Dysregulated Activation of Activator Protein 1 in Keratinocytes of Atopic Dermatitis Patients with Enhanced Expression of Granulocyte/Macrophage-Colony Stimulating Factor

Saveria Pastore,* Maria Laura Giustizieri,* Francesca Mascia,* Alberto Giannetti,† Kenneth Kaushansky,‡ and Giampiero Girolomoni*

*Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS, Rome, Italy; †Department of Dermatology, University of Modena and Reggio Emilia, Modena, Italy; ‡Division of Hematology, University of Washington, Seattle, Washington, U.S.A.

Keratinocytes of patients with atopic dermatitis produce high amounts of granulocyte/macrophage colony-stimulating factor, a factor essential for dendritic cell function and thus for the development of skin immune responses. In contrast to keratinocytes cultured from nonatopic, healthy individuals, granulocyte/macrophage colony-stimulating factor mRNA could be detected in unstimulated cultures of atopic dermatitis keratinocytes, and phorbol myristate acetate induced much greater granulocyte/macrophage colony-stimulating factor mRNA levels in these cells, although the decay kinetics were not altered. Using reporter gene (chloramphenicol acetyl transferase) analysis, a minimal granulocyte/macrophage colony-stimulating factor promoter was shown to confer constitutive and phorbol-myristate-acetate-induced regulation of transcriptional activity in keratinocytes, and significantly higher levels of chloramphenicol acetyl transferase activity were measured in lysates of unstimulated and phorbol-myristate-acetate-treated atopic dermatitis keratinocytes than in control keratinocyte cultures. Electrophoretic mobility shift assays showed that low levels of NF-κB binding activity could be induced by phorbol myristate acetate in both normal and atopic dermatitis keratinocytes. By contrast, activator protein 1 complexes were efficiently induced, and they were invariably present at higher levels in nuclear lysates of atopic dermatitis keratinocytes. Atopic dermatitis keratinocyte nuclear lysates had higher constitutive levels of c-Jun, and phorbol myristate acetate promoted an earlier and stronger expression of c-Jun, JunB, and of the phosphorylated forms of c-Fos. A dysregulated activation of activator protein 1 may be implicated in the molecular mechanisms leading to increased granulocyte/macrophage colony-stimulating factor expression in atopic dermatitis keratinocytes. ***Key words: cytokines/inflammation/skin/transcription factors. J Invest Dermatol 115:1134–1143, 2000***

Atopic disorders comprise diseases such as atopic dermatitis (AD), bronchial asthma, and rhinoconjunctivitis, affecting tissues (i.e., the skin and mucosae) that define boundaries between the host and the environment. They are characterized by IgE hyperresponsiveness to common environmental allergens, and by a peculiar hyperreactivity of the target tissues towards a variety of inflammatory stimuli (Postma *et al*, 1995; Nimer *et al*, 1996). The preferential development of T helper 2 (Th2) immune responses in atopic patients has been associated with abnormal regulation of cytokine genes and signaling pathways (Welter and Eckert, 1995; Khurana Hershey *et al*, 1997; Ray and Cohn, 1999). In contrast, the cellular and molecular bases of the target tissue hyperreactivity have been only marginally investigated (Pastore *et al*, 1997; Sampath *et al*, 1999). Epithelial cells are the outermost component of skin and mucous membranes, and they can be activated by diverse factors to produce cytokines involved in the initiation and amplification of inflammatory and immune responses (Pastore *et al*, 1997; 1998; Albanesi *et al*, 1999).

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine exerting multiple effects on dendritic cells, T cells, monocytes, and eosinophils. In a rat compartmentalized transgene model, a prolonged skin expression of GM-CSF induced changes commonly observed in AD (Xing *et al*, 1997). GM-CSF is readily produced by epithelial cells in response to autocrine interleukin-1α (IL-1α) and tumor necrosis factor α (TNF-α), and to the T-cell-derived cytokines interferon-γ (IFN-γ), interleukin (IL)-4, and IL-17 (Pastore *et al*, 1997; 1998). In the context of atopic diseases, a prominent increased expression of GM-CSF has been documented in nasal and bronchial epithelial cells of rhinitis and asthma patients, respectively (Marini *et al*, 1992; Sousa *et al*, 1993; Nonaka *et al*, 1996; Calderón *et al*, 1997), as well as in peripheral blood mononuclear cells of AD patients (Bratton *et al*,

Manuscript received April 12, 2000; revised June 27, 2000; accepted for publication August 18, 2000.

Reprint requests to: Dr. Saveria Pastore, Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS, via Monti di Creta 104, 00167 Roma, Italy. Email: pastore@idi.it

Abbreviations: AD, atopic dermatitis; AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; Th2, T helper 2.

1995). We have previously shown that GM-CSF is overexpressed in keratinocytes of AD lesions, and that keratinocytes cultured from nonlesional skin of AD patients produce higher levels of GM-CSF, both basally and in response to IL-1α, IFN-γ, or phorbol myristate acetate (PMA), compared with keratinocytes from nonatopic individuals. These findings could have important implications in the differentiation and sustained activation of dendritic cells in AD skin (Pastore *et al*, 1997; Xing *et al*, 1997). GM-CSF expression is regulated by a combination of transcriptional and post-transcriptional control mechanisms (Gasson, 1991). GM-CSF gene is constitutively transcribed, although its mRNA cannot be normally accumulated in the cells, due to instability of this transcript (Malter, 1998). Among stimuli effective in inducing GM-CSF, PMA has been characterized extensively and demonstrated to both increase GM-CSF mRNA stability and directly stimulate GM-CSF gene transcription (Shaw and Kamen, 1986; Malter, 1998). Deletion analysis of the GM-CSF 5′ flanking region showed that a minimal promoter fragment spanning from 91 nucleotides (nt) upstream to 23 nt downstream of the mRNA cap site was sufficient for directing constitutive and inducible transactivation (Kaushansky, 1989; Kaushansky *et al*, 1996). Two regulatory elements within this region, the Conserved Lymphokine Element 2 (CLE2, located between –85 and –76 nt upstream of the cap site) and CLE0 (from –53 to –39 nt), contribute to an efficient GM-CSF gene transactivation in an interdependent and synergistic fashion. The major transcription factors known to be critically responsible for basal as well as induced gene transactivation are NF-κB and activator protein 1 (AP-1) binding CLE2 and CLE0, respectively (Jenkins *et al*, 1995; Kaushansky *et al*, 1996; Nimer *et al*, 1996; Thomas *et al*, 1997). In addition, both activating and repressor elements have been recognized in the region between CLE2 and CLE0, although their specific functions have not been fully characterized (Shannon *et al*, 1997).

In this study, keratinocytes cultured from AD patients were shown to have a higher GM-CSF gene transcriptional activity than keratinocytes cultured from healthy subjects. In addition, AP-1 complexes were over-represented in AD keratinocytes, with higher levels of basal c-Jun and PMA-induced c-Jun, JunB, and phosphorylated forms of c-Fos. Finally, the pattern of AP-1 expression in lesional AD keratinocytes was markedly disturbed. These results provide initial evidence for an important role of AP-1 transcription factors in the pathophysiology of AD.

MATERIALS AND METHODS

Subjects Seventeen adult patients (nine males and eight females; age range, 15–39 y; median age, 26 y) with moderate to severe chronic AD, diagnosed according to standard criteria (Hanifin and Rajka, 1980), participated in the study. Skin involvement ranged from 20% to 60% of the body surface area. Ten patients had associated bronchial asthma and/or rhinoconjunctivitis, whereas seven patients had pure AD. Serum IgE was elevated in 13 patients (120-2000 kU per ml). Patients were not receiving any systemic or topical therapy for at least 2 wk and 1 wk before testing, respectively. The first eight patients had already been included in a previous study (Pastore *et al*, 1997). Nine healthy individuals (five males and four females; age range, 20–50 y; median age, 32 y) served as controls. They had no personal or family history of atopic diseases, and serum IgE levels were within normal limits. Epidermal sheets for keratinocyte cultures were obtained from the roof of suction blisters raised on normal-looking skin of all patients and five control subjects. In four control subjects, skin was obtained during plastic surgery. Four millimeter punch biopsies were taken from lesional skin of the elbow of three AD patients and from normal elbow skin of three nonatopic controls. Informed consent was obtained from all subjects, and the study was approved by the Istituto Dermopatico dell'Immacolata Ethical Committee.

Reagents and antibodies PMA was purchased from Sigma-Aldrich (Milano, Italy). Actinomycin D was from Boehringer Mannheim (Mannheim, Germany), and poly-(dI-dC) from Pharmacia Biotech (Uppsala, Sweden). Protein phosphatase 1 (PP1) was from New England Biolabs (Beverly, MA). Unless otherwise specified, the antibodies were affinity-purified rabbit polyclonal IgG. Anti-NF-κB p50 subunit (SA-170), anti-NF-κB p65 subunit (SA-171), and anti-NF-κB c-Rel (SA-172) antibodies were from Biomol Research Laboratories (Plymouth Meeting, PA). Anti-inhibitor of κBα (IκBα; sc-371), anti-pan-Jun (sc-44), anti-c-Jun (sc-45, goat), anti-JunB (sc-46), anti-JunD (sc-74), anti-pan-Fos (sc-413, mouse monoclonal antibody IgG_{2a}), anti-c-Fos (sc-52), anti-FosB (sc-48), anti-Fra-1 (sc-605), anti-Fra-2 (sc-171), anti-Ets-1 (sc-112), anti-YY1 (sc-1703), anti-Sp1 (sc-59), and anti-C/EBPδ (sc-636) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies employed in Western blot analysis were horseradish peroxidase conjugated antigoat IgG (sc-2020) or antirabbit IgG (sc-2004), from Santa Cruz. For immunohistochemistry, biotinylated antirabbit and antigoat IgG were purchased from Vector Laboratories (Burlingame, CA).

Keratinocyte cultures Primary keratinocyte cultures were established by seeding epidermal cells on a feeder layer of irradiated 3T3/J2 fibroblasts, as described previously (Pastore *et al*, 1997). Second-or third-passage keratinocytes were used in all experiments, with cells cultured in the serum-free medium Keratinocyte Growth Medium (KGM; Clonetics, San Diego, CA), prepared from the essential nutrient solution Keratinocyte Basal Medium (KBM; Clonetics), supplemented with optimal concentrations of epidermal growth factor, hydrocortisone, insulin, bovine pituitary extract, and antibiotics. GM-CSF release was assessed in cells from all subjects, whereas the other assays were repeated on keratinocytes cultured from the same two to four AD patients or control individuals, as indicated in the figure legends.

Enzyme-linked immunosorbent assay (ELISA) Quantitation of GM-CSF was performed on supernatants collected after 48 h treatment with 10 ng per ml PMA of triplicate subconfluent keratinocyte cultures using the GM-CSF Predicta kit from Genzyme (Cambridge, MA). During the assay, keratinocytes were grown in medium devoid of hydrocortisone.

RNA isolation, RNAse protection assay (RPA) and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis Total RNA was extracted with a modified guanidine isothiocyanate acid phenol protocol using Ultraspec RNA Isolation System (Biotecx, Houston, TX), as described previously (Pastore *et al*, 1997). For RPA, the multiprobe template set hCK4 and the complete kit for this assay were purchased from PharMingen (San Diego, CA), and were used according to the manufacturer's instructions. Ten micrograms of RNA was used in each assay. RPA bands were quantified by laser densitometry using an imaging densitometer supported by the Molecular Analyst image analysis software (Bio-Rad, Hercules, CA), with GM-CSF mRNA signals normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For RT-PCR analysis, 1 μg RNA was reverse transcribed and the cDNA was amplified using the GeneAmp RNA PCR kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). The primers for c-Fos amplification were taken from Lane *et al* (1998), and those for the other AP-1 subunits were from Risse-Hackl *et al* (1998). A GAPDH primer set was used as an internal control for the amount of RNA used. The optimal number of amplification cycles for each primer set was deduced from the exponential tract of the line obtained from a semilogarithmic graph in which cycle numbers were plotted against the optical densities of the corresponding PCR band.

Plasmid constructs and keratinocyte transfection The chloramphenicol acetyl transferase (CAT) reporter construct carrying the sequence of the minimal GM-CSF promoter, from 91 nt upstream to 23 nt downstream of the GM-CSF cap site (p91 CAT), has been previously described (Kaushansky, 1989). Site-specific mutations in the native sequence of p91 CAT were introduced to identify its functional elements (Kaushansky *et al*, 1996). Keratinocytes were transfected with plasmids carrying random mutations of five nucleotides within the GM-κB sequence, localized in the CLE2 site (p91μ CAT), two different random trinucleotide substitutions in the CLE0 element, known to bind AP-1 proteins (p91μ6 CAT and p91 Oct^+ CAT), a seven nucleotide mutation in the region between CLE2 and CLE0 (p91μ3 CAT), and finally simultaneous mutations at GM-κB and AP-1 sites (p91μOct^+ CAT) (**Fig 3*A***). The empty expression vector pCAT was used in some experiments as a negative control. Keratinocytes were seeded onto 1.9 cm^2 wells (2×10^5 cells per well), and transfected when approximately 60% confluent, following an established methodology (Efimova *et al*, 1998), with some modifications. Transfection of keratinocytes was performed over a 6 h period in 1 ml KGM containing 12 μl lipofectin (Life Technologies, Gaithersburg, MD), 1 μg CAT test plasmid, and 1 μg pCMV·SPORT-β-galactosidase (β-gal) (Life Technologies), the last used to provide an internal parameter of transfection efficiency (Awane *et al*, 1999). After 6 h, transfection medium was removed, and keratinocytes were allowed to recover in

fresh KGM for 24 h and then cultured for 12 h with KGM in the absence or presence of 10 ng per ml PMA. Cultures were then washed with phosphate-buffered saline and treated with 0.5 ml of lysis reagent (Boehringer Mannheim), and protein concentration was determined according to the method of Bradford (Bio-Rad). CAT and β-gal were measured in aliquots of each lysate as enzyme concentrations (ng per ml) using commercially available ELISA kits (Boehringer Mannheim). CAT activity was expressed as CAT concentration normalized to β-gal concentration × 500, per microgram of protein. All assays were performed in triplicate.

Preparation of cytoplasmic and nuclear extracts Keratinocytes were grown in 75 cm^2 flasks to 70%–80% confluence, and, to reduce background activation and synchronize the cells, culture medium was replaced with KBM supplemented with 0.1% bovine serum albumin for 24 h before stimulation with 50 ng per ml PMA. Keratinocytes and Jurkat cells were lyzed according to Schreiber's method (Schreiber *et al*, 1989). To evaluate the contribution of phosphorylation to the expression of specific transcription factors, aliquots of 10 μg of nuclear lysates were incubated with 1 unit of PP1 per reaction mixture for 1 h at 30°C, in a reaction buffer supplemented with 1 mM $MnCl_2$. Lactate dehydrogenase activity (LDH) was assayed to estimate possible contamination of nuclear extracts with cytoplasmic proteins (Arenzana-Seisdedos *et al*, 1995). Protein samples were diluted to allow determination of initial rates, and values were expressed as variation of optical density at 340 nm per min per mg of protein (ΔOD/min mg).

Electrophoretic mobility shift assay (EMSA) EMSA was performed as described (McDonald *et al*, 1997), with some modifications. In particular, binding reactions were performed using 10 μg nuclear proteins per assay in 25 μl binding buffer (25 mM HEPES pH 7.9, 0.5 mm ethylenediamine tetraacetic acid, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol), supplemented with 100 mM NaCl and 2.5 μg poly-(dI-dC) to detect specific NF-κB complexes. For detection of specific AP-1 complexes, binding buffer was added containing 80 mM NaCl and 5 μg poly-(dI-dC). To ascertain the identity of the complexes, 2 μg of a specific antibody, or a combination of antibodies, was added to the reaction mixture (20 min, 37°C). Finally, 50,000 cpm of the ^{32}P-end-labeled double stranded oligonucleotide was added to the binding mixtures, which were incubated for a further 30 min at 37°C. The sequence of the synthetic oligonucleotide (Life Technologies) used as a probe for NF-κB in EMSA was identical to the sequence −91 to −76 nt of the minimal GM-CSF promoter, added to a repeat of the GM-κB site (underlined): 5′-AGTTCAGGTAGTTCCCGCTGGGTAGTTCCC. AP-1 binding activity was detected taking advantage of a probe homologous to the fragment −63 to −35 downstream of the GM-CSF promoter (AP-1 site underlined): 5′-CATTTTGTGGTCACCATTAATCATTTCCT. In some experiments, we used probes in which the sequence of specific GM-κB and AP-1 binding sites was mutated as in constructs p91μ CAT and p91 Oct^+, respectively (**Fig 3*A***). For cold competition, a 100-fold excess of the double stranded oligonucleotide or mutated oligonucleotide was added to the binding reaction. The resulting samples were electrophoresed on 6% native polyacrylamide gels in 1% TBE. Quantitative analysis of the complexes was performed by laser densitometry.

Western blot analysis Aliquots of the nuclear or cytoplasmic extracts were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and then transferred overnight onto a nitrocellulose membrane (Amersham) at 20 V constant with a Bio-Rad transblot apparatus. Transfer efficiency was visualized by reversible Ponceau Red staining. After exposure to the primary antibody (60 min, 37°C), the membranes were washed and finally incubated for 40 min with horseradish peroxidase conjugated antigoat IgG or antirabbit IgG. The signal was revealed with enhanced chemiluminescence reagents (Amersham), as per the manufacturer's protocol. As a positive migration control for NF-κB/Rel subunits, nuclear extracts from Jurkat T cells were usually included on the gels.

Immunohistochemistry Skin biopsies were fixed in 10% buffered formalin and embedded in paraffin. Three to four micron tissue sections were deparaffinized and then rehydrated. Antigen unmasking was performed by two consecutive 5 min exposures to microwaves (600 W), each time in fresh 10 mM citrate buffer (pH 6.0). Sections were treated with 3% hydrogen peroxide, washed, and then incubated in 10% rabbit or goat serum. Incubation with primary antibody was performed for 1 h at room temperature. Sections were then stained according to an avidin-biotin-peroxidase technique (Vector Laboratories), using 3-amino-9-ethyl carbazole as substrate.

Statistical analysis The Mann–Whitney rank sum test was used to compare GM-CSF release or CAT activity from keratinocyte cultures of atopic and nonatopic subjects. Wilcoxon's signed rank test was applied for within-group comparisons before and after PMA stimulation. Data were expressed as mean ± SD; p values of 0.05 or less were considered statistically significant.

RESULTS

Keratinocytes cultured from AD patients show enhanced GM-CSF release and gene expression compared to keratinocytes from nonatopic controls Extending our previous observations (Pastore *et al*, 1997, 1998) to a larger group of patients, we confirmed that keratinocytes cultured from the majority of AD patients released exaggerated amounts of GM-CSF compared with keratinocytes from nonatopic donors, both in unstimulated cultures and after 48 h treatment with 10 ng per ml PMA (**Fig 1**). Next, we followed GM-CSF mRNA induction in keratinocytes from basal levels to 6 h stimulation with 10 ng per ml PMA, and its subsequent decay following PMA withdrawal (**Fig 2*A***). In contrast to unstimulated control keratinocytes, which had an undetectable GM-CSF mRNA signal, AD keratinocytes with higher constitutive GM-CSF release reproducibly displayed a GM-CSF mRNA band. Moreover, PMA induced a time-dependent increase in GM-CSF mRNA that was much more pronounced in AD keratinocytes than in control cultures. Of note, M-CSF mRNA followed an expression pattern distinct from that of GM-CSF mRNA, and a limited induction following PMA stimulation. After 6 h of PMA treatment, the medium was removed and substituted with fresh medium containing 5 μg per ml actinomycin D, a potent RNA polymerase II inhibitor. Rapid GM-CSF mRNA decay was observed after an initial 15 min interval in which mRNA still accumulated. A calculated half-life of 24 min in keratinocytes from both nonatopic and AD subjects indicated that GM-CSF mRNA overexpression in AD keratinocytes was not due to reduced mRNA degradation (**Fig 2*B***).

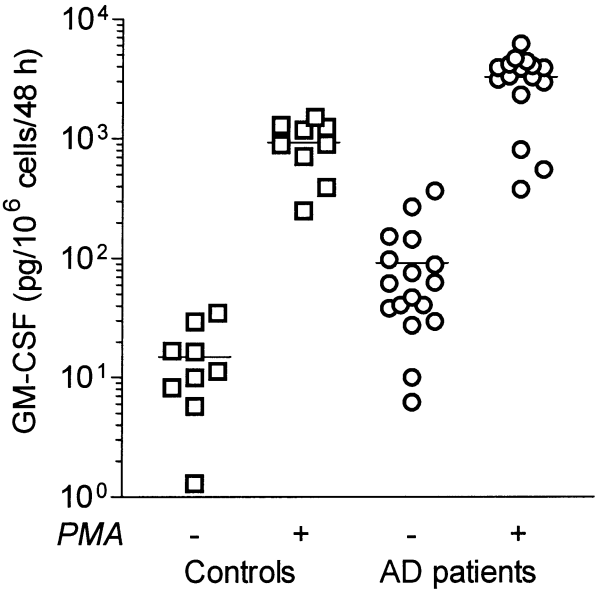

Figure 1. Keratinocytes cultured from AD patients release higher amounts of GM-CSF. Keratinocyte cultures were established from normal appearing skin of 17 AD patients (○) and from nine healthy, nonatopic individuals (□). GM-CSF was measured by ELISA on supernatants collected after 48 h stimulation of subconfluent cultures with medium alone or 10 ng per ml PMA. Both spontaneous and PMA-induced GM-CSF release from AD keratinocytes were significantly higher ($p = 0.045$ and $p = 0.02$, respectively) than from control keratinocytes. Each point represents the mean of triplicate cultures.

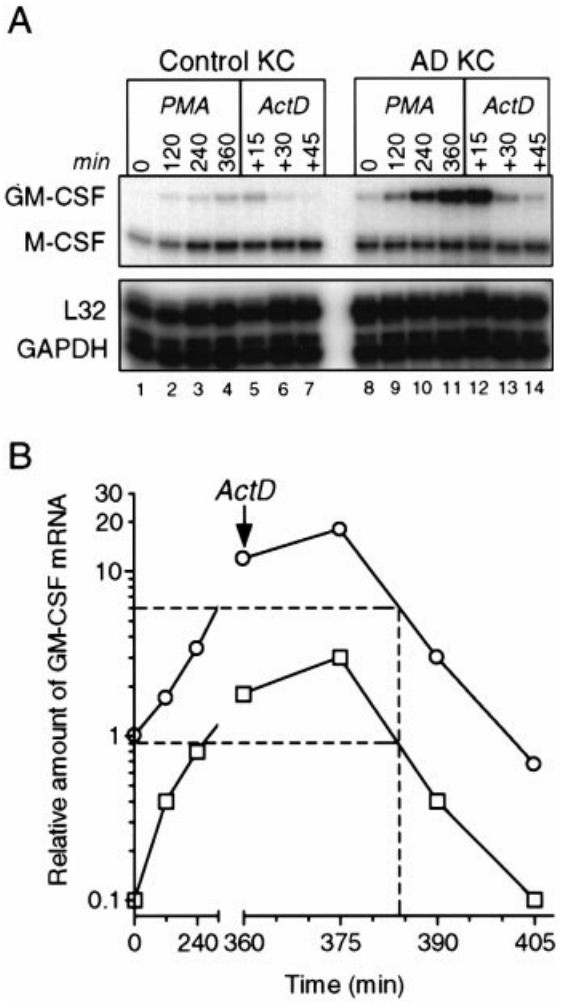

Figure 2. Keratinocytes cultured from AD patients show higher constitutive and PMA-induced GM-CSF mRNA expression, but mRNA decay kinetics are similar to control keratinocytes. (*A*) Total RNA from AD and control keratinocytes stimulated with 10 ng per ml PMA for different time-points was subjected to RPA using a multiprobe template set. After 360 min, cells were washed and then cultured in fresh medium containing 5 μg per ml actinomycin D (ActD). (*B*) RPA bands were quantified by laser densitometry, and GM-CSF mRNA signals normalized to GAPDH mRNA signals were plotted against time. The dashed lines represent the time required for 50% of the GM-CSF mRNA signal present at 360 min of PMA stimulation to decay, in keratinocytes from AD patients (○) and from normal controls (□). Similar results were confirmed in keratinocyte cultures from three AD patients and three controls.

The minimal GM-CSF promoter drives basal and PMA-induced CAT expression in keratinocytes, with higher levels in AD keratinocytes To evaluate the existence of a possible difference in GM-CSF gene transcription efficiency, keratinocytes from AD patients and controls were transiently transfected with a plasmid (p91 CAT) carrying the native sequence of the minimal GM-CSF promoter, known to direct basal and inducible transcription of the reporter gene CAT. A series of plasmids containing site-specific mutations was also employed, as reported in **Fig 3**(***A***) (Kaushansky *et al*, 1996). In all experiments, pCMV·SPORT-β-gal was cotransfected to control for transfection efficiency, and CAT activity was calculated by normalization of raw CAT values to the corresponding β-gal values. As shown in **Fig 3**(***B***), the minimal GM-CSF promoter (p91 CAT) conferred low level constitutive transcriptional activity in unstimulated keratinocytes, which was significantly higher in AD keratinocytes than in control keratinocytes. Any mutation in the native sequence of p91 CAT reduced basal transcriptional activity to undetectable levels, in both AD and control keratinocytes (**Fig 3*B***). Following PMA stimulation, p91 CAT activity increased 10–15-fold, and again AD keratinocytes exhibited much higher levels than control cells (**Fig 3*C***). Transfection with plasmids carrying site-specific mutations in either the GM-κB box (p91μ CAT) or the CLE0 sequence (p91μ6 and p91 Oct$^+$) produced more than 90% reduction of PMA-induced CAT activity, whereas mutation of both sites led to complete loss of activity. Moreover, the introduction of a seven nucleotide mutation in the region between the CLE2 and CLE0 regions, as in p91μ3 CAT, resulted in about a 60% decrease in CAT activity, suggesting that this mutation interfered with specific binding of an activating transcription factor. An Sp1 binding site has been identified adjacent to the GM-κB box, both in murine and human GM-CSF promoters (between −75 and −70 nt in the human sequence) (Shannon *et al*, 1997). Experimental evidence has been provided that mutations in this site potently reduce murine GM-CSF promoter activity (Masuda *et al*, 1994), and it has been suggested that it could have an analogous relevance in the human GM-CSF promoter (Shannon *et al*, 1997). On the other hand, these site-specific mutations abrogated any difference in transcriptional efficiency between AD and nonatopic keratinocytes, indicating that the structural integrity of the minimal GM-CSF promoter was a necessary requisite for functional interdependence and synergism among the transcription factors. They did not help to identify, however, which element of the minimal GM-CSF promoter was responsible for the difference between AD and control keratinocytes in the levels of transcriptional activity.

In subsequent experiments NF-κB and AP-1 complexes were investigated in keratinocyte nuclear lysates. Contamination from cytoplasmic proteins was excluded by LDH assay, with keratinocyte or Jurkat cell cytoplasmic lysates giving LDH values of 30–40 ΔOD/min mg, and nuclear extracts 0.005–0.01 ΔOD/min mg.

NF-κB complexes from keratinocyte nuclear lysates bind the GM-κB region only very weakly EMSA (**Fig 4*A***) showed that GM-κB binding activity in unstimulated Jurkat nuclear lysates was not detectable, but it was potently induced by PMA (*lanes 1–5*). By contrast, PMA induced a poor increase in binding activity both in nonatopic (*lanes 6–10*) and AD (*lanes 11–15*) keratinocyte nuclear lysates, although in the latter a slightly earlier induction was consistently observed (*lanes 12* and *13 vs lanes 7* and *8*). The specificity of the slower-migrating GM-κB DNA binding activity was determined by addition of specific antibodies against NF-κB subunits to the binding reaction, prior to its incubation with the labeled probe. **Figure 4**(***B***) shows that anti-p50 antibody abrogated the complex and anti-p65 antibody strongly impaired its formation. On the other hand, anti-c-Rel, anti-Sp1, or control IgG did not significantly affect the band produced by keratinocyte nuclear lysates, although anti-c-Rel antibody impaired complex formation by Jurkat nuclear lysates (not shown). Moreover, the specific complex was completely competed by a 100-fold excess of unlabeled GM-κB probe, but not of unlabeled oligonucleotide with mutated GM-κB sites (*lanes 9* and *10*, **Fig 4*B***). Western blot experiments (**Fig 4*C***) showed very low levels of p50 and p65 proteins in unstimulated Jurkat cells and keratinocytes, with PMA inducing both p50 and p65, much more strongly in Jurkat cells than in keratinocytes. Noteworthy, a slightly earlier induction of p50 and p65 was invariably noticed in AD than in control keratinocytes (*lanes 10* and *11 vs lanes 5* and *6*). **Figure 4**(***C***) also shows a modest PMA-induced decrease in the level of cytoplasmic IκBα, more evident in Jurkat cells than in keratinocytes, where this inhibitor was present at higher levels. We could not detect IκBα in nuclear lysates, either from keratinocytes or Jurkat cells.

AP-1 complexes are stronger in AD keratinocyte nuclear lysates Nuclear lysates from Jurkat cells and keratinocytes were allowed to form complexes on a labeled duplex oligonucleotide based on the sequence from −63 to −35 bp of the native GM-CSF promoter, which includes CLE0. The resulting binding activity was detectable in quiescent keratinocytes, and was promptly

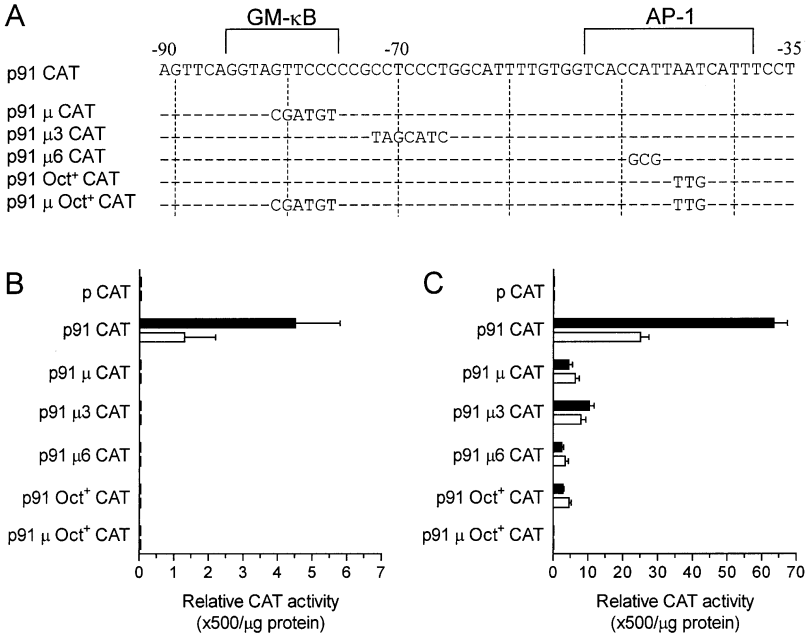

Figure 3. The minimal GM-CSF promoter drives basal and PMA-induced CAT activity in keratinocytes, which is stronger in AD keratinocytes. Keratinocyte cultures were transfected with the indicated minimal GM-CSF promoter constructs (*A*) for 6 h, and after a 24 h recover were cultured for 12 h with or without 10 ng per ml PMA. The cells were then harvested, and extracts were assayed for CAT activity. (*B*) Significantly higher levels of basal CAT activity were measured in lysates of AD keratinocytes (*black bars*) than in those of nonatopic keratinocytes (*white bars*), after transfection of p91 CAT ($p = 0.03$). When keratinocytes from either nonatopic or AD subjects were transfected with constructs carrying site-specific mutations in the native sequence of the minimal promoter, CAT activity decreased to undetectable levels. (*C*) Compared with basal levels, p91 CAT drove a 10–15-fold increase in CAT activity following PMA stimulation, and it was significantly higher in keratinocytes form AD patients ($p = 0.003$). Site-specific mutations identified a prominent reduction in PMA-induced CAT activity. The values represent the mean ± SD of four replicates for each experimental condition. Similar results were obtained with keratinocytes from three additional AD patients and three nonatopic controls.

upregulated by PMA, with the complex more represented in nuclear lysates from AD keratinocytes with high GM-CSF production (**Fig 5*A***). Compared with control keratinocytes, the complex was three to four times more intense in the nuclei of these AD keratinocytes in resting conditions, and it was two to three times stronger at each time-point after PMA stimulation. On the other hand, nuclear lysates of AD keratinocytes that released lower amounts of GM-CSF showed a binding activity similar to controls (data not shown). Moreover, the specific complex was completely abrogated when competed by a 100-fold excess of unlabeled oligonucleotide, but not of unlabeled probe carrying a random mutation in the AP-1 binding sequence (*lanes 16* and *17*, **Fig 5*A***). Addition of anti-pan-Jun antibody to the reaction mixture completely abrogated the complex generated by unstimulated keratinocytes, indicating that it was a specific AP-1 signal (**Fig 5*B***). Binding competition experiments performed by addition of single antibodies specific for each Jun and Fos family member indicated that basal AP-1 complex in normal and AD keratinocytes was composed of JunB and Fra-2, with a small contribution of c-Jun (**Fig 5*B***), whereas anti-Ets1 or anti-YY1 specific antibodies did not alter the constitutive EMSA signal significantly (data not shown). The complex formed by nuclear lysates from PMA-stimulated keratinocytes was completely abrogated by anti-pan-Jun plus anti-pan-Fos antibodies, with competition to AP-1 binding more efficiently accomplished by anti-pan-Jun than by anti-pan-Fos antibody (**Fig 5*C***). In stimulated keratinocytes, c-Jun and Fra-1 were the major components, even though JunB, c-Fos, Fra-2, and to a lesser degree JunD, seemed to participate in complex formation (**Fig 5*D***). The pattern of EMSA complex formation observed in **Fig 5**(***B***)-(***D***) was similar in nonatopic and AD keratinocytes, and did not change significantly at the different time-points after PMA treatment (data not shown).

AD keratinocyte nuclear lysates express higher basal levels of c-Jun, and PMA induces in these cells an earlier and stronger expression of JunB and phosphorylated forms of c-Fos To gain insights into the difference in AP-1 binding activity between AD and nonatopic keratinocytes, we performed Western blot analysis using antibodies specific for each member of the AP-1 family. Except for c-Fos, all AP-1 members were detectable in nuclear extracts from cultured keratinocytes after 24 h starvation in KBM (**Fig 6**). Anti-c-Jun antibody detected a doublet, consisting of a 40 kDa band visibly modulated by PMA and a nonspecific 43 kDa band. Compared to control cells, AD keratinocytes exhibited significantly higher levels of c-Jun at baseline, as well as after PMA stimulation. The highest and the lowest bands of JunB were present in very low amounts in resting cells, and after PMA treatment three discrete bands progressively appeared that were induced at higher levels in AD keratinocytes as soon as 40 min after stimulation (*lane 9 vs lane 3*). In contrast, the levels of JunD specific bands were not affected by PMA, and were equally expressed in nonatopic and AD keratinocytes. With regard to Fos proteins, c-Fos became detectable 20 min after PMA stimulation, followed by the progressive formation of a ladder of distinct heavier bands, with an earlier and stronger induction in AD keratinocytes (*lanes 2–6 vs lanes 8–12*). FosB was detectable in quiescent cells, and was not affected by PMA, both in nonatopic and AD keratinocytes. Finally, both Fra-1 and Fra-2 bands were constitutively present in resting keratinocytes, with the first weakly induced and the second not changed by PMA.

The contribution of phosphorylated forms to Western blot signals of JunB and c-Fos was appreciable in Western blot analysis of nuclear lysate aliquots treated with PP1, an enzyme that specifically hydrolyzes the phosphate groups on serine and threonine (Fetrow *et al*, 1999). **Figure 7** shows that the level of native, nonphosphorylated JunB protein is 2-fold higher in keratinocytes from AD patients than in nonatopic controls (*lane 6 vs lane 3*). By contrast, dephosphorylation of c-Fos gave bands of similar intensity in both keratinocyte types. These data were suggestive of a higher rate of synthesis of JunB, and of a more pronounced phosphorylation of c-Fos.

Semiquantitative RT-PCR analysis indicated that PMA stimulated a transient decrease in steady-state levels of c-Jun mRNA in keratinocytes of both nonatopic and AD patients (**Fig 8**). This decrease was more prominent and prolonged in control cells, where the c-Jun mRNA level recovered in 20 min, compared with a 10 min interval in AD keratinocytes. Also, c-Jun levels reached a higher peak intensity (at 40 min) and were still detectable at 6 h in AD keratinocytes. The JunB mRNA steady-state level was very similar in nonatopic and AD keratinocytes, but underwent some induction following PMA, increasing after 20 min in AD keratinocytes and after 30 min in control cells. Interestingly enough, PMA promoted a transient increase in c-Fos mRNA that was significantly weaker in AD than in control keratinocytes.

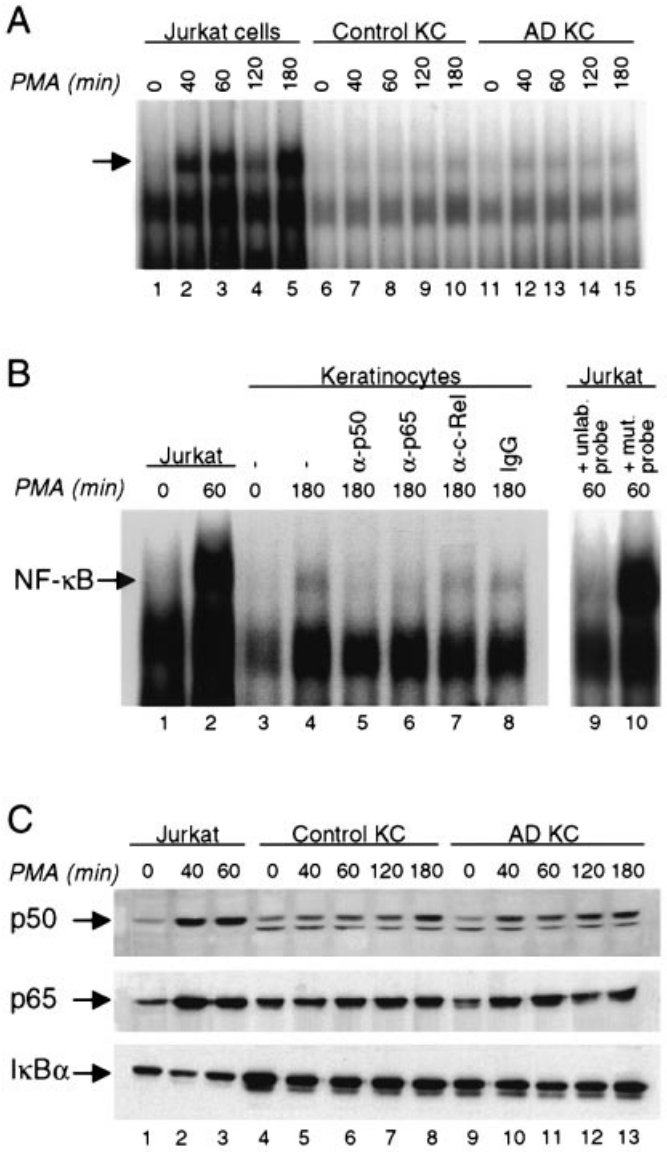

Figure 4. Keratinocyte nuclear lysates bind very weakly to a labeled oligonucleotide probe containing tandem repeats of the GM-κB region. (*A*) EMSA was performed using 10 μg of nuclear proteins from Jurkat cells or keratinocytes, in resting conditions or after stimulation with 50 ng per ml PMA for different time-points. (*B*) GM-κB binding activity of keratinocyte nuclear lysates was efficiently competed by 2 μg per each binding reaction of anti-p50 or anti-p65 specific antibody, but not by anti-c-Rel antibody or control IgG. Competition experiments with 100-fold excess of unlabeled probe abrogated the band, whereas competition with a mutated probe did not affect complex binding. (*C*) Western blot analyses of p50 and p65 induction by PMA were performed on 10 μg aliquots of Jurkat cell or keratinocyte nuclear extracts, whereas IκBα was detected in 10 μg aliquots of cytoplasmic lysates. A nonspecific band at 43 kDa was characteristically present in the p50 blot of keratinocyte nuclear extracts. Identical results were observed in keratinocytes cultured from three AD patients and three controls.

Finally, FosB exhibited a similar delayed induction in both keratinocyte types, whereas Fra-1 and Fra-2 mRNAs did not change significantly upon PMA treatment.

Expression of AP-1 members is markedly perturbed in AD lesional skin Erythematous chronic lesional skin of three patients and normal skin of three nonatopic donors were evaluated for immunoreactivity to AP-1 members, and specificity of staining was checked by its strong reduction after preabsorption of the antibody to a peptide containing the target epitope (not shown).

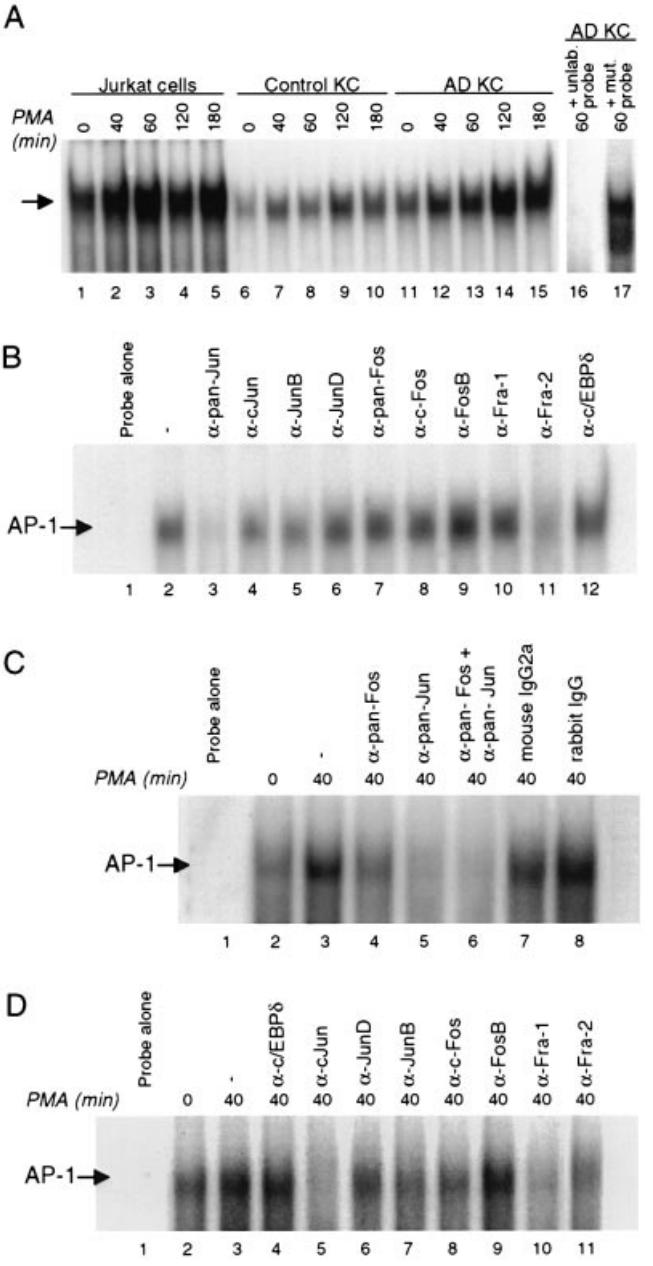

Figure 5. Basal and PMA-induced AP-1 complexes are stronger in AD keratinocyte nuclear lysates. EMSA was performed using 10 μg of nuclear proteins from Jurkat cells or keratinocytes and a labeled oligonucleotide with a sequence based on the region spanning from −63 to −35 bp of the minimal GM-CSF promoter. (*A*) The basal complex formed by unstimulated nuclear extracts of keratinocytes from AD patients (*lane 11*) has an intensity about 3-fold higher than that measured in control keratinocytes (*lane 6*). After PMA stimulation, complexes were invariably stronger in keratinocytes from AD patients. Competition experiments with 100-fold excess of unlabeled probe abrogated the band, whereas competition with a mutated probe did not affect complex binding. (*B*) Basal AP-1 binding activity was completely competed by anti-pan-Jun antibody (*lane 3*). Among Fos proteins, only Fra-2 (*lane 11*) had a relevant role in basal complex formation. (*C*) Jun and Fos were principal constituents of complexes formed by PMA-activated keratinocytes. (*D*) All Jun and Fos proteins (except FosB) had some part in PMA-induced AP-1 complex in keratinocyte lysates. The patterns shown in *panels* (*B*)-(*D*) are representative of both control and AD keratinocytes, and did not change significantly at different time-points of PMA treatment (data not shown). Similar results were observed using keratinocytes from four AD patients and three controls.

Nonimmune rabbit (or goat) IgG gave only minimal nonspecific staining (**Fig 9**). The staining pattern of normal skin was

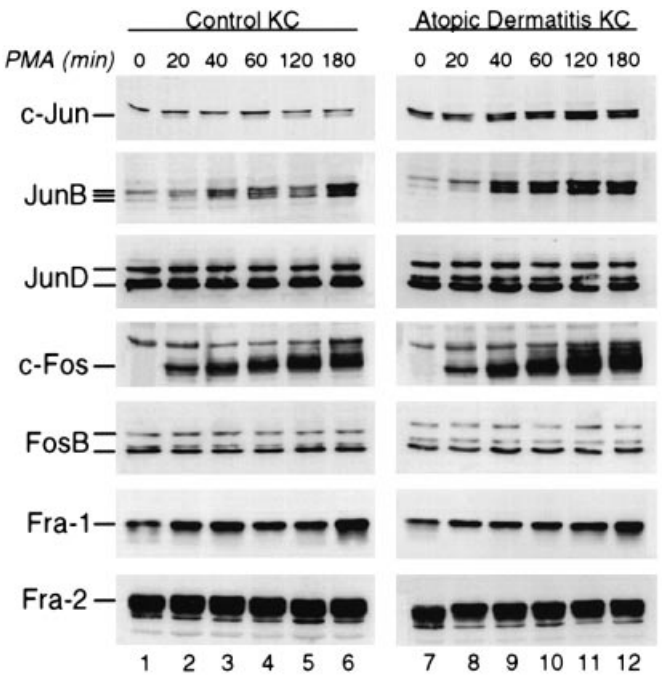

Figure 6. Western blot analysis of keratinocyte nuclear lysates. Unstimulated keratinocytes (*lanes 1 and 7*) contained all AP-1 family members except c-Fos. Moreover, keratinocytes from AD patients basally contained higher levels of c-Jun than nonatopic controls. PMA stimulation induced an earlier and stronger expression of c-Jun and JunB, and also a more pronounced pattern of c-Fos phosphorylated forms. Western blot analyses were performed on 10 μg aliquots of nuclear extracts elecrophoresed on a 10% SDS-polyacrylamide gel. Similar results were obtained using keratinocytes from four distinct AD patients and four controls.

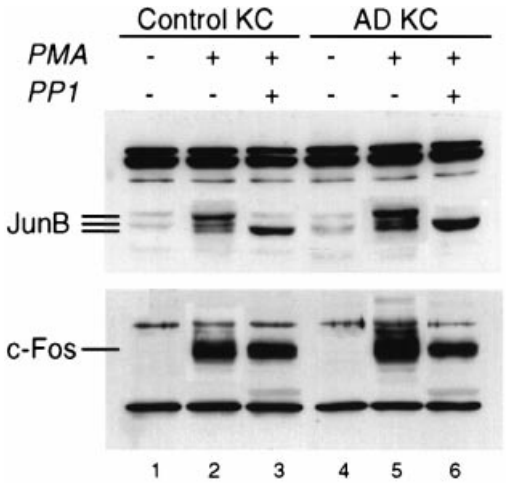

Figure 7. Contribution of phosphorylation to JunB and c-Fos western blot analysis. Dephosphorylation of Ser and Thr residues indicates that the level of native JunB protein is significantly higher in AD keratinocytes, whereas no detectable difference is evident in the levels of nonphosphorylated c-Fos. Ten microgram aliquots of nuclear lysates were treated with 1 unit per reaction mixture of PP1 for 1 h at 30°C, and then subjected to Western blot analysis. Similar results were obtained using keratinocytes from two different AD patients and two control subjects.

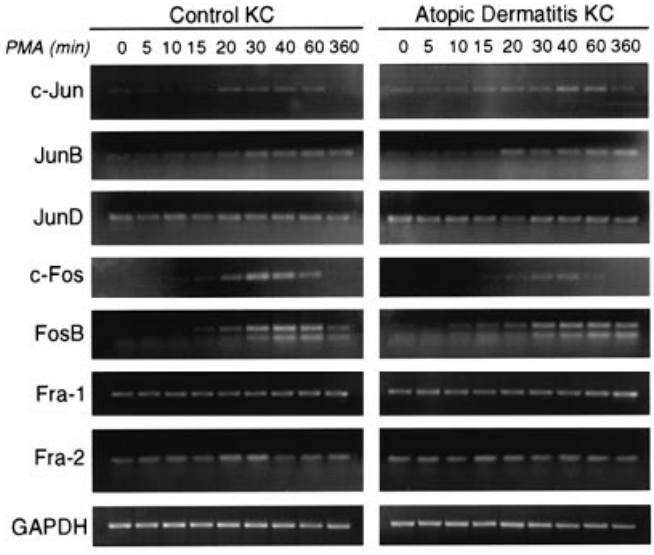

Figure 8. Semiquantitative RT-PCR of AP-1 members. Analysis of specific AP-1 mRNAs indicates that PMA induces a more rapid and pronounced increase of c-Jun and JunB gene expression in keratinocytes of AD patients, whereas the transient appearance of c-Fos mRNA is reduced. RT-PCR analysis was performed on 1 μg of total RNA. An optimal number of amplification cycles was performed to ensure a linear amplification of each reverse-transcribed product. Similar results were observed using keratinocytes from three distinct AD patients and three controls.

substantially in agreement with previous observations (Welter and Eckert, 1995). In particular, staining with anti-c-Jun and anti-JunB antibodies was diffuse to all epidermal layers, with a slightly more intense staining in the granular layer. JunD and c-Fos showed diffuse cytoplasmic staining in all epidermal layers associated with prominent nuclear staining in the basal and spinous layers, respectively. FosB, Fra-1, and Fra-2 exhibited faint, diffuse cytoplasmic and/or nuclear positivity in normal skin, with Fra-1 staining sparing the basal layer. AD lesional skin showed a distinct pattern of AP-1 protein expression that was similar for all the factors investigated, with a peculiar strengthening of cytoplasmic, perinuclear staining (**Fig 9*g***, ***m***), prevalently localized in keratinocytes of the basal and, less prominently, upper spinous and granular layers. In contrast, cytoplasmic staining of the intermediate spinous layer cells was reduced, and nuclear staining, especially for JunD and c-Fos, disappeared. Inflammatory dermal cells showed a strong cytoplasmic positivity for AP-1, particularly for JunB, JunD, FosB, and c-Fos.

DISCUSSION

Perturbation in signal transduction pathways and in the activation of transcription factors can be implicated in the pathogenesis of different aspects of atopic diseases. For example, gain-of-function mutations in the IL-4 receptor have been identified in atopic patients (Khurana Hershey *et al*, 1997). The high IL-4 production and the preferential expansion of Th2 cells have also been associated with polymorphisms in the IL-4 promoter (Song *et al*, 1996) or with alterations in nuclear protein interactions with IL-4 promoter elements (Chan *et al*, 1996; Wierenga *et al*, 1999). Based on experiments in a transgenic mouse model, other authors suggested that an earlier and stronger induction of JunB in differentiating Th2 but not Th1 cells could be responsible for a higher AP-1 transcriptional activity, and consequently for a strong activation of Th2 cytokine gene expression (Li *et al*, 1999). Higher levels of AP-1 DNA binding activity, secondary to increased generation of c-Fos, have been documented in peripheral blood mononuclear cells of corticosteroid-resistant patients with atopic asthma, and glucocorticoid receptor sequestration by AP-1 has been proposed to cause a reduced sensitivity to the anti-inflammatory effects of these drugs (Lane *et al*, 1998). Dysregulated signal transduction could also contribute to set a local condition of abnormal response to cytokine signaling, and thus may direct tissue targeting of the atopic state. A constitutive, abnormal activation of STAT-1 associated with an increased expression of its specific target genes has been selectively detected

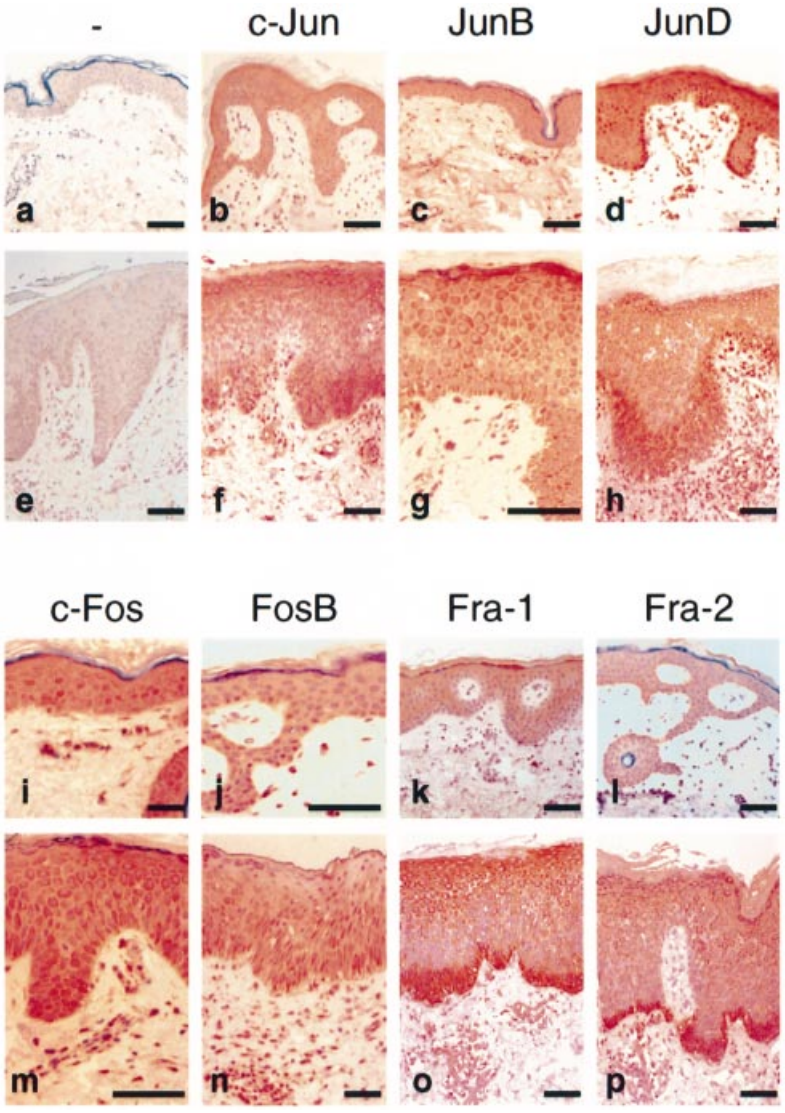

Figure 9. Immunohistochemical localization of AP-1 transcription factors is markedly altered in AD lesional skin. Biopsies were taken from normal skin of nonatopic donors (*a–d* and *i–l*) and from lesional AD skin (*e–h* and *m–p*). Skin sections were stained with antibodies against the indicated AP-1 members using an avidin-biotin-peroxidase technique and 3-amino-9-ethyl carbazole as substrate. Results are representative of staining performed on biopsies from three AD patients and three healthy controls. *Scale bar:* 40 μm.

in bronchial epithelial cells of atopic asthmatic patients, predisposing these subjects to excessive IFN-γ-mediated airway inflammation (Sampath *et al*, 1999). In the case of AD, altered cytokine synthesis by skin cells has been proposed to increase expression of TNF-α, IL-1β, and IL-12 mRNA in the skin of AD patients after contact with detergents or aeroallergens (Jurghans *et al*, 1998). Indeed, keratinocytes cultured from AD patients show enhanced GM-CSF, IL-1, and TNF-α gene expression (Pastore *et al*, 1997, 1998). Increased production of cytokines by resident cells can be very important for initiation and persistence of inflammation and allergic responses in the skin of AD patients.

In this report we propose that an altered control of gene transcription could be implicated in the mechanisms underlying abnormal GM-CSF expression by AD keratinocytes. By analysis of reporter gene expression, we observed that efficient transactivation of the minimal GM-CSF promoter strictly requires cooperation between functional AP-1 and NF-κB binding elements, confirming the existence of a powerful synergism between these two regions of proximal GM-CSF promoter (Tsuboi *et al*, 1994; Jenkins *et al*, 1995; Kaushansky *et al*, 1996; Nimer *et al*, 1996; Thomas *et al*, 1997). GM-CSF gene expression in keratinocytes was crucially controlled by a very modest increase in NF-κB concentration, thus indicating the particular efficacy of NF-κB in stimulating gene transcription (Tanaka, 1996). Of greater interest, we observed upregulated activation of AP-1 in AD keratinocytes producing exaggerated quantities of GM-CSF, suggesting that variations in AP-1 complexes could drive increased transactivation of GM-CSF, and possibly of other inflammatory genes. Western blot experiments demonstrated that nuclear lysates of these AD keratinocytes expressed higher levels of c-Jun, both basally and after PMA stimulation, and phosphorylated forms of JunB and c-Fos accumulated in these cells earlier and to greater levels than in control cells. In resting keratinocytes, c-Jun, JunB, and Fra-2 participated in the formation of AP-1 complexes, so that the higher amounts of c-Jun in quiescent AD keratinocytes could explain their enhanced constitutive AP-1 activity. On the other hand, PMA induces a phosphorylation-dependent reduction of c-Jun ubiquitin-dependent degradation and a prominent increase in c-Jun transcriptional activity, which also leads to increased synthesis of c-Jun itself (Ip and Davis, 1998). Even though participation of JunB in the AP-1 complexes that bound the GM-CSF promoter was relatively modest, higher nuclear concentrations of this transcription factor and its phosphorylated forms could enhance AP-1 activity in AD keratinocytes. JunB exhibits a decreased homodimerization property and a 10-fold weaker DNA binding activity compared to c-Jun, and thus it was initially thought to repress c-Jun-mediated transactivation by competition (Deng and Karin, 1993). There is now evidence, however, suggesting that JunB can be a strong transcriptional activator, and that phosphorylation efficiently provides for its maximal functional activity (Li *et al*, 1999). The wide pattern of c-Fos specific signals in our Western blot experiments indicated the existence of different phosphorylation forms. In fact, c-Fos has a number of activation sites, and phosphorylation at specific Ser and Thr residues in its C-terminus

has been shown to enhance stability and also to increase its transactivation capacity (Bannister and Kouzarides, 1995; Chen *et al*, 1996). In addition, higher levels of active c-Fos, and of AP-1 complexes, are reported to determine a postinductional attenuation of c-Fos expression through a transrepressive feedback mechanism (Cahill, 1997) that could possibly explain the lower levels of c-Fos mRNA expression observed in AD keratinocytes. Apart from being directly responsible for a more pronounced transactivation of AP-1 target genes, higher levels of phosphorylation of Jun and Fos proteins could also improve functional interaction with coactivators, finally leading to a more efficient stimulation of Jun/Fos activity, as previously demonstrated (Bannister and Kouzarides, 1995).

In addition to the specific alterations in AP-1 activation that we found in cultures of AD keratinocytes, immunohistochemical analysis revealed that expression of all AP-1 members was significantly altered in AD skin, suggesting an important role for these transcription factors in AD pathophysiology. Quite interestingly, the staining pattern was remarkably similar for all the factors investigated, with a peculiar increase of cytoplasmic, perinuclear staining predominantly localized in the keratinocytes of the basal and, less evident, the upper spinous and granular layers. Moreover, nuclear staining for JunD and c-Fos was lost. A number of processes linked to the specific function of transcription factors could interfere with epitope availability, and hence with an accurate evaluation of transcription factor levels determined by immunohistochemistry. In particular, appreciation of nuclear staining could be altered by conformational changes induced by the degree and sites of phosphorylation, transcription factor dimerization, or binding to gene promoters. Furthermore, any speculation on the meaning of these alterations in AP-1 signals is complicated by the fact that these factors are involved in the regulation of various keratinocyte-specific genes, including those associated with proliferation and differentiation (Basset-Seguin *et al*, 1990; Welter and Eckert, 1995).

The mechanisms that underlie the selective, excessive activation of c-Jun, JunB, and c-Fos in AD keratinocytes are presently unknown. It is possible, however, that abnormal function of diacylglycerol (DAG)-dependent protein kinase C (PKC) isoforms contributes to enhanced AP-1 activation (Rutberg *et al*, 1996). In fact, the epidermis of AD patients is characterized by a marked decrease in the content of ceramides that causes a dysfunction in the cutaneous permeability barrier (Murata *et al*, 1996). Ceramides can compete with the activating binding of DAGs on distinct PKC isozymes, and interfere with PKC functions (Jones and Murray, 1995). A defect in ceramide generation could therefore result in enhanced PKC activation, leading to an exaggerated AP-1 activation and, eventually, to hyperproduction of GM-CSF and other proinflammatory cytokines by AD keratinocytes. In conclusion, we provide initial evidence that enhanced cytokine production by keratinocytes and skin hyperreactivity of AD patients may be secondary to dysregulated activation of transcription factors of the AP-1 family. Inasmuch as epithelial cells can play an important role in the pathogenesis of atopic diseases, and are a useful target for therapeutic intervention, studies on the mechanisms that regulate expression of inflammatory genes in epithelial cells should ultimately afford new molecular strategies for the control of atopic diseases.

This work was supported by grants from the Consiglio Nazionale delle Ricerche, the Ministero della Sanità, the Associazione Italiana per la Ricerca sul Cancro, the Istituto Superiore di Sanità (AIDS project), and the European Community (BIOMED 2 contract BMH4 CT98–3713).

REFERENCES

Albanesi C, Cavani A, Girolomoni G: Interleukin-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes. Synergistic or antagonist effects with interferon-γ and tumor necrosis factor-α. *J Immunol* 162:494–502, 1999

Arenzana-Seisdedos F, Thompson J, Rodriguez MS, Bachelerie F, Thomas D, Hay RT: Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NF-κB. *Mol Cell Biol* 15:2689–2696, 1995

Awane M, Andres PG, Li DJ, Reinecker H-C: NF-κB-inducing kinase is a common mediator of IL-17-, TNF-α-, and IL-1β-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 162:5337–5344, 1999

Bannister AJ, Kouzarides T: CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J* 14:4758–4762, 1995

Basset-Seguin N, Escot C, Moles JP, Blanchard JM, Kerai C, Guilhou JJ: C-fos and c-jun proto-oncogene expression is decreased in psoriasis: an *in situ* quantitative analysis. *J Invest Dermatol* 97:672–678, 1990

Bratton DL, Hamid Q, Boguniewicz M, Doherty DE, Kailey JM, Leung DYM: Granulocyte/macrophage colony-stimulating contributes to enhanced monocyte survival in chronic atopic dermatitis. *J Clin Invest* 95:211–218, 1995

Cahill MA: c-Fos transrepression revisited. *FEBS Lett* 400:9–10, 1997

Calderón MA, Devalia JL, Prior AJ, Sapsford RJ, Davies RJ: A comparison of cytokine release from epithelial cells cultured from nasal biopsy specimens of atopic patients with and without rhinitis and nonatopic subjects without rhinitis. *J Allergy Clin Immunol* 99:65–76, 1997

Chan SC, Brown MA, Wilcox TM, *et al*: Abnormal IL-4 gene expression by atopic dermatitis T lymphocytes is reflected in altered nuclear protein interactions with IL-4 transcriptional regulatory element. *J Invest Dermatol* 106:1131–1136, 1996

Chen R-H, Juo PC-H, Curran T, Blenis J: Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. *Oncogene* 12:1493–1502, 1996

Deng T, Karin M: JunB differs from c-Jun in its DNA binding and dimerization domains and represses c-Jun by formation of inactive heterodimers. *Genes Dev* 7:479–490, 1993

Efimova T, LaCelle P, Welter JF, Eckert RT: Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP-1 signal transduction. *J Biol Chem* 273:24387–24395, 1998

Fetrow JS, Siew N, Skolnick J: Structure-based functional motif identifies a potential disulfide oxidoreductase active site in the serine/threonine protein phosphatase-1 subfamily. *FASEB J* 13:1866–1874, 1999

Gasson JC: Molecular physiology of GM-CSF. *Blood* 77:1131–1145, 1991

Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Dermato-Venereologica* 92:44–47, 1980

Ip YT, Davis RJ: Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. *Curr Op Cell Biol* 10:205–209, 1998

Jenkins F, Cockerill PN, Bohmann D, Shannon MF: Multiple signals are required for function of the human GM-CSF gene promoter in T cells. *J Immunol* 155:1240–1251, 1995

Jones MJ, Murray AW: Evidence that ceramide selectively inhibits protein kinase C-α translocation and modulates bradykinin activation of phospholipase D. *J Biol Chem* 270:5007–5013, 1995

Jurghans V, Gutgesell C, Jung T, Neumann C: Epidermal cytokines IL-1β, TNF-α, and IL-12 in patients with atopic dermatitis: response to application of house dust mite antigens. *J Invest Dermatol* 111:1184–1188, 1998

Kaushansky K: Control of GM-CSF production in normal endothelial cells by positive and negative regulatory elements. *J Immunol* 143:2525–2529, 1989

Kaushansky K, O'Rork C, Shoemaker SG, McCarthy J: The regulation of GM-CSF is dependent on a complex interplay of multiple nuclear proteins. *Mol Immunol* 33:461–470, 1996

Khurana Hershey GK, Friedrich MF, Esswein LA, Thomas ML, Chatila TA: The association of atopy with a gain-of-function mutation in the a subunit of the interleukin-4 receptor. *N Engl J Med* 337:1720–1725, 1997

Lane SJ, Adcock IM, Richards D, Hawrylowicz C, Barnes PJ, Lee TH: Corticosteroid-resistant bronchial asthma is associated with increased c-Fos expression in monocytes and T-lymphocytes. *J Clin Invest* 102:2156–2164, 1998

Li B, Tournier C, Davis RJ, Flavell RA: Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J* 18:420–432, 1999

Malter JS: Posttranscriptional regulation of mRNAs important in T cell function. *Adv Immunol* 68:1–49, 1998

Marini M, Vittori E, Hollemborg J, Mattoli S: Expression of the potent inflammatory cytokines, GM-CSF, IL-6, and IL-8 in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 89:1001–1009, 1992

Masuda ES, Yamaguchi-Iwai Y, Tsuboi A, Hung P, Arai K, Arai N: The transcription factor Sp1 is required for induction of the murine GM-CSF promoter in T cells. *Biochem Biophys Res Commun* 205:1518–1525, 1994

McDonald PP, Bald A, Cassatella MA: Activation of the NF-κB pathway by inflammatory stimuli in human neutrophils. *Blood* 89:3421–3433, 1997

Murata Y, Ogata J, Higaki Y, *et al*: Abnormal expression of sphingomyelin acylase in atopic drmatitis: an etiologic factor for ceramide deficiency? *J Invest Dermatol* 106:1242–1249, 1996

Nimer SD, Zhang W, Kwan K, Wang Y, Zhang J: Adjacent, cooperative elements form a strong, constitutive enhancer in the human GM-CSF gene. *Blood* 87:3694–3703, 1996

Nonaka M, Nonaka R, Jordana M, Dolovich J: GM-CSF, IL-8, IL-1R, TNF-αR and HLA-DR in nasal epithelial cells in allergic rhinitis. *Am J Respir Crit Care Med* 153:1675–1681, 1996

Pastore S, Fanales-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G:

Granulocyte/macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. *J Clin Invest* 99:3009–3017, 1997

Pastore S, Corinti S, La Placa M, Didona B, Girolomoni G: Interferon-γ promotes exaggerated cytokine production in keratinocytes cultured from patients with atopic dermatitis. *J Allergy Clin Immunol* 101:538–544, 1998

Postma DS, Bleecker ER, Amelung PJ, *et al*: Genetic susceptibility to asthma – bronchial hyperresponsiveness coinherited with a major gene for atopy. *N Engl J Med* 333:894–900, 1995

Ray A, Cohn L: Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *J Clin Invest* 104:985–993, 1999

Risse-Hackl G, Adamkiewicz J, Wimmel A, Schuermann M: Transition from SCLC to NSCLC phenotype is accompanied by an increased TRE-binding activity and recruitment of specific AP-1 proteins. *Oncogene* 16:3057–3068, 1998

Rutberg SE, Saez E, Glick A, Dlugosz AA, Spiegelman BM, Yuspa SH: Differentiation of mouse keratinocytes is accompanied by PKC-dependent changes in AP-1 proteins. *Oncogene* 13:167–176, 1996

Sampath D, Castro M, Look DC, Holtzman MJ: Constitutive activation of epithelial signal transducer and activator of transcription (STAT) pathway in asthma. *J Clin Invest* 103:1353–1361, 1999

Schreiber E, Matthias P, Müller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucl Acid Res* 17:6419–6424, 1989

Shannon MF, Coles LS, Vadas MA, Cockerill PN: Signals for activation of the GM-CSF promoter and enhancer in T cells. *Crit Rev Immunol* 17:301–323, 1997

Shaw G, Kamen R: A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659–667, 1986

Song Z, Casolaro V, Chen R, Georas SN, Monos D, Ono SJ: Polymorphic nucleotides within the human IL-4 promoter that mediate overexpression of the gene. *J Immunol* 156:424–429, 1996

Sousa AR, Paston RN, Lane SJ, Nakhosteen J, Lee TH: Detection of GM-CSF in asthmatic bronchial epithelium and decrease by inhaled corticosteroids. *Am Rev Respir Dis* 147:1557–1561, 1993

Tanaka M: Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators *in vivo*. *Proc Natl Acad Sci USA* 93:4311–4315, 1996

Thomas RS, Tymms MJ, McKinlay LH, Shannon MF, Seth A, Kola I: ETS1, NFκB, and AP1 synergistically transactivate the human GM-CSF promoter. *Oncogene* 14:2845–24855, 1997

Tsuboi A, Muramatsu M, Tsutsumi A, Arai K, Arai N: Calcineurin activates transcription from the GM-CSF promoter in synergy with either protein kinase C or NF-κB/AP-1 in T cells. *Biochem Biophys Res Commun* 199:1064–1072, 1994

Welter JF, Eckert RL: Differential expression of the fos and jun family members c-fos, fosB, Fra-1, Fra-2, c-jun, junB and junD during human epidermal keratinocyte differentiation. *Oncogene* 11:2681–2268, 1995

Wierenga EA, Walchner M, Kick G, Kapsenberg ML, Weiss EH, Messer G: Evidence for suppressed activity of the transcription factor NFTA1 at its proximal binding element *P0* in the *IL-4* promoter associated with enhanced *IL-4* gene transcription in T cells of atopic patients. *Intern Immunol* 11:297–306, 1999

Xing Z, Gauldie J, Tremblay GM, Hewlett BR, Addison C: Intradermal transgenic expression of granulocyte-macrophage colony-stimulating factor induces neutrophilia, epidermal hyperplasia, Langerhans' cell/macrophage accumulation, and dermal fibrosis. *Lab Invest* 77:615–622, 1997