Brief report

Lack of NB1 GP (CD177/HNA-2a) gene transcription in NB1 GP$^-$ neutrophils from NB1 GP–expressing individuals and association of low expression with NB1 gene polymorphisms

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The human neutrophil NB1 glycoprotein (NB1 GP, HNA-2a, CD177) has gained clinical importance for being involved in pulmonary transfusion reactions and immune neutropenias. The NB1 GP shows the unique feature of being expressed only on a neutrophil subpopulation. Recently, we identified splicing defects responsible for an NB1 GP deficiency. In this study, we have investigated the molecular basis of the heterogeneous expression of NB1 GP by separating the 2 neutrophil subpopulations using immunofluorescence followed by single-cell picking or by fluorescence-activated cell sorter. We found a lack of NB1 mRNA in the NB1 GP$^-$ cells that remained constant even after granulocyte colony-stimulating factor (G-CSF) administration. Comparing the cDNA sequences of donors with a large (> 60%) and those with a small (< 40%) NB1 GP–expressing subpopulation, we found 6 polymorphisms. Of the 6, 3 were significantly associated with a small NB1 GP–expressing subpopulation, indicating a genetic basis for NB1 GP nonexpression. (Blood. 2003;102:731-733)

Introduction

The neutrophil-specific NB1 glycoprotein (NB1 GP) was first described in 1971 in a case of neonatal alloimmune neutropenia.[1] Alloantibodies to NB1 GP were later also found in pulmonary transfusion reactions, immune neutropenia after bone marrow transplantation, and drug-induced immune neutropenia. NB1 has been included as HNA-2a in the list of human neutrophil alloantigens (HNAs) and clustered as CD177. NB1 GP has been characterized as a 58- to 64-kDa, glycosylphosphatidyl inositol–linked glycoprotein with 3 N-linked carbohydrate side chains.[2-4] It shows the unique feature of being expressed on a neutrophil subpopulation ranging from 0% to 100%.[5] After identification of NB1 GP deficiency as the result of a splicing defect resulting in mRNA strands containing intron sequences with stop codons, we searched for the reason for its heterogeneous expression.[6] For this, we separated the 2 subpopulations by 2 different methods (laser-assisted microdissection and fluorescence-activated cell sorter), followed by qualitative and quantitative measurements of the mRNA content. Afterward we compared the cDNA sequences of individuals with large and small NB1 GP–expressing subpopulations, searching for differences related to the percentage of NB1-expressing neutrophils.

Study design

After isolation of neutrophils by dextran sedimentation followed by density gradient centrifugation (Ficoll-Paque; Amersham, Uppsala, Sweden) and ammonium chloride lysis of contaminating red cells,[7] we performed immunofluorescence using the NB1 GP–specific monoclonal antibody 7D8, kindly provided by Dr Stroncek (National Institutes of Health, Bethesda, MD). For separation of the 2 subpopulations we used laser-assisted microdissection (PALM laser microbeam system; PALM, Bernried, Germany) and fluorescence-activated cell sorter (Mo Flo; Cytomation, Freiburg, Germany). Using microdissection, 50 to 100 cells were isolated and immediately processed to cDNA synthesis as described elsewhere.[8] After cell sorting, RNA of each subpopulation was isolated by RNeasy Mini Kit (QIAGEN, Hilden, Germany), and after reverse transcription we measured the NB1-specific cDNA: full-length cDNA was amplified using primers positioned in the 3′ and 5′ untranslated regions (bases 4-21 and 1394-1370, respectively; sequence and numbering according to Kissel et al[4]). The polymerase chain reaction (PCR) protocol consisted of denaturation for 6 minutes at 95°C, followed by 40 cycles of a 20-second denaturation at 95°C, a 30-second annealing at 65°C, 3 minutes of elongation at 72°C, and a final elongation of 10 minutes at 72°C (GeneAmp Systems 2400; Perkin Elmer, Weiterstadt, Germany). Sequencing was performed according to the manufacturer's protocols (ABI Prism BigDye Terminator; Perkin Elmer). Quantitative PCR was done on the LightCycler (Roche Mannheim, Germany) technique using primers (positions: 532-552 and 670-647 according to Kissel et al[4]) positioned in the exons around the longest intron of the NB1 gene (intron 5 with 4159 base pair [bp]), avoiding amplification of genomic DNA or incorrectly spliced fragments. Using a hot-start protocol, we started with an initial denaturation of 10 minutes at 95°C followed by 40 cycles with 0 seconds at 95°C, 7 seconds at 64°C, and 10 seconds at 72°C. The products were controlled by melting curve analysis and gel electrophoresis.

Statistical analysis was performed using the Mann-Whitney test for unpaired data. A P value less than .05 was considered significant.

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Table 1. NB1 mRNA copies in NB1 GP$^+$ and GP$^-$ neutrophils

Healthy donors (% NB1 GP$^+$)	n	Median NB1 mRNA formation NB1 GP$^+$ (range)	Median NB1 mRNA formation NB1 GP$^-$ (range)	Median ratio (range)
Unstimulated (20-81)	5	3630 (2382-15 450)	99 (77-308)	28 (12-201)
After G-CSF stimulation (36)	1	5 865 068 (3500 before G-CSF)	223 069 (88 before G-CSF)	26
After G-CSF stimulation (slightly greater than 0)	1	2475 (21 before G-CSF)	2558 (21 before G-CSF)	1

In healthy donors there is a clear difference between NB1 GP$^+$ and NB1 GP$^-$ neutrophils. G-CSF administration causes a 1000-fold up-regulation of NB1 mRNA, but the ratio remains in the magnitude of untreated individuals. G-CSF does not up-regulate the NB1 mRNA content of neutrophils with an extremely low percentage of NB1 GP$^+$ neutrophils. There is no difference between cells with the weakest and the strongest NB1 staining in immunofluorescence.

Results and discussion

NB1 mRNA quantification

We analyzed the neutrophils of 7 different white donors. Of the donors, 5 were healthy individuals with an NB1 GP$^+$ subpopulation ranging from 20% to 81%; the others were investigated after granulocyte colony-stimulating factor (G-CSF) stimulation.

Quantitative LightCycler PCR data (Table 1) showed a large difference in the NB1 mRNA formation of the neutrophil subpopulations from healthy individuals. Compared with the NB1 GP$^-$ polymorphonuclear cell (PMN) supopulation, the NB1 GP$^+$ neutrophils had 28 times (median) more NB1 cDNA, and this was independent of the size (percentage) of the NB1 GP–bearing subpopulation.

After G-CSF stimulation (5 μg/kg body weight given the day before testing), a donor with 36% NB1 GP–expressing PMNs showed an approximately 1000-fold increase in the mRNA content, but the ratio remained constant at 26. Administering G-CSF to a donor with slightly more than 0% NB1 GP$^+$ neutrophils, we found no significant difference in the mRNA content between the PMNs with the strongest and weakest NB1 staining in immunofluorescence. In both individuals the fraction (percentage) of NB1 GP$^+$ neutrophils did not change after G-CSF administration. In an additional individual with 87% NB1 GP–expressing neutrophils the percentage of NB1 GP–expressing PMNs also did not change despite a significant increase in NB1 mRNA formation determined in unseparated neutrophils. The presence of more than 0 copies in NB1 GP$^-$ PMNs can be explained by errors of the cell sorter, which are between 1% and 4%, and nontranslated mRNA fragments.

A qualitative PCR was performed to compare the formation of full-length NB1 cDNA in both neutrophil subpopulations. Complete mRNA formation was found only in the NB1 GP–expressing PMNs but not in the NB1 GP$^-$ neutrophils or the neutrophils from the donor with nearly 0% NB1 GP$^+$ cells (Figure 1). These findings indicate that a lack of (complete) NB1 mRNA formation is responsible for the nonexpression of NB1 GP on a neutrophil subpopulation of NB1 GP$^+$ individuals.

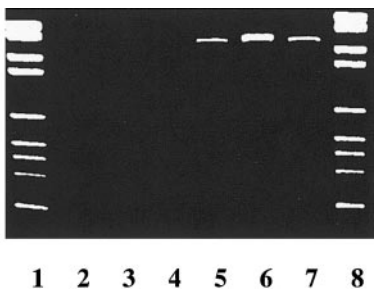

Figure 1. NB1 cDNA in NB1 GP$^-$ and NB1 GP$^+$ neutrophils. Qualitative PCR for full-length NB1 cDNA yielded PCR products with only the cDNA isolated from the NB1 GP$^+$ subpopulation: weight standards (lanes 1 and 8), samples of NB1 GP$^-$ (lanes 2-4), and NB1 GP$^+$ PMNs (lanes 5-7).

Sequence analysis

To answer the question of what may be the cause of the nonexpression of mRNA, we analyzed the full-length NB1 cDNA sequence of several donors who had been characterized as persons with large (> 60%) or small (< 40%) NB1 GP$^+$ subpopulations since distribution of percent NB1 GP$^+$ neutrophils in healthy individuals (n = 46) shows a peak between 10 and 40 and another between 60 and 90. We found 6 polymorphisms causing amino acid exchanges in 5 cases. Of the 5, 3 were significantly associated with the presence of a small NB1 GP$^+$ subpopulation; one other polymorphism was found only in individuals with a large NB1 GP$^+$ subpopulation (Table 2). Since these findings indicated a genetic reason for NB1 GP nonexpression, we analyzed a suspected promotor region including a "tataa" box and spanning from the beginning of exon 1 to 162 bp upstream of exon 1. However, we did not find any polymorphisms associated with the size of the NB1 GP$^+$ subpopulation. Since the cDNA polymorphisms were found in NB1 GP$^+$ neutrophils, it is unlikely that they are the cause of the missing mRNA expression in NB1 GP–lacking PMNs. On the other hand, 5 of the polymorphisms found can cause changes in the tertiary structure of the glycoprotein, which might result in reduced

Table 2. NB1 cDNA polymorphisms

			Polymorphisms					
Donor	% NB1$^+$	mRNA	34 C>G Pro→Ala	119 A>T His→Leu	141 G>A Leu	778 A>C Ile→Leu	1069 G>A Ala→Thr	1318 G>A Gly→Arg
1	83	2212	c/G	a/T	g/A	a/C	g	g
2	81	743	G	a	g	a	g	A
3	81	941	c	a	g	a/C	g/A	g
4	76	1799	G	a	A	a	g	A
5	75	6254	c/G	a	g	a	g	g
6	74	397	G	a	A	a	g	A
7	71	883	G	T	A	a	g	A
8	68	7357	c/G	a	g/A	a/C	g	g
9	63	3074	c	a	g	a	g	g
10	61	6456	c/G	a	g	a	g	g
11	61	3246	c	a	g	a	g	g
12	37	33	G	a	g	a/C	g/A	g
13	36	305	G	a	g	C	A	g
14	32	1386	G	T	A	C	A	g
15	26	272	G	a	g	C	A	g
16	22	66	G	a	g/A	C	g/A	g
17	20	204	G	T	A	C	A	g
18	13	66	G	a	g	C	g/A	g
19	10	463	G	a	g	C	A	g
P		.001	.02	.778	.840	< .001	< .001	.206

NB1 cDNA polymorphisms and their appearance in donors with a large (> 60%, donors 1-11) and a small (< 40%, donors 12-19) NB1 GP$^+$ subpopulation; mRNA: copies/5 × 10^6 cells; 2 letters: heterozygous expression; numbering according to Kissel et al.[6] A significant association can be found between a low percentage of NB1 GP$^+$ neutrophils and the polymorphisms 34C>G, 778A>C, and 1069G>A. The individuals with a small NB1 GP–bearing neutrophil subpopulation show also a significant lower NB1 GP mRNA formation.

antibody binding. The latter may explain the differences observed in the binding of different monoclonal NB1-specific antibodies to neutrophils of some donors.[9-10]

Conclusion

We have shown by NB1 GP mRNA analysis that the NB1 GP⁻ subpopulation results from a transcription defect. This finding, as well as the recently reported finding of NB1 GP mRNA fragments,[6] agrees with the results very recently shown for other newly detected human members of the Ly-6 superfamily, indicating that expression defects are not uncommon in this protein family.[11] The association of a low percentage of NB1 GP–expressing neutrophils with cDNA polymorphisms indicates a genetic basis for the ability to express NB1 GP. We assume that mutations in the promotor region or in the transcription factor binding sites on chromosome 19 are associated with the observed CD177 gene polymorphisms, and that a certain haplotype is responsible for the transcription defect in the NB1 GP⁻ neutrophil subpopulation.

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