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ORIGINAL ARTICLES

Effector Function of CLA+ T Lymphocytes on Autologous Keratinocytes in Psoriasis

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Abstract. *Background*. Cutaneous lymphocyte antigen (CLA) is expressed by a subgroup of memory T cells that exhibit skin homing and are implicated in cutaneous T-cell-mediated diseases.
Material and methods. Expression of genes associated with psoriasis was analyzed in keratinocytes taken from patients and healthy individuals and cultured under different conditions, including activation using supernatants from CLA$^+$ T lymphocytes activated with anti-CD3 and anti-CD28 antibodies.
Results. Keratinocytes from psoriasis patients activated by CLA$^+$ T lymphocytes expressed higher levels of interferon-inducible protein 10, HLA-DR, intercellular cell adhesion molecule 1, and inducible nitric oxide synthase.
Conclusions. Our results suggest that we have developed an in vitro model that will allow analysis of the effector role of CLA$^+$ T lymphocytes on keratinocytes in psoriasis. This model may allow the identification of genes involved in the pathology of psoriasis through induction by CLA$^+$ T lymphocytes.

Key words: psoriasis, cutaneous lymphocyte antigen, keratinocyte, inducible nitric oxide synthase.

FUNCIÓN EFECTORA DE LINFOCITOS T CLA1 SOBRE QUERATINOCITOS AUTÓLOGOS EN PSORIASIS

Resumen. *Introducción*. Los linfocitos T CLA+ representan un subgrupo de linfocitos T de memoria con tropismo cutáneo que están implicados en diferentes enfermedades cutáneas mediadas por células T.
Material y métodos. Se estudió la expresión de algunos genes asociados a la psoriasis en queratinocitos de enfermos y sanos cultivados en diferentes condiciones, entre ellas la activación por sobrenadantes de linfocitos T CLA+ activados mediante anti-CD3/anti-CD28.
Resultados. Los queratinocitos psoriásicos activados por linfocitos CLA+ expresan más IP-10, HLA-DR, ICAM-1 e iNOS.
Discusión. Estos resultados sugieren que hemos establecido un modelo *in vitro* que permite estudiar la función efectora de los linfocitos T CLA+ sobre los queratinocitos en la psoriasis. Este modelo puede permitir la identificación de genes relevantes en la patogenia de la psoriasis al ser inducidos por linfocitos T CLA+ .

Palabras clave: psoriasis, antígeno linfocitario cutáneo, CLA, queratinocito, iNOS.

Introduction

Cutaneous lymphocyte antigen (CLA) is a membrane glycoprotein that is induced in lymph nodes during lymphocyte activation and that confers skin homing on the cells in which it is expressed, facilitating skin infiltration.[1,2] To date, circulating CLA$^+$ T lymphocytes have been implicated in the pathogenesis of chronic T-cell mediated inflammatory skin conditions such as atopic dermatitis, contact eczema, vitiligo, and allergic contact dermatitis.[1,3] In psoriasis, CLA$^+$ T lymphocytes that exhibit skin homing play different pathogenic roles in the acute and chronic

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phases of the disease.[4,5] In the acute phase, they are implicated in triggering the psoriatic lesions by interacting with dendritic cells and keratinocytes.[6] Recently, we have shown a correlation between greater degree of activation of circulating CLA^+ T lymphocytes and increasing psoriasis area severity index (PASI) score and body surface (BSA) area involvement in the acute phases of psoriasis.[7] In the case of chronic lesions, infiltrating T cells have been shown to remain activated and produce factors that can maintain keratinocyte growth and differentiation through mediators derived from dendritic cells.[8]

Given that CLA^+ T lymphocytes account for the majority of T cells present in psoriatic plaques,[9] the study of the interaction of these cells with resident cells such as keratinocytes may help elucidate the pathologic mechanisms of psoriasis. Although the profile of gene expression has been characterized in recent years,[10] the influence of T cells on gene expression in relevant cells such as keratinocytes has yet to be investigated. In this study, we developed an in vitro model that could determine which genes are induced by activated CLA^+ T lymphocytes in psoriatic keratinocytes from the same individual.

Material and Methods

Peripheral blood and skin biopsy samples from 3 adult patients with psoriasis vulgaris were studied (patients with erythroderma, pustular psoriasis, or associated arthritis were explicitly excluded.) A blood sample was taken from all patients for isolation of CLA^+ and CLA^- T-lymphocyte populations. A fresh skin biopsy was taken for cell culture and gene expression studies.

The patients included in the study had all undergone a prior confirmatory biopsy. Disease severity and extension were assessed using the PASI and BSA scores, respectively. Clinical characteristics were reviewed, including possible triggers (streptococcal infection, stress, etc). Samples were obtained after a washout period of at least 6 weeks without any type of treatment. Two healthy control subjects were also included in the study. All patients and controls signed an informed consent.

Purification of Circulating CLA+ T Lymphocytes From Peripheral Blood and Activation

CLA^+ T lymphocytes were purified from cell fractions obtained from 60 mL of peripheral blood by means of Ficoll separation. Three consecutive immunomagnetic separations were then performed using antibodies conjugated to magnetic particles according to a previously reported protocol.[11] The first 2 separations eliminated CD14, CD19, CD16, and CD45RA lymphocytes, to give a cellular suspension of CD45R0 memory T cells. The third separation was able to separate CLA^+ and CLA^- memory cells from the memory T cell population. Purified lymphocytes were incubated in Royal Park Memorial Institute culture medium with 10% fetal calf serum and activated with anti-CD3 and anti-CD28 at a concentration of 500 000 cells/mL.

After 48 hours, supernatants of these cultures were obtained and frozen at −80ºC until subsequent use for activating keratinocyte cultures.

Keratinocyte Culture

Keratinocytes from cutaneous biopsies were cultured according to previously published protocols. The cultured keratinocytes (P2-P4) were incubated for 6 hours with or without stimulus (interferon [IFN]-γ 100 U/mL or anti-CD3/anti-CD28-activated T-lymphocyte supernatant).

The RNA of cultured keratinocytes was extracted using the Gene Elute Mammalian kit (Sigma) and cDNA prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) for subsequent analysis by real-time polymerase chain reaction (RT-PCR) with AB17900HT (Applied Biosystems) and data processing using the SDS version 1.0 analysis program (Applied Biosystems).

Analysis of Keratinocyte Gene Expression by RT-PCR

Eighteen genes in keratinocytes were evaluated (Table 1). The results are presented in terms of relative quality (RQ), which is normalized with respect to an internal housekeeping gene, in this case, the gene coding for glyceraldehyde-3-phosphate dehydrogenase.

Microarray Analysis

Gene expression of psoriatic keratinocytes incubated with CLA^+ T lymphocyte supernatant activated with CD3/CD28 and psoriatic keratinocytes incubated with nonactivated CLA^+ T lymphocyte supernatant was studied using the PIQOR Skin Patho Microarray (Memorec, Cologne, German). The aim was to investigate differences in expression of 1127 genes expressed in skin. This was achieved by obtaining RNA from psoriatic keratinocytes incubated for 6 hours with activated and nonactivated CLA^+ T lymphocytes. The RNA from keratinocytes activated with CLA^+ T lymphocyte supernatant was labeled with cyanine (Cy) 5 and that of those incubated with supernatants of nonactivated CLA^+ T lymphocytes with Cy3.

Results

Activation of Psoriatic and Healthy Keratinocytes by Subpopulations of CLA+ or CLA- Memory T Cells

Analysis of gene expression was done by RT-PCR. Incubation of keratinocytes with supernatants of subpopulations of CLA^+/CLA^- memory cells induced different patterns of gene expression as indicated in Table 2. Of the 18 genes studied (Table 1), those with greatest upregulation in the presence of CLA^+ T lymphocytes compared to CLA^- T lymphocytes were inducible protein (IP)-10, human leukocyte antigen (HLA)-DR, intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-19 (Table 2). The remaining genes studied were not affected during the first 6 hours of culture with T-cell supernatant. Expression of IP-10, HLA-DR, and ICAM-1 was greater in psoriatic patients compared to healthy subjects. IFN-γ was used in these experiments as a control for activation. Activation with IFN-γ indicates that psoriatic keratinocytes are more sensitive to this cytokine compared to keratinocytes from healthy subjects and reaffirms the functionality of keratinocytes used in these studies.

Microarrays

The most pertinent results from the microarray analysis are presented in Figure 1 and Table 3. Figure 1 shows, on a double-logarithmic scale, the signal intensity for the cDNA of each of the genes analyzed. The diagonals identify genes that were upregulated or downregulated. Table 3 shows some of the keratinocyte genes that were upregulated on incubating with activated CLA^+ T lymphocyte supernatant compared to those incubated with nonactivated CLA^+ T lymphocyte supernatant, along with comments on the functional relevance of those genes in the psoriatic process.

Confirmation by Real-Time Polymerase Chain Reaction of the Microarray Results Using Nitric Oxide Synthase

The nitric oxide synthase (iNOS) gene was one of the upregulated genes in the microarray and one that might be preferentially induced by psoriatic CLA^+ T lymphocytes in keratinocytes of patients. To determine the relevance of this gene, confirmation was sought by RT-PCR. Figure 2 shows that supernatants of activated CLA^+ T lymphocytes from psoriatic patients induce greatest gene expression in keratinocytes from psoriatic plaques.

Table 1. Genes Expressed in Keratinocytes and Analyzed Initially by Real-Time Polymerase Chain Reaction

Gene	*Relevance*
IL-7	Cytokine associated with chronic inflammation and T lymphocyte survival
IL-8	Neutrophil-specific chemokine
IP-10	Chemokine specific to activated-T-lymphocytes
IL-15	Cytokine associated with chronic inflammation
VEGF-A	Proangiogenic factor
ICAM-1	Adhesion molecule that triggers keratinocyte activation
TNF-α	A key proinflammatory cytokine in psoriasis
HLA-DR2	Molecule associated with T cell antigen presentation
Psoriasin	Natural antimicrobial peptide
TLR1	Receptor associated with innate immune response
TLR2	Receptor associated with innate immune response
TLR5	Receptor associated with innate immune response
IL-19	Poorly characterized cytokine expressed in psoriasis
IL-20	Poorly characterized cytokine expressed in psoriasis
CCL-27	CLA^+-T-lymphocyte-specific chemokine
Ki67	Marker of cell proliferation
EGF	Epidermal growth factor
ERB-B2	Cell growth signal

Abbreviations: CCL, chemokine (C-C motif) ligand; HLA, human leukocyte antigen; ICAM, intercellular adhesion molecule; IL, interleukin; IP, inducible protein; TLR, toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Discussion

The first step in the formation of psoriatic plaques is dermal infiltration by lymphocytes, followed by epidermal hyperplasia.[12,13] In addition, before epidermal hyperproliferation occurs, significant CLA^+ $CD8^+$ T lymphocyte and $CD45RO^+$ T lymphocyte infiltration has been observed in healthy skin away from the edge of the plaque.[14,15] These results support the hypothesis that CLA^+ T lymphocytes are sequestered during acute phases of psoriasis.[16,17]

Up to now, the influence of T lymphocytes on keratinocytes has been studied by a system that generates T-cell lines or clones from lymphocytes present in biopsies.[18,19] These systems require production of sufficient

Table 2. Gene Expression in Keratinocytes Induced by IFN-γ (Control for Activation) or by CLA+/CLA- T Lymphocyte Supernatants Activated With Anti-CD3/Anti-CD28 Antibodies.[a]

Keratinocyte Genes	*Psoriasis (n=3)*			*Controls (n=2)*		
	IFN-γ	*CLA+*	*CLA–*	*IFN-γ*	*CLA+*	*CLA–*
IP-10	1646 (1281)	1253 (883)	447 (314)	210 (74.9)	722 (1011)	79.7 (83.4)
HLA-DR	244 (166)	53.2 (23.6)	28.6 (17.1)	144 (173)	31.1 (21.8)	7.7 (4.1)
ICAM-1	46.2 (12.7)	63.0 (20.7)	18.7 (1.40)	12.5 (4.70)	14.2 (7.50)	4.5 (3.3)
IL-19	0.4 (0.06)	4.9 (1.9)	0.1 (0.08)	0.5 (0.2)	6.0 (1.9)	0.9 (0.5)

[a]Expression of IP-10, HLA-DR, and ICAM-1 was greater in patients with psoriasis than in healthy controls.
Abbreviations: CLA, cutaneous lymphocyte antigen; IFN, interferon; ICAM, intercellular adhesion molecule; IL, interleukin; IP, inducible protein.

Table 3. Some Genes Found in Microarrays Relevant in Terms of Difference in Expression Between Keratinocytes Incubated With CLA+ T Lymphocyte Supernatant Activated With CD3/CD28 and Psoriatic Keratinocytes Incubated With Nonactivated CLA+ T Lymphocyte Supernatants

Gene	*Increase in Expression*[a]	*Function and Relevance in Psoriasis*
CCL20 (MIP3α) Hs.75498	1.90	MIP3α is a CCR6-specific chemokine. CLA+ T lymphocytes are CCR6+ and are present in psoriatic lesions
CCL28 Hs.334633	2.12	CCL28 is a ligand of CCR10. CLA+ T lymphocytes are CCR10+
iNOS Hs.193788	1.86	iNOS is present in psoriatic lesions and is produced by keratinocytes and dendritic cells
ALCAM (CD166) Hs.10247	1.70	This is a CD6 ligand expressed in T cells. ALCAM is implicated in the interaction between keratinocytes and T cells
Amphiregulin Hs. 270833	2.36	Amphiregulin is a member of the endothelial growth factor family. It is an autocrine factor for keratinocytes. In psoriasis, amphiregulin causes a decrease in the activity of adherens junctions. Transgenic mice expressing amphiregulin have a psoriatic phenotype[26]
Tartrate-resistant phosphatase ACP5 Hs.1211	2.63	Phosphatase resistant to tartrate. This gene may be implicated in antibacterial activities.

[a]Upregulation in keratinocytes induced by activated CLA+ T lymphocyte supernatant compared to keratinocytes incubated with nonactivated CLA+ T lymphocyte supernatants.
Abbreviations: ACP, acid phosphatase; CLA, cutaneous lymphocyte antigen; CCL, chemokine (C-C motif) ligand; CCR chemokine (C-C motif) receptor; iNOS, inducible nitric oxide synthase; MIP, macrophage inflammatory protein.

lymphocytes to be able to perform these studies, as well as artificial activations and the use of antigen-presenting cells, which clearly influence the phenotype of the lymphocytes thereby modifying their characteristics. In our case, study of CLA+ T lymphocytes purified from peripheral blood resolves this problem and allows ex vivo activations to be performed.[20] We have used this model to characterize the effector functions of CLA+ T lymphocytes on keratinocytes (from psoriasis patients and healthy controls) through analysis of gene expression induced by cultures treated with CLA+ T lymphocytes activated by anti-CD3/anti-CD28 antibodies.

The results obtained suggest that CLA+ T lymphocytes induce a profile of gene expression in keratinocytes that is different to that of CLA- T lymphocytes. We have observed that the IP-10, HLA-DR, ICAM-1, and IL-19 genes are expressed significantly in psoriatic lesions.[10] The results of the microarray allowed us to explore the possibilities of our model of gene expression with a greater number of genes in psoriatic keratinocytes induced by CLA+ T lymphocytes. Genes such as CCL20 and CCL28 encode chemokines that attract CLA+ T lymphocytes.[21,22] Amphiregulin is clearly associated with the pathogenesis of psoriasis[23] and the ALCAM gene is implicated in the interaction between

T lymphocytes and keratinocytes.[24] In order to demonstrate that these genes are relevant, it is necessary to confirm their expression by RT-PCR. We did this for iNOS, and found that on comparing the induction capacity of iNOS in keratinocytes, the largest quantities of mRNA for iNOS were generated in the experimental condition with CLA+ T lymphocytes plus autologous psoriatic keratinocytes.

Expression of iNOS and IL-19 in keratinocytes—2 genes previously found to be involved in the pathogenesis of psoriasis—had not been associated previously with effector T cell activity. These genes are associated with the innate immune response and, in our study, were induced by CLA+ T lymphocytes, which form part of the acquired immune response. The effect of IFN-γ on the expression of iNOS and IL-19 is very much lower than the effect of the supernatants of CLA+ T lymphocytes from patients with psoriasis, suggesting that, in addition to IFN-γ, other mediators produced by CLA+ T lymphocytes are able to induce their expression.

iNOS is a nitric oxide (NO) synthase induced after a stimulus. Two different isoforms, both constitutive, can produce NO. These are brain NOS (bNOS) and endothelial NOS (eNOS).[25] In psoriasis, a significant increase in the expression of iNOS has been detected in lesioned skin compared to healthy skin, with a lower expression of bNOS and eNOS (both in healthy and lesioned skin).[26] In situ hybridization and immunohistochemical studies identified iNOS mRNA and protein in the epidermal keratinocytes of psoriatic lesions,[27,28] as well as in the papillary dermis.[29]

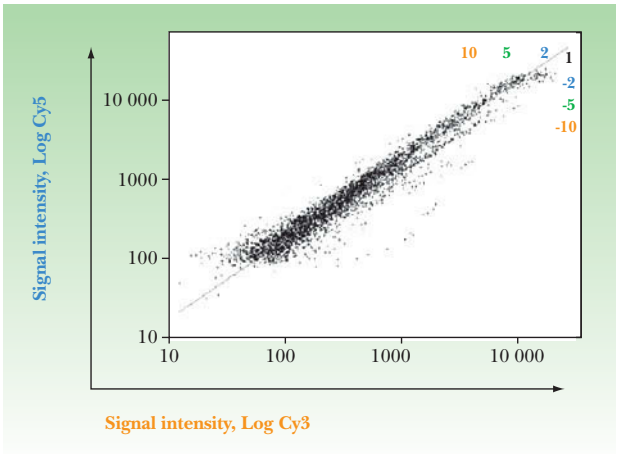

Figure 1. Result of microarray analysis. Cyanine 3 (Cy3) corresponds to RNA of keratinocytes activated with nonactivated cutaneous lymphocyte antigen (CLA)+ T lymphocytes; cyanine 5 (Cy5) to RNA of keratinocytes activated with activated CLA+ T lymphocytes.

Expression of iNOS, including in keratinocytes, seems to be induced by different cytokines, such as IL-8.[26,30]

iNOS synthesizes NO, a free radical that has been implicated in the pathogenesis of different inflammatory diseases, including psoriasis. It has been reported that low levels of NO induce keratinocyte proliferation, whereas higher levels can block this proliferation and trigger cell differentiation, even in keratinocytes.[31,32] In psoriasis, keratinocyte hyperproliferation occurs despite the marked

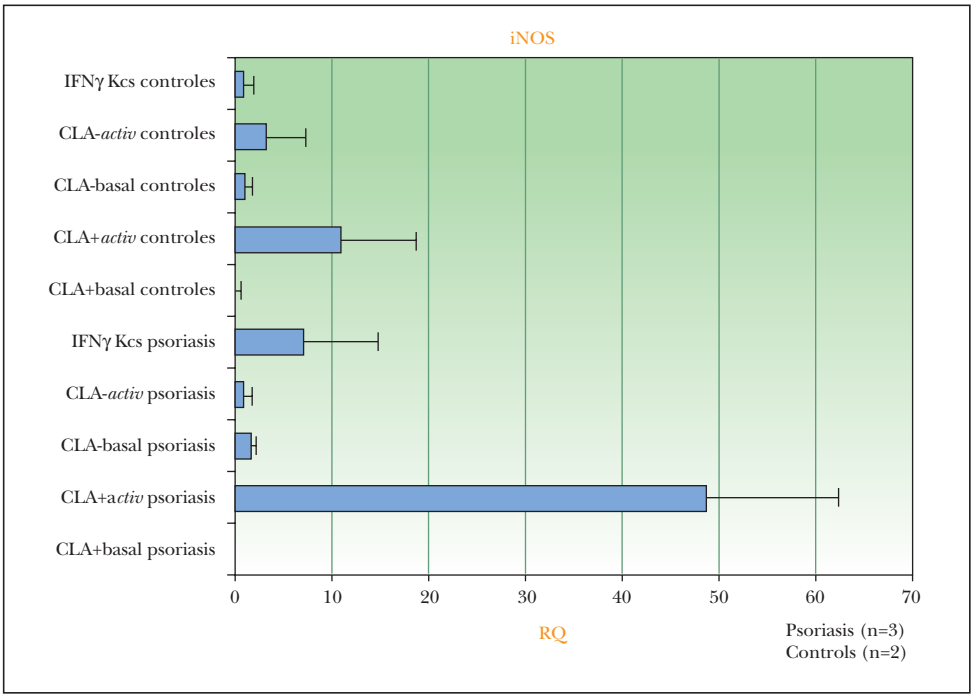

Figure 2. Confirmation by real-time polymerase chain reaction of the result of the microarray in iNOS for different types of cultured healthy/control and psoriatic keratinocytes (Kcs): incubated with interferon-γ, with activated cutaneous lymphocyte antigen (CLA)+/- T lymphocyte supernatants (*activ*) or basal. The experimental condition of psoriatic keratinocytes incubated with activated autologous CLA+ T lymphocyte supernatants generated the largest quantity of iNOS mRNA.

overexpression of iNOS. This would seem to indicate that the activity of the overexpressed iNOS is low, and therefore that the antiproliferative action is not very effective. Bruch-Gerharz et al[27] suggested that arginase 1 (ARG-1) could rationalize this apparently contradictory finding. In psoriatic keratinocytes, ARG-1 is also overexpressed, and colocalized with iNOS. ARG-1 participates in regulating iNOS activity, competing for a common substrate, arginine, and producing L-ornithine as a precursor in the synthesis of polyamines. L-ornithine has been reported to induce keratinocyte proliferation.[31] In addition, competition for substrate would give rise to a low production of NO in keratinocytes. Bruch-Gerharz et al[27] reported an increased NO production through inhibition of arginase activity in cultured keratinocytes, an observation that would confirm the low activity of iNOS in psoriatic keratinocytes. Another possible explanation for the low concentration of NO in keratinocytes would be the overexpression of calcitonin gene related peptide (CGRP) in psoriatic lesions; CGRP appears to suppress NO production, probably through inhibition of iNOS activity.[32] In contrast, some authors have reported increased NO concentrations in psoriatic plaques, and even in the serum of patients with psoriasis.[33] However, those experiments analyzed total NO production in psoriatic skin or NO levels in serum, without specifying the type of cell where NO was produced. In addition to keratinocytes, there are other NO-producing cells that use iNOS (dendritic cells) or other isoenzymes (melanocytes, endothelium, and sweat gland epithelium).[29]

IL-19 forms part of the IL-10 cytokine family and binds to the heterodimer receptor IL-20R1/IL-20R2. In vitro, IL-19 acts as a proinflammatory cytokine or modulator of innate inflammatory response.[34] It is able to induce IL-6 and tumor necrosis factor (TNF)-α in monocytes, giving rise to TNF-α mediated apoptosis.[35] In psoriasis, IL-19 is expressed in the epidermis of affected skin, specifically, in basal and suprabasal keratinocytes localized in the suprapapillary epidermis. It has not been found in monocytes or macrophages, endothelial cells, melanocytes, Langerhans cells, or T lymphocytes from psoriatic plaques.[36] Furthermore, it has been suggested that IL-19 is not only produced by keratinocytes, but that it also acts on these cells.[37] A decrease in IL-19 expression has been demonstrated after treatment with IL-4[38] or cyclosporine.[39] These findings, along with our results pointing to overexpression after stimulation by activated CLA^+ T lymphocyte supernatant, suggest that IL-19 mRNA expression depends on whether IFN-γ-nonproducing T cells are present.

In conclusion, our data suggest that circulating CLA^+ T lymphocytes are able to activate psoriatic keratinocytes, inducing them to express genes that are associated with the innate immune response and that have been previously described in psoriatic lesions. These results support the role of T cells in the pathogenesis of psoriasis. However, further studies are needed to clarify the significance of the differences in gene expression that we have observed, as well as to determine which mediators produced by T lymphocytes are responsible.

Conflict of Interest

The authors declare no conflict of interest.

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