Reconstitution of Human FcγRIII Cell Type Specificity in Transgenic Mice

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Summary

The human low affinity receptors for the Fc domain of immunoglobulin G, FcγRIII, are encoded by two genes (IliA and IIIB) which share >95% sequence identity in both coding and flanking sequences. Despite this extraordinary sequence conservation, IliA is expressed in natural killer (NK) cells and macrophages and is absent in neutrophils, whereas IIIB is expressed only in neutrophils. To determine the molecular basis for this differential expression, we have generated transgenic mice using the genomic sequences of IliA and IIIB. IliA and IIIB transgenic mice show faithful reconstitution of this human pattern of cell type specificity. To determine the cis-acting sequence elements that confer this specificity, we constructed chimeric genes in which 5.8 kb of 5' sequences of the IIIB gene has been replaced with a homologous region from the IliA gene, and conversely, IliA 5' sequences have been substituted for the analogous region of the IIIB gene. Promoter swap transgenic mice that carry IliA 5' flanking sequences express FcγRIII in macrophages and NK cells. In contrast, promoter swap transgenic mice that contain IIIB 5' sequences express FcγRII in neutrophils only. These studies define the elements conferring the cell type-specific expression of the human FcγRIIIA and FcγRIIIB genes within the 5' flanking sequences and first intron of the human FcγRIIIA and FcγRIIIB genes.

Receptors for the Fc domain of IgG, FcγRs, are a family of oligomeric cell surface glycoproteins expressed on effector and lymphoid cells that mediate a variety of activation and inhibitory responses to antigen–antibody complexes. Three classes of FcγRs have been defined based on their different molecular mass, affinity for IgG, and genomic structures: the high affinity FcγRI, and the low affinity FcγRII and FcγRIII (1, 2). In humans, FcγRIII exists in two alternative forms. FcγRIIIA is a heteromultimeric complex composed of a ligand-binding α chain and dimeric γ or ζ chains (3-5). The α chain, encoded by the FcγRIIIA gene, is a type I transmembrane protein that contains the extracellular ligand-binding domain and a distinct cytoplasmic domain (6). The γ and ζ chains are required for assembly and signal transduction of the receptor complex (5). In contrast, FcγRIIB is a monomeric protein anchored to the outer leaf of the plasma membrane by a glycosyl phosphatidylinositol moiety (7, 8). The α chains of IIA and IIIB are >95% identical, conferring indistinguishable ligand binding properties on their respective receptors. The appearance of these two isoforms is the result of a small number of single-nucleotide substitutions in the two genes coding for the α chains of FcγRIIIA and FcγRIIB (9). Remarkably, despite extensive sequence identity extending into flanking sequences and introns, FcγRIIIA is expressed on NK cells and macrophages, where it mediates antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis, whereas FcγRIIB is uniquely expressed on neutrophils, where it functions primarily as a sink for immune complexes and cannot mediate an activation signal in isolation (10).

As a first step toward understanding the molecular basis for the differential expression of human FcγRIII genes, we have generated transgenic mice expressing human FcγRIIIA and FcγRIIB using the intact genomic clones. In addition, promoter swap transgenics were generated in which the putative regulatory sequences have been exchanged: sequences derived from the FcγRIIIA gene 5' flanking region and first intron have been ligated in place of the corresponding sequences of the FcγRIIB gene, and conversely, the analogous regions of FcγRIIIA have been substituted for similar sequences of the FcγRIIB gene. Comparison of the FcγRIII expression on NK cells, macrophages, and neutrophils among these four transgenic lines shows that the cis elements conferring the cell-specific expression of the human FcγRIII genes locate on the 5' flanking sequence and first intron of these genes.

Materials and Methods

Transgene Constructs. Genomic clones encoding human FcγRIIIA and FcγRIIB (c9158 and c7733, respectively) were isolated as previously described (9, 11). The NotI–SalI restriction
fragment containing 5.8 kb of the 5′ flanking sequence of the FcγRIIIA gene including the signal sequence and EC1 exons and the 13-kb SalI–NotI restriction fragment containing the EC2, transmembrane/cytoplasmic (TM/CYT), and 3′ UT sequences of the FcγRIIB gene were isolated by gel purification, ligated, and cloned into the NotI site of cosmid vector pWE15. This yielded promoter swap construct PS19. Similarly, promoter swap construct PS28 was obtained by ligating three DNA fragments: the 19.2-kb NotI–SalI restriction fragment of IIIB containing the 5′ flanking sequences, the 5.8-kb SalI–EcoRI fragment from IIA containing EC1, EC2, TM/CYT, and 3′ UT exons, and the 5-kb EcoRI–NotI of IIIB containing the 3′ flanking sequences (see Fig. 1).

Preparation of DNA Fragments for Microinjection. Large-scale plasmid preparations of transgene constructs were performed according to Sambrook et al. (12). All four constructs were digested with NotI to release the inserts from vector sequences. Digests were fractionated by electrophoresis through 1% agarose gel. The inserts were isolated by excision of a gel slice and electroelution of the DNA fragment into 0.5X Tris–borate–EDTA buffer, followed by CsCl density centrifugation and ethanol precipitation. DNA pellets were washed in 70% ethanol, resuspended in deionized H2O, and diluted to a concentration of 1–2 μg/ml with 1× injection buffer (13).

Transgenic Mice. The transgene constructs were microinjected into fertilized eggs from (C57Bl/6 × CBA/CA) F1 matings, and the injected eggs were transferred to pseudopregnant female CD-1 mice (Charles River Laboratories, Wilmington, MA) for development to term. Transgenic founders were identified by Southern blot hybridization of BamHI-digested tail DNA to a [32P]-labeled annealed oligo spanning the transmembrane and cytoplasmic domain (nucleotides 642–732), which is conserved between IIA and IIIB.

Flow Cytometry Analysis. Single-cell suspensions from bone marrow, spleen, and peritoneal cavity were prepared as described below. Aliquots of 106 cells were stained for 30 min at 4°C in a 100-μl vol with combination of the following antibodies (1 μg/ml): FITC–anti-human CD16 (3G8 F(ab′)2; Medarex, Inc., West Lebanon, NH); PE–anti–mouse granulocyte (R6B-8C1; Pharmingen, San Diego, CA), and anti–mouse Mac-1 (M1/70; Boehringer Mannheim Corp., Indianapolis, IN). Cells were washed twice in PBS containing 1% BSA after each incubation and fixed in PBS and 1% formaldehyde. Fluorescence intensity was measured using a FACScan® (Becton Dickinson & Co., Mountain View, CA). Dead cells were eliminated from the analysis on the basis of forward and sideways light scatter.

Cell Preparation. Mice were injected intraperitoneally with 1 ml of 5% thiglycollate, and peritoneal cells were harvested 3 d later. The cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS and plated in 100 × 15-mm dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) and incubated for 6 h at 37°C in 95% air, 5% CO2. Nonadherent cells were removed by rinsing the monolayers with PBS, and the purified macrophages were subjected to the flow cytometry analysis.

Bone marrow cells were flushed out of the femurs of mice using a 23-gauge needle, resuspended, and then washed in PBS. The erythrocytes were lysed by NH4Cl buffer (160 mM NH4Cl, 17 mM Tris, pH 7.4), and the remaining cells were centrifuged and washed with PBS. The bone marrow-derived neutrophils were then used for the FACS® analysis.

NK cells were prepared as described previously (14). Briefly, spleens were removed aseptically, crushed, and resuspended in NK medium consisting of RPMI 1640 supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT). The erythrocytes were lysed by NH4Cl buffer, and the remaining cells were centrifuged and then passed through a nylon wool column. The nylon-passed cells were resuspended at 5 × 106 cells/ml and cultured in NK medium supplemented with 1,000 U/ml recombinant IL-2 (GIBCO BRL, Gaithersburg, MD) and 1 μg/ml indomethacin (Sigma Chemical Co., St. Louis, MO). Fresh media were added at 1:1 vol every 3–4 d. After 7–10 d of culture, NK cells were harvested and used for the FACS® analysis.

Results and Discussion

Design of Promoter Swap Transgenic Constructs. To determine which sequences are responsible for the cell type–specific expression of human FcγRIII, two promoter swap constructs were generated. As shown in Fig. 1, PS19 contains IIA 5′ flanking sequences and first intron, and most of the IIIB coding and 3′ untranscribed regions. Our first attempt to construct a 5′ IIB–IIA3′ chimeric gene by ligating the

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Figure 1. Maps of the human FcγRIII and promoter swap transgene constructs. Top four lines are physical maps of the transgene constructs with restriction sites designated as follows: NotI (N), HindIII (H), EcoRI (E), BamHI (B), and SalI (S). The open boxes represent the IIA sequences, and the closed boxes represent the IIIB sequences. The intron–exon organization is shown at the bottom. Coding regions are indicated by filled rectangles, untranscribed regions by open rectangles. 3′UT, 5′ untranslated region; S1, first signal exon; S2, second signal exon; EC1, first extracellular exon; EC2, second extracellular exon; TM/C, transmembrane and cytoplasmic domains; 3′UT, 3′ untranslated region; kb, kilobase. The precise 5′ borders have not been determined, as indicated by the jagged lines.
IIIB 5' flanking sequence of the NotI–SalI fragment to the IIA SalI–NotI fragment was not successful, since the resulting insert could not be stably propagated in Escherichia coli. We therefore generated the PS28 construct by ligating three DNA fragments, creating a mosaic gene that contained the IIIB 5' flanking sequences, the IIA coding regions, and IIIB 3' untranslated regions. These two promoter swap constructs were confirmed by TaqI digestion and hybridization with exon-specific probes for the signal sequence and 3' UT, which distinguish between the IIA and IIIB sequences, as previously described (9 and data not shown).

**Figure 2.** Southern blot analysis of the genomic DNA from transgenic mice. Tail DNA was digested with BamHI, electrophoresed, transferred, and blotted with human FcγRIII TM/C probe. The hybridizing bands are ~18 kb for IIA and 4.8 kb for IIIB, PS19, and PS28.

Generation of Transgenic Mice. Each of the four transgene constructs was microinjected into fertilized eggs derived from mating between (C57B1/6J × CBA/CA) f1 mice. Founder mice were identified by hybridizing Southern blots of BamHI-digested tail DNA to a radiolabeled FcγRIII TM/C probe. A representative pattern of restriction fragment is shown in Fig. 2. Hybridizing fragments are ~18 kb for IIA and ~4.8 kb for IIIB, PS19, and PS28. Injection of IIA and PS19 constructs yielded two and six founders, respectively. Five founder mice were generated with the PS28 transgene, and three of them bred. Only one founder was produced with the IIIB transgene. The generated transgenic mice contained between 5 and 50 copies of the transgenes. Human FcγRIII expression was examined in all lines, and all lines carrying a particular transgene construct exhibited qualitatively similar patterns of human FcγRIII expression. Representative data from the following lines are presented below: IIA, No. 1156; IIIB, No. 1373; PS19, No. 4278; PS28, No. 4037. Each of these lines contained between 10 and 30 copies of the injected transgene (Fig. 2 and data not shown).

Expression of Human FcγRIIIA on Macrophages and NK Cells and IIIB on Neutrophils of Transgenic Mice. To determine the surface expression pattern of human FcγRIIIA, macrophages, neutrophils, and lymphocytes were harvested and...
Bone marrow–derived neutrophils were characterized by flow cytometry using 3G8 and a granulocyte marker, Gr-1. As shown in Fig. 4, neutrophils from IIIB- and PS28-transgenic mice were positive for both 3G8 and Gr-1, whereas the macrophages from these mice were negative for 3G8. In contrast, neutrophils from IIIA- and PS19-transgenic mice as well as nontransgenic littermates were not stained with 3G8, yet were positive for the marker Gr-1. Macrophages from nontransgenic littermates of all lines showed no detectable 3G8 staining (data not shown).

Similarly, NK cells prepared from IL-2–activated splenocytes were positive for 3G8 staining on NK cells derived from IIIA- and PS19-transgenic mice, as shown in Fig. 5. 3G8 staining was not detected on splenic T or B cells. No detectable 3G8 staining was found on NK cells from IIIB- and PS28-transgenic mice. These studies localized the macrophage/NK cell restricting element to a 5.8-kb fragment of the IliA gene and a neutrophil-specific element to a 19-kb region included in the 5′ flanking sequences of the IIIB gene. Thus, despite the fact that the mouse expresses a single FcγRIII gene in neutrophils, macrophages, and NK cells, murine cells retained the ability to distinguish between these human sequences that drive cell type specificity.

Functional studies have suggested that IIIA delivers an activation signal upon cross-linking by immune complexes, whereas IIIB occupancy does not result in cellular activation in the absence of a costimulatory signal from another Fc receptor. Preliminary studies using these transgenic mice has confirmed these in vitro observations (Weiner, A., unpublished observations) and will allow for a detailed examination of the role of IIIB in vivo. Finally, the identification of a neutrophil-specific DNA element will facilitate the targeting of gene expression to this important effector cell population and clarify the role of these cells in the inflammatory response.

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