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Variant *FCGR3* Genes: Transcription and Possible Origins

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SUMMARY The Fc receptors for human immunoglobulin G (FcγR) IIIb are encoded by genes clustered on the long arm of chromosome 1 (band q21→24) and exhibit allelic polymorphisms. Several rare *FCGR3B* sequences were identified in both white and black donors. However, the origins of these genomic variants are unknown and their transcription has not yet been investigated. Blood from a donor with known *FCGR3* variants was used to extract DNA from peripheral blood $CD34^+$ cells, $CD19^+$ B-cells, neutrophils and buccal cells, after which *FCGR3* gene sequencing was performed. Additionally, RNA samples from 5 Caucasian individuals containing known variant *FCGR3* genes were reverse-transcribed to cDNA and the *FCGR3* genes were sequenced. Our results showed that the frequencies of variant clones were higher in B-cell preparations than in $CD34^+$ hematopoietic progenitor cells from peripheral blood and neutrophils. Very high variant frequencies were found in buccal cell-derived clones. Variant cDNA sequences were identified in three of five individuals with known *FCGR3* variants. We conclude that *FCGR3* gene variants are differentially transcribed between cell types and tissues, increasing the likelihood of the presence of variant FcγRIII receptors on the cell surface. The significance of the high number of variant clones in buccal cells, however, is unclear.

The Fc gamma receptor IIIb (FcγRIIIb) receptor is expressed on polymorphonuclear neutrophils in humans. It is encoded by the *FCGR3B* gene and exhibits allelic polymorphism. Recently, several rare *FCGR3B* sequences have been identified in white and black donors. Flesch *et al.*[1] and Matsuo *et al*.[2] described 10 different variant genes. We also found several variant genes in a previous study but it is not known whether these variants are caused by somatic mutations or multiple gene loci.[3] Furthermore, it is unclear whether the mutations, observed at the genomic DNA level, are also present at the transcriptional level and thus subsequently translated to protein variants. In this study, we sequenced DNA isolated from peripheral blood hematopoietic progenitor cells, buccal cells and B-cells from a Caucasian individual with variant *FCGR3* forms to obtain further insight as to whether these variants arise due to higher gene loci or by somatic hypermutation. Additionally, *FCGR3* transcripts from neutrophils of individuals with variant *FCGR3* forms were sequenced in order to ascertain if these variants are present at the transcriptional level.

MATERIALS AND METHODS

Subjects

Five healthy Caucasian volunteers were in-

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cluded in the study. Three were females of 28, 45, and 46 years of age while two were males of 42 and 60 years of age. The individual providing the CD34 cells and B cells was male and aged 42. The work was approved by the Ethics Committee of the Medical Faculty of the Christian-Albrechts-University of Kiel, Germany.

Separation of cells

Peripheral blood hematopoietic progenitor cells and B-cells were isolated from a Caucasian individual with known *FCGR3* variants. A concentrated peripheral mononuclear cell fraction was obtained by leukapheresis using the Cobe-Spectra Apheresis System with a special monocyte program. $CD34^+$ hematopoietic progenitor cells were preselected by two passages on anti-HPCA-1 coated magnetic bead columns (CliniMACS cell selection system, Miltenyi Biotec, Germany). Following incubation with a different antibody for CD34 (anti-HPCA-2)-PE, the concentrated $CD34^+$ hematopoietic progenitor cells were obtained by cell sorting (Epics Ultra, Coulter, Germany) to a purity of 78.7%.

The remaining CD34-negative cell fraction was used for the separation of B-cells. Cells were incubated with CD19 Microbeads and then $CD19^+$ B-cells were enriched by two rounds on a CD19 magnetic micro-bead column (Miltenyi Biotec). These cells were further purified by cell sorting (Epics Ultra, Coulter) using an FITC conjugated anti-CD20 antibody. The purity of $CD20^+$ B-cells after sorting was 97.6%. Granulocytes were isolated from peripheral blood by sequential removal of mononuclear cells and red blood cells, with a purity of approximately 95%, as described previously.[6] Buccal swabs were taken from the same individual and used for DNA isolation. The protocol for this study was approved by the Institutional Review Board.

DNA isolation

DNA was isolated from hematopoietic progenitor cells and B-cells using the Nucleospin Blood Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA from buccal cells was extracted with the Purgene DNA Isolation Kit (Puregene, Gentra Systems, Minneapolis, MN, USA).

cDNA preparation

Total RNA was extracted from ~5 x 10^6 granulocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and resolved in 30µl RNase-free water. RNA was reverse-transcribed to cDNA (Reverse Transcription System Kit, Promega, USA) according to the manufacturer's instructions. Subsequently, samples were heated to 95°C for 5 minutes and then incubated at 0°C for 5 minutes.

***FCGR3B* allele-specific DNA amplification**

Allele-specific DNA amplification was performed using an established protocol[1]. Briefly, 100 ng of genomic DNA was added to a mixture containing 0.5 µM each of *FCGR3*-specific sense and antisense primers (Biometra, Göttingen, Germany), 0.125 µM each of human growth hormone sense and antisense primers (Biometra), 0.2 µM dNTPs (Roche, Germany), and 0.75 U of *Taq* DNA Polymerase (Roche, Mannheim, Germany) in a total reaction volume of 20 µl that incorporated a PCR buffer with 1.5 mM $MgCl_2$ (Roche). DNA was amplified using 30 cycles of denaturation (95°C for 30s), annealing (60°C for 1 min) and extension (71°C for 30s). PCR products were fractionated on 1.5% agarose gels and stained by ethidium bromide.

Cloning and sequencing

The *FCGR3B*3*-specific sense primer and *FCGR3B*1*-specific antisense primer spanning the region 5'-95 to 348-3' were used to amplify the coding region of both *FCGR3B* and *FCGR3A*. One hundred nanograms of genomic DNA or 10 µl of cDNA were added to a mixture containing 0.5 µM each of *FCGR3B*3*-specific sense and *FCGR3B*1*-specific antisense primers (Biometra), 0.2 µM dNTPs (Roche) and 0.75 U (2.5 U for cDNA amplification) of AmpliTaqGold Polymerase (PE Applied Biosystems, Germany) in a total volume of 25 µl (100 µl for cDNA amplification) including PCR-buffer (Roche) with 1.5 mM $MgCl_2$ (2.5 mM $MgCl_2$ for cDNA). DNA was amplified via 30 cycles of denaturation (95°C for 30s), annealing (60°C for 1 min) and extension (71°C for 30s) which yielded a 254 bp fragment. Following purification using a QIAquick PCR purification kit (Qiagen), the PCR

product was cloned into the PCR2.1-TOP vector (TOPO TA cloning kit, version K2, Invitrogen, Paisley, UK). Ligations were transformed into chemically-competent *E. coli* TOP10 cells. One hundred microliters of the transformation product was spread onto selective LB agar plates and incubated overnight at 37°C. Blue/white selection was used to inoculate positive colonies in LB medium containing antibiotic. Between 20 to 100 clones per individual cell donor were used for DNA sequencing, as appropriate. Plasmids containing either cDNA or other DNA fragments were extracted from bacterial cultures using a plasmid DNA purification kit (QIAprep Spin Miniprep Kit, Qiagen). Positive recombinant plasmids were confirmed by digestion with *Eco*RI (New England Biolabs, USA) prior to agarose gel electrophoresis (1.5% agarose), and samples producing a 254 bp insert and a 3.9 kb vector were retained for DNA sequencing (BigDye Terminator, PE Applied Biosystems, Weiterstadt, Germany). Sequencing reaction mixtures contained 4 µl of recombinant plasmid DNA (300-500 ng), 1 µl of M13 or T7 sequencing primers (5 pmol/µl, Biometra), 4 µl of terminator ready reaction mix, and 11 µl of deionized water. The sequencing amplification protocol was 2 minutes at 96°C followed by 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 minutes at 60°C. Purifications of the sequencing products were enabled with a DyeEx 2.0 Spin Kit (Qiagen). Sequences were analyzed on an ABI Prism 310 sequencing system (PE Applied Biosystems, Weiterstadt, Germany).

To reduce the probability of reverse transcription and sequencing errors, both experimental procedures were performed twice with DNA extracted from the same individual, which yielded identical results.

Statistical analysis

The Fisher's exact test was used to compare the relative frequencies of variant clones between the buccal cells and the total frequencies from other cells. The total frequencies of variant clones between genomic DNA and cDNA for each proband was also analysed using the Fisher's exact test. A p value < 0.05 was considered to be statistically significant.

RESULTS

DNA sequencing from different cell types

$CD34^+$ hematopoietic progenitor cells and B cells were isolated from a German individual with the *FCGR3B*1-*2+*3-* genotype who carried the *FCGR3* variants 141G, 147C, 227G, 266C, 277A and 141C, 147T, 227G, 266C, 277G. DNA was extracted from the $CD34^+$ hematopoietic progenitor cells and used for *FCGR3B* and *FCGR3A* gene sequencing. Among the 88 clones analyzed, all samples were identified as *FCGR3B*2* and *FCGR3A* clones with the exception of two types of variants, which is identical to previous observations using DNA samples from the same donor's peripheral blood leukocytes (Table 1). The 87 clones derived from B-cells contained these same *FCGR3* variant types, but with a higher relative frequency than in the stem cell preparation (Table 1). Comparable findings were obtained for DNA isolated from peripheral blood granulocytes of the same individual. The buccal swab preparation was devoid of B-cells as adjudged by immunohistologic APAAP-staining with anti-CD19 monoclonal antibody (MAb). Additionally, we could not detect CD16 expression using APPAP (alkaline phosphatase anti-alkaline phosphatase) staining of buccal cells of the same individual or with a gingiva preparation from a control donor (data not shown). Sequencing experiments using buccal cell-derived DNA from the *FCGR3B*2* homozygous individual revealed that 60 from 91 clones contained variant 1 (Table 1) with fewer *FCGR3B*2* and *FCGR3A* clones present than in other cell types.

cDNA sequencing

Five Caucasian individuals with known gene variants were used to generate *FCGR3B* cDNA from granulocytes, which were then sequenced. cDNA variants were found in 4 of the 5 individuals, however the proportions of variants and wild-type genes differed between that of cDNA and genomic DNA samples obtained from the same individual (Table 2). The number of variant clones was much lower than that of the wild-type clones. This was even more pronounced for the cDNA samples, where only 13 variants could be identified out of the 404 clones sequenced from 5 individuals, while genomic DNA samples contained 26 variants out of 124 clones (Ta-

Table 1 Differential *FCGR3*-specific DNA sequences in different cell populations of a *FCGR3B*2* homozygous Caucasian individual

Cell population	nt sequence at positions 141, 147, 227, 266 and 277				
	CTGCA *(FCGR3B*2)*	GCGCG *(FCGR3A)*	CTGCA (var. 1)	CTGCG (var. 2)	Variant clones (%)
CD34$^+$ cells	54	31	2	1	3.4*
B-cells	40	34	6	7	14.9*
Granulocytes	28	10	2	4	13.6*
Buccal cells	21	10	60	0	65.9

The numbers of respective clones are shown for each source of DNA. *$p \leq 0.001$, compared to buccal cells.
nt, nucleotide.

ble 2). The nucleotide exchanges were restricted to the polymorphic positions 141, 147, 227, 266 and 277, which was in keeping with that observed for the DNA variant clones (Tables 1 and 2).

DISCUSSION

The origins and functions of the *FCGR3* variants remain unclear to date. Since FcγR is involved in immune response processes via immune complex binding, one could speculate that variant FcγRs may have altered affinities for immune complexes. In order to determine if variants are present only in mature granulocytes that are actively involved in immune complex binding, or are seen in early progenitor cells, we isolated different cell types from one individual containing known variant *FCGR3* genes. Variant *FCGR3* clones were found within CD34$^+$ progenitor cells but their relative frequencies were clearly lower than in mature granulocytes and B-cells. However, the purity of the CD34$^+$ hematopoietic progenitor cells was low (78.7%) and hematopoietic progenitor cells from peripheral blood may bear the disadvantage of a previous immune complex contact, leaving the question open whether the variants are a product of "affinity maturation". Granulocyte and B-cell derived variant clones were more frequent which led us to suggest that the formation of variant clones predominantly occurs within mature cells. Indeed, changes in CD16 gene expression have been observed during B-cell germinal center transit from centroblasts to centrocytes[7]. We isolated DNA from buccal cells of the same proband to obtain material free of B-cells, which was confirmed by immunohistological staining. Surprisingly, 60 out of a total of 91 sequenced clones derived from buccal cells were identified as variant 1 while only a small minority of *FCGR3A* and *FCGR3B*2* clones were found. The reason for this finding remains unclear. To our knowledge, nothing is known about FcγRIII expression in buccal cells and we were unable to detect FcγRIII expression on these cells. This lack of protein expression makes it difficult to suggest reasons for the existence of these DNA variants.

Should *FCGR3B* variants play a role in altered immune complex binding, it would be necessary to study their transcription or protein expression. Indeed, we could demonstrate that *FCGR3B* variants are present at the cDNA level, albeit with a significantly lower frequency. However, the variant frequencies were not identical at both the genomic DNA and cDNA levels for the same individual. To help eliminate errors during the reverse transcription and sequencing steps, both experimental procedures were performed multiple times using DNA from the same individual with reproducible results. It is possible that some variants were not transcribed into stable RNA and were readily degraded, or were simply not observed in some groups with smaller sample sizes. Nonetheless, our study suggests that some variants in genomic DNA samples also arise when transcribed from RNA into cDNA variants. These

Table 2 Variant *FCGR3*-genomic DNA and cDNA sequences of five Caucasian individuals

Proband no.	PCR typing	Variant design	Variant nt. position					No. of clones		*p** DNA *vs.* cDNA
			141	147	227	266	277	DNA	cDNA	
1	FCGR3B*2	Var. 1	G	C	G	C	A	2/15	1/17	n.s.
2	*FCGR3B*2*	Var. 2	C	T	G	C	G	3/14	0/65	≤ 0.005
3	*FCGR3B*2*	Var. 1	G	C	G	C	A	7/33	0/86	< 0.001
		Var. 2	C	T	G	C	G	2/33	0/86	
		Var. 8	C	C	G	C	A	0/33	1/86	
4	*FCGR3B*1* *FCGR3B*2* *FCGR3B*3*	Var. 1	G	C	G	C	A	3/33	0/129	≤ 0.005
		Var. 2	C	T	G	C	G	0/33	4/129	
		Var. 3	G	C	G	A	A	2/33	0/129	
		Var. 4	G	C	A	C	A	1/33	1/129	
		Var. 6	C	C	A	C	G	2/33	1/129	
		Var. 7	C	C	G	C	G	0/33	1/129	
		Var. 9	G	T	G	C	A	0/33	1/129	
5	*FCGR3B*1* *FCGR3B*2* *FCGR3B*3*	Var. 2	C	T	G	C	G	1/29	3/107	n.s.
		Var. 3	G	C	G	A	A	1/29	0/107	
		Var. 4	G	C	A	C	A	1/29	1/107	
		Var. 5	C	T	G	A	G	1/29	0/107	
		var. 10	C	T	A	C	G	0/29	1/107	

The number of variant clones in correlation to the total number of sequenced clones is given for each individual. The total frequency of variant clones in genomic DNA *vs.* cDNA for each proband was compared. Variation over the total number of genomic and cDNA clones of the five probands was highly significant ($p \leq 0.001$). n.s., not significant.

changes may affect the expression of antigens recognized by HNA-1 and HNA-2 specific antibodies.

Matsuo *et al.*[8] compared HNA-1a and HNA-1b expression in granulocytes in humans possessing both the variant *FcγRIIIB* genes and the wild-type alleles. These researchers showed that HNA-1b expression was affected by polymorphisms in *FcγRIIIB* at position 227 and *FcγRIIIB* at position 277, whereas *FcγRIIIB* polymorphisms at nucleotide positions 141 and 349 appeared more important in the expression of HNA-1a. Studies by Trounstine *et al.*[9] using COS cells (SV40 transformed simian fibroblasts) and hybrid HNA1a-*FcγRIIIB* indicate the roles of polymorphisms at nucleotides 227 and 349 in the expression of HNA-1a and HNA-1b. Substitution of nucleotide 227 in HNA-1a-*FcγRIIIB* with the corresponding nucleotide in HNA-1b-*FcγRIIIB* led COS cells to react strongly with both HNA-1a and HNA-1b MAbs. The replacement of nucleotides at positions 227 and 349 of HNA-1a-*FcγRIIIB* the corresponding residues in HNA-1b-*FcγRIIIB* facilitated a strong reaction of COS cells with HNA-1b-specific MAb while only a weak response was observed with the HNA-1a-specific MAb. Recent studies have shown that Asn65 (227A) and Asp82 (277G) define the HNA-1a antigen while HNA-1b is characterized by Ser65 (227G) and Asn82 (277A-).[10] These results support our findings in that some of the variant *FcγRIIIB* genes could be transcribed and subsequently translated into protein variants. Further investigations are required to define the protein expression levels for these variants. It is not known whether these variants arose due to a natural selection that conferred a selective advantage in the receptor function and immune response or if it is simply the result of random genetic variation. As previously described,[1, 8] the variants are not the result of random mutations but of single nucleotide exchanges at those positions responsible for the *FCGR3* polymorphisms. Because at the respective positions we do not find each of the four possible nucleotides but only those two alternatives present in one of the basic forms, we assume that the variants are the result of recombination events rather than of single nucleotide ex-

changes. We cannot exclude that formation of variant *FCGR3* genes may be caused by *in vitro* artifacts during nucleic acid amplification by PCR. Although these processes can occur both *in vivo* and *in vitro*, the differential frequencies of the variants within different cell types and in genomic and cDNA are more likely to result from *in vivo* recombination events.

New insights into the origins, cellular distribution and transcription of the variant *FCGR3* genes have been provided in the present study. However, the immunologic function and the importance of variant *FCGR3* clones in possible immunization and transfusion-related complications remains unclear, especially in cell types that do not express FcγRIII. Further studies will be necessary in order to shed further light into these unresolved questions.

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