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Atopic Dermatitis Is Associated with a Functional Mutation in the Promoter of the C-C Chemokine RANTES

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Up-regulation of C-C chemokine expression characterizes allergic inflammation and atopic diseases. A functional mutation in the proximal promoter of the RANTES gene has been identified, which results in a new consensus binding site for the GATA transcription factor family. A higher frequency of this allele was observed in individuals of African descent compared with Caucasian subjects (p < 0.00001). The mutant allele was associated with atopic dermatitis in children of the German Multicenter Allergy Study (MAS-90; p < 0.037), but not with asthma. Transient transfections of the human mast cell line HMC-1 and the T cell line Jurkat with reporter vectors driven by either the mutant or wild-type RANTES promoter showed an up to 8-fold higher constitutive transcriptional activity of the mutant promoter. This is the first report to our knowledge of a functional mutation in a chemokine gene promoter. Our findings suggest that the mutation contributes to the development of atopic dermatitis. Its potential role in other inflammatory and infectious disorders, particularly among individuals of African ancestry, remains to be determined. The Journal of Immunology, 2000, 164: 1612-1616.

A topic dermatitis (AD)

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is a chronic inflammatory skin disease that affects 10–15% of the population during infancy. While the molecular basis is currently unclear, infiltration of the skin with eosinophils, T cells, and monocytes has been well documented (1). Several studies have suggested a crucial role of C-C chemokines in the pathogenesis of allergic inflammation. The C-C chemokines preferentially activate and attract eosinophils, basophils, monocytes, and T cell subsets (2). Tissue eosinophilia is pronounced after allergen exposure and is a characteristic finding in allergic inflammation seen in various atopic disorders including AD and asthma (1, 3). Evidence for linkage of asthma to chromosome 17p12-17q11.2 (C-C chemokine cluster) in African American sib pairs has been reported (4), suggesting that functional mutations in C-C chemokine-encoding genes or their regulatory elements may contribute to the pathogenesis of asthma or associated phenotypes.

RANTES is one of the most extensively studied C-C chemokines in allergic and infectious disease. It is a potent chemotactic agent for eosinophils, lymphocytes, monocytes, and basophils (2, 5, 6). RANTES is expressed in activated T lymphocytes, airway epithelial cells, platelets, fibroblasts, and renal epithelial and mesangial cells (reviewed in Ref. 7). Glucocorticoids, the most effective drugs in the treatment of allergic inflammation, decrease the expression of RANTES and other eosinophil-active chemokines in vivo and in vitro (8–11). RANTES has also been studied in the context of HIV infection, because CCR5, a receptor for RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β, has been identified as a coreceptor for macrophage-tropic HIV type 1 strains (12, 13).

Because of the potential role of RANTES in allergic and infectious diseases, we screened populations of both African and Caucasian ancestry for mutations in the proximal promoter region of the RANTES gene that may affect transcriptional activity and subsequently RANTES expression in various cell types. In this communication, we demonstrate a novel functional mutation in the proximal promoter region of the RANTES gene. The mutation is significantly more frequent in individuals of African ancestry than in Caucasian subjects. Moreover, we could demonstrate an association with AD, but not with asthma, in our study populations.

Materials and Methods

Study populations

German population. Two hundred eighty-six unrelated children of the German Multicenter Allergy Study (MAS-90) (14) were genotyped; 188 subjects had been diagnosed with AD at two or more of five visits during the first 3 years of life and were compared with 98 control subjects (50 allergic, 48 nonallergic based on serum IgE Abs >0.35 kU/L to food or inhalant allergens). AD was defined as previously described (15). The definition of AD was based on 1) physician’s diagnosis, 2) manifestation of “dry skin,” and 3) three or more symptoms of AD at three or more areas. Based on these criteria, children were examined once a year (every birth day ± 4 wk), and those who were diagnosed with AD at two or more time points were selected for the present study.

Afro-Caribbean population. Thirty-three nuclear and extended Afro-Caribbean families (n = 713), described in detail previously (16), were recruited in Barbados, West Indies.
African American and Caucasian American populations. Sixty-three predominantly nuclear African American families and 48 nuclear and extended Caucasian families were recruited at the Johns Hopkins Asthma and Allergy Center as part of the Collaborative Study on the Genetics of Asthma (CSGA) (4). No valid assessment of AD has been available for any of these populations of African ancestry. The definition of asthma for Caucasian and African American study populations was based on strict criteria according to the study protocol of the CSGA (4), which included 1) physician’s diagnosis of asthma, 2) a fall in baseline FEV1 by ≥20% at ≥25 mg/ml methacholine or ≥15% increase in FEV1 after bronchodilator use, 3) more than or equal to two asthmatic symptoms (cough, wheeze, dyspnea), and 4) ≤5 pack years of smoking cigarettes. The clinical phenotypes of affected subjects, including their mean age and gender distributions, were described previously in Ref. 4. Subjects of Afro-Caribbean population, asthma was defined as having 1) a reported history of asthma using a standardized questionnaire adapted from the CSGA study (4), and 2) confirmation of asthma by a physician. The asthmatic populations were not described in full detail, because no significant association of the identified mutation with asthma was found in any of the three large populations genotyped.

Single-stranded conformation polymorphism (SSCP)

For sequencing, PCR products from unrelated individuals homozygous for the −401A (n = 3) or −401G allele (n = 3) were first cloned into a TA vector (Original TA Cloning Kit; Invitrogen, San Diego, CA) according to the manufacturer’s directions. Plasmid DNA templates were sequenced using the fluorescent dye terminator method of cycle sequencing on a Perkin-Elmer, Applied Biosystems Division 373A following Applied Biosystems protocols (Perkin-Elmer, Norwalk, CT).

DNA sequencing

For sequencing, PCR products from unrelated individuals homozygous for the −401A (n = 3) or −401G allele (n = 3) were first cloned into a TA vector (Original TA Cloning Kit; Invitrogen, San Diego, CA) according to the manufacturer’s directions. Plasmid DNA templates were sequenced using the fluorescent dye terminator method of cycle sequencing on a Perkin-Elmer, Applied Biosystems Division 373A following Applied Biosystems protocols (Perkin-Elmer, Norwalk, CT).

Transient transfections and cell culture

Two reporter constructs containing either the −401A- or the −401G-expressing RANTES promoter fused to the luciferase structural gene were used for transient transfections of human mast cell line HMC-1, Jurkat, and BEAS-2B cells. Base pairs −885 to +64 of the RANTES gene (−401A, a generous gift of Dr. T. Schall) were cloned into the KpnI site of pGL2 basic vector (Promega, Madison, WI). Site-directed mutagenesis at position −401 (A to G) was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions with oligonucleotides containing the region between base pairs −416 and −386 of the RANTES promoter (−416-GAGGGAGTAAAGTCTCTGAT-386 and its complement; bold indicated where the base pair exchange occurs). Plasmid DNA was obtained with double-cesium chloride purification (BioServe Biotechnologies, Laurel, MD). HMC-1 cells were cultured at 0.5 × 10^6/ml in RPMI 1640 in the presence of 10% FCS 24 h before transient transfections. Jurkat cells were cultured at 0.5 × 10^6/ml in RPMI 1640 containing 2% FCS before transfection. BEAS-2B cells were cultured as previously described (8). The SuperFect reagent (Qiagen, Santa Clarita, CA) was used for transient transfections of Jurkat and HMC-1 cells according to the manufacturer’s directions. Two micrograms of plasmid DNA and 10 μl SuperFect reagent were used for transfection of 1 × 10^6 Jurkat cells. Three microliters of Fugene reagent (Boehringer Mannheim, Mannheim, Germany) and 1 μg plasmid DNA were used for transient transfection of BEAS-2B. Transfections with the promoterless pGL2 basic, RANTES −401A/pGL2, and RANTES −401G/pGL2 were always performed in duplicates.

Luciferase assay

Luciferase expression was monitored by chemiluminescence of cell lysates 12–72 h after transfections using the Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratory, Ann Arbor, MI) as recommended by the manufacturer. Total protein content of cell lysates was determined with Bio-Rad protein assays (Bio-Rad, Hercules, CA). Luciferase activity was measured in a luminometer analyzer (Monolight 3010; Analytical Luminescence Laboratory).

EMSA

Protein extraction from the nuclei of Jurkat and HMC-1 cells and EMSAs were performed as previously described (17). The probes for EMSAs were two 18-bp double-stranded oligonucleotides containing the RANTES promoter sequence between base pairs −409 and −392 of the −401A (5′-GAAAGGAGATAAGTCG-3′ and its complement) or −401G (5′-GAAAGGGGATAAGTCG-3′ and its complement). Abs to GATA-1, GATA-2, and GATA-3 proteins and competitor oligonucleotides (GATA, OCTA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analyses

Pearson χ^2 tests were used to compare genotype frequencies. Wilcoxon nonparametric tests were performed to assess differences in transcriptional activities of −401A and −401G RANTES promoter-driven constructs. Sib-pair analysis and the transmission disequilibrium test for qualitative traits were conducted as described before (16).

Results

Mutational analysis of RANTES promoter

Using SSCP and DNA sequencing, we identified a point mutation in the RANTES promoter at base pair −401 (A −401G) that results in an additional consensus binding site for the GATA transcription factor family (Fig. 1). Frequency of mutant allele and association with AD

We screened three populations of African descent with varying degrees of Caucasian admixture and three Caucasian populations for both promoter alleles using SSCP. Significant ethnic differences in genotype frequencies were observed: 15% of Afro-Caribbeans and African Americans were homozygous for the −401A allele compared with ≤2.1% in either Caucasian population (p < 0.00001; Table I). Although excess allele sharing at this two-allele
marker was observed among both African American and Afro-Caribbean asthmatic sib pairs (mean identical-by-descent 0.54/0.27 for 143/27 full/half sibs; \( p = 0.016 \)), no higher transmission rates of the mutant allele to asthmatic offspring were detected using the transmission disequilibrium test. These findings suggest that a mutation different from, but in close proximity to, the RANTES gene may contribute to asthma pathogenesis in populations of African descent.

However, the −401A allele was more frequent in 188 German children with AD compared with 98 control subjects (\( p < 0.037 \), Table II). The distributions of −401A allele frequency in the control populations were consistent with the expected Hardy-Weinberg equilibrium. All tested children were participants of the German Multicenter Allergy Study (MAS-90), a noninterventional, prospective, birth cohort study (14). Valid assessments of AD have not yet been performed in any of the other genotyped study populations.

Functional analysis of RANTES promoter polymorphism

To test for functional differences between the two promoter variants, we performed transient transfections in two human cell lines with luciferase-reporter constructs driven by either the −401A- or −401G-expressing RANTES promoter. Both mast cells and T lymphocytes express GATA-binding transcription factors (18). HMC-1 and Jurkat cells express RANTES mRNA (7, 19) and were therefore selected for comparative studies of transcriptional activities of the two RANTES promoter constructs. In both cell lines, we detected significantly higher constitutive transcriptional activities of the −401A-expressing promoter (Fig. 2, A and B). We were not able to identify stimuli that differentially up-regulated transcriptional activity from the two reporter constructs, possibly due to the either large number of other cis-regulatory elements in the RANTES promoter or to constitutively elevated nuclear expression of GATA family members in these cells. Transient transfections of the bronchial epithelial cell line BEAS-2B with the same promoter constructs did not show differences in transcriptional activities (\( n = 4 \), mean fold difference 1.1, \( p = 0.47 \); Fig. 2C).

EMSAs showed clear cut differences in binding of nuclear proteins extracted from both HMC-1 and Jurkat cells to probes containing the sequence between base pairs −409 and −392 of the wild-type and the mutant RANTES promoter. Using HMC-1 extracts, treatment of the complexes with Abs directed against GATA-1 or -2 resulted in supershifts of two DNA-protein complexes, formed exclusively with the mutant (−401A) probe, whereas anti-GATA 3 had no effect on the pattern of complex formation (Fig. 3). Using Jurkat extracts, supershifts were observed when complexes (−401A probe) were treated with anti-GATA-3, whereas anti-GATA -1 and -2 had no effect on the pattern of complex formation (Fig. 4). Taken together, these findings are consistent with published GATA binding protein expression patterns in T lymphoid cells and mast cells (18). As expected, very weak binding, and no difference in the pattern of complex formation with the two probes, was observed using nuclear extracts from BEAS-2B (data not shown).

Discussion

The genetic basis of AD is currently unclear. It is thought to contain highly interactive and polygenic components, each with varying degree of genetic influence. In this study, we identified a novel, functional mutation in the proximal promoter region of the RANTES gene. The point mutation at base pair −401 results in a new consensus binding site for the GATA transcription factor family and is strikingly more frequent in individuals of African descent compared with Caucasian subjects. We observed an association of the mutation with AD in a longitudinally well-characterized German cohort, but no association with asthma in any study population, suggesting that the mutation contributes, in part, to the development of AD in Caucasian populations. Additional studies,
FIGURE 3. Binding of HMC-1 nuclear proteins to the GATA binding element in the −401A RANTES promoter. Radiolabeled oligonucleotides incorporating the region from base pairs −409 to −392 of the −401A and −401G RANTES promoter alleles were used in EMSAs with nuclear extracts from unstimulated HMC-1 cells. Clear cut differences in binding to the two probes were observed (lanes 1 and 2). Furthermore, addition of anti-GATA-1 and -2 Abs resulted in supershifts of two DNA-protein complexes (lanes 3 and 4).

FIGURE 4. Binding of Jurkat nuclear proteins to the GATA binding element in the −401A RANTES promoter. Radiolabeled oligonucleotides incorporating the region from base pairs −409 to −392 of the −401A (A) and −401G (B) RANTES promoter alleles were used in EMSAs with nuclear extracts from unstimulated Jurkat cells. Clear cut differences in binding to the two probes were observed (lane 1 in A and B). Furthermore, addition of anti-GATA-3 Abs resulted in a supershift of the specific DNA-protein complex (lane 4, A). Non-specific binding (NS) was observed in both assays. Reduction or elimination of the bands resulting from non-specific binding was seen after competing with nonradioabeled DNA fragments (100-fold molar excess) that were specific (GATA; lanes 6, A and B) or non-specific (OCTA; lanes 7, A and B) for GATA binding proteins.

are expressed in eosinophils (21), mast cells, basophils, and megakaryocytes (22, 24).

The expression of RANTES is differentially regulated in various cell types, and a large number of putative cis-acting elements have been described in the promoter region (7). A recent study showed that disruption of any of four putative binding sites for NF-kB in the upstream region of the RANTES gene resulted in markedly reduced promoter activity in T cell and monocytic cell lines (25), indicating that multiple binding sites for a specific transcription factor may cooperate in the enhancement of promoter activity. Therefore, it is of interest that in addition to the GATA binding motif that we identified at base pair −401, two additional GATA consensus elements are located 755 and 786 bp upstream of the RANTES transcription start site (7), further implicating GATA-binding transcription factors in the regulation of RANTES gene expression. Furthermore, functional analyses of the mutant and wild-type alleles in HMC and Jurkat T-cell lines, both of which express RANTES and GATA binding proteins, showed that 1) a significantly higher transcriptional activity of the −401A mutant allele is found in both cell lines, and 2) differential binding of GATA transcriptional factor family is seen between HMC and Jurkat cells.

Airway epithelial cells are a major source of RANTES (8). However, no significant differences in transcriptional activity between wild-type and mutant RANTES promoters were observed in transiently transfected BEAS-2B cells. GATA expression in epithelial cells has not been reported, which may explain these in vitro findings. In contrast, increased RANTES production in T cells, mast cells, or megakaryocytes, cell types that constitutively express GATA binding proteins (18, 20) and RANTES (6, 26, 27), may contribute to the pathogenesis of AD. Up-regulation of RANTES expression in the skin could enhance the recruitment of eosinophils, lymphocytes, and monocytes to the sites of allergic inflammation. Severe pruritus and excoriations are hallmarks of AD associated with bleeding and subsequent activation of platelets. Because megakaryocytes express GATA binding proteins and significant quantities of RANTES, increased RANTES content of platelets of carriers of the −401A mutation could further explain the association of the mutant allele with AD.

A strikingly higher frequency of the RANTES −401A allele was observed in individuals of African ancestry compared with Caucasian subjects. In light of these ethnic differences, it is intriguing that basic differences in C-C chemokine receptor biology have been described in individuals of African vs Caucasian descent. First, a 32-bp deletion in the CCR5 receptor, a common receptor for RANTES, MIP-1α, and MIP-1β, and a co-receptor for macrophage-tropic HIV strains (28), is found in >10% of Caucasians, whereas this mutation is absent in African populations (29). Second, in contrast to Caucasian individuals, the Duffy Ag receptor for chemokines (DARC) is not expressed on RBC in the vast majority of African people, while it is uniformly expressed on RBC in Caucasians. Lack of DARC on RBC confers an evolutionary advantage because DARC-negative RBC are resistant to infection by Plasmodium vivax (30).

Therefore, it would be of interest to determine the biological role of the −401A-expressing RANTES promoter in AD and other inflammatory and infectious disorders in populations of African descent.

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**Note added in proof.** A recent publication by Liu et al. (1999 *Proc. Natl. Acad. Sci USA* 96:4581) showed an identical polymorphism as we identified in our study populations. However, the numbering of the polymorphic residue is different due to the fact that two additional nucleotides were found in the promoter region of RANTES in their study population. Our numbering was based on the original publication by Nelson et al. (7) and by GenBank database (accession no. S64885).

**References**