD3^+CD56^+$ NK T cells, we separated T cells from the peripheral blood of healthy controls and of patients with psoriasis. These T cells were stimulated for 6 h using anti-CD3 monoclonal antibodies, and then flow cytometric analysis was performed. On the FSC/SSC dot-plot of separated and stimulated T cells obtained from healthy controls a

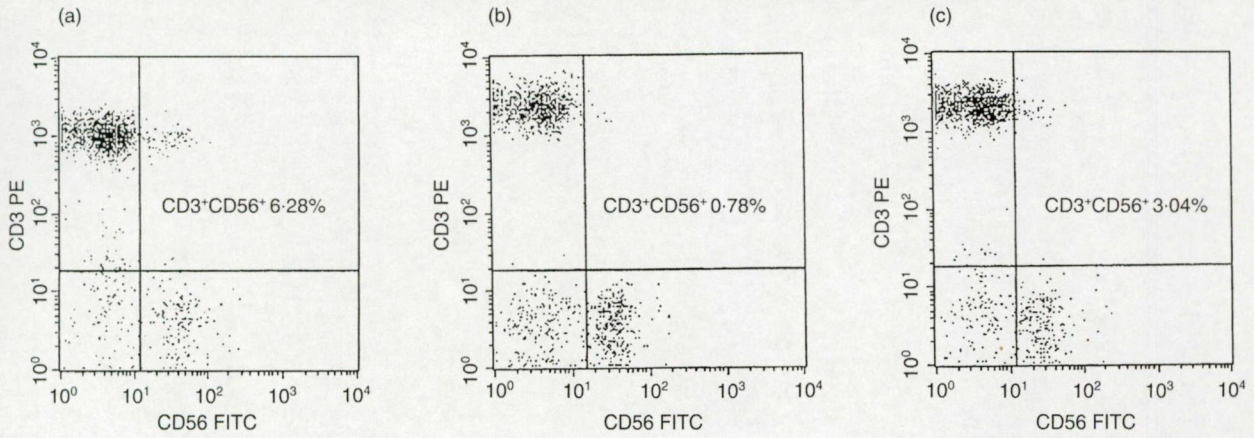

Fig. 1. Representative dot-plot diagrams of peripheral blood cells stained with PE conjugated anti-CD3 monoclonal antibodies and PE conjugated anti-CD56 monoclonal antibodies. $CD3^+CD56^+$ NK T cells in a healthy control (a), in a patient with psoriasis before treatment (b) and in the same patient after treatment (c) are in the upper-right quadrants of each dot-plot diagrams. $CD3^+CD56^+$ NK T cells are significantly decreased in the peripheral blood of patients with psoriasis (b, 0·78%), the percentage of these cells increases after therapy (c, 3·04%), but remains significantly lower than in healthy controls (a, 6·28%).

distinct cell population was recognized, which was not present as a distinct population on the dot-plot of unstimulated $CD3^+$ T cells (Fig. 2). These cells showed a marked granulated pattern. Analysis of stimulated T cells was performed by using two gates: R1 for these granulated cells and R2 for the other cells. All the granulated cells (gate R1) expressed the surface molecule CD56, but only a minority of the other cells (gate R2) expressed this molecule (Fig. 3). Thus the granulated cells were NK T ($CD3^+CD56^+$) cells. More than half of NK T cells (gate R1) expressed the CD4 marker (71·25%), while very few of the less granulated $CD3^+CD56^+$ NK T cells (gate R2) were $CD4^+$ (0·58%) (Fig. 3). The CD8 molecule was expressed by more than half of the less granulated $CD3^+CD56^+$ NK T cells (gate R2) and by about one-third of the granulated NK T cells (gate R1) (Fig. 4). The low affinity receptor for IL-2, an early activation marker for T cells, was detected with an anti-CD25 FITC labelled monoclonal antibody. Almost all the granulated NK T cells (gate R1) expressed CD25 molecules (93·2%), indicating that they were activated cells. Among the less granulated cell population (gate R2) only 59·65% of the cells expressed the low affinity IL-2 receptor (data not shown). After analysing the separated and stimulated T cells collected from patients with psoriasis we found that $CD56^+$ T cells with marked granulated pattern were almost absent. Scattered $CD56^+$ T cells were present between the cells with normal granulation pattern, characteristic for lymphocytes (data not shown). These findings are in concordance with the low levels of $CD3^+CD56^+$ NK T cells that were detected in the peripheral blood samples of patients with psoriasis by flow cytometric analyses of unseparated cells.

In each experiment controls staining of separated and stimulated T cells was performed after 24 h of culture in medium alone, using FITC conjugated anti-CD3 antibodies. The percentage of separated cells that expressed the surface molecule CD3 was 95–98% (data not shown). The vitality of separated and stimulated cells was tested using trypan blue, and was always above 95%.

DISCUSSION

NK T cells are phenotypically and functionally diverse [28]. Initially, NK T cells were described as cells that express an invariant TCR Valpha14 in mouse and Valpha24 in humans [29]. Recently NK T cells expressing diverse TCRs have been also recognized [22,23,30]. The $CD3^+CD56^+$ cells represent one of these NK T cell subpopulations.

Our results show that the number of $CD3^+CD56^+$ NK T cells is significantly decreased in the peripheral blood of patients with psoriasis and that the percentage of these cells increases after different therapies used in psoriasis, but remains significantly lower than those found in healthy controls. The full relevance of this finding is still speculative. The decrease in the number of $CD3^+CD56^+$ cells may represent an intrinsic characteristic feature of patients with psoriasis. This hypothesis is supported by the fact that NK T cells do not reach the values found in healthy controls, so it is possible that the percentage of this cell population is permanently decreased in the peripheral blood of patients with psoriasis. Another possible cause that leads to decreased number of NK T cells may be represented by the early activation of these cells by antigens involved in the relapse of the disease, followed by apoptosis [31]. Since we did not study NK T cells in the skin we could not exclude that the decrease in NK T cells in the peripheral blood might come from the differential homing of NK T cells to the skin lesions. Further studies are needed to elucidate the direct role of NK T cells in the skin of patients with psoriasis.

In our study we found that $CD3^+CD56^+$ NK T cells were capable of rapid activation. After stimulation of $CD3^+$ T cells one population of activated NK T cells appeared. NK T cells are able to recognize non-peptide antigens [32]. *In vivo* administration of synthetic ceramide induces the secretion of both type 1 and type 2 cytokines [24], but repeated doses polarize NK T cells towards Th2 cytokine synthesis [33,34]. Thus IL-4 produced by the NK T cells plays a major role in promoting the differentiation of Th0 cells into Th2 cells [34,35]. Although the precise role of NK T cells

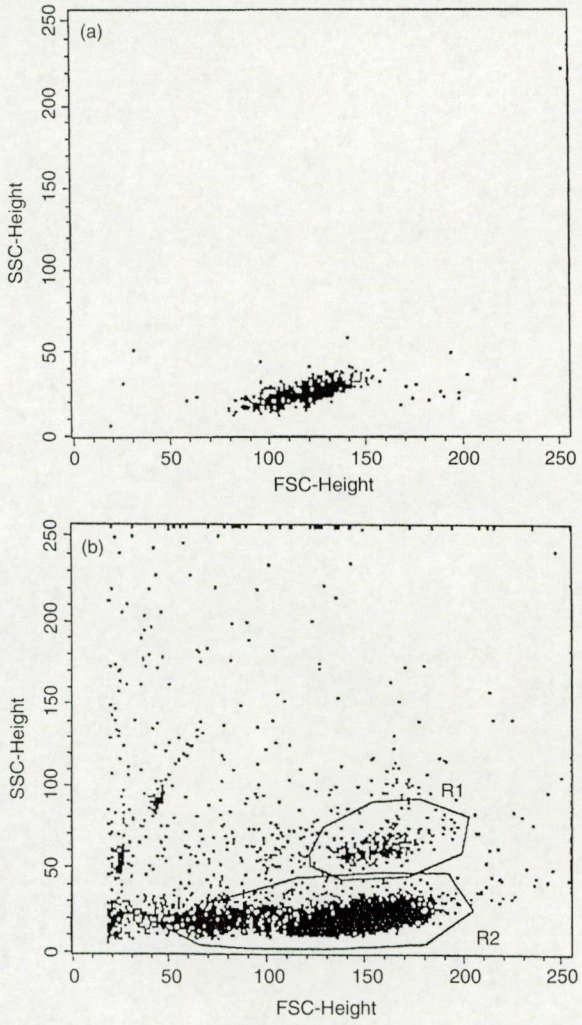

Fig. 2. Light scatter analysis (FSC *versus* SSC) of unstimulated peripherial blood $CD3^+$ T cells (a) and of separated and stimulated $CD3^+$ T cells (b). Peripheral venous blood sample was obtained from a healthy control. Cell separation and stimulation was performed using magnetic beads coated with anti-CD3 monoclonal antibodies. After stimulation of the $CD3^+$ T cells a distinct population of granulated cells appeared (R1).

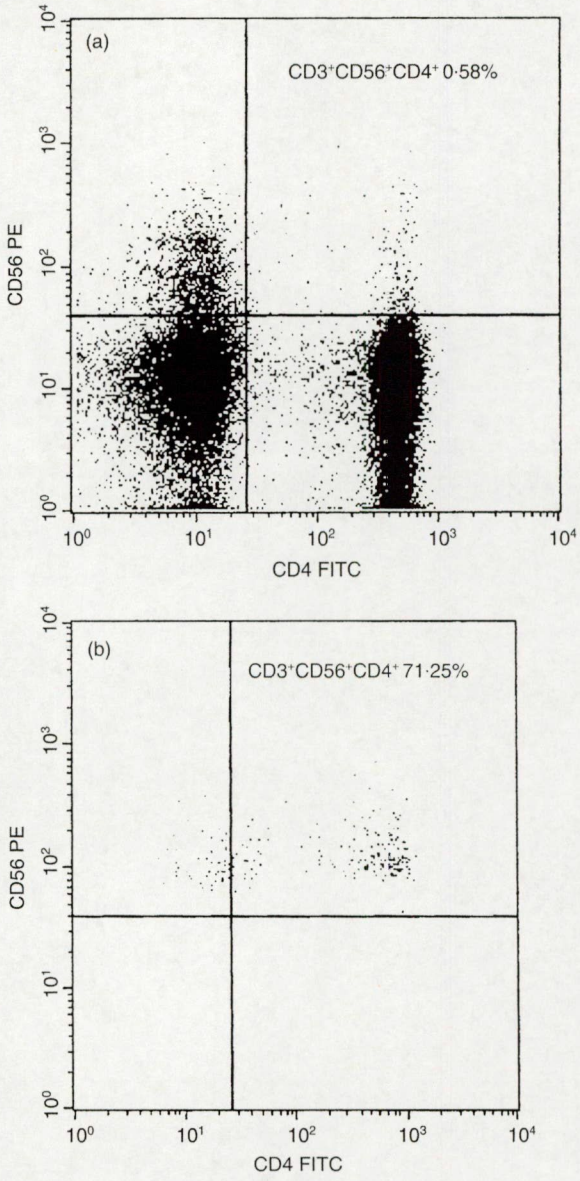

Fig. 3. Phenotypic analyses of separated and stimulated $CD3^+$ T cells. T cells were stained with PE conjugated anti-CD56 and FITC conjugated anti-CD4. The expression of CD56 and CD4 surface molecules on granulated $CD3^+$ T cells, gate R1 in Fig. 2b and on the less granulated $CD3^+$ T cells, gate R2 in Fig. 2a are shown. All the granulated $CD3^+$ T cells express the surface molecule CD56 (b, upper left and right quadrants), but of the less granulated $CD3^+$ T cells only a minor population express this molecule (a, upper left and right quadrants). 71·25% of granulated $CD3^+CD56^+$ cells express the CD4 marker (b, upper right quadrant), at the same time very few (0·58%) of the less granulated T cells express both CD56 and CD4 on the surface (a, upper right quadrant).

is not yet elucidated, they can be a regulatory cell type playing a pivotal role in the development of peripheral tolerance and in the modulation of immune responses by inducing a shift in early activation and consequently in the cytokine secretion of classical T cells [36,37]. Psoriasis is an autoimmune disease in which type 1 cytokine secretion pattern can be demonstrated in T cells derived from lesional skin and from peripheral blood [8,14]. It is possible that this feature is a consequence of an inefficient type 2 response, because of lack of NK T cells. This issue might be clarified by comparing the IL-4 producing capacity of $CD3^+CD56^+$ NK T cells from psoriatic patients with that from healty controls. However, the study of the cytokine production in psoriasis on a per cell basis was beyond the scope of our present work. Our results are the first clear-cut evidence showing that low NK T cell counts are a characteristic of psoriasis patients.These findings raised the question of whether NK T cell deficiency might result in the missing contraregulatory signals needed for the development of a normal immune response upon antigen stimulation, and favours excessive activation of Th1 cells. Type 2 cytokines are also important in the development of tolerance [38]; thus deficit in these cytokines can favour the development of autoimmunity.

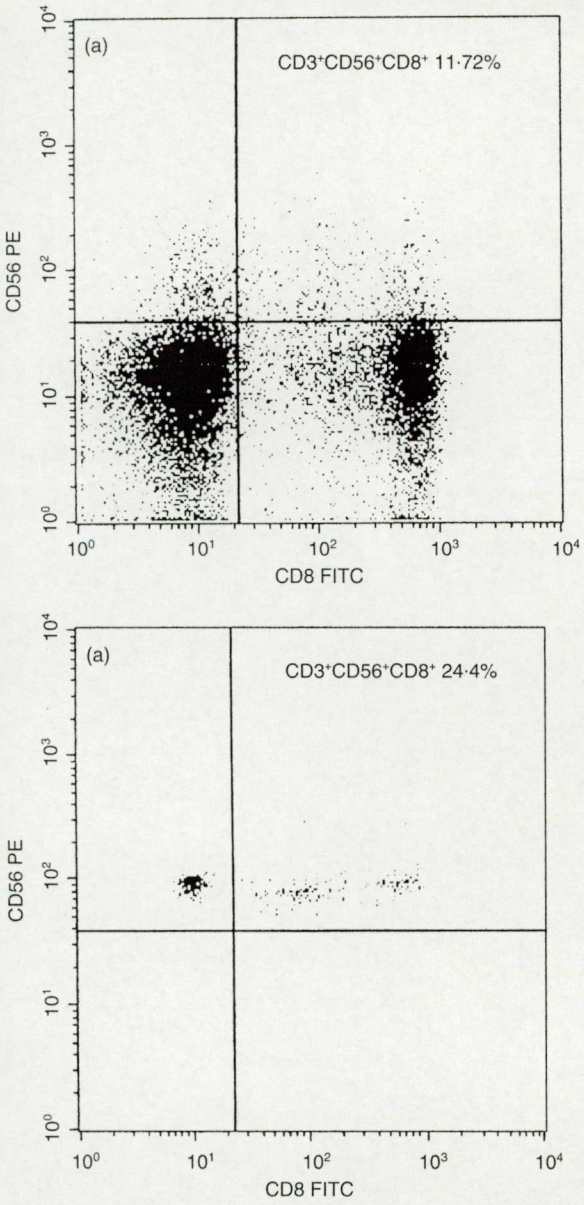

Fig. 4. Phenotypic analyses of separated and stimulated $CD3^+$ T cells. T cells were stained with PE conjugated anti-CD56 and FITC conjugated anti-CD8. The expression of CD56 and CD4 surface molecules on granulated $CD3^+$ T cells, gate R1 in Fig. 2b and on the less granulated $CD3^+$ T cells, gate R2 in Fig. 2a are shown. 24·4% of granulated $CD3^+CD56^+$ cells express the CD8 marker (b, upper right quadrant), at the same time 11·72% of the less granulated T cells express both CD56 and CD8 on the surface (a, upper right quadrant).

The dysfunction of NK T cells correlates with the pathogenesis of other T cell-mediated autoimmune diseases [39,40]. In lpr/lpr mice, in which a spontaneous autoimmune syndrome resembling human systemic lupus erythematosus occurs, NK T cells disappear from the periphery by the time the autoimmune disease develops. Selective experimental depletion of NK T cells from the peripheral blood results in early onset and exacerbation of the autoimmune phenomena [39]. The selective reduction of NK T cells has been also detected in non-obese diabetic mice [40,41]. Studies in humans have showed that decreased number of NK T cells are present in the peripheral blood of patients with rheumatoid arthritis, systemic sclerosis and insulin-dependent diabetes mellitus [27,42,43].

Other lymphocyte populations were also investigated. We found no significant differences between total T cells, total B cells, T helper cells, T cytotoxic cells and NK cells in patients with psoriasis and healthy controls. These results are in concordance with observations of other authors and highlight further the significance of $CD3^+CD56^+$ NK T cells in the pathogenesis of psoriasis [44–46].

We found increased percentage of memory T cells and decreased percentage of naive T cells. However, these changes were not statistically significant, they might be related to the chronic activation of the immune system in patients with psoriasis. Increased numbers of memory T cells have been found in lesional skin and in the synovial tissue of patients with psoriatic arthritis [38,47]. It is interesting that the treatments used in this study had no effect on memory and naive T cells. It is possible that the persistence of the increased number of memory T cells is one of the factors that contribute to the relapses observed in psoriasis, but these assumptions are still speculative and need further investigations.

In conclusion, our results suggest that $CD3^+CD56^+$ NK T cells have a role in the pathogenesis of psoriasis and that reduced NK T cells can be of importance in the development of psoriasis, a Th1 mediated autoimmune disease.

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III.

Low-dose dithranol treatment and tape stripping induce tolerance to dithranol in a mouse ear oedema model

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Short title: Dithranol tolerance in mouse ear

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Summary

It is well known from clinical practice that repeated treatment with dithranol leads to the development of tolerance to the drug. In the present study we investigated the characteristics and mechanism of dithranol tolerance. The mouse ear was pretreated with a low dose of dithranol or croton oil, or on previously sensitised animals by the application of dinitrofluorobenzole (DNFB). 24 hours later irritative dermatitis was elicited by painting the mouse ear with high dose of dithranol, croton oil oil DNFB, and the dermatitis was characterised by measurement of the ear thickness. It was shown that low-dose dithranol significantly suppressed dithranol-induced oedema, whereas it had no effect on croton oil or DNFB-induced dermatitis, suggesting that dithranol-induced tolerance is rather specific. Tolerance to dithranol could not be induced by pretreatment of the mouse ear with a low dose of croton oil or DNFB. Mild stripping of the mouse ear also inhibited the inflammatory effect of dithranol applied 24 hours later. Superoxide dismutase (SOD) treatment abolished the tolerance-inducing effect of low-dose dithranol or stripping. These results suggest that superoxide anion radicals are involved not only in the inflammatory effect of the drug, but also in the induction of dithranol tolerance.

Key words: dithranol, tolerance, tape stripping, SOD

Introduction

Dithranol is one of the most effective local treatment of psoriasis, but its use is hampered by its side-effects of irritancy and staining.[1] In clinical practice, treatment with dithranol is started at low concentration in order to prevent severe perilesional inflammation. The concentration of the drug is then slowly increased every 2-3 days until mild perilesional irritancy is observed, suggesting that the therapeutically effective concentration has been reached. However, after such an "optimal" concentration has been found, repeated treatment with the same concentration results within several days in a decline of both the irritative but also the antipsoriatic effect. Therefore, to see further improvement, the concentration of dithranol should again be increased. This results that, in addition to the irritative and staining side-effects of the drug, the adaptation of the skin to dithranol further complicates its therapeutic use.

The antipsoriatic and inflammatory actions of the drug are related probably to its redox activity, leading to the production of oxygen radicals;[2,3] however the mechanism of the increased tolerance of the skin to dithranol during repeated treatment is not known. The increased tolerance of the skin during the repeated application of dithranol was first proved experimentally by Brandt and Mustakallo. They presumed that continuous depletion of the inflammatory mediators of the skin resulted in the observed decreased inflammatory effect of the drug.[4] In contrast, Lawrence and Schuster hypothesised that this phenomenon might be caused by an active adaptive mechanism,[5] as they found that tape stripping (removing the stratum corneum with an adhesive tape) and application of a very low dose of anthralin also induced tolerance of the skin to the drug. We earlier studied the effects of repeated dithranol treatment on ear thickness in mice. It was suggested that an active adaptation process is responsible for the increased tolerance observed after low-dose dithranol treatment.[1] Parslew and Friedmann recently demonstrated that pretreatment of human skin with a subirritant concentration of dithranol induced a clear adaptive response, which proved to be rather specific as it had no effect on croton oil-induced inflammation.[6] Although these human

studies revealed the adaptive response of the skin to dithranol, the underlying mechanisms could not be elucidated.

In the present study, we investigated the characteristics and mechanism of tolerance to dithranol in a mouse ear oedema model. Our results suggest that the dithranol-induced increased tolerance to the drug is an active process, and oxygen radicals might be responsible for the induction of this adaptive response.

Materials and methods

Animals

The animals used were inbred 8-12-week-old male CFLP mice (25-35 g). They were caged in groups of 10 and fed on standard mouse pellets and water ad libitum.

Dithranol pretreatment in dithranol, croton oil and DNFB dermatitis

Groups of mice were treated by applying 20 µl 0.025% dithranol (Hermal, Reinbek, Germany) or acetone to the mouse ear. 24 hours later 20 µl 0.5% dithranol, 5% croton oil (Sigma, St. Louis, USA) or 0.15% DNFB (Sigma, St. Louis, USA) in previously DNFB-sensitised animals was applied to the mouse ear. Dithranol, croton oil and DNFB were dissolved in acetone. To achieve the DNFB sensitisation, the abdominal skin of the animals was shaved and 10 µl 0.2% DNFB dissolved in acetone was dropped onto the skin once daily for 2 days. The allergic contact dermatitis was elicited 3 days later.

DNFB pretreatment in dithranol and croton oil dermatitis

Groups of mice were treated with by applying 20 µl 0.05% DNFB or acetone onto the mouse ear on previously sensizitised animals. For DNFB sensitisation, 10 µl 0.1% DNFB was dropped to the shaved abdominal skin of the animals. In each DNFB and acetone-pretreated mouse group, 20 µl 0.5% dithranol or 5% croton oil was applied 24 hours later to the mouse ear to generate irritative dermatitis.

Croton oil pretreatment in dithranol, croton oil and DNFB dermatitis

The mouse ear was treated with 20 µl 0.5% croton oil or acetone,. 24 hours later 20 µl 0.5% dithranol, 5% croton oil or 0.15% DNFB (in previously DNFB-sensitised animals) was applied to the mouse ear. The DNFB sensitisation procedure was the same as described above.

SOD and dithranol pretreatment in dithranol dermatitis

Two mouse groups received 0.1 ml (20 mg kg^{-1}) Superoxide dismutase (SOD, Grünenthal GmbH, Germany) or physiological sodium chloride solution intraperitoneally. One hour later, 20 µl 0.025% dithranol or acetone was applied to the mouse ear. Twenty-four hours after the dithranol or acetone treatment, inflammation was induced in the mouse ear with 0.5% dithranol.

The effect of tape stripping in dithranol dermatitis

Both ears of the mice were covered with adhesive tape, which was removed and applied again 5 times (stripping). One, 2 or 7 days later, 0.5% dithranol was applied to the mouse ear. Other groups of mice received 0.1 ml (20 mg kg^{-1}) SOD intraperitoneally before stripping

Measurement of mouse ear oedema

The extent of oedema induced by the skin irritants was characterised by measuring the mouse ear thickness with dial gauge.

Statistical analysis

Data are presented as the mean $\pm$ S.D. Student's t-tests were used for analyses comparing the different groups, with statistical significance considered if $P < 0.05$.

Results

Effects of dithranol pretreatment on dithranol, croton oil and DNFB-induced mouse ear oedema

The ear thicknesses of 0.025% dithranol or acetone pretreated mice were not significantly different 24 hours after application ($19.3 \pm 0.7 \times 10^{-2}$ mm and $18.9 \pm 0.7 \times 10^{-2}$ mm, respectively, $p>0.05$; n=30).

After the mouse ear had been pretreated with 0.025% dithranol for 24 hours, the 0.5% dithranol-induced ear thickness was 40% less than that in the acetone treated group. Dithranol pretreatment (0.025%) of the mouse ear did not influence the 5% croton oil or 0.15% DNFB-induced ear oedema: there was no any significant difference between the ear thicknesses in the dithranol or acetone pretreated mice (Fig. 1). Intraperitoneal SOD pretreatment 1 hour before the application of 0.025% dithranol eliminated the induction of dithranol tolerance (Table 1).

Effects of DNFB pretreatment on dithranol and croton oil induced mouse ear oedema

After 0.1% DNFB senzitization, the ear thicknesses of 0.05% DNFB or acetone pretreated mice were not significantly different 24 hours after application ($19.2 \pm 0.9 \times 10^{-2}$ mm and $19.1 \pm 0.8 \times 10^{-2}$ mm, respectively, $p>0.05$; n=30). 0.05% DNFB pretreatment of the mouse ear for 24 hours did not influence the 0.5% dithranol or 5% croton oil-induced ear oedema (Fig. 2).

Effects of croton oil pretreatment on dithranol, croton oil and DNFB-induced mouse ear oedema

The ear thicknesses of 0.5% croton oil or acetone pretreated mice is not significantly different 24 hours after application ($19.4 \pm 0.8 \times 10^{-2}$ mm and $19.1 \pm 0.7 \times 10^{-2}$ mm, respectively, $p>0.05$; n=30). 0.5% croton oil pretreatment of the mouse ear for 24 hours did not influence the 0.5% dithranol, 5% croton oil or 0.15% DNFB-induced ear oedema: there was no significant difference in ear thickness between the croton oil or acetone-pretreated mice (Fig. 3).

Effects of tape stripping on 0.5% dithranol-induced ear oedema

The extent of ear oedema in the animals pretreated with dithranol directly after stripping did not differ from that in the control group. When dithranol was applied 24 hours after stripping the change in oedema was significantly less than that in the control group. Two days after stripping, the increase in ear thickness was still less than that in the control group, but stripping did not influence the inflammation induced by dithranol applied 7 days later (Table 2). Intraperitoneal SOD pretreatment 1 hour before stripping eliminated the inhibitory effect of stripping on the ear thickness change observed 24 hours later (Table 3). SOD pretreatment did not affect the inflammatory effect of dithranol applied 48 hours after stripping.

Discussion

In the present study we could model the tolerance inducing effect of dithranol in mice. Our results indicate that pretreatment of the mouse ear with low-dose dithranol concentrations significantly decreases the oedema-inducing effect of higher dithranol concentrations applied later, but does not effect the extent of croton oil or DNFB dermatitis. This suggests that this type of tolerance is specific for dithranol dermatitis. When the mice were pretreated with SOD 1 hour before low-dose dithranol pretreatment, the tolerance inducing effect disappeared. As SOD is responsible for the elimination of superoxide anion radicals,[7] it is very likely that free oxygen radicals are involved in the induction of dithranol tolerance. We demonstrated that tape stripping induced a tolerance to dithranol similar to that following low-dose dithranol pretreatment on the mouse ear, and this tolerance could be reduced with an SOD injection 1 hour before stripping. Since SOD cannot penetrate the plasma membrane, it exerts its effect on the extracellular space;[8] it is probable that superoxide radicals generated extracellularly are responsible for the tolerance induction.

It is known from clinical practice that repeated treatment with dithranol leads to a tolerance to the drug in the skin. Brandt and Mustakallo presumed that this tolerance is due to the continuous depletion of the inflammatory mediators.[4] Our results suggest that, even after treatment of the mouse ear with croton oil in higher concentrations, the quantity of inflammatory mediators remaining in the skin is still sufficient to evoke inflammation. We presume that the dithranol induced tolerance of the skin is caused by an an active adaptive mechanism. The superoxide anion radicals which are generated extracellularly on the autooxidation of dithranol may be involved in triggering this adaptive mechanism, which defends the organism from the toxic action of free radicals.

The studies of Lawrence and Schuster indicated that tape stripping moderates the inflammatory effect of dithranol applied 24 hours later, wheras it has no effect on the irritation induced by dithranol applied immediately after stripping.[5] They supposed that stripping triggers an

unknown adaptive mechanism. Our indirect results suggest that superoxide anion radicals generated in the skin during tape stripping induce adaptive mechanisms. In the studies by Juhlin[9] and Misch et al.[10] UV irradiation was found to moderate the erythema-generating effect of dithranol applied 24 hours later. They supposed that UV irradiation induced increased autooxidation of dithranol resulted the less inflammatory effect of the drug. However, it is well known that UV irradiation induces free radical production in the skin.[11,12] We suggest that the inhibitory effect of UV irradiation on dithranol dermatitis is also connected with the adaptive mechanism triggered by superoxide anion radicals.

The adaptive adaptive mechanism that eliminates free radicals generated during repeated treatment with dithranol is not fully known. Our results indicate that the adaptive response might be triggered by the extracellularly localised superoxide anion radicals. Earlier we have shown that in dithranol induced inflammation free radicals played an important role[13]. Free radicals have been shown to induce the activity of antioxidant enzymes[14], we suppose that one of the extracellular antioxidant enzymes might be responsible for the observed adaptive mechanism induced by dithranol or tape stripping. The extracellular SOD and thioredoxinreductase[15] are the two important extracellular antioxidant enzymes. The activity of the extracellular SOD is very low and probably protects only the endothelial cells[8], it is unlikely that this enzyme would be responsible for the observed protective effect. The intensity of dithranol inflammation is higher in nonlesional skin than in the lesions[16], and indeed dithranol-induced inflammation occurrs characteristically only in the perilesional area. It has been shown that the activity of thioredoxinreductase is much higher in psoriatic plaques compared to nonlesional skin[17], suggesting that thioredoxinreductase might be responsible for the induction of dithranol tolerance.

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Table 1.

Effect of SOD plus 0.025% dithranol pretreatment on 0.5% dithranol-induced mouse ear oedema

	Control	Dithranol	SOD+Dithranol	SOD
Increase in mouse ear thickness				
(x 10^{-2} mm)	18.9	11.2	18.2	19.1
$\pm$ SD	1.34	1.52	1.48	1.46
p		0.001	ns	ns

Values represent mean $\pm$ SD, n=20, Student's unpaired t test

p: significance compared to the control

ns: non-significant

SD: standard deviance

SOD: Superoxide dismutase

Table 2.

Effect of tape stripping on 0.5% dithranol-induced mouse ear oedema

		Days after tape stripping			
	Control	0	1	2	7
Mouse ear thickness					
(x 10^{-2} mm)	18.9	19.2	11.3	14.5	19.1
$\pm$ SD	0.82	1.34	1.55	1.42	1.27
p		ns	0.001	0.05	ns

Values represent mean $\pm$ SD, n=20, Student's unpaired t test

p: significance compared to the control

ns: non-significant

SD: standard deviance

Table 3.

Effect of SOD plus tape stripping (TS) pretreatment on 0.5% dithranol-induced mouse ear oedema

	Control	TS	SOD+TS	SOD
Increase in mouse ear thickness				
(x 10^{-2} mm)	19.2	12.2	18.8	19.3
$\pm$ SD	1.44	1.31	1.63	1.52
p		0.001	ns	ns

Values represent mean $\pm$ SD, n=20, Student's unpaired t test

p: significance compared to the control

ns: non-significant

SD: standard deviance

SOD: Superoxide dismutase

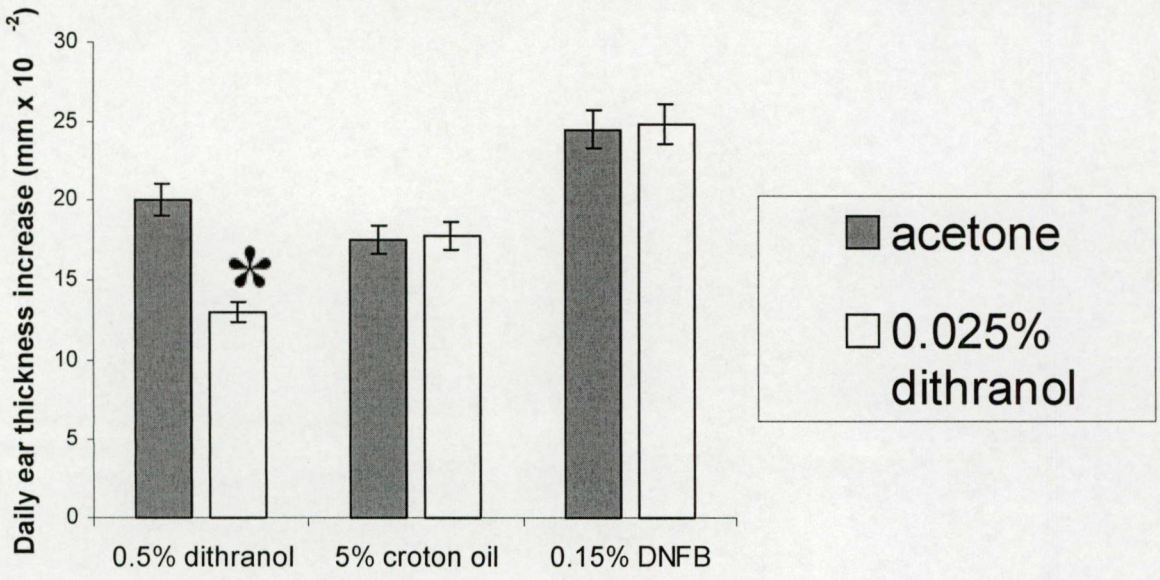

Fig. 1.

Effects of dithranol pretreatment on dithranol, croton oil and DNFB-induced mouse ear oedema

0.5% dithranol, 5% croton oil or 0.15% DNFB-induced ear oedema after 0.025% dithranol or acetone pretreatment for 24 hours. (Values represent mean $\pm$ SD, n=20, *p <0.05 significant differences between the dithranol or acetone pretreated groups, Student's unpaired t test)

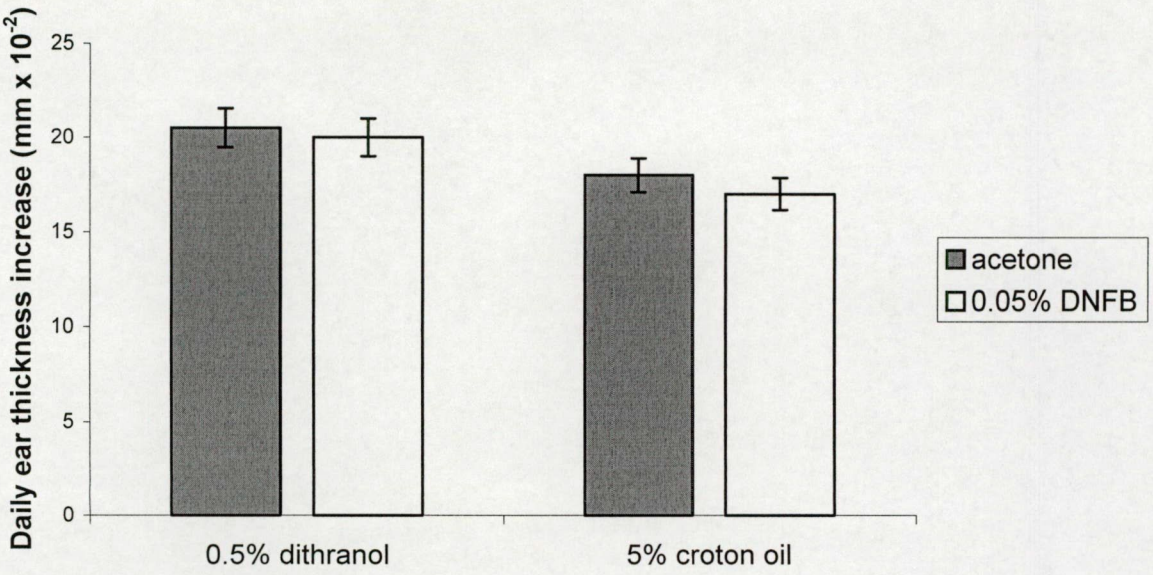

Fig. 2.

Effects of DNFB pretreatment on dithranol and croton oil induced mouse ear oedema

0.5% dithranol or 5% croton oil-induced ear oedema after 0.05% DNFB or acetone pretreatment for 24 hours. (Values represent mean ± SD, n=20, $p > 0.05$ no significant differences between the DNFB or acetone pretreated groups, Student's unpaired t test)

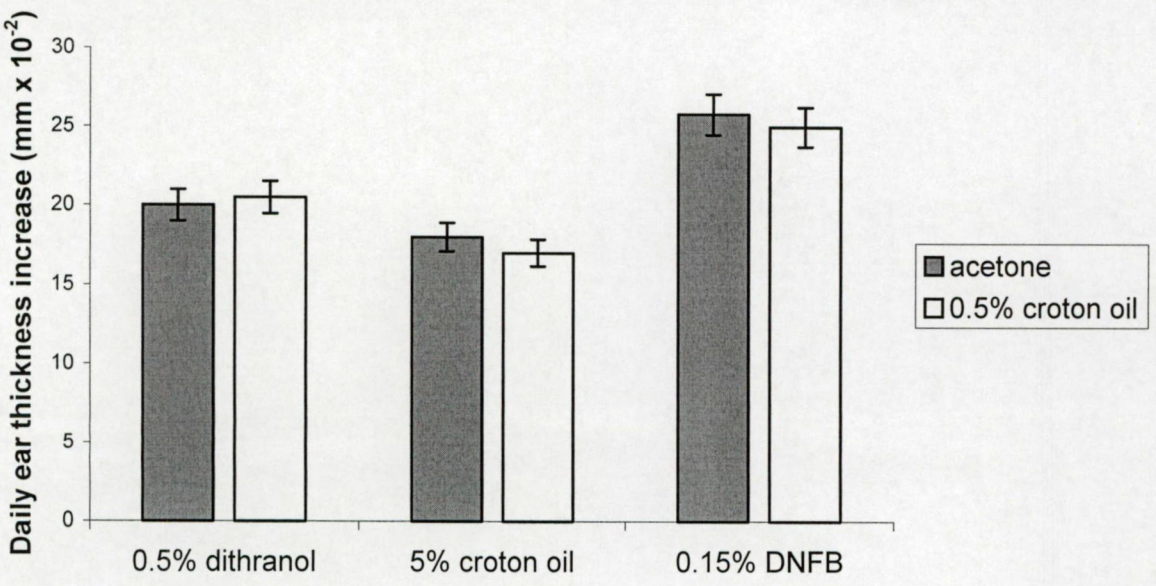

Fig. 3.

Effects of croton oil pretreatment on dithranol, croton oil and DNFB-induced mouse ear oedema

0.5% dithranol, 5% croton oil or 0.15% DNFB-induced ear oedema after 0.5% croton oil or acetone pretreatment for 24 hours. (Values represent mean $\pm$ SD, n=20, $p > 0.05$ no significant differences between the croton oil or acetone pretreated groups, Student's unpaired t test)

IV.

Ethanol and Acetone Stimulate the Proliferation of HaCaT Keratinocytes. The Possible Role of Alcohol and a Metabolite Relevant to Alcohol Metabolism in Exacerbating Psoriasis.

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ABSTRACT Alcohol abuse can precipitate the hyperproliferative skin disease psoriasis vulgaris. The mechanism by which alcohol affects this disease is still elusive. So far there are no reports describing the effects of metabolites relevant to alcohol metabolism on the growth of human keratinocytes. In the present study we examined the effect of ethanol and acetone, which exceeds its normal endogenous level in the blood of heavy drinkers, on the proliferation of human immortalized keratinocytes (HaCaT cells), which are commonly used as a model to study psoriasis. HaCaT cells were incubated for 48 and 72 hours in the presence of different ethanol (2.1375 μM-1,71 mM) and acetone (6,81 μM-1,36 mM) concentrations and the number of viable cells was determined by the MTT assay. The effect of ethanol and acetone on the IL-8 mRNA production and on mRNA levels of genes characteristic for proliferating keratinocytes such as α5 integrin, keratinocyte growth factor receptor and cyclin D1 was studied by RT-PCR. Our data showed that both ethanol and acetone induced the proliferating potential of HaCaT cells. The maximum increase was observed at 4,275 μM ethanol and at 13,6 μM acetone concentration. The α5 integrin, keratinocyte growth factor receptor and cyclin D1 mRNA levels were higher compared to the controls already 0.5 hours after ethanol treatment and 2 hours after acetone treatment of the cells. Ethanol and acetone did not alter the IL-8 production of HaCaT cells. Our results suggest that ethanol and also acetone enhances the proliferation of human immortalized keratinocytes. The stimulatory effect of ethanol and acetone used in physiologically relevant concentrations on human keratinocytes may be one of the reasons why psoriasis can be precipitated by alcohol misuse.

Introduction

Psoriasis is a common genetically determined skin disorder characterized by epidermal hyperproliferation and inflammation. Genetic predisposition is essential but exogenous inducing factors are required to precipitate the disease. (1) It is a well known phenomenon that increased alcohol consumption leads to an exacerbation or treatment unresponsiveness in psoriasis. (2-8) The mechanism by which alcohol affects psoriasis is still elusive.

Numerous studies indicate that acetone exceeds its normal endogenous level in the blood of heavy drinkers (9-12). So far there are no reports about the possible effects of acetone on psoriasis.

The pathologic features observed in psoriasis are partly caused by the highly elevated amounts of stimulatory signals. In psoriasis the regulation for interleukin-8 (IL-8) (13;14) and its type I receptor (IL-8RA) (15) is altered. IL-8 is chemotactic for neutrophils (16), T lymphocytes (17) and keratinocytes (18). It promotes the proliferation (19) and induces human leukocyte antigen-DR (HLA-DR) expression of keratinocytes (20). Several antipsoriatic compounds have inhibitory effects on IL-8 binding to cultured keratinocytes (21).

The epidermal hyperplasia in psoriasis is characterized by an increased percentage of normally quiescent basal keratinocytes in the proliferative phases of cell cycle. (22) Progression to the cell cycle is regulated by cyclin dependent kinases, which are activated by specifically binding to cyclins. (23;24) Keratinocytes express cyclin D1 when they traverse from G0 into cell cycle prior to G1. (25) Cyclin D1 is associated with epithelial hyperproliferation (26) and is overexpressed in psoriasis. (27;28) Furthermore there is increasing evidence of the therapeutic role of cyclin inhibitors in psoriasis. (29;30)

In addition to cell cycle regulation molecules soluble mitogens such as growth factors and their receptors regulate cell growth.

Keratinocyte growth factor (KGF), a fibroblast-derived member of the fibroblast growth factor (FGF) family acts on epithelial cells (31). It has a potent mitogenic activity on human keratinocytes and is involved in the control of epithelial proliferation and differentiation (32). KGF binds to the tyrosine kinase KGF receptor (KGFR) which is a splicing transcript variant of the fibroblast growth factor receptor 2 (FGFR2) (33;34). An increased KGF and KGFR expression, associated with lesional psoriatic tissue might play a role in epidermal hyperproliferation in this disease (35).

Insoluble extracellular molecules play an important role in the regulating the growth of ancorage-dependent cells. (36-38)

Integrins are a large family of cellular surface molecules interacting with the extracellular matrix. Keratinocyte integrins are important epidermal regulatory molecules, they are involved in adhesion, migration, growth and differentiation (39) Fibronectin can regulate keratinocyte growth through the fibronectin receptor ($\alpha5\beta1$). Alterations in expression, topography and function of integrin receptors have been reported in lesional and also in nonlesional psoriatic keratinocytes. (40;41) Nonlesional psoriatic skin expresses higher $\alpha5$ integrin levels relative to normal keratinocytes, which may lead to the hyperresponsiveness of psoriatic keratinocytes to proliferation signals provided by lymphokines produced by intralesional T lymphocytes. (42)

HaCaT cells are commonly used as model system to study hyperproliferative skin diseases such as psoriasis. (43-48)

HaCaT keratinocytes are proper candidates for studying external regulators of proliferation and differentiation (49). Depending on the in vitro conditions HaCaT cells represent different types of keratinocytes. In contact-inhibited, serum-starved culture these cells resemble suprabasal nonproliferated differentiated keratinocytes, after release from contact inhibition and the addition of serum they resemble the activated, differentiated transiently amplifying keratinocytes. (50)

In this study we investigated the effects of ethanol and acetone used in physiologically relevant concentrations on human HaCaT keratinocytes.We found that both ethanol and acetone exerts a proliferative effect on keratinocytes as determined by number of viable cells. In HaCaT cells ethanol and acetone did not alter the strong IL-8 mRNA expression but upregulated the mRNA levels of cell cycle regulation-related genes: α5 integrin, KGFR and cyclin D1. These results indicate that ethanol and acetone may have a role in precipitating psoriasis.

Materials and methods

Cell culture

The spontaneously transformed human keratinocyte cell line HaCaT (kindly provided by N.E. Fusenig, Heidelberg, Germany) was cultured in Dulbecco's modification of Eagle's medium (DMEM; Gibco, Eggstein, Germany) containing 10% fetal calf serum (FCS; Gibco), L-glutamine, penicillin/streptomycin and fungizone (Sigma, Budapest, Hungary). The cells were maintained at 37°C in a humid 5% CO_2 atmosphere incubator.

Ethanol and acetone treatment

The subconfluent cultures were washed with phosphate buffered saline (PBS) under sterile conditions and were incubated for 30 minutes at 37°C with increasing concentrations of ethanol (2,13 μM – 1710 μM) or acetone (6,81 μM – 1360 μM) in DMEM containing 0.5% FCS. Control cells were incubated in DMEM containing 0.5% FCS for 30 minutes.

Determination of cell viability

HaCaT cells were plated at a density of 1x104 cells/well in 96-well microtiter plates. Cells were grown to subconfluence. After incubation with different ethanol and acetone concentrations for 30 minutes in the presence of DMEM containing 0.5% FCS cells were washed with PBS and DMEM containing 10% FCS was added to each well. 72 hours after ethanol treatment or 48 hours after acetone treatment HaCaT cell viability was measured by the MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (51)

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Cells were grown to subconfluence in 6-well plates. After different concentrations of ethanol or acetone treatment HaCaT keratinocytes were washed with PBS and were incubated in DMEM containing 10% FCS at 37°C in a humid 5% CO_2 atmosphere incubator before total cellular RNA isolation according to the TRIzol® protocol (Gibco).

First-strand cDNA was synthesized from 0.5 μg of total RNA in 20 μl final volume (MBI Fermentas, Vilnius, Lithuania). After reverse transcription (RT) semiquantitative polymerase chain reaction (PCR) was performed using Taq DNA polymerase and dNTP set (MBI Fermentas, Vilnius, Lithuania). 2 μl aliquot of the RT solution was used as a template for the β-actin, IL-8, KGFR, cyclin D1, and α5 integrin specific PCR reactions.

The following primer sequences were used: β-actin: 5'-AGA GAT GGC CAC GGC TGC TT-3' and 5'- ATT TGC GGT GGA CGA TGG AG -3', IL-8: 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3' and 5'-TCT CAG CCC TCT TCA AAA ACT TCT T-3', KGFR: 5'-AGT TTA AGC AGG AGC ATC GCA TT-3' and 5'-CTG TTA CCA CCA TAC AGG CGA TTA A-3', cyclin D1: 5'-AGG AGA ACA AAC AGA TCA-3' and 5'-TAG GAC AGG AAG TTG TTG-3', α5: 3'-ATT ATC AGA GCA AGA GCC GGA TAG A-5' and 5'-GGA GAT GAG GGA CTG TAA ACC GA-3' at a final concentration of 0.66 pmol/μl. The following cycling times and temperatures were used to amplify the cDNA: β-actin (25 cycles of 94°C for 60 s, 60 °C for 60 s and 72°C for 60 s), IL-8 (35 cycles of 94°C for 60 s, 59 °C for 90 s and 72°C for 90 s), KGFR (30 cycles of 94°C for 60 s, 60 °C for 60 s and 72°C for 60 s), cyclin D1 (35 cycles of 94°C for 60 s, 60 °C for 60 s and 72°C for 60 s), α5 integrin (30 cycles of 94°C for 60 s, 60 °C for 60 s and 72°C for 60 s). After PCR amplification products were visualized by EtBr-stained agarose gel electrophoresis.

Statistical analysis

Data are presented as the mean $\pm$ SEM. Student's t-tests were used for analyses comparing the different groups, with statistical significance considered if $P < 0.05$.

Results

HaCaT cells were incubated for 48 and 72 hours in the presence of different ethanol (2.1375 μM-1,71 mM) and acetone (6,81 μM-1,36 mM) concentrations and the number of viable cells was determined by the MTT assay. Our data showed that both ethanol (Fig. 1) and acetone (Fig. 2) induced the proliferating potential of HaCaT cells.

The effect of ethanol and acetone on the mRNA levels of genes characteristic for proliferating keratinocytes such as α5 integrin, KGFR and cyclin D1 was studied by RT-PCR, α5 integrin, KGFR and cyclin D1 mRNA levels were higher compared to the controls already 0.5 hours after the ethanol treatment and 2 hours after the acetone treatment of the cells. Ethanol and acetone did not alter the IL-8 production of HaCaT cells . (Fig. 3)

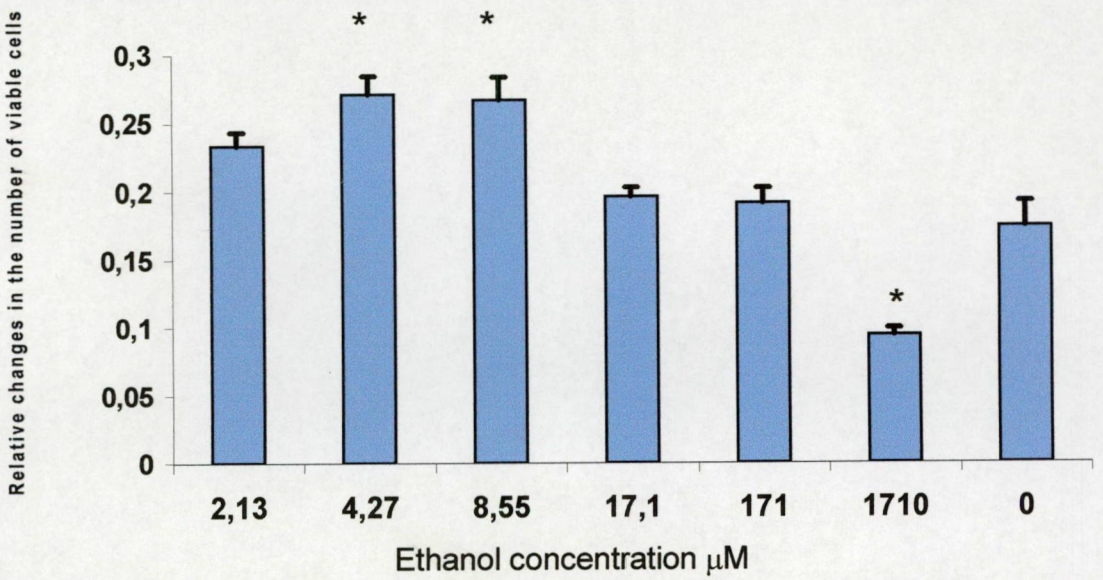

Fig. 1. Relative changes in the number of viable cells determined by the MTT assay 72 hours after ethanol treatment. At 4,27 μM or 8,55 μM concentration ethanol significantly increased the number of viable HaCaT cells compared to the control (0 μM ethanol). At 1710 μM concentration ethanol proved to be toxic and significantly decreased the number of viable HaCaT cells compared to the control (0 μM ethanol). Bars represent means of 3 independent experiments ± SEM, (*$p<0,05$).

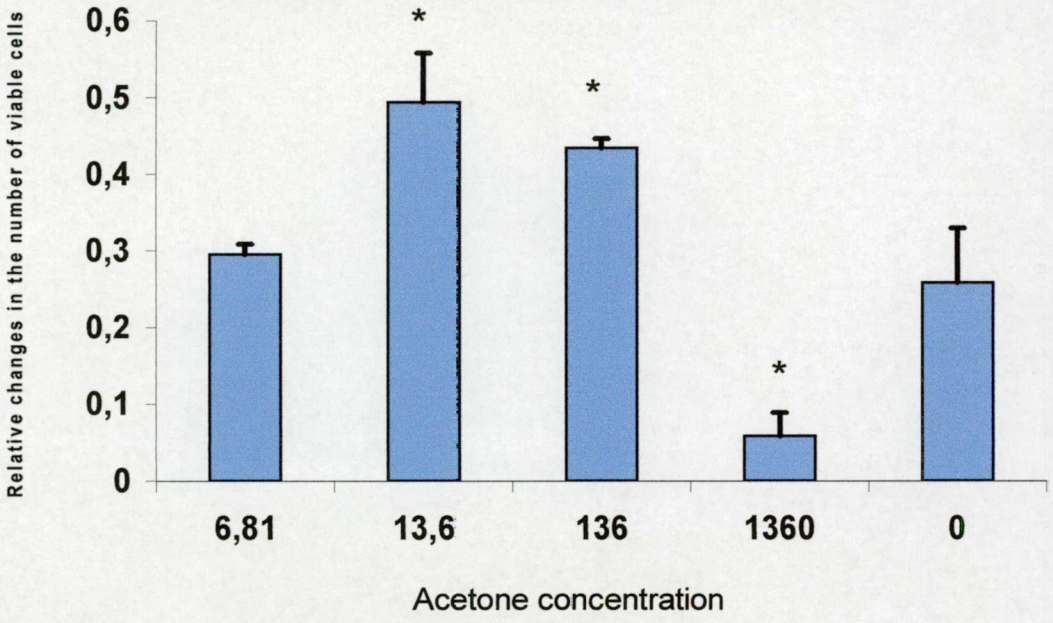

Fig. 2. Relative changes in the number of viable cells determined by the MTT assay 48 hours after acetone treatment. At 13,6 μM or 136 μM concentration acetone significantly increased the number of viable HaCaT cells compared to the control (0 μM acetone). At 1360 μM concentration acetone proved to be toxic and significantly decreased the number of viable HaCaT cells compared to the control (0 μM acetone). Bars represent means of 3 independent experiments ± SEM, (*p<0,05).

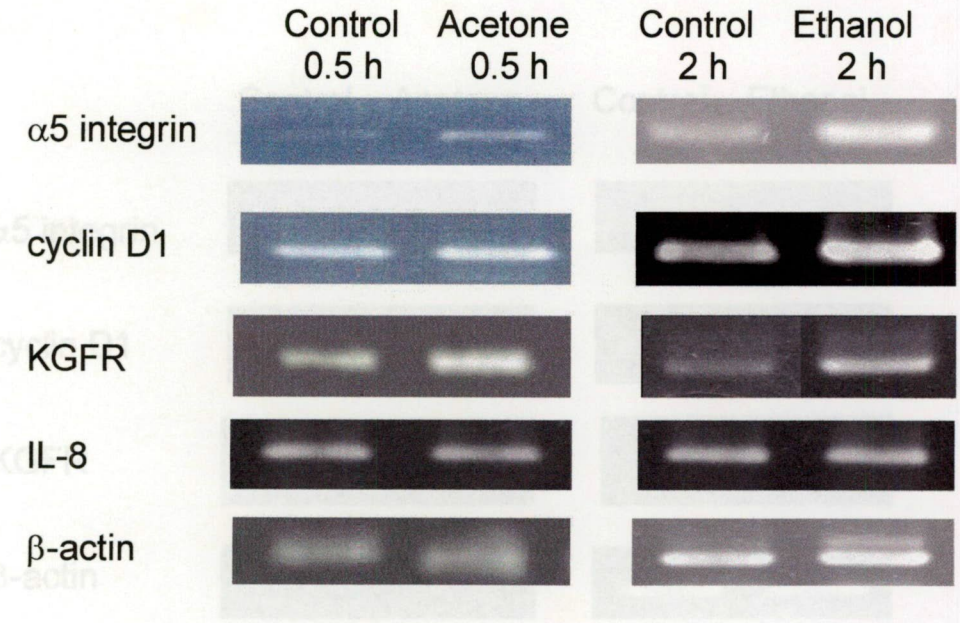

Fig. 3. RT-PCR analyses of IL-8 and cell cycle regulation-related genesThe α5 integrin, cyclin D1 and KGFR mRNA levels were higher compared to the controls after 2 hours of ethanol and 0.5 hours of acetone treatment of HaCaT cells. Ethanol and acetone did not alter the IL-8 mRNA expression of HaCaT cells

Discussion

Psoriasis is a one of the commonest inflammatory dermatoses. Genetic predisposition is indispensable but exogenous precipitating factors are also required to trigger the disease such as trauma, infections, drugs, stress, endocrine and metabolic factors. (1) Alcohol misuse has emerged as and additional factor, which may be associated with psoriasis (2-8), but controversy still exists over this topic.

Protein kinase C (PKC) isoenzymes play an important part in signal transmission regulating cellular growth, differentation, cytokine production and adhesion molecule expression. In psoriasis down regulation of several PKC isoenzymes occurs. In vitro studies show that ethanol can both inhibit or enhance PKC activity, depending on the experimental conditions. (52)

Decreased cyclic AMP (cAMP) levels or an increase in the CGMP/CAMP ratio causes stimulation of cell activity. These changes are important in the hyperproliferation associated with psoriasis. (53) Acute ethanol exposure increases the amount of cAMP (54-57) and inhibits the accumulation of cyclic GMP (58), thus showing an antipsoriatic effect. Chronic alcohol exposure showed opposite effects. (59)

Extracellular lipids of the stratum corneum ere essential for the epidermal permeability barrier function, which is an important factor in regulating epidermal DNA synthesis. (60;61) Alterations in epidermal lipid content and metabolism have been observed in psoriasis. (62;63) Most of the investigations indicate that ethanol enhances cholesterol synthesis, increases the activity of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase, and induces an elevation of triglicerides, high-density lipoprotein, and cholesterol levels in serum and liver. (64-67) However agents known to induce psoriasis generally inhibit cholesterol synthesis and lipid metabolism.

Therefore these findings make it very unlikely that effects of ethanol on psoriasis are mediated through the above mentioned regulatory systems (1).

Some studies indicate that alcohol alters the immune system. It is known that alcoholics are more susceptible to various infections. (68) Because streptococcal infections are trigger factors for psoriasis this increased susceptibility may be involved in the onset and progress of the disease. Ockenfels et al. developed a co-culture model with psoriatic keratinocytes and a T-lypmphoma cell line (HUT-78). It was found that interleukin-6 (IL-6), transforming-growth-factor-α (TGF-α) and interferon-γ (IFN-γ) levels were elevated in the ethanol-treated psoriatic co-culture system indicating an enhanced Th-1 type response in the presence of ethanol. (69)

Among heavy drinkers blood acetone level is elevated. It is widely accepted that blood acetone concentration is good indicator of alcohol consumption. So far there were no reports on possible role of blood acetone concentration in evoking psoriasis.

IL-8 is one of the several proinflammatory mediators that have been reported in lesional skin in psoriasis. (70) The induction of the epidermal IL-8 pathway is characterized by the keratinocytic overexpression of IL-8 and its receptor CXCR2 in psoriatic lesions. (71;72) The chemoattractive properties of keratinocyte-derived IL-8 may contribute to the epidermal accumulation of T cells and neutrophils in lesional skin. IL-8 is involved in the stimulation of epidermal cell growth (73) induction of major histocompatibility complex class II expression (74) and in the upregulation of the inducible nitric oxide synthase (75). In our study we found that ethanol and acetone did not alter the strong IL-8 gene expression of HaCaT cells.

The hallmark of psoriasis is the high epidermal cell turnover. By binding to CDKs, cyclins initiate the G0-G1 transition. Cyclin D1 binding with CDK4/6 starts the G1 phase.(23;24) Cyclin D1 is associated with epithelial hyperproliferation (26) and is overexpressed in psoriasis. (27;28) Our results showed that after ethanol and acetone treatment the ratio of HaCaT cells traversing from G0

into cell cycle prior to G1 is increased. This is similar in psoriasis where the ratio of cycling cells compared to the total number of germinative cells is high.

Soluble mitogens such as growth factors and their receptors are important in regulating cell growth. KGF is a paracrine mediator of epithelial cell growth. (76) KGF elicits its activity through binding to the tyrosine kinase KGFR. (33;34) Alterations in the KGF signaling pathway might be involoved in the epidermal hyperplasia associated with psoriasis. Increased expression of KGF and KGFR is characteristic for lesional psoriatic tissue. (35) Our results demonstrate that KGFR expression in HaCaT keratinocytes is upmodulated after ethanol and acetone exposure, thus suggesting an increased KGF signaling in our model system.

Insoluble extracellular molecules also play an important role in the regulating the growth of ancorage-dependent cells. (77)

Integrins are a large family of cellular surface molecules interacting with the extracellular matrix. Keratinocyte integrins are important epidermal regulatory molecules, they are involved in adhesion, migration, growth and differentation (39) Fibronectin can regulate keratinocyte growth through the fibronectin receptor ($\alpha5\beta1$). The expression of $\alpha5$ integrin in HaCaT keratinocytes is related to an undifferentiated, intensely proliferating state of these cells indicating that HaCaT cells are appropriate to study external regulators of proliferation and the underlying molecular mechanisms (50) We observed a strong expression of $\alpha5$ integrin in ethanol and acetone treated HaCaT cells. Alterations in expression, topography and function of integrin receptors have been previously reported in lesional and also in nonlesional psoriatic keratinocytes. (40;41;78) It is known that nonlesional psoriatic skin expresses higher $\alpha5$ integrin levels relative to normal keratinocytes, which may lead to the hyperresponsiveness of psoriatic keratinocytes to proliferation signals provided by lymphokines produced by intralesional T lymphocytes. (42) It is possible that the effect ethanol and acetone on $\alpha5$ integrin expression of HaCaT cells increases their proliferating capacity.

In the present study we found that, at non-cytotoxic concentrations, ethanol and acetone enhances the proliferation of HaCaT cells. Our results also indicate that the effect of ethanol and acetone is partly mediated by genes characteristic for proliferating keratinocytes such as α5 integrin, KGFR and cyclin D1. Our findings provide further data to understand how alcohol misuse may contribute to exacerbating psoriasis.

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