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Arginase 1 Overexpression in Psoriasis

Limitation of Inducible Nitric Oxide Synthase Activity as a Molecular Mechanism for Keratinocyte Hyperproliferation

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Abnormal proliferation of keratinocytes in the skin appears crucial to the pathogenesis of psoriasis, but the underlying mechanisms remain unknown. Nitric oxide (NO), released from keratinocytes at high concentrations, is considered a key inhibitor of cellular proliferation and inducer of differentiation *in vitro*. Although high-output NO synthesis is suggested by the expression of inducible NO synthase (iNOS) mRNA and protein in psoriasis lesions, the pronounced hyperproliferation of psoriatic keratinocytes may indicate that iNOS activity is too low to effectively deliver antiproliferative NO concentrations. Here we show that arginase 1 (ARG1), which substantially participates in the regulation of iNOS activity by competing for the common substrate L-arginine, is highly overexpressed in the hyperproliferative psoriatic epidermis and is coexpressed with iNOS. Expression of L-arginine transporter molecules is found to be normal. Treatment of primary cultured keratinocytes with Th1-cytokines, as present in a psoriatic environment, leads to *de novo* expression of iNOS but concomitantly a significant down-regulation of ARG1. Persistent ARG1 overexpression in psoriasis lesions, therefore, may represent a disease-associated deviation from normal expression patterns. Furthermore, the culturing of activated keratinocytes in the presence of an ARG inhibitor results in a twofold increase in nitrite accumulation providing evidence for an L-arginine substrate competition in human keratinocytes. High-output NO synthesis is indeed associated with a significant decrease in cellular proliferation as shown by down-regulation of Ki67 expression in cultured keratinocytes but also in short-term organ cultures of normal human skin. In summary, our data demonstrate for the first time a link between a human inflammatory skin disease, limited iNOS activity, and ARG1 overexpression. This link may have substantial implications for the pathophysiology of psoriasis and the development of new treatment strategies. ***(Am J Pathol 2003, 162:203–211)***

Psoriasis is an inflammatory disease of the skin characterized by localized areas of epidermal hyperproliferation.[1] Although the etiology and pathogenesis of psoriasis remain primarily unknown, it is generally assumed that unbalanced immune responses contribute to the disease process.[2,3] The exact sequence of events, as well as the molecular mediators that lead to hyperproliferative responses, are yet to be defined. As a potent regulator of keratinocyte growth and differentiation,[4,5] the multifunctional signaling molecule nitric oxide (NO) has been considered to be a strong candidate in the pathogenesis of psoriasis.[6–9] However, its role in psoriatic epidermal hyperproliferation remains to be elucidated. To determine the contribution of NO to the hyperproliferative disease state of psoriasis, we previously studied the response of primary cultures of human keratinocytes to different concentrations of exogenous NO and described a biphasic growth-regulatory effect of NO: low NO levels promote keratinocyte proliferation; high levels, however, arrest cell proliferation and initiate differentiation.[4] These results suggested that sustained production of large quantities of NO might attenuate the pathophysiological sequelae of psoriatic hyperproliferation *in vivo*. As psoriatic keratinocytes express inducible NO synthase (iNOS) mRNA and protein,[6–9] potentially capable for high-output NO synthesis, one possible hypothesis for explaining the keratinocyte hyperproliferation is that iNOS activity in psoriasis lesions is too low to effectively deliver anti-proliferative NO concentrations. On the contrary, deficiencies in local NO synthesis might contribute to a proliferative or injury

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repair program of keratinocytes that is associated with low amounts of NO.[10]

One of the factors that may limit the extent and duration of NO synthesis by reducing substrate supply for iNOS are cationic amino acid transporters, which mediate L-arginine uptake.[11] Recent studies have identified cDNAs encoding two transmembrane proteins, the constitutively expressed CAT-1 and the cytokine-inducible CAT-2, which is constitutively expressed only in hepatocytes.[11,12] Both transporters differ with respect to their capacity as well as affinity for the cationic amino acids, and thus an absence or low expression level may limit the substrate supply for iNOS enzyme activity.[11] In addition, a number of publications have identified a family of enzymes that might crucially participate in the modulation of high-output synthesis of NO by competing for the common substrate, L-arginine.[13,14] This alternative metabolic pathway is catalyzed by arginases (ARGs) that convert L-arginine to L-ornithine and urea within the urea cycle. Mammalian cells express two isoforms, ARG1 and ARG2, that are encoded by different genes and differ with respect to their cellular distribution and mode of regulation. ARG2 is a mitochondrial enzyme with widespread tissue distribution, most prominently in kidney, small intestine, and brain.[15] In contrast, ARG1 is a cytosolic enzyme, which is constitutively expressed in the liver only, but is cytokine-inducible in many cell types. Because of its subcellular localization, ARG1 can limit substrate availability for high-output NO synthesis in cells co-expressing iNOS.[15–17]

It is currently well established that modulation of intracellular L-arginine levels by enzymes of L-arginine transport or catabolism can greatly influence enzyme activity and thus the regulatory actions of iNOS in diverse physiological and pathophysiological conditions.[16–18] Investigations on their role and expression in common skin diseases, however, are lacking. To explore the hypothesis that iNOS is induced but inappropriately active during the hyperproliferative disease state of psoriasis, we have now analyzed the expression of rate-controlling enzymes of L-arginine transport or catabolism *ex vivo* in psoriatic and normal skin specimens and *in vitro* in primary cultures of human keratinocytes. We further examined the functional importance of ARG1 and iNOS for substrate availability and keratinocyte proliferation, because reduction of L-arginine supply may significantly limit high-output NO synthesis, and thereby, determine the natural history of epidermal hyperproliferation in psoriatic skin disease.

Materials and Methods

Patients and Clinical Specimens

Scalpel or punch skin biopsy specimens (6 mm in diameter) were obtained after informed consent from 10 psoriasis patients, 5 patients with basal cell carcinoma, and 5 healthy volunteers. Biopsy specimens were snap-frozen in liquid nitrogen and stored at −70°C until used for RNA preparation or immunohistochemical evaluation.

Antibodies and Reagents

The anti-ARG1 antiserum was raised in rabbits immunized with a synthetic peptide (EGN HKP ETD YLK PPK) representing the amino acids 348 to 362 of ARG1 and crossreacting with the human enzyme. The mouse monoclonal antibody to macrophage-inducible NOS was purchased from Transduction Laboratories (Lexington, KY). Other reagents were obtained from Sigma Chemical Company (Deisenhofen, Germany) unless otherwise specified.

Cell Culture Experiments

Primary epidermal keratinocytes were isolated from reduction mammoplasty specimens by enzymatic dissociation as described.[7] In brief, after overnight treatment of specimens with Dispase (Boehringer, Mannheim, Germany) at 4°C, epidermis was removed from dermis. The epidermal sheet was placed into 0.25% trypsin and incubated for 20 minutes at 37°C. A single cell suspension was then prepared in the presence of 10% fetal calf serum by gentle teasing. The suspension was filtered through a 112-μm nylon mesh and washed. After the final wash, cells were resuspended and propagated in serum-free keratinocyte growth medium (KGM; Bio Whittaker, Taufkirchen, Germany). For cell stimulation, confluent monolayers of cultured keratinocytes were incubated for 24 hours in the presence or absence of 1000 U/ml of γ-interferon, 1000 U/ml of tumor necrosis factor-α, and 1000 U/ml of interleukin-1β (Strathmann, Hannover, Germany). The capacity of these Th1-cytokines to modulate iNOS, ARG1, ARG2, CAT-1, and CAT-2 as well as Ki67 expression was evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. Where indicated, the NOS inhibitor L-*N*(5)-(1-imino-ethyl)ornithine (NIO; 0.25 mmol/L) was added, and for ARG inhibition cell culture media were supplemented with L-valine (VAL; 10 mmol/L).[19]

Skin Organ Culture Experiments

Short-term organ cultures of normal human skin were prepared from reduction mammoplasty specimens and cultured in RPMI 1640 essentially as described.[20] Organ cultures were maintained *in vitro* on plastic substrates under standard culture conditions. For NO exposure, a stock solution of (z)-1-[N-(2-aminoethyl)-N-(2-ammonium-ethyl)amino]diazen-1-ium-1,2-diolat (DETA)/NO at a concentration of 50 mmol/L was prepared by dissolving the compound in sterile phosphate-buffered saline and was diluted in tissue culture medium before use. Skin organ cultures were incubated for 24 hours in the presence or absence of 1 mmol/L of DETA/NO, and then used for mRNA preparation. The capacity of NO to modulate Ki67 expression was evaluated using RT-PCR amplification.

RNA Extraction and RT-PCR Analysis

Total RNA from snap-frozen epidermal cells or tissues was isolated using RNeasy kits according to the manu-

facturer's instructions (Qiagen, Hilden, Germany). First strand cDNA synthesis and PCR amplification were conducted as described[7] using the following primer sequences: iNOS (5′-ATGCCCGATGGCACCATCAGA-3′ and 5′-TCTCCAGGCCCATCCTCCTGC-3′); ARG1 (5′-CTTAAAGAACAAGAGAGTGTGTGATG-3′ and 5′-TTCTTCCTAGTAGATAGCTGAG-3′); ARG2 (5′-CTCCAGTTTGGGCTGCCACC-3′ and 5′-TGTCCCAGCAACACACACTG-3′); CAT-1 (5′-CCAACGTCAATGATAGGACC-3′ and 5′-CTGGTCCAGCTGCATCATGA-3′); CAT-2 (5′-AGCCTGGCTTATCTTACGAC-3′ and 5′-AATCTGACCCAAGTGTCTGC-3′); Ki67 (5′-ACTTGCCTCCTAATACGCC-3′ and 5′-TTACTACATCTGCCCATGA-3′); and glyceraldehyde-3-phosphate-dehydrogenase as housekeeping standard (G3DPH; 5′-ATGCCCGATGGCACCATCAGA-3′ and 5′-TCTCCAGGCCCATCCTCCTGC-3′). Cycle times were: 30 seconds at 94°C, 60 seconds at 60°C, and 60 seconds at 72°C for 40 cycles (iNOS), 38 cycles (ARG2), 28 cycles (CAT-1), 32 cycles (CAT-2), or 38 cycles (Ki67); 30 seconds at 94°C, 60 seconds at 50°C, and 60 seconds at 72°C for 28 cycles (ARG1) for skin specimens or 34 cycles (ARG1) for cultured cells; 30 seconds at 94°C, 30 seconds at 58°C, and 60 seconds at 72°C for 19 cycles (G3PDH). These cycle protocols ensured that RT-PCR amplifications were always within the linear range.

Immunohistochemical Analysis of iNOS and ARG Protein

The immunohistochemical procedures followed methods described previously[7] and were performed on 7-μm frozen sections of keratoma biopsies embedded in OCT medium (Ames, Elkhart, IN). For negative controls, sections were incubated with an irrelevant isotype-matched control antibody or antiserum.

Measurement of Nitrite Production

iNOS activity in cultures of human keratinocytes was determined by measuring the accumulation of nitrite (NO_{2-}), a stable end product of NO metabolism, using a modified Griess reaction as described earlier.[7] Control samples containing media and respective additives without cells were analyzed alongside the experimental samples.

Measurement of Urea Production

ARG activity in primary keratinocytes was analyzed by measuring urea production using an urea nitrogen assay according to the manufacturer's recommendations. This assay was modified to allow the measurement of urea in 20 μl of culture supernatant fluids. In brief, urea was hydrolyzed by urease, and a further reaction of ammonia with alkaline hypochlorite and phenol in the presence of sodium nitroprusside led to indophenol formation. The concentration of urea is directly proportional to the absorbance of indophenol, which was measured spectrophotometrically in a microplate reader at 540 nm.

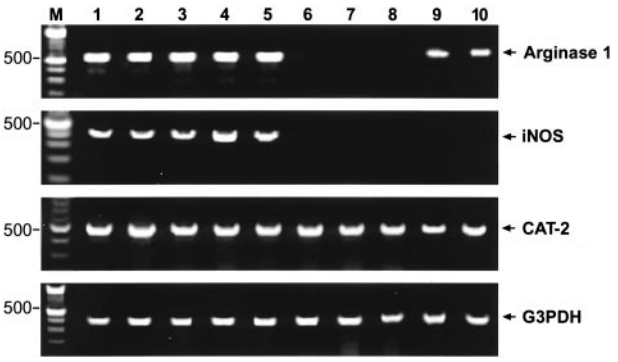

Figure 1. RT-PCR analysis of keratoma biopsies from patients with psoriasis ($n = 10$), basal cell carcinoma ($n = 3$), and from healthy volunteers ($n = 2$). RNA extracted from skin samples was reverse-transcribed and analyzed by PCR with primers specific for ARG1 (550 bp, **lane 1**), iNOS (394 bp, **lane 2**), CAT-2 (457 bp, **lane 3**), and G3PDH sequences (416 bp, **lane 4**). Case numbers are shown above the lanes. ARG1 and iNOS mRNA are present in all biopsies from psoriasis patients (**lines 1** to **5**; shown are 5 of 10 psoriasis specimens). No iNOS-specific signal was found in skin specimens from healthy volunteers (**lines 6** to **8**; shown are three of five normal skin specimens), and a weak signal for ARG1 is seen after six additional amplification cycles only (not shown). In basal cell carcinomas a similar high level of ARG1 mRNA expression was observed (**lines 9** and **10**; shown are two of five basal cell carcinomas) but no iNOS expression. Conversely, CAT-2 gene expression was found in all biopsies investigated.

Statistical Analysis

Each experimental condition was performed in triplicate in the same experiment, and each experiment was repeated at least three times. Data are expressed as mean ± SD. Comparisons were made by Student's *t*-test (two-tailed for independent samples).

Results

ARG1 Is Overexpressed in Psoriatic Skin Lesions

Skin biopsy specimens from 10 patients with psoriasis, control biopsies from 5 healthy volunteers and from 5 patients with basal cell carcinoma were analyzed for expression of iNOS, ARG1, as well as for the expression of the cationic amino acid transporters CAT-1 and CAT-2 at the mRNA level (Figure 1). We demonstrate that gene transcripts for the transporter molecules CAT-1 (not shown) and CAT-2 show no variation in their relative expression intensities between the two skin diseases or as compared to normal skin. These findings suggest that the capacity for cellular transport of L-arginine in psoriasis lesions and basal cell carcinomas appears unchanged relative to normal skin. However, a surprising outcome of our studies is the observation that a very strong expression of both iNOS and ARG1 mRNA is found in all psoriatic skin biopsies, whereas iNOS is not detected in basal cell carcinomas or, under the same experimental conditions, in normal skin. Only after six additional PCR cycles, a weakly positive ARG1 signal is found in normal skin (not shown), an experimental condition, where ARG1 mRNA amplification of psoriatic skin specimens has long ceased to be in the linear phase. In basal cell carcinomas, ARG1 is also expressed at high levels as an indi-

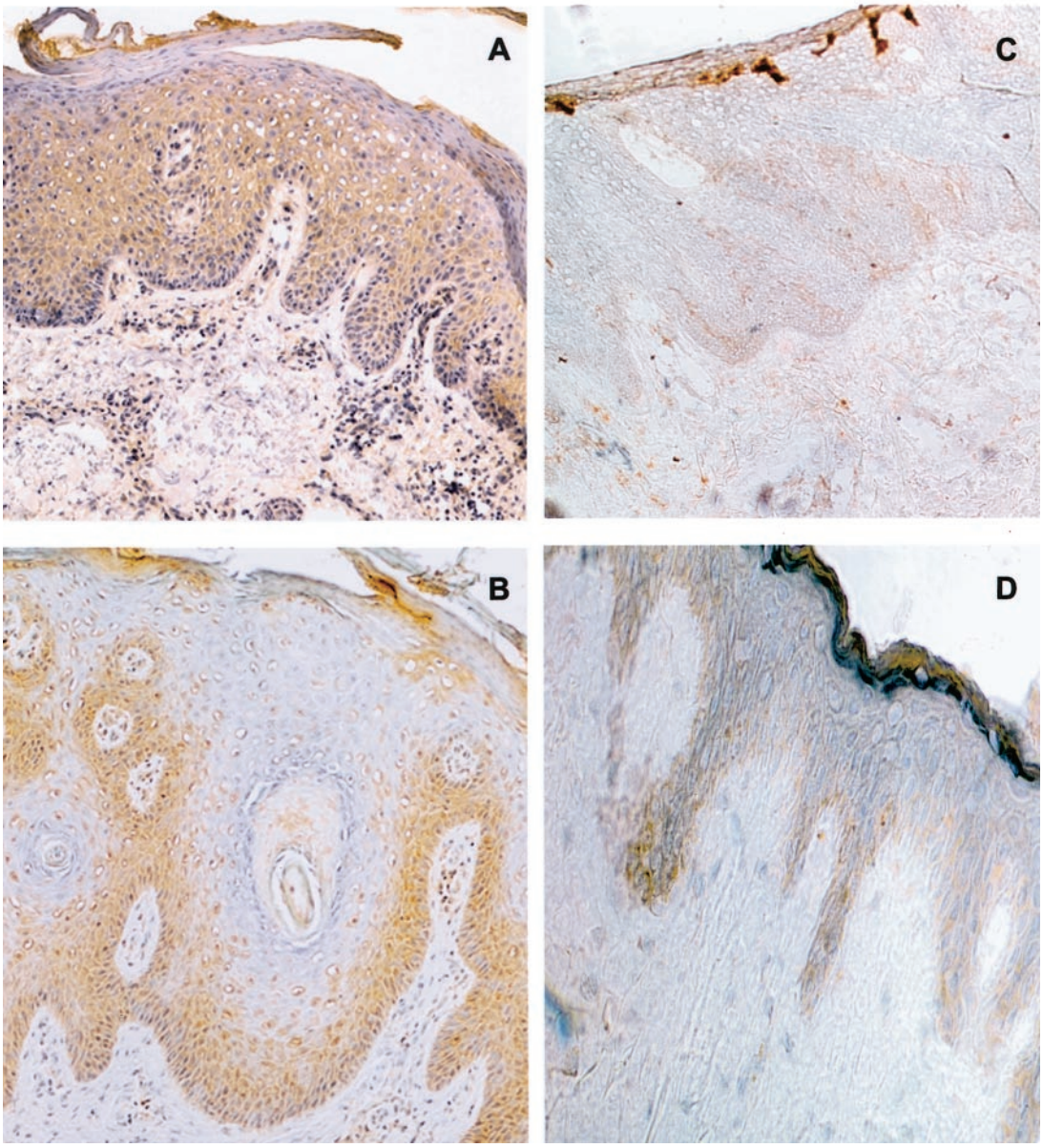

Figure 2. Co-expression of iNOS and ARG1 in skin specimens of psoriasis by immunohistochemistry. In immunolabeling of cryostat sections brown signals are obtained with blue nuclei because of hematoxylin counterstaining. Both ARG1 and iNOS protein are present in the hyperproliferative psoriatic epidermis. In **A** the psoriatic skin ARG1 immunoreactivity is found all over the epidermis, whereas in **B** iNOS is localized to the basal and suprabasal cell layers of psoriatic epidermis. Note the prominent staining of both enzymes in the basal layers of the hyperproliferative epidermis indicating that ARG1 and iNOS are expressed simultaneously. **C:** Control studies performed with isotype-matched control antibodies were uniformly negative. **D:** In normal skin specimens a weak ARG1-specific signal is found all over the epidermis.

cation that ARG1 expression at these high levels is functionally linked to the pathological state of cellular hyperproliferation.[21]

On the basis of these observations, we next studied the localization of iNOS and ARG1 in skin specimens from psoriatic lesions by the use of specific antibodies (Figure 2; A to D). In three independent immunohistochemical experiments, co-expression of ARG1 and iNOS was demonstrated in psoriatic keratinocytes of the basal and suprabasal epidermal layers. Whereas ARG1 immunoreactivity is found in all epidermal layers, that of iNOS is restricted to the basal and a few suprabasal epidermal cell layers. These results demonstrate that hyperproliferating psoriatic keratinocytes in the basal epidermal layers co-express at high levels both L-arginine-metabolizing enzymes.

Primary Keratinocytes Express iNOS, ARG1, and CAT-2 mRNA

To further examine the functional importance of ARG1 overexpression in the pathological state of psoriatic keratinocyte hyperproliferation, we performed *in vitro* experiments using primary cultures of human keratinocytes (Figure 3). Our data show that in resident keratinocytes iNOS-specific mRNA is not detectable. Activation of

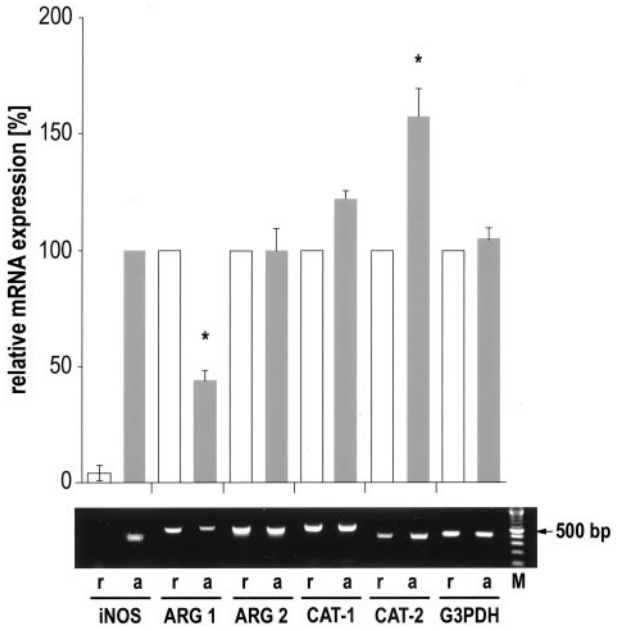

Figure 3. RT-PCR detection of iNOS, ARG1, CAT-1 and CAT-2 mRNA in resident or cytokine-challenged cultured human keratinocytes. Primary keratinocytes were incubated for 24 hours in the presence or absence of 1000 U/ml of γ-interferon, 1000 U/ml of tumor necrosis factor-α, and 1000 U/ml of interleukin-1β. Resident keratinocytes (r) constitutively express ARG1 mRNA and ARG2 mRNA as well as the cationic amino acid transporters CAT-1 and CAT-2. Cytokine activation of primary keratinocytes (a) leads to *de novo* expression of iNOS mRNA, up-regulation of CAT-2 mRNA by a factor of 1.56 ± 0.1 (*, $P < 0.02$) and down-regulation of ARG1 mRNA to a level of 45 ± 12% (*, $P < 0.001$) of resident cells. CAT-1 and ARG2 were not significantly influenced at their mRNA expression level. The figure shows data compiled from three experiments. Relative signal intensities (ie, amplification products per housekeeping gene) were set to 100%, with the exception of iNOS.

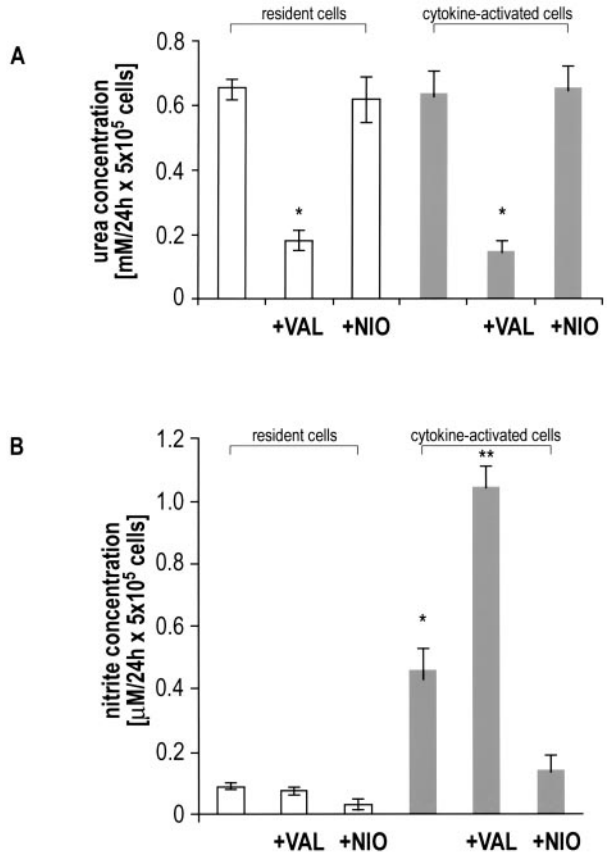

Figure 4. Inhibition of ARG activity during cytokine challenge significantly increases NO production. **A:** ARG activity as determined by urea concentrations in culture supernatants is high in resident cells. Within 24 hours of cytokine activation ARG activity remains unchanged. Supplementation with L-valine (VAL, 10 mmol/L) inhibits ARG activity in resident (**white bars**) as well as in activated cells (**gray bars**) (*, $P < 0.005$), whereas a NOS inhibitor (NIO, 0.25 mmol/L) does not influence ARG activity. **B:** Resident cells produce background levels of nitrite only (**white bars**), and after 24 hours of cytokine-challenge (**gray bars**) the increase in nitrite concentrations (*, $P < 0.001$ as compared to resident cells) demonstrates iNOS activity. Inhibition of NOS decreases nitrite production to background levels, but supplementation with L-valine increases nitrite formation by a factor of 2.1 ± 0.1 (**, $P > 0.01$) as compared to activated cells. Bars represent the mean ± SD of three experiments.

these cells with a proinflammatory cytokine mixture of interleukin-1β, tumor necrosis factor-α, and interferon-γ (1000 U/ml each) leads to expression of iNOS mRNA within 24 hours of culture in accordance with numerous earlier reports. In contrast, ARG1 mRNA is found to be constitutively expressed in resting cultured keratinocytes and cytokine treatment resulted in a significant decrease of mRNA to 45 ± 12% relative to resident cells. In addition, ARG2 mRNA and mRNA of both amino acid transporter molecules CAT-1 and CAT-2 were also found to be constitutively expressed in human keratinocytes. However, although neither ARG2 nor CAT-1 expression exhibited any changes after cytokine challenge, the expression of CAT-2 significantly increased by a factor of 1.56 ± 0.1 in the presence of the proinflammatory cytokines. These results demonstrate the inverse effect of proinflammatory Th1 cytokines on the expressional levels of iNOS and ARG1 mRNA in normal human keratinocytes. Furthermore, this is the first description of a constitutive expression of the inducible-type amino acid transporter CAT-2 in human keratinocytes and its up-regulation by proinflammatory cytokines.

Inhibition of ARG Leads to Increased NO Production in Keratinocytes

To further strengthen the notion that ARG1 and iNOS co-expression is involved in the pathogenesis of psoriatic hyperproliferation, we also examined the possible inhibitory effect of ARG activity on iNOS-derived NO synthesis (Figure 4, A and B). We find that ARG activity, as measured by urea accumulation in culture supernatants of resident keratinocytes, is in accord with the constitutive expression of the enzyme. Within the first 24 hours of cytokine challenge, urea production is unaltered despite the significant decrease in ARG1 mRNA expression. This is apparently because of the known long half-life of this protein, thus a decreased urea production can be expected after several days of activation only. However, NO production of primary keratinocytes, as measured indirectly via nitrite accumulation in culture supernatants, can be significantly augmented by L-valine-mediated inhibition of ARG activity. In contrast, urea synthesis is not affected by NOS inhibition. Thus, despite the presence of abundant amounts of L-arginine (culture media contain ~10 times higher L-arginine concentrations than normal blood) epidermal ARG1 activity can significantly inhibit

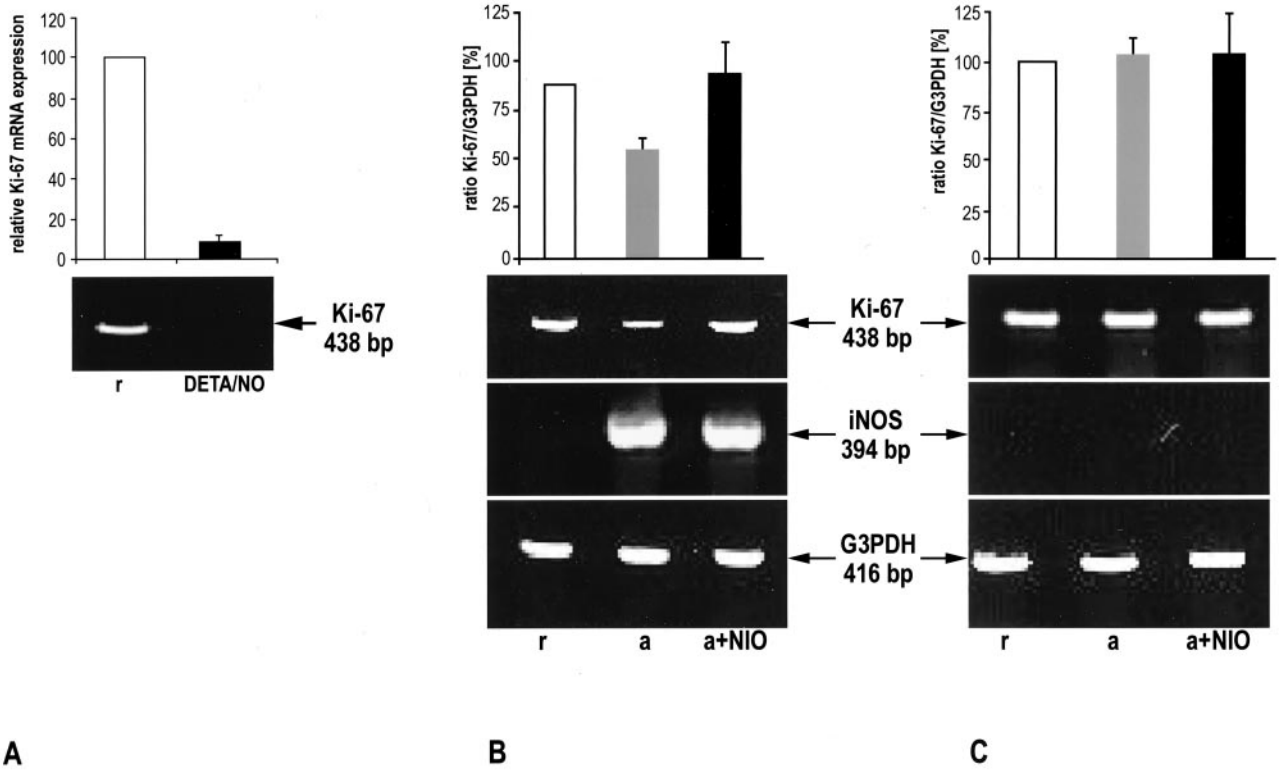

Figure 5. Endogenous and exogenous NO down-regulates proliferation in organ cultures of human skin and cultured keratinocytes. **A:** Maintenance of normal skin specimens for 24 hours in the presence of DETA/NO (1 mmol/L) leads to a strong reduction of Ki67 mRNA expression (**black bar**) to 8 ± 2.5% of the control (*, $P < 0.001$). The figure shows data compiled from three experiments. Relative amplification products are normalized to Ki67 expression of control cultures (r). **B:** Primary keratinocyte cultures (r) were activated with proinflammatory cytokines as in Figure 3 for 24 hours in the presence (a + NIO) or absence (a) of the NOS inhibitor NIO (0.25 mmol/L) for induction of iNOS mRNA. In the absence of NIO Ki67 mRNA expression is reduced to 61 ± 9% (**gray bar**; *, $P < 0.02$). Activation of cells in the presence of NIO blocks this decrease (**black bar**). **C:** A control experiment in medium containing hydrocortisone, which inhibits iNOS mRNA expression, no reduction of Ki67 mRNA expression is found. The figure shows data compiled from three experiments. Relative amplification products are normalized to Ki67 expression of resident control cultures.

iNOS activity. Our data, therefore, unequivocally demonstrate that ARG1 activity will restrict the rate of iNOS-derived NO production in activated epidermal keratinocytes. In psoriatic keratinocytes, low NO production because of ARG1 overexpression could thus have important implications for disease pathogenesis.

The Impact of iNOS and ARG Activities on Human Keratinocyte Proliferation

To determine the potential of NO in modulating epidermal keratinocyte proliferation, we previously studied the response of primary cultures of human keratinocytes to different concentrations of exogenous NO and described a biphasic growth-regulatory effect of NO: low NO levels promote keratinocyte proliferation; high levels, however, arrest cell proliferation and initiate differentiation.[4] To extend these results to the *in vivo* situation, we also examined expression of the proliferation marker Ki67 in short-term organ cultures of normal human skin (Figure 5A). Expression of Ki67 mRNA is easily detected in untreated skin, whereas a strong reduction of Ki67 mRNA was observed after culture in the presence of the NO donor DETA/NO (1 mmol/L) for 24 hours, a decrease not because of cell death. Thus, the previously demonstrated growth-inhibiting activity of NO at higher concentrations also accounts for skin cells within their normal tissue surroundings.

In further investigations, we determined whether and how ARG1 and iNOS enzyme activities will alter cellular proliferation in primary human keratinocyte cultures (Figure 5, B and C). To this end, we analyzed the expression of the proliferation marker Ki67 by RT-PCR amplification in resting and Th1 cytokine-activated cells. As expected, untreated keratinocytes in culture express Ki67 mRNA. Within 24 hours of activation with proinflammatory Th1-cytokines, we observed concomitant with iNOS up-regulation and ARG1 down-regulation a significant reduction in Ki-67 mRNA expression to 61 ± 9% relative to sham-treated cultures. Inhibition of iNOS by the NOS inhibitor NIO completely reverses this effect. As a control, cytokine challenge was performed in medium containing hydrocortisone, which does not allow for iNOS induction despite the presence of cytokines. If then activated, cells do not express iNOS and Ki67 mRNA levels remain unaltered despite the presence of cytokines. This demonstrates that indeed endogenous iNOS-derived NO will induce growth arrest in human keratinocytes in complete accordance to our previous findings with exogenously applied NO. Moreover, our data underscore the potent

growth-regulatory action of NO in human keratinocytes. We also maintained resident keratinocytes under continuous inhibition of ARG activity and monitored their growth rate. We find a small decrease in cellular proliferation (78 ± 8.1% relative to controls) but no evidence for differentiation induction (not shown). From these observations, it becomes evident that NO is essential for reprogramming human keratinocytes toward a proliferation stop.

Discussion

NO synthesized by iNOS plays an important regulatory role in a variety of inflammatory, autoimmune, and hyperproliferative conditions.[3,22,23] The exact contribution of iNOS-derived NO to the pathogenesis of hyperproliferative skin diseases such as psoriasis, however, is still ill-defined. Our previous studies indicate that despite expression of iNOS mRNA and protein in hyperproliferative psoriatic keratinocytes, enzyme activity might be too low to deliver an effective growth-inhibiting signal to keratinocytes in diseased skin.[3,10] The direct measurement of NO synthesis in psoriasis plaques might help to unravel this question. Indeed, slightly increased NO levels have been measured by chemiluminescence above psoriatic plaques as compared to uninvolved skin.[24] However, two problems make such a result currently difficult to interpret: first, as all three NO synthase isotypes are expressed in the skin, the relative contribution of iNOS is impossible to determine; and, second, there is currently no information on NO levels generated by a fully active iNOS *in vivo*. Moreover, a significant contribution could potentially come from the numerous infiltrating immunocytes in the dermis, if NO really permeates so far. We therefore undertook studies to analyze the expression of rate-controlling enzymes of L-arginine transport or catabolism in the context of iNOS expression *ex vivo* in psoriatic lesions and *in vitro* in primary cultures of human keratinocytes.

The data now presented demonstrate for the first time that exceedingly high levels of ARG1 mRNA and protein are co-expressed with iNOS in skin lesions from psoriasis patients. ARG1 immunoreactivity is found in all epidermal layers, whereas that of iNOS is restricted to the basal and a few suprabasal epidermal cell layers. Thus, hyperproliferative psoriatic keratinocytes will metabolize L-arginine by the two alternative pathways, via iNOS and ARG1. In contrast to the co-expression of iNOS and ARG1 in psoriatic skin lesions, expression of high levels of ARG1 only was observed in basal cell carcinomas, which lack concomitant iNOS expression. It thus seems that co-expression of iNOS and ARG1 is functionally linked to the pathological state of epidermal hyperproliferation in psoriasis. Our *ex vivo* findings, therefore, propose a new role for ARG1 in psoriatic lesions, because this L-arginine-catabolizing enzyme may limit high-output NO synthesis in hyperproliferative psoriatic keratinocytes because of the known substrate competition.

It is currently well established that enzymes of L-arginine transport or catabolism are involved in the regulation of iNOS activity, especially in macrophages but also other mammalian cells.[18,25,26] Such a regulation of iNOS activity in cells resident to the skin, however, has not been investigated so far nor is there currently a link to a specific human disease. To further strengthen the notion that a co-expression of iNOS and ARG1 contributes to the pathogenesis of psoriasis, we performed *in vitro* experiments using primary cultures of human keratinocytes. We find that resident human keratinocytes constitutively express ARG1, ARG2, CAT-1, and CAT-2B mRNA. In light of these experiments, it is noteworthy that an identical expression pattern has been found in human hepatocytes.[13] Our new observation indicates that a constitutive expression of the inducible or liver type ARG (ie, ARG1) and CAT (ie, CAT-2) might also be characteristic for other epithelial cells. As published previously by us and others,[6,7] *in vitro* exposure of normal human keratinocytes to proinflammatory Th1 cytokines leads to the induction of iNOS. Here we demonstrate a concomitant up-regulation of CAT-2 mRNA and a significant decrease in ARG-1 mRNA expression, which is in accord with previous observations indicating that ARG1 is expressed in the context of a Th2-type cytokine expression. Thus, ARG-1 overexpression in psoriasis skin plaques, where abundant production of proinflammatory Th1 cytokines is well-characterized,[2,27–29] appears to represent an abnormal and disease-associated expression pattern. Interestingly, our attempts to up-regulate ARG1 expression in epidermal keratinocyte cultures with various agents reported to mediate such an increase all failed. Therefore, the signal(s) leading to ARG1 overexpression in psoriasis appear(s) to involve factors not yet identified, but must be capable of overriding the abundant proinflammatory signals normally down-regulating ARG1 expression. In addition, our data suggest that a constitutive expression of CAT-1 and CAT-2 in skin cells play a physiological role in the maintenance of L-arginine supply for keratinocytes. In addition, the observation that CAT-2 is modified by Th1 cytokines indicates that this transporter molecule is involved in increased L-arginine supply during cutaneous inflammatory processes.

To further elucidate the functional importance of ARG1 overexpression for psoriatic keratinocyte hyperproliferation, we examined the possible inhibitory effect of ARG activity on iNOS-derived NO synthesis in cultures of human keratinocytes. Our experimental data demonstrate that ARG1 activity can indeed restrict intracellular substrate availability for iNOS and thereby significantly hamper the rate of NO production in epidermal keratinocytes. In conclusion, these findings together with our previous analyses of psoriatic disease pathogenesis indicate that ARG1 overexpression could be a molecular mechanism for keratinocyte hyperproliferation in psoriasis by limitation of iNOS activity. Moreover, our observations raise the interesting possibility that inappropriately low iNOS activity because of ARG1 co-expression would permit uncontrolled and thus chronic inflammatory response, because high-output NO synthesis has an important homeostatic role in limiting immune-mediated inflammatory processes. This contention is supported by previous findings demonstrating that inhibition of NO synthesis exacer-

bates chronic inflammatory and immune-mediated processes.[22,23,30]

Although the precise mechanism of keratinocyte hyperproliferation in psoriasis is not clear, it is generally believed that unbalanced immune responses play an important role. Indeed, an altered activation status of nuclear factor-κB and differences in apoptosis resistance have been observed in psoriatic keratinocytes, both factors also being essential in immunoregulation.[31,32] In this context, an antagonistic interaction between iNOS and ARG1 in psoriatic keratinocytes is mechanistically intriguing, because the balance between both enzymes is normally reciprocally regulated by Th1 and Th2 cells in diverse settings of immunopathology, including cutaneous wound healing processes.[33] Thus, Th1 cytokines induce iNOS and down-regulate ARG expression, whereas Th2 cytokines induce ARG and suppress iNOS.[34,35] In psoriasis, the co-expression of iNOS and ARG1 thus appears to represent a pathophysiological state and an aberrant regulation of Th1- and Th2-type responses.

In view of the assumed deleterious role of NO in a number of pathological conditions, both inside and outside the skin, a great deal of effort is being made to develop therapeutic strategies aimed at suppressing the action or production of NO. Our data suggest that attempts to inhibit NO in chronic inflammatory diseases, including psoriasis, should be re-evaluated, particularly in view of a number of studies indicating that high NO levels are of functional importance for the resolution of chronic inflammatory processes.[36] On this basis, a better understanding of the mechanisms that restrict NO production in pathophysiological situations may pave the way for the design of therapeutic approaches to treat and prevent psoriatic skin disease.

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